Characterisation of salt and cold stress response mechanisms in *Listeria monocytogenes* as revealed by whole genome microarray analysis

by

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BBiomedSc (Hons)

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

University of Tasmania

October 2010

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October 2010

Thesis Abstract

This study was conducted to investigate the genetic adaptation of *Listeria monocytogenes* to hyperosmotic (created by high NaCl concentrations) conditions and low-temperature stress. *L. monocytogenes* is an opportunistic pathogenic bacterium capable of causing serious systemic illness, with a high mortality rate in susceptible individuals. Its environmental abundance significantly increases the likelihood of carry-over contamination in food processing surroundings leading to possible contamination of finished products. In addition frequent exposure to environmental stresses during its saprophytic existence renders *L. monocytogenes* able to overcome stresses often used in food production to limit microbial proliferation and to extend shelf-life. Therefore a more detailed understanding of molecular adaptations in this organism to stresses it encounters as a saprophyte may provide insights to minimise proliferation in foods.

To broaden the understanding of *L. monocytogenes* stress resistance a population study, unique in its magnitude, was conducted on growth parameters obtained for a large assortment of isolates of diverse origin, subjected to hyperosmotic stress and, independently, to cold-temperature. On the basis of generation times, no direct correlation (r=0.4) between tolerance to either hyperosmotic- or cold-stress, was evident between isolates, but strains of animal origin were on average found to grow faster when exposed to ether of the two stresses. Strains of serotype 4b appeared to have shorter average generation times of 6.6±0.25 h in BHI at 12.5% (w/v) NaCl and 25°C, whereas serotype 4a strains had an average generation time of 19.4±1.0 h at 4°C in 0.5% (w/v) NaCl BHI, which was faster than other serotypes examined.

Hyperosmotic and cold stress factors, though quite different in terms of physiochemical stress on bacterial cells, revealed many parallels in terms of gene expression in three strains of *L. monocytogenes* studied. The results suggest that a broadly similar genetic regulatory mechanism could be operating in response to cold and hyperosmotic stresses.

Osmoadaptation of four *L. monocytogenes* strains, possessing different tolerances to NaCl, revealed a clear pattern in terms of genomic expression. Prolonged exposure to high levels of NaCl was coupled with activation of genes associated with the bacterial cell envelope, DNA repair and protein synthesis. Repression of genes associated with carbohydrate up-take and metabolism was evident in

osmo-adapted cells reflecting the overall suppression of cellular metabolism characterised by reduced growth rate of these cells. In addition the initial stage of osmo-adaptation, was examined in strain ATCC 19115 (serotype 4b) post short exposure to 10.0% (w/v) NaCl to investigate continuous spectrum of gene expression in response to osmotic stress in this organism. Gene enrichment analysis revealed a prominent, almost reverse gene expression profile in response to shock, compared to the adaptive response to the same concentration of NaCl. This study is the first to strongly highlight such distinction in gene expression between phases of hyperosmotic adaptation, further emphasising the complexity of this response.

Cold adaptation in three *L. monocytogenes* strains, possessing distinctly different growth rates at 4°C, lead to activation of gene sets associated with ribosomes, fatty acid and peptidoglycan biosynthesis and cell division and suppression of carbohydrate transport and metabolism related genes. Cold adapted strains did not activate a SigB or PrfA regulatory responses, suggesting that SigB-mediate stress responses are not closely involved in low temperature adaptation. A similar response was observed during hyperosmotic adaptation in *L. monocytogenes*.

Strain specific response to stress factors has been overlooked in previous whole genome analyses, and although this study suggests a clear correlation in overall response to cold and hyperosmotic stress in this species, strain specific and phase specific responses are also crucial in understanding fully its mechanisms of stress tolerance, survival and persistence. The importance of strain-specific variations in genetic response to stress should be considered particularly if genetic targeting is to be applied to controlling *L. monocytogenes* proliferation in food products. This study is the first to assess genetic stress adaptation in such depth, significantly contributing to the understanding of *L. monocytogenes* physiology.

Acknowledgements

I would like to express my sincere gratitude to A/Prof. John Bowman my primary supervisor, without whom this work would not have occurred. His constant guidance, support and patience have helped me throughout the project. Thank you for giving me the opportunity and for believing in me!

I would also like to thank Melanie O'Keefe and staff at Australian Genome Research Facility Ltd (AGRF) for technical support with the micro-array analysis.

A special thank you must go to A/Prof. Tom Ross, Prof. Tom McMeekin and Moya Durack who contributed enormously to the final version of the manuscript, for their ongoing support and wisdom throughout the project.

I would also like to thank the staff and students within the School of Agricultural Science, University of Tasmania, in particular Dr Jimmy Twin, Dr Alison Dann, Mark De'Pannone and Dr Lyndal Mellefont for sharing the experience and countless cups of coffee. Especially I would like to thank Dr Claudio Bittencourt for his encouragement, support, friendship, and willingness to share a laugh at any, and every moment.

I am very grateful to Sally Jones for her significant contribution to printing the manuscript in addition to her ongoing support and encouragement throughout my involvement with the TIAR.

I would like to thank my family Evghenia, Nikolai, Moya, Patrick, Louise and Margarita for their encouragement, moral support, for their willingness to listen and for believing in me. A very big thank you to all my friends for putting up with me, brightening my spirits and pushing me through to the end.

Finally I would like to thank Paul Durack for his patience, continuous support and for being a constant source of encouragement (and computing support). Thank you for sharing this journey with me and for being such a big part of my life!

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Commonly Used Abbreviations and Terms

AGRF	Australian Genomic Research Facility Ltd.
ATP	Adenosine triphosphate
a_w	Water activity
BCAA	Branched chain amino acids
BCFA	Branched chain fatty acids
BHIA	Brain Heart Infusion Agar
BHIB	Brain Heart Infusion Broth
BSA	Bovine serum albumin
CFU	Colony forming unit
CI	Confidence Interval
CSP	Cold shock protein
DEPC	Diethyl pyrocarbonate
FAB	Fatty acid biosynthesis
GB	Glycine betaine
GMP	Guanosine monophosphate
GT	Generation time
GTP	Guanosine 5'-triphosphate
HSP	Hot shock protein
LI	Lineage I
LII	Lineage II
LIII	Lineage III
LIPI	Listerial Pathogenicity Island
LR	Log Ratio
MZ	Motility zone
KEGG	Kyoto Encyclopedia of Genes and Genomes
OD	Optical density
ORF	Open reading frame
PBP	Penicillin binding protein
PTS	Phosphotransferase System
RNAP	RNA polymerase
RT	Room Temperature
SDS	Sodium dodecyl sulfate
TIAR	Tasmanian Institute of Agricultural Research
TTC	2,3,5-triphenyltetrazolium chloride

Chapter 1

An overview of Listeria monocytogenes osmotolerance and cryotolerance

The bacterial pathogen *Listeria monocytogenes* is well adapted to life in the soil as a saprophyte, where it is thought to live off decaying plant material. Its environmental abundance significantly increases the likelihood of carry-over contamination in food processing surroundings leading to possible contamination of finished produce. Ingestion of contaminated ready-to-eat foods that support *L. monocytogenes* growth by susceptible individuals may cause serious and often fatal infections. This overview focuses on the molecular aspects of cellular mechanisms behind *L. monocytogenes* virulence and adaptation to hyperosmotic as well as cold stresses.

1.1 Genus Listeria

The genus *Listeria* comprises a group of gram-positive, non-spore forming, facultative anaerobic bacilli of low G+C content (36-42%) closely related to genera containing some major human pathogens including *Bacillus*, *Clostridium*, *Enterococcus*, *Streptococcus* and *Staphylococcus* (Allerberger, 2003; Hain *et al.*, 2006; Vazquez-Boland *et al.*, 2001). The genus *Listeria* consists of six known species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. grayi*. *Listeria* species are divided into two major genetic lineages as revealed by a number of sequence-based studies, including 16S and 23S-rRNA coding genes, *prs*, *ldh*, *vcl*A, *vcl*B, *iap* (Hain *et al.*, 2006; Schmid *et al.*, 2005). *L. monocytogenes* and *L. innocua* form one group and the second group includes the remainder of species with *L. grayi* forming a separate deep branch within the genus. The next closest relative is genus *Brocothrix*. Only two *Listeria* species, *L. monocytogenes* and *L. ivanovii* are considered pathogenic in animals and humans, with *L. monocytogenes* being predominantly associated with human illness.

1.2 Listeria monocytogenes

L. monocytogenes is isolated from a wide variety of ecosystems including soil, water (fresh and saline), sewage, fertilisers, animal feeds and asymptomatic human and animal carriers. The natural habitat of this organism is thought to be decomposing plant matter, in which it lives as a saprophyte.

L. monocytogenes has developed efficient mechanisms for growth under stressful conditions such as starvation; wide variations in temperature, ranging from 0 to 45^{o}C; pH as low as 4.2 and as high as 9-9.5 (Farber & Peterkin, 1991) and high salt concentrations, up to 15% NaCl (Cetin *et al.*, 2004; Duché *et al.*, 2002; Volokhov *et al.*, 2002).

1.2.1 Implications of *L. monocytogenes* for the Food Industry

A high degree of adaptability as well as the ubiquitous distribution of *L. monocytogenes* makes the species potentially able to survive in the food processing environment. Conservative treatments used in food processing and preservation often utilise stressing agents and parameters to which this pathogen is habituated, thus rendering these treatments ineffective against *L. monocytogenes.* This pathogen is of particular importance for safety of refrigerated and ready-to-eat (RTE) food products which have long refrigerated shelf life and are consumed without reheating. According to the Australian Food Safety Standard (Standard 1.6.1 Issue 78) there is a 'zero tolerance' (absence in 25g of sampled food) for *L. monocytogenes* in food products that are able to support its growth (http://www.foodstandards.gov.au/). Included in this category are soft and semi-soft cheeses, smoked fish products, meat pate and cooked cured/salted meat, other milk-based products and butter. All other RTE foods, that do not support growth of the pathogen are permitted up to 100 cfu/g without recall being required if detected, which allows commercial distribution of food because such low levels of *L. monocytogenes* in foods are considered to pose an insignificant health risk to consumers(FAO/WHO, 2004). Consumption of *L. monocytogenes* contaminated food by healthy individuals usually results in asymptomatic faecal secretion and, in worst-case scenarios, gastroenteritis resulting in self limiting dysentery (Muller, 1990). Real problems associated with this opportunistic pathogen arise following consumption of contaminated food by a sub-group of individuals who are predisposed to developing further complications collectively referred to as listeriosis.

1.2.2 Listeriosis

The transmission of *L. monocytogenes* by contaminated food was first demonstrated in 1983 and since has been linked to a number of both sporadic cases and outbreaks of listeriosis (Kathariou, 2002). Even though the statistics indicate a decline in the incidence of listeriosis since the implementation of zero-tolerance policy (Goulet *et al.*, 2006; Tappero *et al.*, 1995), outbreaks and contaminated product recalls continue to occur. Listeriosis primarily affects pregnant women, neonates, the elderly and immunocompromised individuals manifesting in septicaemia, meningitis,

encephalitis, spontaneous abortion and still-birth (Doumith *et al.*, 2004; Graves *et al.*, 2005). Listeriosis may assume greater significance in the future with a continuous high prevalence of HIV, an increasingly elderly population, and numerous organ transplant and prosthesis recipients, and those undergoing chemotherapy, all of which are associated with reduced immunity, thus the number of people who are susceptible to this condition is growing. Current epidemiological evidence in Europe suggests that this may already be occurring, with an increase in listeriosis among the older population relative to all other at risk groups (Burki, 2009; Cairns & Payne, 2009; Goulet *et al.*, 2008).

The minimum infective dose of *L. monocytogenes* required to cause clinical infection in humans has not been determined. Variation in the incidence of systemic listeriosis is thought to be due to the degree of virulence of a corresponding strain, bacterial load of the contaminated food and the predisposition of an individual involved, all of which play a major role in disease manifestation and its outcome (Jeffers *et al.*, 2001; Ward *et al.*, 2004; Yildirim *et al.*, 2004). Although rare when compared to other food-borne diseases, a significant feature of listeriosis is the high mortality rate (30% and higher despite early antibiotic treatment), which makes *L. monocytogenes* an important human pathogen and ranks it among the microorganisms that most concern the food industry. Due to the relatively long incubation time outbreaks of listeriosis are often diffuse in time and difficult to recognise and to trace back to contaminated food, thus making outbreaks more difficult to contain.

1.2.3 Pathogenesis and Virulence

Contaminated food is the major source of Listerial infection in both epidemic and sporadic cases, thus the gastrointestinal tract is thought to be the primary site of entry of *L. monocytogenes* into a human body. Before *L. monocytogenes* can cause infection it must survive various hurdles presented by the body's natural defences. The high acidity of the stomach is one such barrier and is capable of destroying a significant proportion of cells ingested with contaminated food, thus minimizing the number of viable organisms able to cause subsequent infection. Once in the small intestine *L. monocytogenes* is thought to cross the intestinal barrier *via* the M cells of the Peyer's patches and is then carried by the lymph or blood to the mesenteric lymph nodes, spleen and liver where the organism proliferates and spreads to cause systemic infection (Vazquez-Boland *et al.*, 2001).
Entry into host cells is induced by binding of the bacterial surface proteins internalin A (InlA) and InlB to the receptors on host cells (Gray *et al.*, 2006; Hamon *et al.*, 2006; Lecuit, 2005). Both internalins recognize different receptors on the host cells thus allowing invasion of different cell types.
Following internalization, the bacterium escapes from the membrane-bound phagocytic vacuole by secreting two phospholipases, PlcA and PlcB, and the pore-forming toxin listeriolysin O (LLO),

thereby gaining access to the cytoplasm where it is free to replicate (Gray *et al.*, 2006; Hamon *et al.*, 2006; Lecuit, 2005). The intracytoplasmic bacteria use the actin of the host cell, in conjunction with their ActA protein to promote cell-to-cell spread where the bacterium, upon contact with the plasma membrane, induces pseudopod-like protrusions that are engulfed by adjacent host cells. After their uptake by adjacent cells, bacterial cells escape the double membrane-bound vacuole, repeating the cycle and infecting yet more neighbouring host cells.

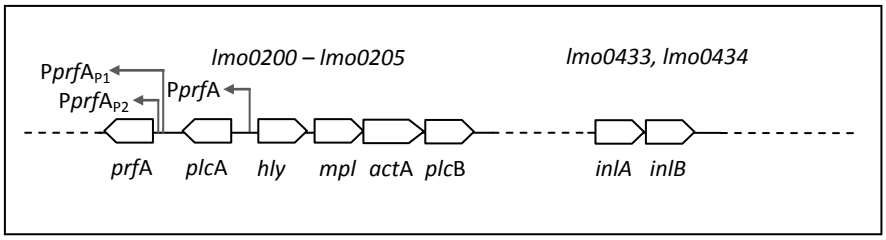


Figure 1.1 Schematic representations of *L. monocytogenes* major virulence genes.

Six of the virulence factors responsible for the intracellular proliferation of *L. monocytogenes* are clustered together in a 9-kb chromosomal island referred to as the PrfA-dependent virulence gene cluster (see Fig. 1.1Figure 1) also known as LIPI-1 (*Listerial* pathogenicity island 1), which is bordered by two housekeeping genes: the *ldh* (encoding lactate dehydrogenase) and *prs* (encoding phosphoribosyl synthetase). LIPI-1 comprises of *prfA*, *plcA* (which encodes a phosphatidylinositol-specific phospholipase C or PI-PLC), *hly* (encoding a haemolysin LLO), *mpl* (encoding a zinc-metalloprotease Mpl that processes PlcB to its mature form), *actA* (encoding an Actin-Polymerising Protein ActA) and *plcB* (encoding the second PI-PLC). The *hly* occupies the central position in this locus, downstream from it and transcribed in the same orientation is the operon, comprising the *mpl*, *act*A and *plc*B genes. The *plc*A-*prf*A operon is located upstream from *hly* and is transcribed in opposite orientation (Fig. 1.1). Two further genes, *inl*A and *inl*B also form an operon independent of the LIPI-1, both of which are transcribed in the same orientation (Gray *et al.*, 2006).

Most of the known virulence proteins in *L. monocytogenes* are controlled by a transcriptional regulator, PrfA, a 27kDa site-specific DNA-binding protein, which itself is regulated by a number of environmental conditions. PrfA-mediated transcription required the binding of PrfA to a conserved 14bp sequence located about 40 bp upstream of the transcriptional start site of each PrfA-regulated gene (Böckmann *et al.*, 2000; Dussurget *et al.*, 2004; Lalic-Mülthaler *et al.*, 2001; Vazquez-Boland *et al.*, 2001). The *prf*A gene is transcribed from two promoters, located immediately upstream of *prf*A (P*prf*A$_{P1}$and P*prf*A$_{P2}$; Fig. 1.1) , into a monocistronic mRNA, or from the PrfA-dependent *plc*A

promoter (P*prf*A) into a bicistronic *plc*A/*prf*A mRNA (Böckmann *et al.*, 2000; Dussurget *et al.*, 2004; Lalic-Mülthaler *et al.*, 2001). PrfA positively regulates its own expression through the activation of *plc*A and negatively regulates its transcription from P*prf*A_{P1} and P*prf*A_{P2} promoters as the transcripts directed by these two promoters appear to be marginally increased in the absence of functional PrfA (Eiting *et al.*, 2005; Greene & Freitag, 2003; Lalic-Mülthaler *et al.*, 2001). The P*prf*A_{P1} is a sigma A (σ^A)-dependent promoter transcripts of which are produced by actively growing unstressed *L. monocytogenes* cells: the resulting *prfA* transcript contains a thermo-sensitive structure that inhibits translation of PrfA at temperatures lower than 30^{o}C (Gray *et al.*, 2006). The leader region of the *prf*A messenger RNA (transcribed by the P*prf*A_{P1}) forms a stable secondary structure that masks the ribosome-binding site at low temperature, thus the PrfA is not translated despite the synthesized transcript. At high temperature (37^{o}C) this thermo-sensor structure partially melts to expose the ribosome-binding site, thereby allowing translation to occur.

The second prfA promoter region, P*prf*A_{P2}, comprises both σ^A and σ^B (stress response regulator) - dependent promoters (Gray *et al.*, 2006). The P*prf*A_{P2}–directed transcript lacks the thermo-sensitive secondary structure, thus allowing expression of PrfA in low temperature environments and cells exposed to environmental stress conditions such as high osmolarity, oxidative stress and carbon starvation. Such post-transcriptional regulation of *prf*A allows rapid expression of the PrfA protein and consequently the expression of virulence factors necessary for pathogenesis as soon as the bacterium enters the host.

Chemical signals from the environment play an important role in the control of the PrfA system. PrfA-dependent genes seem to be weakly expressed in nutritionally rich culture media (such as Brain Heart Infusion broth). The abundance of readily metabolized carbohydrates in such media tend to inhibit transcription of PrfA-dependent virulence genes without affecting PrfA protein levels. On the other hand PrfA-dependent LLO production and *act*A expression are both activated in iron-depleted media. The exact mechanism of environmental sensing is not as yet known, however it has been suggested that *L. monocytogenes* utilizes quorum sensing mechanisms to control the state of PrfA activation. Quorum sensing , is cell-density dependent and is believed to be regulated by small autorepressor molecules which are produced during exponential growth thus preventing a premature or unnecessary release of toxic virulence factors by *L. monocytogenes* cells until required for intracellular proliferation (Ermolaeva *et al.*, 2004).

1.2.4 Biodiversity

Epidemiological, population genetics and evolutionary studies of *Listeria monocytogenes* using various subtyping methods have been vital in establishing means of tracking the transmission of this pathogen from the environment through foods to humans. *L. monocytogenes* strains display both genetic and serotypic diversity. Serotypic diversity arises from combinations of somatic (O) and flagella (H) antigens, resulting in 13 recognized serotypes within the species; 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e and 7 (Allerberger, 2003; Liu *et al.*, 2006). Despite that virulence gene clusters can be found in the genome of all the known *L. monocytogenes* serotypes, infectivity or transmission rates of various serotypes appear to be non-randomly distributed, since most cases of human listeriosis (>98%) are attributed to serotypes 1/2b and 4b (Call *et al.*, 2003; Doumith *et al.*, 2004; Gorski *et al.*, 2006; Nightingale *et al.*, 2005). Furthermore, all major food-borne outbreaks of listeriosis, as well as the majority of sporadic cases have been attributed to serovar 4b strains (Call *et al.*, 2003; Doumith *et al.*, 2004; Gorski *et al.*, 2006; Nightingale *et al.*, 2005).

1.2.5 *L. monocytogenes* genome overview

L. monocytogenes contains one circular chromosome of 2.9-3.0 Mb with an average G+C content of 39% consisting of 2850-3000 protein-coding genes (Glaser *et al.*, 2001; Nelson *et al.*, 2004).

To date a number of whole genome sequence analyses are available for various *L. monocytogenes* strains, EGD-e being the first sequenced and most widely referred to in literature.
Where known the genome sequences of *L. monocytogenes* strains are generally very similar in gene content and organisation, although strain specific and serotype specific genes have been identified, e.g. Nelson *et al*. (2004) identified 51, 97, 69 and 61 strain specific genes from strains F2365 (4b), F6854 (1/2a), H7858 (4b) and EGD-e (1/2a) respectively, and 83 and 51 genes were restricted to the serotype 1/2a and 4b respectively. The majority of the genomic differences consisted of phage insertions, transposable elements, scattered unique genes and islands encoding proteins with mostly unknown function (Nelson *et al.*, 2004). In addition to strains EGD-e (Glaser *et al.*, 2001) and F2365, also referred to as ATCC19115 (Nelson *et al.*, 2004), whole genome sequence for two other strains Clip81459 (serotype 4b) and HCC23 (4a) are available on the Kyoto Encyclopaedia of Genes and Genomes (KEGG database; http://www.genome.jp/kegg/). Collectively, 171 genes which vary between strains have been identified upon analysis of available genome sequence data (Table A.1, Appendix A).

1.2.5.1 *L. monocytogenes EGD-e genome*

Strain EGD-e was the first *L. monocytogenes* to be fully sequenced and, as such, represents the base-line of Listerial research, with a number of full genome expression studies being based on the available genome sequence.

As noted above, the genome of *L. monocytogenes* EGD-e (serovar 1/2a) consists of 3,000 genes, 331 of which encode different transport proteins (11.6% of all predicted genes). Of these, 88 (26%) are associated with carbohydrate transport mediated by phosphoenolpyruvate-dependent phosphotransferase systems (PTS), nearly twice as many PTS permeases as present in *Escherichia coli* and three times as many as previously identified in *Bacillus subtilis* (Glaser *et al.*, 2001). A large number of surface and secreted proteins (133 and 86 respectively) were also identified in the same study. Among these 41 proteins belong to the LPXTG protein family (including InlA) which are covalently attached to the cells wall by a sortase (Dussurget *et al.*, 2004). In addition to LPXTG surface proteins *L. monocytogenes'* genome encodes 9 glycine-tryptophan dipeptide GW surface proteins (including InlB) which are non-covalently anchored to the bacterial cell wall, 11 hydrophobic tail proteins (including ActA), 4 p60-like proteins and 68 lipoproteins (Dussurget *et al.*, 2004). Such abundance of surface proteins reflects the ability of this pathogen to interact with a large variety of surfaces in the environment and to colonise many cell types in the host.

Another interesting feature of *L. monocytogenes* genome is the exceptionally large number of genes (209) that encode transcriptional regulators (7.3% of all predicted genes). This most likely provides a means for coordinated expression of genes in response to the diverse environmental conditions this organism experiences and endures.

1.2.5.2 *Interpretations of EGD-e based full genome analysis*

As additional information about other *L. monocytogenes* genomes is becoming available a better understanding of the species as a whole is being attained. Strain specificity in evaluating genome expression is also becoming apparent. For instance 173 EGD-e specific genes are known to exist, (Table A.2, Appendix A). These must be taken into account when analysing whole genome expression of non-EGD-e strains, unless a full sequence is available and the presence of such genes has been established.

A significant down-side of EGD-e based full genome expression analysis lies in the inability to evaluate the expression of genes which are absent in EGD-e genome but might be present in other strains. At present 564 such genes are identifiable from the available sequenced genomes (Table A.3, Appendix A), and is likely to increase with additional strains being sequenced.

Although the above hurdles must be considered when evaluating EGD-e based genome expression, the effectiveness of analysis should not be doubted considering that the core genome of the species is proving to be remarkably similar in its gene content and organisation (Nelson *et al.*, 2004).

1.3 Stress tolerance of *L. monocytogenes*

Food-borne pathogens are commonly stressed during food processing. During sausage fermentation, for example, bacterial contaminants in raw meat undergo a series of hurdles which include added salt and acid developed during fermentation. In cheese manufacture bacterial contaminants existing in raw milk are exposed to heat during pasteurization, hydrogen peroxide that maybe added to raw milk, acid produced by the starter culture during fermentation, and salt added to the curd in addition the effect of salt is increased due to removal of water through syneresis, cutting and cooking of the cheese curd or exerting pressure on the curd. Therefore understanding the effects of environmental stress on the physical tolerance of *L. monocytogenes* is important in order to assess and minimize the risk of food-borne illness; particularly since exposure to hostile environments is known to provide cross protection against additional stress in this organism (Faleiro *et al.*, 2002; Lou & Yousef, 1996; Lou & Yousef, 1997).

1.3.1 Salt as a food preservative

NaCl is an important food additive used not only as a flavour enhancing agent but also as a food preservative. High NaCl concentrations suppress bacterial growth by decreasing water activity (a_w) of a growth medium thus enhancing plasmolysis and consequently resulting in decreased intracellular turgor pressure. Reduction in turgor pressure inhibits bacterial growth, as turgor pressure provides the essential force that expands the cell thus contributing to bacterial cell growth and division (Amezaga *et al.*, 1995). In addition to increasing osmotic pressure of a growth medium NaCl decreases electrochemical potential across the cell membrane thus disturbing ATP production by oxidative phosphorylation (Shabala *et al.*, 2006).

1.3.2 Osmoadaptation in *L. monocytogenes*

Osmoadaptation includes both the genetic and physiological manifestations of adaptation to low water environments (Galinski, 1995). Osmoadaptation in *L. monocytogenes* as for the majority of bacteria, is a biphasic process consisting of primary and secondary response mechanisms.

1.3.2.1 Primary phase of osmoadaptation

Following a sudden increase in salinity, cells maintain turgor within physiologically accepted boundaries by first increasing the uptake of potassium ions (K^+) and glutamate as its counter anion and then replacing part of the accumulated K^+ with compatible solutes in a second stage of osmoadaptation (Sleator *et al.*, 2003a).

L. monocytogenes possesses two K^+ transporters, a high affinity KdpABC transporter system, which exhibits significant similarity to Kdp of *Escherichia coli* and *Bacillus subtilis* as wells as a low-affinity system encoded by *lmo0993* gene which resembles both the KtrII system of *Enterococcus hirae* and the TrkG and TrkH subunits of *E. coli* Trk system (Ballal *et al.*, 2007; Brøndsted *et al.*, 2003; Kallipolitis & Ingmer, 2001; Sleator *et al.*, 2003a).

1.3.2.2 Secondary phase of osmoadaptation

Generally, bacteria adapt to osmotic stress by active accumulation or *de novo* synthesis of osmotically active compounds interchangeably known as compatible solutes, osmolytes or osmoprotectants. Compatible solutes are low molecular weight, highly-soluble, compounds that bear no net ionic charge at physiological pH (Gardan *et al.*, 2003a; Wemekamp-Kamphuis *et al.*, 2002b). These osmolytes counteract water efflux restoring cell volume while also stabilising protein structure and function at elevated osmolarity (Chattopadhyay, 2001; Sleator *et al.*, 2003a; Wemekamp-Kamphuis *et al.*, 2002b).

Survival of *Listeria monocytogenes* at high salt concentrations appears to be attributed mainly to the accumulation of three compatible solutes glycine betaine, carnitine and proline (Fraser *et al.*, 2000; Gardan *et al.*, 2003a; Sleator *et al.*, 2001a; Sleator *et al.*, 2003a). More recent studies have suggested that other compounds such as acetylcarnitine, gamma-butyrobetaine, alanine, glutamate and 3-dimethylsulfoniopropionate provide additional osmoprotection in this organism (Angelidis *et al.*, 2002; Sleator *et al.*, 2003a; Wemekamp-Kamphuis *et al.*, 2002b).

1.3.2.2.1 Glycine betaine

Glycine betaine (N,N,N-trimethylglycine, 'GB'), a compound which is present at relatively high concentrations in foods of plant origin (Sleator *et al.*, 1999; Sleator *et al.*, 2003a), is the most effective osmolyte not only in *L. monocytogenes* but also in many other Gram-positive, as well as Gram-negative, bacteria (Dreux *et al.*, 2008; Galinski, 1995; Mendum & Tombras Smith, 2002; Sleator *et al.*, 1999; Sleator *et al.*, 2003a). In addition to restoring intracellular osmolarity accumulated GB is believed to stabilize protein molecules in a chaperone-like manner (Bourot *et al.*, 2000; Chattopadhyay, 2001). GB is an osmoprotectant that is completely excluded from the water hydration shell of proteins and, unlike other chaperones, does not bind to proteins (Arakawa & Timasheff, 1985; Bourot *et al.*, 2000). Instead it is believed to assist protein folding by a thermodynamic driven force created by the accumulated glycine betaine which drastically displaces the unfold-fold equilibrium toward the conformation whose surface exposure to water molecules is reduced thus promoting folding of protein molecules.

Bacteria such as *Bacillus subtilis* are able to synthesize a number of compatible solutes *de novo*; *L. monocytogenes* however lacks the required mechanisms for synthesis of either glycine betaine or carnitine. Accumulation of these is therefore achieved solely by transport from the environment. Two distinctive and highly specific permeases devoted to glycine betaine transport have been extensively studied in this organism.

1.3.2.2.1.1 Glycine betaine porter I

GB porter I (BetL) is a product of a *bet*L gene (Fig. 1.2) consisting of a single large open reading frame with a TTG start codon encoding a 507-residue integral membrane-bound protein containing 12 transmembrane domains (Sleator *et al.*, 1999). The BetL of *L. monocytogenes* is a high affinity secondary transporter of GB and is homologous to the secondary transporter OpuD of *Bacillus subtilis*, BetT and CaiT of *Escherichia coli* and BetP of *Corynebacterium glutamicum* (Angelidis *et al.*, 2002; Mendum & Smith, 2002; Sleator *et al.*, 1999; Sleator *et al.*, 2003a; Wemekamp-Kamphuis *et al.*, 2002b). Up-take of GB *via* BetL is known to be coupled to the influx of sodium ions where a GB molecule is co-transported with a Na^+ ion. BetL is rapidly activated by osmotic up-shift to provide immediate up-take of GB thus assisting in the primary stage of osmoadaptation. However, while this transporter does provide some long term protection against low levels of salt stress it fails to do so under high salt concentrations (Mendum & Tombras Smith, 2002; Sleator *et al.*, 2003b).

1.3.2.2.1.2 Glycine betaine porter II

The second glycine betaine transport system in *L. monocytogenes* is the GB porter II also known as Gbu, which is encoded by the *gbu* gene. Gbu belongs to the binding-protein-dependent ATP binding cassette super family of transporters and is homologous to both OpuA of *Bacillus subtilis* and ProU of *E. coli* (Mendum & Smith, 2002; Sleator *et al.*, 2003a; Wemekamp-Kamphuis *et al.*, 2002b). The *gbu* operon consists of three open reading frames (ORFs) which are oriented in the same direction and are known as *gbu*A, *gbu*B and *gbu*C (Ko & Smith, 1999) (Fig. 1.2).

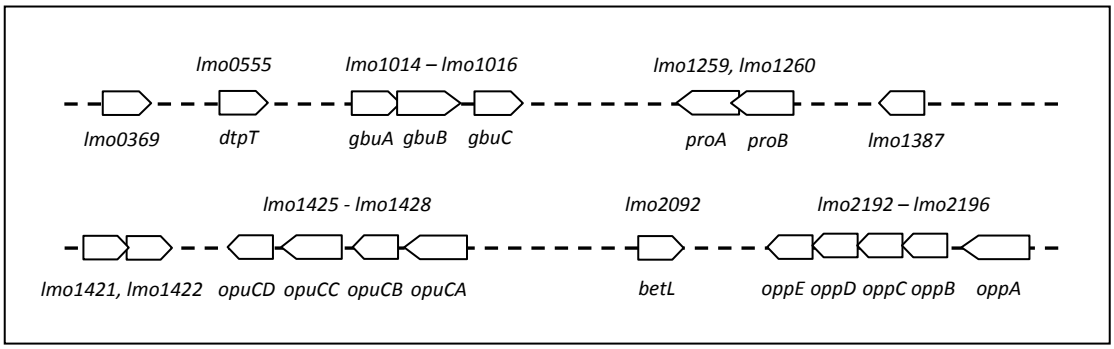


Figure 1.1 Schematic representation of compatible solute associated genes and their relative position and orientation on *L. monocytogenes* chromosome.

The first open reading frame (ORF1), *gbu*A is 1,194 bp in length and encodes a highly hydrophilic protein of 397 amino acid residues forming the ATPase subunit of Gbu transporter. The ORF2, *gbu*B, is 849 bp long and encodes a hydrophobic protein 282 amino acid residues in length which forms a six-helix transmembrane channel protein. The final open reading frame, *gbu*C is 903 bp in length, encoding a hydrophilic protein 300 amino acid residues long that forms the substrate binding protein of the Gbu transporter. The end of *gbuA* overlaps the beginning of *gbuB* by 8bp, and the intergenic distance between *gbuB* and *gbuC* is 13bp, suggesting that the three open reading frames are genetically arranged in an operon. The ATP-dependent Gbu transporter is believed to play a dominant role in long-term osmoadaptation in *L. monocytogenes*, particularly at higher osmolarity as it is able to concentrate GB at much higher level than the ion/metabolite symporter BetL (Sleator *et al.*, 2003a; Wemekamp-Kamphuis *et al.*, 2002b).

1.3.2.2.2 Carnitine

Carnitine (β-hydroxy-γ-[tri-methyloammonio] butyrate) is the second most important osmoprotectant in *L. monocytogenes* after glycine betaine (Mendum & Smith, 2002; Sleator *et al.*, 2001c). The longer carbon chain length of carnitine is thought to decrease its osmoprotective function in comparison to a shorter carbon chain of glycine betaine (Becker *et al.*, 2000). Although it

is not as effective as glycine betaine in ameliorating osmotic stress, the relative abundance of carnitine in mammalian tissue makes it the most readily available and thus possibly the most important osmolyte contributing to the survival of *L. monocytogenes* in foods of animal origin (Sleator *et al.*, 2001c; Sleator *et al.*, 2003a).

L. monocytogenes cannot synthesise carnitine *de novo* and therefore accumulates it from the environment through an ATP-dependent specific transporter OpuC and to some extend the glycine betaine transporter Gbu. Accumulation of carnitine is influenced directly by the osmolarity of the growth medium, and its uptake is subject to a negative regulation by pre-accumulated solute (Fraser *et al.*, 2000).

1.3.2.2.2.1 OpuC transporter

The OpuC transporter encoded by *opu*C operon, belongs to the ATP binding cassette (ABC) substrate binding protein-dependent transporter superfamily and is homologous to the carnitine transporter OpuC in *B. subtilis* (Mendum & Smith, 2002; Sleator *et al.*, 2001c; Sleator *et al.*, 2003a). The *opu*C gene consists of four open reading frames *opuCA*, *opuCB*, *opuCC* and *opuCD* closely positioned to form an operon. ORF1, *opuCA* has close sequence to *opuCA* in *B. subtilis*, and encodes an ATPase subunit (Fig. 1.2). The 3' end of *opuCA* is in a close proximity to the initiation codon of ORF2 separated by only 6bp, including the TAA stop codon. The second ORF, *opuCB*, encodes a membrane pore protein with strong sequence homology to the product of the *opuCB* gene in *B. subtilis* which acts as a permease subunit in the OpuC solute transporter (Angelidis *et al.*, 2002; Fraser *et al.*, 2000). The3' end of *opuCB* is separated from the initiation codon of ORF3, *opuCC*, by 4 bp. OpuCC is a 303-residue protein which acts as an extracellular substrate binding subunit of the OpuC transporter. The 3' end of *opuCC* is separated from the initiation codon of the ORF4 by 17 bp, this final subunit, *opuCD*, encodes a membrane pore protein which acts as the second permease subunit in the OpuC solute transporter in conjunction with OpuCB (Angelidis *et al.*, 2002; Fraser *et al.*, 2000).
The OpuC transporter is highly specific and appears not to participate in accumulation of either betaine or choline compounds which are closely related to carnitine in their chemical structure (Angelidis *et al.*, 2002; Fraser *et al.*, 2000).

1.3.2.2.2.2 Gbu transporter

The major glycine betaine transporter, Gbu has also been identified as an important transporter of carnitine. When carnitine levels are low, the dedicated carnitine OpuC porter functions as a sole

transporter of this compatible solute. As carnitine levels increase, however, the Gbu porter is believed to take over the transport of carnitine as it is, overall, a more energy efficient transporter. This transporter is thought to be the primary means of accumulation of carnitine in an environment abundant with this osmolyte (Angelidis *et al.*, 2002; Fraser *et al.*, 2000).

1.3.2.2.2.3 Potential OpuB transporter

Recent analysis of the *L. monocytogenes* EGD-e genome revealed a possible fourth osmolyte transporter with significant homology to the high affinity choline uptake system OpuB in *B. subtilis* (Wemekamp-Kamphuis *et al.*, 2002a). Consisting of two open reading frames *lmo1421* and *lmo1422*, this operon is located approximately 2.4 kb downstream of *opuC* on the listerial chromosome (Fig. 1.2) with significant sequence similarity to *opuC* (Wemekamp-Kamphuis *et al.*, 2002a). It has been suggested that this operon may encode the low-affinity carnitine uptake system, however this hypothesis remains to be experimentally established.

1.3.2.2.3 Proline

Proline is the third most effective osmolyte in *Listeria monocytogenes,* accumulation of which occurs as a result of proline synthesis from glutamate, as no osmolyte transport systems dedicated to the uptake of proline has yet been described for this organism. Bacterial proline synthesis occurs in a four step reaction catalysed by gamma-glutamyl-kinase (GK) a product of *proB* gene, γ-glutamyl phosphate reductase (GPR) encoded by *pro*A and Δ^1-pyrroline-5-carboxylate (P5C) encoded by *proC* (Fig. 1.2). The remaining, third, step in the sequence is thought to occur spontaneously without a facilitator (Sleator *et al.*, 2001a; Sleator *et al.*, 2001b; Sleator *et al.*, 2003a). The *proBA* operon consists of two open reading frames which originate in the same direction and overlap by 17 nucleotides (Sleator *et al.*, 2001a). The first ORF, *proB*, encodes a 276-residue protein that expresses significant similarity to other proteins in a family of GKs such as the ProB in *E. coli*. The second ORF, *proA*, encodes a 415-residue protein with significant homology to ProA in *Bacillus halodurans* a protein related to GPRs. The tight genetic organization of the overlapping *proB* and *proA* genes suggests that both ORFs constitute an operon, which is distant to *proC*, a gene coding for the P5C enzyme required for the last reaction in the proline biosynthetic pathway. No such gene has been identified in *L. monocytogenes* to date, however analysis of listerial genome revealed two potential *proC* genes, *lmo0396* and *lmo1387*, encoding proteins with similarities to P5CR2 and P5CR3 of *B. subtilis* (Sleator *et al.*, 2003a).

In the absence of effective osmolyte transport, proline accumulation as a function of ProBA has been shown to play a critical role in the growth of *L. monocytogenes* at elevated osmolarities by providing sufficient amount of this compound to act as an effective osmolyte (Bayles & Wilkinson, 2000; Beumer *et al.*, 1994).

1.3.2.2.4 Peptide transport

In a manner similar to that observed for other bacteria, in addition to accumulation of the three compatible solutes mentioned above, *L. monocytogenes* has been shown to increase the internal pools of peptides and free amino acids in hyperosmotic environments. These pools have been shown to consist of peptides containing glycine, hydroxyproline, and proline, such as prolyl-hydroxyproline, prolyl-glycyl-glycine, and prolyl-glycine (Borezee *et al.*, 2000; Verheul *et al.*, 1995; Verheul *et al.*, 1998; Wouters *et al.*, 2005). These intracellular peptides not only serve as a mechanism of maintaining cellular turgor but also as a nutritional supplement for protein synthesis following their breakdown into amino acids by internal peptidases. *L. monocytogenes* possesses two distinct peptide transport systems, allowing internalization of peptides of up to eight residues, a proton motive force-dependent di- and tri-peptide transport system, and an ATP-dependent oligopeptide transport system.

1.3.2.2.4.1 Di- and tri-peptide transport

The di- and tri-peptide transport system in *L. monocytogenes* is encoded by a single gene (*dtpT*). This 1,479 bp gene is thought to encode a single protein of 492 amino acid residues (Wouters *et al.*, 2005). The *dtpT* gene shows 53 and 48% identity to the *yclF* gene of *Bacillus subtilis* and *dtp*T gene of *Lactococcus lactis* respectively (Wouters *et al.*, 2005).

The DtpT polypeptide consists of a bundle of 12 transmembrane α-helices with the N and C termini located internally. This transporter has been shown to have a broad specificity with regard to the nature of amino acids that constitute the peptide (Verheul *et al.*, 1995). Transport of a variety of ti- and tri- peptides is coupled to the proton motive force (PMF), much like the DtpT of *L. lactis*. The DtpT of *L. monocytogenes* has been shown to have a high affinity for proline containing peptides (Verheul *et al.*, 1995), suggesting an involvement of this transporter in osmoadaptation.

1.3.2.2.4.2 Oligopeptide transport

Only one oligopeptide permease operon, the *oppA* has been characterised to date in *L. monocytogenes*. This operon encompasses five genes which display the same genetic organisation and whose products are homologous to those of several *opp* operons previously identified in other bacteria (Borezee *et al.*, 2000; Sleator *et al.*, 2003a). The first gene of the *L. monocytogenes* operon, *oppA,* is separated by a terminator from the downstream genes (*oppB*, *oppC*, *oppD* and *oppF* ; Fig. 1.2), which suggests that although all the *opp* genes are transcribed as a part of an operon, *oppA* is predominantly expressed alone. The encoded 62-kD protein of 558 amino acids, OppA is a lipoprotein anchored to the external part of the cytoplasmic membrane exhibiting substrate binding domain of the oligopeptide transporter protein (Borezee *et al.*, 2000; Sleator *et al.*, 2003a). Both OppB and OppC are predicted to form integral membrane proteins, while the remaining ORFs, encoding OppD and OppF, exhibit high similarity to several ATP-binding proteins. Collectively the Opp permease is an ATP-dependent non-specific oligopeptide transporter capable of translocation of a variety of peptide residues of various sizes.

1.3.2.3 Other osmotolerance related genes

Little information is available concerning other mechanisms that *L. monocytogenes* engages to cope with salt stress, especially when compatible solutes are scarce in the environment. Recently *rel*A was identified as a gene involved in osmotolerance *via* a mechanism different from that involving accumulation of compatible solutes (Gardan *et al.*, 2003a; Okada *et al.*, 2002). Listerial *rel*A gene is 2,214 bp in length and expresses a high homology to *rel*A of *Bacillus subtilis*, which encodes guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), collectively known as (p)ppGpp synthetase (Okada *et al.*, 2002; Sleator *et al.*, 2003a). In many organisms, (p)ppGpp is known as a stress response-related factor and is accumulated in bacterial cells under nutrient limited conditions, such as depletion of amino acids or carbon source limitations. In addition to being a nutritional alarmone in *L. monocytogenes* this protein is also involved in the growth of this organism under high osmotic pressure, regulation of which is yet to be fully elucidated (Gardan *et al.*, 2003a; Okada *et al.*, 2002; Sleator *et al.*, 2003a).

The general stress protein Ctc of *L. monocytogenes* has also been linked to osmotolerance in the absence of any compatible solutes in the environment. This 207-residue protein, a product of the *ctc* gene, is similar to the Ctc protein of *Bacillus subtilis*, which belongs to the L25 family of ribosomal proteins (Gardan *et al.*, 2003b). The function of the Ctc protein of *L. monocytogenes* is unknown,

however there is evidence to suggest that this protein belongs to a novel system utilized by this organism to adapt to osmotic up shift in the absence of other known osmoprotectants.
Two genes, *clp*C and *clp*P, encoding proteins of the Clp (caseinolytic protein) family have also been linked to osmoadaptation in *L. monocytogenes*. These genes are known to encode general stress proteins, ClpC ATPase and ClpP serine protease, which function as chaperones assisting the proper folding, refolding, or assembly of proteins and proteases and processing those that cannot be refolded. Inactivation of these genes confers a general stress sensitivity phenotype, including salt and cold stress sensitivity of the corresponding mutants (Gaillot *et al.*, 2000; Gardan *et al.*, 2003a; Nair *et al.*, 1999; Nair *et al.*, 2000).

The RNA-binding protein Hfq was shown to contribute to the tolerance of osmotic stress in *L. monocytogenes* by affecting its membrane integrity. The protein encoded by *lmo1295* gene (designed *hfq*) consists of 77 amino acids and shows close homology to the *E. coli* Hfq protein (Christiansen *et al.*, 2004). In *L. monocytogenes hfq* is located downstream from *miaA*, which encodes a protein similar to tRNA isopentenylpyrophosphate transferase, and upstream from *lmo1296* gene, which encodes a putative GTP-binding protein HflX. This locus organisation is similar to that in *E. coli* (Christiansen *et al.*, 2004). An increase in transcription of *hfq* in *L. monocytogenes* has been observed following exposure to various stress conditions, including high osmolarity, thus further emphasising the importance for functional Hfq in mediating survival of this organism in unfavourable conditions.

1.3.3 Cryo-tolerance in *L. monocytogenes*

L. monocytogenes is well known for its intrinsic ability to survive and successfully proliferate at refrigeration temperature as low as -0.1°C (Walker *et al.*, 1990). Cryotolerance is a critical aspect of *L. monocytogenes* importance as food-borne pathogen.

Refrigeration temperature is a well accepted means of controlling bacterial contamination of foods as it inhibits growth of most mesophilic food-borne pathogens by severely inhibiting bacterial protein synthesis (Hébraud & Potier, 1999). This, unfortunately, also acts as an effective selective enrichment for *L. monocytogenes*. A detailed understanding of the physiology of *L. monocytogenes* during exposure to refrigeration temperature is vital for designing effective strategies to minimise its proliferation in ready-to-eat foods consequently reducing the number of human listeriosis cases.

Low temperature has profound effects on all aspects of microbial cell structure and function, involving the structural integrity of macromolecules, macromolecular assemblies, protein synthesis and nutrient uptake (Angelidis & Smith, 2003; Zhu *et al.*, 2005). Some of the known mechanisms that *L. monocytogenes* use to adapt to low-temperature conditions include the accumulation of compatible solutes, expression of cold shock proteins and alterations in membrane lipid composition.

1.3.3.1 Accumulation of compatible solutes

Carnitine and glycine betaine are the predominant compatible solutes in *L. monocytogenes* that accumulate post exposure to low-temperature environments. Activation of both the carnitine ATP binding cassette transporter system, OpuC and the glycine betaine Gbu porter has been observed in this organism as a response to low- temperature stress (Angelidis & Smith, 2003; Mendum & Tombras Smith, 2002).

During growth at low temperature the ABC transporter Gbu represents the major pathway for glycine betaine uptake, and a minor route for carnitine uptake, while the OpuC is the dominant porter for carnitine uptake at low temperature (Angelidis *et al.*, 2002; Angelidis & Smith, 2003; Mendum & Tombras Smith, 2002). In general it is thought that glycine betaine is significantly more effective than carnitine in providing cryo-tolerance despite that higher levels of intracellular carnitine have been detected in *L. monocytogenes* at low temperature compared to those of GB (Angelidis *et al.*, 2002; Angelidis & Smith, 2003; Becker *et al.*, 2000; Wemekamp-Kamphuis *et al.*, 2004a). In addition to carnitine and glycine betaine accumulation upon exposure to low-temperature, *L. monocytogenes* has also been shown to activate transcription of *oppA* consequently increasing the up-take of peptides from the environment.

1.3.3.2 Expression of cold shock proteins

Cold shock proteins are typically around 7-kDa in size. Their synthesis increases following temperature downshift. They are essential for bacterial survival and have been identified in nearly all bacteria (Wemekamp-Kamphuis *et al.*, 2002a). A large number of cold-shock proteins (CSPs), have been isolated and studies of *E. coli* and *B. subtilis* have shown them to function as RNA chaperones. They minimize secondary folding and also act as transcription regulators and mediators of message stability, thereby stimulating production of non 7-kDa cold-induced proteins (Angelidis & Smith,

2003; Liu *et al.*, 2002; Wemekamp-Kamphuis *et al.*, 2002a). There are three genes on *L. monocytogenes* chromosome that encode CSPs, *cspB*, *cspD* and *cspL*; induction of which has been observed in bacteria following exposure to low temperature environments. Inactivation of ribosome function following a cold shock is thought to be the main trigger for induction of *csp* genes in this organism (Wemekamp-Kamphuis *et al.*, 2002a).

A number of studies have been conducted to examine the cold shock responses of *L. monocytogenes*, resulting in the identification of a number of other proteins as cold shock induced. One such protein is ferritin (Fri) which is thought to be involved in iron storage, expression of which has been observed to increase significantly during low-temperature exposure (Hébraud & Guzzo, 2000). The expression of Fri is also induced by low temperature in other organisms such as *Anabaena variabilis* and is involved in protection against oxidative stress in *Campylobacter jejuni* (Liu *et al.*, 2002). The specific contribution of Fri to survival of *L. monocytogenes* in a cold environment is still unclear.

1.3.3.3 Expression of cold acclimation proteins

A number of genes and their analogous proteins have been identified in contributing to survival and growth of *L. monocytogenes* at low temperature.

Increased transcription of *fla*A (*lmo0690*) was detected in *L. monocytogenes* grown in a cold temperature environment (10°C), which is believed to encode a major flagellin protein FlaA (Dons *et al.*, 1992; Liu *et al.*, 2002). Increased motility and flagellum production have long been associated with the growth of this organism at low temperature; however its significance is still unclear. It has been suggested that increased motility could be linked to listerial biofilm formation and consequently its survival in food processing environment and enhanced virulence.

A number of genes involved in regulatory response have been linked to *L. monocytogenes* growth in a cold environment; these include *lhkA*, *bglG*, *celD*, *psr* and *rpoN*.

L. monocytogenes cells grown at low temperature also showed an increased transcription of *lhkA* (*lmo1508*) mRNA levels, a gene encoding production of a histidine kinase, which is thought to be an important environmental sensor in a cold adaptation response (Dons *et al.*, 1992).

Increase in transcription of *bgl*G (*lmo0501*) was also linked to cold adaptation in *L. monocytogenes*; this gene encodes a BglG protein (Liu *et al.*, 2002) which functions as a transcriptional anti-terminator acting within the beta-glucoside operon in *E. coli.* While its function in *L. monocytogenes* remains to be determined, based on its position within the EGD-e genome it is likely to also be involved in regulation of expression of carbohydrate metabolism genes (Gorski *et al.*, 2003).

CelD is a protein encoded by *celD* (*lmo1719*). It is homologous to the CelD of *Bacillus stearothermophilus* and is involved in the breakdown of the beta-glucoside polymer cellulose in this organism. Its function in *L. monocytogenes* is unknown although it is thought to be involved in the regulation of gene expression by beta-glucoside availability assisting in regulating the cold adaptive response in this organism (Liu *et al.*, 2002).

The increased expression of a putative peptide methionine sulfoxide reductase regulator, encoded by *psr* (*lmo0443*), following exposure to low temperature (10°C) suggests a role of this protein in a cold adaptive response of *L. monocytogenes*. Listerial Psr is homologous to the Psr repressor protein of *Enterococcus hirae*, MsrR of *Staphylococcus aureus* and CpsA of *Streptococcus pyogene*se and is thought to be involved in a response to oxidative stress adaptation possibly brought about by the increased solubility of oxygen at low temperature (Liu *et al.*, 2002).

L. monocytogenes rpoN (*sigL*) encodes the alternative sigma factor σ^{54}, which is thought to contribute to the regulation of transcription of genes encoding products involved in the utilization of carbohydrates, energy metabolism, RNA modification, chemotaxis, flagellation, electron transport and expression of other alternative sigma factors. The function of this regulon in *L. monocytogenes* cold shock regulation is not known, however its increased transcription during bacterial growth at low temperature suggests a role in a cold acclimation process. It has been speculated that σ^{54} may be involved in regulating the expression of *L. monocytogenes fla*A gene and other genes which have been found to be actively transcribed during cell growth at low temperature (Arous *et al.*, 2004; Liu *et al.*, 2002).

Three genes (*ltrA*, *ltrB* and *ltrC*) collectively known as low-temperature requirement genes have also been reported to be essential for survival of *L. monocytogenes* in cold environments, although their exact role in a cold stress response is unclear (Zheng & Kathariou, 1995).

1.3.3.4 Cold activated general stress response

Protein structure and function is extremely sensitive to alterations in physiological temperature. Consequently bacterial growth at low temperature is characterised with higher proportion of denatured proteins and other macromolecules. In *L. monocytogenes*, for instance, instability in the 70S ribosomal particle is found upon cold shock, which must be overcome to allow normal protein synthesis (Wemekamp-Kamphuis *et al.*, 2002a). Increased expression of general stress proteins functioning as chaperone proteases GroEL, ClpP and ClpB in addition to chaperonins and compatible solutes may be required to increase the proportion of proteins and other catalytic macromolcules in the active, properly folded, conformation (Ratkowsky *et al.*, 2005).

GroEL is a highly conserved protein that functions together with GroES to manage misfolded, damaged, or aggregated proteins as well as to produce proper protein folding (Liu *et al.*, 2002). Increased *groEL* (*lmo2068*) expression at low temperature is, thus, thought to be involved in the maintenance of protein solubility and function in the cytoplasm and is most likely a part of a general stress response to adverse environmental conditions signalled by multiple stimuli.

Clp proteins form large protein complexes that have both proteolytic and chaperone activities and are involved in the degradation of damaged polypeptides and the salvage of amino acids. ClpP is a serine protease that is able to cleave polypeptides of six amino acids or less, forming a more efficient enzyme complex upon association with ClpA or ClpX ATPases. Levels of *clpP* transcription during bacterial growth at low temperature tended to increase, suggesting that the encoded enzyme may be involved in the degradation of abnormal polypeptides or truncated peptides that arise following exposure to low temperature. ClpB mRNA levels increased during *L. monocytogenes* growth at low temperature. Its role is unknown in this organism, however, it is known to play a critical role in cold acclimation in *Synechococcus spp.* (Liu *et al.*, 2002).

L. monocytogenes also showed increased transcription of other genes following exposure to low temperature environment including *eutB* and *mleA*, as well as genes so far unique to *L. monocytogenes lmo0599*, *lmo0170*, *lmo0719* and *lmo1535* which encode hypothetical proteins.

L. monocytogenes eutB is similar to the gene found in *E. coli,* which encodes a heavy chain of ethanolamine ammonia lyase, the first enzyme involved in the degradation of ethanolamine to acetaldehyde and ammonia (Liu *et al.*, 2002). Listerial *mleA* encodes the malolactic enzyme, which is involved in malolactate fermentation in some lactic acid bacteria (Liu *et al.*, 2002). Function of these

two proteins during growth of *L. monocytogenes* in cold environments is unknown, but Liu *et al*. (2002) suggest that induction of these genes is not incompatible with the microaerophilic or facultatively anaerobic life style of *L. monocytogenes* in its natural environment.

Increased levels of transcript *trx*B encoding thioredoxin reductase were evident in cells of *L. monocytogenes* grown at low temperature (Liu *et al.*, 2002). Thioredoxin reductase, along with thioredoxin and NADPH constitutes a thiol-dependent oxidation-reduction system that catalyses the reduction of certain proteins. This system, along with the Fri maintains a reducing environment in the bacterial cytoplasm.

Increased transcription of genes encoding products involved in the biosynthesis of aromatic compounds tryptophan and histidine, *aroA*, *trpG* and *hisJ*, was observed in *L. monocytogenes* cells exposed to low temperature (Liu *et al.*, 2002). Liu et al (2002) suggest that *L. monocytogenes* cells grown in low temperature environment may become starved for certain amino acids, at least partially through feedback inhibition and respond by inducing biosynthetic enzymes for histidine and aromatic amino acids.

1.3.3.5 Re-composition of the cell membrane

Low temperature significantly reduces membrane fluidity and causes membrane phase transition from liquid-crystalline state to more rigid gel-like state. To restore membrane functionality at low temperature, fatty acids with low melting points are incorporated into lipids. *L. monocytogenes increase* membrane fluidity by incorporating unsaturated and branched-chain (*anteiso*) fatty acids, which have lower melting points than the corresponding saturated straight-chain (*iso*) fatty acids, into its lipids (Zhu *et al.*, 2005).

In bacteria, the branched-chain portion of fatty acids derives primarily from branched chain amino acids. Moreover, incorporation of a methyl branch can only be achieved by *de novo* biosynthesis of fatty acids. Two enzymes in the pathway from branched-chain amino acids to branched-chain fatty acids have been well studied in *B. subtilis*; these are the branched chain α-keto acid dehydrogenase (Bkd) and β-ketoacyl-acyl carrier protein synthase III (FabH) (Zhu *et al.*, 2005). Amino acids are initially transaminated by branched-chain amino acid transaminase, and are subsequently oxidatively decarboxylated by the Bkd complex, resulting in formation of acyl-CoA derivatives which are then utilised by FabH as starting units to initiate branched-chain fatty acid biosynthesis.

A Bkd protein has also been established as essential for growth of *L. monocytogenes* at low temperature. In *L. monocytogenes*, Bkd is a multi-enzyme complex encoded by the *bkd* cluster consisting of six genes, *ptb*, *buk*, *lpd*, *bkd*A1, *bkd*A2 and *bkd*B organised in an operon and subsequently co-transcribed. This organisation is similar to that described for *Pseudomonas putida*, *B. subtilis* and *E. faecalis* (Zhu *et al.*, 2005).

In *B. subtilis* modification of membrane fatty acid composition as a response to environmental stimuli is aided by a FabH, a transcript of the *fab*H gene (Choi *et al.*, 2000). A homologue of *fabH* encoded by *lmo2202,* in addition to two similar genes *fab*D and *fab*G , has been identified in *L. monocytogenes*, however the exact role of these in fatty acid biosynthesis is unclear. They are likely to play a similar role in fatty acid biosynthesis thus assisting cryotolerance response of this organism.

Recently a single gene, *lmo1078* that encodes a putative UDP-glucose phosphorylase catalysing the formation of a nucleotide sugar UDP-glucose, an important substrate of bacterial cell envelope structures, has been linked to both cold and osmotic stress tolerance (Chassaing & Auvray, 2007). They found that mutants lacking this gene were less tolerant of osmotic stress and were unable to proliferate at 4°C.

1.3.4 Transcriptional regulation of genes involved in stress-tolerance

Bacteria require the ability to respond rapidly to changes in the environment: these responses are frequently regulated at the transcriptional level. Transcription of DNA into mRNA in bacteria is catalysed by RNA polymerase (RNAP), which consists of a core enzyme and a sigma factor. This sigma factor component of RNAP is required for recognition and binding of the polymerase to specific promoter sequence upstream of a gene (Sue *et al.*, 2003). Global changes in transcription are therefore coordinated by levels of specific sigma factors and activities of which fluctuate in response to environmental conditions. Five sigma factor-encoding genes have been identified in the genome of *L. monocytogenes* EGD-e. These genes are *rpoD* encoding SigA (σ^A); *sigH* encoding σ^H; *rpoN* encoding σ^{54} (SigL); *sigC* (*lmo*0423) and *sigB* (Glaser *et al.*, 2001).

The alternative sigma factors σ^A, σ^C, σ^H and σ^L, have been studied less extensively than σ^B in *L. monocytogenes* and their function in stress tolerance of this organism is poorly understood.

Direct involvement of σ^A to stress tolerance in *L. monocytogenes* is not clear, although a number of stress related genes are known to be at least partly under transcriptional control of this sigma factor, including *betL* (Cetin *et al.*, 2004) and *fri* (Olsen *et al.*, 2005).

Lineage II-specific σ^C regulates transcription of genes involved in extracytoplasmic function (Helmann, 2002; Kazmierczak *et al.*, 2005), induction of which has been observed during exposure of *L. monocytogenes* to temperature up-shift (Zhang *et al.*, 2005). The specific function of σ^H in *L. monocytogenes* is unknown, however it has been shown to contribute to growth of this organism in alkaline conditions and minimal media (Rea *et al.*, 2004). In *L. monocytogenes* σ^L regulates genes involved in carbon and amino acid metabolism (Arous *et al.*, 2004). Transcription of *sigL* (*rpoN*) appears to be induced in cells grown at low temperature (10°C) as compared to those cultivated at 37°C (Liu *et al.*, 2002).

Transcriptional regulation of genes involved in stress-tolerance is a complex network, the inter-relationships of which are only just beginning to be revealed. Only σ^B has been extensively studied in *L. monocytogenes*.

1.3.4.1 Stress inducible alternative sigma-factor B (SigB)

In a number of Gram positive genera, the stress inducible sigma factor σ^B plays a central role in regulating the transcription of genes required for protection against environmental stress such as high osmolarity and low-temperature, oxidative stress and carbon starvation (Chan *et al.*, 2007a; Wemekamp-Kamphuis *et al.*, 2004b). The *sigB* gene of *L. monocytogenes* lies seventh in the σ^B operon, which includes σ^B regulatory proteins *rsbR*, *rsbS*, *rsbT*, *rebU*, *rsbV*, *rsbW*, and *rsbX* (Chaturongakul & Boor, 2004; Ferreira *et al.*, 2004; Wemekamp-Kamphuis *et al.*, 2004b). The *SigB* operon of *L. monocytogenes* is homologous to that of *Bacillus subtilis*. Regulation of σ^B by the Rsb proteins is achieved through a complex cascade in response to various cellular stimuli. Under exponential growth conditions, RsbW, an anti-σ factor forms a complex with σ^B preventing the association of the later with RNAP. RsbV is inactive as an anti-anti- σ factor when it has been phosphorylated on a conserved serine residue by the kinase activity of RsbW (Chaturongakul & Boor, 2004; Ferreira *et al.*, 2004; Wemekamp-Kamphuis *et al.*, 2004b). The affinity of RsbW for its antagonists, σ^B and RsbV, can change depending on environmental factors. RsbU dephosphorylates RsbV upon exposure to an environmental insult. RsbV is then able to bind to RsbW, thus freeing σ^B, which then becomes available to bind RNAP and initiate transcription of a required gene.

Transcription of the listerial *sigB* has been found to be strongly dependent on the osmolarity of the medium, with induced transcription detected under conditions of hyperosmotic stress. Osmotic up shift was found to be one of the most potent stimulators of σ^B activity, further emphasising its involvement in osmoregulation (Cetin *et al.*, 2004; Wemekamp-Kamphuis *et al.*, 2004b). Involvement of σ^B in transcription regulation of cold stress genes is less direct, as both σ^B–dependent and σ^B – independent regulation mechanisms have been shown to contribute to *L. monocytogenes* survival and proliferation at low temperatures (Chan *et al.*, 2007a; Moorhead & Dykes, 2004). Alternative sigma factor σ^B has been reported to be at least partly involved in transcriptional regulation of compatible solute accumulation. Sequence analysis of the DNA sequences upstream from the translation initiation codons of *betL* and *opuCA*, opu*B* , *proBA* and *lmo*1421 revealed the presence of σ^B promoter motifs (Fraser *et al.*, 2003; Kazmierczak *et al.*, 2003; Sleator *et al.*, 2001a; Sue *et al.*, 2003) suggesting an involvement of the alternative sigma factor in regulation of these genes. Regulation of the *gbuA* operon is not very clear as it is thought to be transcribed from dual promoters, a σ^B–independent $gbuAP_1$ and a second promoter $gbuAP_2$ which was found to be σ^B-dependent, but slightly different in structure to the *opuC* promoter (Cetin *et al.*, 2004). The di- and tripeptides transporter encoding gene, *dtp*T is also positively up-regulated by σ^B (Hain *et al.*, 2008).

In a recent study, DNA micoarray analysis of *L. monocytogenes* 10403S revealed 168 genes positively regulated by σ^B under environmental stress conditions (Raengpradub *et al.*, 2008). Some of the genes positively regulated by σ^B included *fri* (iron binding protein), *ctc* (general stress response), *ltrC* (cold stress response), *gadA* (glutamate decarboxylase), *hfq* (RNA-binding protein), and *clpC* (general stress protein) among many others (Raengpradub *et al.*, 2008). A large number of genes involved in carbohydrate biosynthesis and uptake have also been shown to be σ^B-dependent (Hain *et al.*, 2008).

The alternative sigma factor σ^B plays a central role in regulating the transcription of *L. monocytogenes* stress response genes, either by directly controlling transcription of certain genes or through a network with other transcriptional regulators. A direct network between σ^B and the two negative transcription regulators HrcA (Hu *et al.*, 2007a) and CtsR (Hu *et al.*, 2007b) has been established in *L. monocytogenes*, an interaction which is required for appropriate expression of stress response genes in this organism under different environmental conditions. HrcA-regulated genes include the *groESL* operon, a number of ribosomal proteins and proteins that contribute to DNA maintenance, transcription and translation, while CtsR directly suppresses *clp* genes (*clpP*, *clpE*, *clpB* and *clpC*). Regulation of a stress response in *L. monocytogenes* appears to be a complex multi-factorial network which remains to be fully comprehended.

Thesis Objectives

The ability of *Listeria monocytogenes* to adapt to various food and food- processing environments has been attributed to its robustness, persistence and prevalence in the food supply chain. The overall aim of the thesis is to improve the present understanding of molecular mechanisms involved in hyperosmotic and low-temperature stress adaptation of *L. monocytogenes*, facets important for survival of the species within the environment in which it dwells and that contribute to it persisting in the food supply chain.

The objectives of this study were to:

1. **Increase understanding of the distribution of tolerance to high salinity and low temperature of a large, genetically diverse, set of *L. monocytogenes* strains.**

Tolerance to high salinity-induced hyperosmotic stress and low-temperature conditions, defined on the basis of growth rate data, were investigated in a large number of *L. monocytogenes* of different genetic groups and obtained from several different sources. This survey was undertaken to determine whether genotype or source correlate to different tolerance capacities (Chapter 2).

2. **Evaluate changes in gene expression of multiple strains of *L. monocytogenes* that have quantitative differences in tolerance, following adaptation to hyperosmotic stress.**

Analysis of whole-genome expression between strains with different tolerance capacities to salt – induced hyperosmotic stress was conducted to aid in understanding of hyperosmotic adaptation in this organism. Hyperosmotic stress was investigated in terms of tolerance to salinity (NaCl) owing to its environmental significance and tendency to define microbial communities. Both osmoadaptive (Chapter 3) and osmotic shock (Chapter 3) responses to high salinity were investigated.

3. **Evaluate changes in gene expression of multiple strains of *L. monocytogenes* adapted to cold-temperature (4°C).**

The ability to proliferate at refrigeration temperature is an important attribute of *L. monocytogenes*, and the exact mechanisms allowing it to adapt to low-temperature environments is not fully understood. Changes in gene expression of multiple cryo-adapted strains with distinct growth characteristics at 4°C were examined (Chapter 5).

4 Evaluate the effect of hyperosmotic and cold stress on motility in *L. monocytogenes*.

Peritrichous flagella provide the ability of *L. monocytogenes* to mobilise, seek nutrients and has important roles in attachment process. The effects of high salinity induced hyperosmotic stress and low temperature on motility were investigated for a diverse range of strains using metabolic stain indicator systems. This assessment was used to determine the variation between genetically diverse strains and to correlate a phenotypic property with the gene expression datasets (Chapter 6).

5 Evaluate common pathways of adaptation to hyperosmotic and cold stresses in *L. monocytogenes*.

Exposure to stress in this organism has been shown to provide cross-protection against other stress stimuli, suggesting a common pathway of stress-adaptation. To aid in understanding of stress tolerance in this organism the molecular adaptive responses to hyperosmotic and cold stress were compared (Chapter 7). Better understanding of stress adaptation may potentially provide information useful for preventing proliferation of this organism if food and food processing environments.

Chapter 2

Cold and salt stress resistance variation in culture broth among Listeria monocytogenes strains of various serotypes and origins.

Listeria monocytogenes prevalence in food-processing, distribution and storage environments has long been established. Serotype 1/2a is most frequently isolated from food-related environments, although the majority of reported human listeriosis cases are generally most linked to serotype 4b and 1/2b. The reasons for this ecological distribution of genetic groups is still unclear but could be potentially linked to stress tolerance variations. Tolerance to hyperosmotic and cold temperature stresses was evaluated in 152 strains of diverse origin. Tolerance (i.e. ability to grow) to 12.5% (w/v) NaCl concentration was observed in 60.5% of strains, with an average generation time of 8.0±2.5 h, ranging from 5.3 to 17.9 h, at 25°C. Strains of serotype 4b appeared to have shorter average generation times of 6.6±0.25 h at 12.5% (w/v) NaCl, whereas serotype 4a had the shortest average generation time at 4°C (19.4±1.0 h). There was no significant correlation between growth at 12.5% (w/v) NaCl and growth at 4°C for most genetic groups though a weak correlation (r^2= 0.63) was observed for serotype 1/2a strains. However, strains isolated from animals, on average, had shorter generation times when exposed to either 12.5% (w/v) NaCl or 4°C than isolates from other sources. This suggests salinity and low temperature stress do not influence the broad distributions of *L. monocytogenes* lineages but could influence the genetic diversity of strains present in environmental samples that may be mobilised to later stages of food supply chain.

2.1 Introduction

Listeria monocytogenes prevalence in food-processing, distribution and storage environments has long been established. The intrinsic stress adaptation capabilities of this organism make contamination control in food manufacturing environments a great challenge. It is widely presumed that food products are contaminated by *L. monocytogenes* that occasionally pass from food processing environments, including work-contact surfaces, equipment, aerosols and dust, and water. Among strains recovered from foods or food processing plants, serotype 1/2a are over represented (Nelson *et al.*, 2004) and are also among the three most common serotypes associated with human listeriosis. Although there is insufficient evidence to characterise health risks as strain specific,

because all strains of *L. monocytogenes* are considered pathogenic, there are strong indications of virulence heterogeneity among serotypes of this organism (FAO/WHO, 2004). As noted in Chapter 1, the majority of reported human cases (>90%) are associated with serotype 4b and 1/2b, and less frequently with serotype 1/2a (Doumith *et al.*, 2004; Wiedmann, 2002). *L. monocytogenes* lineage-specific characteristics have also confirmed this trend. It appears that lineage I strains are significantly over-represented among human clinical listeriosis cases, compared to their prevalence among animal listeriosis cases, and contaminated foods (Nightingale *et al.*, 2005).

One of the most important strategies to reduce the incidence of food-borne listeriosis is the prevention of growth of *L. monocytogenes* to high numbers in contaminated foods (FAO/WHO, 2004; ILSI, 2005). It is therefore important to understand how this organism is able to adapt its cellular physiology and efficiently overcome various food and food- processing related stresses, and to resist current control measures. Such information may be relevant for developing better ways of preventing contamination and minimising growth in food.

Strain selection is an important consideration in the design and execution of experimental procedures to determine the behaviour of pathogenic bacteria in food products or in systems simulating food-related environments. To select strains with unique stress tolerance characteristics to determine the mechanisms of this behaviour, it is essential to establish the common stress response of a given bacterial population. The current study was designed to investigate the growth potential of *L. monocytogenes* at elevated sodium chloride levels and refrigeration temperature of 4°C, and to select strains with unique stress tolerance for consequent microarray analysis.

2.2 Methods

2.2.1 Bacterial strains

A total of 152 strains of diverse origin were use in this study (Table C.1, Appendix C). The collection contained 28 isolates of human origin, 30 isolates from food and food processing environments and 84 isolates of animal origin. This group consisted of 39 serotype 1/2a, 8 serotype 1/2b, 9 serotype 1/2c, and 29 serotype 4b strains as well as a number of uncharacterised strains. Strains were stored at -80°C in brain heart infusion broth (BHIB) containing 15.0% (v/v) sterile glycerol.

2.2.2 Growth study

2.2.2.1 Sodium chloride stress

L. monocytogenes strains were routinely cultivated on Brain Heart Infusion Agar (BHIA, Oxoid) at 25°C for 24 h. One colony from each strain was inoculated into 10 ml of BHI broth (BHIB, Oxoid) in duplicate and incubated for 24 h at 25°C. Ten microlitres of this culture were then aseptically transferred into L-shaped spectrophotometer tubes ('L-tubes') containing 10ml BHIB supplemented with 12.5% (w/v) NaCl (2.14M NaCl). Cultures were placed in a shaking incubator (model TN3; Advantec, Toyi Roshi international) in a constant temperature room (25 ±1°C) and absorbance was monitored every 1 hr at 600nm (Spectronic 20, Milton Roy Co) against a sterile BHIB blank until stationary phase was reached. Each L-tube inoculum was checked for purity by visual examination of the BHIA purity plate following incubation at 25°C for 48 h after the cessation of each set of growth experiments. Absorbance data obtained were analysed using a modified logistic model (Zwietering *et al.*, 1990) within the program LISREL software (Scientific Software International, SSI) to solve for μmax (h^{-1}) and maximum cell density and determine root mean square deviation. The modified logistic equation used is:

$$y = \frac{D}{1 + \exp\left((4\mu_{max}/D)(\lambda - t + 2)\right)}$$

Whereby, *y*=ln (*cell concentration* at t/cell concentration at t=0),

Equation parameters included:

D=limit of In Abs/Abs (t=0)
λ= lag phase duration (hours)
μ_{max} = maximum growth rate (h^{-1})
t = time (hours)

2.2.2.2 Growth at 4°C

L. monocytogenes strains were routinely cultivated on BHIA at 25°C for 24 h. One colony from each strain was inoculated into 10 ml of BHIB and incubated for 24 h at 25°C. The cultures were diluted in a fresh BHIB to a cell density of approximately 10^4 CFU/ml in quadruplicate. Microtiter trays (96 well, Eppendorf, Soth Pacific Pty.Ltd) were then inoculated with 200 µL of this suspension. Trays were sealed with sterile PCR adhesive film (Abgene) and incubated at 4±1°C, with temperature being monitored with a data logger. Change in absorbance was monitored using a BioRad Benchmark microplate reader at 540 nm until stationary phase was reached. Each well was plated onto BHIA plate and incubated at 25°C for 48 h for visual examination of inoculums' purity following cessation of the growth experiment. Growth curves, following a log conversion of the OD data, were

generated using the curve-fitting (Baranyi & Roberts, 1994) DMFit software package (Institute of Food Research, IFR, UK). Generation time (GT) was established from the output data using the following calculation:

$$GT = \ln(2)/Mu$$

Mu=maximum specific growth rate (=dln(abs)/dt)
t=time (h)

2.2.2.3 Data analysis

2.2.2.3.1 Statistical analysis:

Estimated generation times were further analysed by analysis of variance (ANOVA) (Excel SPC Software for Excel®). Statistical correlation between the two stresses was evaluated using the Pearson Correlation calculated using Excel SPC Software for Microsoft Excel®.

2.2.2.3.2 Bootstrap analysis

Experimental generation times for both stresses were re-sampled randomly with replacement using bootstrap technique (Efron & Tibshirani, 1991). Bootstrap data sets of GTs were generated separately for each serotypes 1/2a, 4a, 4b, 1/2b, 1/2c, for each of the 3 lineages and origin of isolates in a multistep procedure described below.

A single GT bootstrap value was generated randomly from the original experimental data sample, defined as an array for each of the above mentioned sub-groups, in Microsoft®Excel using the following algorithm:

INDEX(*group*,ROWS(*sample*)*RAND()+1,COLUMNS(*group*)*RAND()+1)
group= array of original experimental observations for each group $x=(x_1,x_2...,x_{n)}$.

A mean GT value was then generated from 10 individual GT bootstrap values summarised by the following equation:

$$\bar{x_b} = \frac{\sum x_b}{b}$$

x_b = GT bootstrap samples (x_1,x_2...x_n)
b = number of GR bootstrap values= 10

This was performed in Microsoft®Excel using function 'AVERAGE' and repeated for a total of 5000 average values.

A new data set consisting of 5000 mean GT replicates was thus generated for each sub-group of the experimental population for each of the two environmental stresses.

2.2.2.3.3 Population analysis

Bootstrapped population analysis was evaluated by constructing histograms based on percentage frequency of a mean GT within the 5000 bootstrap replicates.

Confidence level was determined using the following formula:

$$CL=100(1-2\alpha)$$

α = 0.05

The confidence interval (CI) of the bootstrap statistics with unknown distribution was evaluated using the following algorithm:

$$CI = \left[\left(N(1-\alpha)\right)^t, (N\alpha)^t\right]$$

α = 0.05

This was performed in Microsoft®Excel using the function 'SMALL', which selects the lowest value in the array.

Population distribution information based on serotype, lineage and origin of isolates for both stresses is presented in Appendix C.

2.3 Results

2.3.1 12.5% Sodium chloride generation time analysis

A total of 152 *L. monocytogenes* isolates of diverse origin were screened for growth at near growth preventing NaCl concentrations. The selected 2.14M (12.5% (w/v)) NaCl concentration permitted growth of 60.5% of the strains screened in this study. The 92 strains that were able to grow at this NaCl concentration had an average generation time of 8.0 ± 2.5 h (Table C.1, Appendix C), ranging from 5.3 h (strain FW03/0035) to 17.9 h (strain Δ*oppA,* deletion mutant for the *oppA* gene in strain LO28).

To better understand the demographics of this particular collection of *L. monocytogenes* strains, growth data were analysed by examining a variation in generation times, with respect to the assigned lineage, strain and origin of the isolates. In addition to analysing the experimental observations, original data samples were also re-sampled using a bootstrap procedure and the bootstrap results also analysed for statistical significance of differences between strains grouped as described.

2.3.1.1 Generation time analysis of L. monocytogenes isolates at 12.5% (w/v) NaCl based on lineage of isolates.

L. monocytogenes strains from lineage II on average appeared to have the slowest generation times (μ of 8.8 ± 2.4 (SD=2.4 h) for observed data and ±0.76 (SD=0.76 h) for bootstrapped data) when compared to the other two lineage groups (Fig. 2.1).

There was a statistically significant difference in the average generation time values between the three lineage groups examined in this study ($P<0.05$). *L. monocytogenes* strains from LI and LIII groups had faster GTs with a mean value of 7.5 ± 1.7 and 6.9 ± 0.8 h respectively (Fig. 2.1). The average generation times of strains from these two lineages showed no significant statistical difference based on experimental observations ($P>0.05$), suggesting these strains comprise isolates from the same pool of experimental strains.

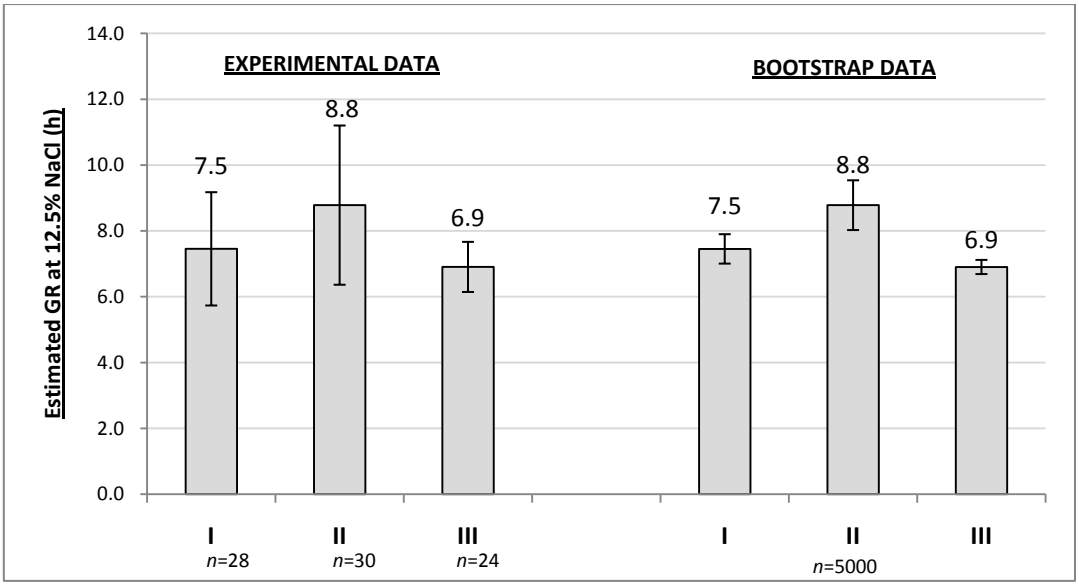


Figure 2.1 Average generation time values at 12.5% (w/v) NaCl for *L. monocytogenes* isolates grouped into three lineages. Error bars indicate standard deviations.

The bootstrap technique allowed for a significant decrease in the estimated standard deviation within grouped observations (Fig. 2.1); thus allowing for more specific variance estimation between the grouped data. Bootstrapped data analysis produced a statistically significant difference between the mean values of LI and LIII groups at as little replication as 50 bootstrap samples ($P<0.001$), suggesting that the two groups originate from two independent pools of *L. monocytogenes* population.

The generated histogram of GT population demographics for each lineage groups further revealed a clear difference between the three lineage groups. The 90% confidence intervals (CI) of GTs for LI salt tolerant isolates were determined to be 6.7 and 8.2 h (Appendix C, Fig. C.1); LII had 90% CI of 7.6 to 10.1 h (Appendix C, Fig. C.2); and LIII had 90% CI of 6.6 to 7.3 h (Appendix C, Fig. C.3).

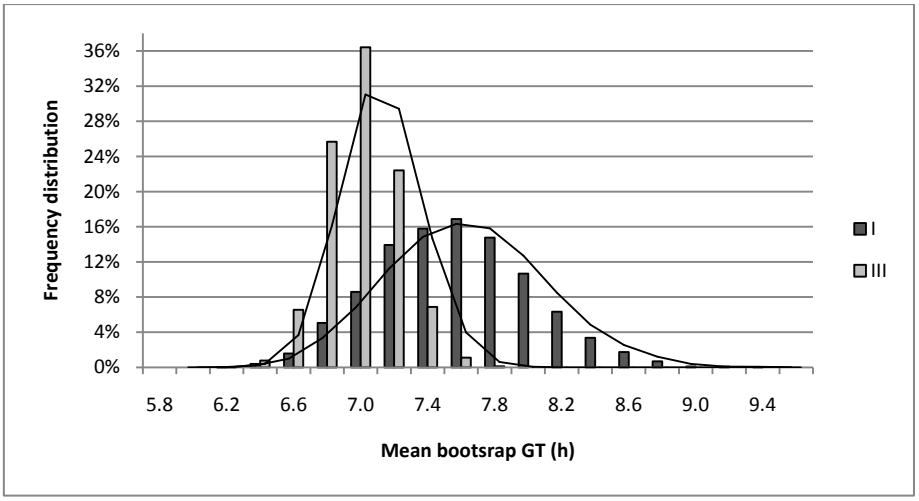


Figure 2.2 Histogram overlaying GT population distributions derived from bootstrap analyses of *L. monocytogenes* lineage I and III salt tolerant isolates at 12.5% (w/v) NaCl

Population analysis showed LI and LIII isolates forming closely overlapping, but clearly separate populations with LI cluster forming a much broader histogram than the less widely spread LIII strains which had a higher frequency of shorter average GTs (Fig. 2.2).

2.3.1.2 Generation time analysis at 12.5% (w/v) NaCl based on serotype of isolates.

Average GT distributions based on serotype of isolates in this *L. monocytogenes* collection showed significant variation. Serotypes 4e, 3a and 4c were very poorly represented in this population with 3, 1 and 1 strains respectively, and were therefore removed from subsequent statistical analysis.

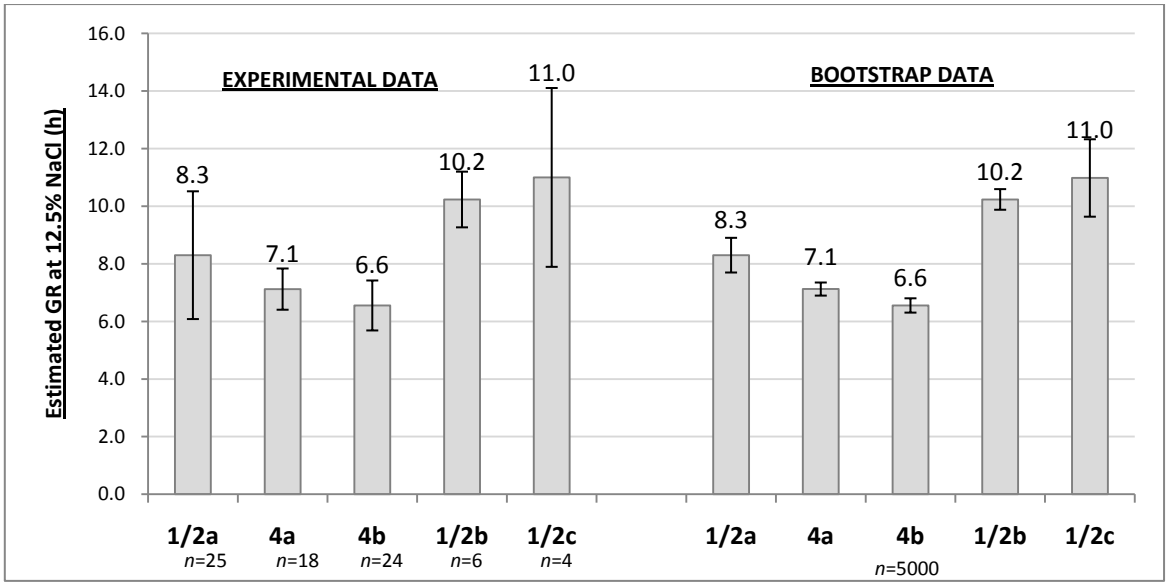


Figure 2.3 Average generation time values for *L. monocytogenes* isolates at 12.5% (w/v) NaCl grouped on the basis of assigned serotypes. Error bars indicate standard deviations.

Isolates of serotypes 1/2b and 1/2c were under-represented within the collection (eight and nine isolates each) and consequently within the salt tolerant population with six and four strains respectively. They showed the slowest average GTs of the serotype groups examined (Fig. 2.3). These two sub-groups appeared to form overlapping but significantly distinct populations, with 90% confidence intervals of 9.6-10.8 h for serotype 1/2b (Appendix C, Fig. C.4) and 8.7-13.2 h for serotype 1/2c (Appendix C, Fig. C.5).

The majority of strains able to proliferate at relatively high salt concentration (i.e., 2.14M NaCl) were of serotype 1/2a, 4b and 4a, with 15, 24 and 18 strains respectively. These groups showed statistically significant variation in average GTs from the original observations ($P<0.05$), and this was also the case for bootstrapped data. Overall, strains of serotype 1/2a appeared to have slower generation times of 8.3 ± 2.2 h for the original observations and 8.3 ± 0.6 for the bootstrapped data, when compared to serotypes 4a and 4b (Fig. 2.3). This serotype group of isolates had a 90% CI from 7.4 to 9.3 h (Appendix C, Fig. C.6).

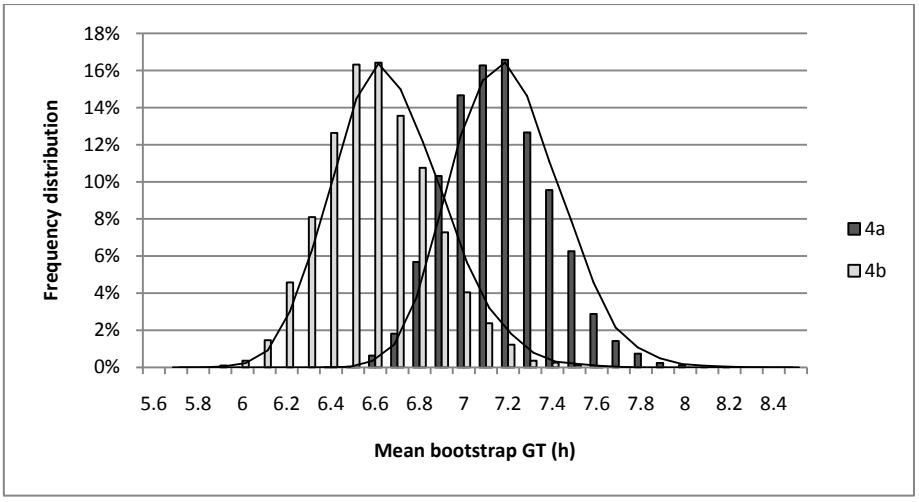


Figure 2.4 Histograms and overlaying of GT distributions of bootstrapped data for *L. monocytogenes* serotypes 4a and 4b salt tolerant isolates.

Serotype 4a and 4b strains formed statistically significant separate clusters ($P<0.05$). Strains of serotype 4b appeared to have marginally faster generation times (6.6 ± 0.87 h for experimental observations and 6.6 ± 0.25 h for bootstrapped data), than serotype 4a strains (average GT of 7.1 ± 0.72 h and 7.1 ± 0.23 h bootstrapped) (Fig. 2.3). Isolates within this sub-group formed a cluster with the 90% CI of 6.8 and 7.5 h (Appendix C, Fig. C.7), while serotype 4b isolates had 90% CI of 6.2 to 7.0 h (Appendix C, Fig. C.8). These two groups formed overlapping but clearly significantly different populations with the majority of 4b isolates exhibiting shorter generation times at 12.5% (w/v) NaCl (Fig. 2.4).

2.3.1.3 Generation time analysis at 12.5% (w/v) NaCl based on origin of isolates.

The majority of the salt tolerant isolates (70%) appeared to have originated from animal sources. This group displayed the shortest average GT of 7.7 ± 2.2 h and 7.7 ± 0.56 h bootstrapped (Fig. 2.5).

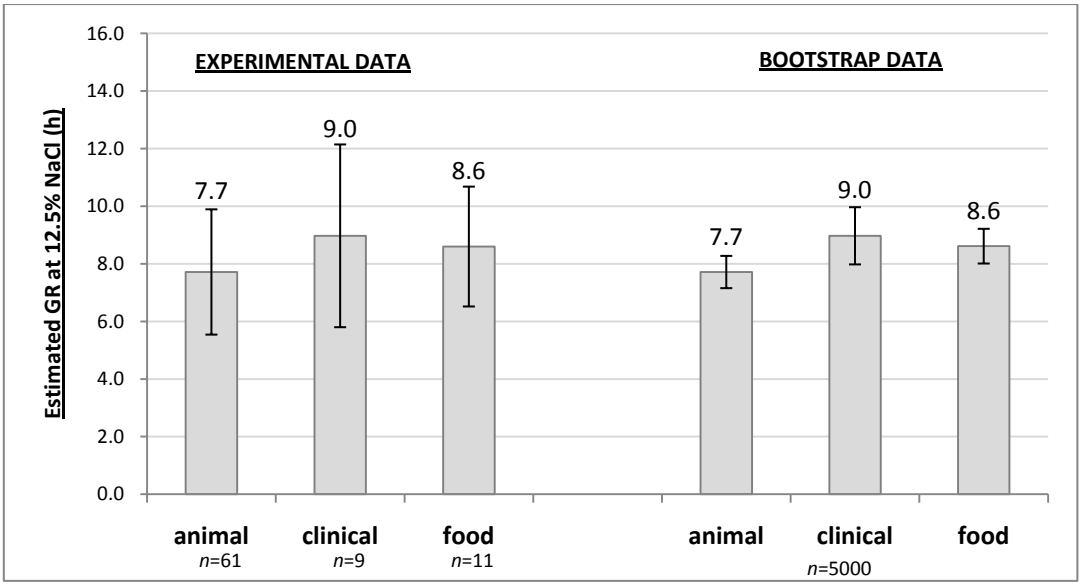


Figure 2.5 Average generation time values for *L. monocytogenes* strains at 12.5% (w/v) NaCl grouped on the basis of origin of isolates. Error bars indicate standard deviations.

Animal isolates formed a distinct cluster with 90% CI of 6.9 to 8.7 h (Appendix C, Fig. C.9) which showed strong statistical difference, from other isolate sources ($P<0.01$) with as little as 50 bootstrap replicates.

The food isolates subgroup GT estimates were characterised by a 90% CI from 7.6 to 9.5 h (Appendix C, Fig. C.10). Clinical isolate GTs showed a 90% CI from 7.4 to 10.7 h (Appendix C, Fig. C.11).

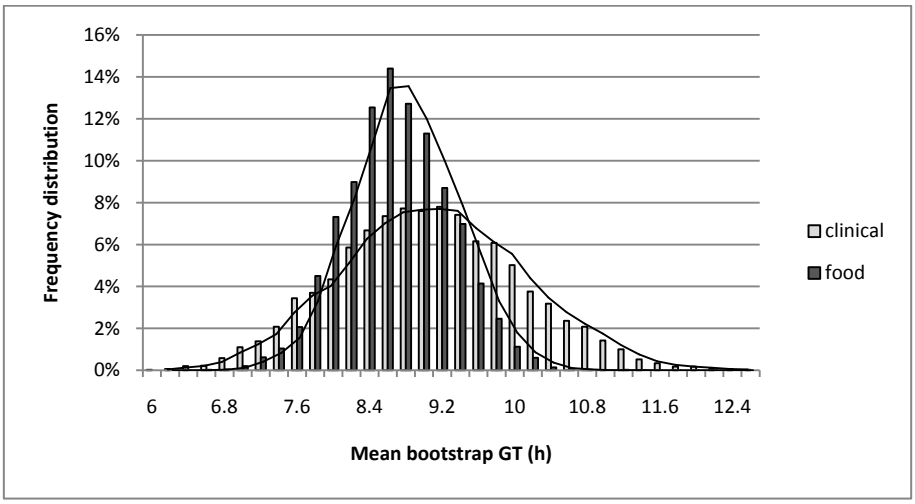


Figure 2.6 Histogram of overlaid generation time distributions of *L. monocytogenes* salt tolerant clinical and food isolates at 12.5% (w/v) NaCl.

Salt tolerant isolates from food and clinical sources appeared to originate from an homogenous *L. monocytogenes* population (P>0.05) even though the clinical isolates, upon first examination, appear to have longer average generation times at 12.5% (w/v) NaCl (Fig. 2.6).

2.3.2 Generation time analysis at 4°C

Generation times of *L. monocytogenes* isolates at refrigeration temperature(4 ±1)°C varied significantly from as short as 9.9 ± 0.6 h for strain 76-1854, to 60.5 ± 5.2 h for strain LO28 ∆*oppA* , with a mean GT of 22.4 ± 7.4 h (Appendix C, Table C.2).

2.3.2.1 Generation time analysis at 4°C based on lineage of isolates.

Observed experimental GTs for isolates of different lineages showed no significant statistical difference between the three groups, with average GTs of 21.3 ± 6.2 h for LI, 20.9 ± 5.7 h for LII and 21.2 ± 6.9 h LIII (Fig. 2.7).

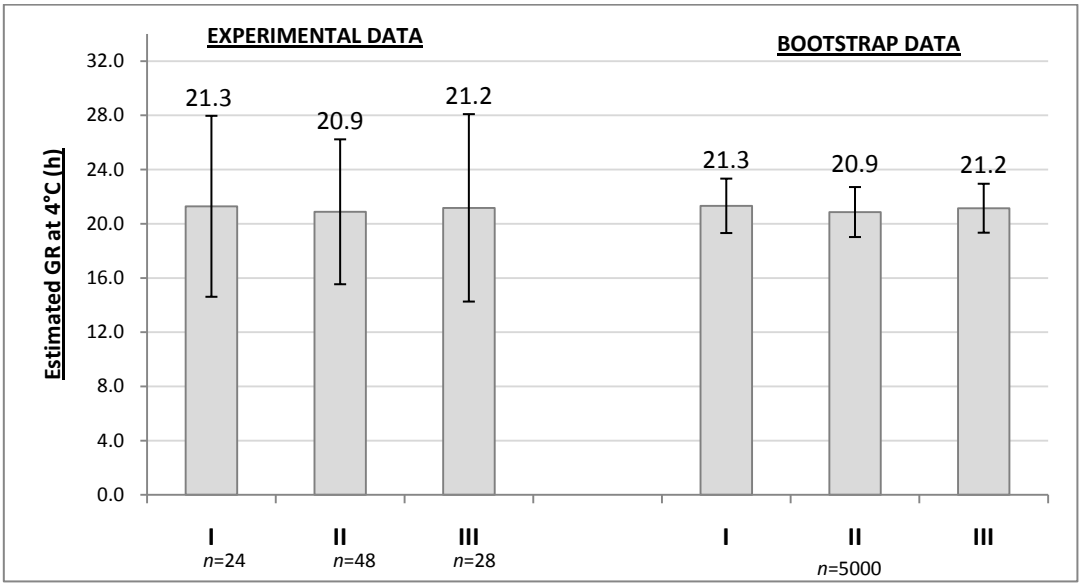


Figure 2.7 Average generation time values for *L. monocytogenes* isolates at 4°C grouped on the basis of assigned lineages. Error bars indicate standard deviations.

Assessment of sub-group population statistics clearly showed homogenous *L. monocytogenes* populations with LI, LII and LIII producing very similar GT spread with 90% CI of 18.2-24.8 h, 17.9-24.0 hand 18.4-24.3 h respectively (Appendix C, Fig. C.12, Fig. C.13 and Fig. C.14 respectively).

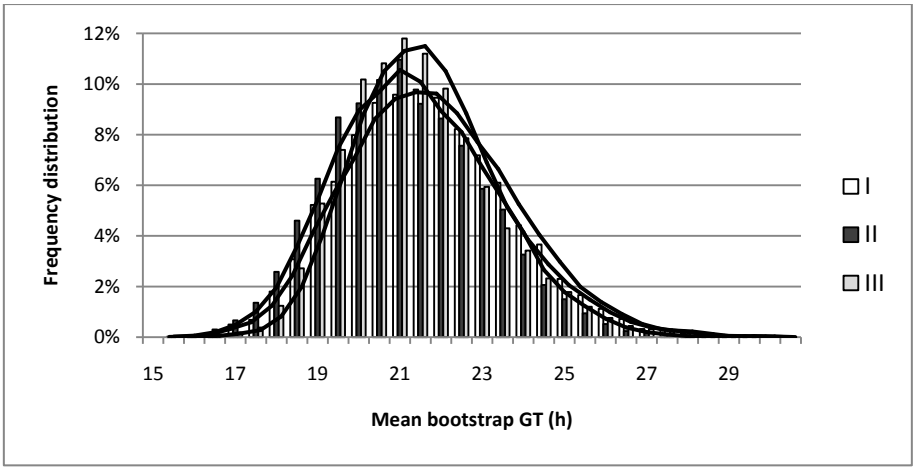


Figure 2.8 Histogram of the bootstrapped GT distributions of *L. monocytogenes* lineage I, II and III isolates at 4°C.

The histogram of the mean-spread within the lineage groups (Fig. 2.8) further confirmed the homology of GT at 4°C among the three lineage groups of *L. monocytogenes* isolates. Although not identical the GT population distributions in Fig. 2.8 appeared to be very similar. It is noted that all distributions are asymmetric with longer right hand tails.

2.3.2.2 Generation time analysis at 4°C based on serotype of isolates

Evaluation of experimental GTs showed no significant difference between serotype groups (P>0.05), suggesting that isolate serotype has no influence on GTs of *L. monocytogenes* at 4°C.Reducing the standard deviation of observed GTs by re-sampling, however, revealed a statistically significant variance between the average GT values of serotype groups.

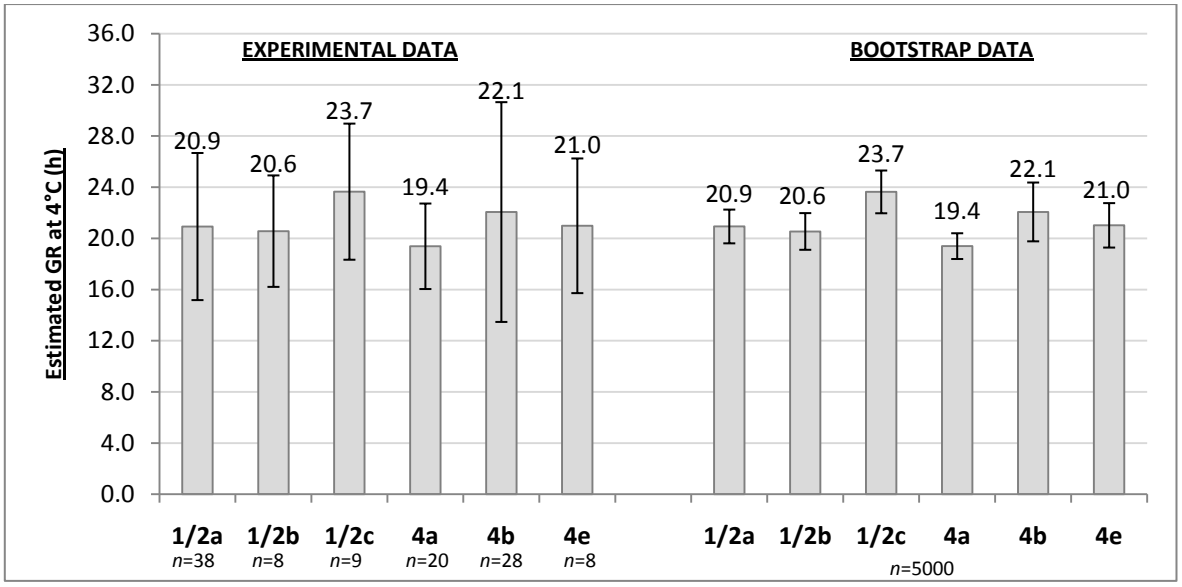


Figure 2.9 Average generation time values for *L. monocytogenes* isolates at 4°C grouped on the basis of assigned serotypes. Error bars indicate standard deviations.

Serotype 4a appeared to have on average slightly shorter bootstrapped GTs 19.4 ± 1.0 h (Fig. 2.9, bootstrapped data) which are statistically distinct from either 1/2a or 1/2b serotypes ($P<0.01$) at bootstrap replication of 50 samples. Bootstrapped population analysis showed the 90% CI to be from 17.6 to 21.1 h for serotype 4a isolates (Appendix C, Fig. C.15).

Serotype 1/2a and 1/2b isolates showed no significant generation time difference at 4°C (bootstrapped n=100). These appeared to originate from an homogenous population with 90% CI of 18.8 and 23.2 h for serotype 1/2a and 18.4 and 23.0 h for serotype 1/2b (Appendix C, Fig. C.16 and Fig. C.17 respectively). It appeared that isolates from serotype 4a, 1/2a and 1/2b form closely overlapping populations based on GT at 4°C, although the majority of 4a isolates are faster growing than either 1/2a or 1/2b (Fig. 2.10).

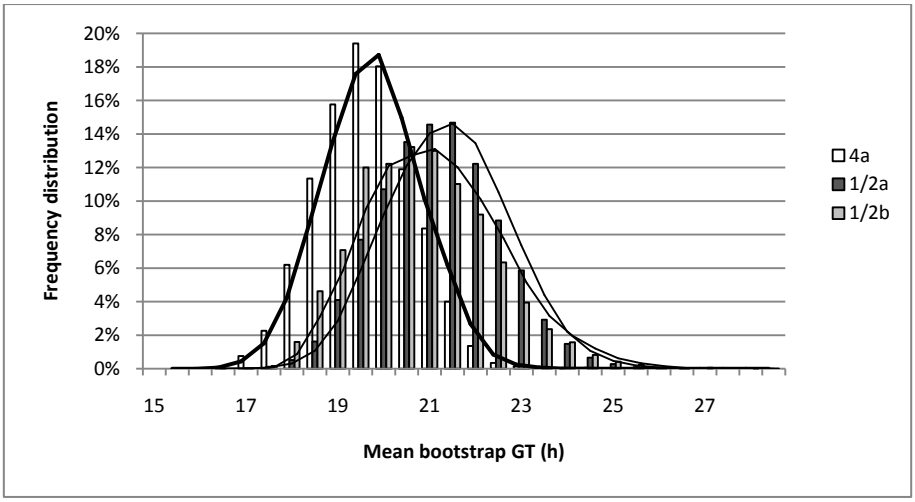


Figure 2.10 Histogram and overlaying bootstrapped GTs distributions of *L. monocytogenes* serotype 4a, 1/2a and 1/2b isolates at 4°C.

Serotypes 4e and 4b showed no significant difference in average bootstrapped GTs at 4°C ($P>0.05$, n=50). Population analysis of the bootstrapped samples showed the 90% CI to be between 18.5 and 24.0 h for serotype 4e and 18.6-25.9 h for serotype 4b isolates (Appendix C, Fig. C.18 and C.19 respectively). Analysis of variance also revealed no significant difference between average GT of serotypes 4e and 1/2a or 1/2a and 4b suggesting that all four strains originate from an homogenous population of *L. monocytogenes* at refrigeration temperature.

Serotype 1/2c strains had the longest average generation time 23.6 ± 1.7 h and was statistically significantly different ($P<0.001$) from other groups even at replication of ≤ 100. Bootstrap population statistics suggested that the 90% CI was from 21.2 to 26.6 h (Appendix C, Fig. C.20).

2.3.2.3 Generation time analysis at 4°C based on origin of isolates

Isolate origin had a clear influence on GT of *L. monocytogenes* strains at refrigeration temperature (4 ± 1.0°C), ($P<0.05$). Strains of clinical origin had an average GT 27.2 ± 7.1 (± 2.0 bootstrapped) h, which was significantly slower than those isolated from food or animal sources (Fig. 2.11). Bootstrapped population analysis revealed that 90% of the population had an average generation time between 23.9 and 30.4 h (Appendix C, Fig. C.21).

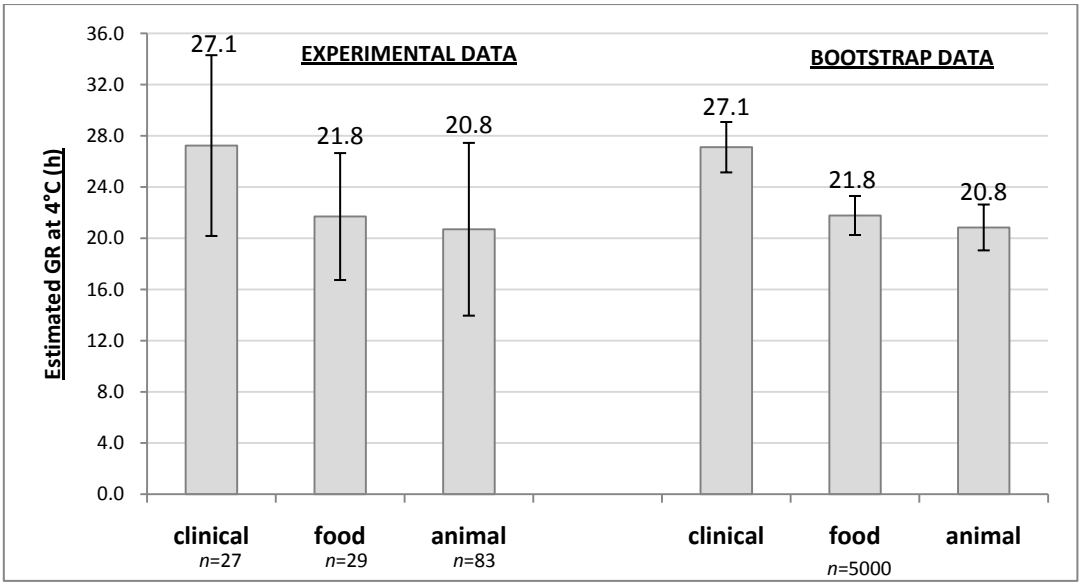


Figure 2.11 Average generation time values for *L. monocytogenes* isolates at 4°C grouped on the basis of isolate origin. Error bars indicate standard deviations.

Isolates from either food or animal origin had, on average, similar GTs of 21.7 ± 5.0 and 20.7 ± 6.7 h respectively, however appeared to come from heterogeneous populations ($P<0.05$ at bootstrap replication of n=50). Population analysis revealed the 90% CI residing between 19.4 and 24.3 h for food isolates and 18.1 - 24.0 h for animal isolates (Appendix C, Fig. C.22 and Fig. C.23 respectively).

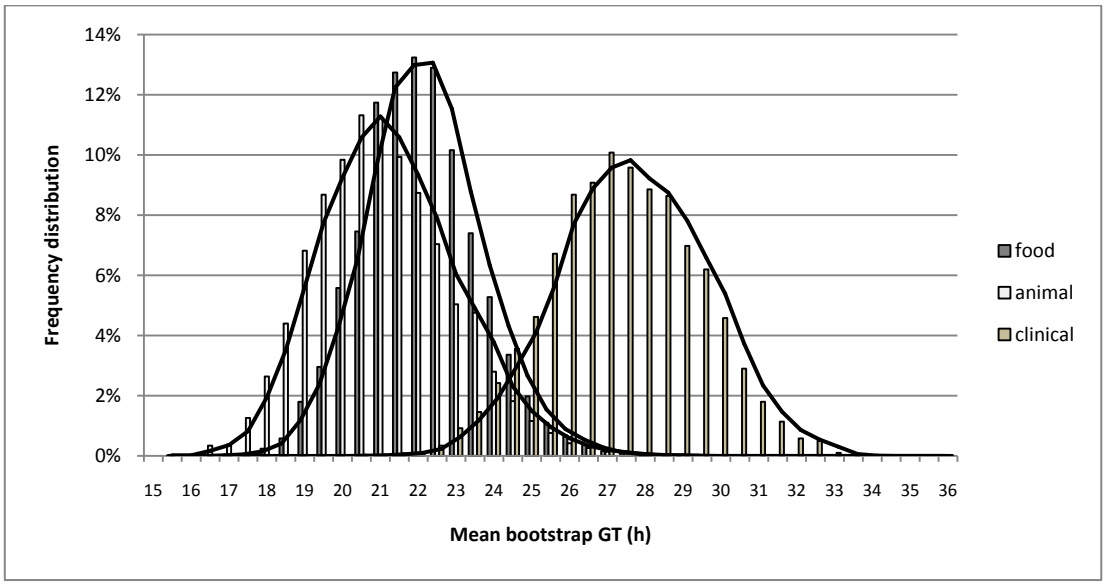


Figure 2.12 Histogram of the overlaying bootstrapped GT distributions of *L. monocytogenes* from food and animal sources.

These two groups produced a significant overlap in mean GT distribution (Fig. 2.12), although animal isolates had an average GT at refrigeration temperature slightly faster that those originating from food.

2.3.3 Theoretical correlation between rates of growth of *L. monocytogenes* isolates independently evaluated at 12.5% (w/v) NaCl and 4°C.

There appeared to be no significant correlation between GTs of *L. monocytogenes* at 12.5% (w/v) NaCl and growth of the same strains at 4°C, Pearson Correlation of 0.4 (*r*=0.4) (Fig. 2.13).

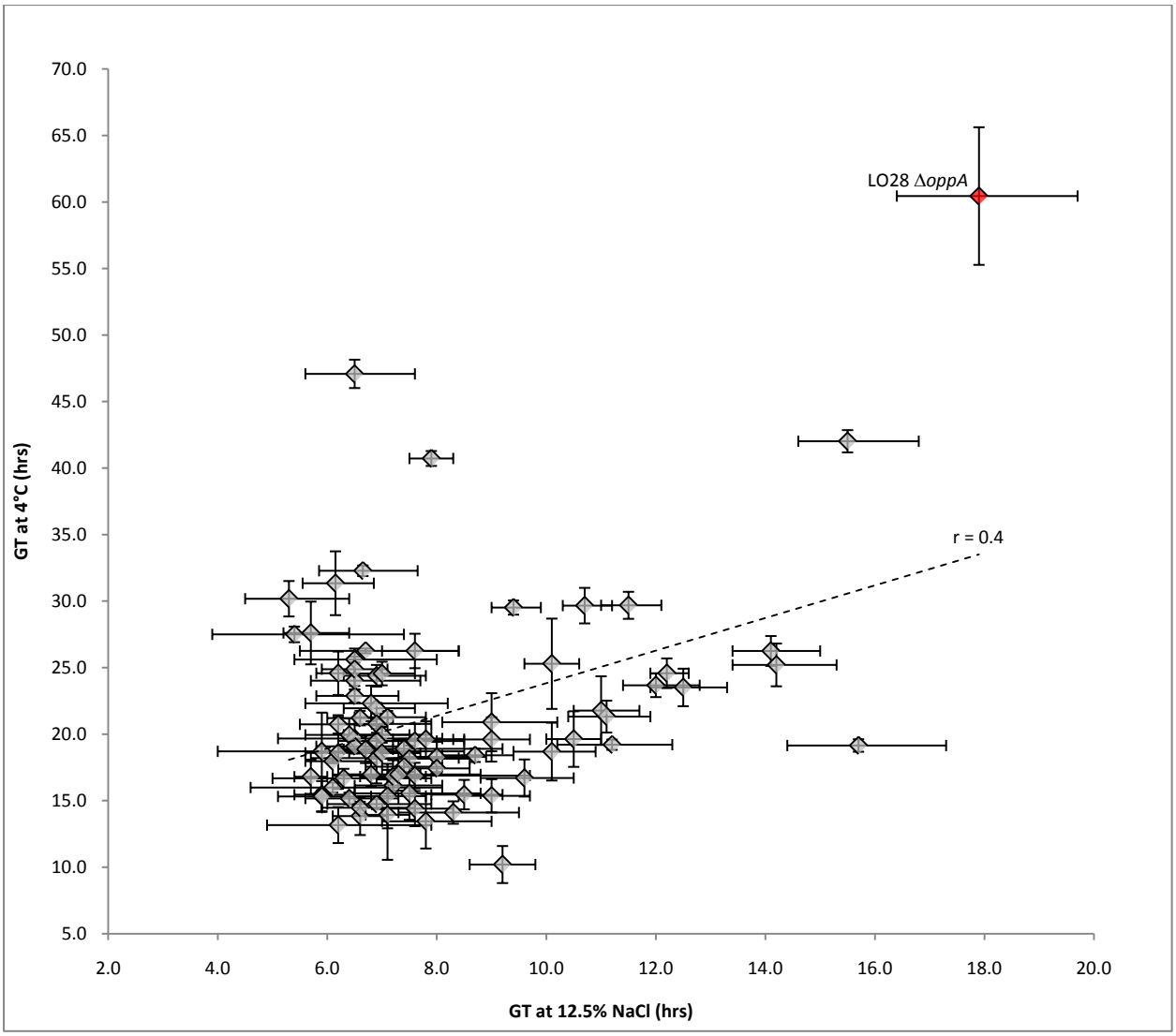


Figure 2.13 Scatter plot of *L. monocytogenes* isolates relationship between generation times independently obtained at 12.5% (w/v) NaCl and 4°C.

There was no significant correlation observed between LI isolates cultivated under 12.5% (w/v) NaCl and the same isolates at 4°C (r=-0.03) (Fig.2.14). *L. monocytogenes* LII isolates, however, showed a slight positive correlation between GTs observed at 12.5% NaCl and 4°C with Pearson Correlation coefficient of 0.61 (Fig. 2.14), suggesting that isolates of this lineage with relatively short GTs observed at high salt concentrations also produced shorter GT at refrigeration temperature of 4°C and vice versa.

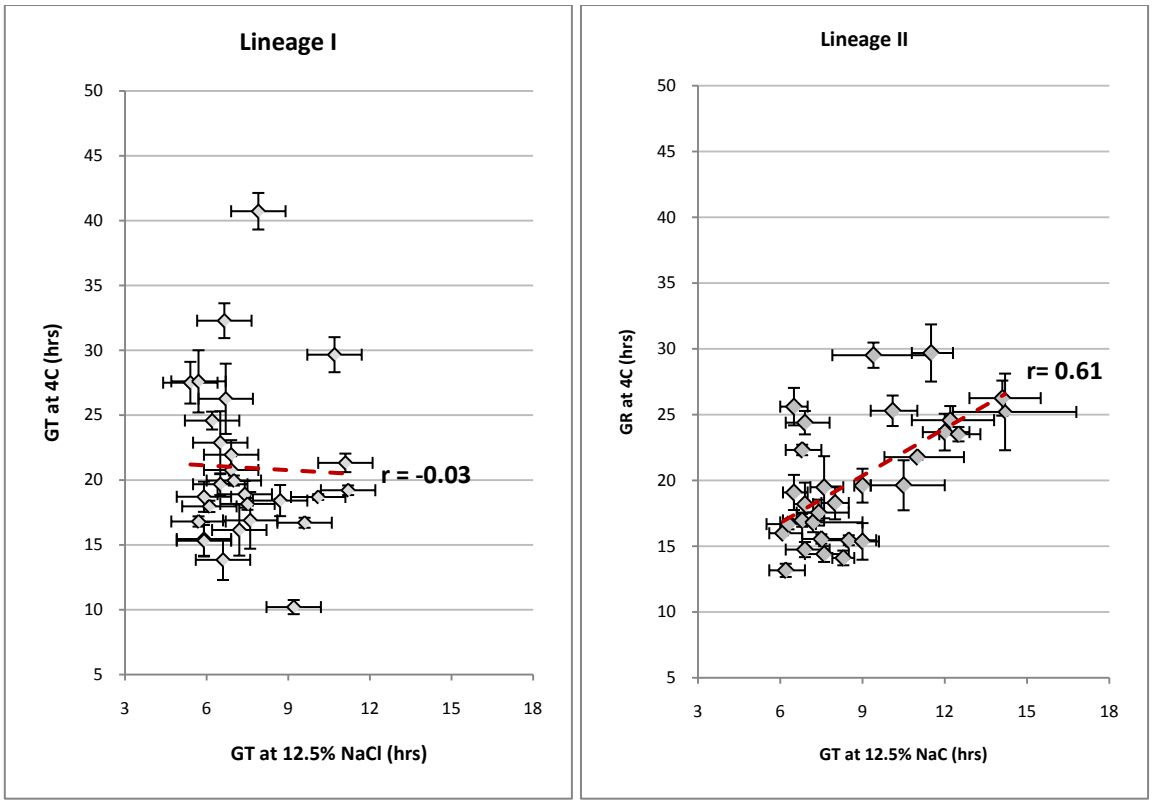


Figure 2.14 Scatter plots of *L. monocytogenes* lineage I and II isolates generation times independently obtained at 12.5% (w/v) NaCl and 4°C.

No correlation was observed in GT at 12.5% (w/v) NaCl or GT at 4°C for isolates of lineage III, with Pearson Correlation coefficient of -0.32 (Fig. 2.15).

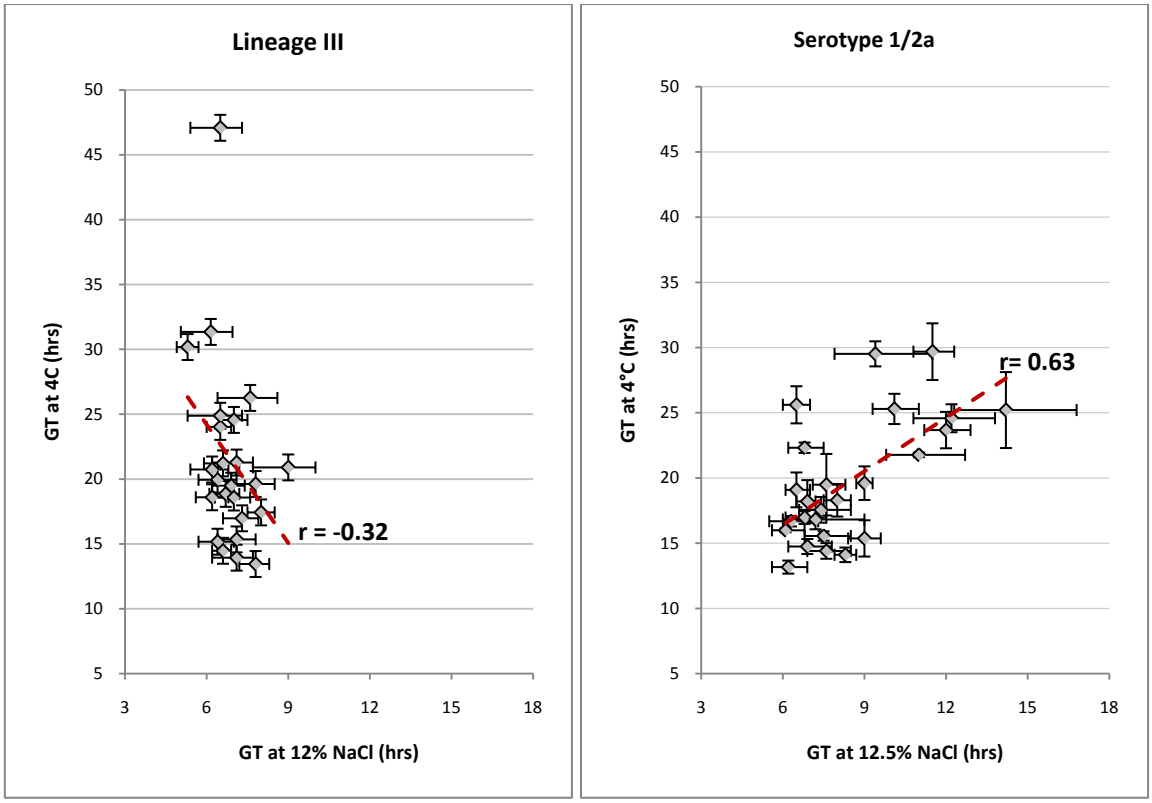


Figure 2.15 Scatter plots of *L. monocytogenes* lineage III and serotype 1/2a isolates generation times independently obtained at 12.5% (w/v) NaCl and 4°C.

Isolates of serotype 1/2a showed a slight positive correlation between generation times at 12.5% (w/v) NaCl and those at 4°C with Pearson Correlation coefficient of 0.63 (Fig.2.15). This suggests that isolates of this serotype with faster growth at high salt concentration also grow faster at 4°C and vice versa.

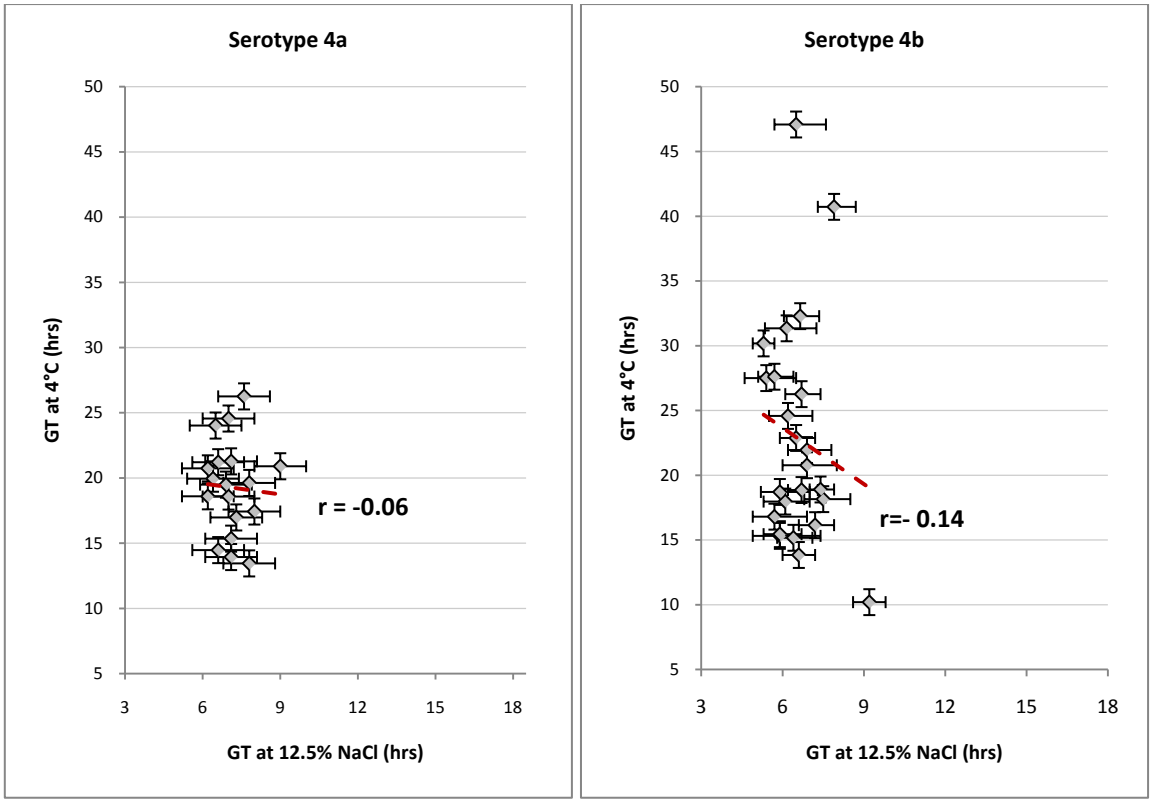


Figure 2.16 Scatter plots of *L. monocytogenes* serotype 4a and 4b isolates generation times independently obtained at 12.5% (w/v) NaCl and 4°C.

No correlation in growth rate at 12.5% NaCl or 4°C was evident for either serotype 4a or 4b strains (Fig. 2.16).

2.4 Discussion

2.4.1 General method overview

This screening study was conducted in order to investigate the growth parameters of *L. monocytogenes* isolates of various serotypes and origins for growth at 12.5% (w/v) NaCl and independently growth at 4°C in a rich medium closely resembling a food matrix. This screen was designed to select for highly tolerant strains from the majority of this population. Optical density was chosen for growth evaluation over viability count on the basis of speed and convenience as viable counts are labour and time-consuming. Optical density growth estimates are known to be generally less sensitive than viable count estimates, able to register change in absorbance at only relatively high concentrations (approximately 10^7 CFU/mL) therefore resulting in generally slower generation times (Augustin *et al.*, 1999). This was not an issue in this study as a sole interest was to elucidate stress tolerant strains over the stress susceptible strains in the culture collection. Microtiter trays were implemented to assess generation times of *L. monocytogenes* at 4°C. The method had been evaluated previous to this study (data not shown) and was found to be a satisfactory screening tool. The advantage of this relatively crude method was the ability to screen a large number of isolates at one time with high replication - up to 8 for each strain screened.

Generation time data was also bootstrapped prior to analysis; this data was examined in parallel with experimental observations. Bootstrapping refers to the use of the original data set to generate larger data sets. This approach is used for estimating the sampling distribution from limited data sets (Lee *et al.*, 2007; Schaffner, 1994). It is a useful statistical tool which significantly improves the discriminatory power and the robustness of the relationships between observed groups.

Mean bootstrap GT estimates for each group examined were identical to observational averages for the same groups. Significant change however was observed with the variance estimates within each group, which in turn allowed for more detailed evaluation of each independent group of isolates. For instance, statistical analysis of experimental data showed no difference in average generation times between lineage I and III (Fig. 2.1), however, when bootstrapped the same data set revealed a difference between the two groups even with small replication.

Additionally, analysis of the bootstrap frequency distribution allowed for a visual examination of the representative populations. Visualising the data is often useful to aid in interpretation of statistical analysis, and can also reveal when the statistics are misleading. Frequency distribution histograms revealed that GT were asymmetrical and not normally distributed, which agrees with previous observations of microbial growth rate data being heteroscedastic (Ratkowsky *et al.*, 1996; Schaffner, 1998).

Interpretation of the bootstrap data should however be treated with caution because the error values decrease with increased replication and falsely significant variances between groups may be created, as was observed with cold data grouped into lineages. Frequency distribution histograms proved very useful in analysing bootstrap data in combination with the statistical analysis, as these provided an insight, *via* visualisation of the data, into relationships between the group mean values. In the case of the analysis of lineage groups grown at 4°C, analysis of the mean distribution revealed a clear overlap between the three lineage groups (Fig.2.8) indicating the subgroups originate from homogenous populations.

The bootstrapping technique undertaken in this study has allowed for evaluation of growth variation between relatively small subgroups of *L. monocytogenes* isolates; thus aiding in better understanding of the demographics of these subgroups in terms of growth under environmental stress.

2.4.2 Growth population evaluation

Growth variation among *L. monocytogenes* strains under different stress conditions has been demonstrated in several investigations (Barbosa *et al.*, 1994; Buncic *et al.*, 2001; Lianou *et al.*, 2006; Shabala *et al.*, 2008; Van der Veen *et al.*, 2008). To better understand bacterial response to any given stress it is important to investigate a given stress effect at extreme ends of a normal distribution. It is therefore important to establish the normal distribution of generation times in a given population to select for outliers. This was the exact aim of the current investigation.

2.4.2.1 Generation time variation of L. monocytogenes strains at 12.5 % (w/v) NaCl

Salt is traditionally an important food preservative and is still widely used to restrict bacterial growth by lowering the water activity of a food matrix. *L. monocytogenes* has a high tolerance for NaCl and has been reported to grow at NaCl as high as 14 % (Farber *et al.*, 1992). In this particular study a moderately high level of NaCl was selected as a proxy for assessing low water activity tolerance of *L. monocytogenes.* The selected level of NaCl challenge was based on reported 13% NaCl (wt/vol) growth limits of *L. monocytogenes* in complex media (Ross *et al.*, 2000; Shabala *et al.*, 2008). Although a small proportion of *L. monocytogenes* strains screened by Shabala *et al* 2008, previously habituated to 8% NaCl were able to grow at 13.9% NaCl (wt/vol). In the current study isolates screened by Shabala *et al* 2008 for maximum NaCl tolerance at 20°C as well as additional isolates were examined for fitness or tolerance to 12.5% (w/v) NaCl assessed in terms of generation times at 25°C in aerobic conditions.

The selected salt concentration of 12.5% (w/v) (equivalent to a_w of 0.92 at 25°C) was sufficient to identify, and prevent the growth of, the salt sensitive isolates, and to notably challenge the salt tolerant isolates. Generation time varied significantly within the salt tolerant isolates ranging from as fast as 5.3 h to as slow as 17.9 h.

Variation in generation times at low water activity, between isolates of different lineages, serotypes and origin was evaluated in this study to obtain a better understanding of hyperosmotic tolerance in *L. monocytogenes*.

Lineage III isolates were found to have faster average generation times at low water activity conditions than lineage I and II isolates, with generation times of the 24 isolates able to grow at this salt concentration ranging from 5.3 h (strain FW03-0035, serotype 4b) to 9 h (strain 78-3536, serotype 4a). Comprising of three serotypes 4a, 4c and 4b, lineage III isolates are thought to be relatively rare compared to the other two lineages. Isolates of this lineage are relatively more prevalent among strains isolated from animals than among clinical (Jeffers, 2001) or food (Gray, 2004) isolates. Previous studies based on small sample size have found that lineage III isolates were less resistant to heat and cold stress (7°C) than isolates belonging to lineage I and II (De Jesus, 2003). It has been proposed that the low frequency of clinical cases associated with this lineage may be due

to reduced ability to survive and multiply under stress conditions typically found in food (Roberts, 2006). Results obtained for salt tolerance in this study potentially refute the assumption made by Roberts (2006).

Lineage II isolates appeared to have the slowest average generation times at 12.5% (w/v) NaCl ranging from 6.1 h (strain 72-0039) to 14.2 h (strain 70-0421) (Appendix C, Table C.1). This lineage includes serotypes 1/2a, 1/2c and 3a with significantly greater prevalence among food isolates and animal clinical cases than among human listeriosis cases, although serotype 1/2a and 1/2c have been linked to sporadic human disease (Nightingale *et al.*, 2005). The relevance of slower GTs for isolates of this environmentally adapted lineage is unclear. It could be concluded, however, that based on the observed results isolates of LII are less fit to tolerate low water activity in comparison to LI or LIII isolates.

Serotype specific influence was observed in the generation time data obtained in this study, with serotype 4b strains exhibiting a faster average generation time (6.6 ± 0.25 h) in the low water activity environment than other serotypes evaluated. GTs varied from 5.3 h (strain FW03-0035) to 9.2 h (7902336/3) (Appendix C, Table C.1) within this sub-group of isolates, 90% of estimated generation times fell within the range 6.2 to 7.0 h (Appendix C, Fig. C.8). This finding agrees with a previous observation of relatively high stress tolerance in this serotype. In one such study a group of serotype 4b strains showed higher tolerance to high osmotic conditions at pH 7.4 (at 30°C) than a group of serotype 1/2a strains and 1/2b strains (Van der Veen *et al.*, 2008). Serotype 4b accounts for nearly all the outbreaks of human food-borne listeriosis even though it is not found as commonly in foods or the environment as other serotypes (Farber & Peterkin, 1991; Jeffers *et al.*, 2001; McLauchlin, 1990). The exact benefit of an enhanced tolerance of this serotype to such low water activity stress is not clear; however it strongly implies a more robust serotype.

Interestingly serotype 4a isolates had the second shortest average GT of 7.1 ± 0.23 h, exhibiting slight overlap in mean values with serotype 4b population. Isolates of this serotype are thought to be rare with somewhat dampened virulence potential (Sokolovic *et al.*, 1996). Serotype 4a isolates are thought to be of environmental origin and are most often isolated from animals. It is possible that during their saprophytic existence strains of this serotype may have acquired tolerance to high levels of osmotic stress through constant exposure to decreased water potential in the environment. Soil

microorganisms are commonly subjected to extremely low water potentials (Halverson *et al.*, 2000; Kieft *et al.*, 1987) which may occur in dry conditions where solutes become concentrated.

Serotype 1/2a has been reported to predominate among food isolates (Cabrita *et al.*, 2004; Nelson *et al.*, 2004) however in this study had a relatively slow average generation time (8.3 ± 0.6 h) with the 90% CI ranging between 7.4 and 9.3 h. It appears that there is no direct correlation between the relatively slow GT of serotype 1/2a in hyperosmotic conditions and the rate of isolation of this serotype from food products. This is most likely due to the fact that most food products do not contain such low water activity, minimizing exposure to extreme hyperosmotic conditions for the otherwise food-dominant serotype. However over-representation of 1/2a serotype in the food isolates may also be due to its abundance in the environment, thus resulting in more frequent contamination of food than less common serotypes.

Serotypes 1/2c and 1/2b isolates produced on average longest generation times at the selected level of osmotic stress, however these were significantly underrepresented in this collection of isolates (9 and 8 strains respectively). There is a reluctance to make generalisations about GT characteristics for these two groups of isolates based on observational results in this study as the small sample size may not be a true representation of the underlying behaviour of those subpopulations.

Isolates of clinical and food origin appeared to have slower average GT at the selected low water activity than animal isolates. Clinical and food isolates appeared to originate from homogenous populations, which is not surprising, considering that scientific interest in *L. monocytogenes* is as a serious food-borne pathogen and most clinical isolate are assumed to have originated from food. The majority of the osmo-tolerant *L. monocytogenes* isolates examined in this study originated from animal sources, which also showed a shorter average generation time of 7.7 ± 0.56 h (90% CI of 6.9 to 8.7 h), suggesting higher tolerance for hyperosmotic stress. Animal isolates of *L. monocytogenes* presumably reach the affected animal from the natural environment, from water or feed. The saprophytic existence of *L. monocytogenes* in natural environments may mean that strains isolated from animal were already selected for stress tolerance due to variable and often adverse environmental conditions as discussed earlier.

2.4.2.2 Variation in generation times of L. monocytogenes at refrigeration temperature

One of the most prominent features of *L. monocytogenes* as a food-borne pathogen is its ability to grow at refrigeration temperatures, thus allowing refrigeration to serve as an effective enrichment for this organism in contaminated foods with long refrigerated shelf lives.

Extensive variation in GTs was observed among tested isolates at 4°C, ranging from 9.9 ± 0.55 h (strain 76-1854) to 60.45 ± 5.2 h (strain LO28 Δ*oppA*) (Appendix C, Table C.2) with an average GT of 22.42 ± 7.44 h. These observations are in agreement with other studies in terms of a wide GT distribution (Lianou *et al.*, 2006; Nufer *et al.*, 2007).

Lineage of isolates appeared to have no significant effect on the average generation time of *L. monocytogenes* strains at 4°C with all three lineages exhibiting similar generation time distributions (Fig. 2.8).

Serotype 4a appeared to have shorter average GT at refrigeration temperature compared to other serotypes examined in this study (average GT of 19.4±1.0 h). Isolates of this serotype are thought to predominate in the environment and as such are likely to be exposed to low temperature stress in their natural habitat where shorter GT times might provide an advantage to the overall organism survival.

Strains of clinical origin exhibited significantly slower GTs of 27.2 ± 2.0 h at 4°C than either animal or food isolates. This is the first unambiguous indication of a strong association between growth at 4°C and isolation source of the strains of *L. monocytogenes,* potentially due to a large number of isolates tested in this study. Strains of clinical origin in general appear to not benefit from fast GTs at low temperature. Ability to grow at refrigeration temperature is an important attribute of *L. monocytogenes* pathogenesis, however even the slowest growing strains are unlikely to be overgrown at 4°C by other pathogens or probiotic bacteria potentially present in food, minimising competition. It is possible these strains derive advantage, and are therefore indirectly selected, on the basis of other factors such as virulence potential or an ability to grow faster at physiological

temperature; these factors are more likely to lead to establishment of infection, irrespective of an ability to grow fast at 4°C.

Animal isolates in comparison produced faster GTs on average at 4°C suggesting adaptation to this temperature likely due to frequent exposure of these strains to cold stress in the environment. An ability to rapidly multiply in a cold environment is of benefit to *L. monocytogenes,* competing for nutrients with other microorganisms. Thus, high bacterial loads are achieved faster, largely increasing the chance of animal exposure to these strains via contaminated feed or water. Animals come in contact with *L. monocytogenes* much more frequently than humans, without necessarily developing infection (Esteban *et al.*, 2009; Lyautey *et al.*, 2007). A high exposure frequency inevitably increases the chance of developing infection even with potentially less virulent strains of *L. monocytogenes* in susceptible animals. Therefore strains able to outgrow other microorganisms in the environment are much more likely to be isolated from either sick or asymptomatic animals.

Strains originating from food produced average GT very similar to those observed for animal isolates, though exhibiting slightly slower generation times, suggesting a close link between the two origins. It is likely that food contaminant strains originate either from affected animals or directly from the environment, such as through contaminated water or soil.

Although all strains originate either directly or indirectly from environmental source, it appears that the origin selects certain traits in isolates that predominate in their sub-populations. Strains isolated from clinical source for instance appear to not benefit from the ability to tolerate environmental stress such as non-physiological temperature of 4°C but, presumably, enhance other potentially more advantageous attributes such as virulence factors.

2.4.2.3 Theoretical correlation of tolerance between cold and salt stresses

In general, there appeared to be little correlation between growth rates of *L. monocytogenes* strains cultivated at 12.5% (w/v) NaCl or 4°C. Lineage I and III isolates showed no correlation between generation times under these stressful conditions. Lineage II isolates however, showed a slightly positive correlation between generation time at 12.5% and 4°C. Serotype overall had no influence on

generation time correlation between the two stresses either, except for strains of serotype 1/2a which showed a slight positive correlation between growth rate under each of the stresses. Both observations with lineage II and serotype 1/2a might be of significance to the food industry. Lineage II comprises of serotypes 1/2a and 1/2c which are the serotypes most commonly associated with food and food processing environments. Serotype 1/2c was significantly under-represented in this study therefore the most predominant and thus most influential serotype in lineage II in the collection of isolates from this study was serotype 1/2a. It is not surprising that both groups showed a similar correlation between the two stresses. It makes sense that ability to tolerate more than one stress is beneficial to strains isolated from foods and which are challenged by numerous stresses utilised in food manufacturing and food preservation procedures. Although serotype 4b showed faster generation times at 12.5% (w/v) NaCl than 1/2a strains, it might be less adapted to the food environment than the latter serotype. The observed tendency of 1/2a strains that grow relatively quickly at 12.5% (w/v) NaCl to also have shorter generation times at 4°C, may explain the predominance of this serotype among food isolates.

Interestingly an *oppA* gene mutant of strain LO28 had the longest GT of all the isolates tested. The estimated GT at 12.5% (w/v) NaCl for Δ*oppA* LO28 was 17.9 hrs, compared to 14.1 hrs of the parent strain LO28 and 60.5 hrs at 4°C compared to 26.3 of LO28. This observation strongly suggests an involvement of the encoded oligopeptide transporter in both osmotic and cold temperature tolerance (discussed further in chapters 3 and 5), which when impaired results in a stress-sensitive phenotype.

2.5 Summary and Conclusions

Results of this study emphasise the contribution of *L. monocytogenes* strain variation to growth behaviour of this organism under hyperosmotic stress induced by 12.5% (w/v) NaCl or at refrigeration temperature of 4°C in a rich medium resembling a food matrix.

Implementation of the bootstrap technique on the original observations in this study allowed for more detailed evaluation of each independent subgroup of isolates by significantly reducing standard deviations between subgroups. Additionally, construction of frequency distribution histograms proved useful in evaluating populations within each subgroup by visual means.

Tolerance to 12.5% (w/v) NaCl concentration was observed in 60.5% of strains, with an average generation time (GT_{ave}) of 8.0±2.5 h, ranging from 5.3 to 17.9 h, at 25°C. Lineage III and I isolates showed overlapping populations with the former being characterised by shorter GTs .Strains of serotype 4b produced shorter GT_{ave} of 6.6±0.25 h than other serotypes examined, suggesting strains of this serotype are in general more tolerant to this form of stress.

Extensive variation in GTs was also observed among tested isolates at 4°C, GT_{ave} of 22.42±7.44h, ranging from 9.9±0.55h to 60.45±5.2h. While isolate lineage appeared to have no significant correlation with GTs at this temperature, serotype 4a strains produced shorter GTs than isolates of other serotypes, suggesting acclimatisation to low-temperature by frequent exposure to such stress in the environment where this serotype predominates.

Although no direct correlation (r=0.4) between tolerance to either hyperosmotic- or cold-stress, on the bases of GTs, was evident between isolates, strains of animal origin were on average found to have shorter GTs when exposed to ether of the two stress agents. Natural adaptation to low water activity and low-temperature in the environment most likely accounts for this observation.

In order to evaluate the mechanisms of tolerance of *L. monocytogenes* to hyperosmotic and hypothermal stresses a number of strains representing the whole spectrum of response based on the results presented in this chapter, were selected for subsequent whole genome microarray analysis.

Chapter 3

Changes in gene expression in Listeria monocytogenes adapted to hyperosmotic stress induced by NaCl.

Listeria monocytogenes is able to adapt to a variety of stresses that allow it survive within the food supply chain. Understanding the mechanisms of stress tolerance in this pathogen may aid in minimising its proliferation in food and reduce incidence of listeriosis. In this study the gene expression responses linked to osmo-adaptation was investigated in four *L. monocytogenes* strains, possessing different tolerances to NaCl. Gene expression trend analysis revealed a clear and consistent pattern in terms of genomic expression between all strains. Prolonged exposure to high levels of NaCl was coupled with activation of genes associated with the bacterial cell envelope, DNA repair and protein synthesis. Repression of genes associated with carbohydrate up-take and metabolism was evident in osmo-adapted cells reflecting the overall suppression of cellular metabolism characterised by reduced growth rates. The SigB, PrfA and CodY regulons was suppressed in most strains and could suggest cells conserve energy while osmo-adapted.

3.1 Introduction

The bacterial pathogen *Listeria monocytogenes* is well adapted to both life in the soil and life inside eukaryotic host cells. During its saprophytic life this bacterium can acquire tolerance to a vast array of physical and physiochemical stresses. These include stresses (e.g. cold, salt, pH) often used in food production to limit microbial proliferation as well as to extend food shelf-life. This creates a potential dilemma for controlling proliferation of this organism in contaminated food to large enough numbers able to cause food-borne infection in susceptible individuals.

Understanding the mechanisms of stress tolerance in this pathogen may aid in minimising its proliferation in contaminated food. One such stress tolerances is hyperosmotic environment which has been a topic of numerous investigations both from a physiological basis and more recently from a genomic perspective (Sleator *et al.*, 2003a). Most publications focus on either a single strain of *L. monocytogenes* or a mutant of a specific gene in a single strain (Liu *et al.*, 2005; Liu *et al.*, 2006;

Raengpradub *et al.*, 2008). *L. monocytogenes* tolerance and subsequently response to environmental stress can be highly strain specific.

The goal of this study was to investigate changes in the gene expression profile of *L. monocytogenes* expressed after adaptation to hyperosmotic environment, induced by NaCl (salt), where responses to the initial shock (high osmotic pressure) have occurred and where microbial growth has been re-established.

3.2 Methods

3.2.1 Strains

Strains used in this study were obtained from the TIAR collection and were part of the set of strains examined for salt and cold tolerance experiments detailed in Chapter 2 .The four strains investigated here included those possessing a range of NaCl tolerances. Strains ScottA and FW03-0035 (isolated from processed meat), both of serotype 4b possess comparatively high NaCl tolerance; clinical strain ATCC19115 (serotype 4b, isolated from cerebral spinal fluid), has moderate NaCl tolerance; and ovine strain 70-1700 (serotype 4e) possesses relatively low NaCl tolerance.

3.2.2 Media and Growth Conditions

3.2.2.1 Control Cultures

All four *L. monocytogenes* strains were grown in duplicate in 100 ml BHIB at 25°C to a transmittance of approximately 60% (late exponential growth phase). Approximately 25 ml of the culture was centrifuged at 1500xg for 10 min and the pellet resuspended in a smaller volume (*ca* 2 ml) of the supernatant. The suspension was then treated with 4 ml RNAprotect Bacterial reagent (as described in section 3.2.3.1).

3.2.2.2 Adaptive NaCl Stress Cultures

L. monocytogenes strains were grown in duplicate in 10 ml BHIB supplemented with 6.0% (w/v) sodium chloride (NaCl) at 25°C for 24 h; 100 μl of this culture was used to inoculate 50 ml BHIB supplemented with set NaCl concentrations depending on the strain NaCl level tolerance level (ScottA and FW03-0035 at 12.0% (w/v), ATCC19115 at 10.0% (w/v) NaCl and 70-1700 at 8.0% (w/v) NaCl) and inoculated at 25°C and incubated till late exponential growth phase (OD_{540} 0.6).

Approximately 25 ml of the culture was centrifuged at 1,500xg for 10 min and the pellet resuspended in a smaller volume of the supernatant and then treated with RNAprotect Bacterial reagent (as described in section 3.2.3.1).

3.2.3 RNA Extractions

3.2.3.1 RNAprotect Bacterial Reagent Treatment

To stabilise cellular RNA for extraction two volumes of RNAprotect bacterial reagent (Qiagen) were added to one volume of culture and mixed by vortexing for approximately 5 seconds, then incubated at room temperature for 5 min. This suspension was then centrifuged at 7,500xg for 10 min. The supernatant was discarded and the cell pellet was stored at -80°C until subsequent RNA extractions were undertaken.

3.2.3.2 RNA extraction

Cells were defrosted on ice and underwent a 6 h enzymatic treatment in 10 mM Tris-1 mM EDTA buffer (pH 8.1) containing 20 mg/ml lysozyme and 10 mg/ml proteinase K at 25°C. Cells were fully lysed by bead beating using 0.1 mm zirconium-silica sand in 4 ml of RNeasy Midi RNA Extraction kit (Qiagen) lysis buffer that was supplemented with 0.1% β-mercaptoethanol (Sigma-Aldrich). The tubes were centrifuged at 7,500xg for 10 min and supernatant transferred into a clean 15 ml Falcon tube and combined with 2.8 ml of absolute ethanol. This suspension was applied in 4 ml aliquots to the RNA Midi column and centrifuged at 7500xg for 10 min. The column was washed twice with 4 ml RWI buffer (RNeasy Midi RNA Extraction kit, Qiagen) and centrifuged at 7500xg for 10 min. each time the flowthrough was discarded. The column was then washed three times with 2.5 ml RPE buffer (RNeasy Midi RNA Extraction kit, Qiagen) by centrifugation at 7500xg for 10 min, each time discarding the flow through. The column was then dried completely by centrifugation at 7500xg for 5 min in a clean falcon tube. The dried column was transferred into a clean 15 ml falcon tube and RNA was eluted with 250 µL of RNase-free water by centrifugation at 7500xg for 10 min. The elution step was repeated with the first eluate. The extracted RNA was stored at -80°C.

3.2.3.3 RNase-free water preparation

RNase-free water was prepared by adding diethylpyrocarbonate (DEPC) to autoclaved milliQ water to achieve a final concentration of 0.1% and incubated overnight at 37°C. The mixture was then autoclaved to degrade the DEPC.

3.2.3.4 Formaldehyde agarose (FA) gel electrophoresis

RNA quality and quantity was assessed by running the RNA samples on a FA (1.2%) agarose gel. The gel was prepared by dissolving 0.24 g of agarose in 18.2 ml 1x FA buffer, diluted 1 in 10 from a solution containing 200 mM 3-[N-morpholino]propanesulfonic acid (MOPS)**,** 50 mM sodium acetate**,** 10 mM EDTA (pH 7.0). After cooling to approximately 60°C, 1 µl of ethidium bromide and 1.8 ml of 37% formaldehyde were added to the gel before it was poured into the gel apparatus and allowed to set. The samples were prepared by mixing 4 volumes of RNA sample to one of 5x RNA loading buffer and incubated at 65°C for 5 min. The loading buffer consisted of 16 µl saturated bromophenol blue solution, 80 µl 500 mM EDTA at pH 8.0, 720 µl 37% formaldehyde, 2 ml 100% glycerol, 3084 µl formamide, 4 mL 10xFA buffer, volume was adjusted to 10 ml with RNase-free water. Samples were then chilled on ice before being loaded onto a dry gel. Gel was run at 5-7 V/cm in 1xFA gel running buffer for 30 min and then visualized on UV transilluminator.

3.2.4 Microarray Analysis

3.2.4.1 Microarray slides

The microarray slide utilised2857 x 70 bp oligonucleotides (AROS v. 1, Eurofins MWG Operon, Huntsville, Al, USA), representing all predicted protein coding genes and pseudogenes of the complete, published genome of *L. monocytogenes* EGD-e (GenBank accession number. AL591824). Oligonucleotides were arrayed onto glass slides using quill pens at the Australian Genomic Research Facility Ltd. (Walter & Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia) with each spot possessing on average a 12 µm diameter.

3.2.4.2 First-strand cDNA synthesis

3.2.4.2.1 First-strand cDNA synthesis reaction

Approximately 5-20 µg of total RNA was combined with 2µl of Anchored Oligo$(dT)_{20}$ Primer (Invitrogen) in 1.5 ml microcentrifuge tube. The final volume was adjusted to 18 µl with DEPC-

treated water and the mixture incubated at 70°C for 5 minutes followed by chilling on ice. Tubes remained on ice while 6 µl of 5X First Strand buffer (250 mM Tris-HCl pH 8.3; 375 mM KCl; 15 mM $MgCl_2$), 1.5 µl 0.1 M DDT, 1.5 µg dNTP mix, 1 µl RNaseOUT and 2 µg of SuperScript III Reverse Transcriptase (all supplied in SuperScript III RT Kit (Invitrogen)) was added to the reaction mix. Tubes were incubated at 46°C for 3 h.

3.2.4.2.2 Alkaline hydrolysis and neutralization

The original RNA was degraded by addition of 15 µl of 1 N NaOH to the reaction tube containing the cDNA and incubated at 70°C for 10 min. Afterwards the reaction mixture was neutralised with addition of 15 µl of 1 N HCl. After which 20 µl 3 M sodium acetate (pH5.2) was added to the mixture and mixed.

3.2.4.3 First-strand cDNA Purification and Labelling

3.2.4.3.1 QIAquick column purification

The neutralised cDNA (from section 3.2.4.2.2) was mixed by vortexing with 375 µl of buffer PB (QIAquick PCR Purification Kit, Qiagen). The sample was loaded onto a Qiaquick column, placed in a collection tube, and centrifuged at 9,000xg at room temperature for 1 min; the flow-through was discarded. The column was then treated with 700 µl of buffer PE (QIAquick PCR Purification Kit, Qiagen) and centrifuged at 9000xg for 1 minute, discarding the flow-through. The wash strep was repeated one more time and flow-through discarded. The column was then dried by centrifugation at 9,000xg for 60 seconds. The air dried column was placed in a fresh 1.5-ml microcentrifuge tube and directly used for subsequent labelling without delay (section 3.2.4.3.2).

3.2.4.3.2 Labelling cDNA with fluorescent CyDye

The dyes were prepared by addition of 15 µl 0.1 M $NaHCO_3$ (pH 9.0) directly to the Cy3 and the Cy5 dye vials (CyDye Post-Labelling reactive Dye Pack, Amersham Bioscienses). CyDye aliquots were then applied to the dried columns (from section 3.2.4.2.1) containing the designated reaction (Cy3 dye solution was loaded onto a column containing the cDNA control suspension and the Cy5 dye solution was applied to the column containing the cDNA test suspension). Loaded columns were incubated at room temperature in the dark for 1 h. Following incubation the dye-coupled cDNA solution was

eluted with 80 µl of water by centrifugation at 9,000xg for 60 sec. Each eluate was then diluted with 400 µl of buffer PB (Qiagen) and the used columns were discarded.

3.2.4.3.3 QIAquick column purification and Cy3/Cy5 labelled sample coupling

The Cy3 labelled control sample eluate suspension (from section 3.2.4.2.2) was applied to a fresh Qiaquick column and centrifuged at 9,000xg at room temperature for 1 min; the flow-through was discarded. The column was then loaded with a corresponding Cy5 labelled test sample suspension and centrifuged at 9,000xg at room temperature for 1 min discarding flow-through. The column was washed twice with 700 µl volumes of buffer PE (Qiagen) by centrifugation at 9,000xg for 1 min, following by additional spin for 1 min to dry the column. The dry column was then loaded with 30 µl of DEPC-treated water, allowed to stand at room temperature for 1 min followed by centrifugation at 9,000xg RT for 1 min. The elution step was then repeated with additional 30 µl of DEPC-treated water. Coupled samples were dried in a vacuum drier and kept in the dark until required for hybridisation.

3.2.4.4 Hybridisation procedure

3.2.4.4.1 Slide Prehybridisation

Custom *L. monocytogenes* strain EGD-e microarray slides were incubated in a Coplin jar containing prehybridisation solution (10mg/ml BSA, 25% formamide, 5XSSC, 0.1% SDS) for 45 min at 42^{o}C. These were then rinsed twice with distilled water and air-dried.

3.2.4.4.2 Hybridisation

Dried samples from section 3.2.4.3.3 were resuspended in the appropriate amount of water depending on the coverslip size (table below).

Coverslip size	**22x25mm**	**40x22mm**	**60x25mm**
Sample re-suspended in water (µl)	6.5	11.0	16.2
2x Hybridisation buffer[#]	12.0	20.0	30.0
Cot1 DNA (5 µg/µl) (Invitrogen)	2.0	3.3	5.0
PolyA (10 mg/ml) (Invitrogen)	1.5	2.5	3.8
Salmon sperm DNA (10mg/ml) (Sigma)	2.0	3.3	5.0
Total volume (µl)	24.0	40.0	60.0

Hybridisation buffer (500µl formamide, 500µl 10xSSC, 20µl 10% SDS)

Samples were heated to 100°C for 2 min and then allowed to cool to room temperature. Prehybridised array slides were covered with Lifter Slip (Grale Scientific) and loaded with the cooled sample mixture. Loaded sides were place into a humid hybridisation chamber, sealed and incubated for 16-20 h in a 42°C water bath.

3.2.4.4.3 Washing

Slides were placed in a staining dish containing a 1xSSC/0.2% SDS (17.5 ml 20xSSC, 7 ml 10% SDS and 325 ml dH_20) wash solution at room temperature. These were washed on a shaker for 5 min and placed into a fresh staining dish with 0.1xSSC/0.2% SDS (1.75 ml 20xSSC, 7 ml 10% SDS and 340 ml dH_20) washing solution. Slides were agitated for 5 min at room temperature. These were then transferred into a staining dish containing 0.1xSSC (1.75 ml 20xSSC in 350 ml dH_20) wash buffer, agitated for 2 min and washed again for additional 2 min with a fresh 0.1xSSC solution. Slides were dried in a plate centrifuge at 1,000xg for 10 min and scanned using a GenePix 4000B scanner (Axon Instruments).

3.2.4.5 Data analysis

Downstream processing used the GenePix-Pro 3 software package to generate gpx filed from TIFF array images. Normalization of raw data and subsequent statistical analysis was performed with the WebArray Online platform (Xia *et al.*, 2005). Within-array normalization used the global LOESS procedure. Between each array quantile normalization was used to insure intensities had the same empirical distribution across arrays and across channels. The significance of differential expression was analysed using linear modal statistical analysis (Smyth, 2004). Oligonucleotides that showed negligible or no hybridization when compared with background hybridization were excluded from analyses. In addition genes only known to be found in the majority of *L. monocytogenes* genomes were considered in the analysis (e.g. if present in strains EGD-e, F2635 and Clip 81459 as shown in Appendix A) if the given 70-bp oligonucleotide sequence had a similarity level of at least 92%.

3.2.4.6 Gene set enrichment analysis

Gene designations, predicted functions and categorization of coded proteins into defined sets from the *L. monocytogenes* EGD-e genome was based on information obtained from published literature, Kyoto Encyclopaedia of Genes and Genomes (http://www.genome.ad.jp/kegg/) and ListiList

(http://genolist.pasteur.fr/ListiList/). A *t*-test based procedure was utilised to score the changes in expression of predefined sets of genes (Boorsma *et al.*, 2005; Bowman *et al.*, 2008). The significance of the T-value score was established by using the associated two-tailed *p*-value determined by using the TDIST function in Microsoft Excel.

3.3 Results and Discussion

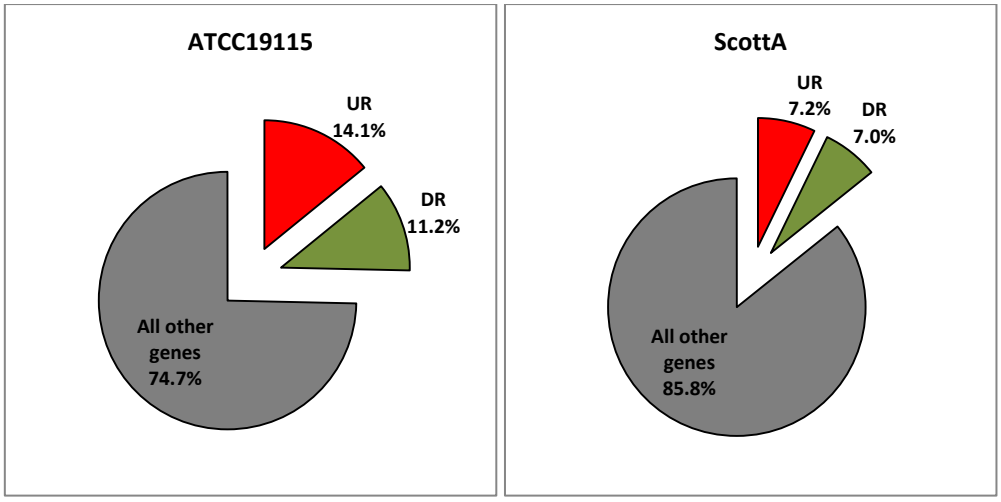
3.3.1 Overview of osmo-adapted response of four *L. monocytogenes* strains

Prolonged growth under stressful conditions results in cells which have re-established the disrupted homeostasis with their external environment, resuming normal cellular functions such as DNA and protein synthesis, cell growth and cellular division. In terms of the food safety cells in an osmo-adapted physiological state cause the most threat as these are able to multiply in the food matrix.

L. monocytogenes cellular adaptation to active growth under high salinity conditions, also known as the osmoadaptive response, was examined in four strains. A large number of *L. monocytogenes* genes showed strain specific differential expression stimulated by post adaptive response to sub-lethal levels of NaCl induced hyperosmotic stress. Variation in gene expression (change is transcription of >twofold, $p<0.05$) was in particular evident in the relatively salt sensitive strain 70-1700.

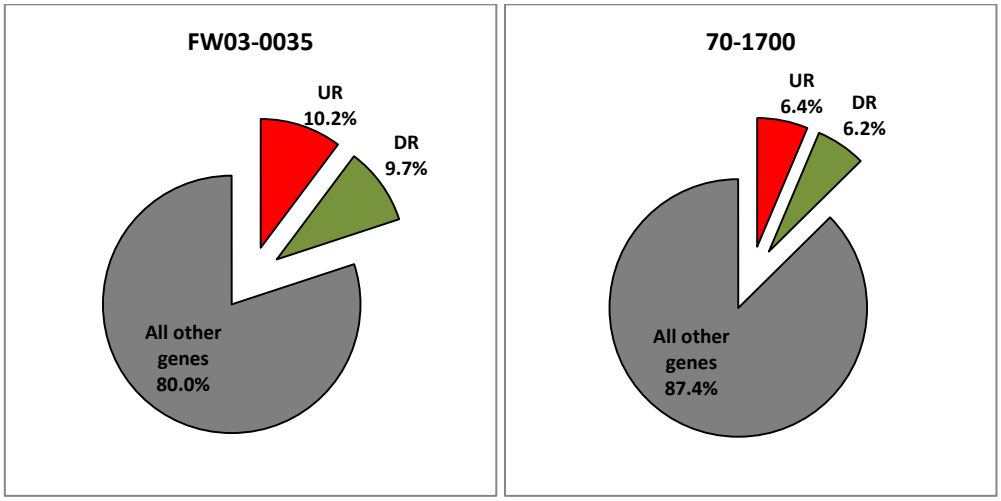
Following adaptation to hyperosmotic stress induced by 10.0% (w/v) NaCl strain ATCC19115 showed ≥two-fold up-regulation of 404 genes and down-regulation of 325 genes out of 2857 total number of homologous genes from the *L. monocytogenes* EGD-e genome (Fig. 3.1).

Individual gene expression of a relatively salt tolerant strain, ScottA showed ≥ two-fold up-regulation of 206 genes and ≥two-fold down-regulation of 201 following adaptation to hyperosmotic environment induced by 12.0% (w/v) NaCl (Fig. 3.1).



UR: up-regulated genes (LR>1, P<0.05); DR: down-regulated genes (LR<-1, P<0.05). Percentages calculated based on total number of genes in EGD-e genome (5827).

Figure 3. 1 Schematic representation of overall transcription results for hyperosmotic adaptive response observed in *L. monocytogenes* strains ATCC19115 and ScottA.



UR: up-regulated genes (LR>1, P<0.05); DR: down-regulated genes (LR<-1, P<0.05). Percentages calculated based on total number of genes in EGD-e genome (5827).

Figure 3. 2 Schematic representation of overall transcription results for hyperosmotic adaptive response observed in *L. monocytogenes* strains FW03-0035 and 70-1700.

Strain FW03-0035, at the same level of hyperosmotic pressure was found to increase expression of 292 genes and suppress the expression of 278 genes (Fig. 3.2). Up-regulation of only 182 and down-regulation of 178 genes was observed in a relatively salt sensitive strain 70-1700 following prolonged exposure to 8.0% (w/v) NaCl (Fig. 3.2). Adaptation to hyperosmotic stress induced significant expression variation in several gene functional categories and sub-categories, as established using the *T-value* scoring procedure (Boorsma *et al.*, 2005; Bowman *et al.*, 2008). The *T-value* scoring results are summarized in Table 3.1.

Table 3. 1 Gene enrichment response of four *L. monocytogenes* strains to NaCl stresses organized into functional gene sets.

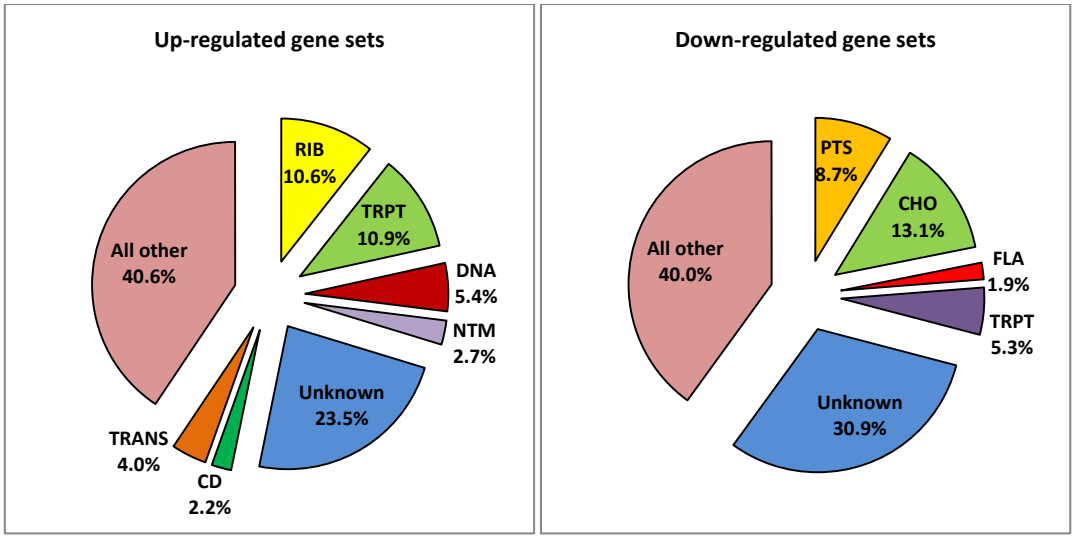
Gene categories and subcategories (no. of genes)	ATTC19115		ScottA		FW03-0035		70-1700	
	T*	P#	T*	P#	T*	P#	T*	P#
Amino acid metabolism								
Aromatic amino acids biosynthesis (23)	**2.15**	0.0437	**2.15**	0.0437	**2.68**	0.0141	**2.08**	0.0498
Branched chain amino acids biosynthesis (16)	**1.95**	0.0720	**1.11**		**0.51**		**2.92**	0.0112
Lysine biosynthesis (16)	**1.64**		**1.75**		**1.94**	0.0726	**1.77**	0.0980
Urea cycle and metabolism of amino groups (12)	**1.03**		**1.11**		**1.15**		**1.73**	
Peptidases (46)	**0.75**		**1.60**		**2.81**	0.0074	**2.10**	0.0417
Glycine/serine/threonine metabolism (26)	**0.58**		**1.52**		**0.91**		**1.86**	0.0759
Alanine/aspartate metabolism (28)	**-0.02**		**-0.11**		**1.40**		**1.12**	
Histidine metabolism (14)	**-0.73**		**-0.92**		**-2.23**	0.0452	**-2.60**	0.0234
D-Alanine metabolism (7)	**-0.81**		**-1.99**		**-1.27**		**-1.84**	
Branched amino acids metabolism (7)	**-1.34**		**-1.71**		**-0.69**		**-1.61**	
Other amino acid-related metabolism (14)	**-1.66**		**-0.82**		**-1.62**		**-2.28**	0.0417
Carbohydrate Metabolism								
Aminosugars metabolism (15)	**1.42**		**-0.20**		**1.32**		**1.41**	
Nucleotide sugars metabolism (11)	**-0.13**		**-1.59**		**-0.63**		**-1.49**	
Pyruvate metabolism (32)	**-0.84**		**-2.10**	0.0440	**0.79**		**0.21**	
Glycolysis/Gluconeogenesis (37)	**-0.97**		**-3.38**	0.0018	**-0.41**		**-0.80**	
Other carbohydrate-related metabolism (49)	**-1.66**		**-2.28**	0.0271	**-3.11**	0.0032	**-2.44**	0.0187
Pentose phosphate pathway (34)	**-3.08**	0.0042	**-2.83**	0.0081	**-2.28**	0.0296	**-0.16**	
Galactose metabolism (16)	**-3.17**	0.0069	**-1.46**		**-3.38**	0.0045	**1.29**	
Butanoate metabolism (19)	**-3.27**	0.0045	**-5.24**	6.6E-05	**-1.08**		**-0.32**	
Fructose/Mannose metabolism (68)	**-4.39**	4.2E-05	**-5.14**	2.7E-06	**-5.76**	2.4E-07	**0.39**	
Pdu/Eut operon (36)	**0.64**		**-3.28**	0.0024	**1.40**		**4.31**	1.3E-04
Cell division								
Cell division (30)	**3.37**	0.0022	**4.72**	6.0E-05	**4.36**	0.0002	**2.27**	0.0311
Septal ring (9)	**2.92**	0.0225	**3.86**	0.0062	**2.87**	0.0240	**2.52**	0.0397
Cell motility								
LPXTG motif (22)	**-1.18**		**-1.99**	0.0609	**-2.85**	0.0099	**-3.06**	0.0062
Type III secretion system (11)	**-1.80**		**-3.18**	0.0112	**-2.38**	0.0415	**-2.61**	0.0284
Flagellar assembly (27)	**-3.16**	0.0041	**-6.57**	6.9E-07	**-4.45**	0.0002	**-3.78**	0.0009
Cell wall associated								
Gram-positive anchored protein (39)	**-0.60**		**-2.67**	0.0112	**-2.91**	0.0061	**-3.33**	0.0020
Teichoic acid biosynthesis (16)	**-0.82**		**-1.96**	0.0702	**-2.11**	0.0530	**-2.76**	0.0153
DNA repair/recombination								
DNA repair/recombination (72)	**2.26**	0.0271	**2.92**	0.0048	**3.09**	0.0028	**1.90**	0.0619

Gene categories and subcategories (no. of genes)	ATTC19115		ScottA		FW03-0035		70-1700	
	T*	P#	T*	P#	T*	P#	T*	P#
DNA replication complex (20)	**1.45**		**2.04**	0.0567	**2.25**	0.0373	**1.80**	0.0881
Energy Metabolism								
Oxidative phosphorylation (25)	**2.90**	0.0080	**1.39**		**1.56**		**-0.18**	
Lipid Metabolism								
Fatty acid biosynthesis (14)	**2.19**	0.0490	**2.78**	0.0166	**2.63**	0.0218	**2.11**	0.0569
Biosynthesis of steroid/terpenoid (16)	**2.07**	0.0573	**2.63**	0.0199	**2.36**	0.0332	**0.43**	
Peptidoglycan biosynthesis (16)	**1.72**		**2.65**	0.0189	**3.31**	0.0052	**2.13**	0.0519
Glycerolipid metabolism (15)	**-1.65**		**-2.36**	0.0347	**-1.39**		**-0.77**	
Membrane transport								
ABC Transporter General (171)	**3.60**	0.0004	**3.54**	0.0005	**3.90**	0.0001	**3.52**	0.0006
Transporter (iron) all (19)	**3.41**	0.0034	**2.10**	0.0511	**1.89**	0.0756	**0.83**	
ABC Transporter (peptides) (10)	**2.84**	0.0217	**3.45**	0.0087	**3.61**	0.0069	**2.35**	0.0467
Transporters All General (287)	**2.79**	0.0056	**3.17**	0.0017	**2.83**	0.0050	**2.41**	0.0166
Transporter (sodium) (12)	**1.73**		**2.69**	0.0226	**2.88**	0.0165	**1.67**	
Transporter (zinc) (7)	**1.52**		**1.32**		**0.94**		**-0.02**	
Transporters All (amino acids) (18)	**1.47**		**0.53**		**0.78**		**0.84**	
Protein export (19)	**0.56**		**2.24**	0.0385	**2.10**	0.0513	**0.87**	
Transporter (compatible solutes) (8)	**0.32**		**0.25**		**1.33**		**3.69**	0.0102
ABC Transporter (sugars) (25)	**-0.64**		**0.96**		**-0.38**		**1.27**	
PTS system (90)	**-11.45**	4.0E-19	**-6.44**	6.1E-09	**-9.60**	2.4E-15	**-1.27**	
Metabolism of Cofactors and Vitamins								
Pantothenate/CoA biosynthesis (15)	**2.67**	0.0193	**2.15**	0.0512	**2.23**	0.0436	**1.59**	
Isoprenoid quinone biosynthesis (9)	**2.48**	0.0422	**1.94**	0.0935	**3.30**	0.0132	**1.47**	
Nicotinate/nicotinamide metabolism (13)	**1.52**		**0.09**		**1.87**	0.0877	**1.96**	0.0755
Vitamin B6 metabolism (6)	**0.37**		**-1.41**		**-1.24**		**0.78**	
One carbon pool by folate (11)	**-0.21**		**1.37**		**1.05**		**0.74**	
Folate biosynthesis (16)	**-1.57**		**-1.14**		**-1.69**		**-1.28**	
Nucleotide Metabolism								
Purine biosynthesis/metabolism (58)	**3.17**	0.0025	**4.09**	0.0001	**4.61**	2.3E-05	**3.20**	0.0022
Purine and Pyrimidine metabolism (82)	**3.06**	0.0030	**4.10**	9.8E-05	**4.55**	1.9E-05	**2.66**	0.0094
Pyrimidine metabolism (36)	**2.96**	0.0055	**2.57**	0.0146	**3.76**	0.0006	**2.16**	0.0382
Pyrimidine biosynthesis/metabolism (44)	**2.27**	0.0285	**2.12**	0.0395	**3.13**	0.0032	**1.27**	
Purine metabolism (44)	**2.05**	0.0464	**2.51**	0.0159	**3.56**	0.0009	**2.98**	0.0047
Purine biosynthesis (15)	**1.78**	0.0992	**3.93**	0.0017	**3.25**	0.0063	**1.44**	
Transcription and Translation								
Ribosome (58)	**10.19**	2.3E-14	**11.58**	1.7E-16	**14.43**	1.6E-20	**9.62**	1.8E-13
Translation/ribosome associated (56)	**3.00**	0.0041	**4.37**	5.7E-05	**4.52**	3.4E-05	**3.86**	0.0003

Gene categories and subcategories (no. of genes)	ATTC19115		ScottA		FW03-0035		70-1700	
	T*	P#	T*	P#	T*	P#	T*	P#
DNA polymerase (10)	**2.65**	0.0295	**1.83**		**1.36**		**1.16**	
Translation factors (12)	**2.61**	0.0259	**2.94**	0.0147	**3.04**	0.0125	**1.74**	
Transcription associated (9)	**2.24**	0.0598	**1.69**		**2.94**	0.0217	**2.51**	0.0402
Aminoacyl-tRNA biosynthesis (24)	**2.04**	0.0534	**3.50**	0.0020	**3.31**	0.0032	**2.30**	0.0312
GTP-binding protein (22)	**0.93**		**2.64**	0.0158	**3.38**	0.0030	**2.76**	0.0121
Protein folding/processing (18)	**0.77**		**0.42**		**2.06**	0.0557	**2.09**	0.0531
Transcription regulation								
Transcription regulation (BglG family) (15)	**-2.27**	0.0408	**-1.56**		**-2.95**	0.0114	**-0.74**	
Transcription regulation (MarR family) (14)	**-1.53**		**-1.71**		**-2.02**	0.0662	**-1.94**	0.0759
Transcription regulation (AraC family) (6)	**-0.82**		**-0.84**		**-1.04**		**1.31**	
Transcription regulation (crp/fnr family) (15)	**-0.63**		**-0.69**		**-0.97**		**-1.78**	0.0988
Transcription regulation General (231)	**-0.44**		**-2.07**	0.0397	**-3.42**	0.0007	**-2.48**	0.0139
Transcription regulation (DeoR family) (8)	**1.72**		**-1.47**		**-0.08**		**-0.24**	
Transcription regulation (GntR family) (17)	**1.73**		**0.73**		**-0.36**		**0.86**	
Signalling								
Two-component signal transduction system (39)	**1.49**		**-0.37**		**1.00**		**1.62**	
Chemotaxis (13)	**-1.30**		**-3.41**	0.0058	**-1.90**	0.0832	**-1.19**	
Other signal transduction (23)	**-2.18**	0.0408	**-1.02**		**-0.36**		**-0.56**	
Virulence								
TN916 (13)	**-1.30**		**-1.81**	0.0984	**-0.69**		**-2.70**	0.0207
Transposases (8)	**-1.16**		**-1.60**		**-2.17**	0.0735	**-2.98**	0.0248
Virulence-related (14)	**0.41**		**-0.33**		**-1.70**		**0.01**	
Autolysin/cell wall hydrolase (13)	**0.82**		**1.83**	0.0938	**1.08**		**0.30**	

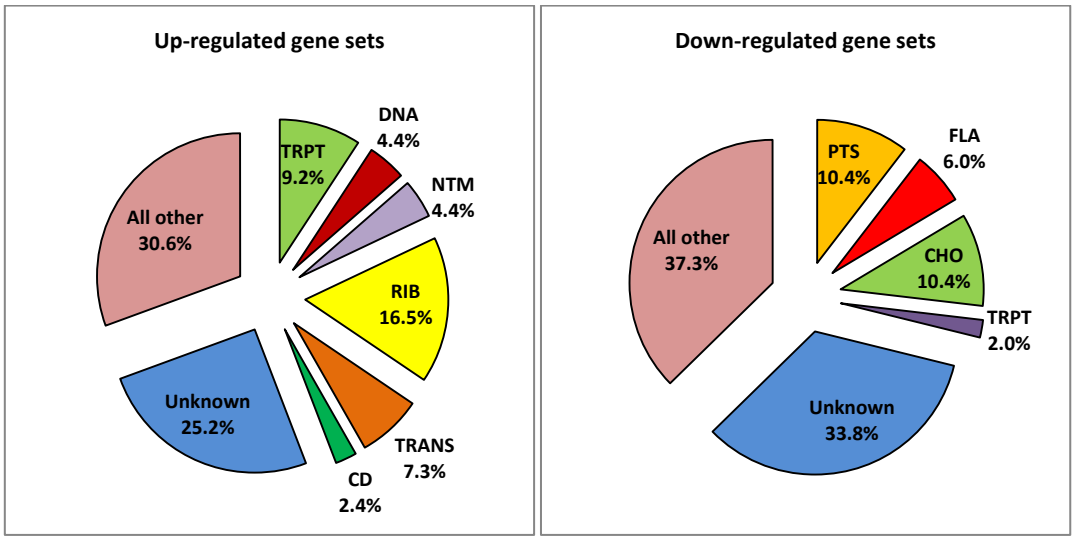
*T-value scores were determined from the expression data using the approach of Boorsma *et al*. (2005).
P-values not shown are >0.1

The most significant up-regulated gene sets observed in all four strains exposed to hyperosmotic stress included ribosomes with *T-value* scores ranging from 9.62 in relatively salt sensitive strain 70-1700 to 14.43 in relatively salt tolerant strain FW03-0035, (Table 3.1). Genes classified in gene sets in the overall subgroup associated with transcription and translation included a large number of up-regulated genes with 14.6% of all significantly up-regulated genes in strain ATCC19115 (Fig. 3.3), a collective value of 23.8% in strain ScottA (Fig. 3.3), 21.6% in FW03-0035 (Fig. 3.4) and 14.2% of all significantly up-regulated genes observed in a relatively salt sensitive strain 70-1700 (Fig. 3.5).



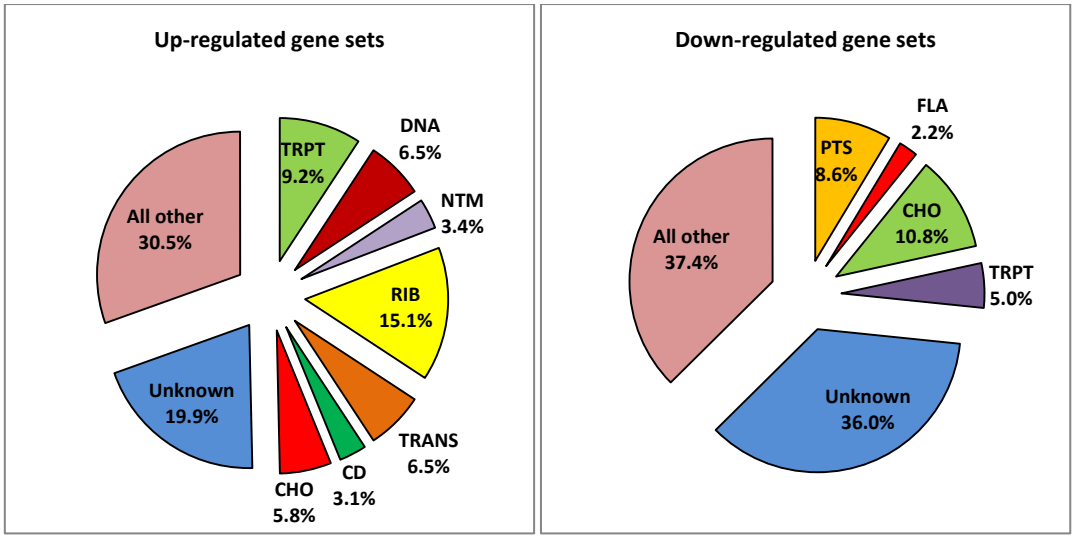
DNA-group of genes associated with DNA repair and recombination and transcription; TRANS- genes associated with translation, TRPT-genes encoding various transporters; RIB-genes encoding ribosomal proteins; CHO- genes associated with carbohydrate metabolism; PTS-genes associated with phosphotransferase system, FLA-genes encoding flagella assembly proteins, CD-genes associated with cell division, NTM- genes related to nucleotide metabolism.

Figure 3.3 Schematic representation of overall gene expression profile results for strain ATCC19115 adapted to hyperosmotic stress induced by 10.0% (w/v) NaCl.



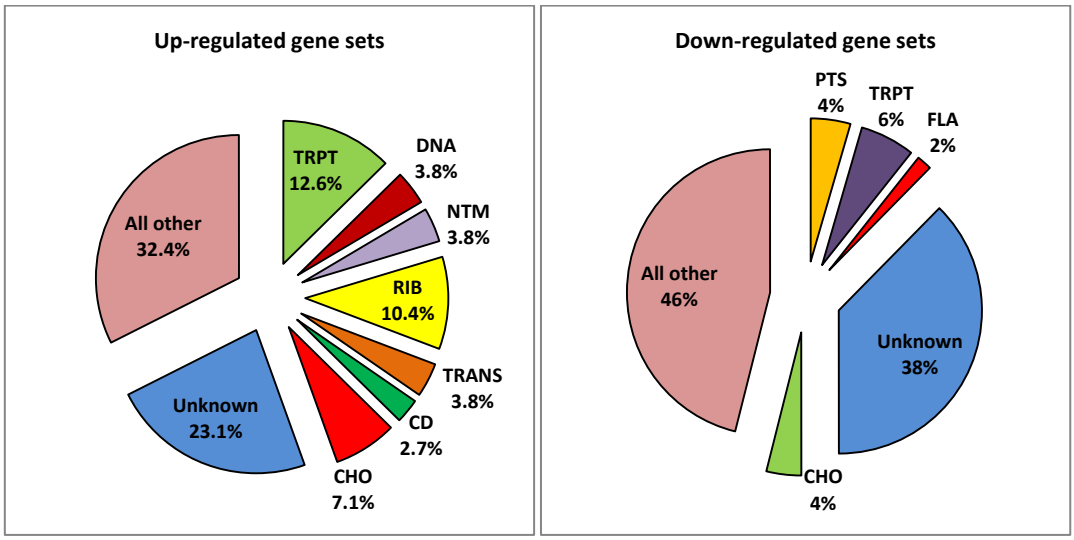
DNA-group of genes associated with DNA repair and recombination and transcription; TRANS- genes associated with translation, TRPT-genes encoding various transporters; RIB-genes encoding ribosomal proteins; CHO- genes associated with carbohydrate metabolism; PTS-genes associated with phosphotransferase system, FLA-genes encoding flagella assembly proteins, CD-genes associated with cell division, NTM- genes related to nucleotide metabolism.

Figure 3.4 Schematic representation of overall gene expression profile results for strain ScottA adapted to hyperosmotic stress induced by 12.0% (w/v) NaCl.



DNA-group of genes associated with DNA repair and recombination and transcription; TRANS- genes associated with translation, TRPT-genes encoding various transporters; RIB-genes encoding ribosomal proteins; CHO- genes associated with carbohydrate metabolism; PTS-genes associated with phosphotransferase system, FLA-genes encoding flagella assembly proteins, CD-genes associated with cell division, NTM- genes related to nucleotide metabolism.

Figure 3.5 Schematic representation of overall gene expression profile results for strain FW03-0035 adapted to hyperosmotic stress induced by 12.0%(w/v) NaCl.



DNA-group of genes associated with DNA repair and recombination and transcription; TRANS- genes associated with translation, TRPT-genes encoding various transporters; RIB-genes encoding ribosomal proteins; CHO- genes associated with carbohydrate metabolism; PTS-genes associated with phosphotransferase system, FLA-genes encoding flagella assembly proteins, CD-genes associated with cell division, NTM- genes related to nucleotide metabolism.

Figure 3.6 Schematic representation of overall gene expression profile results for strain 70-1700 adapted to hyperosmotic stress incused by 8.0% (w/v) NaCl.

Significant up-regulation was also observed in gene sets associated with nucleotide metabolism, cell division, DNA repair/recombination, lipid metabolism in particular those involved in peptidoglycan synthesis (not included in charts due to overall small percentage). A large proportion of up-regulated

genes had an unknown function with 23.5% of all significantly up-regulated genes in ATCC19115 (Fig. 3.3), 25.2% strain ScottA (Fig. 3.4), 19.9% in FW03-0035 (Fig. 3.5) and 23.1% in 70-1700 (Fig. 3.6).

Most down-regulated gene sets were associated with phosphotransferase system (PTS) dedicated to carbohydrate up-take, with *T-value* scores ranging from -1.27 to -11.45 (Table 3.1). Overall PTS associated genes represented 8.8% of all significantly down-regulated genes in strain ATCC19115 (Fig. 3.3), 10.4% in strain ScottA (Fig. 3.4), 8.6% in FW03-0035 (Fig. 3.5) and 4.0% in 70-1700 (Fig. 3.6). Down-regulation was also evident in gene sets related to flagella assembly, carbohydrate metabolism in particular fructose/mannose metabolism and pentose phosphate pathway. Interestingly a significant proportion of genes associated with carbohydrate metabolism were among the activated genes in the osmotolerant strain FW03-0035, representing 5.8% of all of its significantly up-regulated genes (Fig. 3.5). That proportion was 7.1% of all significantly up-regulated genes in a relatively salt sensitive strain 70-1700 which was significantly higher than the number of significantly down-regulated genes, found to be at 4.0%, in the same functional category. A large proportion of down-regulated genes were uncharacterized, these represented 30.9-38.0% of all significantly down-regulated genes (Fig. 3.3 to 3.6).

3.3.2 Evaluation of osmo-adaptive response of four *L. monocytogenes* strains.

3.3.2.1 Effect of osmoadaptation on membrane transporter genes.

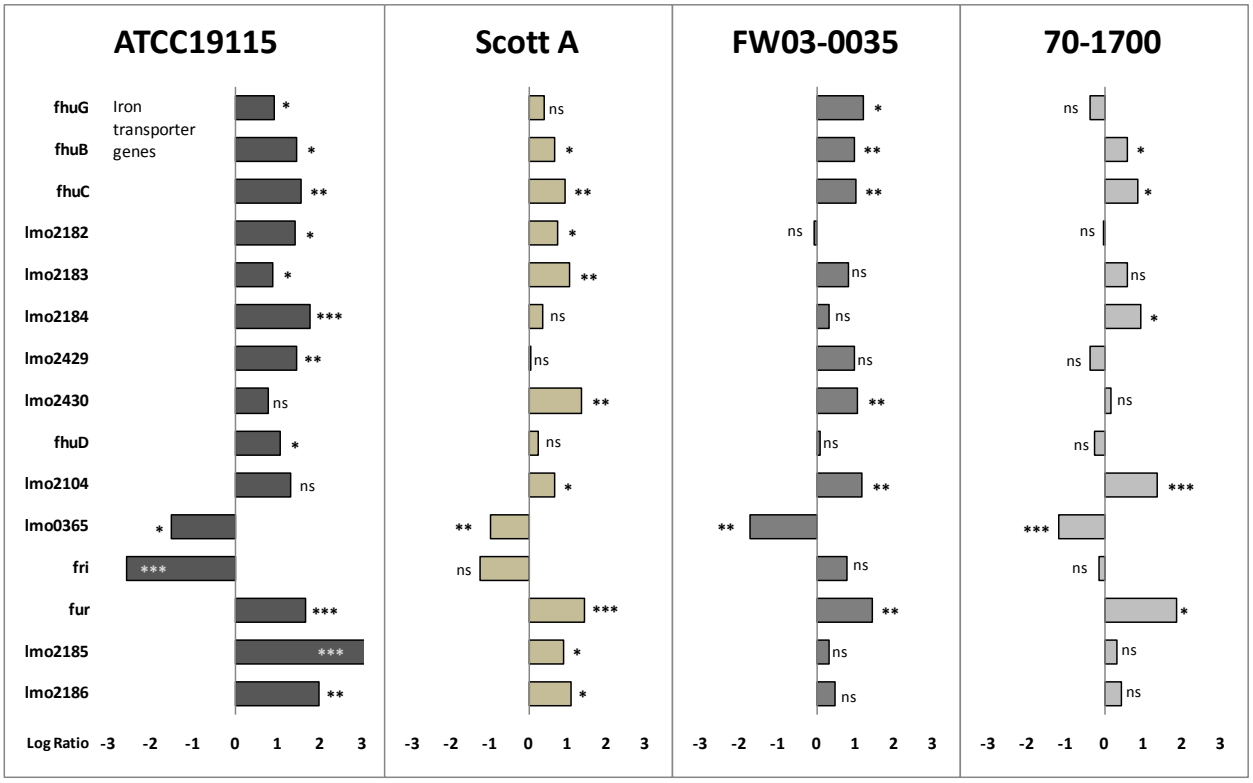
Osmoadaptation resulted in an overall up-regulation of general membrane transport with gene sets encoding all general and ABC-type transporters being up-regulated with *T-value* scores of 2.41 to 3.17 and 3.52 to 3.90, respectively, for the four strains investigated (Table 3.1).

3.3.2.1.1 Hyperosmotic adaptation enhanced iron transport genes.

Genes encoding iron transporters showed significant up-regulation in three strains with *T-value* scores of 3.41, 2.10, and 1.89 for ATCC19115, ScottA and FW03-0035 respectively. The less tolerant strain 70-1700 showed no significant change in transcription of this gene group (*T-value* 0.83, Table 3.1). Microorganisms require iron as a cofactor for indispensable metabolic pathways, including glycolysis, energy generation, DNA synthesis, detoxification of oxygen radicals, and also functions as

a stimulus capable of regulating virulence gene expression in pathogenic bacteria (Neirlands, 1995). Production and extracellular secretion of siderophores is used by majority of bacteria to overcome iron sequestration (Neirlands, 1995). *L. monocytogenes* is unable to secrete iron chelators. Rather, it utilizes the ferric siderophores of other organisms or other iron-containing compounds including catecholamines, haemin, and haemoglobin to obtain iron (Jin *et al.*, 2006).

The *fhu*GBC operon (*lmo1957*, *lmo1958*, *lmo1960*) was on average consistently up-regulated in all strains, with *fhu*C (*lmo1960*) showing significant up-regulation of ≥ two-fold in all four strains (Fig. 3.7).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.7 Expression of genes associated with transport and storage of iron in four osmo-adapted *L. monocytogenes* strains.

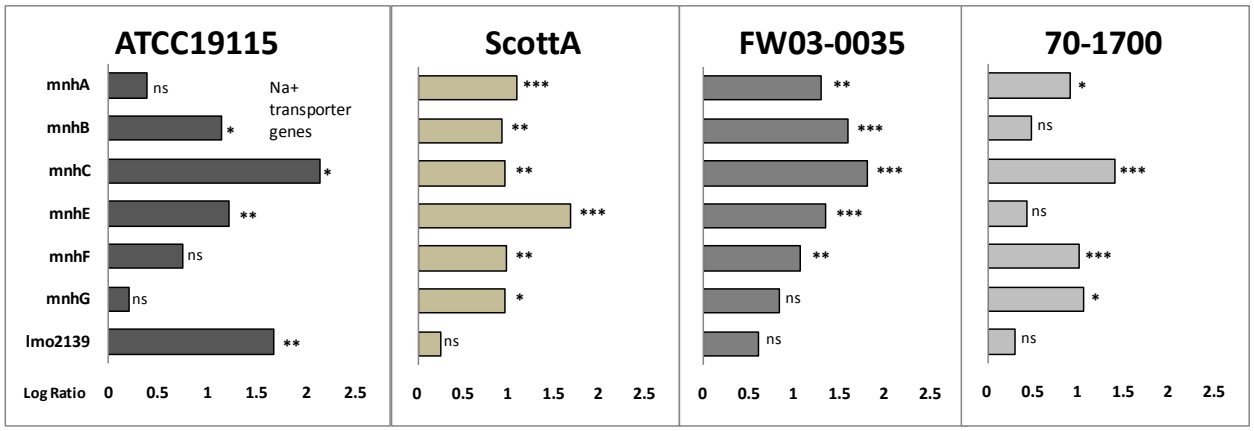
In *L. monocytogenes* this locus codes a highly specific ABC transporter for ferric hydroxamates, a type of siderophores produced by other organisms (Jin *et al.*, 2006). Up-regulation of *lmo2429-2430* was observed clearly in ATCC19115 (Fig. 3.7), this gene has been shown to encode a permease system specific for uptake of haemin and haemoglobin (Jin *et al.*, 2006). A difference in gene

expression between the strains may indicate a strain specific preference in iron containing substance for growth under osmotic stress. Induction of iron transporter genes suggests that cells grown under osmotic stress experience iron limitation a phenomenon also observed in *B. subtilis* (Steil *et al.*, 2003). Increase in iron demand of bacterial cells cultivated in osmotically stressful environment could be due to an increased turnover of iron being utilized as a cofactor in enhanced cellular maintenance processes observed in these cells. A ferric up-take regulator *fur* (*lmo1956*) showed ≥ two-fold up-regulation in all four strains (Fig. 3.7). Fur proteins are believed to monitor intracellular iron levels in bacteria including *L. monocytogenes* and negatively regulate iron uptake genes (Harvie *et al.*, 2005; Ledala *et al.*, 2007; Newton *et al.*, 2005; Stojiljkovic *et al.*, 1994; Xiong *et al.*, 2000). However its function in *L. monocytogenes* iron transport regulation has not been elucidated fully. It is possible that Fur negative regulation of iron transporter genes in this organism is highly selective repressing genes, such as *lmo*0365 (Fig. 3.7), which showed significant down-regulation ≥ two-fold in all strains, but not directly effecting or perhaps positively regulating other genes. Fur regulation could be essential for other metabolic pathways not exclusively for control of iron transport. For instance in *E. coli* Fur regulates genes involved in purine, pyrimidine and methionine biosynthesis (Stojiljkovic *et al.*, 1994).

Intracellular iron in bacteria is stored in inactive form bound to a ferritin molecule, to protect the cell from the toxic nature of free iron molecules. A single ferritin gene, *fri* (*lmo0943*) exists in *L. monocytogenes* (Dussurget *et al.*, 2005; Olsen *et al.*, 2005). Fri has been shown to promote stress tolerance to oxidative stress, heat and cold stresses in this organism (Olsen *et al.*, 2005). However it showed no up-regulation during adaptive osmotic stress response in any of the four strains (Fig. 3.7). This suggests that the internal concentration of this iron carrier maybe sufficient perhaps reflecting lack of saturation of Fri ferritin with iron in osmotically stressed cells. However, it is unknown whether *L. monocytogenes* possesses other iron carrier molecules which may be involved in intracellular iron storage, which may be more efficient than Fri under stressful osmotic conditions. A *fri* mutant lacking functional Fri protein was shown to maintain growth, albeit impaired, under iron limited conditions, suggesting the possible involvement of other proteins in intracellular iron storage of this organism (Olsen *et al.*, 2005).

3.3.2.1.2 Evidence of enhanced transcription of sodium transport genes in osmo-adapted cells.

Activation of sodium transporters was evident in all four strains of *L. monocytogenes* following prolonged exposure to salt stress, with *T-value* scores ranging from 1.67 to 2.88 (Table 3.1) with an average log ratio of the small number of genes in the set ranging from 1.1 to 1.5 (Fig. 3.8).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.8 Expression of genes associated with transport of sodium ions in four osmo-adapted *L. monocytogenes* strains.

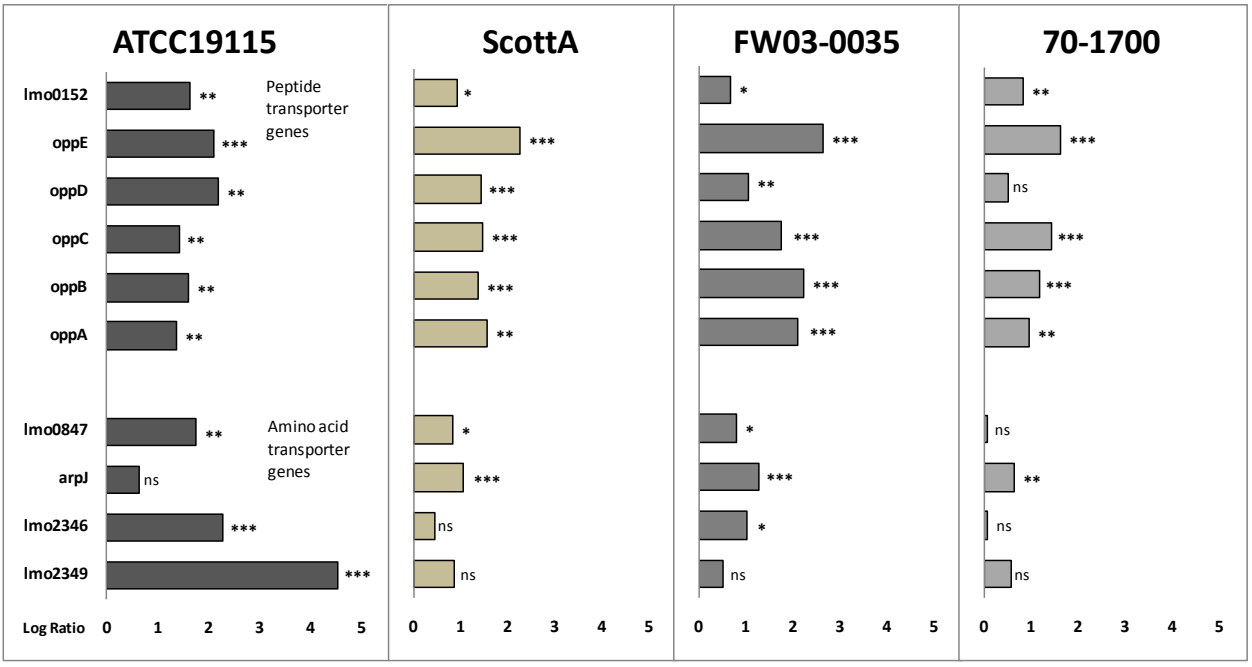
Sodium proton antiporters are vital in transporting Na^+ across the cytoplasmic membrane of all living cells (Krulwich *et al.*, 2001). In bacteria, the antiporter extrudes Na^+ in exchange for H^+. The Na^+/H^+ antiporters have several functions, such as maintenance of intracellular pH homeostasis, regulation of cell volume, the establishment of electrochemical potential of Na^+ and detoxification of cells from toxic sodium cations (Krulwich *et al.*, 2001). The later function of antiporters appeared to be of significance in cells grown under hyperosmotic stress. Up-regulation of antiporter genes has been linked to salt tolerance in *E. coli* and *Cyanobacteria spp*. (Blanco-Rivero *et al.*, 2005; Padan & Schuldiner, 1994). *L. monocytogenes mnh* operon encodes a Na^+/H^+ antiporter, which showed an overall up-regulation in all four strains (Fig. 3.8). It appears that as the extracellular concentration of Na^+ increases more Na^+ ions enter the cytosol down the concentration gradient, thus to sustain homeostasis *L. monocytogenes* activates sodium transporters which actively remove the intracellular Na^+ ions. From the gene expression pattern obtained in this study one may speculate that salt tolerance in *L. monocytogenes* may be linked to the efficiency of the Na^+/H^+ antiporters, as the more tolerant strains, Scott A and FW03-0035, appeared to increase expression of active sodium transporter-related genes to a greater degree (Fig. 3.8). Alternatively the enhanced sodium

transporter genes may reflect the osmotic composition of the medium. The more NaCl tolerant strains were cultivated under greater osmotic pressure, and as such the maintenance of cytoplasmic homeostasis was potentially more active in these cells.

3.3.2.1.3 Transport of peptides and amino acids in osmo-adapted cells.

Growth under hyperosmotic stress appeared to significantly activate gene sets associated with transport of peptides from extracellular environment in all four strains tested with *T-value* scores ranging from 2.35 to 3.61 (Table 3.1).

The *opp* operon (*lmo2192-lmo2196*) showed significant up-regulation in all the strains tested (Fig. 3.9). This operon encodes an ATP-dependent oligopeptide permease, expression of which has been linked to survival of *L. monocytogenes* at low temperature (Borezee *et al.*, 2000).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.9 Expression of genes associated with transport of peptides and amino acids in four osmo-adapted *L. monocytogenes* strains.

A gene encoding a presumed oligopeptide ABC transporter, *lmo0152* was also up-regulated ≥two-fold in all four strains (Fig. 3.9) further emphasizing the impending importance of peptide transport in this organism cultivated under hyperosmotic pressure.

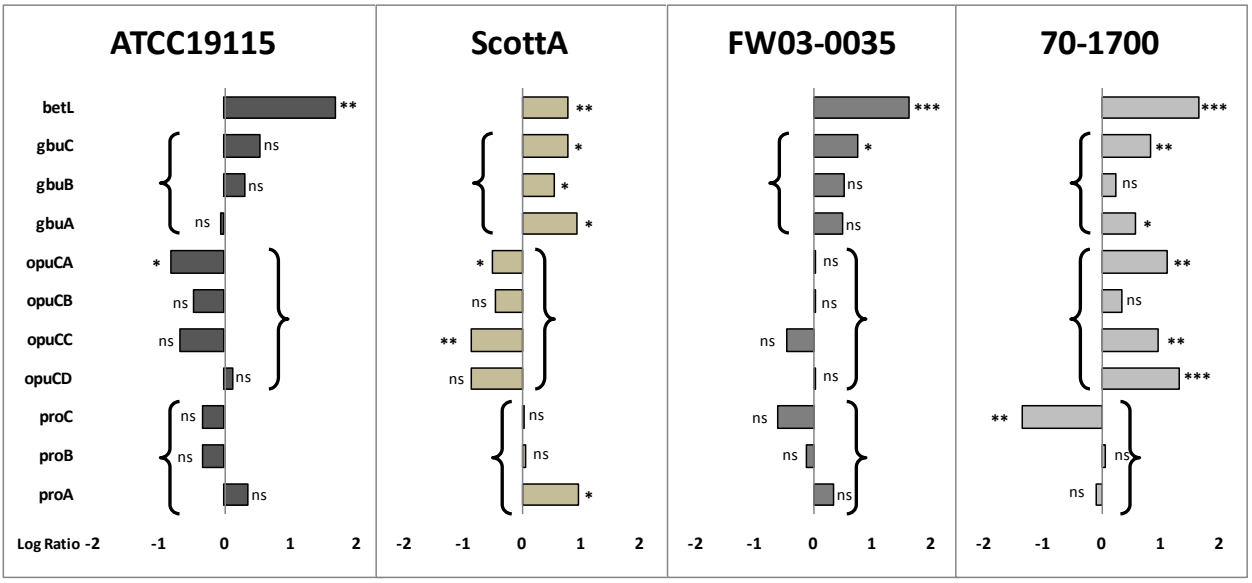
Interestingly, the expression of a proton motive force-dependent di- and tripeptide transporter (DtpT) encoded by *lmo0555* was not significantly affected by hyperosmotic stress, suggesting a preference for energy driven ABC transporters for acquisition of peptides under the experimental conditions of this study.

The exact benefit of activation of oligopeptide transcorters in *L. monocytogenes* cells under high osmotic pressure is unclear. Uptake of exogenous peptides containing glycine, hydroxyproline and proline has previously been shown to enhance osmotolerance of *L. monocytogenes*, leading to intracellular accumulation of peptides and free amino acids (Amezaga *et al.*, 1995). In addition, *L. monocytogenes* can utilize oligopeptides containing up to eight residues, which are ultimately hydrolyzed by internal peptidases to serve as a source amino acids essential for growth (Borezee *et al.*, 2000; Verheul *et al.*, 1998). Activation of oligopeptide transcorters may also function in maintaining the integrity of the cellular envelope, in Gram-negative bacteria for instance oligopeptide transporters have been shown to be involved in recycling the cell wall peptides for synthesis of new peptidoglycan (Borezee *et al.*, 2000).

Amino acid transporters encoded by *lmo0847*, *lmo2346*, *lmo2349* and *arpJ* (*lmo2250*) encoding an arginine transporter showed significant activation in one or more strains tested (Fig. 3.9). The discrepancy in gene expression observed between strains may be due to strain specific requirement and or preference for certain amino acids. For instance the Scott A strain is known to require six amino acids (Leu, Ile, Arg, Met, Val and Cys) where as others only require methionine and cysteine for optimal growth (Hsiang-Ning & Hodgson, 2003; Premaratne *et al.*, 1991). Enhanced up-take of amino acids during growth in hyperosmotic environment could reflect the intracellular demand for increased protein synthesis in such stressed cells, while also assisting in maintaining cellular osmolarity. The intracellular pools of both amino acids and peptides in *L. monocytogenes* have been shown to increase with external osmolarity in a manner consistent with a role in osmoprotection (Amezaga *et al.*, 1995).

3.3.2.1.4 Transport of compatible solutes

L. monocytogenes adaptation to high osmolarity results from accumulation of a number of compatible solutes, so termed due to their compatibility with vital cellular processes at high internal concentrations. Only one of the four strains examined, 70-1700 showed an overall significant up-regulation in genes associated with uptake of compatible solutes with a *T-value* score of 3.69 (Table 3.1). Due to the nature of the *T-value* scoring, individual gene expression of this gene set was examined for a better understanding of individual transporter involvement in the hyperosmotic response of this organism.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.10 Expression of genes associated with transport of compatible solutes in four osmo-adapted *L. monocytogenes* strains.

Glycine betaine has long been established as the preferred and most effective compatible solute for *L. monocytogenes* (Angelidis *et al.*, 2002). The *betL* (*lmo2092*) showed significant up-regulation in all strains (Fig. 3.10). The BetL transporter is the secondary betaine transporter in *Listeria*, driven by the membrane potential of the cell, transporting an ion in symport with the osmolyte. The level of permease activity has been shown to be controlled by the ionic strength of the growth medium (Gerhardt *et al.*, 1996; Sleator *et al.*, 2003b). Up-regulation of transcript expression of this gene is of benefit to bacteria grown under hyperosmotic stress as it is less energy costly than the ATP-dependent Gbu transporter. BetL expression is independent of σ^B and it is known to be responsible for the majority of glycine betaine uptake in *L. monocytogenes* immediately following osmotic shock,

and long-term protection for low levels of stress (Cetin *et al.*, 2004; Mendum & Tombras Smith, 2002; Sleator *et al.*, 2003b). In order to save the much needed energy to drive other important ATP-dependent reactions, *L. monocytogenes* osmotically adapted cells appeared to continuously transport the compatible solute glycine betaine via the BetL permease even at high concentrations of NaCl, previously reported to suppress this transporter (Sleator *et al.*, 2003b).

The *gbu* operon, encoding the primary GB transporter showed significant up-regulation in ScottA only with an average of two-fold increase in transcription in this strain and a somewhat less profound response in FW03-0035 and 70-1700 with overall 1.5-fold up-regulation (Fig. 3.10). The *opu* operon responsible for the uptake of carnitine (second most preferred compatible solute) showed up-regulation on average of ≥two-fold in singular strain 70-1700 (Fig. 3.10). Both Gbu and Opu are ATP-dependent ABC family of transporters and are under control of the stress response regulator σ^B with *gbu*A being transcribed from dual promoters and *opu*C being entirely σ^B – dependent (Cetin *et al.*, 2004). Activation of *gbu* and *opu* operons suggests increased up-take of GB and carnitine via these transporters by strains ScottA and 70-1700 under prolonged osmotic stress. In addition to acquisition of GB via the BetL transporter it appears that ScottA and to a lesser extent FW03-0035 also engaged the energy costly Gbu transporter mechanism, perhaps reflecting the level of stress experienced by these cells grown at 12.0% (w/v) NaCl. One may speculate that additional accumulation of this compatible solute may aid in overcoming extreme osmotic stress. This is further accentuated by the observed gene expression in a relatively less salt tolerant strain 70-1700 in which both Gbu and Opu transporters were activated to protect against NaCl stress experienced by this strain.

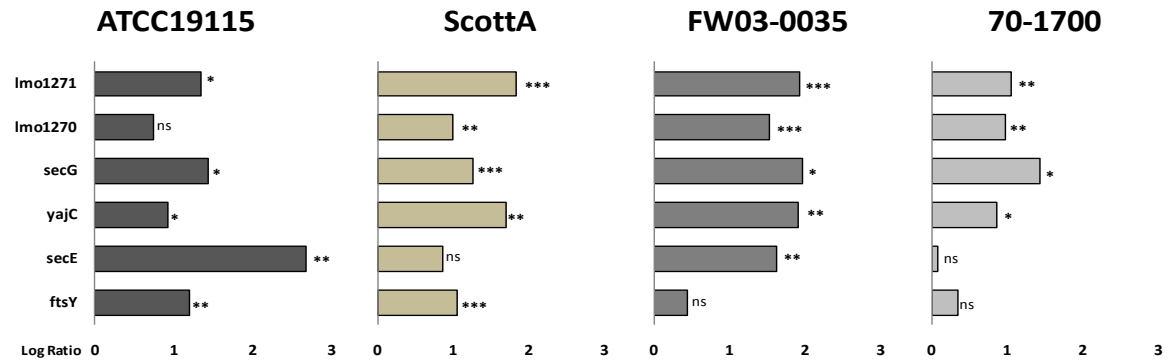
The marginal gene expression of compatible solute transporter genes observed was unexpected, as according to numerous studies acquisition of compatible solutes is a critical step in osmoadaptation in *L. monocytogenes* and a much stronger response was anticipated. It is possible that activation of these genes occurred much earlier in the adaptive response, provided that both of the primary ABC-transporter gene clusters are activated by the σ^B and consequently not captured in this experiment. The observed gene expression might be responsible for the ongoing top up of the cytosol with compatible solutes in these strains and not so much the initial uptake of these molecules.

Proline synthesis genes appeared to have no overall significant change in transcription levels when compared to the cells grown under control conditions. Synthesis of this potential compatible solute either occurred earlier in the adaptation response or played no part in osmoregulation in strains examined in this experiment. Proline on its own has been characterised as a weak compatible solute in *L. monocytogenes* (Amezaga *et al.*, 1995), it is much more effective when present in di-or tri-peptide compounds, up-take of which is most likely facilitated by the OppA oligopeptide transporter.

3.3.2.1.5 Hyperosmotic adaptation effects on protein export.

Bacterial surface proteins have a variety of functions one of which is to protect the cell from environmental challenges. In Gram-positive bacteria the first step in localization of proteins to the cell surface is transport through the cell membrane a process referred to as protein export. Proteins are transported in an unfolded form through the membrane channels formed by the highly conserved proteins of the Sec family, SecY and SecE, SecG, and YajC (Scott & Barnett, 2006). Prolonged growth under hyperosmotic stress appeared to activate genes associated with protein export in two strains grown at 12.0% (w/v) NaCl ScottA and FW03-0035 with *T-value* scores of 2.24 and 2.10 respectively (Table 3.1). It is possible that strains grown at higher osmolarity experience more 'stress' in terms of surface protein damage due to higher ionic strength of the growth medium. These cells experience more external damage and consequently require more proteins being secreted to repair the surface of these cells.

Individual gene expression pattern revealed significant up-regulation of *sec*G (*lmo2451*) and *yajC* (*lmo1529*) in all four strains (Fig. 3.11). It is believed that proteins secreted in bacteria via the Sec pathway are cleaved and consequently activated by type I signal peptidase during the process of secretion (Scott & Barnett, 2006; Yamane *et al.*, 2004).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.11 Expression of genes associated with protein export in four osmo-adapted *L. monocytogenes* strains.

Indeed two genes *lmo1270* and *lmo1271* encoding such peptidases, SipY and SipZ, were up-regulated in all four *L. monocytogenes* strains (Fig. 3.11). This hints to a certain involvement of the Sec family of transporters in adaptation to osmotic stress in *L. monocytogenes*. However the Gram-positive protein export mechanism is as yet poorly understood. It is therefore difficult to elaborate the exact involvement of the Sec family of transporters in hyperosmotic stress.

It is worth noting that the twin arginine translocation system encoded by the *tat*C (*lmo0361*) and *tat*A (*lmo*0362), responsible for transport of pre-folded proteins were suppressed during growth in hyperosmotic conditions in all the strains. This suggests a strong preference for the Sec family of transporters for protein export in the strains under the defined experimental conditions.

3.3.2.1.6 Carbohydrate transport in osmo-adapted cells.

Transport of carbohydrates showed overall suppression in osmo-adapted cells. Gene sets associated with PTS showed significant down-regulation in osmo-adapted cells of the three relatively salt tolerant strains with *T-value* scores ranging from -6.44 in ScottA to -11.45 in ATCC19115 (Table 3.1). The down-regulation of this gene group in a relatively salt sensitive strain 70-1700 was not statistically significant (Table 3.1). A number of genes showed significant down regulation up to 1136-fold (Table 3.2). It appears that intracellular intake of sugars from the growth medium is not a major priority in salt adapted cells, perhaps reflecting the reduced metabolism and growth rate of these cells relative to the control cultures.

Table 3. 2 Gene expression patterns of four *L. monocytogenes* strains associated with phosphotransferase system (PTS) observed during adaptive hyperosmotic stress response.

Gene	ATCC19115		ScottA		FW03-0035		70-1700		Function
	LR[#]	P	LR	P	LR	P	LR	P	
fruA	**1.12**	0.007	**-3.93**	1.5E-05	**0.92**	0.001	**0.99**	0.029	similar to PTS system, fructose-specific IIABC component
lmo0027	**-1.52**	0.008	**0.78**		**-1.47**	0.044	**-0.61**		similar to PTS system, beta-glucosides-specific IIABC component
lmo0373	**-1.16**	0.009	**-1.16**	0.004	**-1.25**	0.008	**-1.14**	0.001	similar to PTS system, beta-glucoside/cellobiose-specific IIC component
lmo0543	**-1.04**	0.050	**-0.78**	0.022	**-0.48**		**-0.55**	0.034	similar to PTS system, glucitol/sorbitol-specific IIB component
lmo0544	**-3.04**	0.001	**-1.31**	4.1E-04	**-2.05**	0.001	**-1.01**	0.009	similar to PTS system, glucitol/sorbitol-specific IIC component
lmo0781	**-1.45**	0.019	**-2.03**	5.4E-05	**-1.78**	3.3E-04	**0.15**		similar to PTS system, mannose-specific IID component
lmo0782	**-2.43**	0.001	**-2.63**	1.3E-05	**-1.85**	4.5E-04	**0.27**		similar to PTS system, mannose-specific IIC component
lmo0875	**-2.13**	1.4E-04	**-0.73**	0.015	**-1.48**	4.0E-05	**-1.06**	0.002	similar to PTS system, cellobiose-specific IIB component
lmo0901	**-2.44**	0.022	**-0.74**	0.006	**-1.47**	0.001	**-1.48**	2.0E-04	similar to PTS system, cellobiose-specific IIC component
lmo0914	**-1.57**	0.012	**-0.48**		**-0.81**	0.009	**-0.02**		similar to PTS system, cellobiose-specific IIB component
lmo0916	**-2.65**	0.022	**-0.39**		**-0.02**		**0.31**		similar to PTS system, cellobiose-specific IIA component
lmo1255	**-1.02**		**1.49**	0.001	**-0.76**		**0.79**	0.018	similar to PTS system, trehalose-specific IIBC component
lmo1719	**-4.12**	3.7E-06	**-1.72**	1.6E-04	**-1.72**	0.020	**0.47**		similar to PTS system, lichenan/cellobiose-specific IIA component
lmo1720	**-2.87**	0.001	**-1.58**	5.0E-04	**-2.24**	0.001	**0.29**		similar to PTS system, lichenan/cellobiose-specific IIB component
lmo2000	**1.79**	0.021	**1.02**	0.001	**0.17**		**0.29**		similar to PTS system, mannose-specific IID component
lmo2001	**1.08**		**0.59**		**-0.48**		**0.69**	0.048	similar to PTS system, mannose-specific IIC component
lmo2002	**-0.16**		**0.63**	0.022	**-0.62**		**1.50**	2.0E-05	similar to PTS system, mannose-specific IIB component
lmo2096	**0.19**		**-1.05**	0.014	**-1.10**	0.030	**1.19**	0.003	similar to PTS system, galacitol-specific IIC component
lmo2097	**1.51**		**-0.76**		**-0.53**		**1.46**	3.5E-05	similar to PTS system, galacitol-specific IIB component
lmo2098	**-0.17**		**-1.22**	0.013	**-1.38**	7.7E-05	**0.98**	0.003	similar to PTS system, galacitol-specific IIA component
lmo2259	**1.10**	0.008	**0.73**	0.036	**0.80**	0.006	**0.60**	0.038	similar to PTS system, beta-glucoside/cellobiose-specific IIA component
lmo2373	**-0.21**		**-2.29**	0.003	**-1.90**	0.025	**-1.08**	0.028	similar to PTS system, beta-glucoside/cellobiose-specific IIB component
lmo2649	**-4.76**	3.4E-04	**0.16**		**-0.98**	0.016	**-0.26**		similar to PTS system, mannitol/ascorbate-specific IIC component
lmo2650	**-3.92**	2.3E-05	**0.08**		**-1.17**	0.003	**-0.51**		similar to PTS system, mannitol/ascorbate-specific IIB component
lmo2651	**-2.18**	0.020	**0.54**	0.036	**1.01**	0.036	**0.46**		similar to PTS system, mannitol/ascorbate-specific IIA component
lmo2665	**-6.09**	1.4E-07	**-0.78**	0.039	**-3.26**	0.001	**0.17**		similar to PTS system, galacitol-specific IIC component
lmo2666	**-7.71**	1.6E-05	**-1.59**	0.007	**-3.99**	0.001	**0.05**		similar to PTS system, galacitol-specific IIB component
lmo2667	**-6.53**	0.004	**-1.96**	6.8E-05	**-3.61**	0.001	**-0.64**	0.032	similar to PTS system, galacitol-specific IIA component
lmo2683	**-10.15**	1.1E-07	**-1.50**	0.004	**-2.64**	0.002	**-0.76**	0.042	similar to PTS system, cellobiose-specific IIB component
lmo2684	**-9.46**	4.1E-07	**-3.09**	0.001	**-5.05**	0.001	**-1.58**	0.007	similar to PTS system, cellobiose-specific IIC component
lmo2685	**-8.68**	9.9E-08	**-2.01**	1.3E-04	**-3.71**	7.3E-05	**-1.23**	3.5E-04	similar to PTS system, cellobiose-specific IIA component
lmo2697	**-2.01**	0.002	**-1.75**	0.001	**-1.58**	2.7E-04	**-0.34**		putative PTS-dependent dihydroxyacetone kinase, phosphotransfer subunit
lmo2708	**-8.90**	2.8E-07	**-1.93**	0.001	**-1.85**		**-0.65**		similar to PTS system, cellobiose-specific IIC component
lmo2780	**-5.77**	1.7E-06	**-1.34**	0.031	**-2.13**	0.024	**-0.13**		similar to PTS system, cellobiose-specific IIA component
lmo2782	**-5.03**	1.2E-04	**-1.26**	0.015	**-2.42**	9.8E-05	**-0.79**	0.003	similar to PTS system, cellobiose-specific IIB component

Gene	ATCC19115		ScottA		FW03-0035		70-1700		Function
	LR#	P	LR	P	LR	P	LR	P	
lmo2783	**-2.12**	0.023	**-0.76**	0.022	**-0.85**	0.018	**-0.93**	0.007	similar to PTS system, cellobiose-specific IIC component
lmo2799	**-1.08**	0.039	**-0.60**	0.020	**-0.10**		**-1.17**	0.007	similar to PTS system, mannitol-specific IIBC component
mptA	**-0.90**		**-1.07**		**-1.25**	0.003	**-0.53**		similar to PTS system, mannose-specific IIAB component
mptC	**0.47**		**-1.56**	0.049	**-0.70**		**-0.08**		similar to PTS system, mannose-specific IIC component
mptD	**-1.75**	0.001	**-1.36**	0.016	**-1.44**	7.0E-05	**-0.62**		similar to PTS system, mannose-specific IID component

LR- Log Ratio; P values not shown are >0.05.

A number of genes putatively associated with cellobiose/beta-glucoside transport such as *lmo2683-2685* showed significant down regulation. Genes encoding proteins putatively specific for up-take of fructose such as *lmo2780*, *lmo*2782, and *lmo*2783, as well as those encoding galacitol transporter genes *lmo2665* and *lmo2667*, mannose specific transporter genes *lmo*0781 and lmo*0782* also showed down-regulation (Table 3.2). Perhaps osmolarity in *L. monocytogenes* has an adverse effect on PTS activity much like that observed in *Corynebacterium glutamicum* and *E. coli*, accounting for both decreased rates of both sugar up-take in concordance with growth rate (Gourdon *et al.*, 2003) (Roth *et al.*, 1985).

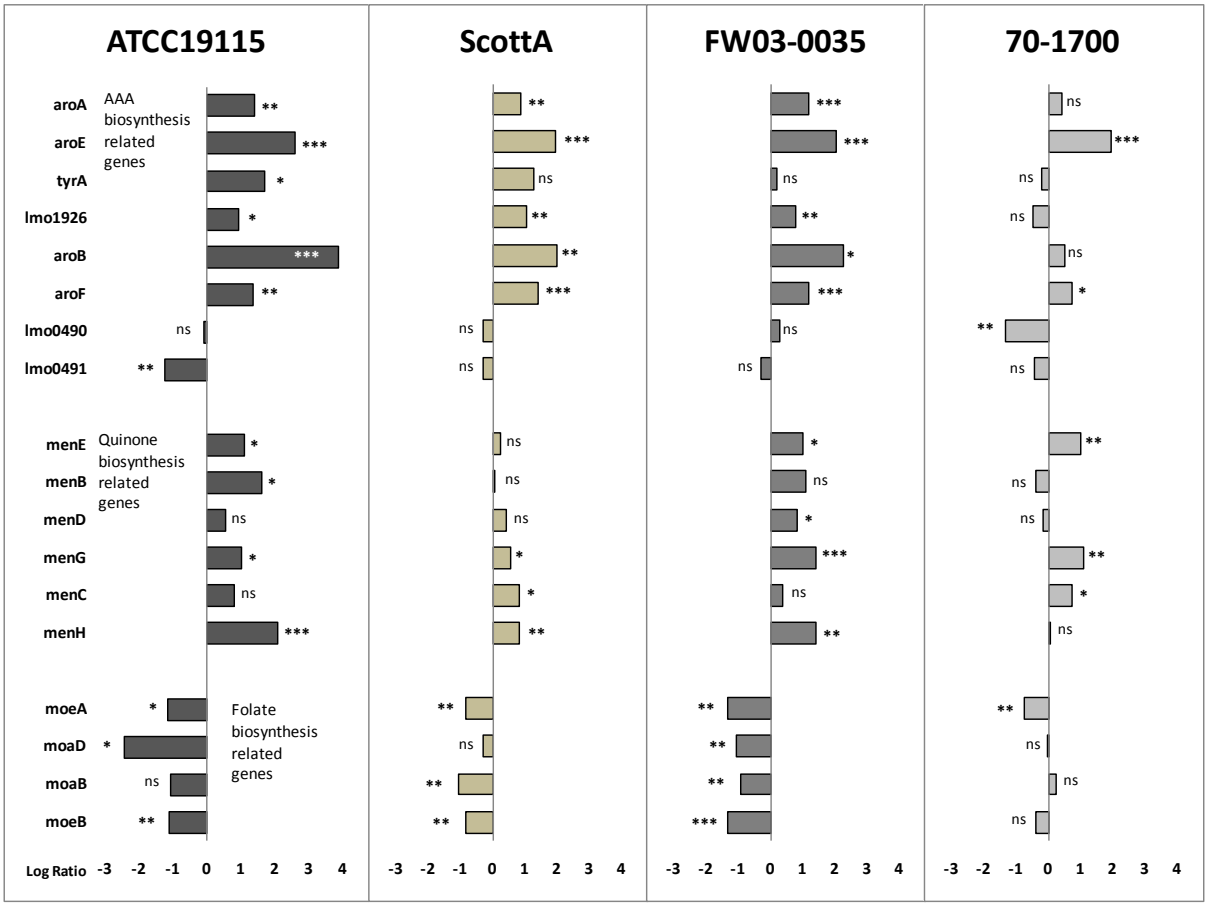
The other carbohydrate transporter genes showed no significant change in transcription levels from that of a control. Unlike the importance of sugar transporters seen during the hyperosmotic shock (section 4.3.1.4) response this mechanism appeared to have little impact on the survival of *L. monocytogenes* strains in hyperosmotic conditions during prolonged exposure to stress.

3.3.2.2 Effect of osmoadaptation on amino acid metabolism.

3.3.2.2.1 Evidence for activation of chorismate and menaquinone biosynthesis

L. monocytogenes strains appeared to significantly activate genes associated with aromatic amino acid biosynthesis when osmo-adapted with *T-value* scores of ≥ 2 in all four strains (Table 3.1). Biosynthesis of the three aromatic amino acids in bacteria namely phenylalanine, tyrosine and tryptophan start with a common pathway. Leading from phosphoenolpyruvate (PEP) and erythrose 4-phosphate through 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) and shikimate to chorismate, later dividing into separate pathways specific for each of the aromatic amino acid (Panina *et al.*, 2003). Genes encoding enzymes involved in the early 'common' stage of aromatic amino acid biosynthesis showed up-regulation in all for strains (Fig. 3.12). These included *aroE*

(*lmo1923*) and *aroF* (*lmo1928*) genes encoding 5-enolpyruvylshikimate-3-phosphate synthase and chorismate synthase respectively.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.12 Expression of genes associated with biosynthesis of aromatic amino acids (AAA), quinine and folate in four osmo-adapted *L. monocytogenes* strains.

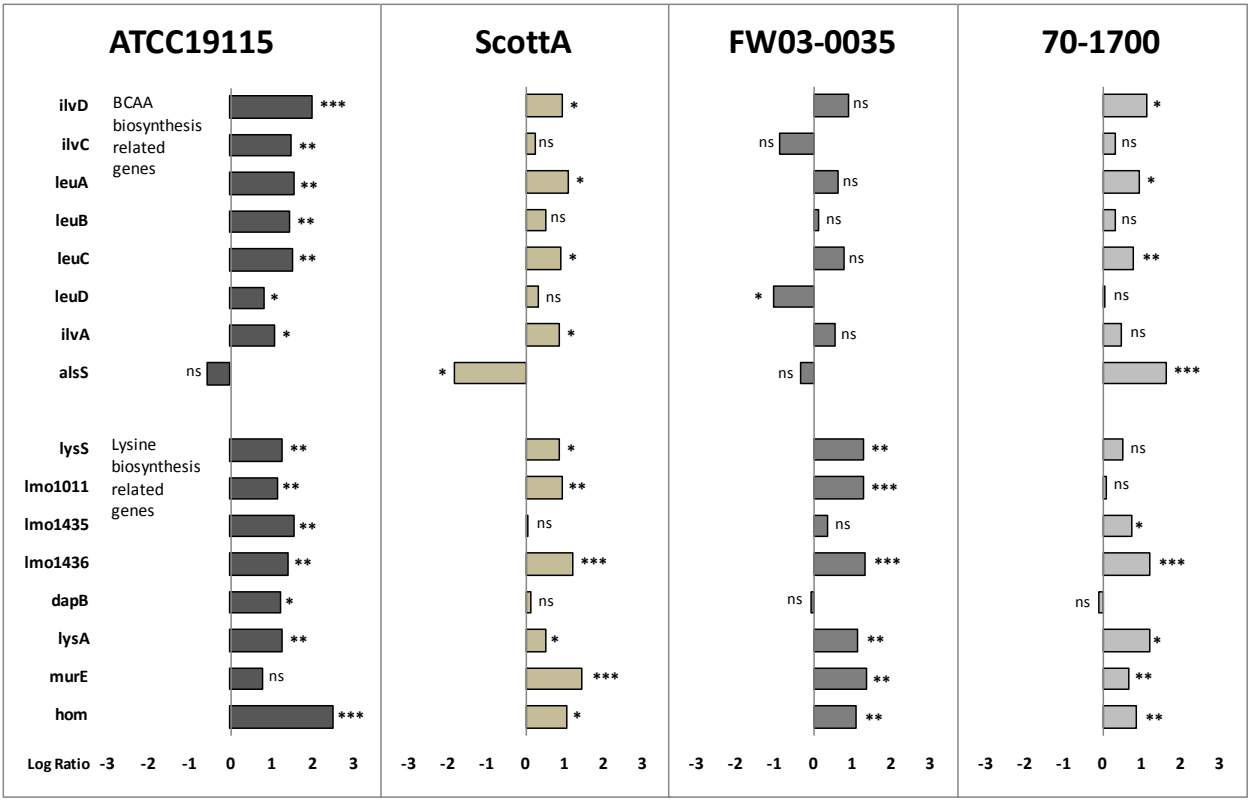
Genes, *aroA* (*lmo1600*) and *aro*B (*lmo1927*), encoding DAHP synthase and 3-dehydroquinate synthetase respectively, showed significant up-regulation of ≥ two-fold in all strains except 70-1700 (Fig. 3.12). This suggests an increased demand for aromatic amino acid precursors in *L. monocytogenes* cells following prolonged exposure to high levels of NaCl. Interestingly biosynthesis of tryptophan appeared to not be affected by hyperosmotic stress, with *trp* genes (*lmo1627-lmo1632*) encoding enzymes responsible for conversion of chorismate via a cascade of reactions into tryptophan, showing no significant change in transcription in all four strains. Chorismate is converted to prephenate by a bifunctional chorismate mutase enzyme encoded by *aroA*. Its further transformation to phenylpyruvate, a precursor for phenylalanine, is facilitated by the actions of prephenate dehydratase coded by *pheA* (*lmo1536*). In *L. monocytogenes* strains adapted to high

levels of salt, no significant change in transcription levels of the *pheA* gene were detected, indicating no requirement for increase in phenylalanine biosynthesis by these strains under salt stress. Prephenate is converted to tyrosine in a series of reactions involving a number of enzymes, two of which showed significant up-regulation of ≥two-fold, *tyrA* (*lmo1924*) and *his*C (*lmo1925*) in two strains ATCC19115 and ScottA, with only the later being up-regulated in FW03-0035. This gene expression pattern suggests that under prolonged hyperosmotic stress *L. monocytogenes* ATCC19115 and ScottA favour biosynthesis of tyrosine.

In *L. monocytogenes* chorismate serves as a precursor for not only the aromatic amino acid biosynthesis but also for folate and menaquinone biosynthesis (Stritzker *et al.*, 2004). Genes associated with folate biosynthesis showed slight down-regulation in all four strains (*T-values* -1.14 to -1.69;Table 3.1, for individual gene expression refer to Fig. 3.12); however gene sets associated with menaquinone (isoprenoid) biosynthesis showed up-regulation in all four strains (*T-value* scores of 1.47 to 3.30, Table 3.1). In *L. monocytogenes* the genes *menEBHDFA*, *menG* and *menC* (*lmo1672-1677*, *lmo1931* and *lmo2520*) are involved in ubiquinone/menaquinone biosynthesis, which exhibited an overall increase in transcription following hyperosmotic stress (Fig. 3.12). Isoprenoid quinones are an essential component of the respiratory chain in bacteria and play an important role in oxidative phosphorylation (Stritzker *et al.*, 2004). Requirement for extra energy generation via the oxidative phosphorylation in *L. monocytogenes* strains adapted to high osmolarity activate menaquinone biosynthesis potentially explaining the up-regulation of genes involved in chorismate biosynthesis.

3.3.2.2.2 Effect of hyperosmotic adaptation on branched chain amino acid biosynthesis.

The least NaCl tolerant strains, ATCC 19115 and 70-1700 exhibited increased expression of genes associated with biosynthesis of branched chain amino acids (BCAA) Table 3.1). BCAA, comprising of valine, leucine and isoleucine, are an important group of amino acids in bacterial proteins. Genes with ≥ two-fold increase in transcription levels included *ilv*D (*lmo1983*) in all four strains, while *leuA* (*lmo1987*), *leuB* (*lmo1988*) and *leuC* (*lmo1989*) also exhibited a trend of up-regulation in all strains (Fig. 3.13).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.13 Expression of genes associated with biosynthesis of branched chain amino acids (BCAA) and lysine in four osmo-adapted *L. monocytogenes* strains.

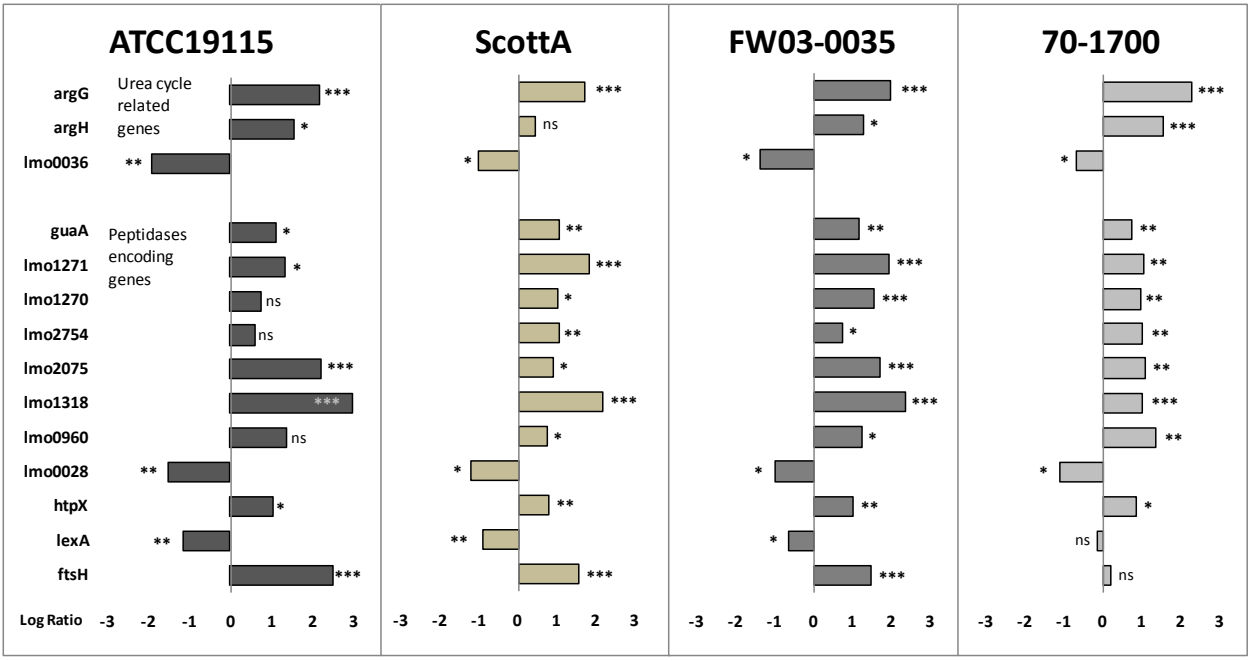
In *L. monocytogenes* BCAA biosynthesis provides precursors for the synthesis of branched-chain fatty acids (BCFA) essential for ensuring membrane fluidity and transport functions in response to environmental stress such as temperature, pH and high levels of salt (Chan *et al.*, 2007b; Chihib *et al.*, 2003; Giotis *et al.*, 2007; Zhu *et al.*, 2005). Up-regulation of genes associated with BCAA biosynthesis is most likely driven by requirement of these in BCFA biosynthesis, to modulate the cell membrane lipid composition. Genes associated with fatty acids (FA) biosynthesis were also found to be significantly up-regulated during hyperosmotic stress in all four *L. monocytogenes* strains and will be discussed further in section 3.3.2.3. Interestingly, a gene encoding α-acetolactate synthase, *alsS* (*lmo*2006) showed significant up-regulation in only the relatively salt sensitive strain 70-1700 (Fig. 3.13). This enzyme functions to convert pyruvate into acetolactate, an important precursor of all three BCAA. Unlike the shock response gene expression pattern of ATCC19115 strain in which both *alsS* and *alsD* (*lmo1992*) showed up-regulation (section 4.3.1.5), in 70-1700 strain adapted to hyperosmotic stress *alsD* showed no significant change in transcription levels, thus implying the activation of acetolactate synthesis may not be related to conversion to acetoin. Instead it appears that acetolactate in strain 70-1700 is utilized more exclusively for BCAA biosynthesis, implying a

potentially greater requirement for the production of these amino acids in order to survive under hyperosmotic stress.

3.3.2.2.3 Biosynthesis of other amino acids

Gene sets associated with biosynthesis of lysine showed an overall up-regulation in all four strains with *T-value* scores ranging from 1.64 to 1.94 (Table 3.1). Genes with most significant up-regulation in transcription levels in all four strains included *lysA*, *lmo1436* and a gene encoding homoserine dehydrogenase, *hom* (Fig. 3.13). While *lysS* was up-regulated significantly in three stains it showed no evidence of change in transcription levels in 70-1700 (Fig. 3.13). Accumulation of intracellular lysine levels has been observed in *E. coli* under salt stress most likely functioning as an osmolyte (Shahjee *et al.*, 2002).

Gene sets associated with urea cycle and metabolism of amino groups showed up regulation following prolonged exposure to salt stress in all four strains studied, the P- value where >0.05, however this group was very small, consisting of 12 genes and therefore the P-value score was not a true indication of significance of these results due to the nature of the calculation of these values. Two genes showed significant up-regulation of >twofold *argG* in all strains and *argH* in all strains except ScottA (Fig. 3.14).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.14 Expression of genes associated with urea cycle and peptidases in four osmo-adapted *L. monocytogenes* strains.

Gene sets associated with peptidases showed significant up-regulation in only two of the strains tested with *T-value* scores of 2.81 for FW03-0035 and 2.10 for 70-1700 (Table 3.1). This group of proteolytic enzymes plays a crucial role in protein quality control removing any misfolded or incompletely synthesized proteins and is also highly important for many regulatory processes within the cell.

Integrity of membrane and membrane proteins is vital for all living cells. Mal-folded, misassembeled and damaged membrane proteins become a major burden on cells grown under stressful conditions protein quality control therefore becomes very important in such circumstances. In both *E. coli* and *B. subtilis*, FtsH, a membrane-bound and ATP-dependent zinc metalloprotease, has been shown to play a central role in the degradation of unstable membrane proteins (Sakoh *et al.*, 2005; Zweers *et al.*, 2008) Increased expression of the *ftsH* gene has been detected in both *B. subtilis* and the wine malolactic fermentation bacterium *Oenococcus oeni* post exposure to hyperosmotic stress, while a *L. lactis ftsH* mutant grew poorly under increased osmotic pressure (Bourdineaud *et al.*, 2003; Deuerling *et al.*, 1995; Nilsson *et al.*, 1994). In addition to FtsH, the heat-inducible, membrane-bound metallo protease HtpX has been implicated in the quality control of *E. coli* membrane proteins, having cellular roles that complement or overlap those of FtsH (Sakoh *et al.*, 2005; Zweers *et al.*,

2008). In *L. monocytogenes* both proteases appeared induced in hyperosmoticaly adapted cells. The *htpX* (*lmo0963*) gene showed up-regulation of ≥ two-fold in all four strains and *ftsH* (*lmo0220*) was up-regulated significantly in all but 70-1700 (Fig. 3.14). Maintenance of the membrane proteins is evidently important in *L. monocytogenes* exposed to prolonged salt stress and may potentially contribute to salt tolerance as the less tolerant strain did not to increase a level of transcription of *ftsH* under stress. A gene encoding a membrane associated metalloprotease with a predicted function similar to that of *htpX* and *ftsH*, *lmo*1318 also showed significant up-regulation of 7.8-fold in ATCC19115, 4.5-fold in ScottA, 5-fold in FW03-0035 and two-fold in 70-1700 (Fig. 3.14); thus further emphasizing the importance of this type of enzymes in an osmoadaptive response of *L. monocytogenes.*

A number of genes encoding proteins with proteolytic activity involved in regulating metabolic processes also showed activation in *L. monocytogenes* cells exposed to hyperosmotic stress. The gene encoding GMP synthase (glutamine hydrolyzing), *guaA* (*lmo1096*) showed significant up-regulation of ≥ two-fold in all four strains (Fig. 3.14), which has also been observed in *Lactococcus lactis* under acid stress and under acid and heat stresses in *Lactobacillus reuteri* (Dal Bello *et al.*, 2005; Rallu *et al.*, 2000). This enzyme is crucial in a number of metabolic pathways in *L. monocytogenes* including purine and glutamate metabolism, increased production of which appears to be beneficial in cells exposed to prolonged hyperosmotic stress.

A gene encoding a membrane bound D-alanyl-D-alanine carboxypeptidase (penicillin binding protein), *lmo2754* showed significant up-regulation in three strains of *L. monocytogenes* examined apart from ATCC19115 (Fig. 3.14). This gene is similar in function to a *dacA* gene of *E. coli*, essential for maintenance of cell shape and contour, mutants lacking this gene had a markedly reduced and weakened peptidoglycan layer and over production of PBP5 has been shown to induce spherical cell formation (Santos *et al.*, 2002).

A gene encoding O-sialoglycoprotein endopeptidase, *gcp* (*lmo2075*) showed significant up-regulation of up to 5-fold in all four strains. Activation of *gcp* has been documented during response to stress in other bacteria. In *Lactococcus lactis*, *gcp* up-regulation was observed during exposure to heat stress, but not acid or salt stresses (Xie *et al.*, 2004). Mutation in *gcp* gene significantly reduced

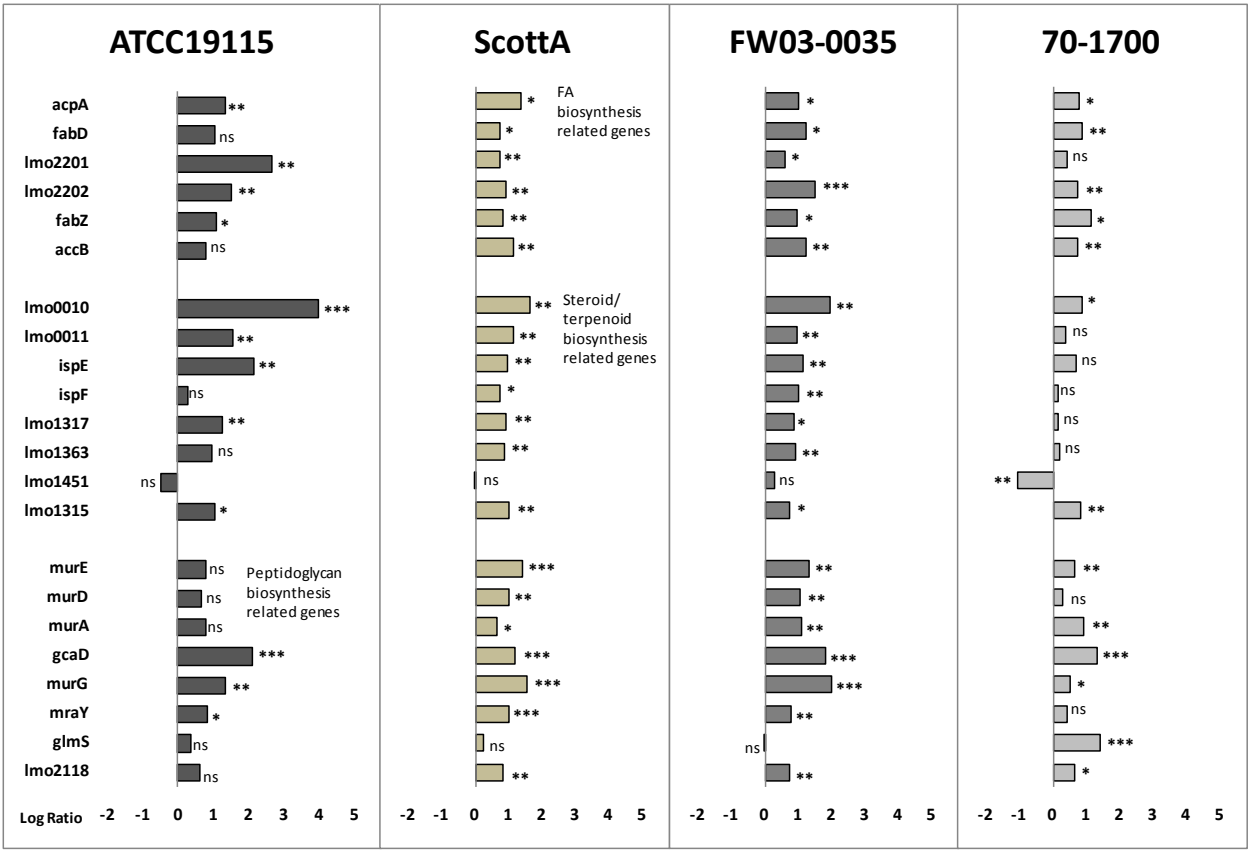
salt tolerance in cyanobacterium *Synechococystis sp*. suggesting significant involvement in salt tolerance of this organism (Karandashova *et al.*, 2002).

Increased expression of amino acid biosynthetic genes observed in all four stains correlates with increased expression of protein synthesis associated genes. Under high osmotic pressure conditions the energy required for the maintenance of cellular integrity increases, while biomass formation decreases (Varela *et al.*, 2004). Thus it appears that cells cultivated under high osmotic pressure channel energy into maintaining cell integrity resulting in greater expression of genes necessary for sustaining the integrity of cellular proteins.

3.3.2.3 Prolonged salt exposure effect on genes associated with lipid metabolism and bacterial cell wall modifications.

Bacterial lipid homeostasis and an ability to adjust membrane lipid composition to acclimatize the cell to different environments are crucial for bacterial existence. Viscosity of the bacterial membrane, which directly governs the membrane permeability, depends in large part on the fatty acid structures that are incorporated into the component phospholipids and is essential in adaptation of bacteria to stressful environmental challenges (Zhang & Rock, 2008).

During prolonged exposure to hyperosmotic environment *L. monocytogenes* gene sets associated with fatty acid biosynthesis (FAB) showed an overall up-regulation in all four *L. monocytogenes* strains with *T-value* scores ranging from to 2.11 to 2.79 (Table 3.1). A gene encoding an acyl carrier protein (Acp) a central molecule which carries all of the FAB pathway intermediates, *acpA* (*lmo1806*) was among the most significantly activated genes in all four strains (Fig. 3.15).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.15 Expression of genes associated with fatty acid (FA) biosynthesis, steroid/terpenoids biosynthesis and biosynthesis of peptidoglycan in four osmo-adapted *L. monocytogenes* strains.

Other up-regulated genes in all four strains included *fabZ* (*lmo2524*) and *lmo2202* a gene homologous to *fabH* in *E. coli* and *B. subtilis*. The *fabD* (*lmo1808*) and *accB* (*lmo1356*) genes showed significant up-regulation in three strains but not ATCC19115 (Fig. 3.15). Whereas *lmo2201* a gene homologous to *fabF* in *E. coli*, showed up-regulation in all strains but 70-1700. *In B. subtilis* both FabF and FabH proteins are induced following salt stress and FabZ activation has been linked to alkaline tolerance in sulfate-reducing bacterium *Desulfovibrio vulgaris* (Hoper *et al.*, 2006; Stolyar *et al.*, 2007).

Expression profile of genes associated with synthesis of isoprenyl units, common precursors required for synthesis of isoprenoid, steroid and terpernoid, was found to be activated in osmotically adapted *L. monocytogenes* strains (Table 3.1).

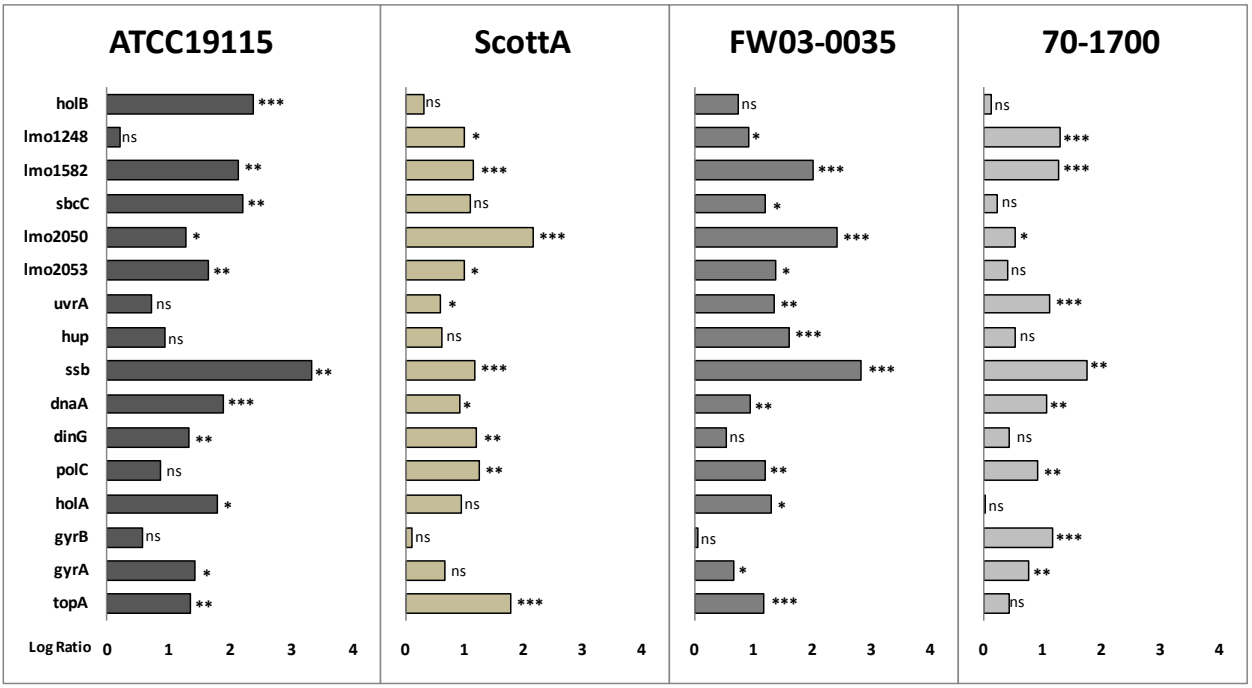
Gene sets associated with biosynthesis of steroid/terpenoid showed significant up-regulation in three relatively salt tolerant strains ATCC19115, ScottA and FW03-0035 with *T-value* scores of 2.07 to 2.63 (Table 3.1). Strain 70-1700 showed no significant change in overall expression of genes in this group; however two genes *lmo*0010 (homogenous to *mva*K1 is *S. aureus*) encoding mevalonate kinase and *lmo1315* showed up-regulation in all strains (Fig. 3.15). The later gene is homologous to *uppS* in other bacteria and encodes undecaprenyl pyrophosphate (C_{55}-P) synthase required for the synthesis of C_{55}-P, an important carrier lipid involved in biosynthesis of peptidoglycan and a variety of other cell-wall polysaccharide components (Bouhss *et al.*, 2008). Genes which were significantly up-regulated in strains ATCC19115, ScottA and FW03-0035 included *lmo0011* (similar to *mvaD* in *S. aureus*), *ispE* (*lmo0190*) and *lmo1317* (homologous to *dxr* in *E. coli*). Two genes in this group showing significant up-regulation of ≥two-fold in only the relatively salt tolerant strains ScottA and FW03-0035 included *ispF* (*lmo0236*) and *lmo1363* (homologous to *ispA* in *E. coli*). Products of these genes play a major role in mevalonate and mevalonate-independent pathways resulting in assembly of isopentenyl diphosphate, a precursor for biosynthesis of hopanoids, which in membrane models have been shown to influence membrane fluidity and stability (Rohmer, 1999). The presence of hapanoids in bacterial cell membranes was correlated with the tolerance towards high temperature of *Alicyclobacillus acidocaldarius* and towards ethanol and high osmotic pressures of *Zymomonas mobilis* (Rohmer, 1999). It could be speculated that in *L. monocytogenes* activation of genes and consequently gene products involved in steroid and terpernoid biosynthesis pathways may provide extra membrane stability when exposed to hyperosmotic stress. Consequently lack or inefficiency of adequate synthesis of these important lipids may compromise the ability to tolerate high levels of osmotic stress, as indeed was observed in gene expression profile of a relatively salt sensitive strain of *L. monocytogenes* 70-1700 (Fig. 3.15).

Activation of peptidoglycan biosynthesis which comprises a major component of listerial cell wall, was evident in all strains of *L. monocytogenes* following prolonged exposure to hyperosmotic stress with *T-value* scores ranging from 1.72 to 3.31 (Table 3.1). Individual gene expression patterns showed evident variation among the four strains examined in this study, driven by a combination of different osmotic pressure and inherent strain variation. Two genes were up-regulated in all four stains, *gcaD* (*lmo0198*) from 2.3- to 4.3-fold and *murG* (*lmo2035*) of up to 4.0-fold (Fig. 3.15). In addition to these genes, strain ATCC19115 only showed significant up-regulation of one more gene, *mraY* (*lmo2037*). Strain 70-1700 gene expression profile showed additional up-regulation of *murE*, *murA*, *glmS* and *lmo2118* (gene homologous to *glmM* in *E. coli*). In the gene profile of the two

relatively salt tolerant strains, ScottA and FW03-0035, *murE*, *murD* (*lmo2036*), *murA*, *mraY*, and *lmo2118* also showed significant up-regulation of ≥two-fold in transcription levels (Fig. 3.15). Changes to the peptidoglycan structure of the cell wall due to osmotic pressure in bacteria such as *B. subtilis* and *S. aureus* is well recognized and documented. Adaptation to hyperosmotic environment leads to apparent thickening of the cell wall by altering composition of peptidoglycan structure, resulting in reduced lysozyme sensitivity, a phenomenon also observed in *L. monocytogenes* (López *et al.*, 1998; Palomino *et al.*, 2009; Zhang & Rock, 2008).

3.3.2.4 Prolonged salt exposure effect on information processing and storage genes.

Osmotically adapted *L. monocytogenes* cells exhibited increased expression of several genes involved with DNA polymerase and DNA repair and replication complexes as indicated by significant *T-value* scores (Table 3.1). Four genes showed significant up-regulation in all four hyperosmotically adapted strains (Fig. 3.16): *lmo1582* (up to 4.4-fold), *ssb* (*lmo0045*, up to 10-fold), *lmo2050* (up to 4.4-fold), and *dnaA* (*lmo0001*, up to four-fold).



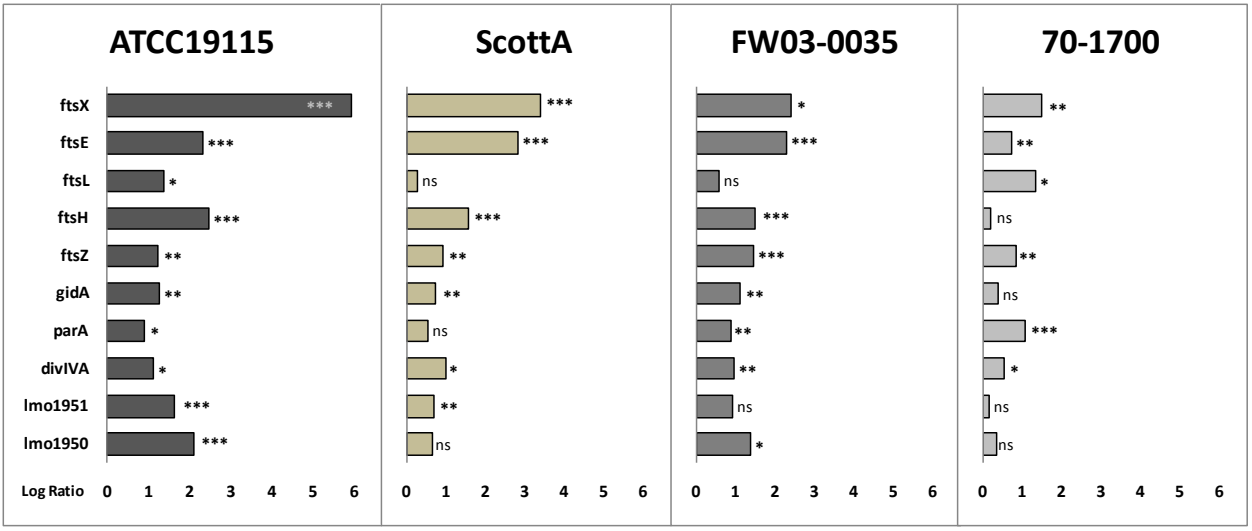
*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.16 Expression of genes associated with DNA polymerase, DNA repair/recombination and DNA replication complex in four osmo-adapted *L. monocytogenes* strains.

DnaA is required for activation of genes encoding nucleotide biosynthesis and in initiating DNA replication and cell division (by directly activating *ftsZ*), it also functions in promoting expression of components necessary for successful chromosome duplication (Susin *et al.*, 2006) and is induced in *E. coli* under cold stress (Atlung & Hansen, 1999). Accumulation of a single stranded-DNA-binding protein, Ssb has been shown to occur in *E. coli* cells after an increase of the extracellular NaCl concentration and is believed to play a role in protection and repair of chromosomal DNA under cellular stress (Weber *et al.*, 2006). A number of significantly up-regulated genes showed strain variation in expression, some of these included *holB* (*lmo0162*, 5.2-fold up-regulation in ATCC19115), *lmo1248* and *uvrA* (*lmo2488*), which showed increase in transcription in three strains excluding ATCC19115; *lmo2553* and *topA* (*lmo1275*) were up-regulated in all but 70-1700 s whereas *gyrB* (*lmo0006*) was up-regulated in 70-1700 strain only; *sbcC* (*lmo1645*) was up-regulated up to 5-fold in ATCC19115 and FW03-0035 (Fig. 3.16). Alterations to binding affinities and rates of binding of transcriptional regulatory proteins to their target site on a DNA molecule has previously been linked to the bacterial intracellular electrolyte concentration (Csonka, 1989). It has in fact been suggested that cations bind to phosphate groups on the DNA molecule decreasing its hydrophilic property which in fact leads to changes in the structure of the molecule (Kas'yanenko, 2006). A negatively charged DNA molecule is vulnerable to attack by Na^+ ions, which by binding to the DNA changes the interaction of DNA molecule with transcriptional regulatory proteins. Activation of genes associated with DNA polymerase and DNA repair may help to stabilize DNA-protein interactions.

3.3.2.5 Adaptive salt response effect on genes associated with cell division.

Overall, cell division associated genes showed significant up-regulation in all four *L. monocytogenes* strains following adaptation to hyperosmotic stress with *T-value* scores ranging from 2.27 to 4.72 (Table 3.1). Hyperosmotic stress has been shown to impair cell division in this organism (Jøsrgensen *et al.*, 1995). Of the known cell-division proteins in *E. coli* (Weiss, 2004), expression of homologues to a number of these found in *L. monocytogenes* was observed to increase under prolonged osmotic stress. These included *ftsZ*, *ftsE* and *ftsX* in all four of the strains examined, while *ftsH* showed up-regulation in thee of the strains excluding 70-1700 (Fig. 3.17); while the *ftsL* gene was found to be up-regulated in strains ATCC19115 and 70-1700.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.17 Expression of genes associated with cell division and septal ring in four osmo-adapted *L. monocytogenes* strains.

FtsZ is the central regulator for bacterial cell division a crucial function performed by determining the site and progression of septation (Mukherjee *et al.*, 2005; Powell & Court, 1998; Varma & Young, 2004; Weiss, 2004). FtsZ has been shown to interact with PBP5 (encoded by *lmo2754*, which also showed up-regulation and has been previously mentioned in section: 3.3.2.2.3) to influence the cell shape of *E. coli* (Varma & Young, 2004). Environmental stress, including high-osmotic strength medium has been shown to affect FtsZ assembly and bundling in bacteria (Mukherjee *et al.*, 2005; Powell & Court, 1998). A poorly functional FtsZ has been shown to result in bacterial cells with morphological abnormalities. A possible way of overcoming a suppression of cell division due to defective cell division machinery is by activating the expression of genes encoding cell-division proteins.

FtsX and FtsE in *E. coli* and are thought to act as stability factors assisting in assembly and stability of the septal ring (Schmidt *et al.*, 2004) From results here they appear to be particularly important when cells are subjected to hyperosmotic conditions.

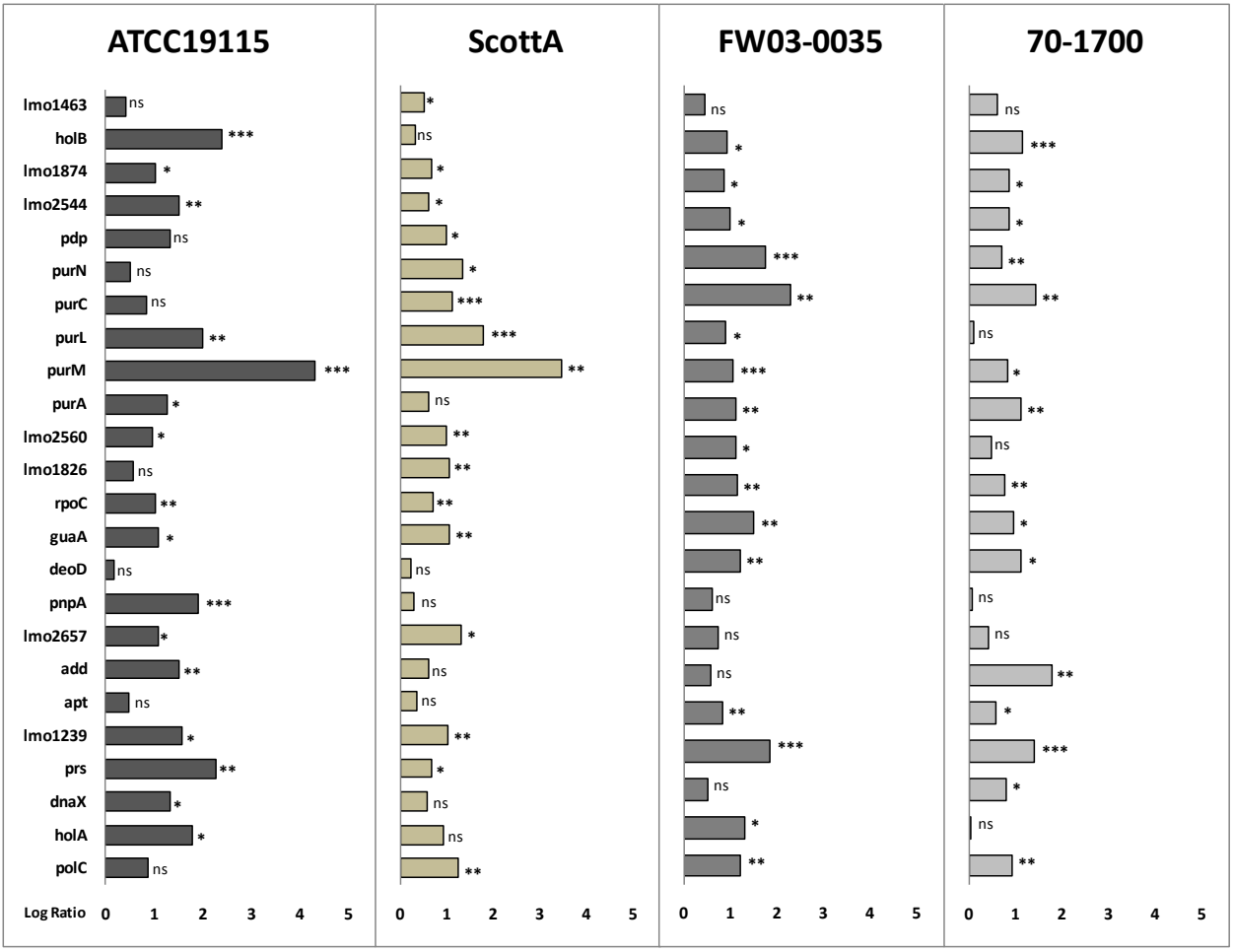
Other genes which were found to be up-regulated in *L. monocytogenes* salt tolerant strains included *gidA* (not up-regulated in strain 70-1700) the exact function of which in bacteria is unknown, but is believed to encode an enzyme involved in tRNA modification. Mutations of this gene render cells with impaired translation efficiencies in *S. pyogenes* (Caldon *et al.*, 2001; Cho & Caparon, 2008).

ScpA and ScpB encoding genes *lmo1951* and *lmo1950* showed an overall up-regulation in three relatively salt tolerant strains (no up-regulation observed in strain 70-1700, Fig. 3.17). Both of these proteins are essential for chromosome segregation in bacteria, abnormalities in either of the proteins in *B. subtilis* resulted in temperature-sensitive growth and production of cells lacking normal chromosome structure (Soppa *et al.*, 2002).

Gene sets associated with nucleotide synthesis (biosynthesis and metabolism of both purine and pyrimidine) showed significant up-regulation in all four *L. monocytogenes* strains with *T-value* scores ranging from 1.78 to 4.61 (Table 3.1). A number of genes showed strain variation (Fig. 3.18), for instance a gene encoding adenine phosphoribosyltransferase, *apt* (*lmo1524*) was induced in only one strain 70-1700. Another gene, *hol*B (*lmo0162*) was significantly induced in strain ATCC19115 whereas no significant up-regulation of genes such as *pdp* (*lmo1993*), *purN* (*lmo1766*), *purC* (*lmo1772*) and *polC* (*lmo1320*) was evident in this strain, which were found to be induced in the three other strains.

Genes displaying up-regulation in transcription levels in all four strains included *purM* (*lmo1767*), *purL* (*lmo1770*), *lmo2560*, *lmo1239*, *prs* (*lmo0199*) and *guaA* (*lmo1096*). PurM showed the most up-regulation in this sub group of genes and has been found to be up-regulated in pathogenic bacterium *Tropheryma whipplei* under cold stress (Crapoulet *et al.*, 2006).

In *B. subtilis* inhibition of GMP (guanosine monophosphate) synthase (encoded by *guaA*) lowered cellular GTP levels (guanosine 5' – triphosphate) and has shown to decrease translation efficiency (Duwat *et al.*, 2002). GTP is a precursor for (p)ppGpp (guanosine pentphosphate), an alarmone responsible for induction of the stringent response. Amino acid or carbon starvation triggers the stringent response by inhibiting energy-consuming replication and transcription in bacteria and has been shown to confer stress resistance to a number of pathogenic bacteria such as *Salmonella typhimurium* and *Vibrio cholerae* (Duwat *et al.*, 2002).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.18 Expression of genes associated with nucleotide synthesis in four osmo-adapted *L. monocytogenes* strains.

Activation of stringent response was not evident in *L. monocytogenes* following adaptation to hyperosmotic stress, as the gene responsible for the conversion of GTP to (p)ppGpp, *relA* (*lmo*1523) showed no significant up-regulation in either of the strains examined. Thus, potentially, a preferential involvement of *guaA* in nucleotide interconversion occurs in this particular response. Interestingly, *guaB* (*lmo2758*), which has previously been shown to have an increased expression in *L. monocytogenes* LO28 following adaptation to hyperosmotic conditions (Duché *et al.*, 2002) showed significant 22-fold down-regulation in expression in strain ATCC19115, and roughly a 2-fold up-regulation in ScottA, FW03-0035 and 70-1700 strains (data not shown).

Overall, induction of nucleotide metabolism genes in osmo-adapted *L. monocytogenes* cells suggests an increase in demand for both biosynthesis and recycling of nucleotides for RNA synthesis and DNA repair.

3.3.2.6 Effect of hyperosmotic stress adaptation on translation-apparatus-related genes.

A large number of genes directly involved with translation and ribosomes exhibited increased expression following prolonged exposure to hyperosmotic stress. Ribosome structure integrity is significantly impaired by ionic strength of a medium (Brigotti *et al.*, 2003), which in turn stalls translation. To contradict impaired translation *L. monocytogenes* appears to activate genes encoding numerous ribosomal proteins (Table 3.3).

Translation initiation factors encoding genes *infC* (*lmo1785*) and *infA* (*lmo2610*) both showed ≥ two-fold increase in transcription levels in three relatively salt tolerant *L. monocytogenes* strains (Table 3.3), whereas in strain 70-1700 *infA* showed no significant change in transcription. The translational elongation cycle is catalysed by three main elongation factors EF-Tu encoded by *tufA* (*lmo2653*), EF-Ts encoded by *tsf* (*lmo1657*) and EF-G encoded by *fus* (*lmo2654*), all of which showed up-regulation in the three relatively salt tolerant strains of *L. monocytogenes* following adaptation to hyperosmotic stress and only EF-Tu failed to show significant up-regulation in strain 70-1700 (Table 3.3).

Table 3. 3 Gene expression patterns of four *L. monocytogenes* strains associated with translation and ribosome proteins observed during adaptive hyperosmotic stress response.

Gene	ATCC19115		ScottA		FW03-0035		70-1700		Function
	LR[#]	P	LR	P	LR	P	LR	P	
cca	**1.26**	0.009	0.37		**1.15**	0.002	**1.48**	0.001	tRNA nucleotidyltransferase (CCA-adding enzyme)
efp	0.87	0.022	0.77	0.004	**1.07**	0.011	0.23		elongation factor EF-P
frr	**2.00**	0.000	**1.07**	0.011	**1.45**	1.4E-04	0.46		similar to ribosome recycling factor
fus	**2.28**	0.000	**1.19**	1.7E-04	**1.42**	3.1E-04	**1.10**	0.002	elongation factor EF-G
gatA	0.09		**1.24**	0.002	**1.21**	0.008	0.42		aspartyl/glutamyl-tRNA amidotransferase A subunit
infA	**1.20**	0.017	**1.19**	0.006	**1.21**	0.002	0.18		translation initiation factor IF1
infC	**1.30**	0.019	**1.21**	0.003	0.78		0.61		translation initiation factor IF3
lmo0227	**3.21**	7.3E-05	**2.68**	4.1E-05	**3.22**	2.0E-06	**1.32**	0.003	putative tRNA-dihydrouridine
lmo0935	**1.53**	0.002	0.81	0.003	**1.10**	0.001	**1.00**	0.001	putative rRNA methylase
lmo1238	**1.40**	0.016	0.74	0.020	**1.44**	0.004	0.91	0.016	similar to ribonuclease PH
lmo1530	0.68		**1.19**	0.001	**1.42**	0.005	**1.01**	0.005	similar to queuine tRNA-ribosyltransferase

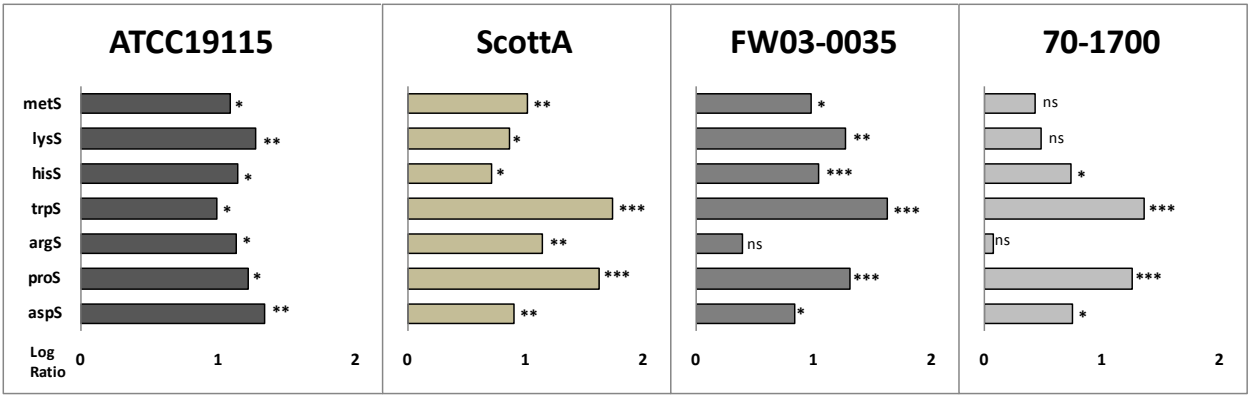
Gene	ATCC19115		ScottA		FW03-0035		70-1700		Function
	LR[#]	P	LR	P	LR	P	LR	P	
lmo1543	0.63		0.56	0.030	**1.22**	0.003	0.61	0.032	similar to ribonuclease G
lmo1698	**2.06**	0.000	0.96	0.002	0.58		0.96	0.006	ribosomal-protein-alanine N-acetyltransferase
lmo1703	**1.38**	0.015	0.65		0.94	0.014	0.62	0.008	putative 23S rRNA (uracil-5-)-methyltransferase
lmo1709	**1.60**	0.019	0.65	0.019	0.37		0.54	0.014	similar to methionyl aminopeptidase
lmo1722	**1.04**		**1.09**	0.002	0.91	0.013	0.70	0.013	putative ATP-dependent RNA helicase
lmo1949	**1.14**	0.041	0.73		**1.05**	0.031	-0.32		similar to 23S RNA-specific pseudouridine synthase B
lmo2448	**1.46**	0.004	**1.02**	0.004	0.70	0.022	0.19		SsrA (tmRNA)-binding protein
rplA	**3.37**	0.001	**1.90**	7.7E-05	**2.93**	3.3E-05	**1.08**	0.002	ribosomal protein L1
rplB	0.91		0.81	0.019	**1.26**	0.003	0.16		ribosomal protein L2
rplC	**1.69**	0.002	0.82	0.012	**1.40**	0.002	**1.06**	0.015	ribosomal protein L3
rplD	**2.08**	0.000	**1.31**	3.8E-04	**2.12**	1.4E-06	0.94	3.6E-04	ribosomal protein L4
rplE	**1.24**	0.007	**1.26**	4.6E-04	**2.03**	4.2E-05	**1.40**	3.0E-05	ribosomal protein L5
rplF	**1.22**	0.009	**1.05**	0.016	**1.90**	3.4E-04	0.48		ribosomal protein L6
rplJ	**2.50**	1.8E-04	**2.11**	6.5E-05	**2.99**	4.4E-05	**1.52**	2.8E-04	ribosomal protein L10
rplK	**3.36**	0.001	**1.49**	4.8E-04	**2.49**	3.6E-04	**1.79**	1.5E-04	ribosomal protein L11
rplL	**2.88**	0.001	**1.96**	4.0E-04	**2.58**	3.2E-04	**1.21**	0.001	ribosomal protein L7/L12
rplM	**0.81**		**1.54**	0.001	**1.44**	0.018	0.73		ribosomal protein L13
rplN	**1.96**	0.001	**1.45**	0.001	**3.18**	8.8E-07	**1.54**	2.6E-04	ribosomal protein L14
rplR	**1.90**	0.001	**1.34**	0.002	**2.37**	1.9E-04	**1.37**	2.5E-05	ribosomal protein L18
rplS	**1.94**	0.001	**1.29**	2.2E-04	**1.79**	2.8E-05	0.78	0.017	ribosomal protein L19
rplT	**1.29**	0.006	0.90	0.007	0.84	0.005	0.48		ribosomal protein L20
rplU	**2.74**	4.31E-05	**2.41**	1.6E-04	**2.28**	3.5E-05	0.89	0.015	ribosomal protein L21
rplV	**1.81**	0.001	**1.33**	0.003	**2.01**	1.4E-04	0.80	0.001	ribosomal protein L22
rplW	**2.05**	0.002	0.85		**1.53**	0.005	0.32		ribosomal protein L23
rplX	**1.13**	0.014	**1.03**	0.013	**1.58**	0.001	0.41		ribosomal protein L24
rpmA	**1.31**	0.003	**1.53**	0.001	**1.96**	1.8E-05	0.91	0.021	ribosomal protein L27
rpmB	**3.27**	9.02E-05	**3.01**	0.001	**3.52**	8.1E-07	**1.84**	4.1E-04	ribosomal protein L28
rpmC	**1.03**	0.015	**1.20**	0.002	0.99	0.012	**1.24**	0.008	ribosomal protein L29
rpmD	**2.44**	4.2E-04	**1.62**	0.001	**2.13**	0.001	**1.51**	0.005	ribosomal protein L30
rpmE	**2.59**	4.3E-04	**1.95**	1.1E-04	**2.89**	4.7E-07	**1.91**	7.1E-05	ribosomal protein L31
rpmI	**2.39**	1.1E-04	**1.80**	3.3E-04	**2.36**	1.1E-06	**1.38**	9.0E-05	ribosomal protein L35
rpmJ	**1.52**	0.009	**1.66**	0.002	**1.06**	0.033	0.02		ribosomal protein L36
rpsB	**2.22**	3.6E-04	**2.21**	2.0E-04	**1.45**	0.002	0.30		ribosomal protein S2
rpsC	**1.40**	0.004	**1.01**	0.024	**1.18**	0.009	0.94	0.021	ribosomal protein S3
rpsD	**3.21**	0.001	**1.03**	0.001	**2.34**	0.001	**1.48**	4.7E-04	ribosomal protein S4
rpsE	**1.11**	0.006	0.84	0.003	**1.39**	3.9E-04	0.78	0.002	ribosomal protein S5
rpsG	**3.41**	2.6E-05	**1.61**	0.003	**2.68**	2.2E-07	0.79	0.048	ribosomal protein S7
rpsH	**1.43**	0.005	**1.08**	0.007	**2.86**	3.7E-06	**1.69**	1.1E-04	ribosomal protein S8
rpsJ	**2.07**	0.002	**1.40**	0.004	**1.55**	0.006	0.30		ribosomal protein S10
rpsK	**1.36**	0.003	0.67		**1.19**	1.2E-04	0.55	0.025	ribosomal protein S11
rpsL	**4.10**	5.9E-06	**1.81**	4.6E-04	**2.59**	3.6E-08	**1.43**	2.0E-04	ribosomal protein S12
rpsM	**1.73**	0.001	**1.44**	0.004	**1.27**	2.7E-04	0.32		ribosomal protein S13
rpsN	**1.68**	0.001	**1.74**	0.001	**1.80**	3.6E-05	0.71	0.004	ribosomal protein S14
rpsQ	**1.15**	0.007	**1.30**	4.6E-04	**1.74**	2.5E-04	0.86	0.002	ribosomal protein S17

Gene	ATCC19115		ScottA		FW03-0035		70-1700		Function
	LR[#]	P	LR	P	LR	P	LR	P	
rpsS	**2.24**	0.001	**1.30**	0.001	**2.21**	2.0E-05	**1.18**	9.9E-05	ribosomal protein S19
rpsT	**1.23**	0.007	0.33		0.81		**1.66**	0.024	ribosomal protein S20
tsf	**1.46**	0.001	**1.28**	4.7E-05	**1.52**	4.1E-04	**1.03**	2.6E-04	elongation factor EF-Ts
tufA	**2.02**	0.000	**1.29**	0.002	**1.58**	2.6E-03	0.79		elongation factor EF-Tu

LR= Log Ratio; P values not shown were >0.05

EF-Tu is a small GTP-binding protein and in combination with a GTP molecule is responsible for transporting and binding of the appropriate codon-specified aminoacyl-tRNA to the acceptor site on the ribosome. EF-Ts is a guanine nucleotide exchange factor acting on EF-Tu, allowing its reactivation by mediating GDP/GTP exchange. In addition to its role in translation and elongation EF-Tu has been shown to have protective influence on newly synthesized proteins in terms of protein folding and renaturation after stress (Caldas *et al.*, 1998; Duché *et al.*, 2002). EF-G is a small GTP-binding protein, which is required for translocation of ribosomes on the mRNA molecule. ATCC19115, ScottA and FW03-0035 also showed increase in transcription of *efp* (*lmo1355*) a gene encoding the less well known elongation factor EF-P (Table 3.3). EF-P is believed to be involved in a formation of a first peptide bond of a protein molecule (Ganoza *et al.*, 2002; Hanawa-Suetsugu *et al.*, 2004). The gene encoding ribosomal recycling factor, *frr* (lmo*1314*) was up-regulated in ATCC19115, ScottA and FW03-0035 strains and showed no significant change in transcription in 70-1700 (Table 3.3). Ribosome recycling factor in conjunction with EF-G is required for dissociation of post-termination ribosomal complex after release of a newly synthesized polypeptide (Pai *et al.*, 2008; Toyoda *et al.*, 2000).

Aminoacyl-tRNA synthetases are key components of the protein translation machinery that catalyse activation of amino acids via the formation of aminoacyl adenylates and linking the activated amino acid to the cognate tRNAs (Hausmann & Ibba, 2008; Wolf *et al.*, 1999). Genes in this subgroup showed significant up-regulation in all four strains of *L. monocytogenes* with *T-value* scores ranging from 2.04 to 3.50 (Table 3.1). Activation of this group of genes has also been observed in *B. sublilis* following adaptation to osmotic stress (Hahne *et al.*, 2010).Four genes in this group showed up-regulation in all four strains of *L. monocytogenes*, these were *hisS* (*lmo1520*) encoding histidyl-tRNA synthetase, *trpS* (*lmo2198*) encoding tryptophanyl-tRNA synthetase, *proS* (*lmo1319*) encoding prolyl-tRNA synthetase and *aspS* (*lmo1519*) encoding aspartyl-tRNA synthetase (Fig. 3.19).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.19 Expression of genes associated with aminoacyl-tRNA biosynthesis in four osmo-adapted *L. monocytogenes* strains.

Increased transcription in cells adapted to hyperosmotic stress is evident from the observed gene expression profiles and is suggestive of a requirement of these cells for amplified protein synthesis for utilization in cell-modifications to assist in repair of NaCl induced cell damage.

The expression of RNA polymerase delta (δ) factor (*lmo*2560) has shown on average 2-fold increased in all four strains following adaptation to hyperosmotic stress (Fig. 3.18). The exact role of bacterial δ-factor is still very poorly understood, although it has been shown to have a global affect on gene expression in *B. subtilis*, where it is thought to participate in maintaining transcriptional specificity and RNA polymerase recycling (Lopez de Saro *et al.*, 1999; Seepersaud *et al.*, 2006). A mutation in δ has also been reported to affect the ability of human pathogen *S. aureus* to recover from nutrient starvation and increase its sensitivity to acid stress (Watson *et al.*, 1998). Association of δ-factor with RNA polymerase reduces binding to DNA templates containing non relevant promoter sites, which may be of benefit in an organism straggling to maintain normal physiological function when exposed to environmental stress such as hyperosmotic stress. Prevention of non-specific RNA polymerase binding to DNA may reduce energy wastage by increasing the amount of free RNA polymerase within the bacterial cell.

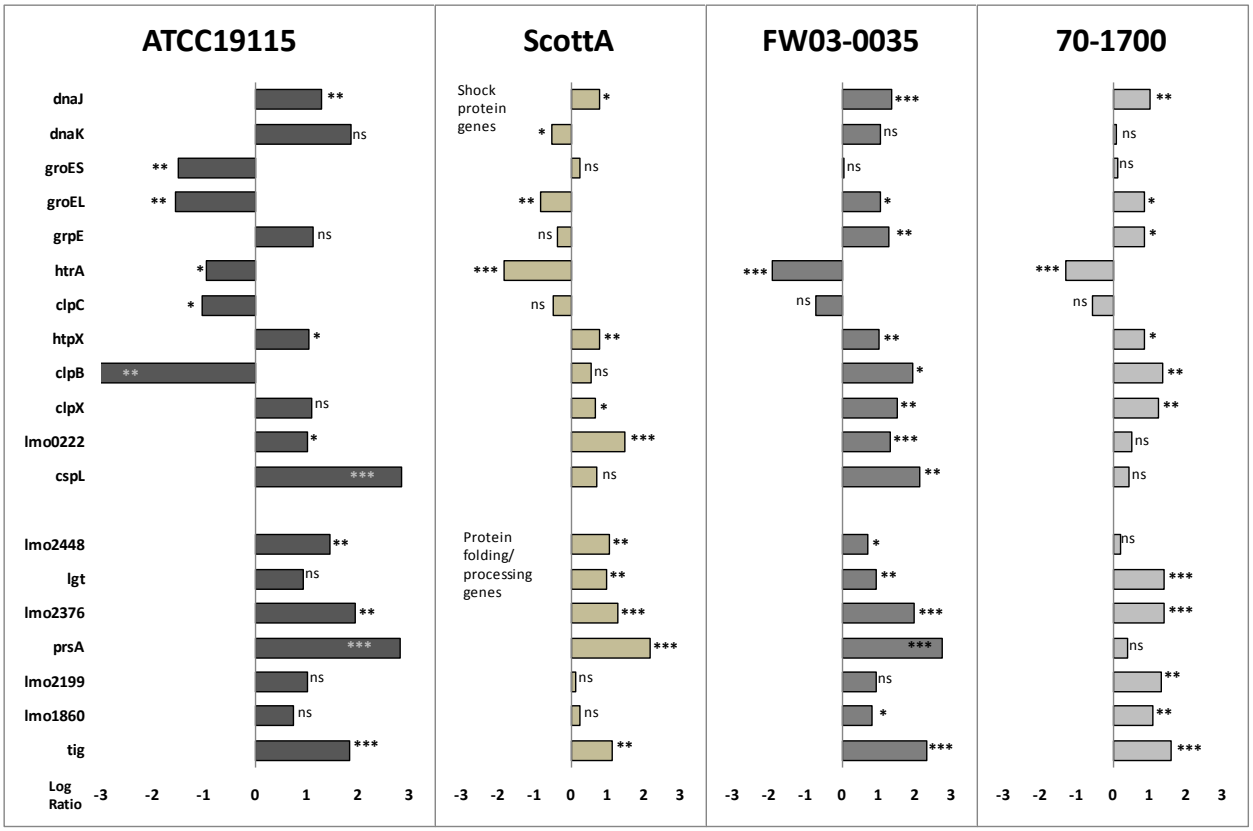
Defective messages are frequently produced during growth and can be critical for cell survival especially when bacterium is under stress. A number of mechanisms exist in bacteria which control the quality of the message pre- and post-translation, the *trans*-translation reaction involving transfer-messenger RNA (tmRNA) is one such mechanism. The *trans*-translation reaction directs

incompletely synthesized peptides to degradation and releases stalled ribosomes and assists in the adaptation to stress (Fujihara *et al.*, 2002; Gillet & Felden, 2001; Withey & Friedman, 2003). In both *E. coli* and *B. subtilis*, tmRNA has been shown to assist bacterial cells in adaptation to environmental change, including elevated temperatures and to exposure to ethanol and cadmium chloride (Fujihara *et al.*, 2002; Gillet & Felden, 2001; Withey & Friedman, 2003). The *ssrA* gene (*lmo2448*) to date has not been linked to stress adaptation in *L. monocytogenes*. Marginal increase in the level of tmRNA conscript was observed in three out of four strains of *L. monocytogenes* following exposure to prolonged hyperosmotic stress in this study (Table 3.3). It appears that the quality control mediated by tmRNA might be of benefit in ensuring that the proteins allowing growth under hyperosmotic stress are produced and the consequences of translational errors that are expected to increase are corrected.

3.3.2.7 Osmotic adaptation effect on stress response genes.

Maintenance of protein stability is essential for viability of all living cells. The proper functioning of proteins depends on their structural integrity, inherent instability and external stress may cause proteins to misfold and aggregate. A number of bacterial stress proteins or chaperones are responsible for stabilizing proteins by aiding protein folding. Dysfunctional proteins need removing from the cell to prevent unnecessary accumulation which may interfere with normal cell function. This crucial function is dedicated to a small number of stress response proteases, which degrade improperly folded proteins and restore amino acid pools.

A number of chaperones and proteases show induction in transcription levels in osmo-adapted cells. Neither the heat shock protein coding genes nor the folding catalyst protein-coding genes exhibited significant expression alterations (data not shown). Genes associated with protein folding/processing showed up-regulation in strains FW03-0035 and 70-1700 with *T-value* scores of 2.06 and 2.09 respectively, although the statistical significance was borderline (Table 3.1). As a number of genes in the same function group also showed significant down-regulation the overall expression was annulled rending a less significant result. This phenomenon in gene expression just emphasizes the complexity of hyperosmotic stress adaptation response especially when strain specificity is also taken into consideration.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.20 Expression of genes associated with protein folding/processing and shock proteins in four osmo-adapted *L. monocytogenes* strains.

Two genes, products of which are associated with protein folding and processing, showed significant up-regulation following hyperosmotic adaptation in all four strains examined in this study, *tig* (*lmo1267*) and *lmo2376* (Fig. 3.20). In *L. monocytogenes tig* encodes a trigger factor (Tig), an ATP-dependent chaperone involved in the prevention of the misfolding and aggregation of nascent chains as they are translated by the ribosome (Bigot *et al.*, 2006; Reyes & Yoshikawa, 2002; Wong & Houry, 2004). Mutations in *tig* has been linked to susceptibility to cold shock and ethanol stress in *L. monocytogenes*, but had no effect on osmotic stress susceptibility in this organism (Bigot *et al.*, 2006). From the results obtained in this study Tig appears to be of significance during hyperosmotic stress in *L. monocytogenes* strains examined in this study. Mutagenesis studies fail to reliably exclude an involvement of a particular gene in the stress response, due to the multifactorial nature of such responses. As numerous other gene products may compensate for the silenced protein thus camouflaging the exact function of a mutated gene. Observed activation of genes associated with translation during prolonged exposure to sub-lethal levels of NaCl would require transcription activation of supporter proteins, such as Tig to aid in stabilizing proteins during the transcription process. Gene *lmo*2376 encodes a putative peptidyl-prolyl cis-trans isomerase (PPIase). PPIase

catalyse the cis-trans isomerization of propyl peptide bonds, an essential step in protein folding process, thus facilitating protein folding within cells (Fischer *et al.*, 1998). Hyperosmotic adaptation also induced expression of *prs*A (*lmo2219*) a gene encoding a post-translocation molecular chaperone homologous to that in *B. subtilis* in ATCC19115, ScottA and FW03-0035 ranging from 5- to 7-fold up-regulation in gene expression (Fig. 3.20). PrsA is a major extracytoplasmic folding factor of the PPIase family in *B. subtilis* and is believed to be involved in the synthesis and maintenance of the bacterial cell wall, assisting in the folding and stability of newly synthesized cell wall proteins in the post-translocational phase of secretion at the membrane-cell wall interface (Wahlström *et al.*, 2003). Interestingly, the serine protease HtrA encoding gene *htr*A (*lmo0292*) showed significant down-regulation in all four osmo-adapted strains of *L. monocytogenes*. This was unexpected as this enzyme has previously been shown to induce stress tolerance, including osmotolerance in *L. monocytogenes* (Stack *et al.*, 2005; Wonderling *et al.*, 2004). Results obtained from this study demonstrate that HtrA has no direct effect on osmotolerance in osmo-adapted cells. It does however appear to be of significance during the initial response to hyperosmotic stress. It is crucial to make the distinction between the involvement of a particular gene and corresponding protein in the stress tolerance response phases. Clear understanding of the stress tolerance process will assist in illuminating *L. monocytogenes* from food products by selective targeting of particular gene products essential for bacterial survival under stressful conditions. Developing HtrA inhibitors for instance to reduce the survivability of this pathogen in foods as proposed by Wonderling *et. al*. (2004), may not work efficiently as the bacteria contaminating the food in most cases would already be adapted to the stressful environment, a physiological state which does not appear to require HtrA.

Out of the heat-shock inducible proteins only *htpX* (*lmo0963*) showed up-regulation of ≥two-fold in all four strains of *L. monocytogenes* examined in this study (Fig. 3.20). HtpX is a heat inducible endopeptidase in *E. coli* involved in the quality control of membrane proteins and degradation of some cytoplasmic proteins, with cellular roles that complement those of FtsH (Sakoh *et al.*, 2005; Shimohata *et al.*, 2002).

From the gene expression patterns obtained during this experiment it appears that prolonged hyperosmotic shock has a dramatic effect on the outside layer of the cell, consequently cell wall

proteins require more constant maintenance thus requiring increased expression of 'house keeper' proteins such as FtsH, HtpX and PrsA.

A gene encoding heat shock protein 33 (Hsp33), *lmo0222* showed ≥two-fold up-regulation in all strains except 70-1700 (Fig. 3.20). In an oxidized state Hsp33 protein is highly efficient chaperone holdase that binds tightly to substrate proteins, thereby preventing their irreversible aggregation (Kang *et al.*, 2007; Kim *et al.*, 2001; Winter *et al.*, 2005). Some of the substrate proteins for this chaperone include proteins involved in fatty acid biosynthesis as well as proteins involved in transcription and cell division (Winter *et al.*, 2005). Hsp33 has a redox regulated activity; it responds to changes in redox environment by sensing intracellular levels of H_2O_2 and becomes activated (oxidized) to function as a molecular chaperone. In *E. coli* over expression of Hsp33 provides greater tolerance to oxidative stress and in *Bacillus psychrosaccharolyticus* Hsp33 transcription levels were found to increase during oxidative and solvent stresses (Kang *et al.*, 2007; Kim *et al.*, 2001). Involvement of Hsp33 in hyperosmotic adaptation in *L. monocytogenes* cannot be excluded; on the contrary it maybe speculated that expression of this chaperone in osmotically challenged cells renders cells which can withstand higher levels of osmotic pressure. Induction of oxidative-stress responsive genes under continuous osmotic stress has been observed in *E. coli* (Gunasekera *et al.*, 2008) but was not evident in *B. subtilis* (Steil *et al.*, 2003). There was no clear evidence of intracellular peroxide stress in NaCl challenged strains of *L. monocytogenes* as none of the H_2O_2 regulatory machinery showed activation. This included the PerR, a peroxide stress response regulator encoded by *lmo1683*, which showed no apparent change in transcription levels, *kat* (*lmo2785*) a gene encoding catalase showed no change in transcription(data not shown), and *sodA* (*lmo1439*) and *osmC* (*lmo2199*) only showed activation in relatively salt sensitive strain 70-1700. Inactivation of machinery responsible for redox regulation in these cells suggests that a trigger other than oxidative stress activates transcription of Hsp33 in hyperosmotically adapted cells of *L. monocytogenes* grown in a rich medium. Strains unable to respond to such trigger may result in a less salt tolerant phenotype, possibly observed in strain 70-1700.

Osmotic-stress induced transcription of *clp*B (*lmo2206*) which encodes ClpB a heat induced endopeptidase. Transcription of this gene showed strain specific pattern with 9-fold down-regulation observed in strain ATCC19115, no change in transcription in ScottA and up-regulation of 4-fold in FW03-0035 and two-fold in 70-1700 (Fig. 3.20). ClpX encoding gene *clpX* (*lmo1268*) showed

≥ two-fold up-regulation in strains ScottA, FW03-0035 and 70-1700, while its increased expression in ATCC19115 was not statistically significant. Transcription of another *clp* gene, *clpE* was found to be significantly reduced in all four strains ranging from two-fold down-regulation in 70-1700 to 54-fold in strain ATCC19115 (data not shown). Other heat induced chaperones also showed strain specific transcription patters, including *grpE* (*lmo1474*) with ≥two-fold up-regulation observed in strains FW03-0035 and 70-1700 and *groEL* (*lmo2068*) showing ≥two-fold down-regulation in strains ATCC19115 and ScottA and ≥two-fold up-regulation in strains FW03-0035 and 70-1700 (Fig. 3.20). DnaJ encoding gene *dnaJ* (*lmo1472*) showed up-regulation in all four strains, while *dnaK* (*lmo1473*) expression was found to be statistically insignificant (Fig. 3.20). DnaK involvement in hyperosmotic stress of *L. monocytogenes* have been previously assigned as an initial stress chaperone only (Duché *et al.*, 2002).

Out of the three cold shock proteins found in *L. monocytogenes* only one showed activation in hyperosmotically adapted cells be it in only two strains examined in this study. Transcription of CspL encoded by *cspL* (*lmo1364*) was found to be up-regulated 4-fold in strain FW03-0035 and 7 fold in ATCC19115 (Fig. 3.20). Activation of cold shock proteins have previously been linked to osmotic tolerance in *L. monocytogenes* (Schmid *et al.*, 2009). These CspA-like RNA-binding proteins are thought to function as RNA chaperones, preventing secondary structure formation during RNA transcription that, if uncorrected, could potentially stall transcription (Jiang *et al.*, 1997).

3.3.2.8 Prolonged salt exposure effect on genes associated with chemotaxis, flagella assembly and extracellular proteins.

Osmo-adapted cells of *L. monocytogenes* showed significantly diminished transcription of genes associated with flagella assembly as all four strains showed repression of transcription of genes in this subgroup with *T-value* scores of -3.16 to -6.54 (Table 3.1). Transcription of flagella motility genes is under control of three regulators a temperature dependent repressor MogR, a response regulator DegU and an anti-repressor GmaR (Mauder *et al.*, 2008; Shen *et al.*, 2006). Flagella gene transcription is thought to be repressed by MogR at physiological temperature, however at low temperature DegU activates transcription of GmaR which in turn inhibits MogR repression activity thus allowing for transcription of flagella genes to occur. Flagella synthesis anti-repressor gene *gmaR* (*lmo0688*) showed no significant up-regulation in osmo-adapted cells, on the contrary its transcription was evidently down-regulated in two strains ATCC19115 and ScottA (Table 3.4).

Table 3. 4 Gene expression patterns of four *L. monocytogenes* strains associated with flagella assembly and LPXTG motif observed during adaptive hyperosmotic stress response.

Gene	ATCC19115		ScottA		FW03-0035		70-1700		Function
	LR[#]	P	LR	P	LR	P	LR	P	
gmaR	**-1.12**	0.022	**-1.08**	0.022	-0.40		0.51		similar to glycosyltransferases
fliR	**-1.33**	0.005	**-1.29**	0.008	**-1.24**	0.010	**-1.21**	0.001	flagellar biosynthesis pathway protein
fhlB	**-1.53**	0.006	-0.71	0.040	-0.67		**-1.15**	0.001	flagellar biosynthesis pathway protein
flhF	0.63		-0.42		**-1.16**	0.003	-0.40		flagellar GTP-binding protein
motA	-0.23		**-1.03**	0.007	**-1.16**	0.002	-0.58		similar to motility protein (flagellar motor rotation)
flaA	**-3.43**	2.0E-05	**-4.70**	1.6E-05	**-1.07**	0.002	-0.45		flagellin
flgE	**-1.01**	0.015	**-1.12**	0.001	**-1.22**	0.009	-0.90	0.006	flagellar hook protein
fliN	-0.40		**-1.68**	0.004	-0.48		-0.51		similar to flagellar motor switch protein
flgK	**-1.89**	0.003	-0.95	0.008	**-1.48**	0.002	-0.81	0.020	flagellar hook-associated protein
fliD	**-1.26**	0.014	-0.74	0.010	-0.70	0.014	**-1.03**	0.010	flagellar capping protein
fliS	-0.91	0.043	**-1.28**	0.003	-0.96	0.023	-0.89	0.002	flagellin-specific chaperone
flgC	-0.68		**-1.70**	0.022	-0.63		-0.17		flagellar basal body rod protein
fliE	-0.50		**-1.79**	8.4E-05	-0.95	0.005	-0.34		flagellar hook-basal body protein
lmo1602	**-1.20**	0.005	-0.31		-0.04		-0.16		protein with divergent methyl-accepting chemotaxis-like domain
cheV	-0.94		**-1.21**	4.3E-04	**-1.02**	0.013	-0.94	0.004	similar to CheA activity-modulating chemotaxis protein
cheY	-0.85		**-1.55**	3.0E-04	-0.21		0.29		chemotaxis response regulator
inlA	**-1.98**	0.002	**-2.66**	2.5E-04	**-1.30**	0.041	**-1.02**	4.2E-04	internalin A
lmo0610	**-1.67**	0.028	**-1.69**	1.3E-04	**-1.59**	4.3E-04	-0.73	0.048	putative peptidoglycan bound protein
lmo0514	**-1.33**	0.006	-0.68		-0.79		-0.94	0.021	putative peptidoglycan bound protein
lmo0331	-0.74		-0.79	0.035	-0.71	0.078	**-1.46**	3.2E-05	putative peptidoglycan bound protein
lmo0725	-0.85		**-1.04**	0.007	**-1.27**	0.001	**-1.57**	2.0E-05	putative peptidoglycan bound protein (LPXTG motif)
lmo0627	**-2.34**		-0.71		-0.46		-0.75	0.011	peptidoglycan bound protein (LPXTG motif) similar to adhesin
lmo1799	-0.08		-0.21		**-1.22**	0.002	-0.04		putative peptidoglycan bound protein (LPXTG motif)
lmo0409	**-1.35**	0.007	-0.54	0.045	-0.76	0.025	**-1.30**	2.1E-04	putative peptidoglycan bound protein (LPXTG motif)
lmo0549	-0.69		-0.61	0.027	**-1.14**	0.010	**-1.11**	0.003	putative peptidoglycan bound protein (LPXTG motif)
lmo0842	**-3.08**	0.001	**-1.49**	0.001	**-2.29**	1.5E-06	**-1.39**	0.002	putative peptidoglycan bound protein (LPXTG motif)

LR=Log Ratio; P values not shown were >0.05

Both *mogR* (*lmo0674*) and *degU* (*lmo2515*) showed no significant change in transcription (data not shown). Transcription of *flaA* (*lmo0690*) encoding flagellin was found to be down-regulated up to 26-fold in the three salt tolerant osmo-adapted cells (Table 3.4). Hyperosmotic conditions have previously been shown to inhibit the synthesis of flagellin and expression of flagella in *E. coli* (Li *et al.*, 1993), *B. subtilis* (Steil *et al.*, 2003) and *Enterobacter sakazakii* (Riedel & Lehner, 2007). A large amount of energy and cell resources (in terms of amino acids) is required for synthesis of flagella, energy that might be channelled into other cellular processes that aid the survival of the organism in hyperosmotic condition. The exact mechanism of flagellin gene transcription inhibition is unknown

although it is believed that CodY represses the expression of these genes in *B. subtilis* in response to the availability of amino acids (Steil *et al.*, 2003).

Chemotaxis and type III secretion system (T3SS) gene sets also showed overall down-regulation during hyperosmotic stress with *T-value* scores ranging from -1.30 to -3.41 (Table 3.1). Chemotaxis proteins may not be needed so late in the adaptive response to salt in bacteria especially since the cells appear to have reduced motility. T3SS is associated with creation of the bacterial flagella basal body and as a consequence appeared to not be required in osmo-adapted cells.

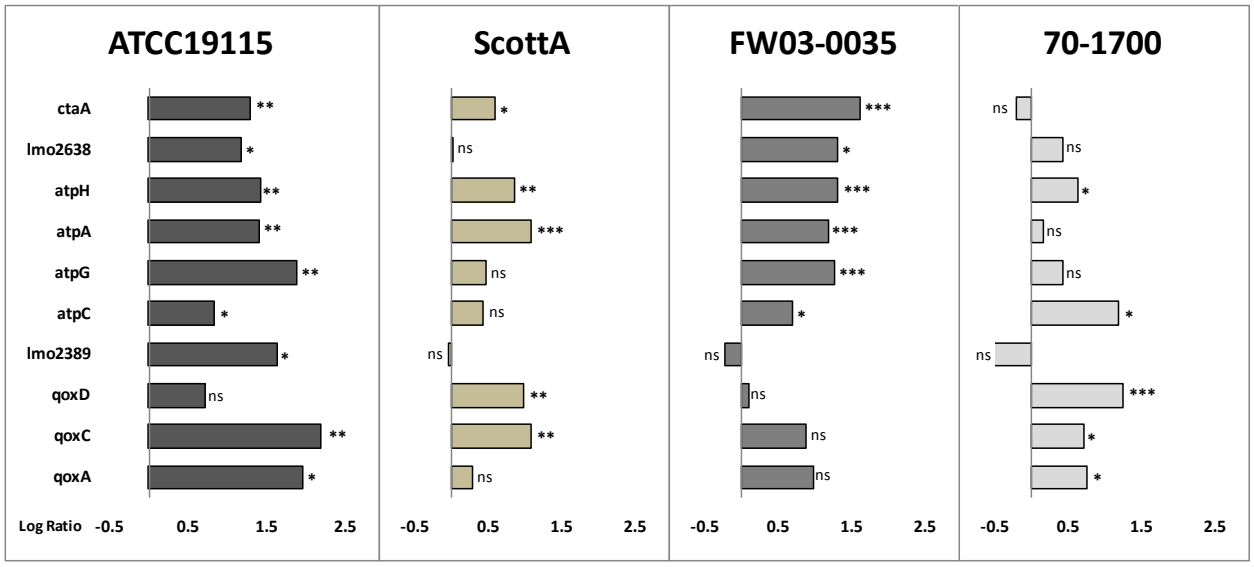
Gene sets encoding surface proteins were found to be down-regulated in NaCl-adapted cells. Gram-positive anchored proteins encoded genes were on average significantly down-regulated in three strains of *L. monocytogenes* with *T-values* ranging from -2.67 to -3.33 (Table 3.1). Genes in this group down-regulated in all four strains included *inlA* (encoding internalin A) and *lmo0610* (Table 3.4). A large number of cell wall anchoring proteins have a conserved LPXTG motif, which acts as a recognition site for cell wall sorting machinery responsible for attaching the translocated protein to the bacterial cell wall. A large number of genes encoding such 'tagged' proteins showed down-regulation in osmo-adapted cells with genes such as *lmo*0842 showing down-regulation of up-to 9-fold (Table 3.4). Surface proteins fulfil a variety of functions which may be of benefit to the organism although it appears that following adaptation to hyperosmotic stress cells de-emphasize such proteins. It is possible that expressing a large number of surface proteins may be a burden in a hyperosmotic environment. These could constantly get physically damaged by NaCl molecules and an ongoing replacement would be too energy costly for the bacterial cell. Evident thickening of bacterial cell wall during hyperosmotic adaptation as a defence mechanism against high osmotic pressure may significantly alter the surface topology of the bacterial cell wall completely or partly obscuring binding sites of surface proteins.

3.3.2.9 Prolonged salt exposure effect on genes associated with energy production and carbohydrate metabolism.

Bacterial cells undergo metabolic changes to cope with a demand for energy as osmotic pressure of the extracellular medium increases. Cells grown under high osmotic pressure have higher energy demands to form and sustain ionic gradients across the cell membrane and to counteract ion

diffusion into the cytoplasm (Ohwada *et al.*, 1994; Oren, 1999; Varela *et al.*, 2004). Consequently the intracellular ATP content has been observed to increase in various organisms under conditions of osmotic stress (Ohwada *et al.*, 1994; Varela *et al.*, 2004). In addition to higher demand for energy in osmotically challenged bacterial cells, NaCl has been found to significantly diminish the rate of ATP production via oxidative phosphorylation by decreasing the electrochemical potential across bacterial cell membrane (Shabala *et al.*, 2006).

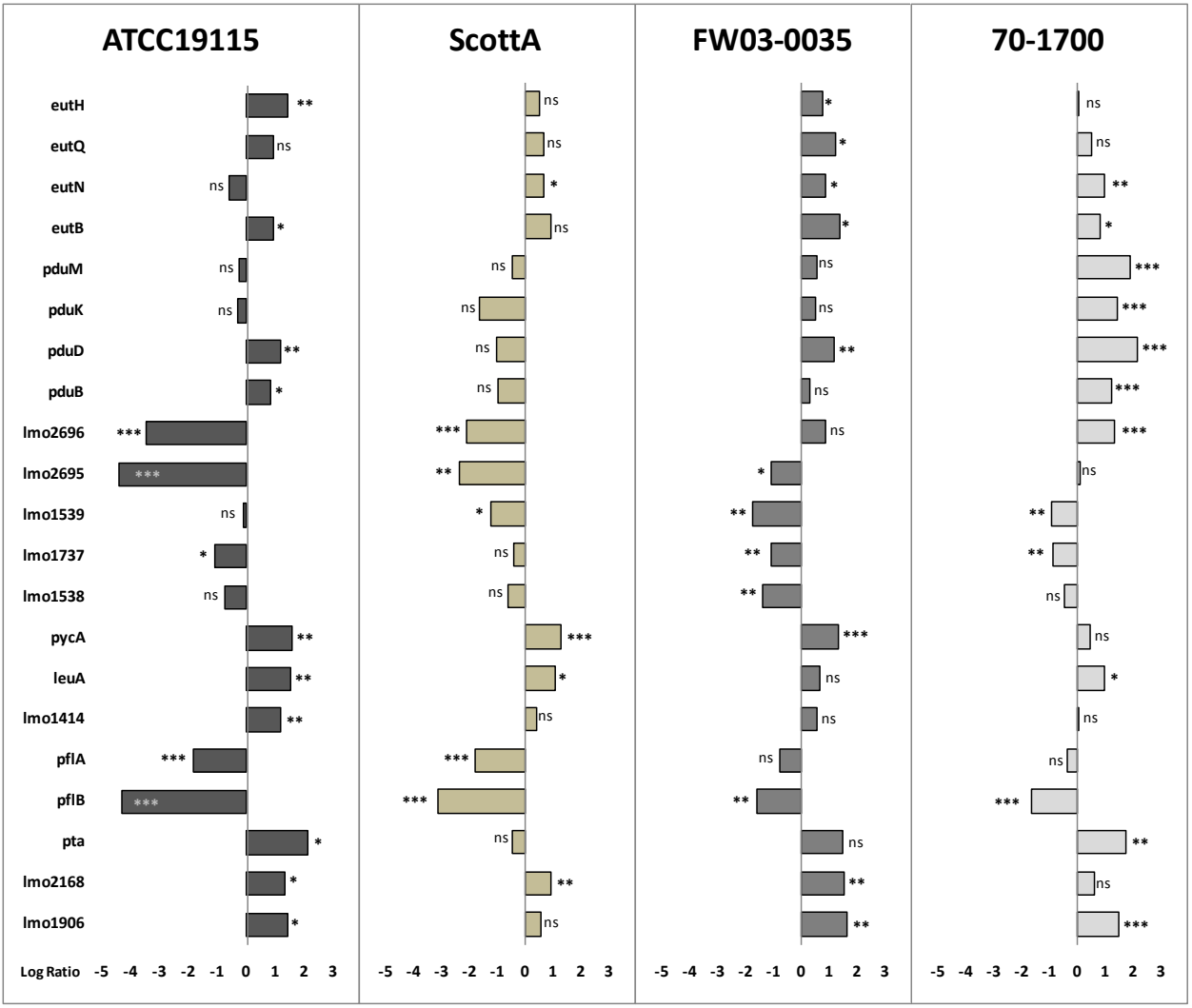
Energy pool management appeared to be of critical importance in osmo-adapted *L. monocytogenes* cells suggested by an increase in transcription levels of a number of genes associated with oxidative phosphorylation. Genes such *atpC*, *atpG*, *atpA* and *atpH* encoding F1F0-ATPase (Fig. 3.21) as well as NAD oxidase/ dehydrogenase genes *lmo2389* and *lmo2471* (Fig. 3.21) showed significant activation in cells adapted to hyperosmotic stress.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.21 Expression of genes associated with oxidative phosphorylation in four osmo-adapted *L. monocytogenes* strains.

Overall there appeared to be a suppression of genes associated with carbohydrate metabolism with genes such as *pflB* and *pflA* showing up to 20-fold decreases in transcription levels in at least two of the strains examined in this study (Fig. 3.22).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

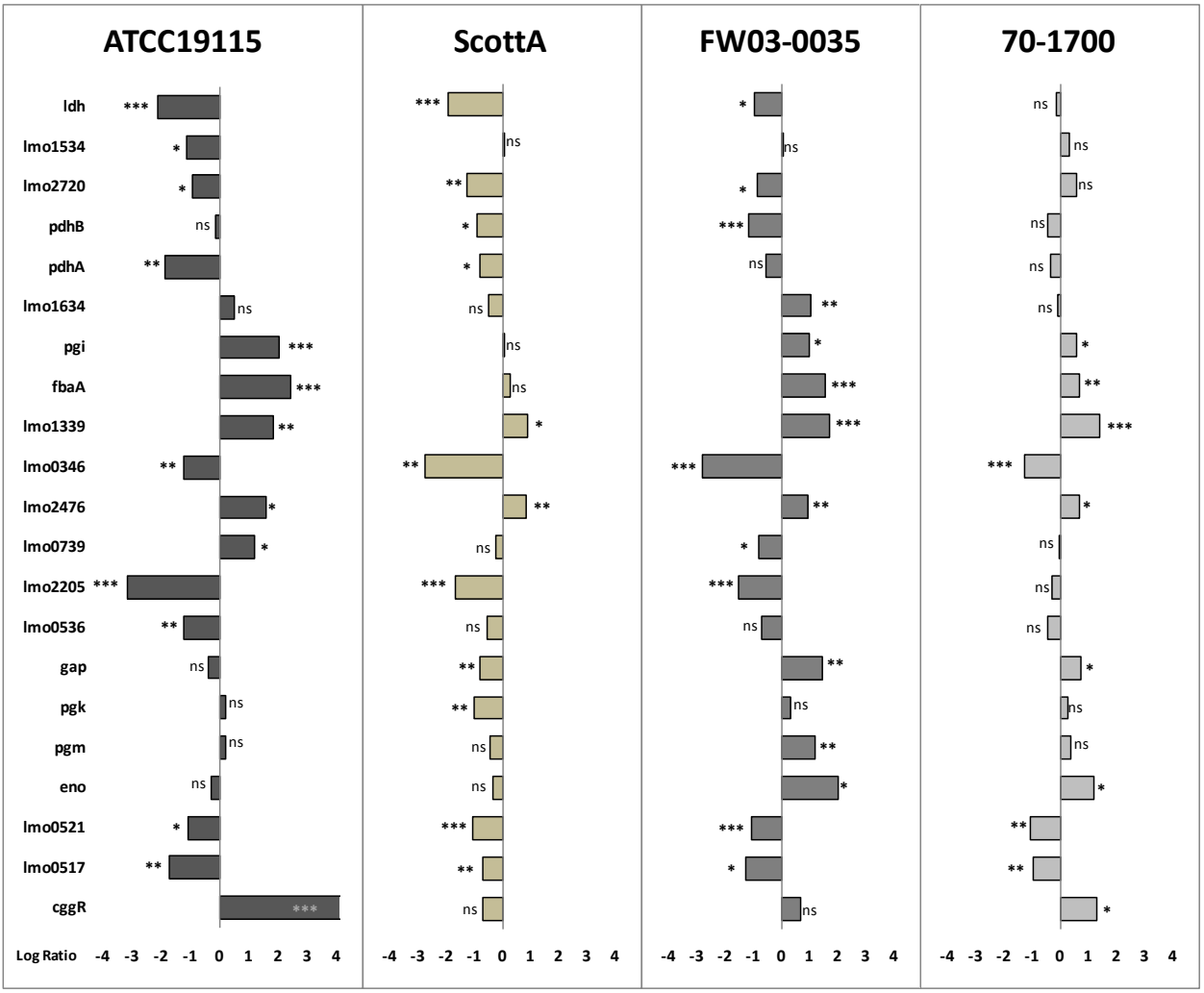
Figure 3.22 Expression of genes associated with pyruvate and glycerol metabolism of four osmo-adapted *L. monocytogenes* strains.

While pyruvate carboxylase encoded by *pycA* (*lmo1072*), is involved in pyruvate metabolism and citrate cycle in addition to alanine and aspartate metabolism pathway, which directly feeds the aminoacyl-tRNA biosynthesis pathway. The later has been found to be induced in osmo-adapted cells, with genes such as *aspS* (*lmo1519*) showing up-regulation (Fig. 3.19).

Listerial *pdu* genes are orthologous to genes necessary in *Salmonella sp*. for the co-enzyme B_{12}-dependent degradation of propanediol as an energy source for growth (Bobik *et al.*, 1999; Buchrieser *et al.*, 2003; Chen *et al.*, 1994; Liu *et al.*, 2007). Collectively these showed up-regulation in a singular strain, 70-1700 during the prolonged exposure to salt stress with a *T-value* score of 4.31

(Table 3.1). Some of the genes that were significantly up-regulated included *pduB* (*lmo1152*), *pduD* (*lmo1154*), *pduK* (*lmo1158*), *pduM* (*lmo1162*) (Fig. 3.22).

A number of genes encoding proteins involved in glycolysis showed down-regulation in all four strains such as *lmo0346*, *lmo0517* and *lmo0521*, while others such as *lmo2205* and *ldh* were significantly down-regulated in strains ATCC19115, ScottA and FW03-0035 but showed no change in transcription in strain 70-1700 (Fig. 3.23).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 3.23 Expression response of genes associated with glycolytic pathway following in four osmo-adapted *L. monocytogenes* strains.

Surprisingly the gene encoding catabolite control protein CcpA (*lmo1599*), showed no change in transcription levels in all four strains examined in this study (data not shown). CcpA controls the pathways of carbon catabolism and has previously been shown to be induced in *L. monocytogenes* strain LO28 during prolonged exposure to salt stress (Duché *et al.*, 2002). A gene encoding a protein similar to a central glycolytic genes regulator protein CggR of *B. subtilis* (Doan & Aymerich, 2003) *lmo2460* showed significant activation in only two strains of *L. monocytogenes* 20-fold up-regulation in strain ATCC19115 and 3-fold up-regulation in 70-1700 (Fig. 3.23).

In *B. subtilis* high levels of intracellular fructose-1,6-bisphosphate (FBP) has been shown to inhibit CggR DNA-binding activity thus having a positive effect on glycolytic pathway (Doan & Aymerich, 2003). Interestingly FBP is a product of a *fruB* gene, which as described previously was up-regulated in all but ScottA strains. Perhaps the activation of *fruB* and consequently post-transcriptional inhibition of CggR serve as an indicator of stress experienced by some stains of *L. monocytogenes* more than others when cultivated in a hyperosmotic medium, especially since it has been established that transcription of *cggR* in this organism is positively influence by stress response regulator σ^B (Raengpradub *et al.*, 2008). The exact interaction of FBP and CggR has not been studied in *L. monocytogenes* and it is unclear whether such extrapolations can be made. It is also unclear, why *cggR* showed no significant change in transcription in strains ScottA and FW03-0035 (Fig. 3.23), both of which were exposed to 12.0% (w/v) NaCl. It is possible that high levels of salt somehow disturb the normal metabolic regulation processes, or perhaps activate another glycolysis pathway inhibitor not yet elucidated.

The fructose/mannose metabolism gene set also showed significant down-regulation in the three relatively-salt tolerant strains with no significant change in transcription levels in strain 70-1700 (Table 3.1). A number of genes were found to show significant down-regulation in all four strains, these included *lmo*0401, *lmo*0506 and *lmo*0346 (Table 3.5).

Table 3. 5 Gene expression patterns of four *L. monocytogenes* strains associated with Fructose/Mannose metabolism observed during adaptive hyperosmotic stress response.

Gene	ATCC19115		ScottA		FW03-0035		70-1700		Function
	LR[#]	P	LR	P	LR	P	LR	P	
fbaA	**2.41**	9.4E-05	0.28		**1.55**	0.000	0.67	0.009	fructose-bisphosphate aldolase
fruK	**1.45**	0.008	**-3.19**	0.002	**1.37**	0.001	**2.36**	5.9E-06	fructose-1-phosphate kinase
lmo0346	**-1.26**	0.004	**-2.75**	0.002	**-2.80**	0.000	**-1.29**	1.5E-04	similar to triosephosphate isomerase
lmo0401	**-3.69**	1.4E-05	-0.71	0.017	**-2.55**	2.4E-06	-0.60	0.039	putative alpha-mannosidase
lmo0429	-0.67		-0.79	0.019	-0.87	0.001	**-1.29**	0.001	putative alpha-mannosidase
lmo0506	**-1.24**	0.028	-0.81	0.011	**-1.30**	0.002	-0.69	0.009	similar to polyol (sorbitol) dehydrogenase
lmo0544	**-3.04**	0.001	**-1.31**	0.000	**-2.05**	0.001	**-1.01**	0.009	similar to PTS system, glucitol/sorbitol-specific IIC component
lmo0781	**-1.45**	0.019	**-2.03**	5.4E-05	**-1.78**	0.000	0.15		similar to PTS system, mannose-specific IID component
lmo0782	**-2.43**	0.001	**-2.63**	1.3E-05	**-1.85**	0.000	0.27		similar to PTS system, mannose-specific IIC component
lmo2000	**1.79**	0.021	**1.02**	0.001	0.17		0.29		similar to PTS system, mannose-specific IID component
lmo2001	**1.08**		0.59		-0.48		0.69	0.048	similar to PTS system, mannose-specific IIC component
lmo2002	-0.16		0.63	0.022	-0.62		**1.50**	2.0E-05	similar to PTS system, mannose-specific IIB component
lmo2014	**1.17**	0.005	0.72	0.034	0.27		**1.41**	7.4E-05	putative alpha-mannosidase
lmo2095	0.88		-0.74	0.006	0.09		**1.56**	2.9E-04	similar to 1-phosphofructokinase
lmo2110	**2.92**	0.000	0.30		**1.57**	0.001	**1.45**	4.2E-05	mannose-6-phosphate isomerase
lmo2475	0.80		**1.05**	0.002	**1.16**	0.003	0.93	0.009	phosphomannomutase
lmo2649	**-4.76**	0.000	0.16		-0.98	0.016	-0.26		similar to PTS system, mannitol/ascorbate-specific IIC component
lmo2650	**-3.92**	2.3E-05	0.08		**-1.17**	0.003	-0.51		similar to PTS system, mannitol/ascorbate-specific IIB component
lmo2651	**-2.18**	0.020	0.54	0.036	**1.01**	0.036	0.46		similar to PTS system, mannitol/ascorbate-specific IIA component
lmo2663	**-5.28**	0.009	**-1.15**	0.011	**-3.92**	1.5E-05	0.24		polyol dehydrogenase
lmo2664	**-7.36**	1.9E-05	**-1.60**	0.006	**-3.73**	0.000	-0.05		similar to sorbitol dehydrogenase
lmo2734	-0.29		-0.55		**-1.24**	0.004	-0.49	0.044	putative alpha-mannosidase
lmo2799	**-1.08**	0.039	-0.60	0.020	-0.10		**-1.17**	0.007	similar to PTS system, mannitol-specific IIBC component
lmo2849	-0.37		-0.42		**-1.36**	0.005	-0.28		similar to rhamnulokinase
mptA	-0.90		**-1.07**		**-1.25**	0.003	-0.53		similar to PTS system, mannose-specific IIAB component
mptC	0.47		**-1.56**	0.049	-0.70		-0.08		similar to PTS system, mannose-specific IIC component
mptD	**-1.75**	0.001	**-1.36**	0.016	**-1.44**	7.0E-05	-0.62		similar to PTS system, mannose-specific IID component

LR=Log Ratio; P values not shown are >0.05.

A number of repressed genes also included those involved in transport of these sugars, as described previously in section 3.3.2.1.6. A few genes in this subgroup showed up-regulation although in a case of *lmo2014*, which was found to be up-regulated in strains ATCC19115, ScottA and 70-1700 (Table 3.5), however strong down-regulation in all four strains also occurred for *lmo0401* encoding a protein with an analogous alpha-mannosidase function. A gene encoding mannose-6-phosphate isomerase, *lmo2110* showed ≥two-fold up-regulation in three strains of *L. monocytogenes* examined excluding ScottA (Table 3.5). Interestingly ScottA strain showed significant up-regulation of PTS-related gene for trehalose uptake (*lmo1255*) as well as the gene encoding a trehalose-6-phosphate

hydrolase (*lmo1254*), which converts trehalose to glucose. This perhaps reflects strain-related preference for certain sugars under high levels of stress that requires further investigation.

Overall, carbohydrate metabolism in osmo-adapted cells was evidently suppressed. This suppression may reflect the slowed growth rate of NaCl stressed cells when compared to non-stressed cells with abundant nutrients present in an enriched BHI medium. It appears that instead of channelling energy into cell division and replication (normal metabolic processes) cells focus their energy primarily on cell maintenance.

3.3.2.10 Adaptive hyperosmotic response effect on genes with unknown function.

A large proportion of salt-stress activated genes were found to encode proteins of unknown function. Some of the stronger up-regulated genes included *lmo*2223, *lmo*2056, *lmo*2079, *lmo*2048, *lmo*1541, *lmo*1922, *lmo*1008 and *lmo*2522 with up-regulation in all four strains (Table 3.6).

Table 3. 6 Gene expression patterns of *four L. monocytogenes* strains encoding proteins of unknown function up-regulated during adaptive hyperosmotic stress response.

Gene	Function	ATCC19115		ScottA		FW03-0035		70-1700	
		LR[#]	P	LR	P	LR	P	LR	P
lmo0247	unknown protein	**1.31**	0.014	0.57		0.53		0.08	
lmo0289	similar to uncharacterized conserved proteins	**1.78**	0.001	**1.18**	0.002	**1.01**	3.2E-04	0.53	0.049
lmo0485	similar to nitroreductase-like family protein	**1.80**	0.018	**1.07**	0.004	0.17		-0.40	
lmo0581	similar to predicted SAM-dependent methyltransferases	**1.35**	0.020	0.45		-0.03		0.21	
lmo0663	similar to predicted hydrolases of the HAD superfamily	**2.13**	2.5E-04	-0.09		0.64		0.26	
lmo0763	similar to predicted phosphohydrolases	**1.65**	0.003	**1.82**	0.000	0.89		-0.16	
lmo0952	unknown protein	**1.37**		0.56	0.039	**1.34**	0.019	**-1.04**	0.009
lmo1008	unknown protein	**1.25**	0.009	**2.09**	0.003	**2.06**	0.001	0.90	0.002
lmo1183	unknown protein	-0.08		**1.31**	0.003	0.88	0.026	0.98	0.001
lmo1240	similar to predicted phosphoesterase	**1.34**	0.033	0.76	0.011	0.82	0.009	0.91	0.003
lmo1245	unknown protein	**1.57**	0.007	**1.30**	3.7E-04	**1.28**	3.4E-04	0.73	0.007
lmo1282	unknown protein	**1.83**	0.043	0.45		**1.31**	0.020	0.05	
lmo1306	similar to uncharacterized conserved proteins	**3.18**	0.005	**3.21**	0.001	**2.66**	4.1E-04	0.91	
lmo1333	similar to aminodeoxychorismate lyase family proteins	**1.71**	0.008	0.96	0.006	0.73	0.025	0.55	
lmo1541	predicted ribosomal protein	**2.33**	0.001	**1.40**	1.8E-04	**1.63**	0.001	**1.08**	0.001
lmo1626	unknown protein	**1.21**	0.014	**1.54**	4.8E-04	**1.49**	0.005	-0.59	
lmo1650	similar to Bacillus CodC protein (cyotchrome c defective protein)	**1.12**		**1.41**	0.001	**1.09**	0.033	0.93	0.010
lmo1707	unknown protein	**1.95**	4.5E-04	**1.10**	2.5E-04	**1.14**	0.001	**1.08**	2.0E-04

Gene	Function	ATCC19115		ScottA		FW03-0035		70-1700	
		LR[#]	P	LR	P	LR	P	LR	P
lmo1743	unknown protein	**1.40**	0.008	**1.37**	0.001	**1.40**	3.1E-04	**1.10**	0.001
lmo1760	predicted phosphate-binding enzyme	**2.18**	0.001	0.37		**1.69**	0.001	**1.46**	0.002
lmo1810	similar to uncharacterized protein possibly involved in aromatic compounds catabolism	**2.66**	0.010	**1.11**		0.10		-0.67	
lmo1815	similar to uncharacterized conserved proteins	0.93		**3.25**	0.002	0.04		-0.28	
lmo1921	unknown protein	**2.13**	0.004	**1.10**	0.002	**1.68**		**1.58**	3.1E-04
lmo1922	similar to pilus assembly protein	**1.74**	0.006	**1.75**	0.000	**1.94**	5.9E-06	**1.94**	3.7E-07
lmo1941	similar to uncharacterized conserved proteins	**1.56**	0.003	**1.13**	0.007	0.96		0.11	
lmo1976	simialr to short-chain dehydrogenases of various substrate specificities	**1.61**	0.001	0.75	0.022	**1.35**	0.001	-0.11	
lmo1979	similar to uncharacterized conserved proteins	**2.07**	0.001	**1.28**	0.001	**1.26**	0.005	0.65	0.026
lmo2005	similar to predicted oxidoreductases (related to aryl alcohol dehydrogenases)	**1.99**	0.007	**1.30**	0.016	0.75		**1.38**	5.3E-05
lmo2013	similar to uncharacterized conserved proteins	**1.66**	0.010	0.69	0.049	-0.10		0.81	0.011
lmo2029	predicted integral membrane protein	**1.52**	0.002	**1.05**	0.021	**1.23**	0.001	0.77	0.009
lmo2048	similar to uncharacterized conserved proteins	**2.70**	3.9E-04	**2.59**	5.4E-07	**2.47**	2.8E-07	**1.64**	5.9E-05
lmo2051	similar to predicted secreted protein	**1.39**	0.002	**1.21**	1.7E-04	**1.40**	0.001	0.88	0.004
lmo2056	similar to uncharacterized conserved proteins	**1.43**	0.004	**1.66**	1.2E-05	**1.70**	1.7E-04	**1.44**	0.001
lmo2071	Unknown protein	**1.69**	0.001	0.98	0.022	**1.57**	2.3E-05	**1.20**	7.2E-05
lmo2079	putative lipoprotein	**1.87**	3.4E-04	**1.92**	2.7E-05	**1.57**	2.2E-05	**1.23**	3.3E-04
lmo2080	putative lipoprotein	**2.07**	3.4E-04	0.58	0.035	0.81	0.010	0.62	
lmo2083	similar to uncharacterized conserved proteins	**1.11**	0.015	0.42		**1.15**	0.041	**1.41**	0.002
lmo2089	similar to esterase/lipase family proteins	**1.65**	0.012	0.39		0.94		0.60	0.010
lmo2112	putative DNA-binding protein	-0.15		-0.09		0.17		**1.82**	1.9E-05
lmo2120	similar to uncharacterized conserved proteins	**3.52**	1.7E-05	**1.63**	0.007	**1.93**	0.007	1.00	
lmo2187	unknown protein	**1.57**	0.007	0.78	0.028	0.97	0.006	0.98	0.002
lmo2197	unknown protein	**1.69**	0.002	0.68	0.020	0.71	0.024	**1.05**	0.006
lmo2208	similar to predicted hydrolases of the HAD superfamily	**1.20**	0.018	0.93	0.020	**1.54**	0.001	**2.05**	7.7E-07
lmo2223	similar to uncharacterized conserved proteins	**2.41**	1.3E-04	**2.74**	0.004	**3.13**	2.6E-07	**1.37**	2.3E-04
lmo2247	similar to aldo/keto reductases, related to diketogulonate reductase	**1.21**	0.033	0.48		**1.43**	0.027	0.62	
lmo2261	similar to uncharacterized conserved proteins	**1.94**	0.011	**1.18**	2.7E-04	0.54		-0.21	
lmo2351	similar to NADH-dependent FMN reductases	**2.07**	2.6E-04	0.88	0.041	0.23		0.68	0.021
lmo2359	similar to predicted hydrolases of the HAD superfamily	**1.71**	0.001	**1.22**	1.7E-04	0.89	0.004	0.66	0.041
lmo2450	similar to esterase/lipase family proteins	**1.79**	0.001	**1.23**	0.001	**1.02**	0.003	0.28	
lmo2472	similar to uncharacterized conserved proteins	**1.38**	0.041	0.30		0.52		0.54	
lmo2479	similar to uncharacterized conserved proteins	**1.44**		**1.14**	0.001	**1.60**	0.001	**1.47**	0.001
lmo2504	similar to membrane-bound metallopeptidases	**2.46**	9.9E-05	**2.62**	2.6E-05	**2.54**	4.7E-06	0.80	0.037
lmo2508	similar to uncharacterized conserved proteins	**1.67**	0.001	**1.67**	7.8E-05	**2.52**	0.002	0.89	0.030
lmo2522	similar to uncharacterized conserved proteins	**2.11**	0.009	**3.90**	1.1E-04	**3.66**	1.2E-04	**3.52**	7.8E-07
lmo2658	acetyltransferase, GNAT family	**1.75**	0.006	0.91	0.034	**1.39**	0.016	0.97	0.007
lmo2843	similar to uncharacterized protein involved in cytokinesis	**2.47**	3.0E-04	**1.36**	0.001	**1.45**	0.003	0.47	

LR= Log Ratio; P values not shown are >0.05.

Activation of these genes appeared to be beneficial for *L. monocytogenes* adaptation to hyperosmotic environments their direct involvement in the adaptive response will most likely be elucidated as more research is carried out on the physiology of this pathogen in the future. A few genes showed up-regulation in the three relatively-salt tolerant strains and not 70-1700, such as *lmo2120*, *lmo1306* and *lmo2450*, suggesting a potential involvement in the salt tolerance.

Genes such as *lmo*2226, which showed up-regulation of 16-fold in strain ScottA only (data not shown) could provide extra advantage during NaCl challenge. This gene may encode a protein similar to RbbA (ribosome bound ATPase) in *E. coli*. The exact function of RbbA in translation is unknown, however it is believed to assist EF-Tu (Ganoza *et al.*, 2002). Activating transcription of RbbA may provide a means of distributing energy more efficiently during transcription. Another gene with a similar expression pattern included *lmo1815* (Table 3.6).

Considering the well accepted fact that salt stress suppresses overall metabolism of bacterial cell it was no surprise that a large number of genes showed down-regulation during adaptive response to high levels of NaCl. This was also true for a large number of unknown genes which showed significant down-regulation (Table 3.7). Repressed genes in all four strains included *lmo0602*, *lmo0629*, *lmo0654*, and *lmo2813*. Ability to turn-off unnecessary genes in stressful environments to save cellular energy renders an overall more adapted and arguably more stress-tolerant cell. It is therefore important to take into account suppressed gene profile in a given stress response.

Table 3. 7 Gene expression patterns of four *L. monocytogenes* strains encoding proteins of unknown function down-regulated during adaptive hyperosmotic stress response.

Gene	Function	ATCC19115		ScottA		FW03-0035		70-1700	
		LR[#]	P	LR	P	LR	P	LR	P
lmo0057	predicted membrane protein	-0.67		-0.44		-0.80		**-1.52**	3.8E-05
lmo0086	unknown protein	**-1.48**	0.019	-0.99	0.005	**-1.96**	0.002	**-1.45**	0.001
lmo0133	similar to uncharacterized conserved proteins	-0.86		**-2.14**	0.001	**-1.15**	0.044	**-1.09**	1.6E-04
lmo0134	similar to acetyltransferase (GNAT) family proteins	-0.85		**-2.01**	0.004	-0.96		-0.15	
lmo0267	similar to predicted ring-cleavage extradiol dioxygenases	**-2.29**	1.3E-04	**-1.04**	0.004	-0.84	0.030	-0.60	
lmo0324	Unknown protein	**-1.23**	0.027	**-1.44**	0.005	-0.83	0.040	-0.84	0.002
lmo0344	similar to dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	**-1.34**	0.012	**-2.11**	0.008	**-2.43**	1.9E-04	-0.32	
lmo0350	Unknown protein	**-1.54**	0.002	**-1.91**	0.043	**-2.59**	9.5E-05	-0.08	

Gene	Function	ATCC19115		ScottA		FW03-0035		70-1700	
		LR[#]	P	LR	P	LR	P	LR	P
lmo0393	unknown protein	**-2.00**	0.001	**-1.07**	0.001	**-1.43**	3.7E-05	0.17	
lmo0419	similar to uncharacterized conserved proteins	**-1.56**	0.003	-0.61	0.023	-0.95	0.011	**-1.24**	4.0E-04
lmo0546	oxidoreductase family protein	**-1.06**	0.039	-0.83	0.004	**-1.49**	0.002	-0.88	0.023
lmo0602	similar to sortase and related acyltransferases	**-1.38**	0.003	**-1.94**	1.7E-04	**-1.77**	5.6E-05	**-1.02**	0.003
lmo0625	similar to SGNH_hydrolase family proteins	**-1.05**	0.012	**-1.08**	0.002	**-1.41**	1.3E-04	**-1.00**	0.004
lmo0629	hydrolase, isochorismatase family	**-1.37**	0.007	**-1.98**	7.3E-05	**-1.75**	1.5E-04	**-1.63**	1.9E-05
lmo0654	unknown protein	**-1.18**		**-3.50**	1.6E-07	**-2.58**	5.8E-05	**-1.17**	7.8E-05
lmo0771	unknown protein	-0.12		-0.35		**-1.47**	1.7E-04	**-1.32**	4.7E-04
lmo0779	similar to uncharacterized conserved proteins	**-1.15**	0.026	**-1.10**	0.001	**-1.68**	3.1E-05	-0.82	0.007
lmo0794	similar to putative NADH-flavin reductases	-0.97	0.022	**-2.10**	0.001	-0.99	0.002	-0.23	
lmo0800	similar to uncharacterized conserved proteins	**-4.04**	0.001	**-3.35**	0.001	**-3.07**	0.001	-0.14	
lmo0850	unknown protein	-0.16		**-1.00**	0.012	**-1.82**	1.1E-04	-0.83	0.007
lmo0878	similar to predicted oxidoreductases (related to aryl alcohol dehydrogenases)	**-1.99**	0.009	-0.81	0.009	**-1.14**	0.001	-0.22	
lmo0905	similar to predicted protein tyrosine phosphatase	-0.38		-0.92	0.015	**-1.14**	4.3E-04	**-1.51**	3.2E-06
lmo0937	unknown protein	**-2.07**	0.002	**-1.16**	0.001	**-1.57**	0.003	-0.72	
lmo0953	putative lipoprotein	-0.27		**-1.15**	0.002	**-1.73**	3.1E-05	**-2.61**	6.2E-06
lmo0994	unknown protein	**-2.27**	1.3E-04	-0.78	0.016	**-1.37**	4.9E-04	-0.78	0.025
lmo0999	putative membrane protein	**-2.08**	0.001	-0.65	0.007	-0.82	0.006	-0.86	0.003
lmo1037	similar to uncharacterized conserved proteins	**-1.50**	0.002	**-1.16**	0.005	**-1.64**	0.002	-0.89	0.007
lmo1690	similar to predicted membrane-bound metal-dependent hydrolases	-0.88		**-1.59**	0.007	**-1.04**	0.013	0.74	0.005
lmo1718	similar to uncharacterized conserved proteins	-4.21	1.9E-06	**-1.54**	0.005	**-2.21**	2.0E-04	-0.69	
lmo2065	Unknown protein	**-1.54**	0.002	0.12		-0.45		0.09	
lmo2066	Unknown protein	**-3.54**	5.4E-05	-0.23		**-1.86**	2.8E-04	-0.35	
lmo2156	unknown protein	-0.48		-0.77		**-3.12**	0.002	0.75	0.020
lmo2175	similar to dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	**-2.57**		**-1.24**		**-2.26**	0.002	0.81	0.037
lmo2210	unknown protein	0.47		**-1.14**	0.006	**-2.13**	1.8E-04	**-2.68**	1.4E-05
lmo2229	similar to membrane carboxypeptidases (penicillin-binding proteins)	**-2.92**	1.7E-04	-0.89	0.040	**-1.48**	0.001	-0.67	0.015
lmo2454	unknown protein	**-1.88**	0.053	**-3.54**	0.003	**-2.20**	0.033	-0.71	
lmo2567	unknown protein	-0.38		**-1.22**	0.034	**-1.43**	4.1E-04	-0.69	0.023
lmo2646	similar to uncharacterized conserved proteins	**-4.18**	3.3E-06	-0.21		**-1.36**	4.7E-04	-0.07	
lmo2648	similar to predicted metal-dependent hydrolases with the TIM-barrel fold	**-4.93**	1.5E-05	0.14		-0.86		0.25	
lmo2675	unknown protein	**-1.09**	0.010	-0.59	0.026	**-1.72**	1.4E-05	-0.32	
lmo2707	unknown protein	**-10.26**	6.8E-08	**-2.68**	3.3E-05	**-2.68**		-0.83	
lmo2731	unknown protein	**-4.33**	0.003	0.90	0.003	-0.19		-0.91	
lmo2742	similar to uncharacterized conserved proteins	**-1.53**	0.005	**-1.11**	0.017	**-1.26**	0.001	-0.32	
lmo2755	similar to predicted acyl esterases	**-6.78**	2.0E-07	-0.39		**-2.62**	3.9E-04	-0.23	
lmo2778	unknown protein	**-6.20**	3.8E-05	0.30		**-1.85**	4.6E-05	**-1.07**	0.001
lmo2803	unknown protein	**-3.98**	0.001	-0.06		**-1.05**		-0.38	
lmo2804	similar to putative lipases	**-6.26**	0.001	-0.70		-0.67		-0.03	
lmo2813	unknown protein	**-1.45**	0.002	**-1.25**	0.002	**-1.33**	0.002	**-1.15**	0.002

Gene	Function	ATCC19115		ScottA		FW03-0035		70-1700	
		LR[#]	P	LR	P	LR	P	LR	P
lmo2828	unknown protein	**-6.42**	5.2E-05	-0.86	0.025	**-2.84**	2.2E-04	-0.30	
lmo2852	similar to uncharacterized conserved proteins	**-4.27**	0.001	0.18		-0.75	0.015	0.46	

LR= Log Ratio; P values not shown are >0.05.

3.3.2.11 Regulation of gene response in osmo-adapted cells.

Prolonged exposure to high levels of NaCl had an antagonistic effect on genes positively influenced by σ^B with *T-value* scores ranging from -12.77 in ScottA to merely -1.46 in 70-1700 (Table 3.8).

Table 3. 8 Gene response of four *L. monocytogenes* strains to prolonged exposure to NaCl stress organized into sets based on known regulons.

Regulon	ATTC19115		ScottA		FW03-0035		70-1700	
	T[*]	P[#]	T[*]	P[#]	T[*]	P[#]	T[*]	P[#]
HrcA+[¥]	**5.50**	3.9E-06	**3.16**	0.0033	**6.07**	6.9E-07	**4.77**	3.4E-05
HrcA-	**0.45**		**0.95**		**2.08**		**1.95**	
CtsR-	**-2.60**	0.0407	**-2.66**	0.0373	**-1.14**		**-0.61**	
SigB-	**4.87**	3.2E-06	**3.33**	0.0011	**4.25**	4.1E-05	**2.84**	0.0052
SigB+	**-7.79**	4.6E-13	**-12.77**	3.4E-27	**-7.49**	2.8E-12	**-1.46**	
RpoN+	**-0.56**		**-1.26**		**-0.85**		**-0.79**	
RpoN-	**-3.26**	0.0018	**-1.91**		**-1.21**		**-2.26**	0.0273
PrfA-	**-1.55**		**0.63**		**-1.83**		**-0.43**	
PrfA+	**-4.24**	7.4E-05	**-8.02**	3.0E-11	**-3.63**	0.0006	**-0.04**	
VirR+	**0.29**		**-0.93**		**-1.27**		**0.33**	
CodY-	**-7.02**	5.9E-10	**-4.26**	5.3E-05	**-6.64**	3.2E-09	**-0.96**	
CodY+	**-1.63**		**-5.51**	2.8E-07	**-4.68**	9.2E-06	**-0.48**	

*T-value scores were determined from the expression data using the approach of Borsma *et al*. (2005).
[#] P-values not shown are >0.05
[¥] The gene sets for the given regulatory proteins are designated from empirical deletion studies on the basis of what genes are positively influenced ("+") or negatively influence ("-"), The influence of the regulators in many cases are not direct thus T-profile data only gives a general trend in terms of expression responses.

The set of genes whose expression is suppressed (either directly or indirectly) by σ^B showed overall activation with *T-value* scores ranging from 2.84 in strain 70-1700 to 4.87 in strain ATCC19115 (Table 3.8). This strongly suggests that stress response regulator σ^B is not involved in the late stages of salt tolerance response.

The SigB and PrfA regulons include 20 genes that are influenced by both regulators, consequently PrfA positively controlled gene group also showed suppression in majority of osmo-adapted *L. monocytogenes* strains with *T-value* scores of -4.24 in strain ATCC19115, -8.02 in ScottA, -3.63 in FW03-0035, with no evident change in transcription levels in strain 70-1700 (Table 3.8). PrfA is the main regulator of virulence in *L. monocytogenes* (Greene & Freitag, 2003; Lalic-Mülthaler *et al.*, 2001; Nadon *et al.*, 2002). Together with σ^B, CodY and other transcription regulators PrfA is also involved in controlling genes associated with PTS, various metabolic pathways and a number of as yet uncharacterized genes. Some of the strongly down-regulated genes in the three relatively salt tolerant strains which are thought to be under positive control of PrfA included *lmo2695*, *lmo2391*, *lmo2573*, *lmo2673*, *lmo0263*, *lmo0781*, *lmo0782*, *lmo0784* and *lmo2697*. Some of the genes which showed down-regulation in all four strains included *inlA* (*lmo*0433), *lmo*0654, *lmo*0602, and *lmo2213*.

Hyperosmotic adaptation caused significant activation of *hrc*A regulon, with genes positively controlled by this regulator showing up-regulation with *T-value* scores of 3.16 to 6.07 (Table 3.8). The HrcA regulon is part of an integrated network of transcriptional regulators contributing to stress response systems in *L. monocytogenes* (Hu *et al.*, 2007a). Genes that are activated by HrcA include ribosomal proteins, DNA replication and those responsible for transcription and translation processes (Hu *et al.*, 2007a) all of which showed significant activation in osmo-adapted strains of *L. monocytogenes*. Some of the genes strongly activated by HrcA included *ssb*, *fur* and numerous ribosome associated genes such as *rplK*, *rplA* and *rpsF*, to name a few.

The influence of CodY or the rpoN on regulation of hyperosmotic stress response was not very straight forward, most likely due to interaction of other regulators, as a large number of genes in both regulons are under control of multiple regulators and not solely CodY or RpoN. In *B. subtilis* CodY aids in controlling smooth transition from rapid exponential growth to the stationary growth phase (Sonenshein, 2005). In *L. monocytoges* CodY controls genes associated with amino acid biosynthesis, carbohydrate metabolism and transport, motility and chemotaxis and has a major role in monitoring the energetic capacity and nutritional state of the cell (Bennett *et al.*, 2007; Sonenshein, 2005). Overall CodY regulon showed repression, suggesting a shift into an energy conservation mode might be invoked in osmotically challenged cells.

Genes under positive control of CodY showed seemingly random expression patterns, some were up-regulated in all strains such as *arg*G (*lmo2090*) ranging from 3-fold in ScottA to 5-fold in 70-1700, some were strongly down-regulated in all strains such as *fliS* (*lmo0708*) or *lmo2664* which showed down regulation in three relatively salt tolerant strains ranging from 169-fold in ATCC19115 to 2-fold in ScottA, while no significant change in transcription was observed in strain 70-1700. This result emphasises the complexity of transcription regulation in *L. monocytogenes*, in which the same gene might be influenced by various response regulators.

RpoN has been shown to assist in regulation of carbohydrate metabolism in *L. monocytogenes* (Arous *et al.*, 2004), a metabolic process which was evidently suppressed in osmo-adapted cells. Genes under control of this regulator too showed varied patters in expression up-regulated in some instances such as seen with *topA* (*lmo*1275) gene which showed activation in all strains, and down-regulated such as gene *lmo*1350 which showed down-regulation in strains ATCC19115, ScottA and FW03-0035. Transcriptional control by both CodY and RpoN seems to take a secondary role in osmo-adapted cells, overridden it appears by other control mechanisms.

3.4 Summary and Conclusions

Osmoadaptation of *L. monocytogenes* in a rich medium showed a clear pattern of gene expression among the four strains examined with only minimal variations in individual gene expression between strains. Most strain-dependent differences were observed with a relatively salt sensitive strain 70-1700.

Prolonged exposure to high levels of NaCl was associated with an overall activation of membrane transport associated gene sets such as iron transporter genes (*fhuC* and *fhuB*) and sodium transporter genes (*mnh* operon). There was an indication of enhanced peptide up-take with up-regulation of the *opp* operon, either utilised in maintaining the integrity of the cellular envelope or as compatible solutes. Strong unanimous up-regulation of *betL*, implied a passive up-take of glycine betaine driven by the influx of Na^+ ions in osmo-adapted cells, previously thought to be involved only in initial stages of hyperosmotic stress response. Carbohydrate uptake and subsequent metabolism was suppressed in these cells, reflecting the overall decrease in cellular metabolism. Increased energetic demand for maintaining cellular integrity and stability through changes to membrane composition was indicated by activation of gene sets associated with fatty acid biosynthesis (*acpA*, *fabZ*), peptidoglycan biosynthesis (*gcaD*, *murG*) and membrane-bound proteases (*ftsH*, *lmo1318*, *htpX*). Thickening of bacterial cell wall in response to osmotic stress alters the surface topology of the bacterial cell, likely obscuring binding sites of surface proteins such as flagella and InlA, transcription of which was suppressed. Up-regulation of genes associated with DNA repair (*ssb* and *dnaA*) numerous translation associated genes, cell division, and related chaperones (*tig* and *dnaJ*) suggested destabilisation of DNA, ribosomes and the assembly of septal rings. Sodium-induced decrease in electrochemical potential across bacterial cell membrane appeared to stimulate transcription of genes associated with oxidative phosphorylation.

Adaptation to hyperosmotic stress in *L. monocytogenes* is a biphasic process consisting of primary shock response and secondary adaptive response mechanisms. In order to comprehend the overall osmoadaptive response in this organism both stages of the response mechanism must be investigated. The hyperosmotic shock response was investigated in chapter 4.

Chapter 4

Sodium chloride induced shock response changes in gene expression of Listeria monocytogenes strain ATCC 19115 (serotype 4b).

Overcoming osmotic shock is an important step in osmo-adaptation in bacteria. To aid overall understanding of hyperosmotic stress responses in *Listeria monocytogenes* changes in gene expression following salinity-induced hyperosmotic shock was investigated in serotype 4b strain ATCC19115. Microarray-based data analysed using gene expression trend analysis revealed a prominent, almost reverse gene expression profile compared to corresponding adaptive responses to exposure to 10.0% (w/v) (1.8 M) NaCl. Hyperosmotic shock induced gene sets associated with carbohydrate metabolism and transport, suggesting an increased demand for energy, whilst the majority of other metabolic functions were significantly reduced with a significant decrease in transcription of gene sets associated with transcription, fatty acid biosynthesis and cell division and accompanied by increased expression of genes influenced by the SigB, PrfA and VirR regulons.

4.1 Introduction

Listeria monocytogenes is an opportunistic pathogen with an ability to survive, and even proliferate, under a variety of stressful environmental conditions including elevated osmolarity (Cole *et al.*, 1990; Dykes & Moorhead, 2000; Liu *et al.*, 2005; Sleator *et al.*, 2000). This characteristic of the organism, coupled with an increasing demand for ready to eat products, increases concerns within the food industry to minimise food contamination by this pathogen.

It is important to understand the mechanism of *L. monocytogenes* survival in stressful environments, because this pathogen continues to be of significant public health concern and continues to cost the food industry a significant amount. Only recently an outbreak in Toronto, Canada, which has been linked to ready to eat meat products recalled by Maple Leaf Foods plant has resulted in 14 deaths out of 38 confirmed cases of listeriosis. Over 191 Maple Leaf products were recalled, with an estimated cost of US $18.7 million. Significant costs to the food industry may be provided by better comprehension of the stress adaptations of this bacterial species.

Salt is the oldest food additive, which has been and to present day is still used, not only as flavouring but also as a preservative to control microbial contamination in food. Salting is an essential step in cheese making, cured meat product manufacturing and salami making, just to mention a few, all of which have been linked to *L. monocytogenes* contamination (Degenhardt & Sant'Anna, 2007; Gianfranceschi *et al.*, 2006; Larson *et al.*, 1999; Uyttendaele *et al.*, 1999).

Overcoming a sudden increase in osmolarity is an important step in hyperosmotic tolerance in bacteria. To date genetic expression during the initial stage of the osmotolerance response has only been investigated in a *ΔsigB* mutant of *L. monocytogenes* (Raengpradub *et al.*, 2008). In this study hyperosmotic shock response of *L. monocytogenes* strain ATCC19115 was examined using whole genome microarray analysis. To aid overall understanding of hyperosmotic stress response change in gene expression, observed in the same strain of *L. monocytogenes*, during both stages of the response was investigated.

4.2 Methods

4.2.1 Strains

Strain ATCC19115 used in this study is of serotype 4b and was originally isolated from cerebral spinal fluid from a clinical listeriosis case. This strain was shown to exhibit a relatively moderate NaCl tolerance (Chapter 2).

4.2.2 Media and Growth Conditions

4.2.2.1 Bacterial Cultures

Listeria monocytogenes strain ATCC19115 was grown in duplicate in 100 ml BHI broth at 25°C to a transmittance of approximately 80%. The culture was split between four falcon tubes, each receiving approximately 25 ml. The cell suspensions were then centrifuged at 1,500xg for 10 min. Two of the four falcon tubes were treated as controls where the cell pellets were resuspended in fresh BHI and incubated at 25°C for 10 min. The other two falcon tube cell pellets were resuspended in BHI supplemented with 10.0% (w/v) NaCl and incubated at 25°C for 10 min. All falcon tubes were treated with RNA protect bacterial reagent as described in section 3.2.3.1.

4.2.3 RNA Extractions

4.2.3.1 RNAprotect Bacterial Reagent Treatment

Two volumes of RNAprotect bacterial reagent (Qiagen) were added to one volume of culture and treated as described in section 3.2.3.1. Following stabilisation of RNA the cell pellet was stored at -80°C until subsequent RNA extractions were undertaken.

4.2.3.2 RNA extraction and quantification.

Bacterial cell pellets were thawed on ice and treated with lysozyme and proteinase K as described previously in section 3.2.3.2 for a duration of 4 hours at 25°C. These were then mechanically lysed by bead beating using 0.1 mm zirconium-silica sand in 4 ml of RNeasy Midi RNA Extraction kit (Qiagen) lysis buffer that was supplemented with 0.1% β-mercaptoethanol (Sigma-Aldrich). RNA extraction was performed with RNeasy Midi RNA Extraction kit (Qiagen) in compliance with the manufacturer's instructions previously documented in detail in section 3.2.3.2 and the extracted RNA was stored at -80°C.

Extracted RNA was assessed by examination of formaldehyde agarose gel described in section 3.2.3.4.

4.2.4 Microarray Analysis

4.2.4.1 Microarray slides hybridisation.

Oligonucleotides were arrayed onto glass slides using quill pens at the AGRF (Walter & Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia) with each spot possessing a 12 µm diameter. The array included 2857 x 70 bp oligonucleotides (AROS v. 1, Eurofins MWG Operon, Huntsville, Al, USA), representing all predicted protein coding genes and pseudogenes of the complete, published genome of *L. monocytogenes* EGD-e (GenBank accession number. AL591824).

RNA was converted to cDNA as described previously in section 3.2.4.2 and labelled with a fluorescent CyDye according to the procedure described in section 3.2.4.3. Custom *L.*

monocytogenes strain EGD-e microarray slides were hybridised with CyDye labelled cDNA according to procedure summarised in section 3.2.4.4.

4.2.4.2 Data analysis

Downstream processing used the GenePix-Pro 3 software package to generate gpx filed from TIFF array images. Normalization of raw data and subsequent statistical analysis was performed with the WebArray Online platform (Xia *et al.*, 2005). Within-array normalization used the global LOESS procedure. Between each array quantile normalization was used to insure intensities had the same empirical distribution across arrays and across channels. The significance of differential expression was analysed using linear modal statistical analysis (Smyth, 2004). Data analysis otherwise followed methods described in section 3.2.4.5.

4.2.4.3 Gene set enrichment analysis

Gene designations, predicted functions and categorization of coded proteins into defined sets from the *L. monocytogenes* EGD-e genome was based on information obtained from published literature, Kyoto Encyclopaedia of Genes and Genomes (http://www.genome.ad.jp/kegg/) and ListiList (http://genolist.pasteur.fr/ListiList/). A *t*-test based procedure was utilised to score the changes in expression of predefined sets of genes (Boorsma *et al.*, 2005; Bowman *et al.*, 2008). The significance of the *T-value* score was established by using the associated two-tailed *p*-value.

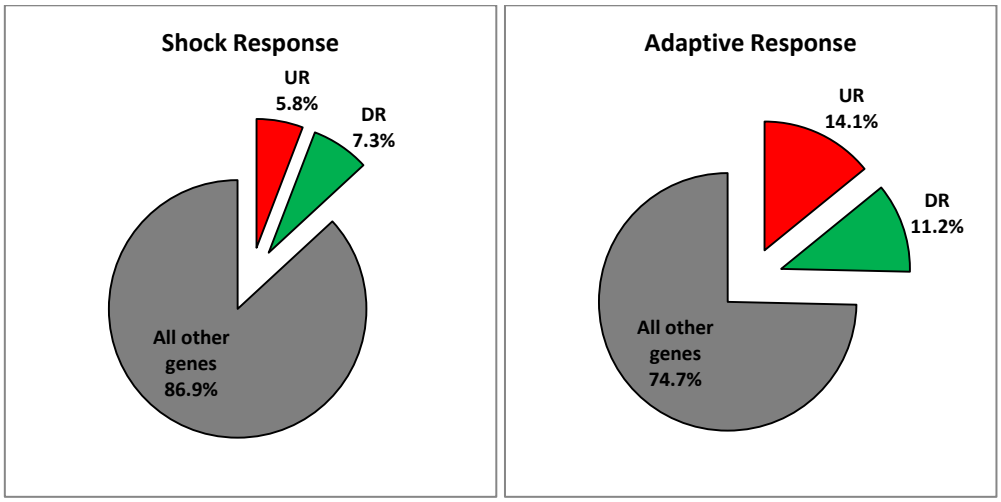
4.3 Results and Discussion

4.3.1 The effect of 10.0 % (w/v) NaCl shock on gene expression of *L. monocytogenes* strain ATCC19115

The responses of *L. monocytogenes* to high salinity levels can be examined in two distinct physiological phases. These include an initial reaction to a sudden rise in salinity, also known as osmotic shock, and the subsequent cellular adaptation leading to resumption of active growth under high salinity conditions, also known as the osmo-adaptive response. Osmotic shock itself is biphasic with initiation occurring within seconds of stress exposure (de Nadal & Posas, 2010; Wood, 1999) and associated with cessation of respiration and transport, cytoplasmic decrease in a_w and gradual increase in ATP levels. The second phase may continue from 2 to 60 min (Wood, 1999) and involves

the up-take of potassium ions and compatible solutes which then gradually transitions into the adaptive response. In this study the former of these physiological responses was examined for associated changes in the transcriptional profile of *L. monocytogenes* ATCC19115.

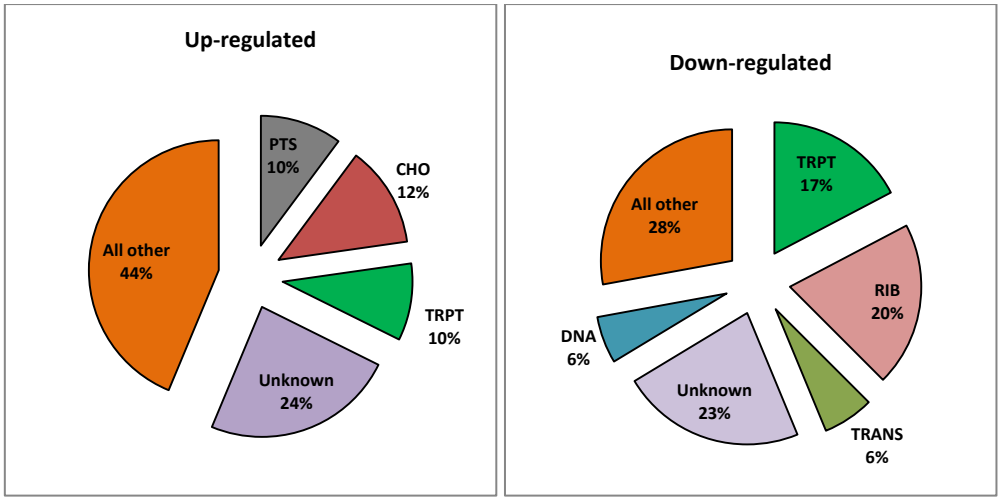
Overall changes in relative gene expression of *L. monocytogenes* strain ATCC19115 induced by hyperosmotic shock with 10.0% (w/v) NaCl resulted in a completely different gene expression profile to the counterpart adaptive response following osmotic stress; in many cases an opposite response was observed in expression trends. Only 167 genes showed up-regulation of ≥ two-fold following hyperosmotic shock treatment and 208 were down-regulated ≥two-fold (Fig. 4.1). Osmo-adaptive response for the same osmotic stress resulted in ≥two-fold up-regulation of 404 genes and down-regulation ≥two-fold of 320 genes showed down regulation (Fig. 4.1) and has been addressed in more detail in chapter 3.



UR: up-regulated genes (LR>1, P<0.05); DR: down-regulated genes (LR<-1, P<0.05). Percentages calculated based on total number of genes in EGD-e genome (5827).

Figure 4.1 Schematic representation of overall transcription results for hyperosmotic shock and adaptive response observed in *L. monocytogenes* strain ATCC19115

The majority of all up-regulated genes observed during the shock response had an unknown function, followed by a group of genes associated with carbohydrate metabolism and genes encoding various transporters, especially phosphotransferase systems (Fig. 4.2).



DNA-group of genes associated with DNA repair and recombination; TRANS- genes associated with translation, TRPT- genes encoding various transporters; RIB-genes encoding ribosomal proteins; CHO- genes associated with carbohydrate metabolism; PTS- genes associated with phosphotransferase system transporters;

Figure 4.2 Schematic representation of all up-regulated and all down-regulated genes separated into functional categories for hyperosmotic shock response observed in *L. monocytogenes* ATCC19115.

Down-regulated genes in shock response included those associated with ribosomal proteins and other proteins involved in translation, DNA repair, including those involved in transcription, and various transporters. The majority of all the significantly suppressed genes in shock response included again genes with generally unknown functions (Fig. 4.2).

Hyperosmotic stress induced significant expression variation in several gene functional categories and sub-categories, as established using the *T-value* scoring procedure (Boorsma *et al.*, 2005; Bowman *et al.*, 2008). The *T-value* scoring results are summarised in Table 4.1Table 4.1.

Table 4.1 Response of *L.monocytogenes* strain ATCC19115 genes to NaCl shock and adapted stresses, organized into gene functional categories.

Gene categories and subcategories (no. of genes)	Shock Response		Adapted Response	
	T-value*	P-value#	T-value*	P-value#
Amino Acid Metabolism				
D-Alanine metabolism (7)	3.17	0.0249	-0.81	
Cysteine metabolism (13)	2.72	0.0198	-0.27	
Other/unknown amino acid-related metabolism (14)	1.42		-1.66	
Arginine/proline metabolism (22)	1.40		0.18	
Methionine metabolism (13)	1.15		-0.64	
Alanine/aspartate metabolism (28)	1.03		-0.02	

Gene categories and subcategories (no. of genes)	Shock Response		Adapted Response	
	T-value*	P-value#	T-value*	P-value#
Urea cycle and metabolism of amino groups (12)	1.02		1.03	
Branched chain amino acids biosynthesis (16)	0.91		1.95	0.0720
Aromatic amino acids biosynthesis (23)	0.50		2.15	0.0437
Lysine biosynthesis (16)	-0.47		1.64	
Branched amino acids metabolism (7)	-0.69		-1.34	
Carbohydrate Metabolism				
Fructose/Mannose metabolism (68)	5.83	1.8E-07	-4.39	4.2E-05
Glycolysis/Gluconeogenesis (37)	4.43	8.9E-05	-0.97	
Butanoate metabolism (19)	4.07	0.0008	-3.27	0.0045
Pdu/Eut operon (36)	3.43	0.0016	0.64	
Starch/sucrose metabolism (31)	2.92	0.0067	-1.03	
Galactose metabolism (16)	2.76	0.0154	-3.17	0.0069
Inositol/inositol phosphate metabolism (9)	2.32	0.0531	-0.59	
Other/unknown carbohydrate-related metabolism (49)	1.50		-1.66	
Pyruvate metabolism (32)	1.47		-0.84	
Pentose phosphate pathway (34)	1.31		-3.08	0.0042
Aminosugars metabolism (15)	-0.47		1.42	
Cell division				
Cell division (30)	-2.14	0.0416	3.37	0.0022
Other/unknown cell cycle/division-related proteins (16)	-1.53		0.21	
Septal ring (9)	-0.41		2.92	0.0225
Cell Motility				
Flagellar assembly (27)	0.90		-3.16	0.0041
Type III secretion system (11)	-0.30		-1.80	
LPXTG motif (22)	-1.01		-1.18	
DNA repair/recombination				
DNA repair/recombination (72)	-1.10		2.26	0.0271
DNA replication complex (20)	-0.17		1.45	
Energy Metabolism				
Oxidative phosphorylation (25)	-0.54		2.90	0.0080
Lipid Metabolism				
Fatty acid biosynthesis (14)	-4.38	0.0009	2.19	0.0490
Peptidoglycan biosynthesis (16)	-1.87	0.0828	1.72	
Biosynthesis of steroid/terpenoid (16)	-1.80		2.07	0.0573
Membrane Transport				
PTS system (90)	7.95	5.9E-12	-11.45	4.0E-19
Transporter (sugars) (28)	2.84	0.0086	-0.93	

Gene categories and subcategories (no. of genes)	Shock Response		Adapted Response	
	T-value*	P-value#	T-value*	P-value#
ABC Transporter (amino acids) (11)	0.68		1.74	
Transporter (iron) all (19)	0.29		3.41	0.0034
ABC Transporter (peptides) (10)	-1.28		2.84	0.0217
ABC Transporter General (171)	-1.36		3.60	0.0004
Transporter (amino acids) (7)	-1.38		0.18	
Transporter (sodium) (12)	-1.86	0.0928	1.73	
All Transporters (287)	-2.24	0.0258	2.79	0.00555
Protein export (19)	-2.48	0.0239	0.56	
Transporter (metal cations) (7)	-3.50	0.0173	-0.61	
Transporter (compatible solutes) (8)	-2.91	0.0269	0.32	
Metabolism of Cofactors and Vitamins				
Vitamin B6 metabolism (6)	1.78		0.37	
Nicotinate/nicotinamide metabolism (13)	1.23		1.52	
Pantothenate/CoA biosynthesis (15)	0.71		2.67	0.0193
Folate biosynthesis (16)	0.62		-1.57	
Isoprenoid quinone biosynthesis (9)	-0.79		2.48	0.0422
Nucleotide Metabolism				
Pyrimidine biosynthesis/metabolism (44)	-2.45	0.0186	2.27	0.0285
Pyrimidine biosynthesis (8)	-2.38	0.0549	-0.96	
Purine metabolism (44)	-1.73	0.0918	2.05	0.0464
Pyrimidine metabolism (36)	-1.55		2.96	0.0055
Purine and Pyrimidine metabolism (82)	-1.54		3.06	0.0030
Purine biosynthesis/metabolism (58)	-0.98		3.17	0.0025
Purine biosynthesis (15)	1.02		1.78	0.0992
Signaling				
Two-component signal transduction system (39)	2.33	0.0253	1.49	
Other signal transduction (23)	1.54		-2.18	0.0408
Transcription and Translation				
Ribosome (58)	-11.92	5.5E-17	10.19	2.3E-14
Translation factors (12)	-3.33	0.0076	2.61	0.0259
Translation/ribosome associated (56)	-3.04	0.0036	3.00	0.0041
GTP-binding protein (22)	-2.84	0.0101	0.93	
Transcription associated (9)	-1.83		2.24	0.0598
RNA polymerase (10)	-1.33		0.42	
DNA polymerase (10)	-0.30		2.65	0.0295
Folding catalysts (13)	0.20		1.49	
Aminoacyl-tRNA biosynthesis (24)	1.45		2.04	0.0534
Transcription regulation				
Transcription regulation (AraC family) (6)	2.09		-0.82	

Gene categories and subcategories (no. of genes)	Shock Response		Adapted Response	
	T-value*	P-value#	T-value*	P-value#
Transcription regulation (BglG family) (15)	1.98	0.0688	-2.27	0.0408
Transcription regulation (TetR/AcrR family) (11)	1.55		-0.34	
Transcription regulation General (231)	1.38		-0.44	
Transcription regulation (MarR family) (14)	0.13		-1.53	
Transcription regulation (DeoR family) (8)	0.07		1.72	
Transcription regulation (GntR family) (17)	0.01		1.73	
Transcription regulation (MerR family) (7)	-2.84	0.0250	0.42	
Virulence				
Phage (78)	2.11	0.0378	-0.62	
Virulence-related (14)	1.93	0.0781	0.41	
Autolysin/cell wall hydrolase (13)	-1.21		0.82	
TN916 (13)	-4.03	0.0020	-1.30	

*T-value scores were determined from the expression data using the approach of Boorsma *et al.* (2005).
P-values not shown are >0.1

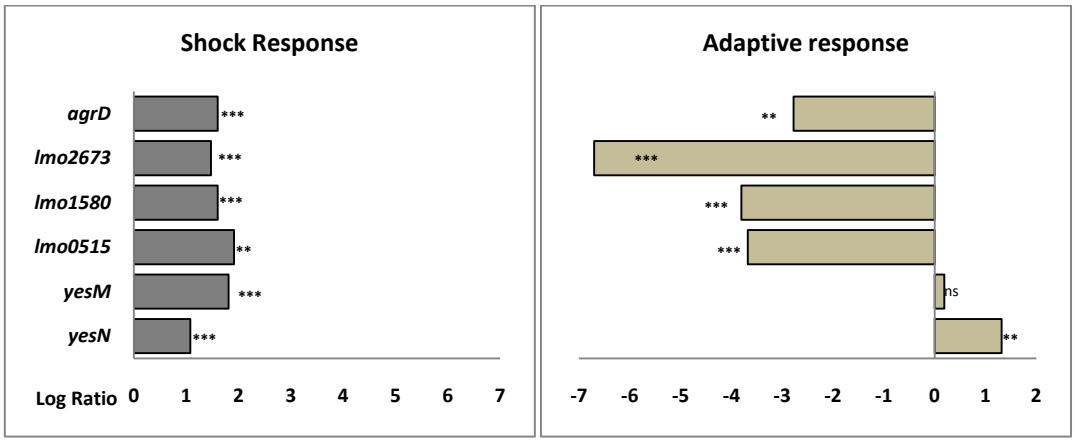
The most significant up-regulated gene sets observed in strain ATCC19115 exposed to hyperosmotic shock included those for PTS systems with *T-value* score of 7.95, fructose/mannose metabolism and glycolysis with *T-value* scores of 5.83 and 4.43, respectively. Most down-regulated gene sets were ribosome-associated genes with a *T-value* score of -11.92 as well as those encoding translation factors, translation/ribosome associated and fatty acid biosynthesis with scores of -3.04, -3.33 and -4.38 respectively (Table 4.1).

Gene sets encoding ribosome proteins were the most significantly up-regulated in the osmo-adapted cells with *T-value* scores of 10.19 (Table 4.1). Other highly up-regulated gene sets include general ABC transporters, transporters of iron, cell division, septal ring and DNA repair/recombination with *T-value* scores of 3.60, 3.41, 3.37, 2.92, and 2.26 respectively. Most down-regulated gene sets included PTS system, fructose/mannose metabolism, butanoate metabolism, galactose metabolism, flagella assembly and pentose phosphate pathway with *T-value* scores of -11.45, -4.39, -3.27, -3.17, -3.16, -3.08.

4.3.1.1 Environmental sensing genes activated during hyperosmotic shock response.

Following a sudden change in osmolarity of the extracellular environment, from approximately 0.5% (w/v) NaCl (BHIB) to 10.0% (w/v) NaCl, *L. monocytogenes* ATCC19115 showed up-regulation of gene

sets associated with a specific two-component signal transduction systems (overall *T-value* score 2.33;Table 4.1). Two-component signal transduction systems mediate the sense of change in bacterial extracellular environment and transforms this information into an appropriate response by amending gene expression (Stock *et al.*, 2000).



. *=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 4.3 Expression of genes associated with environmental sensing in *L. monocytogenes* ATCC19115 following shock and adaptation to 10.0% (w/v) NaCl.

Two genes in this group showed relatively significant up-regulation, *lmo2010* (two-fold) and *lmo 2011* (3.5-fold, Fig. 3.3) homologous to *yesN* and *yesM* respectively of *B. subtilis*, *E. faecium* and *E. faecalis* (Fabret *et al.*, 1999; Hancock & Perego, 2002). While the exact function of the two-component YesN-YesM is not known, the YesM histidine kinase, is involved in signal recognition, activation of which is followed by phosphotransfer to YesN (Fabret *et al.*, 1999; Hancock & Perego, 2002). Response regulator, YesN, upon phosphorylation acts as a transcription regulator responsible for activation or repression of genes involved in an adaptive response (Fabret *et al.*, 1999; Hancock & Perego, 2002).

Expression of *agrD* (*lmo0050*) was activated during hyperosmotic shock response (Fig. 4.3); however none of the other three genes included in the *agr* operon showed any change in transcription. The AgrD is a part of a quantum-sensing system, a precursor of a peptide pheromone that is secreted from the bacteria into the culture medium (Garmyn *et al.*, 2009). The *agr* system plays a significant role in the adaptation of *L. monocytogenes* to environmental conditions (Garmyn *et al.*, 2009). In

addition the *ΔagrD* of *L. monocytogenes* has been shown to produce reduced biofilm formation and virulence (Riedel *et al.*, 2009; Rieu *et al.*, 2007).

Up-regulation of two-component signal transduction system gene sets was less evident during adaptive response, with *T-value* score of 1.49 (Table 4.1). A somewhat lesser response than that observed with the hyperosmotic shock, implying less active involvement of these systems in controlling osmotic equilibrium during prolonged exposure to hyperosmotic stress.

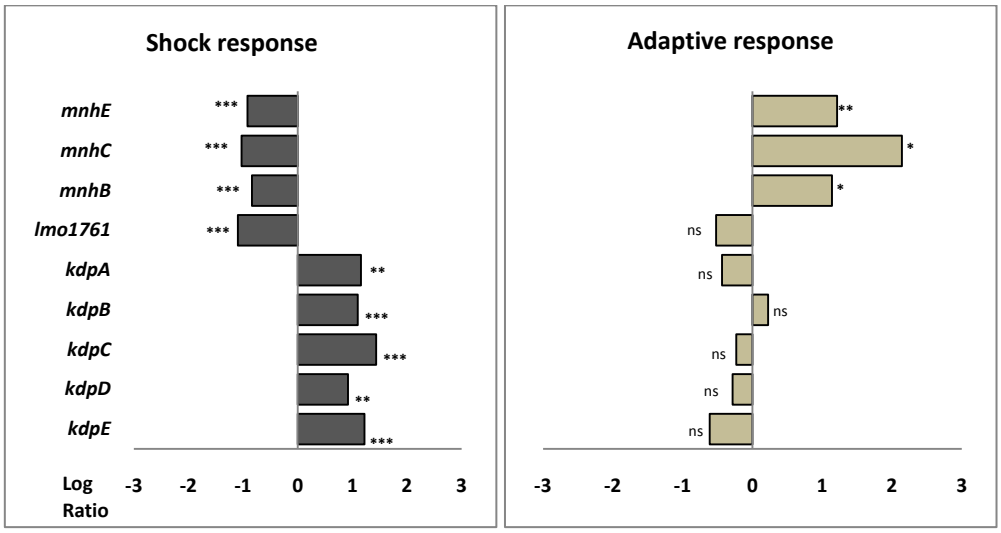
The importance of the environmental sensing mechanism during hyperosmotic shock was further emphasized by an up-regulation of certain genes associated with other signal transduction mechanisms. This group consists mainly of genes of the universal stress protein family (Usp) encoding for small cytoplasmic proteins, expression of which is enhanced following exposure to various stress agents including high levels of NaCl (Kvint *et al.*, 2003; Marceau *et al.*, 2004). In *L. monocytogenes* no *usp* genes have been specifically studied to date, however genes such as *lmo0515*, *lmo1580* and *lmo2673* encode UspA-like proteins and these were all significantly up-regulated (3.8, 3.0, and 2.8-fold respectively, Fig. 4.3) during hyperosmotic shock.

These same putative *usp* genes were significantly down-regulated (12.8, 14.0 and 104-fold respectively) during the adaptive response. The overall gene set associated with other signal transduction mechanisms in osmo-adapted cells was significantly down-regulated (T-value score -2.18, Table 4.1). This suggests complete acclimatization to the hyperosmotic environment, removing the requirement for stress sensing protein production.

4.3.1.2 Potassium ion transport requirement during hyperosmotic shock.

Following a sudden increase in salinity bacterial cells maintain turgor within physiologically acceptable boundaries by increasing intracellular potassium (K^+) content, by uptake of K^+ from the external environment. This is often carried out by a high-affinity potassium uptake system encoded by the *kdp* operon (Ballal *et al.*, 2007; Brøndsted *et al.*, 2003; Kallipolitis & Ingmer, 2001; Laszlo, 1989). The *kpd* operon consists of the sensor histidine kinase, *kdpD*, and a response regulator, *kdpE*. Proteins encoded by these genes are involved in sensing cell turgor and transferring the signal to the

kdpABC promoter, activating its transcription (Ballal *et al.*, 2007; Brøndsted *et al.*, 2003; Laszlo, 1989). This indeed was the case in the hyperosmotic shocked cells which showed up-regulation of all five *kdp* genes, *lmo2678* to *lmo2682* (with an average log ratio of 1.17, Fig. 4.4).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

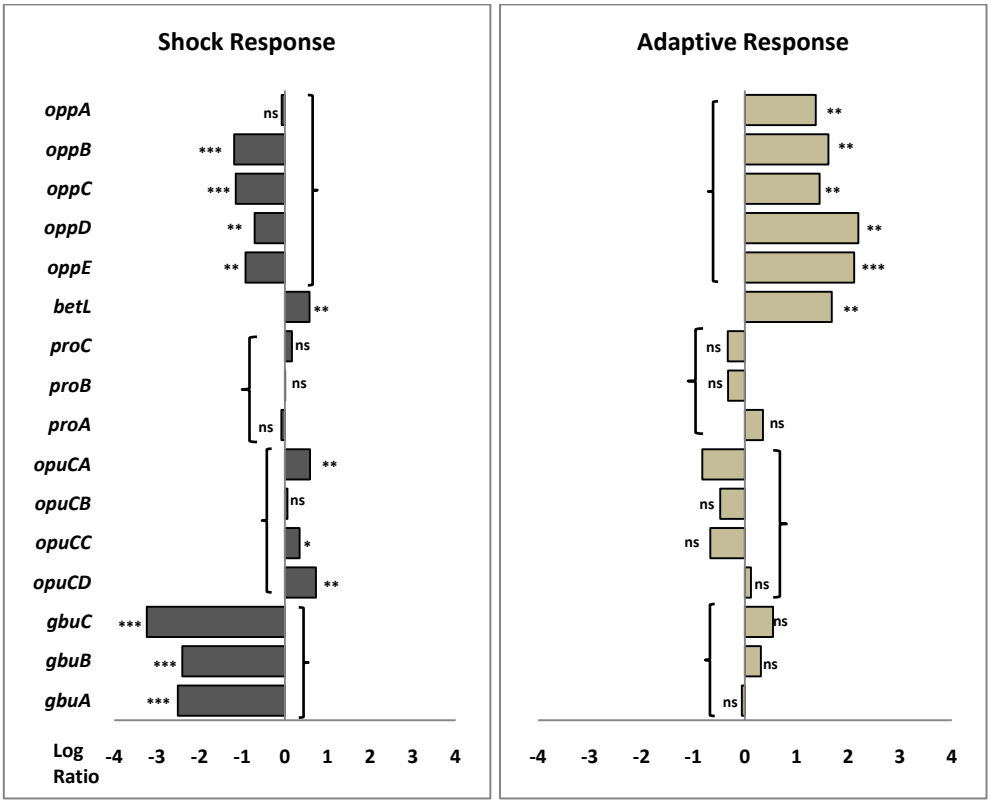
Figure 4.4 Expression of genes associated with various cation transporters in *L. monocytogenes* ATCC19115 following hyperosmotic shock and adaptation.

This implies a requirement for K^+ uptake by the osmotically shocked cells. The electro-imbalance due to influx of K^+ was found to be neutralized by uptake of glutamate from the extracellular medium within minutes of osmotic up shift in a similar manner observed in *E. coli* (Wood, 1999). Activation of the putative glutamate/GABA antiporter which is encoded by the *gadC* gene was evident in osmotically shocked cells (described in detail in section 4.3.1.5).

Due to the stabilized turgor of the osmotically acclimatized cells in the adapted stress response experiment, *kdp* operon genes showed down regulation (Fig. 4.4). This further emphasizes the requirement of K^+ uptake from the external environment during the initial stages of hyperosmotic stress response, and not in the later stages of hyperosmotic exposure. This was clearly demonstrated in a study of the induction of *kdp* in *Salmonella enterica* where the transcription increased at 20 minutes post NaCl shock after which it dropped significantly and plateaued for the rest of the experiment (Balaji *et al.*, 2005).

4.3.1.3 Compatible solute uptake was not evident during hyperosmotic shock

Overall, there was a strong indication of membrane transport suppression following hyperosmotic shock with down-regulation of genes encoding transporter proteins (*T-value* score -2.42) observed. This agrees with previously reported responses to hyperosmotic shock in other bacteria. Within the first couple of minutes following osmotic shock bacterial cell dehydrates and most transport ceases (Wood, 1999).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 4.4 Expression of genes associated with compatible solute and peptide transporters in *L. monocytogenes* ATCC19115 following hyperosmotic shock and adaptation.

During hyperosmotic shock genes encoding compatible solute uptake mechanisms were found to be overall, down-regulated (*T-value* score of -2.91, Table 4.1) with the glycine betaine/proline *gbuABC* (*lmo1014* to *lmo1016*) transporter showing the most down-regulation of (5.7, 5.3 and 9.5-fold respectively, Fig. 4.5). The ion-dependent secondary GB transporter encoding gene *betL* (*lmo2092*) however, showed slight up-regulation (1.5-fold) during hyperosmotic shock, suggesting that this transporter might contribute somewhat to the initial stages of salt resistance in *L. monocytogenes* at relatively high levels of NaCl (10.0% (w/v) in this instance). Rapid activation of *betL* in response to

relatively low salt stress (1 to 2% of NaCl) has previously been demonstrated in this organism, with decreased activity observed at NaCl concentrations of greater than 2% (Sleator *et al.*, 2003b). Results of this study have shown an evidently more pronounced up-regulation of *betL* in the adaptive response to salt stress (Fig. 4.5), suggesting a later involvement of this transporter in the overall response to hyperosmotic stress in this strain. Overall, it maybe concluded that up-regulation of genes responsible for the up-take of GB in strain ATCC19115 in a rich medium following hyperosmotic stress does not occur within the first 10 minutes of the response.

The carnitine transporter operon *opu* (*lmo1425* to *lmo1428*) showed overall an insignificant change in transcription levels in hyperosmotically shocked cells when compared to the control cells (Fig. 4.5). Although transcription of the *opuC* operon was not as strongly suppressed as the *gbu* operon; this is perhaps due to the high abundance of carnitine in animal tissue and consequently in the brain heart infusion growth medium. Control cells might be actively transporting carnitine in a BHI medium independent of NaCl stress. With the addition of NaCl stimulus the requirement for additional activation of genes responsible for up-take of this compound is only slightly altered during the initial stage of hyperosmotic stress response.

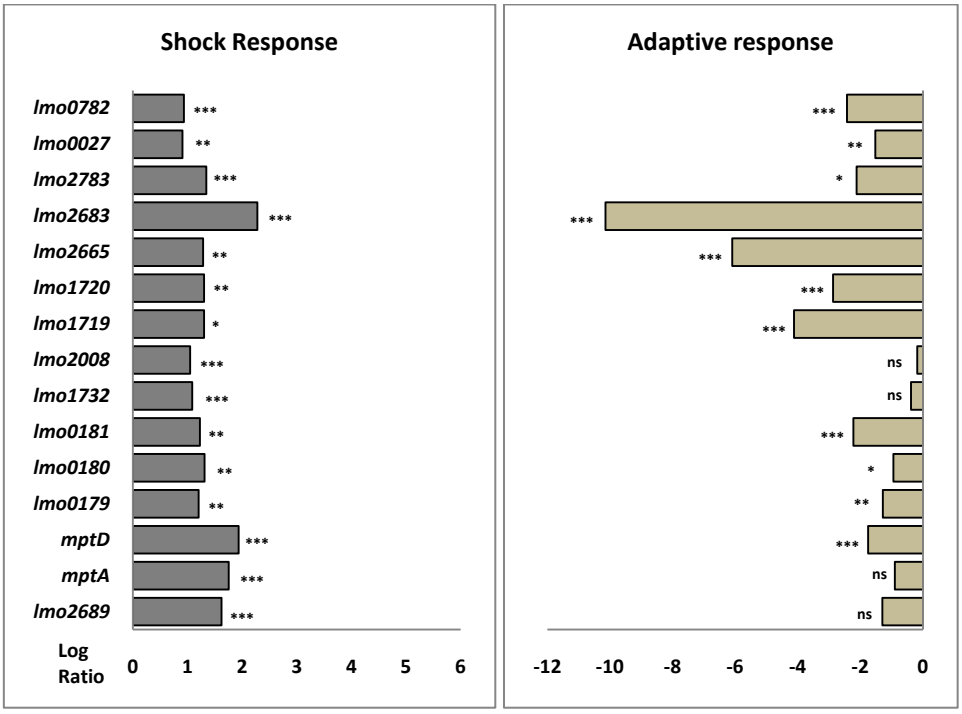
Synthesis of proline from glutamate was not evident during hyperosmotic shock, with the three most important genes for proline synthesis: *proA* (*lmo1259*), *proB* (*lmo1260*) and *proC* (*lmo0396*) showing no significant up-regulation. Proline has previously been shown to benefit *L. monocytogenes* during osmotic stress when present as a polypeptide (Amezaga *et al.*, 1995). On its own proline may not be beneficial in osmotically shocked cells. In addition up-take of oligopeptides was evidently suppressed in hyperosmotically shocked cells with *opp* operon showing significant down-regulation in transcription (Fig. 4.5).

Overall compatible solute accumulation does not appear to pay a major role in the early hyperosmotic shock response of *L. monocytogenes*, at least in an organically rich medium. Compatible solute uptake in bacteria as a response to osmotic up-shift is known to take place in the second stage of osmoadaptation process 20 to 60 minutes post the exposure to hyperosmotic environment (Wood, 1999) and this appears to hold true for *L. monocytogenes* also.

4.3.1.4 Carbohydrate uptake and metabolism activation during hyperosmotic shock

Carbohydrate metabolism was strongly up-regulated during osmotic shock with *T-value* scores as high as 5.83 (Table 4.1).

The uptake of carbohydrates from the environment in *L. monocytogenes* is predominantly mediated by phosphotransferase systems (PTS) (Dills *et al.*, 1980; Larsen *et al.*, 2006; Mitchell *et al.*, 1993). PTS sugar uptake and phosphorylation is achieved through a cascade of enzymatic reactions, in which a phosphate moiety is transferred from phosphoenoelpyruvate (PEP) to the sugar, forming sugar phosphate and pyruvate, an overall reaction which requires Mg^{2+}. PTS were strongly up-regulated during hyperosmotic shock with an overall *T-value* of 7.95 (Table 4.1, see Fig. 4.6 for individual gene expression), and strongly down regulated during the adaptive response *T-value* of -11.45 (Table 4.1).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 4.5 Expression of genes associated with carbohydrate up-take in *L. monocytogenes* ATCC19115 following hyperosmotic shock and adaptation.

Some of the strongly activated genes of the PTS included *lmo0098* and *lmo2683*, which were up-regulated, 3.8 and 5-fold respectively (Fig. 4.6). A magnesium transporter encoded by the *lmo*2689 gene also reflected this expression pattern, it was found to be up-regulated 3-fold during shock

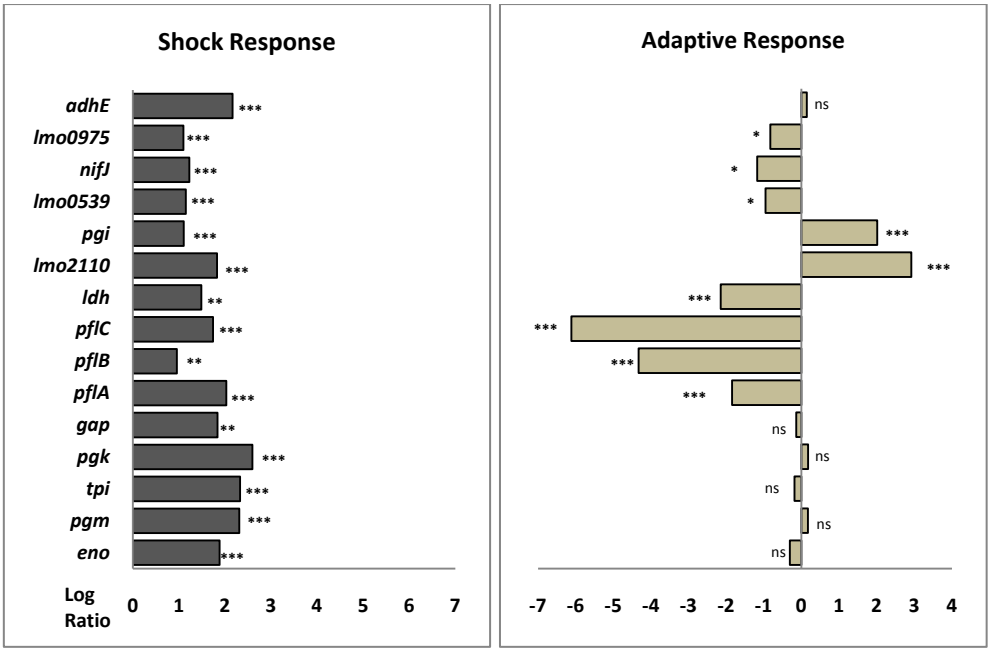
response (Fig. 4.6) and may have a role in supplying Mg^{2+} for PTS system. This gene appeared to be down-regulated 2.5-fold during the adaptive response (Fig. 4.6). A principal high affinity glucose transporter in *L. monocytogenes* is encoded by the *mptACD* operon (*lmo0096* to *lmo0098*), which showed up-regulation 3.4, 4.3, 3.8-fold respectively (Xue & Miller, 2007). Other non PTS sugar transporters also showed up-regulation with *T-value* score of 2.84 (Table 4.1). This group included the PrfA-repressed genes *lmo0179-lmo0181* which are homologous to *ycjNPO* in *E. coli*, encoding a putative ABC sugar transporter (Fig. 4.6).

Some BglG family transcriptional antiterminators are known to be highly involved in regulation of the PTS in *B. subtilis*, *E. coli* and *Staphylococcus carnosus* (Knezevic *et al.*, 2000; Schmalisch *et al.*, 2003). A number of genes belonging to this family were significantly up-regulated during hyperosmotic shock including *lmo2099* (3.3-fold increase in transcription) encoding a galacitol-specific PTS system operon regulator and *lmo2436* (2.3-fold up-regulation) encoding beta-glucoside-specific PTS system operon regulator.

Overall this pattern of expression was suggestive of a rapid uptake of carbohydrates from the BHI broth during the shock response. Carbohydrate transport in *L. monocytogenes* is of critical importance as this facultative, non-respiring organism, depends on carbohydrates for its primary energy source (Parker & Hutkins, 1997). Thus it could be concluded that there is an increase in the cellular energy requirement during the early stage of exposure to hyperosmotic stress in *L. monocytogenes*.

Carbohydrates entering the cell during the hyperosmotic shock appeared to be converted to pyruvate mainly through the process of glycolysis and to a lesser extent through pyruvate metabolism. Genes associated with these processes were up-regulated during hyperosmotic shock (*T-value* scores of 4.43 and 1.47 respectively, Table 4.1).

Genes encoding enzymes that catalyse the five steps of the central glycolysis pathway (conversion of glyceralydehyde-3-phosphate to phosphoenolpyruvate) were significantly up-regulated (3.6 to 6 fold) during shock response (Fig. 4.7).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 4.7 Expression of genes associated with carbohydrate metabolism in *L. monocytogenes* ATCC19115 following hyperosmotic shock and adaptation.

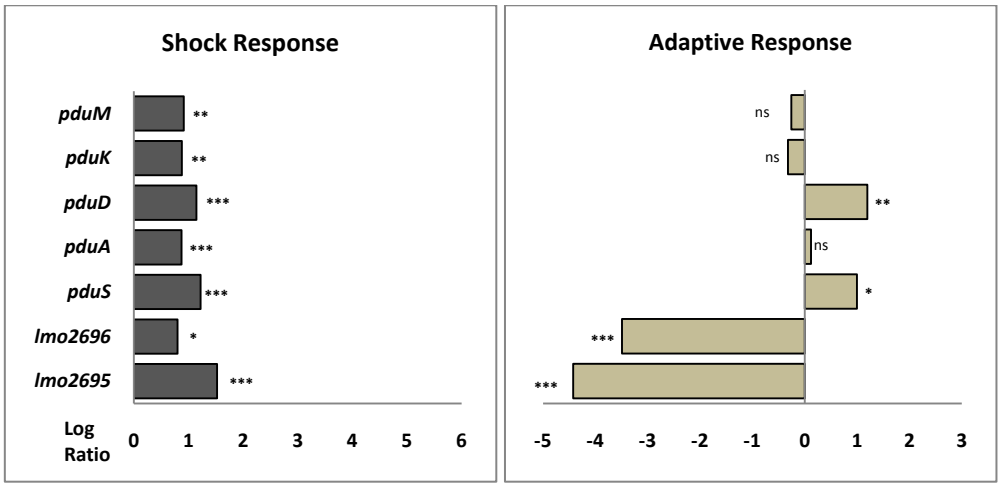
Genes coding for enzymes involved in pyruvate metabolism such as *pflA* (*lmo1917*) and *pflB* (*lmo 1406*) were up-regulated 4.1 fold and 2.0 fold, respectively (Fig. 4.7). These genes code pyruvate formate lyases, which convert pyruvate to formate and acetyl-CoA. The *pflC* gene (*lmo1407*), encoding pyruvate formate lyase-activating enzyme was also up-regulated 3.3 fold (Fig. 4.7). Pfl has been reported to be induced in other bacteria as a result of various stresses; in *E. coli* with increased concentration of NaCl in growth medium, in *Bifidobacterium longum sp.* under bile salt stress and following exposure to acid stress in *Streptococcus oralis* (Rasmussen *et al.*, 1991; Sánchez *et al.*, 2005; Wilkins *et al.*, 2001). Interestingly the *pdhABCD* operon (*lmo1052-1054*) had no significant change in transcription levels, encoding enzymic complex of pyruvate dehydrogenase involved in the transformation of pyruvate to acetyl-CoA. This strongly suggests that the production of acetyl-CoA, is directed towards the formate production pathway. The preference for Pfl mimics that observed in *E. coli* following glucose starvation, which results in decreased production of reduced form of NAD^+ (NADH) and the respiratory chain-mediated generation of free radicals, thus reducing oxidative stress and conserving energy (Moreau, 2004).

Three fold up-regulation of *ldh* (Fig. 4.7) strongly suggests activated conversion of lactate into pyruvate a reaction facilitated by encoded oxidoreductase, lactate dehydrogenase, a process which also generated NADH.

Pyruvate is used for energy production in *L. monocytogenes*, thus it could be concluded that during hyperosmotic shock listerial cells require more energy than cells grown in non-stressful conditions. The energy is most likely required to drive energy costly ABC transporters, such as the *kdp* transporter mentioned earlier and to drive the uptake of other nutrients from the environment, such as carbohydrates. Furthermore pyruvate may aid glutamate in stabilising of the intracellular electro-neutrality following sudden influx of cations into cytosol, as pyruvate dissociates into carboxylate anions. A glutamate GABA antiporter encoding gene *gadC* (lmo*2362*) showed a 16-fold up-regulation following hyperosmotic shock, suggesting an increased up-take of glutamate from the growth medium. Glutamate up-take from the extracellular medium within minutes of osmotic up-shift has previously been observed in *E. coli* as a means of maintaining electro-neutrality (Wood, 1999).

Rapid uptake of carbohydrates from the extracellular environment during hyperosmotic shock could also serve in increasing osmolarity of the cytosol, preventing plasmolysis in *L. monocytogenes*. While sugar up-take has not been directly linked to osmotolerance in this organism, it has been demonstrated in other bacteria. For example uptake of sucrose in response to lowered water activity has been reported in *Sinorhizobium meliloti, E. coli* and *Salmonella sp.,* which served in restoring the cytoplasmic volume after plasmolysis following hyperosmotic stress (Gouffi *et al.*, 1998; Kunte, 2006). Accumulation of mannitol in *Pseudomonas putida* has aided osmotolerance of this organism (Kets *et al.*, 1996). An increase in intracellular tetra-, di- and mono-saccharides concentration was detected in *Lactobacillus rhamnosus* following exposure to hyperosmotic stress (Prasad *et al.*, 2003). Uptake and accumulation of sugars from the environment cannot therefore be excluded as a mechanism of osmoregulation in *L. monocytogenes*. Escalating the intracellular concentration of sugars could potentially elevate the intracellular osmolarity at least temporarily, thus preventing plasmolysis by restoring cellular turgor, until all the cellular machinery is ready for compatible solute uptake.

Genes comprising the *pdu*/*eut* operon were up-regulated during the hyperosmotic shock, *T-value* score of 3.43 (for individual gene expression pattern see Fig. 4.8). Listerial *pdu*/*eut* genes are orthologous to genes necessary in *Salmonella sp*. for the co-enzyme B_{12}-dependent degradation of propanediol and, ethanolamine (respectively) as an energy source for growth (Bobik *et al.*, 1999; Buchrieser *et al.*, 2003; Chen *et al.*, 1994; Liu *et al.*, 2007).



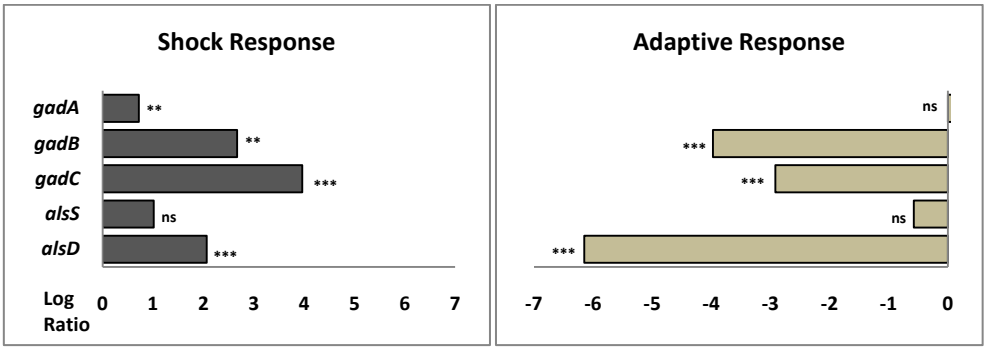
*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 4.8 Expression of genes associated with metabolism of glycerol in *L. monocytogenes* ATCC19115 following hyperosmotic shock and adaptation.

The *pdu* operon encodes genes that convert glycerol in a series of reactions to 1,2-propanediol, which is further converted to 1-propanol and propionic acid, generating ATP and three carbon intermediates (propionyl-CoA), which are fed into central metabolism (Bobik *et al.*, 1999; Liu *et al.*, 2007). Activation of this operon suggested potential involvement of glycerol as energy source in *L. monocytogenes* following hyperosmotic shock (Fig. 4.8). Glycerol utilization in bacteria also occurs through conversion of glycerol to glycerone phosphate in a series of reactions, which is fed into glycolysis pathway generating energy (Abram *et al.*, 2008). Generation of glycerone phosphate involves dihydroxyacetone kinase related proteins encoded by *lmo*2695 and *lmo*2696, which were shown to be up-regulated during the shock response (Fig. 4.8). The *eut* operon was not significantly affected by hyperosmotic shock.

4.3.1.5 Importance of maintaining intracellular pH during hyperosmotic shock.

An involvement of a glutamate decarboxylase (GAD) system was detected during hyperosmotic shock, with up-regulation of the *gad* genes (*lmo2362*, *lmo2363* and *lmo2434*, (Fig. 4.9)). The GAD system is essential for resistance to acid stress and operates by converting a molecule of glutamate to γ–aminobutyrate (GABA), consuming an intracellular proton and consequently preventing acidification of the cytoplasm (Cotter *et al.*, 2001; Cotter *et al.*, 2005). The involvement of this system has not been previously reported in hyperosmotic stress and its mechanism in salt shock is not clear, as no significant pH change could be detected in the external medium, the pH maintenance must therefore be internal. A decrease in intracellular pH has indeed been previously observed in *L. monocytogenes* as an initial response to osmotic shock (Fang *et al.*, 2004).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 4.9 Expression of genes associated with glutamate decarboxylase system and acetoin synthesis in *L. monocytogenes* ATCC19115 following hyperosmotic shock and adaptation.

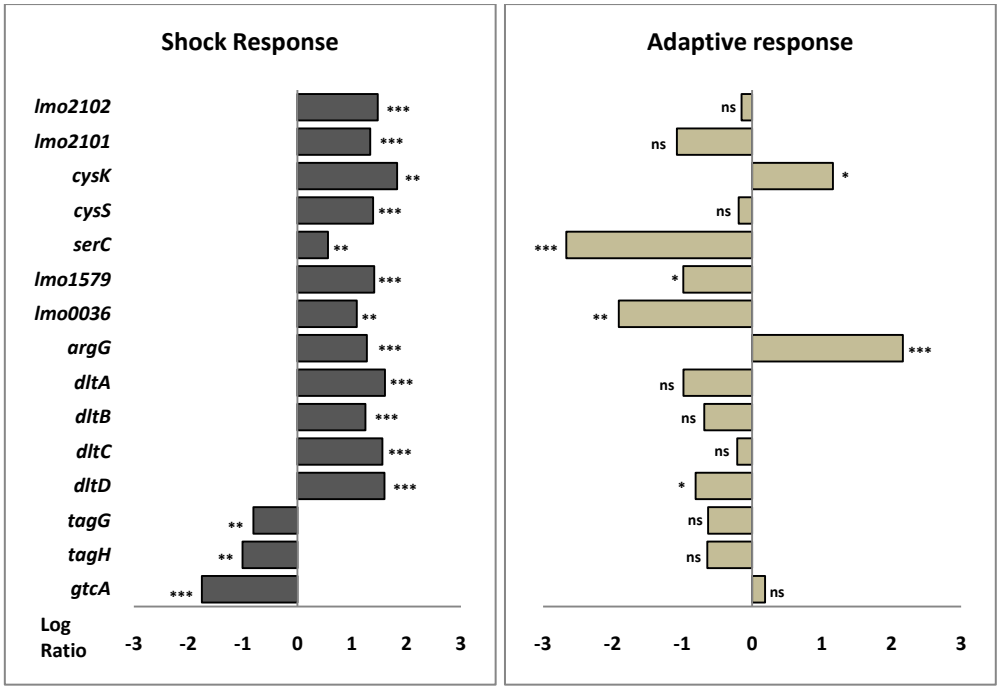
Overactive carbohydrate utilization, as suggested by up-regulation of genes associated with carbohydrate up-take and metabolism following osmotic shock most likely leads to acidification of the bacterial cytosol with glycolysis intermediates such as pyruvate and lactate (*ldh* was up-regulated (Fig. 4.7); responsible for conversion of lactate into pyruvate). Therefore in order to maintain pH homeostasis, while supplying the cell with sufficient energy to overcome the hyperosmotic shock, *L. monocytogenes* potentially engages the GAD system. High intracellular levels of pyruvate not only add to acidification of the cytosol but also are toxic to the cell. One way of removing this substance is to convert it to the neutral metabolite acetoin, which is a pathway, utilized by a number of lactic acid bacteria to maintain pH homeostasis (Cañas & Owens, 1999; García-Quintáns *et al.*, 2008; Romick & Fleming, 1998; Tsau *et al.*, 1992). The *alsDS* (*lmo1992* and *lmo2006*) genes, responsible for the conversion of pyruvate into acetoin were up-regulated in the shock response treatment but strongly down-regulated during the adapted response (Fig. 4.9).

Unlike *B. subtilis* which is able to utilise acetoin as an energy source (Karlin *et al.*, 2004), *L. monocytogenes* lacks this metabolic pathway. Accumulation of acetoin in the cytosol unutilized is most likely the case in *L. monocytogenes* ATCC19115 in response to hyperosmotic shock.

Furthermore, as mentioned previously in section 4.3.1.2, activation of the GAD system may aid in stabilising sudden intracellular influx of additional positively charged ions from the environment. Gene encoding the putative glutamate/GABA antiporter, *gadC* showed up-regulation of 15.6-fold in osmotically shocked cells (Fig. 4.9). Additionally, the MerR family of DNA-binding regulatory proteins showed suppression during hyperosmotic shock with *glnR* (*lmo1298*) being down-regulated up to 10-fold. GlnR negatively regulates the expression of the *glnRA* operon, which encodes glutamine synthetase (GlnA), under conditions of intracellular nitrogen excess in a form of nitrogen containing molecules (Detsch & Stülke, 2003; Doroshchuk *et al.*, 2006). Suppression of *glnR* potentially may promote accumulation of additional intracellular glutamate, by slowing its conversion to glutamine, thus helping maintenance of intracellular homeostasis when initially exposed to a hyperosmotic environment.

4.3.1.6 Amino acid metabolism role in hyperosmotic shock

Although the up-regulation of genes associated with the uptake of amino acids from the extracellular medium was not observed during hyperosmotic shock in strain ATCC19115 (Table 4.1), there was significant up-regulation of genes associated with metabolism of arginine, aspartate, cysteine, methionine, proline and aromatic amino acid biosynthesis (including synthesis of phenylalanine, tyrosine and tryptophan), as well as those involved in urea cycle and metabolism of amino groups.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 4.10 Expression of genes associated with amino acid metabolism in *L. monocytogenes* ATCC19115 following hyperosmotic shock and adaptation.

It appears that following a sudden increase in osmotic pressure *L. monocytogenes* cells channel energy into synthesis of compounds essential for cell maintenance. The overall up-regulation of genes associated with amino acid metabolic pathways could be due to a number of reasons. Cells need to synthesize more transporters, such as those required for transport of compatible solutes, or enzymes required for cell maintenance.

There was a strong up-regulation of D-alanine metabolism genes, with *T-value* score of 3.17 (Table 4.1). This amino acid plays a major role in the structure of the Gram-positive cell wall. The *dlt* operon, consisting of four genes (*lmo0971* to *lmo0974*, Fig. 4.10) codes proteins required for incorporation of D-alanine residues into the lipoteichoic acids (consisting of teichoic acid and lipids) of the cell wall in *L. monocytogenes* (Abachin *et al.*, 2002). In both *L. monocytogenes* and *Staphylococcus aureus* disruption of *dlt* operon, results in a mutant susceptible to a number of cationic polypeptides (Abachin *et al.*, 2002; Ganz, 2001; Jordan *et al.*, 2008). Addition of D-alanine to the cell-wall results in decreased negative charge of the cell wall and depolarization of the cell wall during hyperosmotic shock might be of benefit for the organism, as Na^+ uptake in bacteria is regulated by membrane potential. Reducing the negative charge of the cell-wall reduces its

permeability to toxic Na^+ cations (Apte *et al.*, 1987). Furthermore, genes encoding teichoic acid transporters *tagG* and *tagH* (*lmo1074* and *lmo1075*) were on average down-regulated two-fold in this response, as were a genes *gtcA* (*lmo2549*, down-regulated 3.5 fold) which is involved in teichoic acid biosynthesis (Fig. 4.10).

4.3.1.7 The effect of hyperosmotic shock on metabolism of cofactors

4.3.1.7.1 Vitamin B_6

Up-regulation of some genes involved in vitamin B_6 metabolism was observed during hyperosmotic shock (*T-value* score of 1.78, P-value >0.05, due to the small number of genes present in this group). Two genes *lmo2101* and *lmo2102*, homologous to the *yaaD* and *yaaE* found in *B. subtilis*, were up-regulated more that 2.5 fold (Fig. 4.10). The exact mechanism of the pyridoxine biosynthesis proteins, encoded by these two genes, has not yet been clearly defined in either of the two species. However these are thought to be involved in incorporating glyceraldehyde 3-phosphate (product of glycolysis) into pyridoxal 5'-phosphate (PLP), a biocatalytically active form of vitamin B_6 (Belitsky, 2004; Sakai *et al.*, 2002). PLP represents the most versatile organic cofactor and that plays a central role in amino acid metabolism (Garrido Franco *et al.*, 2001; Percudani & Peracchi, 2003; Schneider *et al.*, 2000). PLP-dependent enzymes are involved in basic metabolic pathways, reactions of which include the transfer of amino group, decarboxylation and inter-conversion of L- and D-amino acids (Percudani & Peracchi, 2003). Up-regulation of this group of genes suggests a greater demand for this essential cofactor perhaps due to increase in amino acid metabolism.

The majority of PLP-dependent enzymes were only slightly up-regulated at the molecular level. Some of these include *serC* (*lmo2825*), encoding phosphoserine aminotransferase –this gene only showed slight (1.47 fold) increase in transcription compared to the control (Fig. 4.10); *trpB* (*lmo1628*) encoding a subunit of tryptophan synthase showed up-regulation of 2.5 fold; and *metB* (*lmo1680*) encoding cystathionine beta-lyase with a 1.42 fold increase in transcription. The marginal increase in transcription of these genes suggests that these are already sufficiently expressed during no-stressed cell growth as PLP-dependant enzymes are essential for normal cell function, and only require a slight adjustment to account for the metabolic changes due to hyperosmotic shock.

However, two PLP-dependent proteins - *gadB* (*lmo2363*) and *cysK* (*lmo0223*) - showed significant up-regulation exhibiting 6.4- and 3.6-fold increases in expression (Fig. 4.10), respectively. The *gadB* gene product, glutamate decarboxylase, has an important role in controlling intracellular pH as described previously (Section 4.3.1.5). This protein is also involved in the metabolism of glutamine, alanine, and aspartate. This gene was found to be highly expressed (with a log intensity of 12.9) indicating its importance in metabolism of this organism. The *cysK* gene was also highly expressed as indicated from the microarray signal data. It encodes cysteine synthase, an enzyme involved in cysteine biosynthesis, and has been reported as a salt shock protein in this organism and was shown to be induced in *B. subtilis* during cold and oxidative stress (Antelmann *et al.*, 2000; Duché *et al.*, 2002). In the study by Duché *et al*, 2002 CysK in *L. monocytogenes* strain LO28 was only expressed in the first 30 minutes following NaCl stress; however in this experiment with strain ATCC19115 *cysK* was up-regulated 2.24-fold in the adaptive response as well as 10 min post NaCl stress (Fig. 4.10), demonstrating potential strain variability in terms of gene expression.

4.3.1.7.2 Nicotinate and nicotinamide metabolism

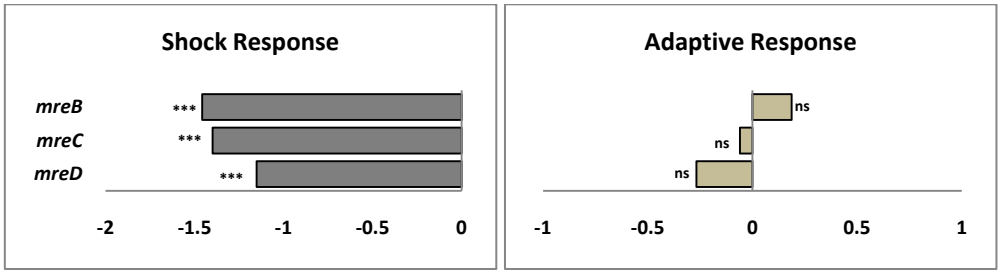
Gene encoding proteins essential for nicotinate and nicotinamide metabolism were up-regulated under both shock and adapted hyperosmotic conditions (Table 4.1). This metabolic pathway provides the means for synthesis of the nicotinamide adenine dinucleotides (NAD+ and NADP+). These molecules are essential cofactors in many oxidation-reduction reactions and NAD+ also serves as a substrate for the enzymatic modification of proteins (Kawai & Murata, 2008; Zhang *et al.*, 2002). Increased demand for NAD in metabolism of amino acids and the production of pyruvate due to NaCl induced hyperosmotic stress appears to lead to up-regulation of NAD synthesis. Alanine dehydrogenase is one of several NAD-dependent enzymes, which has been labelled as a salt shock protein in a study by Duché *et al*, 2002, encoded by *lmo1579* transcription of which was up-regulated 2.7-fold in this study during hyperosmotic shock (Fig. 4.10), this enzyme is responsible for conversion of pyruvate to L-alanine (Duché *et al.*, 2002).

Glyceraldehyde-3-phosphate dehydrogenase is an important enzyme in glycolysis, synthesis of which has previously been observed to increase in *L. monocytogenes* following salt shock (Duché *et al.*, 2002). Expression of Gap following hyperosmotic shock has also been confirmed in this study with a significant 3.6-fold up-regulation of *gap* (*lmo2459*) in osmotically shocked cells (Fig. 4.7). The *ldh* gene, (mentioned previously in section 4.3.1.5) was up-regulated 2.8-fold following hyperosmotic

shock (Fig. 4.7); it encodes a NAD-dependent enzyme lactate dehydrogenase, which was induced in *E. faecalis* under a number of stresses and also in *B. longum* following bile salt stress (Giard *et al.*, 2001; Sánchez *et al.*, 2005).

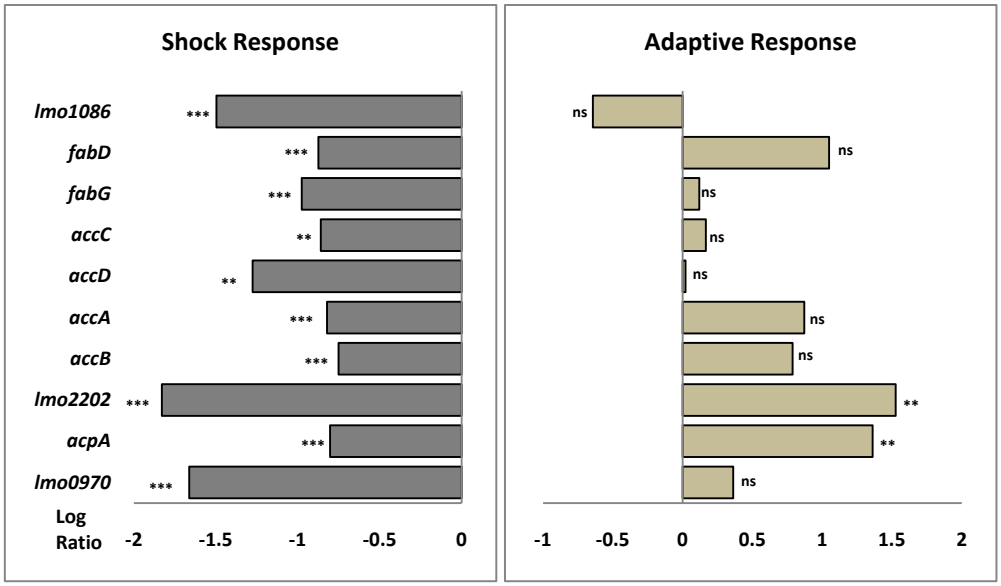
4.3.1.8 Effect of hyperosmotic shock on genes associated with cell division, lipid metabolism, phage, and virulence.

Genes associated with cell division were down-regulated following hyperosmotic shock (*T-value* score of -2.14, Table 4.1), this suggests that the cells were not actively dividing but entering rapidly into a transitory state in order to manage NaCl stress.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 4.11 Expression of genes associated with cell division in *L. monocytogenes* ATCC19115 following hyperosmotic shock and adaptation.

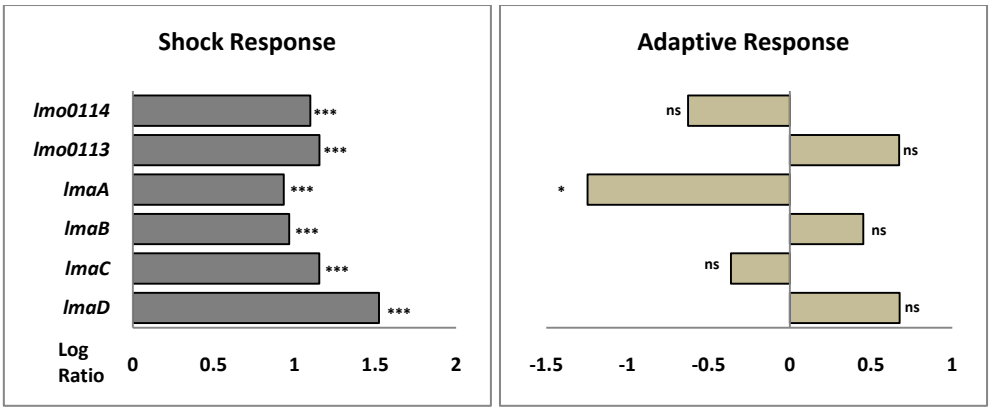


*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 4.12 Expression of genes associated with lipid metabolism in *L. monocytogenes* ATCC19115 following hyperosmotic shock and adptation.

Among genes which were suppressed, only three genes *mreD*, *mreC*, *mreB* (*lmo1546-1548*, Fig.4.11), were down-regulated greater than two-fold, the rest showed only slight down shift in transcription levels.

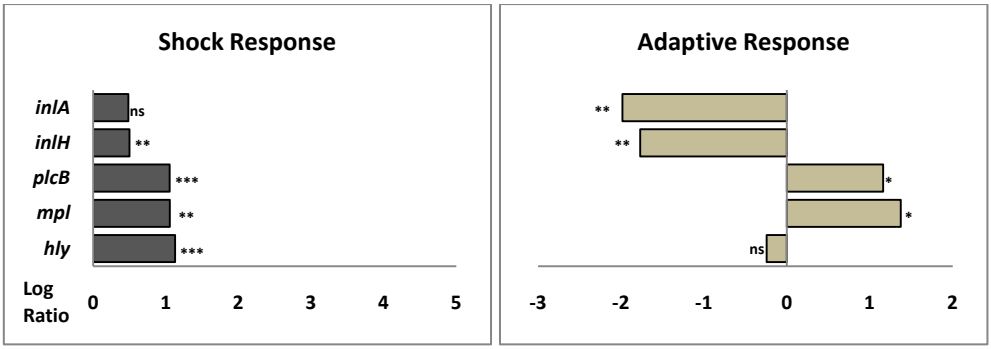
Genes associated with fatty acid biosynthesis and biosynthesis of steroids/terpenoids were down-regulated during salt shock response with *T-value* scores of -4.38 and -1.80 respectively (Table 4.1). Genes down-regulated ≥ two-fold included *lmo*0970, *fabGD* (*lmo1807*, *lmo1808*), *lmo2202*, *accAD* (*lmo1572*, *lmo1573*) and *accBC* (*lmo1356*, *lmo1357*) involved in fatty acid biosynthesis, and *lmo1086* involved in the non-mevalonate pathway for iso-prenoid biosynthesis (Fig. 4.12).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 4.13 Expression of genes encoding phage proteins in *L. monocytogenes* ATCC19115 following hyperosmotic shock and adaptation.

A group of genes with unknown function in this organism, which encode proteins similar to bacteriophage proteins, such as *lmo0113* and *lmo0114*, were up-regulated ≥two-fold during this response (Fig. 4.13). A gene cluster, *lmo0115-lmo0118*, organized in to a *lmaABCD* operon and encoding a *Listeria sp*. specific antigen of bacteriophage origin were up-regulated during hyperosmotic shock response (Fig. 4.13). These are believed to have been deposited in *Listeria* genomes by a phage and persisted though the evolution of the species, their physiological function in this organism is as yet unknown (Hanawa *et al.*, 2002; Schäferkordt & Chakraborty, 1997).



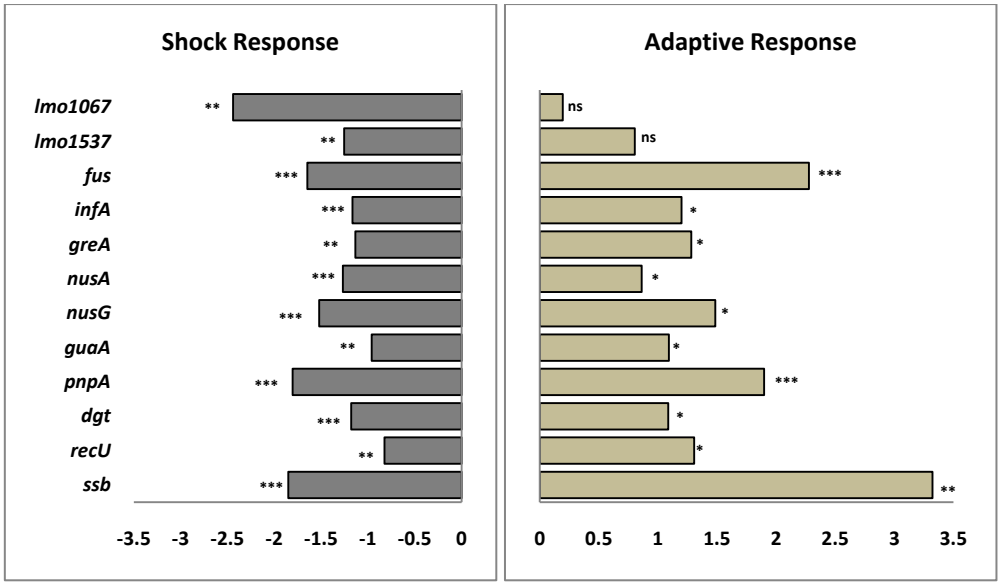
*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 4.14 Expression of virulence related genes in *L. monocytogenes* ATCC19115 following hyperosmotic shock and adaptation.

Hyperosmotic shock overall slightly activated virulence genes with *T-value* score of 1.93, with genes *hly* (*lmo0202*), *mpl* (*lmo0203*), *actA* (*lmo0204*) and *plcB*(*lmo0205*) being up-regulated ≥two-fold (Fig. 4.14). This agrees with a previously observed link between exposure to sublethal environmental stress and induction of virulence genes in *L. monocytogenes* (Garner *et al.*, 2006; Kazmierczak *et al.*, 2003).

4.3.1.9 Effect of hyperosmotic shock on information processing and storage genes

Hyperosmotic shock had an overall negative effect on transcription-associated genes gaining a *T-value* score of -1.83 (Table 4.1). A number of genes showed down-regulation such as *nusG* (*lmo0246*) encoding transcription antitermination factor with 2.9-fold decrease in transcription (Fig. 4.15), both *nus*A (*lmo1322*) and *gre*A (*lmo1496*) encoding transcription elongation factors were over two-fold suppressed. Genes involved in nucleotide metabolism (purine and pyrimidine), such as *pnpA* (*lmo1331*), *guaA* (*lmo1096*) and *dgt* (*lmo2657*) were also found to be correspondingly suppressed (Fig. 4.15).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 4.15 Expression of genes associated with transcription, nucleotide metabolism as well as DNA repair and recombination in *L. monocytogenes* ATCC19115 following hyperosmotic shock and adaptation.

Gene sets encoding GTP-binding proteins with various roles related to ribosomes and protein synthesis were down-regulated (*T-value* of -2.84, Table 4.1). A number of genes showed ≥ two-fold suppression in transcription included *typA* (*lmo1067*; ribosome-associated GTP-binding elongation factor) which showed a down-regulation of 5.4 fold (Fig. 4.15), *fus*, (*lmo2654* – elongation factor G, suppressed 3.2 fold) and *lmo1537* (*obgE*, down-regulated 2.5 fold).

Transcription termination factor, *rho* (*lmo2551*) was up-regulated 2.6-fold (data not shown). Rho factor function in *E. coli* is to ensure that the bacterial cell produces proteins needed for immediate cellular functions, adjusting the level of transcription to the translational needs of the organism, by suppressing transcription of non-essential proteins thus decreasing resource burdens on the cell (Cardinale *et al.*, 2008). It appears that during the hyperosmotic shock many cellular processes associated with normal function are partially suspended, potentially suppressed by the Rho, and only proteins essential for the survival of the organisms are actively expressed.

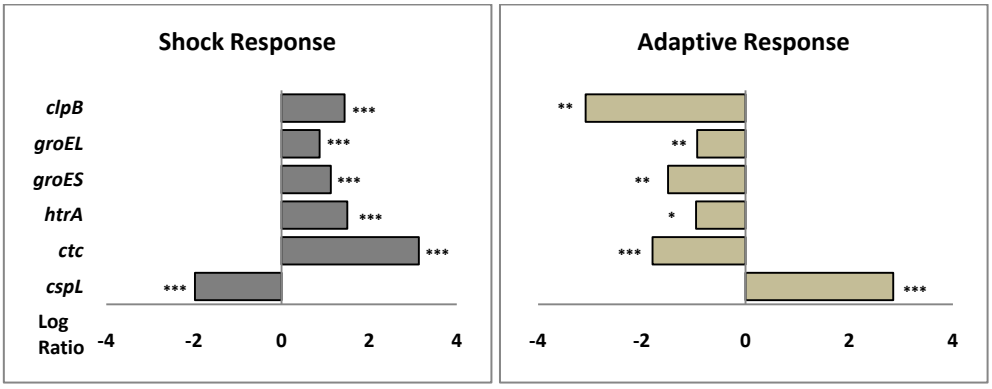
There was no apparent evidence of DNA damage during NaCl shock treatment, with genes involved in DNA repair and recombination being overall down-regulated with *T-value score* of -1.10 (Table 4.1). The *recA* (*lmo1398*) gene was suppressed 2.7-fold (data not shown) in salt shocked cells. RecA, encodes a major activator of the SOS response in *B. subtilis*, the mechanism involved in the repair of

DNA damage (Au *et al.*, 2005; Van der Veen *et al.*, 2007). Some of the genes that showed strong down-regulation included *ssb* (*lmo0045*) which showed a down-shift of 3.6-fold (Fig. 4.15), *radC* (*lmo1549*) with two-fold change (data not shown) and *recU* (*lmo1891*) had a 2.7-fold down shift in expression (Fig. 4.15).

4.3.1.10 Hyperosmotic shock effect on stress response genes

In all living organisms exposure to stressful environmental conditions is followed by the induction of specific patterns of stress response genes.

During hyperosmotic shock a gene encoding a general stress ribosomal protein *ctc* (*lmo0211*) similar to that found in *B. subtilis*, (Schmalisch *et al.*, 2002; Volker *et al.*, 1994), showed up-regulation of 9-fold (Fig. 4.16). The Ctc protein has previously been linked to osmotolerance in *L. monocytogenes* in the absence of osmoprotectants (Duché *et al.*, 2002; Gardan *et al.*, 2003b). Even though compatible solutes are present in the growth medium the bacterial cells lack the mechanism of their acquisition this early in the osmo-adaptive response thus reliance on Ctc for accurate translation under stress conditions could be important.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 4.16 Expression stress protein encoding genes in *L. monocytogenes* ATCC19115 following hyperosmotic shock and adaptation.

Only a few heat shock protein encoding genes were up-regulated ≥two-fold during hyperosmotic stress (Fig. 4.16); these included *htr*A (*lmo0292*), *groES* (*lmo2069*), *groEL* (*lmo2068*) and *clpB* (*lmo2206*). HtrA protein expression is activated by NaCl in *Lactobacillus helveticus* 20 minutes

following exposure with maximum level of mRNA reached at 40 minutes post exposure to stress (Smeds *et al.*, 1998). The expression of *groES* and *groEL* chaperones is activated in *Lactococcus lactis* 10 minutes post exposure to hyperosmotic stress and its importance in salt tolerance has been previously noted in *Caulobacter crescentus*, but not *L. monocytogenes* (Kilstrup *et al.*, 1997; Susin *et al.*, 2006). General stress protein ClpB has not been linked to osmotolerance in *L. monocytogenes* strain LO28, but is activated during hyperosmotic up shift in the halophilic methanogen *Methanohalophilus portucalensis* (Chastanet *et al.*, 2004; Shih & Lai, 2007).

A gene encoding a CspA-like cold shock protein, *cspL* (*lmo1364*) showed significant 4-fold down-regulation during hyperosmotic shock response, which was found to be up-regulated 7.2-fold during adaptive response to the same hyperosmotic stress. Cold shock proteins, including CspL, have previously been linked to osmotic and cold temperature tolerance in *L. monocytogenes* (Schmid *et al.*, 2009). Suppression of the *cspL* gene during hyperosmotic shock observed in strain ATCC19115 suggests that the encoded RNA-binding protein only plays a significant role later in hyperosmotic response. Considering that CspA has been shown to act as RNA chaperone, important for maintaining undisturbed transcription in *E. coli* (Jiang *et al.*, 1997), it is likely that suppression of CspL transcription reflects the overall diminished activity in transcription observed during early phase of hyperosmotic stress response (section 4.3.1.11).

4.3.1.11 Effect of hyperosmotic shock on translation-apparatus related genes.

Overall protein synthesis in bacteria following hyperosmotic shock has previously been shown to be suppressed (Fulda *et al.*, 1999). This also appeared to be the case for *L. monocytogenes* with gene sets encoding ribosomal proteins being significantly down-regulated with *T-value* scores of -11.92 (Table 4.1). Most of the genes in this group showed down-regulation of ≥ two-fold, with *rpmB* (*lmo1816*) showing the strongest down-shift of 7.5-fold; other genes with strong down-regulation included *rpsL* (*lmo2656*) and *rspP* (*lmo1797*) showing 5.3- and 4.7-fold suppression (Table 4.2).

Table 4.2 Response of *L. monocytogenes* ATCC19115 genes associated with translation and ribosome proteins to NaCl shock and adaptive stresses.

Gene	Shock response		Adaptive Response		Function	Functional Category
	Log Ratio	P value	Log Ratio	P value		
rpoA	-1.29	2.9E-05	0.58		RNA polymerase, alpha subunit	RNA polymerase
rpoC	-0.96	0.0050	1.02	0.0098	RNA polymerase, beta subunit	RNA polymerase
rpmB	-2.90	1.2E-05	3.27	9.0E-05	ribosomal protein L28	Ribosome, large subunit
rpsL	-2.40	0.0005	4.10	5.9E-06	ribosomal protein S12	Ribosome, small subunit
rpsP	-2.24	2.0E-05	0.70		ribosomal protein S16	Ribosome, small subunit
rplK	-2.05	0.0005	3.36	0.0005	ribosomal protein L11	Ribosome, large subunit
rplA	-2.03	0.0005	3.37	0.0006	ribosomal protein L1	Ribosome, large subunit
rplJ	-1.66	4.4E-05	2.50	0.0002	ribosomal protein L10	Ribosome, large subunit
rplL	-1.60	1.7E-05	2.88	0.0006	ribosomal protein L7/L12	Ribosome, large subunit
rpmA	-1.76	0.0004	1.31	0.0029	ribosomal protein L27	Ribosome, large subunit
rplU	-2.19	9.9E-05	2.74	4.3E-05	ribosomal protein L21	Ribosome, large subunit
rplT	-1.97	5.7E-06	1.29	0.0058	ribosomal protein L20	Ribosome, large subunit
rpmI	-2.50	0.0001	2.39	0.0001	ribosomal protein L35	Ribosome, large subunit
rplS	-1.66	7.2E-06	1.94	0.0005	ribosomal protein L19	Ribosome, large subunit
rpmF	-1.28	4.4E-05	1.01	0.0132	ribosomal protein L32	Ribosome, large subunit
rpmE	-1.64	0.0001	2.59	0.0004	ribosomal protein L31	Ribosome, large subunit
rplM	-2.34	0.0006	0.81		ribosomal protein L13	Ribosome, large subunit
rpmJ	-1.32	0.0002	1.52	0.0089	ribosomal protein L36	Ribosome, large subunit
rplO	-0.97	0.0006	0.99		ribosomal protein L15	Ribosome, large subunit
rplF	-1.35	7.8E-05	1.22	0.0095	ribosomal protein L6	Ribosome, large subunit
rplE	-1.33	0.0004	1.24	0.0073	ribosomal protein L5	Ribosome, large subunit
rplX	-1.21	0.0002	1.13	0.0145	ribosomal protein L24	Ribosome, large subunit
rplN	-1.27	0.0001	1.96	0.0007	ribosomal protein L14	Ribosome, large subunit
rplP	-1.23	0.0001	0.78		ribosomal protein L16	Ribosome, large subunit
rplV	-1.36	0.0003	1.81	0.0015	ribosomal protein L22	Ribosome, large subunit
rplB	-1.36	8.6E-05	0.91		ribosomal protein L2	Ribosome, large subunit
rplD	-1.50	9.3E-05	2.08	0.0002	ribosomal protein L4	Ribosome, large subunit
rpsD	-1.03	0.0022	3.21	0.0008	ribosomal protein S4	Ribosome, small subunit
rpsB	-1.70	0.0039	2.22	0.0004	ribosomal protein S2	Ribosome, small subunit
rpsI	-2.17	0.0001	0.34		ribosomal protein S9	Ribosome, small subunit
rpsK	-1.38	0.0004	1.36	0.0027	ribosomal protein S11	Ribosome, small subunit
rpsM	-1.30	0.0004	1.73	0.0007	ribosomal protein S13	Ribosome, small subunit
rpsE	-1.11	0.0004	1.11	0.0060	ribosomal protein S5	Ribosome, small subunit
rpsH	-1.27	0.0001	1.43	0.0046	ribosomal protein S8	Ribosome, small subunit
rpsN	-1.40	5.3E-05	1.68	0.0009	ribosomal protein S14	Ribosome, small subunit
rpsQ	-1.22	0.0002	1.15	0.0070	ribosomal protein S17	Ribosome, small subunit
rpsS	-1.17	4.7E-05	2.24	0.0010	ribosomal protein S19	Ribosome, small subunit
rpsJ	-1.92	0.0014	2.07	0.0021	ribosomal protein S10	Ribosome, small subunit
rpsG	-1.94	0.0003	3.41	2.6E-05	ribosomal protein S7	Ribosome, small subunit
raiA	1.77	0.0001	-3.84	5.2E-05	ribosome-associated inhibitor (protein Y)	Translation/ribosome associated
infC	-2.70	0.0001	1.30	0.0192	translation initiation factor IF3	Translation factors

Gene	Shock response		Adaptive Response		Function	Functional Category
	Log Ratio	P value	Log Ratio	P value		
tsf	-1.49	0.0002	1.46	9.9E-05	elongation factor EF-Ts	Translation factors
lmo0227	-1.05	0.0009	3.21	7.3E-05	putative tRNA-dihydrouridine	Translation/ribosome associated
lmo1530	-1.11	0.0009	0.68		similar to queuine tRNA-ribosyltransferase	Translation/ribosome associated
sigH	1.39	2.8E-05	1.02	0.0105	RNA polymerase, specialized sigma subunit	RNA polymerase

P values not shown were >0.05

Ribosome associated proteins and translation factors, were in general down-regulated with *T-value* scores -3.04 and -3.33, respectively. The gene encoding ribosome inhibitor protein RaiA, (*lmo2511*) was up-regulated 3.4-fold following hyperosmotic shock (Table 4.2).

Gene sets encoding RNA-polymerase proteins were also down-regulated during hyperosmotic shock, for example the RNA polymerase main catalytic subunit coded by *rpoA* (*lmo2606*) showed suppression in transcription of 2.4-fold (Table 4.2).

Interestingly a gene coding alternative sigma factor 30, σ^H (*sigH*, *lmo0243*) was up-regulated 2.6-fold (Table 4.2). Sigma H is expressed in *B. subtilis* upon entry into sporulation and has previously been shown to contribute to survival of non sporulating cells of *B. subtilis* under various stress conditions such as growth in acidic media and alkaline conditions (Gaidenko & Price, 1998). In *L. monocytogenes* σ^H factor is essential for growth under alkaline conditions and for efficient growth in a minimal medium (Rea *et al.*, 2004). Its role in osmotic stress has not been studied in either species. It appears to play some regulatory role during osmotic stress response as *lmo0243* is also up-regulated two-fold during the adaptive response; it is however uncertain what that role may be until its regulon is better defined.

4.3.1.12 Regulation of gene responses during hyperosmotic shock

Alternative sigma factor σ^B has previously been shown to contribute to the ability of *L. monocytogenes*, as well as other related organism such as *B. subtilis* and *S. aureus*, to survive under various environmental stress conditions including osmotic stress (Becker *et al.*, 1998; Chan *et al.*, 1998; Kazmierczak *et al.*, 2003; Moorhead & Dykes, 2003; Raengpradub *et al.*, 2008; Volker *et al.*,

1999). The *sig*B gene (*lmo0895*) itself showed no significant change in transcription during hyperosmotic shock from the control state. It is possible that the σ^B activation is almost instantaneous (occurs within seconds following stress) and has been missed in this experiment. Genes that have been shown to be positively up-regulated either directly or indirectly by this alternative sigma factor in *L. monocytogenes* (Abram *et al.*, 2008; Hain *et al.*, 2008; Raengpradub *et al.*, 2008; Wemekamp-Kamphuis *et al.*, 2004b)showed significant up-regulation (*T-value* score of 6.54, Table 4.3) and those genes shown to be more actively expressed in a sigB null mutant were down-regulated (*T-value* score of -3.37, Table 4.3).

Table 4.3 Response of *L. monocytogenes* strain ATCC19115 genes to NaCl shock and adapted stresses based on regulons.

Regulon	Shock Response		Adapted Response	
	T-value*	P-value#	T-value*	P-value#
HrcA+	-5.07	1.4E-05	5.50	3.9E-06
HrcA-	1.11		0.45	
SigB-	-3.31	0.0012	4.87	3.2E-06
SigB+	6.54	6.0E-10	-7.79	
CodY+	1.25		-1.63	4.6E-13
CodY-	1.61		-7.02	5.9E-10
CtsR-	2.19		-2.60	0.0407
RpoN+	3.22	0.0045	-0.56	
RpoN-	4.80	1.1E-05	-3.26	0.0018
PrfA+	5.65	4.0E-07	-4.24	7.4E-05
PrfA-	2.70	0.0354	-1.55	
VirR+	4.98	0.0002	0.29	

*T-value scores were determined from the expression data using the approach of Boorsma *et al*. (2005).
P-values not shown are >0.05
+ indicate +ve regulation by a given regulon, - indicate –ve regulation by a given regulon.
¥ The gene sets for the given regulatory proteins are designated from empirical deletion studies on the basis of what genes are positively influenced ("+") or negatively influence ("-"), The influence of the regulators in many cases are not direct thus T-profile data only gives a general trend in terms of expression responses

PrfA functions as a virulence regulator in *L. monocytogenes* which itself is positively regulated by σ^B (Chakraborty *et al.*, 1992; Kazmierczak *et al.*, 2006; Nadon *et al.*, 2002; Schwab *et al.*, 2005). This transcriptional activator showed no significant change during hyperosmotic shock. Genes positively controlled or influenced indirectly by *prf*A on the other hand showed up-regulation (*T-value* score 5.65, Table 4.3). Additional virulence regulator VirR also showed activation in cold shocked cells with *T-value* score of 4.98 (Table 4.3). A number of studies have previously shown the link between stress response and virulence in this pathogen. In order to cause disease the pathogen has to overcome a number of environmental obstacles, such as acid stress in the stomach, oxidative stress inside

macrophages and osmotic stress inside the gastrointestinal lumen. The regulatory mechanisms for stress response and virulence have evolved to be closely interrelated, thus activating one stimulates the other and vice versa. Interestingly genes suppressed by PrfA also showed overall activation (*T-value* score of 2.70, Table 4.3). This small group of genes mainly encodes for ABC transporters for sugars, it is possible that the influence of other regulatory proteins or non-coding RNAs influences the expression of this set of genes.

There was no evident direct correlation between CodY regulon and the salt shock response (Table 4.3) most likely due to interactions with other regulators as not all genes in its regulon are under sole direct control of CodY. In *B. subtilis* CodY helps to regulate transition from rapid exponential growth to the stationary growth phase (Molle *et al.*, 2003; Shivers & Sonenshein, 2004; Sonenshein, 2005). As *L. monocytogenes* undergoes rapid transition from exponential growth in control medium to suppressed growth (a temporary lag phase) due to encountered NaCl stress, CodY regulon appears to not be directly involved in overcoming the imposed environmental stress, its involvement appears to be more apparent in the adaptive response to hyperosmotic stress with CodY repressed genes showing a strong down-regulation of -7.02 (as discussed previously in section 3.3.2.11). *L. monocytogenes* CodY represses genes associated with metabolism and transport of carbohydrates as well as amino acid biosynthesis (Bennett *et al.*, 2007). CodY also has a major role in monitoring the energetic capacity and nutritional state of the cell (Sonenshein, 2005). During hyperosmotic shock *L. monocytogenes* showed increased demand for energy in terms of carbohydrate uptake and metabolism, which is re-repressed by inactive CodY regulator.

Hyperosmotic shock induced genes activated by rpoN regulator, also known as an alternative sigma factor σ^N or σ^{54}. The σ^{54}–dependent genes have a wide variety of cellular functions in bacteria from nitrogen and carbon utilization to flagella synthesis and virulence (Arous *et al.*, 2004; Buck *et al.*, 2000; Studholme & Buck, 2000). In *L. monocytogenes* σ^{54} has been shown to assist in regulation of mainly carbohydrate metabolism genes (Arous *et al.*, 2004).The *mpt* operon, which is under positive control of σ^{54}, showed up-regulation of over 3.3-fold. This further emphasizes the involvement of alternative sigma factor σ^{54} in carbohydrate metabolism and subsequently its involvement in adaptation to hyperosmotic shock. Negative control of gene expression by σ^{54} was not very straight forward. Some genes showed down-regulation such as *rpl*F (*lmo*2617) which was repressed 2.6-fold. Others showed up-regulation, such as *pfl*A (*lmo*1917) which was activated 4-fold. This suggests that

σ^{54} control of gene expression is somewhat indirect (Arous *et al.*, 2004) and interaction with other regulatory factors during hyperosmotic shock is evident, at least in *L. monocytogenes* ATCC19115.

4.3.1.13 Genes with analogous responses during hyperosmotic shock and osmoadaptation response in L. monocytogenes strain ATCC19115.

The two phases of hyperosmotic stress response in *L. monocytogenes* strain ATCC19115 produced significantly different gene expression profiles, signifying an overall very different physiological response. Only 17 genes showed significant activation during both phases of hyperosmotic stress response (Table 4.4).

Two genes encoded ribosomal proteins, *rplR* and *rpsC* remain active throughout the duration of hyperosmotic response observed in strain ATCC19115 (Table 4.4).

Table 4.4 Genes activated in both shock and adaptive response of *L. monocytogenes* strain ATCC19115 when exposed to hyperosmotic stress induced by 10.0% (w/v) NaCl.

Gene	Shock Response		Adaptive Response		Function
	LR[#]	P	LR	P	
argG	**1.27**	5.5E-05	**2.17**	0.001	argininosuccinate synthase
cggR	**1.38**	0.009	**4.32**	7.1E-06	central glycolytic genes regulator
lmo0095	**1.19**	1.1E-04	**1.20**	0.040	unknown protein
lmo0099	**1.56**	2.0E-04	**1.76**	0.008	putative regulatory protein (similar to L. casei ManO protein)
lmo2005	**1.11**	2.6E-04	**1.99**	0.007	similar to predicted oxidoreductases (related to aryl alcohol dehydrogenases)
lmo2110	**1.83**	0.001	**2.92**	3.9E-04	mannose-6-phosphate isomerase
lmo2447	**1.32**	4.9E-05	**1.85**	0.001	similar to transcription regulator
lmo2843	**1.25**	0.001	**2.47**	3.0E-04	similar to uncharacterized protein involved in cytokinesis
mpl	**1.05**	0.009	**1.37**	0.026	Zinc metalloprotease (elastase) propeptide
pduD	**1.15**	1.0E-04	**1.20**	0.009	similar to cobalamin-dependent diol dehydratase, medium subunit; similar to S. enterica PduD protein
pgi	**1.11**	0.001	**2.01**	0.001	glucose-6-phosphate isomerase
purM	**1.10**	0.001	**4.31**	4.4E-06	phosphoribosylaminoimidazole synthetase
rho	**1.38**	1.2E-04	**1.18**	0.017	transcription termination factor
rplR	**1.06**	0.010	**1.90**	0.001	ribosomal protein L18
rpsC	**1.44**	0.032	**1.40**	0.004	ribosomal protein S3
sigH	**1.39**	2.8E-05	**1.02**	0.010	RNA polymerase, specialized sigma subunit (sigma24 homolog)
yesN	**1.08**	4.1E-04	**1.32**	0.005	two-component response regulator

LR= Log Ratio

Among analogously expressed genes was *sigH*, described in section 4.3.1.11, encoding a regulatory protein similar to SigH in *B. subtilis* responsible for transition from exponential phase to stationary phase, nutrient transport and regulation of other regulatory factors. SigH dependent *L. monocytogenes* genes have not been detected, however a *sigH*-null mutant showed reduced growth in a minimal medium and in alkaline conditions (Rea *et al.*, 2004). It is unclear what function this regulatory factor plays in strain ATCC19115 response to osmotic stress, but it might be involved in regulating nutrient acquisition in stressed cells. A gene encoding a putative regulatory protein similar to ManO in *Lactobacillus casei*, was activated in both stages of stress response in this strain. The exact function of ManO in *L. casei* is not clear but it might be linked to control of glucose uptake in this organism via the *mpt* operon (Yebra *et al.*, 2006). Among other genes encoding regulatory proteins was transcription terminator factor *rho* (described in section 4.3.1.9), *cggR* encoding a protein similar to the central glycolytic genes regulator protein CggR of *B. subtilis* (Doan & Aymerich, 2003), *yesN* encoding a two-component response regulator (section 4.3.1.1) as well as *lmo2447*.

It appears that these proteins may play an important role in regulating *L. monocytogenes* strain ATCC19115 osmotic stress response.

4.4 Summary and Conclusions

The aim of this study was to elucidate the effect of osmotic stress on gene expression in *L. monocytogenes* strain ATCC19115 at an early stage of osmoadaptation induced by sudden exposure to 10.0% (w/v) NaCl.

The initial response to osmotic stress is an important aspect of salt tolerance, as it establishes the essential machinery to withstand the hurdles induced by NaCl stress. Osmotic shock response was only examined in one strain of *L. monocytogenes*; it is therefore not an overview of *listerial* response to hyperosmotic shock *per se* but provides a detailed observation of gene expression variation induced by high levels of NaCl (10.0% (w/v) or 1.7M) on a representative virulent strain. It would be intriguing to evaluate gene expression of other strains when exposed to osmotic shock to obtain a more complete picture of the response in this species, especially comparing strains that show different tolerances to hyperosmotic shock. Microarray gene enrichment analysis revealed a very different, almost reverse gene expression in strain ATCC19115 in response to the adverse stress responses with the same NaCl concentration.

Following a sudden increase in extracellular osmolarity, *L. monocytogenes* ATCC19115 activated transcription of signalling genes (*yesN*, *yesM*, *agrD* and *usp*). Although overall transport-related genes showed suppression, there was enhanced transcription of *kpd* operon and PTS sugar transporter genes. Up-regulation of *gad* genes suggested activation of GAD-system potentially counteracting the acidification of bacterial cytoplasm. Prior to this study GAD-system has not been linked to hyperosmotic stress. Up-regulation of *dlt* operon was evident suggesting potential depolarization of the cell membrane reducing permeability of Na^+ ions. As the cells adjusted to the sudden increase of extracellular osmolarity the normal metabolic functions such as protein and fatty acid synthesis appeared to be temporarily suspended in parallel with activation of the SigB regulon.

Exposure of bacteria, including *L. monocytogenes*, to mild stress is known to provide cross protection against additional stress, including stresses other than that used to induce the initial response (Begley *et al.*, 2002; Flahaut *et al.*, 1996; Hill *et al.*, 2002; Pichereau *et al.*, 2000). The genetic responses to a low-temperature environment was investigated in Chapter 5 to aid in understanding cold tolerance and potentially provide better understanding of overall stress response in this organism.

Chapter 5

Characterisation of the transcriptomes of three cryo-adapted Listeria monocytogenes strains.

The ability of *Listeria monocytogenes* to grow at low temperatures is a critical aspect of its role as a food-borne pathogen. The understanding of cold adaptation mechanisms in *L. monocytogenes* however is not completely elucidated and a better comprehension may result in a novel strategy for controlling the growth of *L. monocytogenes* at refrigeration temperatures. Cryo-adaptation in three *L. monocytogenes* strains, possessing distinctly different growth rates at 4°C, lead to activation of gene sets associated with ribosomes, fatty acid and peptidoglycan biosynthesis and cell division and suppression of carbohydrate transport and metabolism related genes. Cold adapted strains suppressed SigB, PrfA and CodY regulons to different degrees. The data suggests SigB-mediated stress responses though not closely involved in cryo-adaptations in *L. monocytogenes* and strain variation in growth rates at 4°C could derive from differently coordinated genetic networks.

5.1 Introduction

The ability of *Listeria monocytogenes* to grow at cold temperatures is a critical aspect of its role as a food-borne pathogen, since such conditions inhibits growth of most other microorganisms thus acting as a selective agent for this cold-tolerant food pathogen. *L. monocytogenes* encounters numerous forms of cold stress challenge at various stages in food-processing and storage environments; as a means of survival adaptation of molecular responses is essential in order to overcome the faced challenges and proliferate in food products (Tasara & Stephan, 2006).

Cold stress has profound effects on several cellular events. Temperature reduction leads to a general slowdown in cellular metabolic reactions due to an overall reduced enzyme activities. There is decreased fluidity in the lipid bilayer at low temperature, which compromises membrane structural integrity and various other cell-membrane associated functions. An increased stability of RNA and DNA secondary structures and destabilization of ribosomes have previously been associated with cold stress exposure.

Exposure to low temperature is usually associated with an active response by bacteria, typically the synthesis of specific proteins, leading to a transient metabolic adaptation (Panoff *et al.*, 1998). In view of *L. monocytogenes* the response mechanisms described to date involve maintenance of cell-membrane lipid fluidity, intracellular uptake of compatible solutes and production of several cold stress proteins (Chan & Wiedmann, 2009; Tasara & Stephan, 2006).

The understanding of psychrotolerance mechanisms in *L. monocytogenes* however is not completely elucidated and a better comprehension may result in a novel strategy for controlling the growth of *L. monocytogenes* at refrigeration temperatures.

Both transcriptomic and proteomic approaches have been applied in investigating the mechanisms of cold stress response of *L. monocytogenes*, which have revealed a small number of cold induced genes (Bayles *et al.*, 1996; Liu *et al.*, 2002). More recently a genome wide microarray investigation reported 105 and 170 genes differentially expressed in logarithmic- and stationary-phase *L. monocytogenes* 10403S cells during growth at 4°C and 37°C (Chan *et al.*, 2007b).

This study was conducted to investigate a genome wide transcription response in three *L. monocytogenes* strains adapted to growth at 4°C when compared to growth at ambient 25°C.

5.2 Methods

5.2.1 Strains

Strains used in this study included ScottA and ATCC19115, both serotype 4b, and strain 70-1700, serotype 4e of ovine origin obtained from the TIAR collection.

5.2.2 Media and Growth Conditions

5.2.2.1 Control Cultures

All three *L. monocytogenes* strains were grown in duplicate in 100 ml BHI broth at 25°C to a transmittance of approximately 80%.as described in section 3.2.2.1.

5.2.2.2 Adaptive Cold growth Stress Cultures

Listeria monocytogenes strains were grown in duplicate in 10 ml BHIB at 25°C for 24 hours and 100 µl of a 10^{-4} dilution of this culture was used to inoculate a 50 ml BHI sterile sidearm flask and incubated at 25°C until turbidity reached 90%. Subsequently, 100 µl of a 10^{-1} dilution of this culture was used to inoculate a fresh 50 ml BHI and incubated at 15°C for 16 hours, after which the culture was moved to a shaking water bath at 4 °C (±1.0) for a time sufficient to provide late log phase cells, transmittance of roughly 80%., approximately 14 days depending on the strain. Approximately 25 ml of the culture was centrifuged at 1,500xg for 10 minutes at 4°C and the pellet resuspended in a smaller volume of the supernatant, which was immediately treated with RNAprotect Bacterial reagent (as described in section 3.2.3.1).

5.2.3 Microarray generation and data analysis

5.2.3.1 RNAprotect Bacterial Reagent Treatment

To stabilise cellular RNA for extraction two volumes of RNAprotect bacterial reagent (Qiagen) were added to one volume of culture and mixed and processed as described previously in section 3.2.3.1.

5.2.3.2 RNA extraction

Cells were thawed on ice and underwent a 6 h enzymatic treatment in 10 mM Tris-1 mM EDTA buffer (pH 8.1) containing 20 mg/ml lysozyme and 10 mg/ml proteinase K at 25°C. Cells were fully lysed by beat beating using 0.1 mm zirconium-silica sand in 4 ml of RNeasy Midi RNA Extraction kit (Qiagen) lysis buffer that was supplemented with 0.1% β-mercaptoethanol (Sigma-Aldrich). Subsequent RNA extractions were performed using of RNeasy Midi RNA Extraction kit (Qiagen) according to procedure described previously in section 3.2.3.2. Extracted RNA quality and quantity was assessed by running the RNA samples on a FA (1.2%) agarose gel as documented in section 3.2.3.4.

5.2.3.3 Microarray analysis

5.2.3.3.1 Microarray slides

Oligonucleotides were arrayed onto glass slides using quill pens at the AGRF (Walter & Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia) with each spot possessing a 12 µm

diameter. The array included 2857 x 70 bp oligonucleotides (Eurofin MWG Operon , Huntsville, Al, USA), representing all predicted protein coding genes and pseudogenes of the complete, published genome of *L. monocytogenes* EGD-e (GeneBank accession number. AL591824).

5.2.3.3.2 RNA processing

Approximately 5-20 µg of total RNA was converted to cDNA in a series of steps described in detail in section 3.2.4.2.1 through to 3.2.4.2.2. The neutralised cDNA was purified using QIAquick PCR Purification Kit, (Qiagen) as described in section 3.2.4.3.1, then labelled with a fluorescent Cy3/Cy5 dye according to the procedure previously described in detail in section 3.2.4.3.3 and finally hybridised onto the custom microarray slides following the procedure documented in section 3.2.4.4.1 through to 3.2.4.4.3.

5.2.3.4 Microarray data analysis

Downstream processing used the GenePix-Pro 3 software package to generate gpx filed from TIFF array images. Normalization of raw data and subsequent statistical analysis was performed with the WebArray Online platform (Xia *et al.*, 2005). Within-array normalization used the global LOESS procedure. Between each array quantile normalization was used to insure intensities had the same empirical distribution across arrays and across channels. The significance of differential expression was analysed using linear modal statistical analysis (Smyth, 2004). Oligonucleotides that showed negligible or no hybridization when compared with background hybridization were excluded from analyses. Analyses otherwise followed that described in section 3.4.2.5.

5.2.3.5 Gene set enrichment analysis

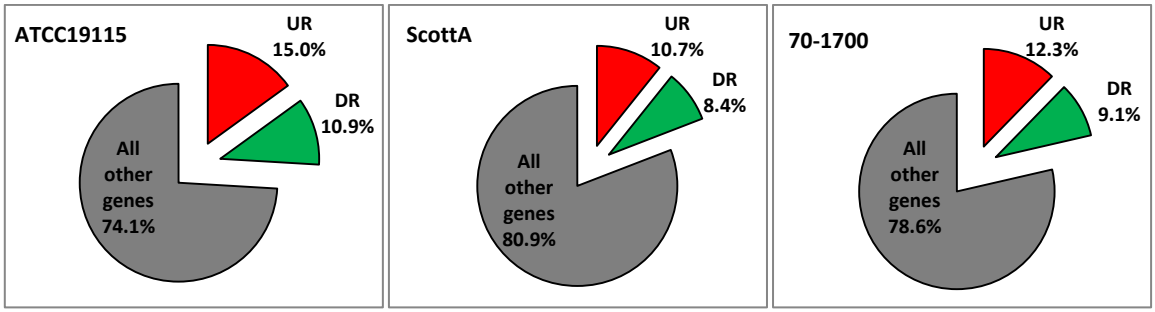
Gene designations, predicted functions and categorization of coded proteins into defined sets from the *L. monocytogenes* EGD-e genome was based on information obtained from published literature, Kyoto Encyclopedia of Genes and Genomes (http://www.genome.ad.jp/kegg/) and ListiList (http://genolist.pasteur.fr/ListiList/). A *t*-test based procedure was utilised to score the changes in expression of predefined sets of genes (Boorsma *et al.*, 2005; Bowman *et al.*, 2008). The significance of the *T-value* score was established by using the associated two-tailed *p*-value.

5.3 Results and Discussion

5.3.1 Gene expression trends characterising the cold-adaptive response of *L. monocytogenes*.

5.3.1.1 Overview of general trend in gene expression observed in cold-adapted cells of three L. monocytogenes strains.

Low temperature has profound effects on all aspects of microbial cell structure and function, involving the structural integrity of macromolecules, macromolecular assemblies, protein synthesis and nutrient uptake (Panoff *et al.*, 1998; Weber & Marahiel, 2002; Zhu *et al.*, 2005). As such it had a dramatic effect on gene expression profiles of the *L. monocytogenes* strains studied following adaption. The gene expression profile of ATCC 19115, a strain that is relatively average in its cold tolerance (in terms of growth rate as indicated in Chapter 2), revealed a ≥ two-fold up-regulation of 510 genes; approximately 18% of the total number of homologous genes from the *L. monocytogenes* EGD-e genome (Fig. 5.1). By comparison 461 (16%) genes were down regulated. At the same temperature strain ScottA, which grows slower at 4°C, was found to increase the expression of 411 (14.0%) genes and suppress ≥ two-fold the expression of 343 (12.0%) genes (Fig. 5.1). Meanwhile ≥ two-fold increased expression of 424 (15.0%) genes and reduced expression of 378 (13.0%) genes was observed for strain 70-1700 (Fig. 5.1).



UR: up-regulated genes (LR>1, P<0.05); DR: down-regulated genes (LR<-1, P<0.05). Percentages calculated based on total number of genes in EGD-e genome (5827).

Figure 5.1 Schematic representation of overall gene expression trend for *L. monocytogenes* strains ATCC19115, ScottA and 70-1700 adaptive response to cold temperature of 4°C.

Adaptation to a cold stress of 4°C induced significant expression variation in several gene functional categories and sub-categories, as established using the *T-value* scoring procedure (Boorsma *et al.*, 2005; Bowman *et al.*, 2008). The *T-value* scoring results are summarized in Table 5.1.

Table 5.1 Gene Response of *L.monocytogenes* strains ATCC19115, ScottA and 70-1700 to 4°C, organized into gene functional categories.

Gene categories and subcategories (no. of genes)	ATCC19115		ScottA		70-1700	
	T-value*	P-value#	T-value*	P-value#	T-value*	P-value#
Amino Acid Metabolism						
Branched chain amino acids biosynthesis (16)	**-1.98**	0.0676	0.03		-0.14	
Other amino acid-related metabolism (14)	**-1.86**	0.0873	-0.92		**-1.62**	
Histidine metabolism (14)	**-1.88**	0.0839	-0.30		**-1.99**	0.0696
Alanine/aspartate metabolism (28)	**-2.03**	0.0532	**-2.67**	0.0129	0.19	
Branched amino acids metabolism (7)	**-1.22**		**-2.23**	0.0758	**-1.00**	
Urea cycle and metabolism of amino groups (12)	-0.28		0.70		**1.05**	
Aromatic amino acids biosynthesis (23)	**1.21**		0.97		0.64	
Peptidases (46)	**2.09**	0.0425	0.40		**2.58**	0.0132
Lysine biosynthesis (16)	**1.75**		0.46		0.62	
D-Alanine metabolism (7)	0.99		**1.70**		0.55	
Glutathione metabolism (7)	0.49		**-1.89**		0.95	
Glutamate metabolism (28)	-1.14		**-1.93**	0.0645	0.19	
Carbohydrate Metabolism						
Nucleotide sugars metabolism (11)	-0.99		**-2.68**	0.0253	**-2.00**	0.0766
Glyoxylate/dicarboxylate metabolism (7)	**-1.17**		-0.78		-0.86	
Starch/sucrose metabolism (31)	**-2.47**	0.0196	0.88		-0.51	
Fructose/Mannose metabolism (68)	**-6.83**	0.0000	**-2.40**	0.0192	-0.72	
Other carbohydrate-related metabolism (49)	**-2.38**	0.0214	**-1.61**		**-1.83**	0.0730
Galactose metabolism (16)	**-4.14**	0.0010	**-1.86**	0.0837	**-1.40**	
Pentose phosphate pathway (34)	**-1.63**		-0.76		0.33	
Inositol/inositol phosphate metabolism (9)	-0.47		**-1.40**		**-1.80**	
Pyruvate metabolism (32)	**-1.02**		**-2.27**	0.0308	0.02	
TCA cycle (8)	-0.50		**-1.16**		0.07	
Aminosugars metabolism (15)	**1.76**		0.35		0.84	
Glycolysis/Gluconeogenesis (37)	-0.82		**-2.18**	0.0358	**-2.20**	0.0343
Butanoate metabolism (19)	**-3.65**	0.0020	**-4.04**	0.0008	**-2.74**	0.0139
Cell division						
Other cell cycle/division-related proteins (16)	0.22		**1.70**		0.87	
Cell division (30)	**5.07**	2.3E-05	**3.20**	0.0034	**3.55**	0.0014
DNA replication complex (20)	**1.51**		**1.53**		0.85	
Septal ring (9)	**2.68**	0.0314	**1.13**		**2.25**	0.0595
Cell Motility						
Flagellar assembly (27)	**-6.51**	8.0E-07	**-6.70**	5.1E-07	**-1.45**	
Type III secretion system (11)	**-1.83**		**-2.81**	0.0203	**-1.51**	
Type II secretion system (14)	**1.03**		-0.14		**1.21**	
Cell wall associated proteins						
Gram-positive anchored protein (39)	**-1.97**	0.0565	**-2.59**	0.0138	**-2.11**	0.0418
LPXTG motif (22)	**-1.66**		**-1.52**		**-2.57**	0.0183
DNA repair/recombination						
DNA repair/recombination (72)	**2.11**	0.0384	**2.23**	0.0290	**1.77**	0.0807
Energy Metabolism						
Oxidative phosphorylation (25)	-0.20		-0.59		**1.10**	
Other energy metabolism (12)	-0.16		-0.36		**-1.23**	

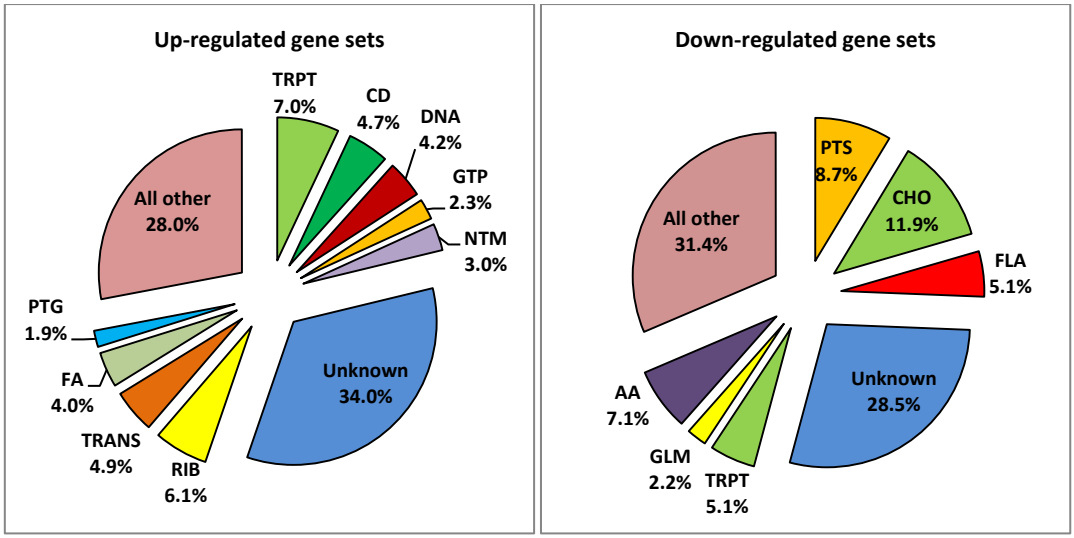
Gene categories and subcategories (no. of genes)	ATCC19115		ScottA		70-1700	
	T-value*	P-value#	T-value*	P-value#	T-value*	P-value#
Nitrogen metabolism (11)	**-1.49**		-0.45		-0.45	
Lipid Metabolism						
Fatty acid biosynthesis (14)	**2.99**	0.0113	**3.06**	0.0099	**2.29**	0.0412
Peptidoglycan biosynthesis (16)	**4.18**	0.0009	**3.51**	0.0035	**1.96**	0.0702
Glycerophospholipid metabolism (12)	0.32		-0.09		-0.48	
Glycerolipid metabolism (15)	**-2.09**	0.0565	**-4.51**	0.0006	**-5.20**	0.0002
Biosynthesis of steroids/terpenoids (16)	**1.94**	0.0725	**1.10**		0.91	
Pdu/Eut operon (36)	0.27		**-2.75**	0.0095	-0.37	
Membrane Transport						
Transporters All General (287)	0.46		**2.43**	0.0155	**1.31**	
ABC Transporter General (171)	0.83		**2.47**	0.0147	**2.42**	0.0165
Transporter (iron) all (19)	0.15		**3.58**	0.0023	**1.67**	
Transporter (amino acids) (18)	0.74		0.81		**1.13**	
ABC Transporter (compatible solutes) (8)	**1.67**		-0.83		**1.87**	
ABC Transporter (peptides) (10)	0.84		**1.30**		**3.20**	0.0126
ABC Transporter (cobalt/nickel) (8)	**-1.49**		**-2.14**	0.0760	**-1.30**	
Transporter all (sugars) (28)	**-1.51**		0.95		-0.65	
PTS system (90)	**-10.88**	0.0000	**-3.71**	0.0004	**-3.79**	0.0003
Ion channel/pore (10)	**-1.63**		**-2.08**	0.0709	**-1.07**	
ABC Transporter (sugars) (25)	**-1.21**		0.98		0.11	
Protein export (19)	**2.30**	0.0345	**2.40**	0.0280	**1.90**	0.0747
Metabolism of Cofactors and Vitamins						
Porphyrin/cobalamin metabolism (34)	**-1.29**		-1.96	0.0588	**-1.74**	0.0919
One carbon pool by folate (11)	-0.36		0.35		0.60	
Pantothenate/CoA biosynthesis (15)	**1.53**		**2.59**	0.0226	**1.11**	
Folate biosynthesis (16)	-0.65		**-1.56**		**-2.09**	0.0558
Thiamine metabolism (9)	-0.97		0.10		**-2.44**	0.0448
Nicotinate/nicotinamide metabolism (13)	**1.65**		0.69		**1.13**	
Isoprenoid quinone biosynthesis (9)	0.82		0.12		**1.78**	
Nucleotide Metabolism						
Purine biosynthesis (15)	0.07		0.78		**1.53**	
Pyrimidine biosynthesis (8)	**-1.60**		**-1.62**		-0.53	
Purine and Pyrimidine metabolism (82)	**2.69**	0.0087	**2.69**	0.0086	**4.08**	0.0001
Pyrimidine metabolism (36)	**2.89**	0.0067	**2.66**	0.0119	**3.38**	0.0018
Purine metabolism (44)	**3.58**	0.0009	**3.40**	0.0015	**3.77**	0.0005
Transcription and Translation						
Ribosome (58)	**6.88**	5.5E-09	**7.95**	9.4E-11	**17.11**	6.6E-24
Folding catalysts (13)	0.16		0.24		0.07	
RNA polymerase (10)	0.96		-0.21		**1.26**	
Heat shock protein (18)	-0.13		**-2.63**	0.0181	**1.17**	
Translation/ribosome associated (56)	**3.45**	0.0011	**3.43**	0.0012	**3.51**	0.0009
Protein folding/processing (18)	0.89		**1.82**	0.0881	0.62	
DNA polymerase (10)	0.16		**1.29**		**1.57**	
GTP-binding protein (22)	**2.84**	0.0100	**3.51**	0.0022	**4.34**	0.0003
Translation factors (12)	**1.91**	0.0858	**1.17**		**3.60**	0.0048
Transcription associated (9)	**2.60**	0.0356	**2.16**	0.0672	**4.23**	0.0039
Aminoacyl-tRNA biosynthesis (24)	**1.03**		**1.13**		**1.47**	

Gene categories and subcategories (no. of genes)	ATCC19115		ScottA		70-1700	
	T-value*	P-value#	T-value*	P-value#	T-value*	P-value#
Transcription regulation						
Transcription regulation (231)	-0.21		**1.20**		**-1.59**	
Transcription regulation (TetR/AcrR family) (11)	-0.63		0.87		-0.92	
Transcription regulation (GntR family) (17)	**1.42**		**1.70**		0.47	
Transcription regulation (BglG family) (15)	**-3.77**	0.0024	-0.98		**-1.58**	
Transcription regulation (AraC family) (6)	**-1.89**		-0.39		-0.50	
Transcription regulation (LysR family) (9)	0.35		**1.44**		-0.46	
Signaling						
Chemotaxis (13)	**-4.54**	0.0008	**-4.09**	0.0018	-0.74	
Two-component signal transduction system (39)	**1.37**		-0.10		**1.14**	
Virulence						
Phage (78)	**-4.32**	4.6E-05	**-3.94**	0.0002	**-3.49**	0.0008
Transposases (8)	**-1.93**		**-1.82**		**-1.79**	
TN916 (13)	**-2.06**	0.0640	**-3.11**	0.0099	**-2.63**	0.0234
Virulence-related (14)	**-1.08**		**-2.22**	0.0466	**-1.99**	0.0698

*T-value scores were determined from the expression data using the approach of Boorsma *et al*. (2005).
P-values not shown are >0.1

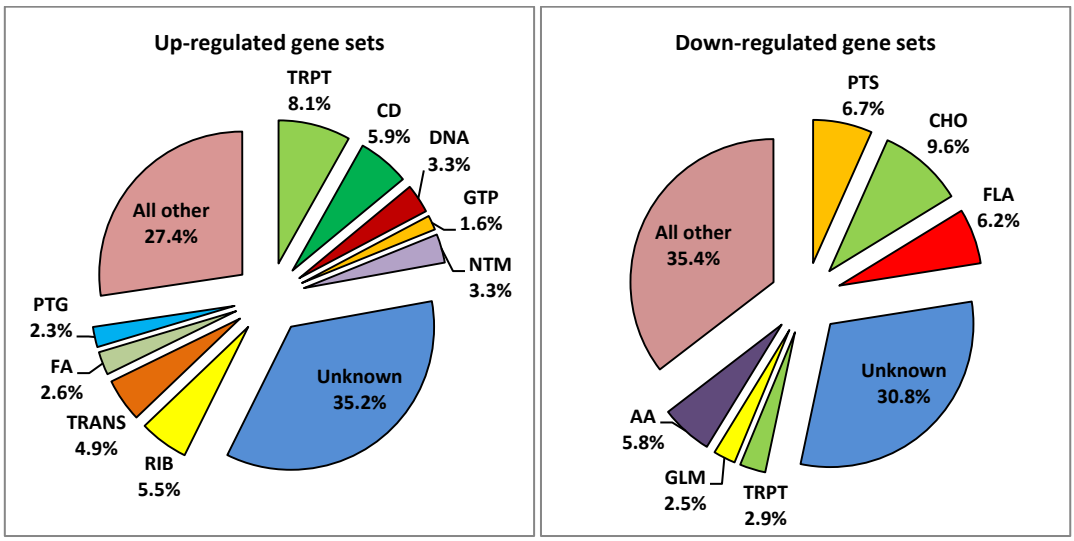
The most significant up-regulated gene sets observed in all four strains adapted to cold temperature included ribosomes with *T-value* scores ranging from 6.9 for strain ATCC19115 to 17.1 for strain 70-1700 (Table 5.1). Other highly activated gene sets included those associated with cell division, peptidoglycan and fatty acid biosynthesis, DNA repair and recombination, GTP-binding proteins, transcription and translation (Table 5.1).

Analysis of individual gene expression revealed that the majority of all significantly up-regulated genes had an unknown function 34.0% of all up-regulated genes in strain ATCC19115 (Fig. 5.2), 35.2% for ScottA (Fig. 5.3) and 22.8% for 70-1700 (Fig. 5.4). Collectively genes associated with ribosomes represented 6.1%, 5.5% and 14.2% of all the up-regulated genes observed in strains ATCC19115, ScottA and 70-1700 respectively. Other subsets of up-regulated genes included those associated with various transporters, lipid metabolism and cell division (Fig. 5.2, Fig. 5.3 and Fig. 5.4).



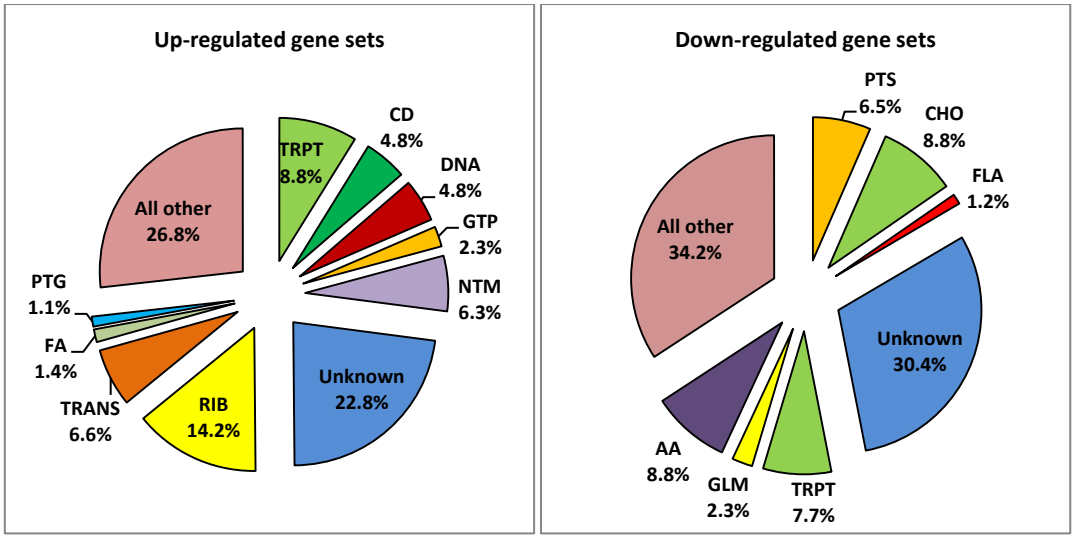
DNA-group of genes associated with DNA repair/recombination and transcription; GTP-genes encoding GTP-binding proteins; CD- genes associated with cell division; NTM- genes associated with metabolism of nucleotides; FA- genes associated with lipid metabolism; TRANS- genes associated with translation, TRPT- genes encoding various transporters; RIB-genes encoding ribosomal proteins; AA- genes associated with amino acid metabolism; FLA- genes associated with flagellar assembly, CHO- genes associated with carbohydrate metabolism; PTG- genes associated with peptidoglycan biosynthesis, PTS- genes associated with phosphotransferase system; GLM- genes associated with glycerolipid metabolism.

Figure 5.2 Schematic representation of overall gene expression profile results for strain ATCC19115 adapted to cold temperature of 4°C.



DNA-group of genes associated with DNA repair/recombination and transcription; GTP-genes encoding GTP-binding proteins; CD- genes associated with cell division; NTM- genes associated with metabolism of nucleotides; FA- genes associated with lipid metabolism; TRANS- genes associated with translation, TRPT- genes encoding various transporters; RIB-genes encoding ribosomal proteins; AA- genes associated with amino acid metabolism; FLA- genes associated with flagellar assembly, CHO- genes associated with carbohydrate metabolism; PTG- genes associated with peptidoglycan biosynthesis, PTS- genes associated with phosphotransferase system; GLM- genes associated with glycerolipid metabolism.

Figure 5.3 Schematic representation of overall gene expression profile results for strain ScottA adapted to cold temperature of 4°C.



DNA-group of genes associated with DNA repair/recombination and transcription; GTP-genes encoding GTP-binding proteins; CD- genes associated with cell division; NTM- genes associated with metabolism of nucleotides; FA- genes associated with lipid metabolism; TRANS-genes associated with translation, TRPT- genes encoding various transporters; RIB-genes encoding ribosomal proteins; AA- genes associated with amino acid metabolism; FLA- genes associated with flagellar assembly, CHO- genes associated with carbohydrate metabolism; PTG- genes associated with peptidoglycan biosynthesis, PTS- genes associated with phosphotransferase system; GLM- genes associated with glycerolipid metabolism.

Figure 5.4 Schematic representation of overall gene expression profile results for strain 70-1700 adapted to cold temperature of 4°C.

Down-regulation of a large number of gene sets was observed in all three strains following a prolonged exposure to cold temperature of 4±1°C. The most significant down-regulated gene sets observed in all four strains adapted to cold temperature included phosphotransferase system (PTS) with *T-value* scores ranging from -10.9 for strain ATCC19115 to -3.7 for strain ScottA (Table 5.1) these represented 6.5% to 8.7% of all the significantly down-regulated genes (Fig.5.2, Fig.5.3 and Fig.5.4). Gene sets associated with flagella, glycerophospholipid metabolism, chemotaxis and metabolism of various carbohydrates also showed significant suppression in cells exposed to cold temperature (Table 5.1).

Carbohydrate metabolism gene sets represented a large proportion of all down-regulated genes, collectively representing 8.8% to 11.9% of all suppressed genes. The majority of the down-regulated genes in the three *L. monocytogenes* strains examined had an unknown function, and represented 28.5% to 30.8% of all suppressed genes (Fig.5.2, Fig.5.3 and Fig.5.4). A number of genes encoding cellular transporters showed up-regulation, with a slightly smaller proportion of genes in this subgroup showing significant down-regulation most likely reflecting the overall decrease in cellular metabolism at low temperature.

5.3.1.2 Cold-adaptation has a profound effect on cell-envelope.

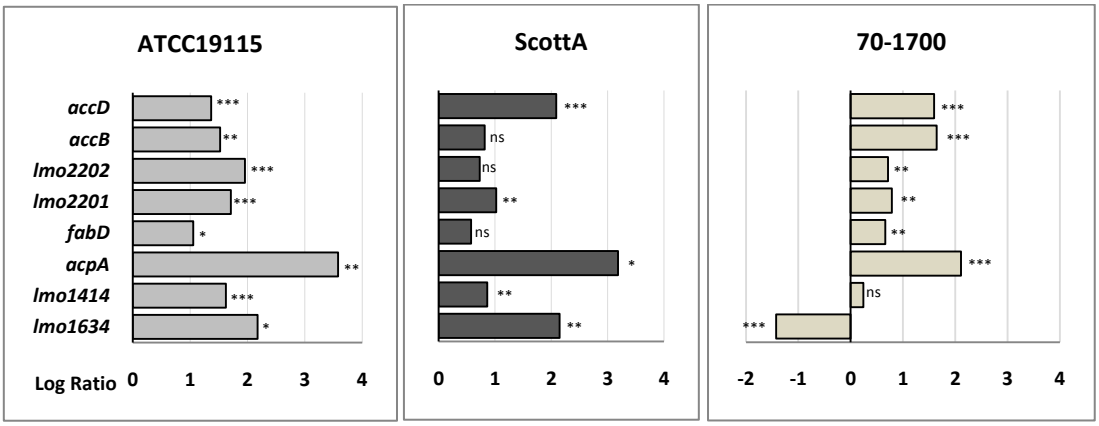
5.3.1.2.1 Gene expression changes related to cold induced cell membrane fatty acid and phospholipid modulation.

The correct physical state of the membrane lipids is critical for optimal structural and functional integrity of cell membranes. Low temperature reduces membrane fluidity of cells causing membrane phase transitions from a liquid-crystalline state to a more rigid gel-like state, interfering with the normal function of the membrane and membrane-associated proteins. To counteract this phenomenon, *L. monocytogenes* has been shown to undergo homeoviscous adaptation by increasing the amount of fatty acids with low melting points being incorporated into membrane lipids. This is thought to be achieved by active odd-numbered fatty acids and short branched-chain fatty acids (BCFAs) synthesis and a switch from iso to anteiso branching patters (Najjar *et al.*, 2007; Zhu *et al.*, 2005). Among the common BCFAs in *L. monocytogenes* anteiso-$C_{15:0}$ has the lowest melting point; it has been demonstrated that as the growth temperature declines, anteiso-$C_{15:0}$ content in the membrane increases to maintain optimal membrane fluidity (Zhu *et al.*, 2005).

Low-temperature adaptation has been known to stimulate fatty acid production to counteract membrane rigidity. Maintenance of cell fluidity has been shown to occur in cold-stressed cells on continuous basis (Najjar *et al.*, 2007). Indeed gene sets associated with fatty acid biosynthesis showed up-regulation of ≥two-fold in all three strains of *L. monocytogenes* with *T-value* scores ranging from 2.3 for strain 70-1700 to 3.1 for ScottA (Table 5.1). The acyl carrier protein encoding gene *acpA* (*lmo1806*) was strongly up-regulated 12-fold in ATCC19115, 9-fold ScottA and 4- fold in strain 70-1700 (Fig. 5.5). Acyl carrier protein (ACP) is a universal and highly conserved carrier of acyl intermediates during fatty acid synthesis (Byers & Gong, 2007). It is thought that ACP acts as a signalling molecule in *E. coli* relating the status of cellular fatty acid metabolism (Battesti & Bouveret, 2006). Other up-regulated genes in all four strains included *accD* (*lmo1573*) and *lmo2201* homologous to *fabF* in *E. coli*. Thermal modulation of FA production in *E. coli* is controlled at the level of β–ketoacyl-ACP synthetase II, product of the *fabF* gene, increased activity of which has been shown to correlate with temperature downshift (Allen & Bartlett, 2000). A gene homologous to *fabH* in *E. coli* and *B. subtilis*, *lmo2202*, in comparison showed up-regulation only in ATCC19115 and 70-1700 (Fig. 5.5). the product of this gene β–ketoacyl-ACP synthesase III catalyses the first step in FA elongation, its regulation therefore prevents initiation of new acyl chains and limits the total number of FA that are produced (Choi *et al.*, 2000; Zhang & Rock, 2008). Strain specific expression variation

observed may reflect differences in the levels of FA metabolism undertaken by different strains during prolonged exposure to cold stress.

The mechanisms of fatty acid metabolism in *L. monocytogenes* is not well understood and only two genes have been classified in this subgroup, *lmo1414* encoding acetyl-CoA acetyltransferase, and *lmo1634* encoding acetaldehyde dehydrogenase. Both of these showed up-regulation in ATCC19115 and ScottA but not in 70-1700 (Fig. 5.5). Involvement of FA metabolism in *L. monocytogenes* cold-adaptive response is not clear, but it may aid in intracellular fatty acid turnover particularly in cold stressed cells.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

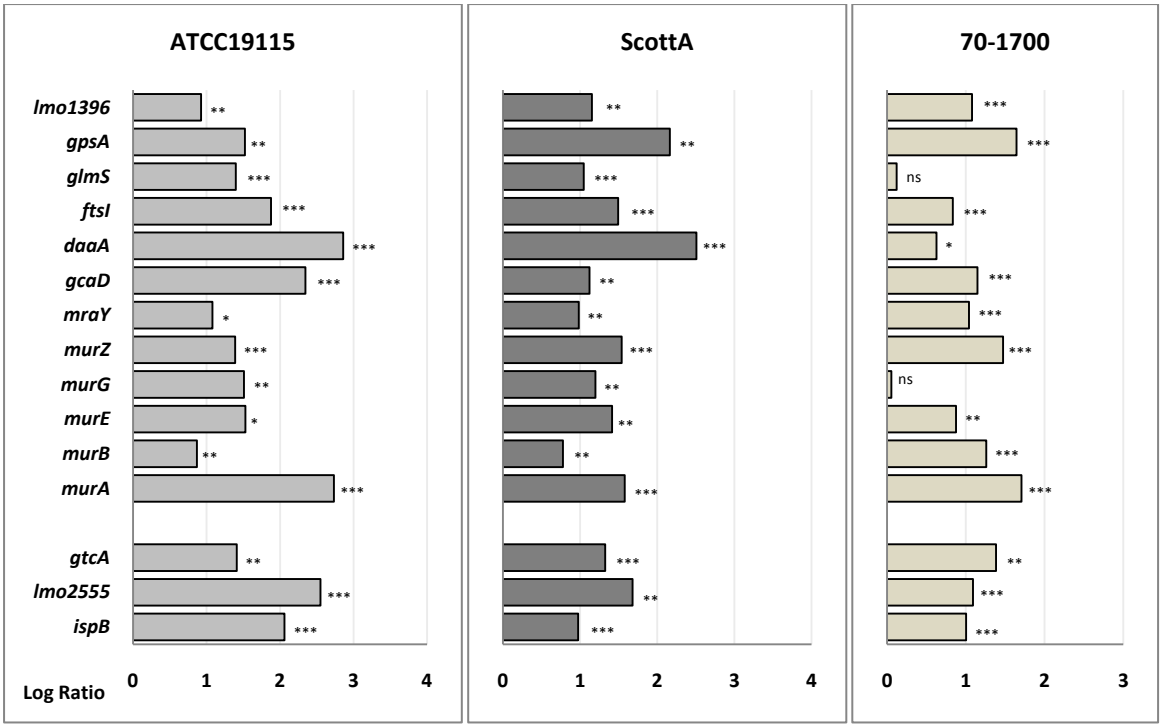
Figure 5.5 Expression response of genes associated with fatty acid biosynthesis in three *L. monocytogenes* strains adapted to cold stress.

Two genes associated with glycerophospholipid metabolism *gpsA* (*lmo1936*) and *lmo1396* homologous to *pgsA* gene in *E. coli* showed up-regulation in all three cold adapted strains of *L. monocytogenes* (Fig. 5.6). Mutations in either of the two genes in *E. coli* lead to inhibition of phospholipid synthesis (Cronan, 2003; Rodionov & Ishiguro, 1996) suggesting a strong involvement of these in cell maintenance rather than de novo synthesis. Cold stress has previously been shown to stimulated transcription of *gpsA* in *L. monocytogenes* strain 10403S in both log- and stationary growth phase cells (Chan *et al.*, 2007b). Consequently up-regulation of these genes in *L. monocytogenes* cold adapted strains may correspond to the increased synthesis of phospholipids. Interestingly the overall gene set associated with glycerolipid metabolism showed significant down-regulation (Table 5.1) signifying that this process is somewhat reduced in cold adapted cells.

5.3.1.2.2 Peptidoglycan biosynthesis and teichoic acid modification associated genes are up-regulated in cryo-adpated L. monocytogenes.

A number of genes associated with aminosugars metabolism also showed up-regulation of ≥two-fold in all three strains of *L. monocytogenes* (Fig. 5.6) these included *murA* (*lmo2526*), *murZ* (*lmo2552*), *gcaD* (*lmo0198*) and lmo1420, a gene homologous to *murB* in both E*. coli* and *B. subtilis*,. Enzymes encoded by these genes are responsible for synthesis of UDP-N-acetylmuramate, the essential precursor for peptidoglycan biosynthesis (Navarre & Schneewind, 1999). Activation of these may potentially be directly linked to stress tolerance as a *murB* mutant of *S. aureus* was sensitive to high temperature stress (Matsuo *et al.*, 2003). Activation of genes associated with aminosugars metabolism is most likely driven by the overall increase in peptidoglycan biosynthesis or turnover requirement observed in cold adapted strains. Evident up-regulation of gene sets related to peptidoglycan biosynthesis was also observed with *T-value* scores of 4.18, 3.51 and 1.96 for strains ATCC19115, ScottA and 70-1700 respectively (Table 5.1). Individual gene expression revealed significant activation of a number of genes in this subset (Fig. 5.6), these included *ftsI* (*lmo1438*), *daaA* (*lmo1619*), *murE* (*lmo2038*), *murA* (*lmo2526*), *gcaD* (*lmo0198*) and *mraY* (*lmo2037*). Strains ATCC1911 and ScottA also showed up-regulation of ≥ twofold of *murG* (*lmo2035*) and *glmS* (*lmo0727*) encoding glucosamine-fructose-6-phosphate aminotransferase, expression of which was not apparent in strain 70-1700 (Fig. 5.6).

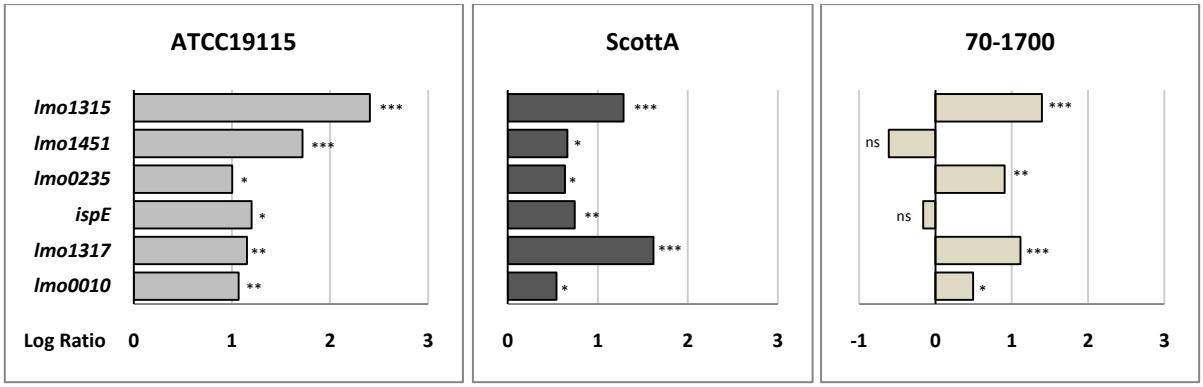
The genes *gtcA (lmo2459)* and *lmo2555* both coding teichoic acid glycosyltransferases as well as an *ispB*-homologous gene (*lmo0927*) coding a putative phosphoglycerol transferase required for commencement of lipoteichoic acid biosynthesis were also activated in cold-adapted cells (Fig. 5.6). These results suggest possible changes of cell membrane teichoic acid components. Interestingly teichoic acid biosynthesis related gene subsets collectively showed no significant change in transcription levels in cold-adapted cells (Table 5.1), suggesting that perhaps the major modifications to the peptidoglycan structure do not involve incorporation of additional teichoic acid. A problem however is that teichoic acid biosynthetic genes in lineage I strains are not homologous with those on the EGD-e genome (located in a cluster from *lmo1077-lmo1091*) thus it is possible teichoic acid biosynthesis is enhanced but further studies will be needed to ascertain this.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.6 Expression response of genes associated with peptidoglycan and teichoic acid biosynthesis in three *L. monocytogenes* strains adapted to cold stress.

Potentially driven by a possible increase in peptidoglycan biosynthesis a number of genes associated with the mevalonate/non-mevalonate biosynthesis pathwaywere significantly up-regulated. Among these the most up-regulated genes in all three strains included *lmo1317* and *lmo1315* (Fig. 5.7). The former is homologous to *dxr* of *B. subtilis* and *E. coli*, and has been shown to increase in transcription levels following cold adaptation in *Vibrio parahaemolyticus* (Lei *et al.*, 2009) although its true function in adaptation response to cold-temperature growth is unclear. On the other hand *lmo1315* a gene homologous to *uppS*, encodes a cell-wall component carrier lipid protein required for the assembly of peptidoglycan, teichoic acid and other cell membrane components in bacteria (Bouhss *et al.*, 2008) and thus its up-regulation is in keeping with aforementioned results.



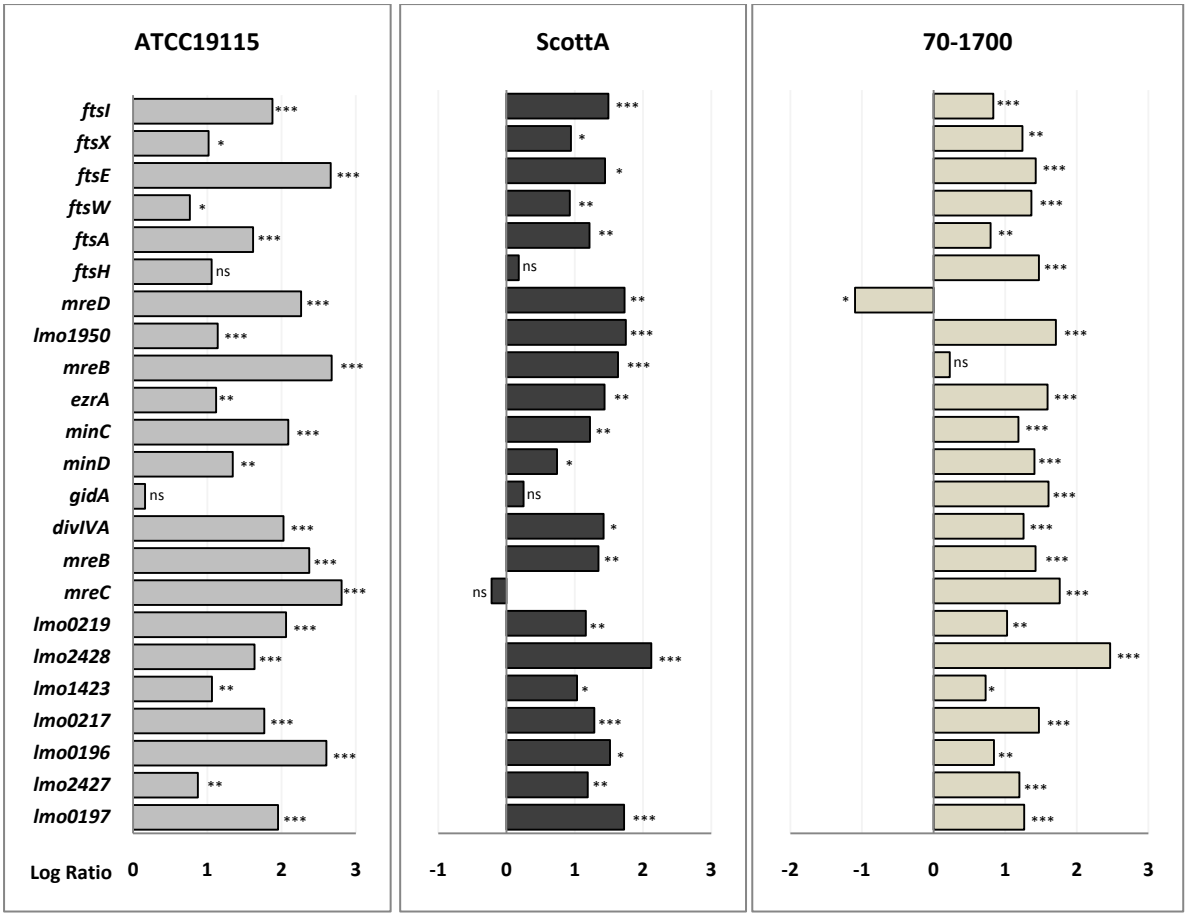
*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.7 Expression response of genes associated with steroids and terpenoids biosynthesis in three *L. monocytogenes* strains adapted to cold stress.

Overall maintaining membrane and cell wall integrity is evidently a crucial component of cold survival in *L. monocytogenes*, a process which appears to be ongoing in cold-adapted cells. Activation of gene sets responsible for either directly or indirectly influencing membrane lipid composition provides the means of overcoming the decreased fluidity in the lipid bilayer at low temperature and preserving membrane structural integrity. In addition modifications to the cell envelope involving presumably increased peptidoglycan and potentially teichoic acid may also be important in maintaining the efficiency of critical cell wall associated functions such as cell division.

5.3.1.3 Cold-adaptation activated cell division gene sets.

Cold temperature adaptation resulted in activation of *L. monocytogenes* genes associated with cell division (*T-value* scores ranging from 3.2 for ScottA to 5.1 for ATCC19115 (Table 5.1)) and septal ring related gene sets (*T-value* scores 1.1, 2.3 and 2.7 for strains ScottA, 70-1700 and ATCC19115 respectively (Table 5.1)). Several genes in each of the categories showed ≥two-fold up-regulation in all three strains; these included *minC* (*lmo1545*), *minD* (*lmo1544*), *ezrA* (*lmo1594*) as well as *lmo1950* a gene homologous to *scpB* in *B. subtilis* and septal ring associated genes *ftsE* (*lmo2506*), *ftsX* (*lmo2507*), *ftsI* (*lmo1438*), and *ftsA* (*lmo2033*) (Fig. 5.8). Proteins encoded by these genes are essential for maintaining functional cell division. Cell division in bacteria is a highly co-ordinated and fine-tuned process and any imbalance leads to abnormal cell division, as indicated in a number of mutagenic studies in various genera.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.8 Expression response of genes associated with cell division in three *L. monocytogenes* strains adapted to cold stress.

It appears that over expression of cell division related genes in *L. monocytogenes* cold-adapted cells is fairly homogenous with majority of genes in the subgroup showing up-regulation. A number of genes also showed strain specific expression, these included *ftsH* which showed no significant change in expression in ScottA (Fig. 5.8); *mreD* showed up-regulation of 5- and 3-fold in strains ATCC19115 and ScottA respectively and a two-fold down-regulation in strain 70-1700 (Fig. 5.8); *mreC* showed 7- and 3-fold up-regulation in ATCC19115 and 70-1700 respectively but showed no significant change in transcription in strain ScottA (Fig. 5.8).

A number of genes encoding as yet uncharacterised cell division-related proteins also showed up-regulation in cold adapted cells. Some of these included *lmo2428* which exhibited 3-, 4- and 6- fold up-regulation in strains ATCC19115, ScottA and 70-1700 respectively (Fig. 5.8); this gene product shares a similarity with a protein RodA which is essential for controlling cell shape and elongation in

both *B. subtilis* and E. *coli* (Herinques *et al.*, 1998; Uehara & Park, 2008). RodA mutants of *E. coli* demonstrated spherical cell morphology when exposed to high temperature stress of 41°C reflecting inactivation of the elongation pathway (Uehara & Park, 2008). Both *lmo0197* and *lmo0196* encode near duplicate proteins homologous to the regulatory protein SpoVG of *B. subtilis,* a protein involved in regulating septation. Cell division initiation protein encoding gene *lmo0217* similar to *divIC* in *B. subtilis* also showed significant up-regulation in all three strains of *L. monocytogenes* (Fig. 5.8). Increase in transcription of DivIC has previously been demonstrated in a cold-adaptation response of *B. subtilis* (Budde *et al.*, 2006).

Overall amplified expression of genes associated with cell division may indicate a potential sensitivity of cell division machinery proteins to low-temperature, i.e. distabilisation. Cold-sensitivity of FtsZ polymerization similar to that of a human homolog tubulin, has been established in *E. coli* (Justice *et al.*, 2000). Although *ftsZ* (*lmo2032*) itself showed no change in transcription in either of the cold-adapted strains (data not shown) activation of the other proteins may be needed to control the overall division process. Assembly of the Z-ring in *B. subtilis* has been shown to be inhibited by MinC and MinD over-expression (Gregory *et al.*, 2008), which also function to control timing of cell division in this organism. It is well accepted that cell replication in bacteria is slow at low-temperature, most likely due to general decrease in cellular metabolic rates and DNA replication; this is also the case for *L. monocytogenes* cold-adapted cells which showed a lack of *ftsZ* activation and over-expression of genes encoding cell division inhibition mainly *minCD* and *divIVA*, suggesting a basis for overall reduced rate of cell replication.

5.3.1.4 Low-temperature adaptation induced changes to transcription-associated genes including general stress proteins.

5.3.1.4.1 Cold-temperature induced activation of ribosome proteins encoding genes.

Ribosomes are considered one of the key cellular structures involved in microbial cold stress adaptation due to their central role in protein synthesis. It is presumed that one of the effects of cold stress exposure is to severely compromise ribosome structural stability and function, thus compromising microbial protein synthesis (Tasara & Stephan, 2006). Cold-adaptation strongly activated gene sets associated with ribosomes (*T-value* scores ranging from 6.9 to 17.1) and other translation associated genes (*T-value* scores ranging from 3.4 to 3.5) in all three strains of *L. monocytogenes* (Table 5.1). From a large number of significantly activated genes, gene *rpmB*

(*lmo1816*) encoding a ribosomal protein L28 expressed the strongest up-regulation of 20-, 12- and 17-fold in strains ATCC19115, ScottA and 70-1700 respectively (Table 5.2). Expression of this protein has not previously been linked to stress adaptation in *L. monocytogenes*, however up-regulation of *rpmB* transcription was observed in an emerging human pathogen *Tropheryma whipplei* following temperature down-shift (Crapoulet *et al.*, 2006). Other strongly up-regulated ribosomal proteins in all three strains included *rplK* (*lmo0248*), *rpmE* (*lmo2548*) and *rpmF* (*lmo0486*). It has been suggested that cold-induced proteins associated with translation , in particular ribosomal proteins, are involved in the adaptation of ribosome function to low-temperature conditions (Graumann & Marahiel, 1999). A number of ribosome encoding genes showed strain specific expression, the most profound of which was *rplR* (*lmo2616*) showing 5-fold down-shift in transcription for ATCC19115 and 5-fold up-regulation for strain 70-1700, while no change in transcription was evident for ScottA (Table 5.2). Overall strain 70-1700 displayed more active ribosomal involvement in the cold adaptive response in comparison to the other two strains, with more genes showing significant up-regulation of ≥twofold. As ribosome stability is thought to be significantly compromised at low temperature, the observed increase in gene expression of ribosome associated proteins may provide the means to overcome the loss ribosomal efficiency thus preventing stalling of protein synthesis at 4°C.

Table 5.2 Expression response of genes associated with ribosomes and other translation related machinery in three *L. monocytogenes* strains adapted to cold stress.

Gene	ATCC19115		ScottA		70-1700		Function
	LR*	P#	LR	P	LR	P	
rplK	**2.25**	4.6E-04	**2.54**	0.006	**3.16**	1.3E-06	ribosomal protein L11
rplA	**1.71**	0.002	**2.01**	0.007	**2.97**	4.1E-06	ribosomal protein L1
rplJ	**1.44**		**1.52**	0.010	**3.03**	1.9E-07	ribosomal protein L10
rplL	0.77		**1.36**	0.002	**2.43**	0.000	ribosomal protein L7/L12
rpmA	**2.20**	0.012	**1.45**		**1.93**	5.0E-06	ribosomal protein L27
rplU	**2.90**	0.005	**1.60**		**2.91**	4.4E-05	ribosomal protein L21
rplT	0.69	0.021	**2.04**	0.004	**1.79**	6.0E-06	ribosomal protein L20
rplS	**1.88**	0.001	**3.05**	0.002	**2.45**	5.7E-05	ribosomal protein L19
rpmB	**4.34**	0.001	**3.58**	0.007	**4.11**	4.2E-06	ribosomal protein L28
rpmE	**2.49**	0.001	**3.32**	0.004	**3.69**	2.1E-06	ribosomal protein L31
rplM	**2.16**	2.1E-04	**1.38**	0.055	**2.26**	1.5E-06	ribosomal protein L13
rplR	**-2.34**	0.023	0.25		**2.21**	3.7E-06	ribosomal protein L18
rplF	-0.13		0.15		**1.76**	1.8E-05	ribosomal protein L6
rplE	0.14		0.34		**2.29**	2.6E-06	ribosomal protein L5
rplX	0.00		0.49		**2.18**	6.5E-06	ribosomal protein L24
rplN	0.36		0.38		**2.23**	4.7E-06	ribosomal protein L14
rpmC	-0.23		0.42		**1.88**	5.3E-05	ribosomal protein L29
rplV	-0.11		0.34		**2.01**	1.3E-04	ribosomal protein L22

Gene	ATCC19115		ScottA		70-1700		Function
	LR*	P#	LR	P	LR	P	
rplB	0.48		0.78		**1.67**	3.0E-05	ribosomal protein L2
rplW	**1.06**		0.23		**2.29**	2.1E-04	ribosomal protein L23
rplD	0.58		0.39		**1.81**	1.5E-04	ribosomal protein L4
rpmI	**1.32**	0.001	**1.66**	0.007	**2.32**	8.5E-07	ribosomal protein L35
rpmG	**1.79**	3.2E-04	**2.59**	2.9E-05	**1.85**	6.5E-06	ribosomal protein L33
rpmF	**3.30**	4.3E-04	**2.93**	0.001	**1.63**	1.9E-04	ribosomal protein L32
rpmD	**2.21**	0.003	0.06		**2.15**	2.5E-06	ribosomal protein L30
rpsF	**1.62**	0.003	0.99	0.003	**1.59**	9.3E-06	ribosomal protein S6
rpsR	**1.38**	0.021	**1.04**	0.002	**1.89**	5.8E-06	ribosomal protein S18
rpsO	**2.00**	0.001	**2.12**	0.001	**1.66**	2.4E-05	ribosomal protein S15
rpsD	**1.32**	0.001	**1.73**	0.020	**1.99**	8.5E-06	ribosomal protein S4
rpsB	**2.34**	4.3E-05	0.51		**1.72**	3.3E-05	ribosomal protein S2
rpsP	**1.54**	0.010	**2.10**	0.001	**3.04**	1.3E-06	ribosomal protein S16
rpsI	**1.43**	0.001	**1.32**	0.049	**2.14**	1.5E-06	ribosomal protein S9
rpsH	0.81		0.23		**2.38**	3.6E-06	ribosomal protein S8
rpsN	-0.07		0.05		**2.10**	7.3E-05	ribosomal protein S14
rpsQ	-0.15		0.28		**2.07**	8.0E-05	ribosomal protein S17
rpsC	-0.16		**1.04**		**1.81**	0.007	ribosomal protein S3
rpsS	0.51		0.45		**2.40**	1.5E-05	ribosomal protein S19
rpsG	**1.39**	0.010	0.62		**2.45**	3.5E-04	ribosomal protein S7
rpsL	**2.06**	3.5E-05	**1.35**		**3.24**	2.5E-07	ribosomal protein S12
rpsA	**1.61**	0.008	**2.00**	1.6E-04	**1.54**	2.6E-05	ribosomal protein S1
rpsU	**1.70**	0.013	**1.26**	0.017	**1.46**	2.7E-05	ribosomal protein S21
rpoE	**1.45**	2.5E-04	**1.75**	4.1E-05	**1.71**	1.2E-05	RNA polymerase, delta subunit
rpoZ	**1.89**	0.007	**2.27**	0.001	**2.09**	2.7E-05	RNA polymerase, omega subunit
efp	**1.64**	0.017	**1.19**	0.028	**1.64**	4.4E-04	elongation factor EF-P
tsf	**1.32**	0.015	0.47		**2.16**	7.6E-06	elongation factor EF-Ts
infC	**2.15**	1.5E-04	**1.86**	0.006	**2.83**	2.8E-07	translation initiation factor IF3
fus	**1.16**	0.001	**1.21**		**2.44**	6.1E-07	elongation factor EF-G
tufA	0.55		**1.30**		**2.41**	1.2E-06	elongation factor EF-Tu
lmo0218	**2.33**	2.1E-05	0.86	0.003	**1.47**	3.2E-05	predicted RNA binding protein
deaD	**2.38**	1.7E-05	0.51		0.32		similar to ATP-dependent RNA helicase
lmo1450	**3.36**	3.5E-06	**1.54**	4.9E-04	**1.47**	1.4E-05	putative ATP-dependent RNA helicase
lmo1530	**1.31**	0.003	**1.10**	0.006	**1.20**	1.7E-04	similar to queuine tRNA-ribosyltransferase
lmo0227	**1.88**	0.001	**1.31**	1.5E-04	**1.64**	1.1E-04	putative tRNA-dihydrouridine
miaA	**1.75**	1.0E-04	**1.23**	0.001	0.88	0.002	tRNA delta(2)-isopentenylpyrophosphate transferase
lmo1366	**1.65**	0.007	**1.84**	0.002	**2.44**	1.0E-05	predicted rRNA methylase; putative hemolysin
lmo1722	**2.19**	2.0E-04	**1.32**	2.4E-04	0.87	0.002	putative ATP-dependent RNA helicase
lmo1843	**1.73**	4.8E-04	**1.19**	0.006	**1.20**	0.014	similar to 23S RNA-specific pseudouridine synthase D
lmo2369	**1.08**	0.003	**1.75**	1.2E-04	0.83	0.005	similar to B. subtilis general stress protein 13 containing a ribosomal S1 protein domain
lmo2811	-0.49		**1.64**	0.033	**1.76**	0.009	similar to tRNA modification GTPase

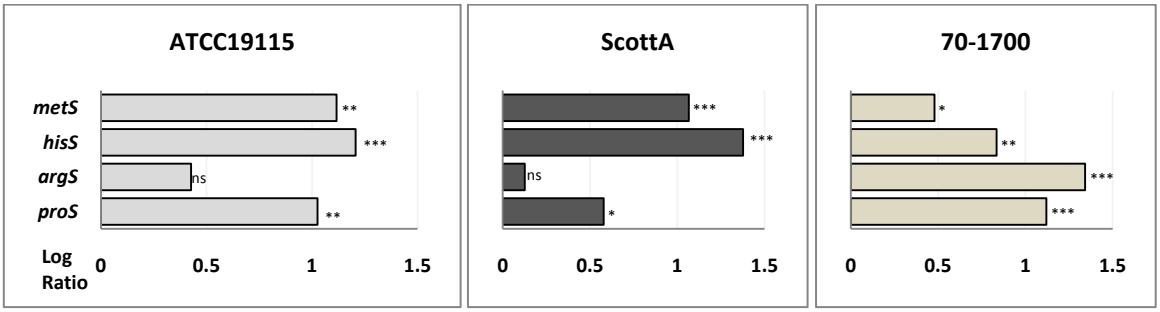
*LR-represents log ratio value.
P-values not shown were >0.05

5.3.1.4.2 Cold-temperature induced activation of other translation related genes.

Other translation related genes also showed significant activation in cold adapted strains of *L. monocytogenes,* further suggesting an increase in requirement of RNA synthesis support machinery and subsequent increase in translation associated protein expression. For instance genes encoding RNA polymerase subunits *rpoE* (*lmo2560*) and *rpoZ* (*lmo1826*) showed ≥twofold up-regulation in all three strains (Table 5.2). Translation initiation factor IF3 encoding gene *infC* (*lmo1785*) showed a 4-fold up-regulation in strains ATCC19115 and ScottA and a 7-fold increase in transcription in strain 70-1700 (Table 5.2). IF3 has been shown to play a vital role in *E. coli* cold-adaptation response (Giuliodori *et al.*, 2007). A number of translation factors showed increased transcription in *L. monocytogenes* cold adaptive response, similar to that observed in *B. subtilis* (Graumann & Marahiel, 1999). Up-regulation of *lmo1067* encoding a protein similar to GTP-binding elongation factor also showed up-regulation (Table 5.2) and has previously been linked to *L. monocytogenes* cold-adaptive response in strain 10403S (Chan *et al.*, 2007b). The same study also identified *lmo1722* and *lmo0866* involvement in cold-adaptation of *L. monocytogenes* strain 10403S (Chan *et al.*, 2007b). A number of RNA helicase encoding genes showed ≥twofold up-regulation in the current study including *lmo1722*, *lmo1450* in all three strains (Table 5.2); however only ATCC19115 showed significant up-regulation of *deaD* (*lmo0866*) with ScottA and 70-1700 expressing no change in transcription levels, further emphasising strain variability in gene expression of *L. monocytogenes*. RNA helicases have previously been linked to cold-adaptation in various microorganisms including *Exiguobacterium sibiricum*, where they were thought to assist in unwinding the RNA secondary structure for efficient translation at low-temperature (Rodrigues *et al.*, 2008).

This pattern of gene expression in cold-adapted *L. monocytogenes* cells indicates a strong requirement for increase in protein synthesis machinery to support cell function at low-temperature environments. As a reflection of an increase in ribosomal protein transcription, genes associated with aminoacyl-tRNA biosynthesis also showed up-regulation. Some of the up-regulated genes included a methionyl-tRNA synthetase encoding gene *metS* (*lmo0177*) showed a two-fold up-regulation in strains ATCC19115 and ScottA (Fig. 5.9); *hisS* (*lmo1520*) encoding a histidyl-tRNA synthetase exhibited a 3-fold activation in strain ScottA and a twofold up-regulation in ATCC19115; *proS* (*lmo1319*) a prolyl-tRNA synthetase encoding gene showed two-fold up-regulation in strains ATCC19115 and 70-1700; where as an arginyl-tRNA synthetase coding gene *argS* (*lmo2561*) showed 3-fold up-regulation in strain 70-1700 only (Fig. 5.9). Aminoacyl-tRNA are utilised during protein

synthesis and their apparent increase in gene transcription suggests an increase in the rate of subsequent aminoacyl consumption as a result of increased peptide synthesis.

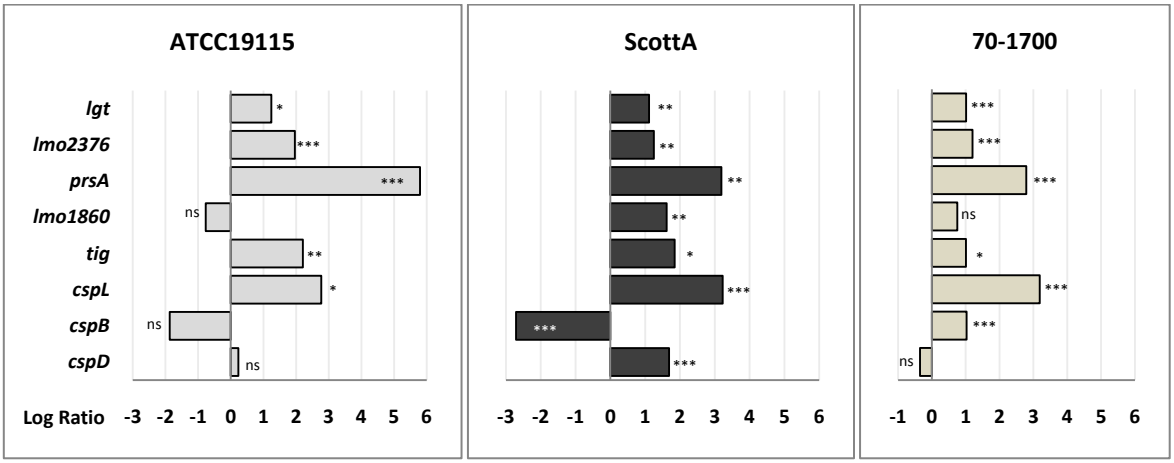


*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.9 Expression response of genes associated with aminoacyl-tRNA biosynthesis in three *L. monocytogenes* strains adapted to cold stress.

5.3.1.4.3 Cold-temperature induced activation of cold shock proteins encoding genes.

Overall, an increase in protein synthesis machinery observed in cold-adapted cells most likely compensates for lowered activity of essential cell enzymes under low-temperature conditions. Consequently a number of genes encoding molecular chaperons showed up-regulation in cold-adapted *L. monocytogenes* cells, suggesting an increase requirement for these support molecules in activated protein synthesis under low-temperature conditions.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.10 Expression response of genes associated with protein folding and processing and cold shock proteins in three *L. monocytogenes* strains adapted to cold stress.

Cold adapted cells exhibited activation of *tig* (*lmo1267*) a gene encoding trigger factor a protein chaperone associated with ribosomes. Activation of *tig* following adaptation to cold shock has been observed in *B. subtilis* (Graumann & Marahiel, 1999). Although a characterisation of *tig* mutants of *L. monocytogenes* strain EGD-e excluded its involvement in cold stress tolerance of this organism (Bigot *et al.*, 2006). Significant 5-fold, 4- and two fold up-regulation of *tig* transcription observed in strains ATCC19115, ScottA and 70-1700 respectively provides sufficient evidence to dispute the conclusion derived by Bigot *et al*. It appears that under the conditions examined trigger factor plays an important role in cold-adapted *L. monocytogenes* cells most likely stabilising ribosomal proteins affected by low-temperature, and it is very likely that in a *Δtig*$^{-}$ background other proteins with chaperone properties could be compensating for the lack of Tig.

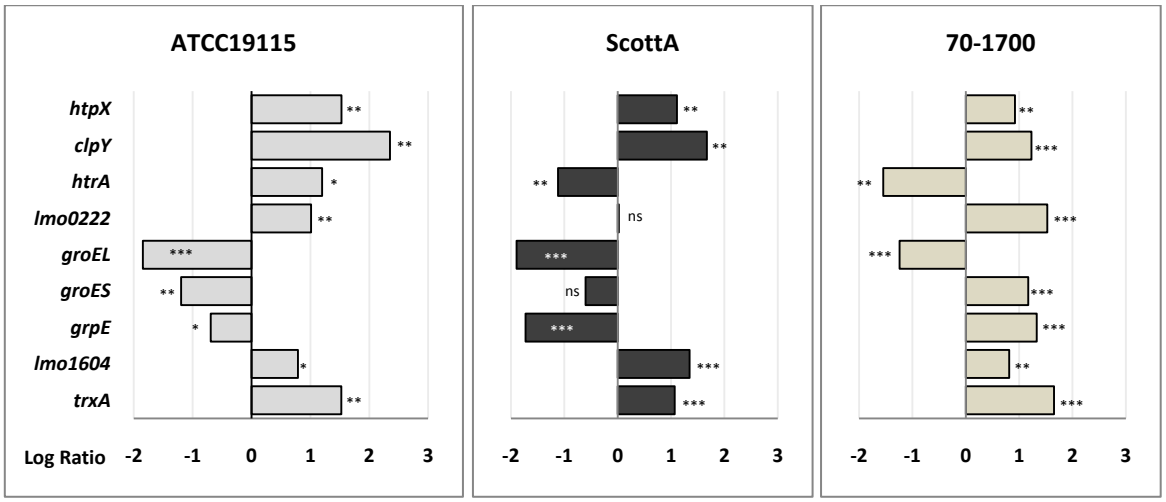
Cold shock protein *cspL* (*lmo1364*) showed significant up-regulation of 7-fold in ATCC19115 and 9-fold in ScottA and 70-1700 (Fig. 5.10). Activation of this cold shock protein in *L. monocytogenes* strain 10403S has been observed in both log- and stationary growth phase cells at 4°C (Chan *et al.*, 2007b). CspL has also been shown to dramatically induced in response to cold shock in *L. monocytogenes* strain LO28 in a similar manner observed for *E. coli* and *B. subtilis*, with maximum induction observed in cold adapted cells after 20hr incubation at 10°C (Wemekamp-Kamphuis *et al.*, 2002a). The remaining two cold-shock proteins exhibited strain specific expression with *cspB* (*lmo2016*) exhibiting twofold up-regulation in transcription levels in strain 70-1700 and a 7-fold down-regulation in ScottA (Fig. 5.10); while *cspD* (*lmo1879*) showed 3-fold up-regulation in ScottA only, with no significant change in transcription observed for ATCC19115 or 70-1700 (Fig. 5.10). Cold stress proteins involvement in cold stress adaptation has not been fully resolved however it is accepted that these proteins assist cell adaptation to low temperature through RNA-chaperone activities (Chattopadhyay, 2006; Ermolenko & Makhatadze, 2002; Tasara & Stephan, 2006). This presumably promotes transcription and translation functions that are hindered under low-temperature stress.

Interestingly, genes encoding three low temperature requirement proteins *ltrA* (*lmo0389*), *ltrB* (*lmo0215*) and *ltrC* (*lmo2398*) showed no significant change in transcription in any of the strains examined. Mutagenesis based studies have previously described *L. monocytogenes* cold-sensitive phenotypes unable to grow at 4°C arising from inhibition of low-temperature requirement genes (Zheng & Kathariou, 1994). It appears that activation of these genes is not essential for *L.*

monocytogenes strains ATCC19115, ScottA , 70-1700 or 10403S (Chan *et al.*, 2007b) proliferation at 4°C at least not when cryo-adapted.

5.3.1.4.4 Cold-temperature induced activation of heat shock proteins encoding genes.

Involvement of heat-shock proteins (HSPs) in cold stress adaptation has previously been shown in *L. monocytogenes* and other microorganisms, as a part of general stress response mechanism. Two HSP genes *htpX* (*lmo0963*) and *clpY* (*lmo1279*) showed significant up-regulation in all three *L. monocytogenes* strains (Fig. 5.11). The exact function of HtpX is unknown in *L. monocytogenes*, but its homolog in *E. coli* is an endopeptidase, substrates of which include membrane proteins (Sakoh *et al.*, 2005). HtpX may potentially assist in quality control of cell-membrane proteins actively being modified in *L. monocytogenes* cold-adapted cells, but its exact role is yet to be elucidated. ClpY encodes a regulatory subunit with ATPase and substrate recognition site of a two-component protease clpYQ. In *E. coli* ClpY functions by binding and unfolding the substrate while transferring it to the catalytic subunit ClpQ (Lien *et al.*, 2009). Gene encoding the ClpQ subunit of the later protease showed no significant change in transcription levels; the exact function of the ClpY in the cold-adapted cells of *L. monocytogenes* is therefore unclear.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

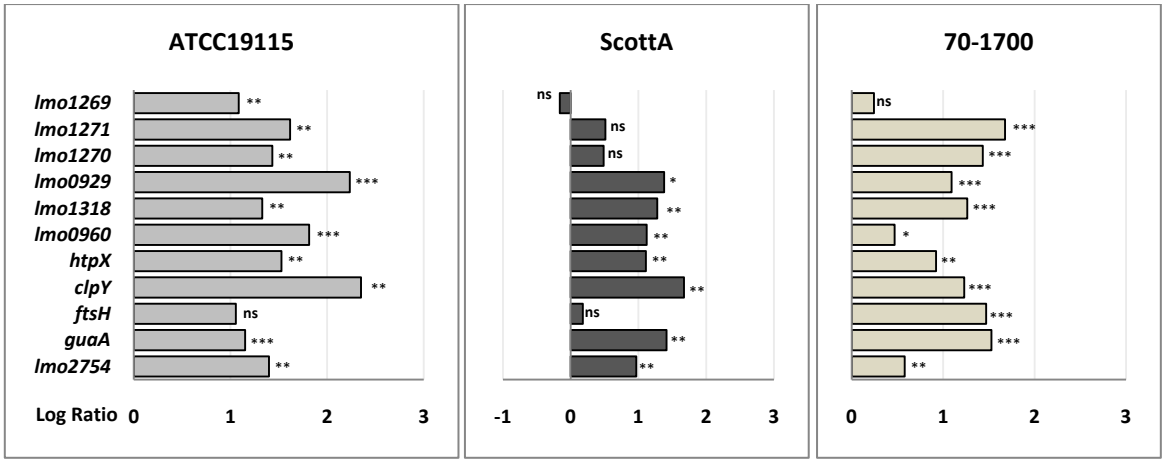
Figure 5.11 Expression response of heat shock genes in three *L. monocytogenes* strains adapted to cold stress.

Strain variability in gene expression was once again evident particularly for *htrA* (*lmo0292*) which showed up-regulation only in strain ATCC19115, *groES* (*lmo2069*) and *grpE* (*lmo1474*) were up-

regulated only in strain 70-1700 (Fig. 5.11). Additionally the phenomenon of strain specific HSP gene expression has previously been demonstrated in this organism with *groES* which revealed significantly decreased transcription in strain 10403S at 4°C (Chan *et al.*, 2007b). A gene homologous to *hslO* in *B. subtilis* (*lmo0222*) encoding HSP33 molecular chaperone showed up-regulation in strains ATCC19115 and 70-1700 (Fig. 5.11). HSP33 belongs to a redox-regulated proteins activity of which is regulated by the redox-state of the cytosol and once activated serves as a highly efficient chaperone able to prevent aggregation of a variety of unfolded proteins (Graf *et al.*, 2004). Cold stress adaptation mechanisms have been linked to oxidative stress in *L. monocytogenes* cells through elevated transcription of *trxB* encoding thioredoxin reductase (Liu *et al.*, 2002; Tasara & Stephan, 2006). This particular gene showed no change in transcription of *L. monocytogenes* strains examined (data not shown), however a gene encoding thioredoxin, *trxA* (*lmo1233*) showed significant up-regulation (Fig. 5.11). Thioredoxin is a part of the thiol-dependent oxidation-reduction system that contributes to maintenance of reducing environments in bacterial cytoplasm (Uziel *et al.*, 2004). In addition a gene *lmo1604* encoding a putative peroxiredoxin showed ≥twofold up-regulation in *L. monocytogenes* cold-adapted cells, further indicating a potential oxidative stress encountered by these cells. Low-temperature induces oxidative stress in bacteria, most likely because it increases the solubility of oxygen (Okuyama *et al.*, 2008).

5.3.1.4.5 Cold-temperature induced activation of various peptidase encoding genes.

Two of the three *L. monocytogenes* cold-adapted strains exhibited overall activation of genes encoding peptidases with T-value scores of 2.09 for strain ATCC19115 and 2.58 for strain 70-1700 (Table 5.1). The gene encoding sortase A, *lmo0929*, showed 5-, 3- and two-fold up-regulation in strains ATCC19115, ScottA and 70-1700 respectively (Fig. 5.12).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.12 Expression response of genes encoding peptidases in three *L. monocytogenes* strains adapted to cold stress.

Sortase A (SrtA) is the main protein in *L. monocytogenes* responsible for covalent attachment of Sec-dependent translocated proteins to bacterial cell wall (Desvaux & Hebraud, 2006). Activation of *srtA* further strengthens the conclusion made previously that enhancement of protein export in cold-adapted cells may in fact be driven by increased requirement for cell-wall related proteins perhaps to assist with the ongoing maintenance of bacterial cell-wall.

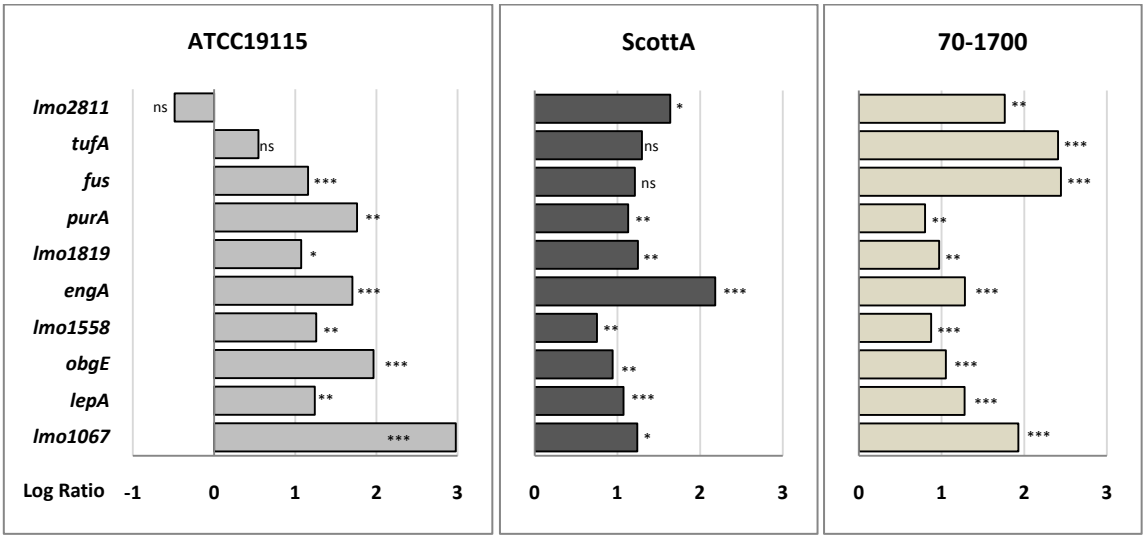
All proteins targeted to the Sec translocon possess an N-terminal signal peptide, which is removed by the action of signal peptidase (SPase) prior to being integrated into bacterial membrane or cell wall *L. monocytogenes* possesses three SPase I SipX, SipY and SipZ (Desvaux & Hebraud, 2006). All three of which showed activation in cold-adapted strain ATCC19115 *sipX* (*lmo1269*) showed two-fold up-regulation and both *sipY* (*lmo1270*) and *sipZ* (*lmo1271*) exhibited 3-fold up-regulation (Fig. 5.12); only two SPase encoding genes showed 3-fold up-regulation in 70-1700 *sipY* and *sipZ* (Fig. 5.12); no up-regulation of SPase encoding genes was evident in strain ScottA.

Peptidases generally contribute to degradation of peptides that cannot be folded by molecular chaperones; as such their activation in stressful conditions such as cold temperature is not surprising. A number of these were activated in all three strains, including previously mentioned *clpY* and *htpX* a peptidase involved in purine metabolism encoded by *guaA* (*lmo1096*) and a gene homologous to *decA* in *B. subtilis* encoding D-alanyl-D-alanine carboxypeptidase involved in peptidoglycan biosynthesis (Fig. 5.12). A gene orthologous to *yebA* in *E. coli*, *lmo1318* showed ≥two-

fold up-regulation in all three strains, its exact function in a cold-adapted response is not clear, however it has been recently identified as one of the factors linked to peptidoglycan splitting process in cell division and thus important for daughter cell separation in *E. coli* (Uehara *et al.*, 2009).

5.3.1.5 Low-temperature adaptation induced changes to transcription of GTP-binding proteins.

Cold-adapted *L. monocytogenes* cells exhibited significant activation of genes encoding GTP-binding proteins, *T- value* scores ranging from 2.8 for ATCC19115 to 4.3 in strain 70-1700 (Table 5.1). GTP-binding proteins are highly conserved signalling molecules involved in diverse cellular processes including cell transduction, protein synthesis and cell cycle regulation as well as protein translocation across membranes (Bourne *et al.*, 1991). An increase in transcription levels of these signalling molecules in cold-stressed cells was therefore not surprising.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.13 Expression response of GTP-binding protein encoding genes in three *L. monocytogenes* strains adapted to cold stress.

Among the highly up-regulated genes were *lmo1067 homologous* to *typA /bipA* in *E. coli*, *engA* (*lmo1937*), *obgE* (*lmo1537*) and *lmo1819* homologous to *rsgA* in *E. coli* (Fig. 5.13). TypA was required for survival of both *E. coli* (Pfenning & Flower, 2001) and a nitrogen-fixing bacterium *Sinorhizobium meliloti* (Kiss *et al.*, 2004) in low temperature conditions with *typA* mutants exhibiting cold-sensitive phenotypes in both organisms. In addition the typA-homolog of *B. subtilis* was found to be up-regulated at 15°C (Beckering *et al.*, 2002). This GTP-binding elongation factor homolog was also

activated in *L. monocytogenes* 10403S cells at 4°C (Chan *et al.*, 2007b), strong up-regulation of *typA* observed in strains ATCC19115, ScottA and 70-1700, suggests a strong involvement in cold-adaptive response, most likely involved in aiding translation elongation at low-temperature.

A GTP-binding protein encoding gene *engA* has been linked to *L. monocytogenes* 10403S 4°C growth (Chan *et al.*, 2007b). It is thought to be involved with ribosome maturation in *E. coli* (Bharat *et al.*, 2006; Hwang & Inouye, 2006). ObgE also shares a similar function in *E. coli* additionally being involved in chromosome partitioning (Sato *et al.*, 2005).

It appears that GTP-binding genes most activated in cold-adapted cells of *L. monocytogenes* examined are either directly involved in controlling normal ribosome maturation or indirectly involved with ribosome associated transcription once again emphasising the need of support molecules for proper ribosome function under such adverse condition.

5.3.1.6 Low-temperature adaptation affected the DNA molecule and subsequently hindered transcription.

There appeared to be an increased expression of DNA repair and recombination genes in strains ATCC19115 and ScottA of *L. monocytogenes* (*T-values* of 2.1 and 2.2 respectively), up-regulation in strain 70-1700 was also noted although it was less significant (Table 5.1). Some of the strongly up-regulated genes included homologues of *hobB*, *recU*, *recA* (not strain 70-1700), *nusG, ssb* (not strain ScottA), *xerD* (not strain 70-1700), *dnaA* and *nusA* (not ScottA), *parE* and *topA* (Table 5.3). The up-regulation of these genes suggests a potential hindering in transcription due to a possible damage to the DNA molecule induced by cold temperature stress. Indeed the degree of superhelicity of DNA molecule in bacteria varies in response to change in the ambient temperature, with an increase in negative supercoiling observed at low temperature (Erriksson *et al.*, 2002).

Table 5.3 Expression response of GTP-binding protein encoding genes in three *L. monocytogenes* strains adapted to cold stress.

Gene	ATCC19115		ScottA		70-1700		Function
	LR*	P#	LR	P	LR	P	
holB	**2.14**	2.7E-05	**2.38**	9.6E-06	**2.01**	6.0E-06	DNA polymerase III delta' subunit
codV	**1.37**	0.002	0.87	0.005	0.80	0.001	similar to site-specific recombinase
lmo1502	**1.36**	0.014	**1.09**	0.001	0.44	0.048	putative Holliday junction resolvase
lmo1582	**2.23**	8.3E-05	0.95		**2.40**	8.9E-07	putative adenine-specific DNA methylase
recU	**1.33**	0.010	**1.75**	4.5E-05	**1.35**	6.7E-05	similar to DNA repair and homologous recombination protein
recA	**1.27**	4.7E-04	**1.62**	5.0E-05	0.29		DNA recombination protein
lmo1449	**1.73**	4.4E-04	0.77	0.058	0.66	0.002	similar to endonuclease IV
xerD	**1.66**	1.1E-04	0.97	0.002	0.56	0.019	similar to integrase/recombinase
lmo2050	0.53	0.033	**1.05**	0.007	**1.58**	2.8E-04	putative excinuclease ATPase subunit
hup	**1.71**	0.054	0.76		**2.58**	5.4E-06	similar to non-specific DNA-binding protein HU
parE	**1.51**	4.5E-04	**1.04**	0.009	**1.56**	2.0E-04	topoisomerase IV, B subunit
topA	**2.11**	0.006	**2.24**	1.3E-05	**1.39**	5.7E-05	topoisomerase IA
ssb	**1.47**	0.009	0.58		**1.52**	1.2E-05	single-stranded DNA-binding protein
dnaA	**1.53**	0.003	**1.04**	0.014	0.81	0.001	chromosomal replication initiator protein
nusA	**1.31**	4.6E-04	0.58	0.014	**1.13**	7.7E-05	transcription elongation factor
nusG	**2.19**	2.5E-05	**1.64**	2.6E-04	**2.23**	5.3E-06	transcription antitermination factor
greA	**2.16**	3.2E-04	0.82		**1.97**	5.6E-06	transcription elongation factor

*LR-represents log ratio value.
P-values not shown were >0.05

Initiation of the SOS response, a mechanism involved in the repair of DNA damage and restart of stalled replication forks (Maul & Sutton, 2005), was observed in strains ATCC19115 and ScottA with >twofold up-regulation of *recA* (*lmo1398*) gene, encoding the major activator of the SOS response, RecA. This protein has previously been linked to cold tolerance in *E. coli* (Panoff *et al.*, 1998).

Significant activation of transcription antitermination factor encoded by *nusG* (*lmo0246*) was observed in all three strains (Table 5.3). NusG is known to be induced by cold shock through transcription anti-termination mediated by CspA and other cold shock proteins in *E. coli* (Bae *et al.*, 2000). This together with activation of transcription elongation factors *greA* (not evident in strain ScottA) and *nusA* (not significant in ScottA), suggests enhanced transcription requirement by cold-adapted cells, perhaps to maintain transcription levels sufficient for normal cell functions.

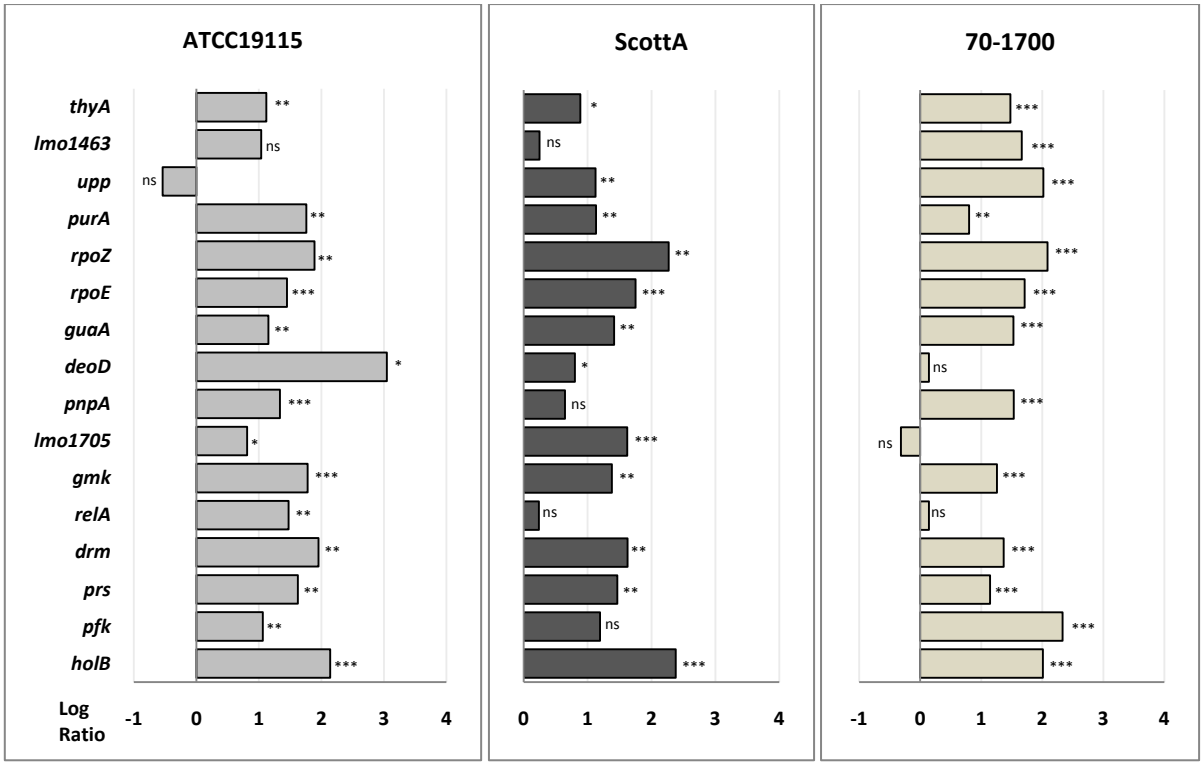
An increase in negative supercoiling of DNA molecule would hinder the transcription machinery in bacterial cells, this needs to be overcome to allow for undisturbed cell metabolism during cold stress. At least in *E. coli* the superhelical tension of DNA molecule encountered at low-temperature is mainly regulated through the two opposing topoisomerase activities (Erriksson *et al.*, 2002). Two

topoisomerase encoding genes were up-regulated in all three *L. monocytogenes* strains at low-temperature *parE* (*lmo1286*) and *topA* (*lmo1275*), suggesting a potential involvement of these in maintaining superhelical tension in this organism under cold stress. Increased expression of *hup* (*lmo1934*) was observed in strains ATCC19115 and 70-1700 (Table 5.3), encoding histone-like protein which may affect DNA topology. In *E. coli*, protein HU, which is very similar to the protein encoded by *hup* has been shown to alter the conformation of DNA, functioning as a transcriptional modulator at the post-transcriptional level (Dame & Goosen, 2002). This functionality may aid in maintaining superhelical tension in cold-adapted cells.

5.3.1.7 Low-temperature adaptation induced transcription of genes associated with nucleotide metabolism.

Cold adapted cells of *L. monocytogenes* strains ATCC19115, ScottA and 70-1700 exhibited activation of genes associated with purine and pyrimidine metabolism with combined *T-value* score of 2.69, 2.69 and 4.08 respectively (Table 5.1). It appears that metabolism rather than biosynthesis of nucleotides is activated following cold adaptation.

Some of the strongly up-regulated genes are summarised in Fig. 5.14; these included *purA* (*lmo0055*), *thyA* (*lmo1874*), *rpoZ* (*lmo1826*), *rpoE* (*lmo2560*), *guaA* (*lmo1096*), *gmk* (*lmo1827*), *drm* (*lmo1954*) and *holB* (*lmo0162*). Gene encoding adenylosuccinate synthetase *purA* has previously been shown to increase transcription in *L. monocytogenes* cultivated at 4°C (Chan *et al.*, 2007b). Impaired low-temperature stress response of a *Streptococcus thermophilus deoD* mutant has previously been observed (Varcamonti *et al.*, 2003). A cold sensitive growth defect in *gmk* mutant had also been reported in *Salmonella enterica* (Beck *et al.*, 2003). Activation of this subset of genes observed in cold-adapted cells implies an important involvement of purine and pyrimidine metabolism in bacterial low-temperature response.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.14 Expression response of genes associated with purine and pyrimidine metabolism in three *L. monocytogenes* strains adapted to cold stress.

Nucleotide salvage pathways may play a significant role in cold adapted cells in maintaining nucleotide turnover, by reutilization of the bases and nucleosides.

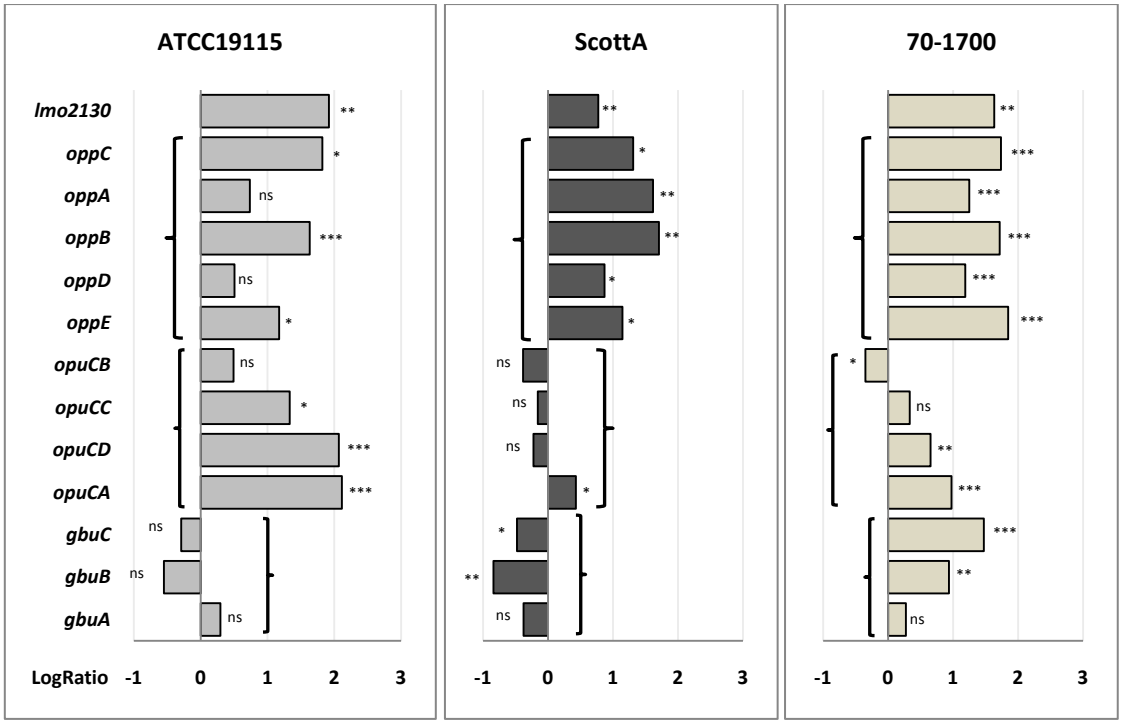
5.3.1.8 Low-temperature adaptation induced transcription of genes associated with various membrane transporters.

Collectively genes encoding membrane transporters showed variation in transcription among the three strains of *L. monocytogenes* examined. Overall general transporter encoding genes (not including the PTS system transporters) were up-regulated only in strain ScottA with a *T-value* score of 2.43 (Table 5.1). The ABC type transporters, in general showed up-regulation in ScottA and 70-1700 with *T-value* scores of 2.47 and 2.42 respectively (Table 5.1). Strain ATCC19115 showed no significant change in overall transcription of transporter genes, perhaps reflecting variation of substance requirement in different strains of this organism under low-temperature stress.

5.3.1.8.1 Cold-adapted cells induced changes in transcription of compatible solute transporter encoding genes.

Cryotolerance in bacteria is accompanied by accumulation of compatible solutes (Angelidis & Smith, 2003; Tasara & Stephan, 2006). The exact function of compatible solute molecules in cold-adaptive response is unclear, although it has been suggested that these molecules (in particular glycine betaine) prevent cold induced aggregation of proteins, assist in stabilisation of enzymatic functions and the cell membrane lipid bilayer at low temperatures (Chattopadhyay, 2002; Lippert & Galinski, 1992). Low-temperature tends to change aqueous viscosity (D'Amico *et al.*, 2006), it is likely that cells cultivated at cold temperature experience pseudo-osmotic stress due to increased viscosity of the cellular matrix. Compatible solutes may potentially help to maintain intracellular fluidity thus allowing normal cellular processes.

L. monocytogenes cold adapted strains exhibited strong variation in regards to expression of genes encoding compatible solute transporters. Carnitine transporter genes (*opuCA*, *opuCC*, *opuCD*) showed significant activation in ATCC19115 (Fig. 5.15). This suggests a potential requirement for an increased uptake of carnitine from the environment by strain ATCC19115 in preference to other compatible solutes when cultivated at 4°C. In comparison strain 70-1700 exhibited two-fold up-regulation of genes *gbuC* and *gbuB* encoding components of a glycine betaine transporter (Fig. 5.15); suggesting a possible preference for glycine betaine uptake at 4°C by this strain. Strain ScottA on the other hand showed no activation of either of the compatible solute transporter genes. This strain was a relatively slow growing at 4°C thus it could be speculated that the rate of growth of this strain reflects a lack of enhanced accumulation of two major compatible solutes glycine betaine and carnitine.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.15 Expression response of genes encoding compatible solute transporters in three *L. monocytogenes* strains adapted to cold stress.

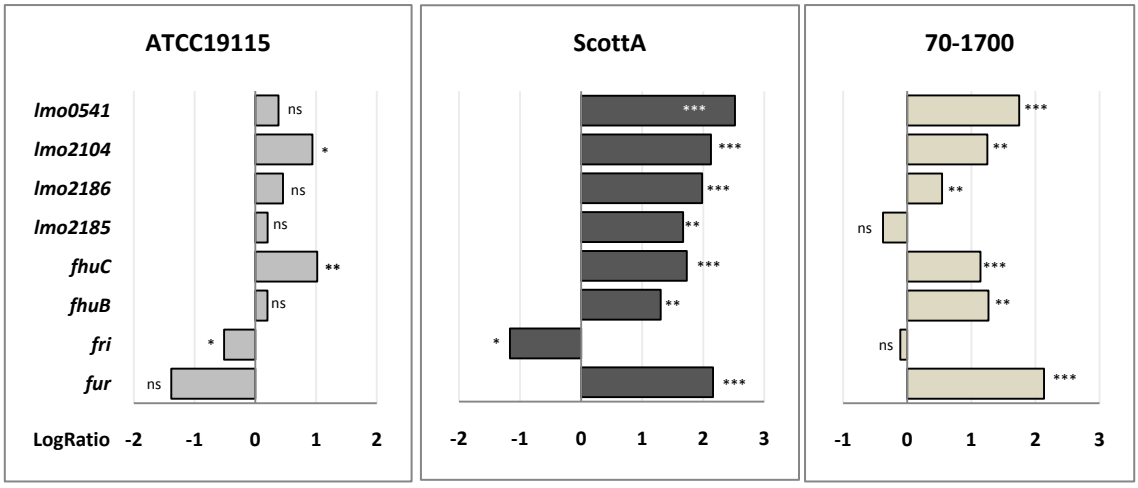
There was no evidence of activation of the secondary glycine betaine transporter BetL with *betL* (*lmo2092*) showing no change in transcription levels (data not shown). Proline synthesis appeared to not play a role in compatible solute acquisition by either of the cold stressed strains of *L. monocytogenes* with *proA*, *proB* and *proC* showing no significant change in transcription levels (data not shown).

Activation of oligopeptide transporter encoding genes was evident in all three cold-adapted strain of *L. monocytogenes* (Fig. 5.15). Accumulation of oligopeptides from the growth medium by means of the OppA was found to be essential for survival of *L. monocytogenes* strain LO28 at 5°C (Borezee *et al.*, 2000). The molecular function of oligopeptide accumulation by cold-adapted cells is unclear. It has been suggested that accumulated short peptides might be involved in activation of certain signal transduction pathways that promote other *L. monocytogenes* cold-adaptation and growth mechanisms at low-temperature (Tasara & Stephan, 2006). Accumulated peptides may have cryoprotective functions similar to proline, uptake of which may confer cold stress tolerance in a similar manner to other compatible solutes. These may also provide substrates that are hydrolysed by internal peptidases providing amino acids which are utilised in other cellular processes. Amino

acid uptake from the growth medium might also be activated during cold-stress owing to the evident up-regulation of a putative amino acid transporter gene *lmo2130* (Fig. 5.15), which could also provide essential amino acids to maintain growth at 4°C.

5.3.1.8.2 Cold-adapted cells induced changes in transcription of iron transporter encoding genes.

Cold adaptation appeared to activate genes encoding iron transporters in strain ScottA with T-value of 3.58 and to a lesser extent in strain 70-1700 (Table 5.1). Some of the strongly activated genes included *lmo0541*, which was up-regulated 6-fold and 3-fold in ScottA and 70-1700 respectively (Fig. 5.16).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.16 Expression response of genes encoding iron transporters and ferric uptake regulator in three *L. monocytogenes* strains adapted to cold stress.

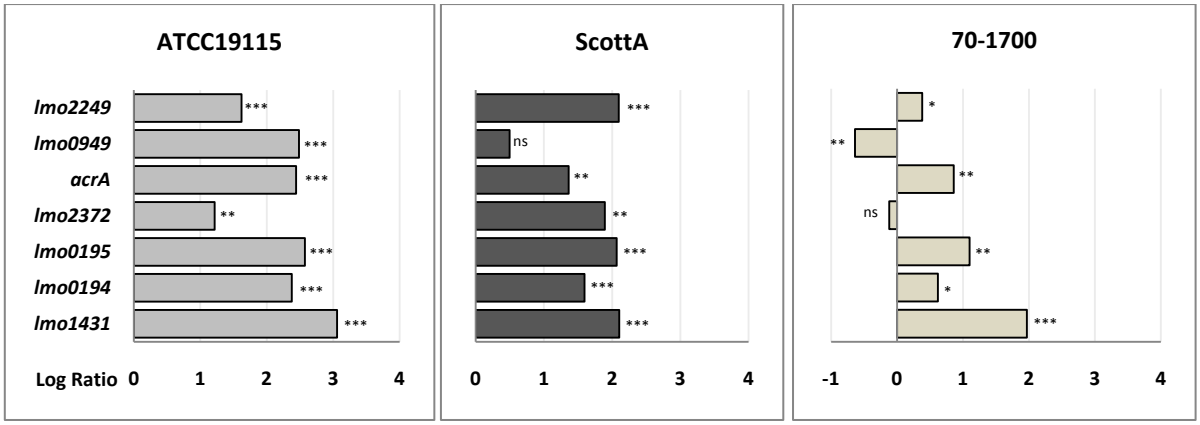
A gene homologous to *feoA* of *E. coli* (*lmo2104*), encoding ferrous iron transport protein A, showed significant up-regulation in all strains (Fig. 5.16). In addition all strains showed up-regulation of *fhuC*, while activation of *fhuB* was only increased in strains ScottA and 70-1700. Interestingly the ferric uptake regulator encoding gene, *fur* showed significant up-regulation in strains ScottA and 70-1700 (Fig. 5.16). This seems contradictory to the Fur function as a supposed negative regulator of iron transporters in *L. monocytogenes* (Ledala *et al.*, 2007). It is unclear what role Fur plays in cold-adaptation of *L. monocytogenes* as strain ATCC19115 showed no significant change in *fur* transcription. It is equally unclear if indeed Fur function in controlling transcription of iron transporter genes, perhaps its true function in *L. monocytogenes* is iron independent much like that

observed in *Bradyrhizobium japonicum*. In *B. japonicum* regulation of many iron-transporter genes is non-Fur regulated, and Fur expression is insensitive to iron (Hamza *et al.*, 1999). The ferritin-like protein encoding gene *fri* (also known as *flp*), is negatively regulated by Fur in *L. monocytogenes* (Fiorini *et al.*, 2008). Fri protein is believed to be the only Dsp protein in this organism capable of storing iron as ferric hydroxide in the protein internal cavity (Fiorini *et al.*, 2008; Olsen *et al.*, 2005). Elevated Fri transcription has previously been observe following cold shock (Hébraud & Guzzo, 2000) and in cold-acclimated *L. monocytogenes* cells (Liu *et al.*, 2002). This was not observed in the current experiment in which *fri* transcription showed significant down-regulation in ScottA cold-adapted strain and no significant change in transcription for either ATCC19115 or 70-1700 strains (Fig. 5.16). This however does not necessarily exclude the involvement of Fri in cold adapted cells of *L. monocytogenes* due to a relative long life of this protein (Fiorini *et al.*, 2008). Overall the observed repression of *fri* transcription coupled with activation of iron transporters in cold-adapted cells strongly suggests a rapid turnover of intracellular iron within the cells.

5.3.1.8.3 Cold-adapted cells induced transcription of other membrane transporter encoding genes.

Cold-adaptation resulted in activation of a number of general transporter genes *acrA* (*lmo0193*), *lmo0194*, *lmo0195*, *lmo2249* and *lmo1431* (Fig. 5.17). The precise function of these transporter genes in cold adaptive response of *L. monocytogenes* is unclear; however there is a strong evidence to suggest their important involvement in the stress response. For instance mutation of *acrA* gene in *Caulobacter crescentus* resulted in a decreased cold-tolerance of the organism (Ricardo *et al.*, 2008).

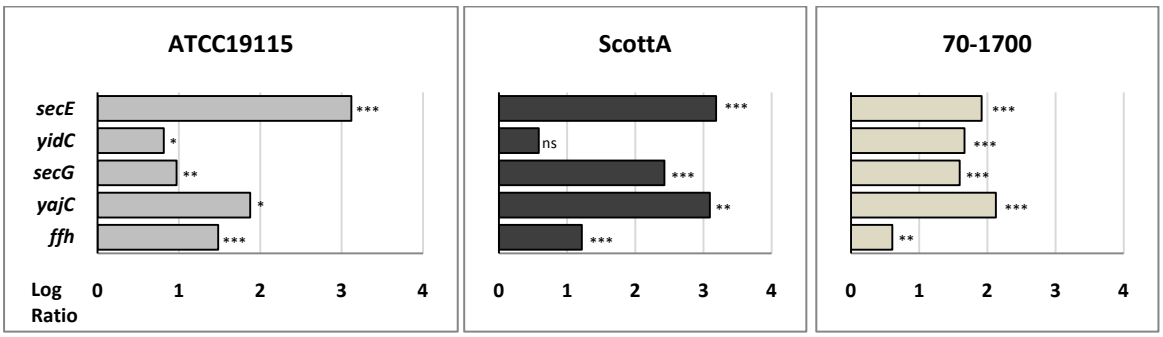
Increased uptake of various substances from the growth medium is critical for survival of *L. monocytogenes* in stressful environmental conditions which may be utilised in energy production or increased intracellular protein turnover observed during stress.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.17 Expression response of genes encoding intracellular transporters in three *L. monocytogenes* strains adapted to cold stress.

Not only uptake but also secretion mechanisms appear to play an important part in *L. monocytogenes* cold-stress adaptation. Cold-adapted cells exhibited significant activation of genes encoding translocase proteins responsible for protein translocation across the cytoplasmic membrane. Genes such as *secE* (*lmo0245*), *secG* (*lmo2451*) and *yajC* (*lmo1529*) showed significant up-regulation in all three cold-adapted strains of *L. monocytogenes* (Fig. 5.18). These genes have long been considered essential for cold-tolerance in *E. coli* in which disruption of *secG* rendered a cold-sensitive mutant (Hanada *et al.*, 1996; Nishiyama *et al.*, 1994), a phenomenon also independently observed in *B. subtilis* (van Wely *et al.*, 1999); inactivation of *secDF-yajC* complex also conferred a cold-sensitive phenotype in *E. coli* (Nouwen & Driessen, 2005).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

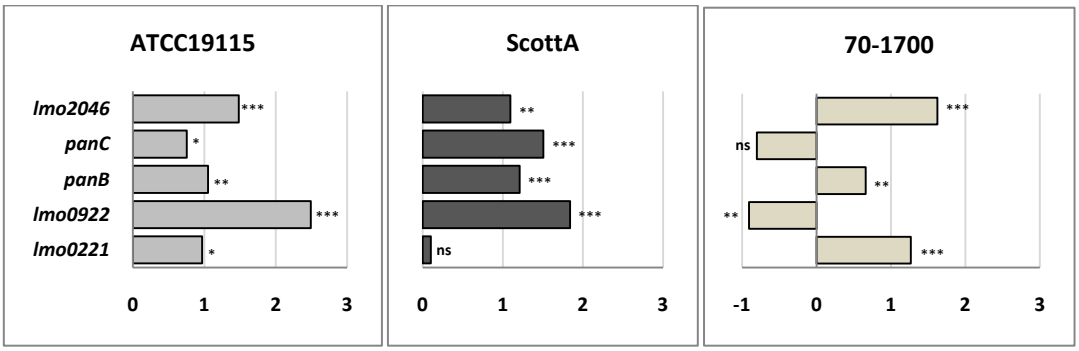
Figure 5.18 Expression response of genes associated with protein export in three *L. monocytogenes* strains adapted to cold stress.

The exact involvement of protein secretion in cold adapted response in *L. monocytogenes* is unclear, however it is most likely associated with translation of newly synthesised membrane and cell wall proteins and thus could be linked to the ongoing cell maintenance of cold stressed cells.

5.3.1.9 Low-temperature adaptation induced changes to cofactor and vitamin metabolism related genes.

5.3.1.9.1 Low-temperature induced activation of Coenzyme A biosynthesis.

Cold-adaptation resulted in activation of genes associated with pantothenate and coenzyme A biosynthesis *T-value* scores of 1.53, 2.59 ($P<0.05$) and 1.11 for strains ATCC19115, ScottA and 70-1700 respectively (Table 5.1); due to a small number of genes classified in this subgroup the respective *T*- and *P*-values were weak, but a clear trend of activation throughout the strains was evident. Coenzyme A is used in a multitude of biochemical reactions as a carrier of activated acyl groups. In most bacteria it is synthesised from pantothenate (vitamin B_5) which in itself can be derived from pyruvate or independently from valine degradation.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.19 Expression response of genes associated with biosynthesis of pantothenate and coenzyme A in three *L. monocytogenes* strains adapted to cold stress.

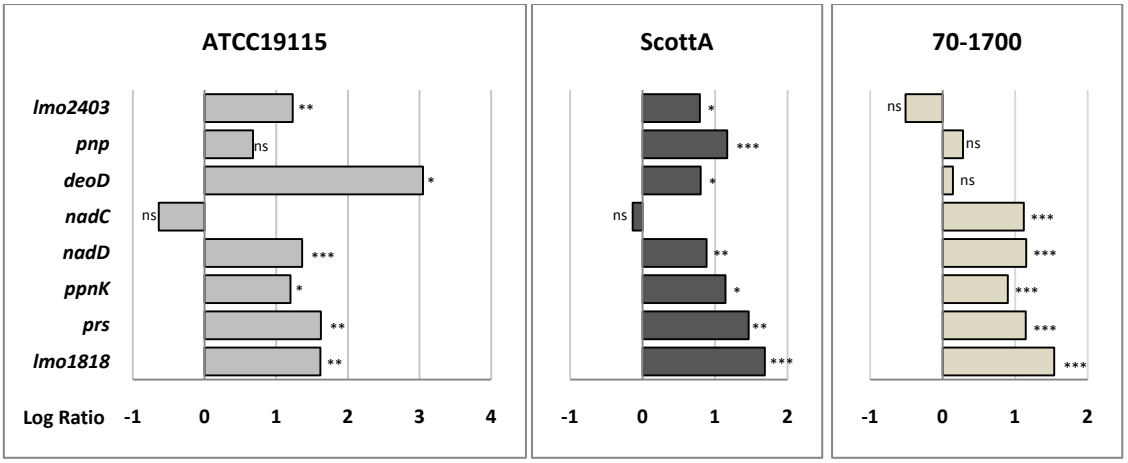
Significantly up-regulated genes of this subgroup found to occur in all three strains included *panB* (*lmo1902*) encoding ketopantoate hydroxymethyl transferase (Fig. 5.19) and *lmo2046* a gene homologous to *panE* in *E. coli* shown to enhance overall pantothenate production (Elischewski *et al.*, 1999). Interestingly genes homologous to *coaA* in *E. coli* encoding different types of pantothenate kinase showed strain specific transcription response in cold-adapted *L. monocytogenes* cells; gene *lmo0922* encoding type I CoaA enzyme showed 6- and 4-fold up-regulation in strains ATCC19115 and

ScottA respectively while transcription was down-regulated two-fold in strain 70-1700 (Fig. 5.19) meanwhile *lmo0221* gene encoding type III pantothenate kinase showed >two-fold up-regulation in strain 70-1700 (Fig. 5.19), a two-fold up-regulation in strain ATCC19115 and no significant change in transcription was evident in strain ScottA. This preference for a certain type of pantothenate kinase particularly in strains ScottA and 70-1700 is intriguing; however its relevance to cold-adaptation is unclear. A gene (*fbaA*, *lmo2556*) encoding an enzyme homologous to 6-phospho-5-dehydro-2-deoxy-D-gluconate aldolase, which is involved in one of the steps of acetyl-CoA synthesis also showed significant up-regulation 11-fold in strain 70-1700, 6-fold in ATCC19115 and 5-fold in ScottA (data not shown).

Overall, an increased demand for CoA biosynthesis observed in cold-adapted *L. monocytogenes* strains is most likely driven by active fatty acid synthesis where CoA is utilised as a cofactor in various enzymatic reactions. CoA dependent enzyme encoding genes include *lmo2202*, *accD* (*lmo1573*), *accB* (*lmo1356*), and *fabD* (*lmo1808*) associated with FA biosynthesis (Fig. 5.5) as well as *lmo1634* and *lmo1414* involved in FA metabolism (Fig. 5.5) all of which have shown activation in one or more of *L. monocytogenes* strains examined in this study.

5.3.1.9.2 Low-temperature induced activation of nicotinate/nicotinamide metabolism

In addition to their role in numerous redox reactions nicotinamide adenine dinucleotide (NAD+/NADH) and nicotinamide adenine dinucleotide phosphate (NADP+/NADPH) are utilised in many metabolic and regulatory processes as consumable co-substrates (Rodionov *et al.*, 2008). In *L. monocytogenes* NAD/NADP are derived from nicotinate/nicotinamide metabolism which exhibited a slight increase in transcription in cold adapted strains. Some of the significantly up-regulated genes in all three strains included *nadD* (*lmo1488*) and *ppnK* (*lmo1586*). Strain specific transcription response was observed with *pnp* (*lmo1953*) which showed >twofold up-regulation in strain ScottA and *nadC* (*lmo2024*) transcription of which was up-regulated >twofold in strain 70-1700 only (Fig. 5.20). A gene encoding purine-nucleoside phosphorylase *nadD* (*lmo1856*) showed 8-fold up-regulation in strain ATCC19115 and two-fold up-regulation in ScottA whilst no increase in transcription was observed for strain 70-1700 (Fig. 5.20).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.20 Expression response of genes associated with nicotinate and nicotinamide metabolism and genes encoding NAD-dependent enzymes in three *L. monocytogenes* strains adapted to cold stress.

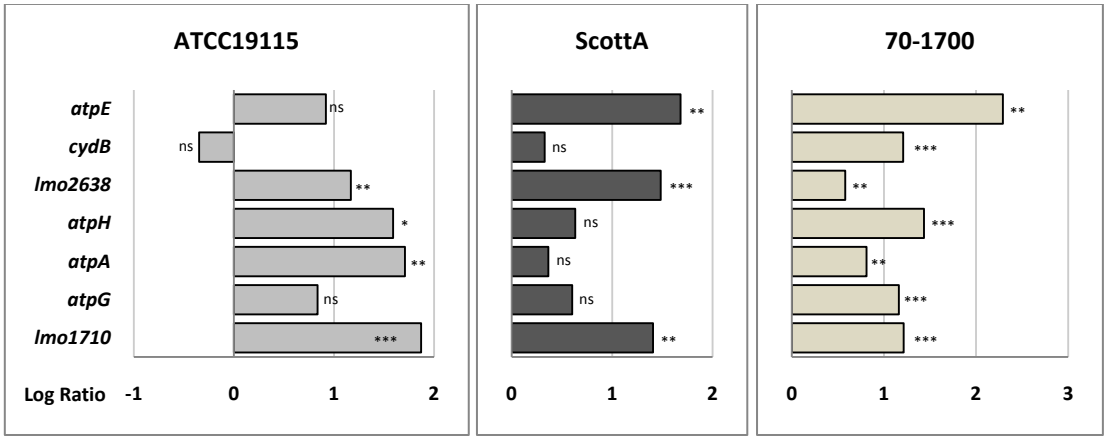
Low temperature induced accumulation of intracellular NAD and NADP has previously been reported for *L. monocytogenes* and *Yersinia pseudotuberculosis* (Somov *et al.*, 1999). Interestingly only a small number of NAD-dependent enzymes exhibited activation in transcription in cold adapted strains of *L. monocytogenes* these included *gpsA* (*lmo1936*) a gene encoding NAD(P)H-dependent glycerol-3-phosphate dehydrogenase an enzyme involved in glycerophospholipid metabolism (Fig. 5.6). Some indirectly involved enzymes such as ribulose-5-phosphate 3 epimerase encoded by *lmo1818* and phosphoribosyl pyrophosphate synthetise encoded by *prs* (*lmo0199*) both of which are involved in the late stages of pentose phosphate pathway and indirectly feeding the purine and pyrimidine metabolic pathways also demonstrated activation (Fig. 5.20).

Low temperature activated hydrogen transport in the respiratory chain and subsequent ATP synthesis has previously been reported in *L. monocytogenes* cultivated at low temperature (Somov *et al.*, 1999) suggesting that NAD/NADP accumulation in cold-adapted cells is utilised as oxidising agent in controlling the influx of H^+ ions into the cell via oxidative phosphorylation.

5.3.1.10 Low-temperature adaptation induced changes to oxidative phosphorylation related genes.

The overall energy metabolism of *L. monocytogenes* cells exposed to prolonged cold-temperature stress appeared to be reduced, most likely reflecting the overall decreased level of metabolism and slow growth rate observed at 4°C.

A number of genes associated with oxidative phosphorylation however showed significant activation in cold-adapted strains, suggesting a requirement for additional ATP in otherwise metabolically sluggish cells. During oxidative- phosphorylation, the membrane embedded F_oF_1–ATP synthases couple the movement of protons down an electrochemical gradient to the synthesis of ATP. A number of genes encoding various types of ATP synthases that in general showed increased expression in ATCC19115 and 70-1700 but less so in ScottA (Fig. 5.21).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.21 Expression response of genes associated with oxidative phosphorylation in three *L. monocytogenes* strains adapted to cold stress.

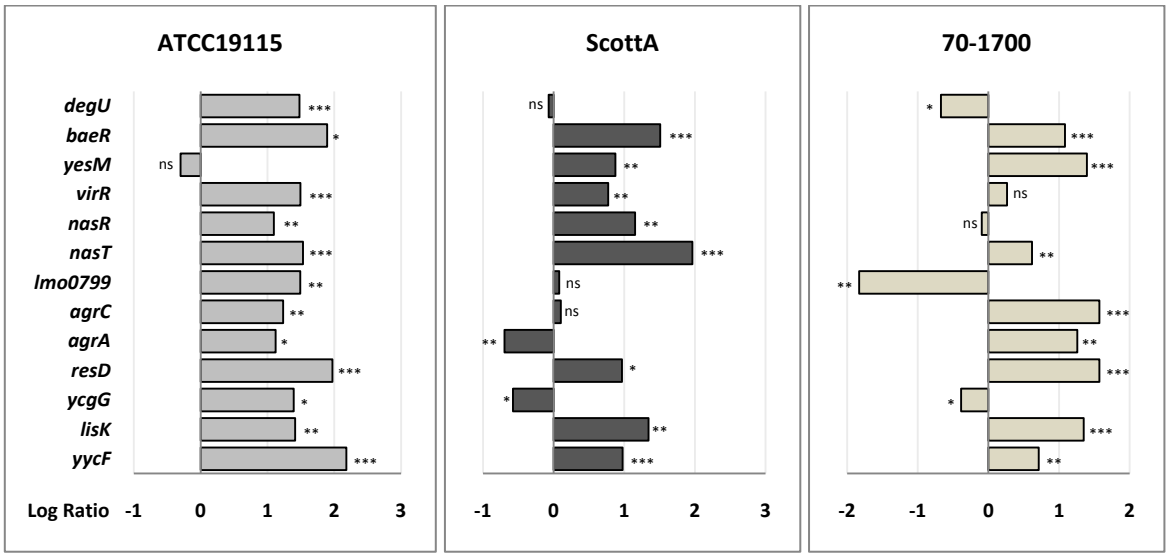
Significantly up-regulated genes in ATCC19115 and ScottA included a gene encoding NADH dehydrogenase *lmo2638* homologous to *ndh* in *E. coli*, however activation in strain 70-1700 was very weak (Fig. 5.21). A gene encoding protein similar to flavodoxin *lmo1710* also showed up-regulation in the three strains of *L. monocytogenes* (Fig. 5.21). Flavodoxins are flavin mononucleotide containing proteins involved in a variety of electron transfer reactions. Flavodoxin is not involved in oxidative phosphorylation, but indirectly involved in energy metabolism.

Psychrophilic microorganisms such as *Psychrobacter cryohalolentis* increased their cellular ATP concentration at decreased temperatures despite having slower growth rates, potentially catalysing the activity rate of a number of enzymatic reactions for which ATP is a substrate (Amato & Christner, 2009). An *E. coli* mutant capable of maintaining an elevated ATP concentration also demonstrated an increased cold tolerance during extended exposure to 0°C (Morrison & Shain, 2008). Potentially

increase in ATP synthesis in *L. monocytogenes* cells exposed to prolonged cold-stress acts as a catalyst of enzymatic reactions, as increase in substrate levels accelerate the rate of reaction in a forward direction.

5.3.1.11 Low-temperature adaptation induced changes to two-component signal transduction system.

A general strategy utilised by bacteria for efficient adaptation to environmental changes involves the reception and transmission of appropriate stimuli by signal transduction. Two-component signal transduction systems (TCSTS) allow for targeted gene expression in response to changes in the environment in order to ensure optimal growth. Although the overall TCSTS gene sets showed no significant activation in transcription (Table 5.1), there was sufficient evidence to suggest its partial involvement in late stage of the cold-adaptive response in *L. monocytogenes*. Individual gene expression revealed activation of a number of genes in all three strains, these included *baeR* (*lmo2422)*, *nasT* (*lmo1172)*, *resD* (*lmo1948*), *lisK* (*lmo1378*) and *yycF* (*lmo0287)* (Fig. 5.22).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.22 Expression response of genes associated with two-component signal transduction system in three *L. monocytogenes* strains adapted to cold stress.

LisK has been identified as an important regulator of listerial osmotolerance (Sleator & Hill, 2005) deletion mutant was reported to have increased sensitivity to acid, ethanol and oxidative stresses

(Kallipolitis & Ingmer, 2001). Whilst activation of *lmo0287* has previously been described in cold-activation response of *L. monocytogenes* strain10403S (Chan *et al.*, 2007b).

5.3.1.12 Low-temperature adaptation induced changes to numerous genes with unknown function.

Cold adapted strains of *L. monocytogenes* exhibited activation in a large number of genes encoding as yet unclassified proteins, these represented > 30% of all up-regulated genes observed in the three strains examined in the study. A significant proportion of these genes encoded proteins with a presumptive membrane associated functions such as *lmo0867*, *lmo2637*, *lmo0047*, *lmo1337*, *lmo1864, lmo0927* and *lmo1690* (Table 5.4).

Table 5.4 Expression response of genes with unknown function in three *L. monocytogenes* strains adapted to cold stress.

Gene	Presumptive function	ATCC19115		ScottA		70-1700	
		LR*	P#	LR	P	LR	P
lmo0047	predicted membrane protein	**2.12**	0.033	**1.88**	3.9E-04	**1.47**	9.8E-05
lmo0186	similar to uncharacterized conserved proteins	**4.61**	1.0E-06	**4.01**	2.3E-06	**3.69**	7.9E-07
lmo0189	similar to uncharacterized conserved proteins	**5.28**	2.1E-06	**4.37**	3.5E-07	**2.65**	1.3E-05
lmo0375	unknown protein	**1.62**	1.5E-04	**3.12**	1.7E-06	**1.15**	0.011
lmo0592	unknown protein	**2.86**	0.001	**2.07**	0.006	**1.93**	2.7E-04
lmo0802	similar to uncharacterized conserved proteins	**2.59**	9.1E-06	**1.72**	1.3E-04	**1.70**	7.5E-06
lmo0836	similar to phosphate-starvation-inducible protein E	**1.39**	0.007	**1.96**	1.1E-04	0.77	0.003
lmo0850	unknown protein	**2.72**	3.2E-05	**1.61**	0.005	**1.57**	0.002
lmo0867	predicted membrane protein	**1.92**	5.2E-05	**2.03**	1.5E-05	0.14	
lmo0950	similar to uncharacterized conserved proteins	**1.92**	0.001	0.56	0.030	0.87	0.001
lmo0952	unknown protein	**2.85**	3.4E-05	**1.56**	9.5E-05	-0.73	0.026
lmo0954	unknown protein	**3.99**	0.001	**1.84**	0.036	**1.57**	1.0E-05
lmo1001	unknown protein	**3.82**	1.6E-06	**2.98**	2.1E-06	-0.25	
lmo1007	unknown protein	**1.02**	0.006	**2.11**	4.3E-04	**1.40**	4.3E-04
lmo1008	unknown protein	**2.15**	0.001	**2.20**	0.002	**1.84**	4.1E-06
lmo1069	unknown protein	**3.12**	4.1E-06	**2.57**	3.6E-06	**1.08**	0.001
lmo1070	unknown protein	**1.41**	0.001	**2.23**	1.1E-04	**2.51**	1.6E-05
lmo1183	unknown protein	**2.09**	7.5E-05	**1.47**	3.2E-04	0.79	0.009
lmo1236	predicted hydrocarbon binding protein	**2.12**	3.1E-05	**1.19**	0.002	-0.18	
lmo1245	unknown protein	**1.41**	2.6E-04	**2.15**	1.0E-04	-0.05	
lmo1257	unknown protein	**1.32**	0.005	**2.43**	4.1E-05	0.99	0.002
lmo1266	unknown protein	**1.96**	6.2E-05	**2.18**	1.9E-04	**2.00**	4.3E-06
lmo1284	predicted membrane protein	**1.47**	2.6E-04	**1.05**	0.003	0.39	0.042
lmo1306	similar to uncharacterized conserved proteins	**3.80**	7.2E-05	**3.53**	0.001	**3.87**	4.6E-07

Gene	Presumptive function	ATCC19115		ScottA		70-1700	
		LR*	P#	LR	P	LR	P
lmo1333	similar to aminodeoxychorismate lyase family proteins	**4.21**	7.5E-07	**2.60**	4.3E-05	0.97	0.001
lmo1334	unknown protein	**4.57**	3.6E-06	**2.86**	3.2E-05	**1.97**	5.2E-06
lmo1337	uncharacterized membrane protein	**2.52**	1.6E-05	**1.51**	2.8E-04	0.85	0.001
lmo1338	unknown protein	**2.51**	4.0E-04	**1.03**	0.008	0.97	0.005
lmo1402	unknown protein	**1.84**	2.6E-04	**1.07**	4.0E-04	0.31	
lmo1432	unknown protein	**2.93**	1.4E-05	-0.04		0.45	
lmo1440	similar to uncharacterized conserved proteins	**2.30**	2.9E-05	**1.20**	0.002	**1.18**	0.001
lmo1486	similar to uncharacterized conserved proteins	**1.89**	1.8E-04	**1.69**	6.1E-05	**1.81**	5.2E-06
lmo1487	predicted HD superfamily hydrolase involved in NAD metabolism	**1.77**	7.3E-05	**1.38**	3.7E-04	0.93	0.001
lmo1495	similar to uncharacterized conserved proteins	**1.31**	3.7E-04	-0.12		**1.70**	0.006
lmo1528	unknown protein	**2.05**	8.7E-05	**1.62**	0.001	0.17	
lmo1541	predicted ribosomal protein	**2.60**	0.003	**1.60**		**2.42**	1.8E-06
lmo1626	unknown protein	**2.02**	2.9E-04	0.99	0.001	-0.28	
lmo1649	unknown protein	**1.74**	0.007	0.38		0.70	0.003
lmo1668	unknown protein	**3.50**	0.008	**1.04**	0.006	0.37	0.031
lmo1690	similar to predicted membrane-bound metal-dependent hydrolases	**3.18**	3.1E-04	**1.34**	0.011	0.83	0.004
lmo1707	unknown protein	**1.56**	0.008	**1.81**	9.3E-05	**1.26**	0.001
lmo1760	predicted phosphate-binding enzyme	**1.97**	6.1E-05	**2.08**	1.0E-04	**1.81**	4.7E-05
lmo1762	unknown protein	**2.23**	2.0E-05	**1.81**	6.9E-05	**1.93**	3.0E-04
lmo1828	similar to uncharacterized stress-induced protein	**1.92**	4.9E-04	**1.02**	0.004	0.12	
lmo1864	predicted membrane protein, hemolysin III homolog	**1.53**	0.002	**1.87**	8.3E-05	**1.09**	6.4E-05
lmo1870	putative membrane protein	**1.69**	1.8E-04	**1.08**	0.002	0.50	0.005
lmo1921	unknown protein	**1.44**	0.004	0.56	0.042	**2.66**	3.6E-06
lmo1941	similar to uncharacterized conserved proteins	**2.16**	3.3E-05	0.62	0.051	0.60	0.002
lmo1966	unknown protein	**2.22**	0.001	**1.19**	3.8E-04	**1.60**	0.001
lmo2048	similar to uncharacterized conserved proteins	**1.15**	0.003	**1.75**	0.001	**2.09**	6.1E-06
lmo2127	CAAX amino terminal protease family protein	**1.24**	0.018	**1.96**	0.005	-0.86	
lmo2187	unknown protein	**0.96**	0.002	**1.79**	2.5E-05	**1.23**	0.001
lmo2204	unknown protein	**2.30**	0.018	0.64		0.36	
lmo2210	unknown protein	**4.83**	2.3E-06	**3.58**	4.1E-06	0.00	
lmo2223	similar to uncharacterized conserved proteins	**2.28**	0.003	**1.89**	0.029	**1.10**	0.001
lmo2359	similar to predicted hydrolases of the HAD superfamily	**3.34**	2.5E-05	**3.42**	1.1E-04	-0.48	
lmo2487	similar to uncharacterized conserved proteins	**2.27**	0.001	**1.11**		**1.75**	4.5E-06
lmo2502	similar to uncharacterized conserved proteins	**1.86**	3.6E-04	0.93	0.018	0.07	
lmo2504	similar to membrane-bound metallopeptidases	**2.65**	1.0E-04	0.62	0.035	0.35	
lmo2508	similar to uncharacterized conserved proteins	**1.84**	1.6E-04	0.61		-0.04	
lmo2514	similar to uncharacterized conserved proteins	**1.91**	7.3E-05	0.31		-0.27	
lmo2522	similar to uncharacterized conserved proteins	**6.55**	9.8E-08	**4.40**	4.3E-07	**4.30**	1.6E-07
lmo2567	unknown protein	**1.99**	0.004	0.21		-0.02	
lmo2568	unknown protein	**2.19**	0.001	0.45		0.93	0.001
lmo2637	membrane-anchored lipoprotein	**2.38**	3.2E-05	**3.49**	1.6E-05	**1.69**	3.5E-05
lmo2692	unknown protein	0.22		**1.28**	0.022	**1.80**	3.7E-05

*LR represents Log Ratio values.
P-values not shown were >0.05

Another gene which showed up-regulation of ≥ two-fold in all three strains was *lmo1864* homologous to *yplQ* of *B. subtilis* encoding a putative integral membrane protein that is homologous to haemolysin III, which previously showed up-regulation in strain 10403S (Chan *et al.*, 2007b). Similarly a gene which has also been shown to increase transcription levels *in L. monocytogenes* under prolong cold temperature stress, *lmo2522* encoding an unknown protein (Chan *et al.*, 2007b), showed significant up-regulation of 93-fold in strain ATCC19115, 21-fold in ScottA and 20-fold in 70-1700. This gene is homologous to *yocH* of *B. subtilis* encodes a hypothetical cell membrane binding protein. Evidently involvement of these two gene products in cold-adaptation of *L. monocytogenes* is supported in both independent studies however the role it plays in this response is so far undefined.

Among other genes with large increases in expression *lmo0186*, which showed a 13 to 24-fold change (Table 5.4), encoded a protein similar to *B. subtilis* cell-wall binding protein YabE, activation of which has previously been described in cold-adapted *B. subtilis* (Budde *et al.*, 2006). A gene (*lmo0189*) homologous to the *B. subtilis* Veg protein showed 6- to 31-fold increased expression. The protein coded by *lmo0189* has an unknown function but was also reported to be apparently important in strain 10403S during exposure to cold-stress (Chan *et al.*, 2007b).

A gene similar to *yneF* in *B. subtilis* (*lmo1306*) exhibited 12 to 15- fold up-regulation. YneF has recently been characterised in *B. subtilis* as a DNA replication inhibitor (Rahn-Lee *et al.*, 2009) and has been previously reported as one of the late-induced proteins in cold-shock response of that organism (Kaan *et al.*, 2002). Mechanisms of controlling DNA replication and coordinating chromosome duplication with the cell cycle are crucial aspects of survival of microorganisms in stressful environments. Pausing DNA replication in metabolically sluggish cells grown in a cold environment until all the other cellular components are assembled may serve as a regulatory mechanism ensuring proper replication of DNA in cells exposed to such environments.

5.3.1.13 Low-temperature adaptation suppressed expression of genes associated with cell metabolism.

Low temperature has a profound synergistic effect on general enzymatic chemical reactions, with reducing temperature resulting in much slower reaction rates. Whether it is due to a reduction in substrate recognition and subsequent decrease in substrate binding, change in enzyme structure due to temperature effect on folding of proteins, or a combination of numerous factors; the end

result is a reduction in reaction rate which has shown to require more energy input to force reactions in forward direction.

Decreases in overall reaction rates renders an overall suppression of metabolism, resulting in slower rate in bacterial protein synthesis and cell division, observed in numerous mesophilic bacteria including *L. monocytogenes*.

5.3.1.13.1 Cold-stress adaptation suppressed expression of carbohydrate transporters and metabolism related genes.

Cold-adaptation overall repressed phosphotransferase system (PTS) transporter gene expression in all three strains of *L. monocytogenes* with *T-value* scores ranging from -3.7 for strain ScottA to -10.9 for ATCC19115 (Table 5.1) most likely due to an overall suppression of metabolism observed in cold-adapted cells.

Table 5.5 Expression response of genes associated with carbohydrate metabolism in three *L. monocytogenes* strains adapted to cold stress.

Gene	ATCC19115		ScottA		70-1700		Function
	LR*	P#	LR	P	LR	P	
cggR	**3.43**	2.1E-06	**1.57**	0.001	0.35	0.047	central glycolytic genes regulator
fruA	**1.24**	0.006	**-1.04**	0.017	**1.15**	0.001	similar to PTS system, fructose-specific IIABC component
gadA	-0.64	0.025	**-2.63**	2.5E-04	0.48	0.047	glutamate decarboxylase
gadB	**-4.26**	3.7E-04	**-4.69**	2.0E-06	**-2.63**	0.003	glutamate decarboxylase
gpmA	**-1.66**	1.1E-04	**-3.13**	1.3E-05	**-1.53**	4.5E-05	phosphoglycerate mutase
lmo0027	**-2.11**	4.0E-04	**1.74**	9.5E-05	0.56		similar to PTS system, beta-glucosides-specific IIABC component
lmo0105	**-2.80**	1.0E-05	**-2.01**	1.8E-05	**-1.85**	0.001	similar to chitinase B
lmo0401	**-3.25**	3.1E-05	0.40	0.035	-0.28		putative alpha-mannosidase
lmo0402	**-2.06**	0.002	0.29		**-2.72**	1.3E-06	fructose-specific PTS system operon regulator
lmo0425	**-1.46**	0.002	-0.89	0.004	**-1.89**	1.6E-04	mannose/fructose-specific PTS system operon regulator
lmo0429	**-1.07**	0.013	-0.23		-0.98	0.002	putative alpha-mannosidase
lmo0506	-0.92	0.008	**-1.45**	0.002	-0.91	2.1E-04	similar to polyol (sorbitol) dehydrogenase
lmo0529	**-1.44**	0.001	**-1.61**	1.9E-04	-0.83	0.004	similar to putative glucosaminyltransferases
lmo0539	-0.32		**-1.69**	2.4E-04	**-1.34**	0.009	tagatose 1,6-diphosphate aldolase
lmo0782	**-1.06**	0.015	**-2.13**	1.2E-04	**-1.45**	0.002	similar to PTS system, mannose-specific IIC component
lmo0813	**-1.73**	0.012	-0.18		0.15		fructokinase
lmo0873	-0.69	0.053	-0.38	0.204	**-1.98**	1.9E-05	putative cellobiose-specific PTS system operon regulator
lmo0875	**-1.48**	0.001	-0.14		**-2.03**	3.6E-05	similar to PTS system, cellobiose-specific IIB component
lmo0982	-0.31		**-1.41**	1.4E-04	0.11		similar to cellulase M and related proteins

Gene	ATCC19115		ScottA		70-1700		Function
	LR*	P#	LR	P	LR	P	
lmo1254	**-1.63**	0.005	**2.00**	1.4E-05	0.46	0.011	similar to alpha,alpha-phosphotrehalase
lmo1255	**-1.91**	0.002	**2.04**	1.2E-05	**1.59**	3.2E-05	similar to PTS system, trehalose-specific IIBC component
lmo1534	**-1.52**	0.004	-0.71	0.005	**-1.09**	9.2E-05	similar to L-lactate dehydrogenase
lmo1719	**-4.35**	2.5E-05	**-1.25**	0.001	**-1.90**	4.7E-06	similar to PTS system, lichenan/cellobiose-specific IIA component
lmo1720	**-3.83**	1.0E-04	**-1.37**	0.007	-0.20		similar to PTS system, lichenan/cellobiose-specific IIB component
lmo2096	**-1.47**	2.2E-04	**-1.15**	0.001	-0.15		similar to PTS system, galacitol-specific IIC component
lmo2098	**-1.17**	0.002	**-1.35**	0.002	0.19		similar to PTS system, galacitol-specific IIA component
lmo2213	**-1.01**	0.002	**-2.36**	4.1E-04	-0.32		similar to uncharacterized enzyme involved in biosynthesis of extracellular polysaccharides
lmo2253	-0.90	0.027	**-1.31**	0.005	-0.09		similar to phosphoglucomutases
lmo2401	-0.79	0.020	**-1.08**	0.003	**-1.52**	2.0E-05	similar to predicted sugar phosphatases of the HAD superfamily
lmo2649	**-3.20**	9.1E-05	0.46	0.022	**-1.28**	2.3E-04	similar to PTS system, mannitol/ascorbate-specific IIC component
lmo2650	**-2.36**	0.001	0.00		**-1.83**	6.7E-05	similar to PTS system, mannitol/ascorbate-specific IIB component
lmo2651	**-2.09**	0.001	0.83	0.073	-0.89	0.008	similar to PTS system, mannitol/ascorbate-specific IIA component
lmo2663	**-5.15**	0.004	-0.80	0.007	-0.62	0.006	polyol dehydrogenase
lmo2664	**-5.26**	0.002	**-1.23**	0.001	-0.90	4.7E-04	similar to sorbitol dehydrogenase
lmo2666	**-6.39**	2.0E-04	**-1.45**	0.002	**-1.17**	1.5E-04	similar to PTS system, galacitol-specific IIB component
lmo2667	**-4.60**	1.2E-04	**-1.09**	0.003	**-2.27**	9.6E-06	similar to PTS system, galacitol-specific IIA component
lmo2668	**-5.86**	1.9E-05	**-1.23**	2.7E-04	**-1.14**	7.5E-05	galacitol-specific PTS system operon regulator
lmo2683	**-2.26**	1.8E-04	0.85		**1.57**	3.7E-04	similar to PTS system, cellobiose-specific IIB component
lmo2684	**-4.14**	4.6E-06	**-1.11**	0.007	**-2.85**	1.8E-07	similar to PTS system, cellobiose-specific IIC component
lmo2685	**-6.00**	1.1E-05	**-1.42**	0.003	**-2.86**	3.8E-07	similar to PTS system, cellobiose-specific IIA component
lmo2697	**-2.54**	9.2E-05	**-3.48**	2.0E-05	**-2.48**	2.2E-06	putative PTS-dependent dihydroxyacetone kinase, phosphotransfer subunit
lmo2720	-0.72	0.023	**-2.47**	7.0E-05	-0.82	0.004	acetyl-CoA synthetase
lmo2733	**-2.21**	0.027	-0.08		-0.20		similar to PTS system, fructose-specific IIABC component
lmo2734	**-1.39**	0.004	-0.16		-0.64	0.008	putative alpha-mannosidase
lmo2780	**-2.53**	2.5E-04	-0.27		**-1.70**	3.5E-05	similar to PTS system, cellobiose-specific IIA component
lmo2781	**-1.58**	1.4E-04	-0.43		0.21		beta-glucosidase
lmo2782	**-4.60**	1.2E-04	-0.93	0.009	**-1.55**	0.001	similar to PTS system, cellobiose-specific IIB component
lmo2849	**-1.41**	0.011	-0.42	0.032	**-1.35**	3.8E-05	similar to rhamnulokinase
mptA	-0.25		**-2.57**	0.003	0.91	0.003	similar to PTS system, mannose-specific IIAB component
mptC	-0.13		**-2.25**	0.013	0.99	2.3E-04	similar to PTS system, mannose-specific IIC component
pdhA	**-2.60**	3.3E-04	**-2.86**	1.8E-06	**-1.10**	0.002	pyruvate dehydrogenase E1 component, alpha subunit
pdhB	**-2.95**	2.6E-05	**-2.54**	3.1E-05	**-1.81**	2.6E-04	pyruvate dehydrogenase E1 component, beta subunit
pdhC	**-3.28**	2.4E-06	**-2.64**	1.6E-05	**-1.72**	2.5E-05	pyruvate dehydrogenase E2 component (dihydrolipoamide acetyltransferase)
pdhD	**-2.66**	8.8E-06	**-2.03**	6.1E-05	**-1.68**	1.5E-04	dihydrolipoamide dehydrogenase, E3 subunit of pyruvate dehydrogenase complex

*LR-represents log ratio value.
P-values not shown were >0.05

Some of the strongest down regulated sugar transporters included those putatively associated with uptake of cellobiose/beta-glucosides, which collectively had a *T-value* score of -12 (P<0.001) for

ATCC19115, -6 (P<0.001) for strain 70-1700 and -3 (P<0.005) for ScottA. Highly repressed genes in this subgroup included *lmo2685*, *lmo2684* and *lmo1719*, ranging from 2- to 64-fold change in transcription (Table 5.5). The majority of all other sugar PTS transporter encoding genes also showed significant down-regulation, these included *lmo2666* and *lmo2667* in all three strains. However some exceptions were evident for PTS-related gene *treB* (*lmo1255*) for trehalose uptake which showed a 4- and 3- fold up-regulation in strains ScottA and 70-1700 respectively and a 4-fold down-regulation in strain ATCC19115; interestingly a gene *treC* (*lmo1254*) encoding trehalose-6-phosphate hydrolase which converts trehalose to glucose only showed significant 4-fold up-regulation in ScottA strain with no significant change in transcription observed for 70-1700 (Table 5.5) indicating a potential accumulation of trehalose by the latter strain. Intracellular accumulation of trehalose has been shown to prevent protein aggregation and denaturation also functioning as a membrane stabaliser in various organisms caused by a variety of stress conditions including desiccation, dehydration and cold temperature (Arguelles, 2000; Elbein *et al.*, 2003). In addition trehalose has been shown to act as a cryoprotectant in freeze-drying of *Lactococcus lactis* with intracellular levels correlating well with cell stress resistance (Termont *et al.*, 2006). It could therefore be speculated that accumulation of trehalose by cold-adapted cells of *L. monocytogenes* may assist in overcoming the adverse affects of cold stress on protein structure and stability of cell membranes. Strain variation in potential trehalose up-take is possible but unproven.

It appears that growth in a rich BHI broth at optimal temperature promotes active expression of many PTS transporters and other carbohydrate-transporting genes in *L. monocytogenes*. Following adaptation to cold conditions expression of these genes appears to be significantly reduced, which inevitably cascades to the energy production metabolism which also appeared to be significantly dampened. Gene sets associated with carbohydrate metabolism showed significant suppression in all three strains of *L. monocytogenes* although the magnitude of response varied significantly with each strain (Table 5.1). The Pdh operon, consisting of *pdhABCD* (*lmo1052-lmo1055*), was among the strongest cold-repressed glycolysis-related genes. A gene very similar to the *B. subtilis* glycolysis *gap* operon repressor gene *cggR* (Meinken *et al.*, 2003), *lmo2460* showed 11- and 3-fold up-regulation in strains ATCC19115 and ScottA respectively (Table 5.5), potentially responsible for the down regulation of glycolysis genes. Up-regulation of this particular repressor gene has previously been linked to presumed catabolism suppression in *L. monocytogenes* during exposure to high hydrostatic pressure (Bowman *et al.*, 2008).

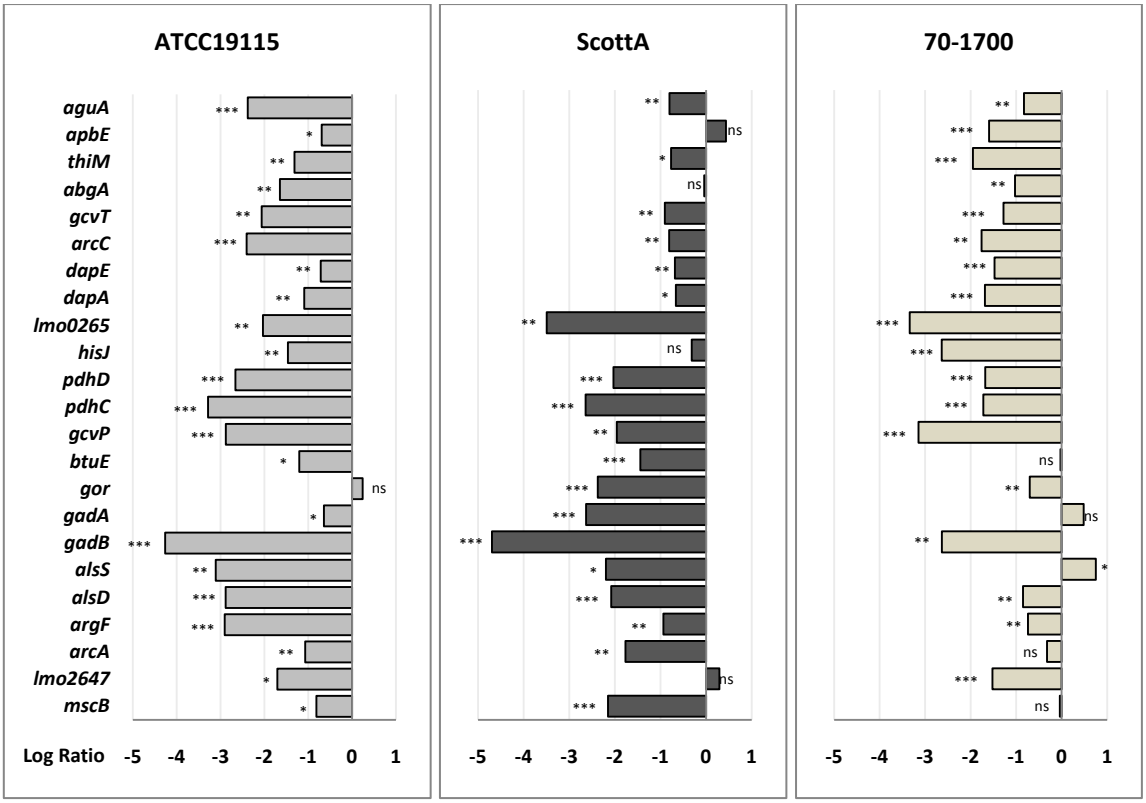
5.3.1.13.2 Cold-stress adaptation suppressed expression of amino acid metabolism related genes.

Genes associated with amino acid metabolism overall showed down-regulation in all three cold adapted strains of *L. monocytogenes*. Although individual *T-value* scores were weak collectively it was clear that metabolism of amino acids in cold adapted cells was significantly reduced compared to optimal control conditions.

One of the strongest down-regulated gene included *gadB* (*lmo2363*); the mRNA level of glutamate decarboxylase genes (*gadA* and *gadB*) were also found to be significantly low in *E. coli* cells grown at 15 °C or below (Yokoigawa *et al.*, 2003). Other down regulated genes included *gcvP* (*lmo1349*) and *lmo0265* homologous to *dapE* in *E. coli* (Fig. 5.23).

Transcription of *aguA* (*lmo0038*), *arcC* (*lmo0039*), *argF* (*lmo0036*) was down-regulated in all three strains, however interpretation of these cannot extend beyond ATCC19115 as these genes have been shown to not be universal in the listerial genome (Appendix A).

Generally suppression of amino acid metabolism in cold-adapted cells reflected the overall decrease in cellular metabolism and growth rates observed for cold cultivated strains.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

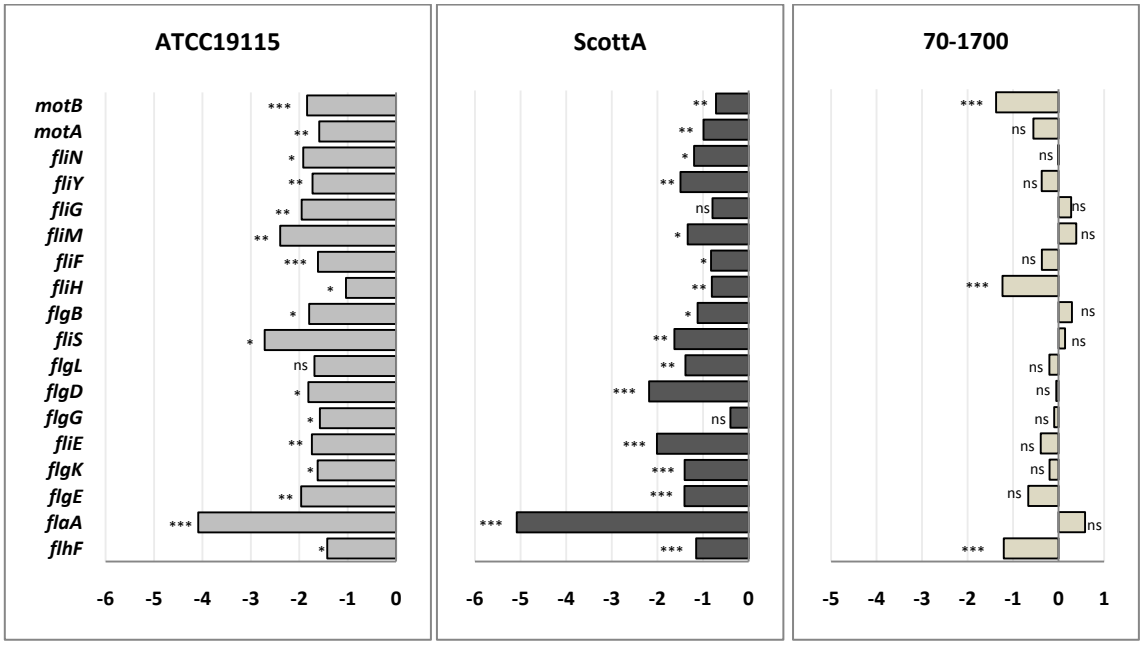
Figure 5.23 Expression response of genes associated with amino acid metabolism in three *L. monocytogenes* strains adapted to cold stress.

5.3.1.14 Low-temperature adaptation suppressed expression of genes associated with flagella assembly.

The expression of flagella-based motility in *L. monocytogenes* is regulated in response to growth temperature, circulating literature mainly particularly focuses on regulation of flagella repression at physiological temperature of 37°C. Regulation of motility in *L. monocytogenes* at 37°C occurs through direct repression of flagella genes by MogR (Shen & Higgins, 2006); at temperatures below 37°C a MogR anti-repressor, GmaR binds directly to MogR thus antagonising repression of flagella gene transcription(Shen *et al.*, 2006). Production of GmaR requires DegU, a protein which is also necessary for proper assembly of flagella (Kamp & Higgins, 2009).

At 4°C transcription of flagella genes appeared to be significantly down-regulated in strains ATCC19115 and ScottA with *T-value* scores of -6.51 and -6.70 respectively (Table 5.1); however in strain 70-1700 this trend was not statistically significant.

The most strongly down-regulated flagella genes was flagellin encoding *flaA* (*lmo0690*) which showed a 17- and 34- fold repression of transcription in strains ATCC19115 and ScottA respectively (Fig. 5.24). Other strongly down-regulated genes included *fliE* (*lmo0712*), *flgD* (*lmo0696*) and *fliS* (*lmo0708*) amongst others (Fig. 5.24). The majority of flagella encoding genes of strain 70-1700 showed no significant change in transcription with only genes *flhF* (*lmo0681*), *fliH* (*lmo0715*) and *motB* (*lmo0686*) exhibiting significant down-regulation.



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.24 Expression response of genes encoding flagella associated proteins in three *L. monocytogenes* strains adapted to cold stress.

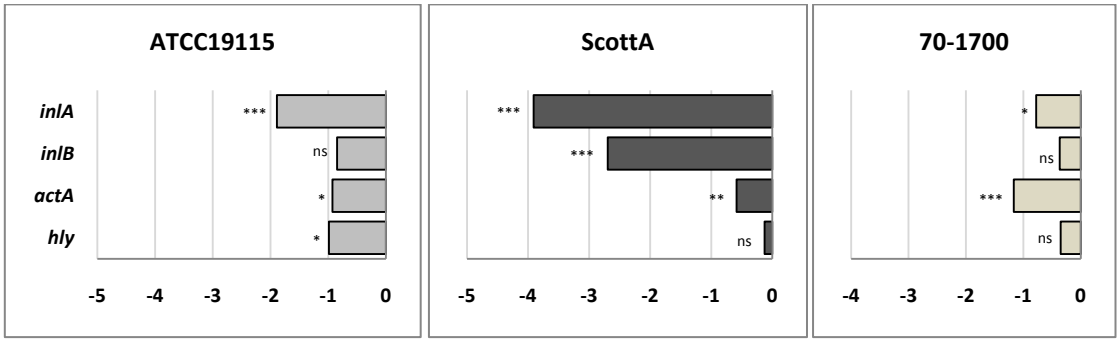
Transcription repression regulation at 4°C is unclear with genes encoding both GmaR and MogR, *lmo0688* and *lmo0674* respectively showing no change in transcription in any of the three strains. Activation of 3-fold was observed for *lmo2515* in strain ATCC19115 encoding a two-component response regulator DegU, with no change in transcription observed for either ScottA or 70-1700. Flagella motility is highly energetically demanding which might be of significant disadvantage for cells struggling to preserve energy for metabolic processes vital for cell survival at low temperature. It is possible that down-regulation of flagella assembly in *L. monocytogenes* at 4°C is controlled by temperature-independent regulation, possibly through cellular energy sensors. The CodY regulator has been shown to repress flagella synthesis in *B. subtilis* (Bergara *et al.*, 2003) and also *L. monocytogenes* (Bennett *et al.*, 2007). Overall, suppression of genes associated with flagella

synthesis observed in *L. monocytogenes* strains during growth at low temperature is most likely linked to the activation of CodY regulon described below in section 5.3.1.16.

Interestingly a strong down-regulation of *uspA* homologues (*lmo0515*, *lmo1580*, *lmo2673* decrease up to 13-fold) was observed in all three cold-adapted *L. monocytogenes* strains. Although the specific function of uspA-like is not defined in *L. monocytogenes* these have been linked to adjusting intracellular metabolism in *E. coli* under various stress conditions as well as regulation of motility (Nachin *et al.*, 2005). It is tempting to speculate that these may have similar regulatory functions in *L. monocytogenes*, thus adjusting cellular motility according to energy requirement of the cell, however further investigations are required to evaluate an existence of such involvement.

5.3.1.15 Low-temperature adaptation suppressed expression virulence-related genes.

Adaptation to cold-temperature reduced transcription of virulence genes, however the magnitude of down-regulation observed was relatively weak most likely due to already weak expression in control cells that were incubated at 25°C. Cellular energy conservation is most likely the reason behind virulence gene suppression observed in bacteria at non physiological temperature, as transcription of these would utilise energy with no apparent benefit to the cell. Internalin A encoding gene *inlA* was among the strongest down-regulated virulence related genes observed in cold adapted strains (Fig. 5.25); decrease in transcription at low temperature of which has previously been observed in this organism (McGann *et al.*, 2007).



*=significance level of Log Ratio for each gene * p<0.05; ** p<0.01,*** p<0.001, ns p>0.05

Figure 5.25 Expression response of genes encoding flagella associated proteins in three *L. monocytogenes* strains adapted to cold stress.

5.3.1.16 Low-temperature adaptation effect on regulatory gene sets.

Cold-adaptation did not induce a SigB response in any of the three strains tested; genes positively influenced by SigB collectively showed significant down-regulation with *T-value* scores of -5.43 for ATC19115, -19.37 for ScottA and -5.92 for strain 70-1700 (Table 5.7). A large number of genes either directly or indirectly activated by SigB showed strong down-regulation in cold-adapted cells, these included *dapD* (*lmo0265*), *uspF* (*lmo1580*), *rpiB* (*lmo2674*), *pdhB* (*lmo1053*), *pdhA* (*lmo1052*), *lmo2454*, *gpmA* (*lmo2205*) and *glpF* (*lmo1539*). In addition genes repressed by SigB (total of 130 genes) showed significant up-regulation in cold-adapted strains with *T-value* scores ranging from 6.18 in ATCC19115 to 8.96 in strain 70-1700 (Table 5.7). Some of the highly activated genes directly or indirectly repressed by SigB included *rpsP* (*lmo1797*), *lmo2522*, *lmo1306*, *rpmF* (*lmo0486*), *secE* (*lmo0245*), *acpA* (*lmo1806*), *cspL* (*lmo1364*), *prsA* (*lmo2219*) and *ybiT* (*lmo1431*).This further accredits an observation of *L. monocytogenes* 10403S σ^B null mutant which had no cold growth defect in rich BHI medium (Chan *et al.*, 2007a) suggesting that SigB stress response is not activated in nutrient abundant environment in cold-adapted cells of *L. monocytogenes*.

Table 5.6 Regulon expression responses in *L. monocytogenes* strains adapted to cold-temperature.

Regulons	ATCC19115		ScottA		70-1700	
	T-value*	P-value#	T-value*	P-value#	T-value*	P-value#
SigB-[¥]	**2.78**	0.006	**6.75**	4.6E-10	**7.20**	4.6E-11
SigB+[¥]	**-5.43**	1.7E-07	**-19.37**	2.2E-46	**-5.92**	1.5E-8
PrfA+	**-2.58**	0.012	**-12.36**	1.3E-18	**-3.80**	3.3E-04
CodY-	**-8.00**	6.8E-12	**-3.46**	0.001	**-1.63**	
HrcA+	**3.94**	3.9E-04	**2.87**	0.007	**6.35**	3.1E-07
HrcA-	**-1.38**		**-3.39**	0.015	**1.33**	
RpoN-	**-4.20**	9.0E-05	**-2.68**	0.009	**-1.06**	
RpoN+	-0.79		**-1.60**		**0.10**	
CtsR-	-0.20		**-1.89**		**-1.30**	
VirR+	-0.04		**1.63**		**1.96**	

*T-value scores were determined from the expression data using the approach of Boorsma *et al*. (2005).
[#] P-values not shown are >0.1
[¥]The gene sets for the given regulatory proteins are designated from empirical deletion studies on the basis of what genes are positively influenced ("+") or negatively influence ("-"), The influence of the regulators in many cases are not direct thus T-profile data only gives a general trend in terms of expression responses.

Directly influenced by suppression of SigB regulon was an evident down-regulation of a relatively small number of PrfA influenced genes (Milohanic *et al.*, 2003) with *T-values* ranging from -2.58 for ATCC19115 to -12.36 for ScottA (Table 5.7). *Listeria monocytogenes* SigB and PrfA are pleiotropic transcriptional regulators that co-regulate a subset of virulence genes through complex network interactions (Ollinger *et al.*, 2009). PrfA itself is the main regulator of virulence in *L. monocytogenes* (Milohanic *et al.*, 2003) and together with SigB is also involved in controlling other specific transport and metabolic genes, including many that have as yet undefined function. There is an overlap of 47 genes being positively controlled by both regulators thus simultaneous strong down-regulation observed for both regulons is not surprising. Some of the stronger down-regulated genes under dual influence included *lmo0994*, *inlA* (*lmo0433*), *manY* (*lmo0782*), *dhaM* (*lmo2697*), *dhaK* (*lmo2695*) and *uspF* (*lmo2673*). Genes positively controlled by an additional virulence regulator, VirR, overall showed no significant change in transcription.

Perhaps relevant to changes in SigB and PrfA regulon expression, it was also observed that cold-adaptation influenced the expression of genes within the HrcA regulon which in a complex interaction with other transcriptional regulators contributes to transcription of stress response genes (Hu *et al.*, 2007a). Genes directly regulated by HrcA showed activation in cold-adapted cells of *L. monocytogenes* strains examined with *T-value* scores ranging from 2.86 to 6.35 (Table 5.7). Some of the strongly activated genes directly controlled by HrcA included *yceD* (*lmo2048*), ribosomal proteins *rplU* (*lmo1542*), *lmo1541*, *rplA* (*lmo0249*) and *rplK* (*lmo0248*) as well as *rplM* (*lmo2597*), *rpmI* (*lmo1784*) and *infC* (*lmo1785*) which are activated by HrcA and repressed by SigB. Genes directly repressed by HrcA collectively only showed down-regulation in strain ScottA (Table 5.7). Some of the down-regulated genes repressed by HrcA included *dnaK* (for ATCC19115 and ScottA), and *DnaJ lmo0669*, and *lmo0670* (ScottA only).

Genes negatively controlled by an alternative sigma factor Sig-54, RpoN, showed down-regulation (Table 5.7) with *pdhD* (*lmo1055*) and *lmo1538* directly down-regulated by RpoN showing strong repression as well as *pdhB* (*lmo1053*) and *glpD* (*lmo1293*) regulation of which is also induced by SigB. It is however unclear whether this regulator contributes to transcription regulation in cold adapted cells as the genes positively controlled by RpoN overall showed no significant change (Table 5.7). RpoN is thought to be involved in cold shock response of *B. subtilis* (Wiegeshoff *et al.*, 2006). RpoN

regulation is thought to only regulate carbohydrate metabolism in *L. monocytogenes* EGD-e, mainly by controlling expression of the *mpt* PTS operon (Arous *et al.*, 2004).

CodY regulon (Bennett *et al.*, 2007) appeared to be activated by cold-adaptive response in *L. monocytogenes* magnitude of this response however was somewhat muted possibly due to interference of other regulators as not all genes are under direct control of CodY. Genes that have an increased expression in a *codY* null mutant (Bennett *et al.*, 2007) repression of which is regulated by CodY showed down-regulation in cold-adapted strains (Table 5.7). In *L. monocytogenes*, repressor CodY controls genes associated with amino acid biosynthesis, carbohydrate metabolism and transport, nitrogen uptake, motility and chemotaxis (Bennett *et al.*, 2007), most of which showed significant down-regulation in cold-adapted cells of *L. monocytogenes* strains examined. A number of genes directly regulated by CodY showed down-regulation these included *gatB* (*lmo2666*), *gatD* (*lmo2664*), *ydjL* (*lmo2663*), *motB* (*lmo0686*) and *lmo0709*, as well as *fliH* (*lmo0715*) which is under dual regulation with SigB. CodY acts as a sensor of the energetic capacity and nutritional status of the cell (Sonenshein, 2005). Repression of CodY activated genes observed suggests that cold temperature imposes restrictions on intracellular energy availability, leading to suppression of some energy consuming processes such as flagella biosynthesis.

5.4 Summary and Conclusions

Overall cold-adaptation to 4°C strongly activated gene sets associated with ribosomes, fatty acid and peptidoglycan biosynthesis and cell division. This suggests that low-temperature strongly compromises protein synthesis by directly affecting the stability of ribosomes. To compensate, *L. monocytogenes* respond by increasing transcription of ribosome related genes thus potentially restoring impaired protein synthesis to a level sufficient to sustain undisturbed cellular metabolic function. Gene sets associated with peptidoglycan and fatty acid biosynthesis processes were activated in cold-adapted cells, possibly as a means of counteracting the known effect of cold temperature on cell membrane stability. Transcription levels of genes linked to cell division were evidently enhanced, most of these, however, appear to restrain premature cell replication.

There was evidence of increase requirement for chaperone molecules associated with transcription suggested by strong induction of genes such as *tig*, *cspL* and *typA*. Cold adaptation also induced oligopeptide transporter *opp* genes, as well as compatible solute transporter encoding genes in two of three strains examined. These showed significant variation between strains, perhaps reflecting strain-specific preference for certain compatible solutes.

As a reflection of an overall cellular metabolism at low-temperature gene sets associated with carbohydrate and animo acid metabolism were suppressed in cold-adapted *L. monocytogenes* cells. Overall, low temperature failed to induce a SigB regulatory response, suggesting that this major stress response regulator is not involved in the maintenance of a cryo-adapted state. However there was evidence of CodY operon involvement most likely associated with regulation of intracellular energy distribution, leading to suppression of some energy consuming processes such as flagella biosynthesis.

Down-regulation of flagella assembly genes, suggested decreased motility of this organism in a cold environment, a phenomenon previously overlooked in this organism. Investigations concerning changes in motility, induced by low-temperature and NaCl, are reported in chapter 6.

Chapter 6

Evaluation of temperature and salinity effect on motility of selected *Listeria monocytogenes* strains.

Flagella motility is an important attribute of *Listeria monocytogenes* especially when it is in its active saprophytic lifestyle phase and also influences its virulence. However, there is little specific information about the effect of environmental stress on flagella motility between different strains. This investigation evaluated the effect of low-temperature and salinity on motility of seven *L. monocytogenes* strains using a qualitative and a quantitative approach depending on reduction of the metabolic dye 2,3,5-triphenyltetrazolium chloride. The observed motility was strain specific, with ATCC19115, LO28 and 86-3009 exhibiting greater motility in almost all experimental treatments. Optimum motility was observed at 25°C in brain heart infusion semisolid agar, with average motility zones of 13.5 (±4.5) mm. Motility decreased with lowering of temperature to an average zone of 10.8 (±5.2) mm at 15°C and 3.7 (±0.7) mm at 4°C. Salt concentration had a strong inhibitory effect on motility at all temperatures. Motility declined from an average of 13.8 (±4.5) mm on un-supplimented BHI agar (BHIA) to 5.8 (±1.5) mm 3.0% (w/v) NaCl BHIA at 25°C, with no motility observed on 5.0% (w/v) NaCl BHIA. The negative effect of low-temperature and salinity on motility of *L. monocytogenes* strains correlated with suppression of flagella assembly associated genes as revealed by transcriptome data.

6.1 Introduction

Flagella motility is a highly advantageous, but energetically demanding survival mechanism utilised by bacteria. Flagella function as a motility device allowing the bacterium to move towards, or retreat from specific environmental conditions, through the process of chemotaxis, thus maintaining the capacity to grow (Armitage, 1999).

Listeria monocytogenes produces five to six peritrichous flagella (O'Neil & Marquis, 2006). The motility of *L. monocytogenes* has been shown to be reduced at temperatures below 37°C. The suppression of motility in *L. monocytogenes* at 37°C occurs through direct repression of flagella biosynthesis genes by MogR (Shen & Higgins, 2006); at temperatures below 37°C a MogR anti-

repressor, GmaR binds to MogR, thus antagonising repression of flagella gene transcription(Shen *et al.*, 2006). Production of GmaR requires DegU, a protein which is also necessary for proper assembly of flagella (Kamp & Higgins, 2009).

Most published literature on flagella proteins and their transcriptional regulation address the involvement of flagella in *L. monocytogenes* virulence. There is limited information, however, regarding the adverse effects of environmental stress such as cold-temperature and especially NaCl on motility of *L. monocytogenes* particularly in nutrient rich environment such as in brain heart infusion (BHI) medium. To correlate the observed flagella structural and biosynthesis-associated gene expression (section 5.3.1.14 and 3.3.2.8) with the physical motility of cells in this study the effect of various levels of NaCl and temperature on the phenotypic motility response of seven *L. monocytogenes* strains from a transcriptomic perspective, was investigated.

6.2 Methods

6.2.1 Strains

Strains used in this study included Scott A and ATCC19115, both serotype 4b and of clinical origin; FW03-0035, serotype 4b, isolated from food; LO28 serotype 1/2c, of clinical origin, 76-1854 serotype 4b from bovine origin; 86-3009 serotype 1/2a from caprine origin and strain 70-1700, serotype 4e of ovine origin. All strains were obtained from the TIAR collection and were stored at -80°C in brain heart infusion (BHI) broth (Oxoid, CM0225B) containing 15.0% (v/v) glycerol.

6.2.2 Media and Growth Conditions

6.2.2.1 Qualitative motility evaluation

6.2.2.1.1 0.2% Semi-solid BHI agar preparation.

BHI broth was reconstituted according to manufacturer's standards (37g in 1L of dH_2O) and supplemented with 0.2% bacteriological agar (Oxoid, LP0011). Various amounts of NaCl were added to the media to achieve salinity levels of 3.0%, 4.0%, 5.0% , 6.0% , 8.0% , 10.0% and 12.0% (w/v) equivalent to 0.5M, 0.7M, 0.9M, 1.0M, 1.4M, 1.7M and 2.0M respectively, prior to sterilisation by autoclaving for 15 minutes at 121°C. BHI agar was allowed to cool to approximately 55°C and further supplemented with a filter sterilised solution of 2,3,5-triphenyltetrazolium chloride (TTC)

solution(Sigma-Aldrich) to achieve a final concentration of 0.05g/L. Six millilitres of this medium were aseptically dispensed into screw-cap sterile glass tubes and allowed to set in the dark.

6.2.2.1.2 Inoculation of test medium and assessment of motility.

Agar tubes were inoculated using one colony of freshly cultured (24hr, 25°C) strains on BHIA by stabbing the centre of the column of medium to approximately half the depth with a sterile straight wire loop. These were incubated in four temperature conditions 4°C, 15°C, 25°C and 37°C in the dark for time intervals ranging from 48 hours to 14 days depending on the conditions, until a visible red coloration was evident in the original line of inoculation. Motility was indicated by a visible red turbidity extending out from the line of stab inoculation; while negative motility was characterised by visible red colour change only along the line of inoculation (Fig. 6.1).

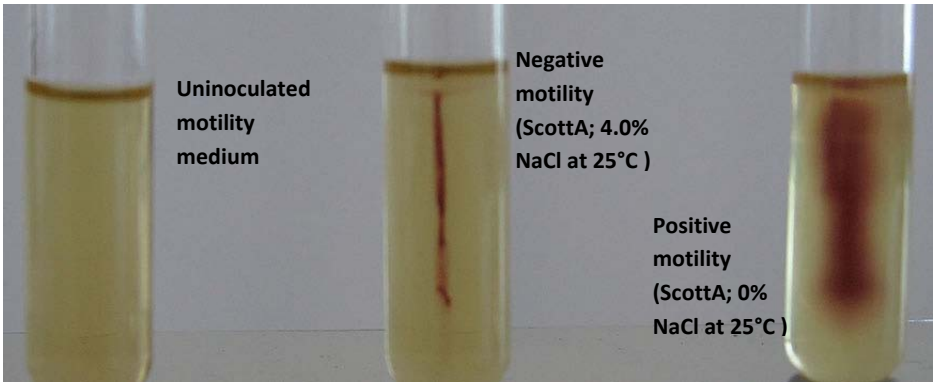


Figure 6.1 Example of motility tubes used in the experiment and subsequent interpretation.

6.2.2.2 Quantitative motility evaluation

6.2.2.2.1 0.3% Semi-solid BHI agar preparation.

BHI broth containing various levels of NaCl and a final concentration of 0.05g/L of TTC was prepared as described in section 6.2.2.1.1 except that the media were supplemented with 0.3% bacteriological agar. Plates were poured with minimum light exposure and stored at 4°C, in the dark.

6.2.2.2.2 Plate inoculation and assessment of motility.

Plates were inoculated with a single colony (24 hr growth on BHIA at 25°C) of one strain, with a maximum of four inoculations per plate. A modified stab-technique was implemented for inoculation using the sterile pointed end of a disposable 100 µl pipette tip. Each strain was tested in

triplicate at various salt concentrations (BHI, 3.0%, 4.0%, 5.0%, 6.0%, 8.0%, 10.0% (w/v) NaCl) with the exception of strain 70-1700 which was not inoculated at salinity greater than 8.0% (w/v) (based on previous NaCl tolerance study) and strains Scott A and FW03-0035 which were additionally inoculated in a 12.0% NaCl containing BHIA + 0.05g/L TTC. Plates were incubated at four temperatures (4°C, 15°C, 25°C and 37°C), in the dark for various time intervals ranging from 48 hours to 14 days depending on the temperature and salinity, until a visible red colour was observed on the original stab. The diameter of the bacterial zone of motility, as represented by the extent of red coloration in the agar due to chemical reduction of the TTC by the bacteria, was measured in millimetres at the widest point that was attained during the incubation. The average diameter of motility zones (MZ) was calculated with an absolute minimum of 1mm which corresponded to the size of the original stab, indicating zero motility.

6.3 Results

6.3.1 Qualitative results.

L. monocytogenes strains were motile at temperatures ranging from 4°C to 25°C and non-motile at 37°C (Table 6.1).

Table 6.1 Motility of *L. monocytogenes* strains in BHI agar supplemented with additional NaCl at 4°C, 15°C, 25°C and 37°C.

4°C	BHI	3%	4%	5%	6%	8%	10%	12%
ATCC19115	+	-	-	-	-	-	-	NA
ScottA	+	-	-	-	-	-	-	-
FW03-0035	+	-	-	-	-	-	-	-
LO28	+	-	-	-	-	-	-	NA
70-1700	+-	-	-	-	-	-	NA	NA
76-1854	+	-	-	-	-	-	-	NA
86-3009	+	+-	-	-	-	-	-	NA

15°C	BHI	3%	4%	5%	6%	8%	10%	12%
ATCC19115	+	+-	-	-	-	-	-	NA
ScottA	+	+-	-	-	-	-	-	-
FW03-0035	+	+-	-	-	-	-	-	-
LO28	+	+-	-	-	-	-	-	NA
70-1700	+	-	-	-	-	-	NA	NA
76-1854	+	-	-	-	-	-	-	NA
86-3009	+	+-	-	-	-	-	-	NA

25°C	BHI	3%	4%	5%	6%	8%	10%	12%
ATCC19115	+	+	-	-	-	-	-	NA
ScottA	+	+	-	-	-	-	-	-
FW03-0035	+	+	-	-	-	-	-	-
LO28	+	+	-	-	-	-	-	NA
70-1700	+	+	-	-	-	-	NA	NA
76-1854	+	+	-	-	-	-	-	NA
86-3009	+	+	-	-	-	-	-	NA

37°C	BHI	3%	4%	5%	6%	8%	10%	12%
ATCC19115	+-	-	-	-	-	-	-	NA
ScottA	-	-	-	-	-	-	-	-
FW03-0035	-	-	-	-	-	-	-	-
LO28	-	-	-	-	-	-	-	NA
70-1700	-	-	-	-	-	-	NA	NA
76-1854	-	-	-	-	-	-	-	NA
86-3009	-	-	-	-	-	-	-	NA

Abbreviations: + indicates motility; +- indicates weak motility; - indicates absence of motility; NA, not tested.
BHI- Brain Heart Infusion medium with no additional NaCl; % NaCl- total salinity level (w/v) in BHI.

Interpretation of motility was not always straight forward with some strains showing weak motility which was slightly greater than observed for the corresponding negative control e.g., for strain 86-3009 in 3.0% (w/v)NaCl BHI at 4°C (Fig. 6.2 far right).

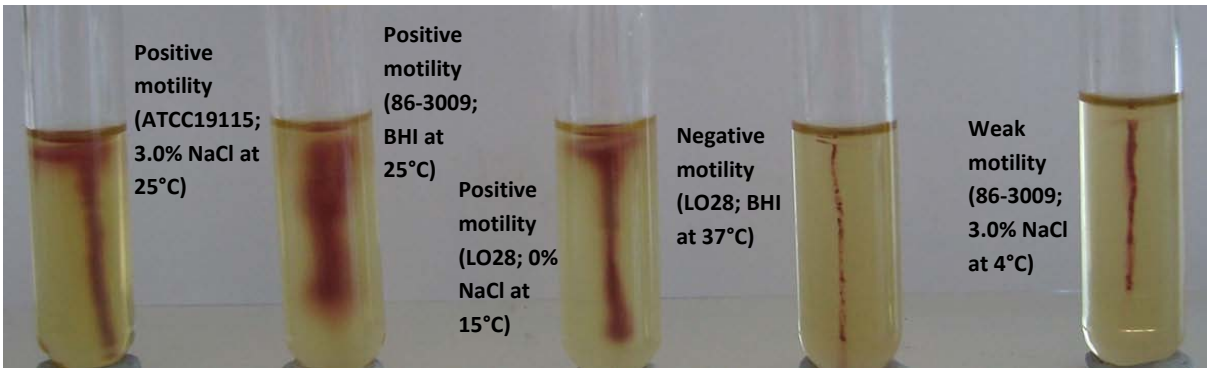


Figure 6.2 Examples of motility observed for a number of *L. monocytogenes* strains at various NaCl concentrations and at different temperature conditions.

Motility appeared to be relatively sensitive to addition of NaCl to the growth medium with reduced motility evident with as little as 3.0% (w/v) NaCl, depending on temperature conditions (Table 6.1).

Tube motility is a qualitative method that is a useful tool in screening for motility in *L. monocytogenes*, however it is a poor method for accurate comparisons between the motility capacity of different strains.

6.3.2 *Quantitative results.*

In order to compare individual strain response a novel quantitative approach was undertaken to estimate the level of motility expressed in terms of zone sizes (Fig. 6.3 and Fig. 6.4).

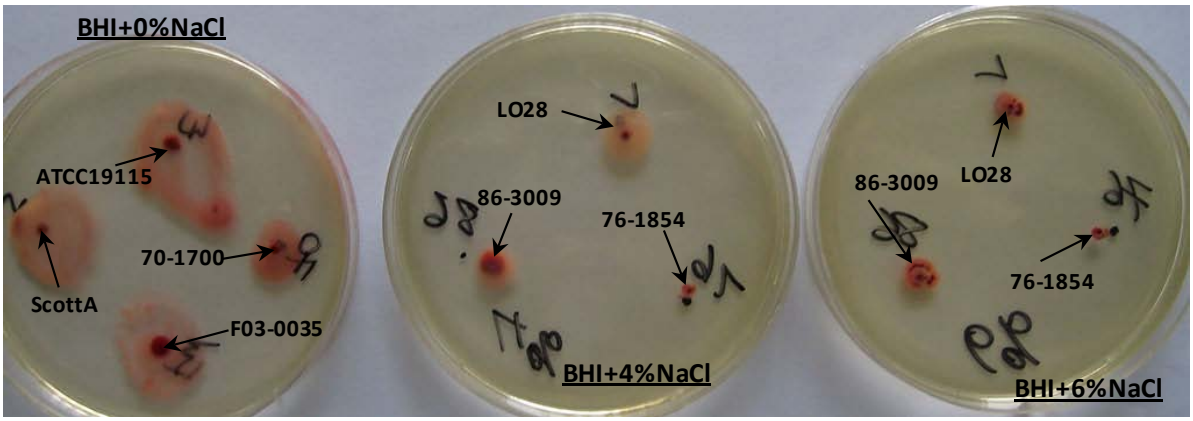


Figure 6.3 Examples of motility zones observed at 25°C for selective strains at different salinity conditions.

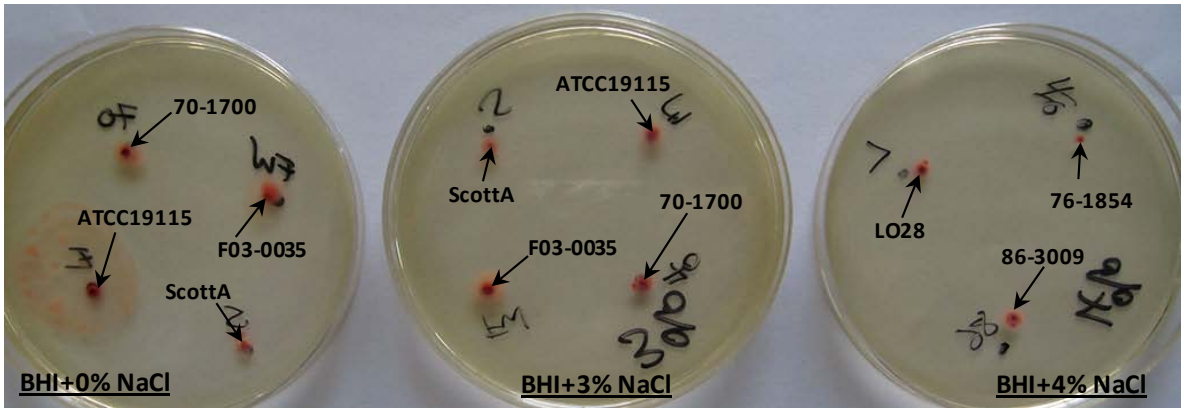


Figure 6.4 Examples of motility zones observed at 15°C for selective strains at different salinity conditions.

Motility was temperature-dependent with most strains exhibiting very minimal zone sizes at 37°C in unsupplemented BHI (Table 6.2); with an average zone size of 2.4 mm. This corresponds to an average 1.4 mm migration through the agar as the original inoculum size was 1mm in diameter. It is possible that the small zone size observed at this temperature may be artificial, resulting from initial migration of the organism directly following inoculation at room temperature before the medium reached a desired 37°C. Small zone sizes were also artefacts of colour change as the yellow dye 2,3,5-triphenyltetrazolium chloride (TTC) is reduced to produce red TTC formazan pigment, indicating a presence of metabolically active bacteria. A motility zone size of <3.0 mm was therefore considered as non-motile.

Table 6. 2 Average motility zone sizes (mm) at 37°C as influenced by medium salinity.

37°C	BHI	3.0%	4.0%	5.0%	6.0%	8.0%	10.0%	12.0%
ATCC19115	2.8 *(±0.3)*	2.5 *(±0.5)*	1.8 *(±0.3)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA
ScottA	2.5 *(±0.5)*	2.2 *(±0.3)*	1.7 *(±0.3)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*
FW03-0035	2.7 *(±0.3)*	2.3 *(±0.3)*	1.8 *(±0.3)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*
LO28	2.7 *(±0.6)*	2.0 *(±0.0)*	1.3 *(±0.3)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA
70-1700	1.3 *(±0.3)*	1.2 *(±0.3)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA	NA
76-1854	1.7 *(±0.3)*	1.5 *(±0.0)*	1.2 *(±0.3)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA
86-3009	2.8 *(±0.3)*	2.2 *(±0.3)*	1.8 *(±0.3)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA

Motility zone size of <3.0 = negative motility. NA- not tested. Values in brackets represent standard deviations.
BHI- Brain Heart Infusion medium with no additional NaCl; % - total salinity level (w/v) in BHI.

Table 6. 3 Average motility zone sizes (mm) of various strains observed on unsupplemented BHI agar plates.

	37°C	4°C	15°C	25°C
ATCC19115	2.8 *(±0.3)*	4.0 *(±1.0)*	15.7 *(±1.5)*	17.3 *(±2.1)*
ScottA	2.5 *(±0.5)*	3.3 *(±0.3)*	6.3 *(±1.5)*	14.0 *(±2.0)*
FW03-0035	2.7 *(±0.3)*	3.3 *(±0.8)*	9.7 *(±0.6)*	14.0 *(±1.8)*
LO28	2.7 *(±0.6)*	4.0 *(±0.5)*	15.3 *(±1.2)*	17.3 *(±2.6)*
70-1700	1.3 *(±0.3)*	3.0 *(±1.0)*	6.7 *(±0.6)*	8.3 *(±2.0)*
76-1854	1.7 *(±0.3)*	3.2 *(±0.8)*	4.5 *(±0.5)*	7.0 *(±1.0)*
86-3009	2.8 *(±0.3)*	5.0 *(±1.0)*	17.3 *(±2.3)*	18.3 *(±1.3)*

Values in brackets represent standard deviations.

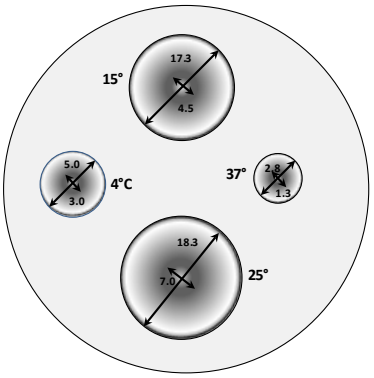


Figure 6.5 Schematic representation of average max and min motility zones (BHI+ 0% NaCl).

Table 6. 4 Average motility zone sizes (mm) at 25°C influenced by medium salinity.

25°C	BHI	3.0%	4.0%	5.0%	6.0%	8.0%	10.0%	12.0%
ATCC19115	17.3 *(±2.1)*	6.0 *(±1.0)*	3.3 *(±0.3)*	2.7 *(±0.6)*	2.3 *(±0.6)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA
ScottA	14.0 *(±2.0)*	6.7 *(±1.5)*	2.3 *(±0.6)*	1.3 *(±0.6)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*
FW03-0035	14.0 *(±1.8)*	6.3 *(±1.0)*	3.0 *(±1.0)*	2.7 *(±0.6)*	2.3 *(±0.8)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*
LO28	17.3 *(±2.6)*	6.7 *(±1.5)*	3.3 *(±1.0)*	2.7 *(±0.6)*	2.3 *(±0.6)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA
70-1700	8.3 *(±2.0)*	5.0 *(±1.0)*	2.5 *(±1.0)*	1.5 *(±0.5)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA	NA
76-1854	7.0 *(±1.0)*	2.7 *(±0.6)*	1.7 *(±0.6)*	1.3 *(±0.6)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA
86-3009	18.3 *(±1.3)*	7.0 *(±1.0)*	3.3 *(±1.3)*	2.7 *(±0.8)*	2.3 *(±0.6)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA

Motility zone size of <3.0 = negative motility. NA- not tested. Values in brackets represent standard deviations.
BHI- Brain Heart Infusion medium with no additional NaCl; % - total salinity level (w/v) in BHI.

Table 6.5 Average motility zone sizes (mm) at 15°C influenced by medium salinity**.**

15°C	BHI	3.0%	4.0%	5.0%	6.0%	8.0%	10.0%	12.0%
ATCC19115	15.7 *(±1.5)*	5.5 *(±0.5)*	2.7 *(±0.3)*	1.8 *(±0.6)*	1.3 *(±0.3)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA
ScottA	6.3 *(±1.5)*	5.5 *(±0.5)*	*2.0 (±0.0)*	1.2 *(±0.3)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*
FW03-0035	9.7 *(±0.6)*	5.8 *(±0.3)*	*2.3 (±0.3)*	1.7 *(±0.3)*	1.3 *(±0.3)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*
LO28	15.3 *(±1.2)*	5.7 *(±0.3)*	3.0 *(±0.5)*	1.8 *(±0.3)*	1.3 *(±0.3)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA
70-1700	6.7 *(±0.6)*	4.0 *(±0.5)*	1.7 (±0.3)	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA	NA
76-1854	4.5 *(±0.5)*	2.5 *(±0.5)*	1.3 *(±0.3)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA
86-3009	17.3 *(±2.3)*	6.3 *(±0.6)*	2.8 *(±0.6)*	1.8 *(±0.3)*	1.3 *(±0.3)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA

Motility zone size of <3.0 = negative motility. NA- not tested. Values in brackets represent standard deviations.
BHI- Brain Heart Infusion medium with no additional NaCl; % - total salinity level (w/v) in BHI.

Table 6.6 Average motility zone sizes (mm) at 4°C influenced by medium salinity.

4°C	BHI	3.0%	4.0%	5.0%	6.0%	8.0%	10.0%	12.0%
ATCC19115	4.0 *(±1.0)*	2.8 *(±0.3)*	2.3 *(±0.6)*	1.3 *(±0.3)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA
ScottA	3.3 *(±0.3)*	1.2 *(±0.3)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*
FW03-0035	3.3 *(±0.8)*	2.5 *(±0.9)*	*2.0 (±0.0)*	1.3 *(±0.6)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*
LO28	4.0 *(±0.5)*	2.8 *(±0.8)*	2.3 *(±0.6)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA
70-1700	3.0 *(±1.0)*	2.7 *(±0.6)*	*2.0 (±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA	NA
76-1854	3.2 *(±0.8)*	1.7 *(±0.6)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA
86-3009	5.0 *(±1.0)*	3.5 *(±0.5)*	2.7 *(±0.3)*	1.7 *(±0.6)*	1.0 *(±0.0)*	1.0 *(±0.0)*	1.0 *(±0.0)*	NA

Motility zone size of <3.0 = negative motility. NA- not tested. Values in brackets represent standard deviations.
BHI- Brain Heart Infusion medium with no additional NaCl; % - total salinity level (w/v) in BHI.

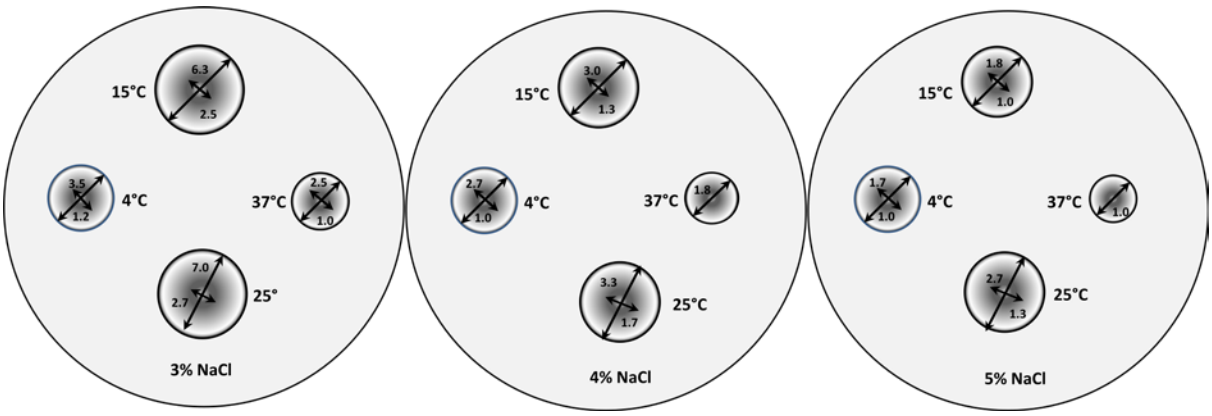


Figure 6.6 Schematic representation of average max and min motility zones on BHI plates with 3.0%, 4.0% and 5.0% NaCl (w/v) concentrations respectively.

A clear pattern emerged from the motility zone (MZ) size observations in response to growth temperature, with an optimum motility observed at 25°C with an average MZ of 13.5 (±4.5) mm and zones ranging from 7.0 mm for strain 76-1854 to 18.3 mm for ATCC19115 (Table 6.3, Fig. 6.5).

Less motility was observed at 15°C on unsupplemented BHIA (i.e. no added NaCl) with an average MZ of 10.8 (±5.2) mm and zones ranging from 6.3mm in strain Scott A to 17.3 mm observed for strain ATCC19115 (Fig. 6.5; Table 6.3). Motility, although evident in all strains, was relatively weak at 4°C on unsupplemented BHIA with an average MZ of 3.7 (±0.7) mm ranging from 3.0mm for strains 70-1700 (which was considered weakly motile by the qualitative tube method) to 5.0 mm for strain 86-3009.

The motility capacity varied between strains, with ATCC19115, LO28 and 86-3009 exhibiting the greatest motility, as evidenced by a larger zone sizes on motility plates in almost all experimental treatments. Strains 76-1854 and 70-1700 produced smaller motility zones indicating relatively weaker motility in comparison to other strains examined.

Salt concentration had a strong inhibitory effect on motility at all temperatures. Addition of NaCl to the growth medium resulted in a significant reduction of zone sizes in all strains examined with a decrease in average MZ from 13.8 (±4.5) mm on BHIA to 5.8 (±1.5) mm on 3.0% NaCl BHIA at 25°C (Table 6.4, Fig. 6.6). Strain ATCC19115, for example, showed a reduction of 11.3 mm in zone size on 3.0% NaCl (w/v) BHIA at 25°C (Table 6.4). In addition strains exhibiting higher motility at optimal conditions at 25°C with no additional NaCl also showed slightly larger MZ on 3.0%NaCl BHIA at 25°C (Table 6.4), namely ATCC19115, LO28 and 86-3009.

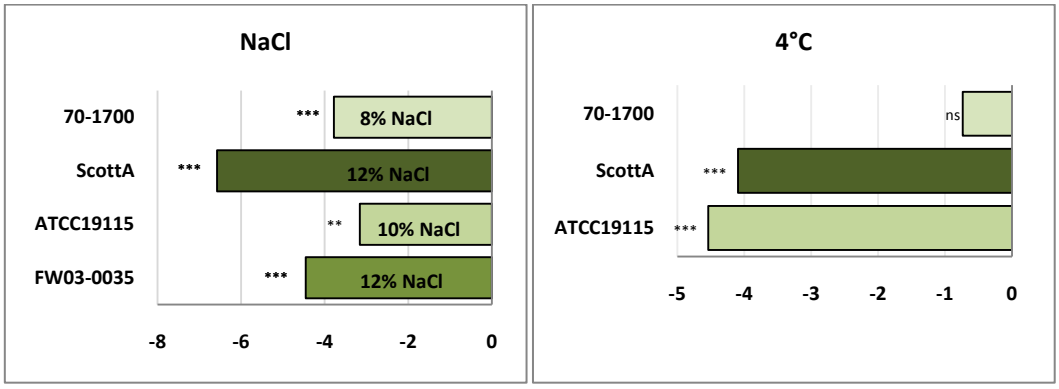
Overall minimal motility was observed at 25°C on BHI supplemented with NaCl to a 4.0% concentration (Fig. 6.6) with an average MZ of 2.8 (±0.6) mm with only 4 strains producing zones of >3.0 mm (Table 6.4). Strains Scott A, 70-1700, and 76-1854 had MZ of <3.0 mm and were considered non-motile. All motility ceased on 5.0% NaCl BHIA at 25°C with all MZ at <3.0 mm (Fig. 6.6). These observations agreed with the results from the qualitative tube method.

A decrease of incubation temperature to 15°C appeared to induce additional stress on motility of *L. monocytogenes* strains simultaneously exposed to NaCl stress. At this temperature motility decreased from 10.8 (±5.2) mm on BHIA+0% to 5.0 (±1.3) mm on 3.0% (w/v) NaCl BHIA (Table 6.5, Fig. 6.6) and was not evident on 4.0% (w/v) NaCl BHIA, with only strain LO28 exhibiting a MZ size of 3.0 mm.

Only one strain, 86-3009 was motile on 3.0% (w/v) NaCl BHIA at 4°C with MZ of 3.5 mm, all the other strains produced zone sizes of <3.0 mm in these conditions (Table 6.6). Strains were non-motile at NaCl concentration of 4.0% (w/v) and above at this temperature.

6.3.3 *Correlation of phenotypic observations with gene expression.*

Using the T-profiler approach (Boorsma *et al.* 2005) gene sets associated with flagella biosynthesis and function showed an overall down regulation in *L. monocytogenes* strains exposed to hyperosmotic stress (Fig. 6.7) and also a temperature of 4°C, although in the case strain 70-1700 grown at 4°C the overall gene down-regulation was found to be statistically non-significant. For individual gene expression refer to Table 3.4 (hyperosmotic stress adaptation) and Fig. 5.24 (cold-stress adaptation).



*=significance level of *T-value* for each gene set ** p<0.01,*** p<0.001, ns p>0.05

Figure 6.7 T-value scores for flagella associated gene sets.

6.4 Discussion

L. monocytogenes cells are non motile at 37°C (Peel *et al.*, 1988), due to MogR repression of flagella biosynthesis-related gene transcription (Gründling *et al.*, 2004; Shen & Higgins, 2006). At temperatures below 37°C, the MogR anti-repressor GmaR, itself activated by DegU, binds directly to MogR relieves this repression (Gueriri *et al.*, 2008; Kamp & Higgins, 2009; Shen *et al.*, 2006). Constriction of motility in *L. monocytogenes* strains to temperatures below 37°C was also evident in the current study. It was interesting to note that motility declined with a decrease in ambient temperature, with cells at 15°C being less motile than those at 25°C (Tables 6.3 to 6.5).

At 4°C motility was substantially reduced in *L. monocytogenes* strains in comparison to activity observed at 15°C and 25°C. Flagella motility in *L. monocytogenes* increases adherence to abiotic surfaces (Lemon *et al.*, 2007; Todhanakasem & Young, 2008; Tresse *et al.*, 2009; Vatanyoopaisarn *et al.*, 2000). Cold temperature has been shown to decrease attachment of this organism to various surfaces such as stainless steel, compared to that occurring at 20°C (Mai & Conner, 2007). Attachment to polystyrene is also significantly lower at 8°C than at 20°C (Tresse *et al.*, 2009), possibly suggesting a direct link between temperature and flagella function. The results suggest that cells once cryoadapted may have fewer flagella per cell than found at the higher growth temperatures.

Hyperosmotic stress induced by NaCl had a strong inhibitory effect on observed motility of *L. monocytogenes* strains. Motility more than halved with an addition of NaCl to BHI growth medium to 3.0% w/v concentration at 25°C and ceased completely at 5.0% (w/v) NaCl. Growth in the presence of inorganic salts and carbohydrates induced loss of motility in *E. coli*, which was correlated with a reduction in the amount of cellular flagellin (Li *et al.*, 1993).

Combination of osmotic and temperature stress had a stronger inhibitory response on motility of *L. monocytogenes* strains than that observed for individual stress factors examined. Cells exposed to cold stress of 4°C combined with 4.0% (w/v) NaCl were found to be non motile.

Strain variation in motility observed was evident throughout the study, with strains ATCC19115, LO28 and 86-3009 proving to exhibit more motility than other strains examined in this study. Strain 86-3009, a serotype 1/2a isolate of animal origin, showed the greatest motility when compared to other strains including serotype 4b strains ATCC19115 and LO28. In addition strain 86-3009 was the only strain exhibiting motility in 3.0% (w/v) NaCl BHI at 4°C, suggesting that motility in this isolate is more tolerant to adverse conditions. Strain specificity in terms of motility has not previously been reported in *L. monocytogenes*. However serotype 1/2a strains were found to be more efficient at forming biofilms than serotype 4b strains (Pan *et al.*, 2009) which is an indirect reflection of a flagella function, as flagella have been shown to be an important factor in biofilm formation (Lemon *et al.*, 2007). Additionally serotype 1/2a strains had higher adhesion to polystyrene compared to other strains (Tresse *et al.*, 2009), though no connection to serotype of isolates tested was actually made by the authors of that study.

Transcriptomic analysis of adaptive response in a number of *L. monocytogenes* strains to hyperosmotic and cold stress has revealed a significant suppression of genes encoding proteins associated with flagella assembly (section 3.3.2.8 and 5.3.1.14). Suppression of transcription of flagella encoding genes strongly suggests a reduction in a number of flagella of these cells which leads to observed decreased motility.

Flagella synthesis is an energetically costly process. Suppression of this cellular process in adverse conditions may help in cellular energy preservation. Energy conservation appears to be important in cells exposed to hyperosmotic and cold stress, as suggested also by suppression of CodY regulon in these cells (section 3.3.2.11 and 5.3.1.16). CodY is responsible for monitoring the overall energy capacity of a bacterial cell by sensing the levels of intracellular GTP (Bennett *et al.*, 2007). Activation of the CodY regulon has been shown to repress flagella synthesis in *B. subtilis* (Bergara *et al.*, 2003).

6.5 Summary and Conclusions

In conclusion *L. monocytogenes* motility is highly temperature-dependent with optimal motility observed at 25°C. Strains exhibited motility at 15°C and 4°C although motility was significantly impaired. Osmotic pressure had a profound inhibitory effect on motility of *L. monocytogenes* with only very weak motility observed in four strains on 4.0% (w/v) NaCl growth medium at 25°C. A combination of low temperature stress and osmotic pressure further decreased motility significantly. Strain variation in motility was evident throughout the study with strains ATCC19115, LO28 and 86-3009 exhibiting on average greater motility and strains 70-1700 and 76-1854 demonstrated the weakest motility in all the conditions addressed in this study. In addition strain 86-3009 was the only strain motile on 3.0% (w/v) NaCl BHIA at 4°C.

A decrease in motility with increasing NaCl concentration and also at 4°C correlates well with the observed repression of flagella associated genes in *L. monocytogenes* strain ATCC19115, ScottA, FW030035 and 70-1700, suggesting decreased synthesis of flagellin in these cells. Suppression in motility is most likely driven by energy conservation in stressed cells induced by activated CodY regulon.

Chapter 7

General stress response of Listeria monocytogenes to hyperosmotic and cold stress factors.

Resistance to stressful environments combined with an ability to thrive at refrigeration temperature has rendered *Listeria monocytogenes* as a serious human pathogen able to survive and proliferate in a numerous food products. Exposure to stress stimuli often provides cross-protection against other stresses, which suggests a common stress adaptation pathway in this organism, which is not well established. Based on transcriptomics data presented in this thesis, adaptation to hyperosmotic and cold temperature stresses has revealed many parallels in terms of gene expression profiles. Gene sets associated with ribosomes and translation, transcription, cell division as well as fatty acid biosynthesis and peptide transport showed activation in cells adapted to either cold or hyperosmotic stress. While repression of genes associated with carbohydrate metabolism and transport as well as flagella was evident in stressed cells. Adaptation to either of the stress stimuli showed down-regulation of SigB and PrfA activated genes suggesting minimal direct involvement of these regulons in transcription regulation of stress adapted cells. The CodY regulon showed activation in stress adapted cells. Although this study suggests a clear correlation in overall response to cold and hyperosmotic stress in this species, strain-specific responses were also evident.

7.1 Introduction

Resistance to stressful environments combined with an ability to thrive at refrigeration temperature has rendered *Listeria monocytogenes* as a serious human pathogen able to survive and proliferate in a numerous food products. A deeper understanding of the resistance mechanisms of this pathogen will assist in controlling it from proliferating in food and food processing environments, thus minimising public health risk and subsequent cost involving product recall.

The food industry is faced with a serious challenge with growing elderly and immunosuppressed populations and thus increased numbers of listeriosis susceptible individuals. It is, therefore, under more pressure to minimize potential risk of food contamination. In addition an increase in health conscious consumer society in recent years with a strong inclination for organic food has prompted

the food industry to implement more natural approaches to food preservation. Health conscious trends in food consumption provide an additional challenge to the food industry in terms of minimizing food contamination, as it involves decreasing not only food preservatives but also the amount of sodium and sugars present in food, thus reducing the osmotic stress experienced by food spoilage organisms in a food matrix. Moreover, there is an increased demand for RTE products with longer shelf lives, which in combination with reduced osmotica of food increases the risk of *L. monocytogenes* proliferation, if present.

Exposure to hostile environments has previously been shown to provide cross-protection against stresses other than that used to induce the original response (Begley *et al.*, 2002; Hill *et al.*, 2002; Lou & Yousef, 1997; Shabala *et al.*, 2008). In depth comprehension of the molecular mechanisms behind the stress adaptation is vital for optimizing control against food contamination with *L. monocytogenes*.

Based on transcriptomics data presented in this thesis adaptation to high levels of NaCl (Chapter 3) and cold temperature (Chapter 5) has many parallels in terms of gene expression profiles; although in most cases salt induced a stronger response.

7.2 General overview of functionally-assigned gene set response to stress in *L. monocytogenes*.

A relatively high proportion of genes (69%) on the *L. monocytogenes* EGD-e genome can be assigned a functional role through comparisons to bioinformatic databases and direct experimental data. Analysis of gene expression utilising a combined ontological- and statistical-based approach allows identification of trends between functionally linked genes providing an insight into physiological adjustments to set conditions. A comparison is made in the next section on functional and regulatory responses between hyperosmotic and low temperature conditions on this basis. The data is based on the mean responses of three separate strains that exhibit quite different growth patterns under stress (Chapter 2) and thus potentially provides a more rigorous analysis of responses to osmotic and cold stress.

7.2.1 Independent adaptation to hyperosmotic and cold stresses induces translation-apparatus –related genes in *L. monocytogenes*.

Adaptation to hyperosmotic and cold stress had a strong positive effect on translation-apparatus related genes. Genes associated with ribosomes were among the most strongly activated gene sets in response to both stresses with average *T-value* scores of 10.46 (±0.58) for salt adapted strains and 10.65 (±3.25) for cold adapted strains (Fig. 7.1). Among a large array of up-regulated ribosomal genes eight showed consistently strong (>two fold) homologous up-regulation in all three strains following independent adaptation to both stresses including *rplK* (*lmo0248*), *rplA* (*lmo0249*), *rplJ* (*lmo0250*), *rpmI* (*lmo1784*), *rpmB* (*lmo1816*), *rpmE* (*lmo2548*), *rpsD* (*lmo1596*) and *rpsL* (*lmo2656*) (Table 7.1). Although different physical stresses, both high Na^+ concentration and low temperature have damaging effect on ribosome structure. While high ionic concentration is thought to cause the ribosome subunits to become displaced from mRNA strands (Brigotti *et al.*, 2003), low temperature alters the structural integrity of ribosomal subunit (Tasara & Stephan, 2006), with an overall reduction in ribosome function. Stress response adaptation of *L. monocytogenes* to both stress factors appears to involve activation of ribosomal gene transcription.

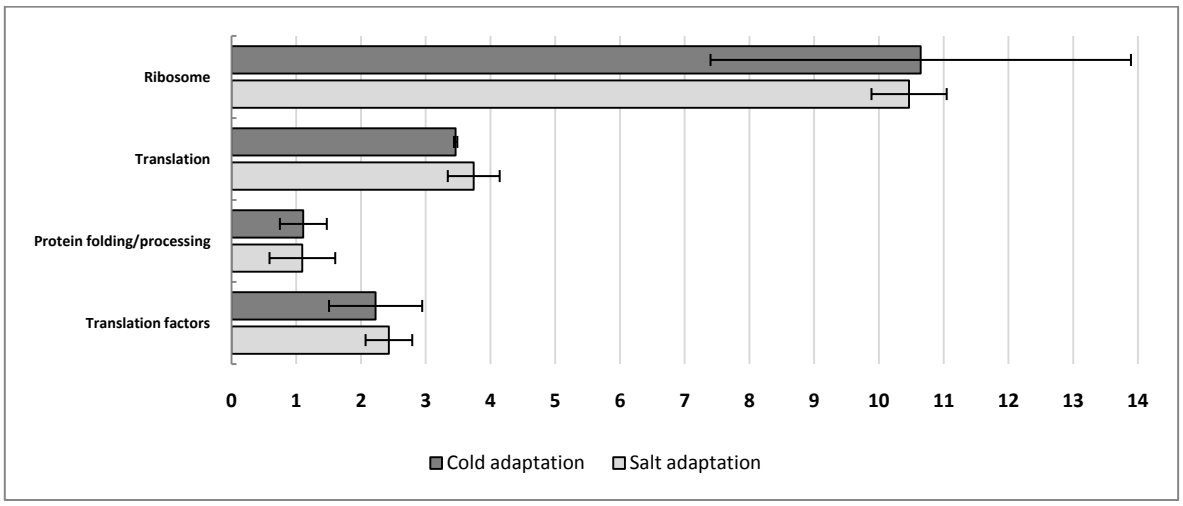


Figure 7.1 Average *T-value* scores for gene sets involved in protein synthesis.

Cold temperature and high osmolarity stress adaptation also had an effect on protein translation, evident by activation of genes associated with this cellular process. Average *T-values* of 3.74 (±0.49) and 3.46 (±0.03) for transcription-related genes and 2.43 (±0.36) and 2.23 (±0.72) for translation factors in salt and cold adapted cells were observed, respectively (Fig. 7.1).

Gene sets encoding proteins involved in assisting protein folding also showed activation in adaptation to both cold (refer to sections 5.3.1.4.3 and 5.3.1.4.4) and hyperomotic stresses (addressed in section 3.3.2.7) suggesting increased protein turnover. This could be due to interference in protein folding, induced independently by high levels of cations and low temperature. Two genes *ppiB* (*lmo2376*) and *tig* (*lmo1267*) were universally up-regulated in cells adapted to either of the two stress factors (Table 7.1). The PpiB protein catalyses the isomerization of peptidylprolyl bonds in *B. subtilis* (Graumann *et al.*, 1996) whilst Tig (Trigger factor) also possesses prolyl isomerase activity (Deuerling *et al.*, 1999; Jones & Inouye, 1994) suggesting that isomerase activity is important in cells for growth when stressed by high levels of NaCl or low temperature alike.

7.2.2 Independent adaptation to hyperosmotic and cold stresses induces information processing and storage genes in *L. monocytogenes*.

The structural integrity of DNA molecules is sensitive to changes in the physiological environment, which partially explains the enhancement of gene transcription associated with DNA repair, recombination and other proteins involved in stabilizing DNA molecule in *L. monocytogenes* exposed to environmental stress (Fig. 7.2). High salt concentration causes dehydration of DNA molecule in which water molecules are displaced by cations, which in turn disturbs electrostatic interactions of the DNA molecule (Kas'yanenko, 2006), whilst low ambient temperature directly alters DNA structure by increasing negative supercoiling (Erriksson *et al.*, 2002).

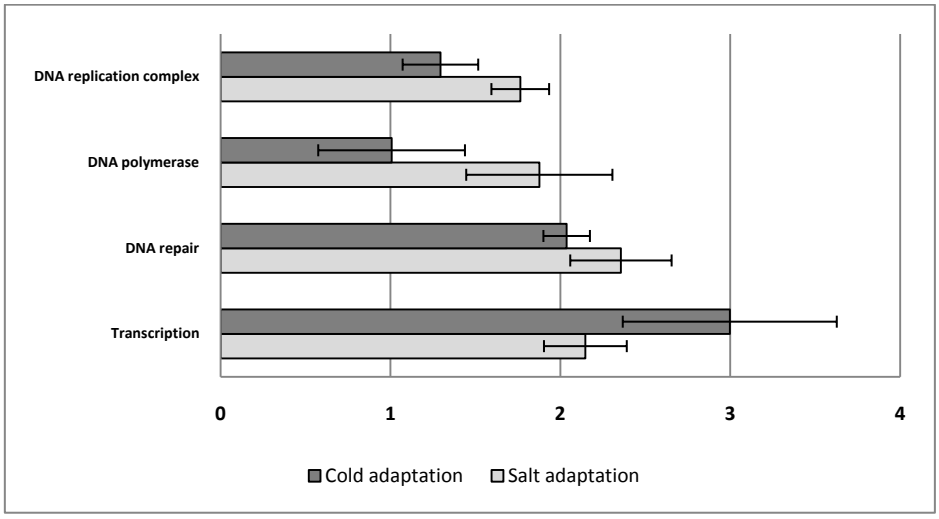


Figure 7.2 Average *T-value* scores for gene sets associated with the DNA structure and integrity.

L. monocytogenes appears to respond to changes to the DNA molecule structure by up-regulating genes that encode support proteins responsible for maintaining the integrity of DNA. Interestingly, both stresses induced activation of different individual genes within this functional group of genes. Whilst activation of *ssb* (*lmo0045*), encoding single-stranded DNA-binding protein, and *hsdM* (*lmo1582*), encoding a DNA methylase, was evident in all strains during adaptation to hyperosmotic stress (described in detail in section 3.3.2.4), growth in cold temperature activated *holB* (*lmo0162*), *recU* (*lmo1891*), *parE* (*lmo1286*) and *cspL* (*lmo1364*) in all strains of *L. monocytogenes* tested (refer to sections 5.3.1.6. and 5.3.1.4.3).

7.2.3 Independent adaptation to hyperosmotic and cold stresses induces changes to cell envelop and membrane transport in *L. monocytogenes*.

Adaptation to hyperosmotic and cold environments was evidently accompanied by a change in the cellular envelope. Gene sets encoding proteins associated with peptidoglycan biosynthesis showed significant activation in *L. monocytogenes* adapted to either hyperosmotic or cold stresses, with *T-value* of 3.22 (±0.66) and 2.17 (±0.27) obtained for cold and salt adaptation (described in detail in sections 5.3.1.2 and 3.3.2.3, respectively). The increase in peptidoglycan biosynthesis gene transcription was also associated with increased expression of the gene set including genes for synthesis of the polyprenoid carrier lipid. In addition genes linked to fatty acid biosynthesis exhibited up-regulation, most likely associated with cell membrane structure modulation occurring in response to both stress factors.

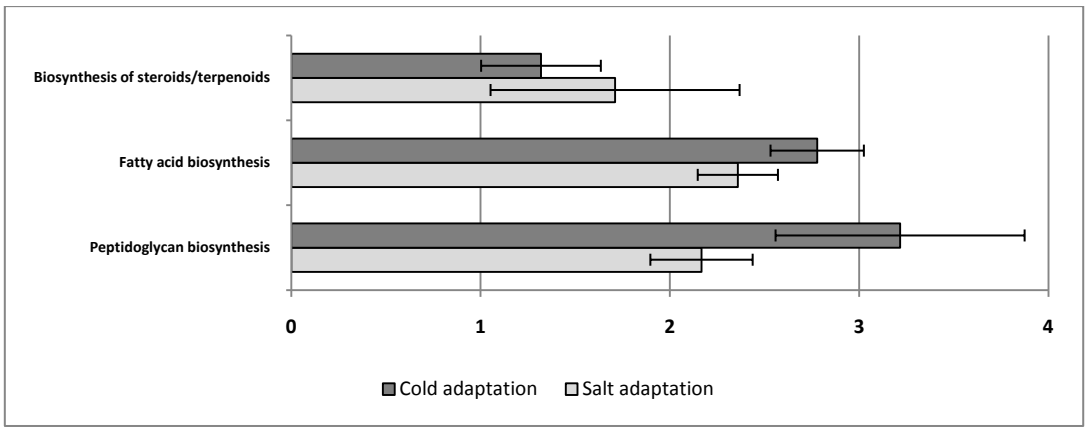


Figure 7.3 Average *T-value* scores for gene subsets associated with maintenance of cellular envelope.

It was interesting to note that one gene, *lmo0198*, homologous to *gcaD* in *B. subtilis* (Hilden *et al.*, 1995), which encodes *N*-acetylglucosamine 1-phosphate uridyltransferase responsible for synthesis of peptidoglycan precursor UDP-*N*-acetylglucosamine, was up-regulated in all strains following adaptation to both stresses. The involvement of this gene transcript in adaptation to cold and salt environments in *L. monocytogenes* has been overlooked in previous studies; however the universal up-regulation of this gene throughout this study strongly suggests an important role in stress adaptation of *L. monocytogenes* in hyperosmotic and cold temperature environments.

A gene with as yet uncharacterised function in *L. monocytogenes*, *lmo2522*, showed a relatively strong analogous up-regulation (up to 93-fold) in strains adapted to both cold and salt stimuli. The encoded protein appears homologous to YocH in *B. subtilis*, and is a cell membrane binding protein the exact function of which has not yet been defined. Its up-regulation has been linked, however, to salt stress response in *B. subtilis* (Eiamphungporn & Helmann, 2009) and cold adaptation in *L. monocytogenes* strain 10403S (Chan *et al.*, 2007b) as well as prolonged exposure to ethanol (Gottschalk *et al.*, 2008). Characterisation of this particular gene product in the future might provide an important component in preventing proliferation of this organism in adverse environmental conditions, in particular high osmotic stress and low temperature.

Bacterial interaction with the environment is controlled by the permeability of the cell membrane. Both environmental stresses examined in this study induced modification of cell membrane composition in *L. monocytogenes*. As a result there was a significant change in transporter related gene transcription observed in adaptive response of this organism to both stresses (addressed in detail in section 3.3.2.1 and 5.3.1.8).

Adaptation to both stresses appeared to activate transcription of genes associated with general transport across the cell membrane, in particular those associated with uptake of peptides (Fig. 7.4). Up-take of peptides from the external environment as a means of hyperosmotic (Sleator *et al.*, 2003a) and cold (Borezee *et al.*, 2000) stress acclimatisation has previously been described in *L. monocytogenes*. Indeed the *oppA* operon overall showed significant up-regulation in all strains adapted to either cold or hyperosmotic stresses, thus further emphasising the involvement of the encoded peptide transporter in aiding adaptation to environmental stress in this pathogen.

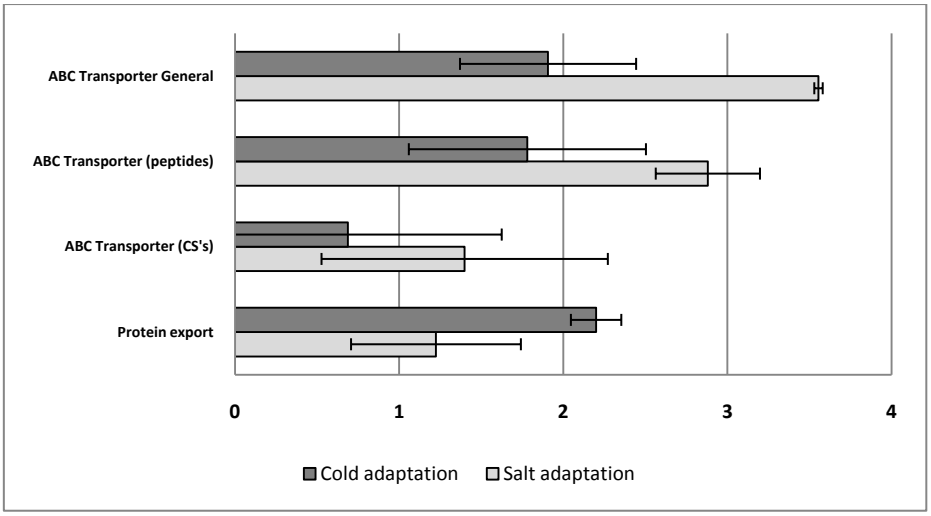


Figure 7.4 Average *T-value* scores for gene sets encoding various transporters.

A mutant of strain LO28 lacking the *oppA* transporter (Δ*oppA* LO28), produced significantly longer generation times when grown at 12.5% (w/v) NaCl and also 4°C (section 2.3.1 and 2.3.2 respectively), further emphasising the importance of this transporter in stress tolerance of this organism. The Opp systems of other bacteria are thought to be involved in nutrient uptake as well as in recycling the cell membrane peptides for synthesis of new peptidoglycan (Goodell & Higgins, 1987). In addition, accumulation of oligopeptides from the growth medium, such as proline derivatives, may aid stress tolerance in a way similar to that observed for compatible solutes.

Accumulation of compatible solutes as a mechanism of overcoming hyperosmotic or chill stress in *L. monocytogenes* has been closely examined in numerous independent studies. It is well accepted that compatible solutes provide stability to enzymes and assist protein folding and other cellular processes vulnerable to environmental stress. Genes encoding compatible solute transporters collectively showed up-regulation in *L. monocytogenes* cells adapted to cold (5.3.1.8.1) and also osmotic stress (3.3.2.1.4), however strain specificity for compatible solute preference was significant and something that has not been previously addressed in stress response adaptation of this pathogen.

Interaction with the external environment is an important aspect of bacterial stress response and it appears that *L. monocytogenes* not only actively accumulates substances from the external environment but also actively exports proteins during adaptation to stressful environments. The

protein secretion systems in *L. monocytogenes* are not well understood, especially those involved in stress adaptation. It is highly likely that activation of protein secretion mechanism, observed in both cold and salt adapted cells, is associated with translocation of membrane proteins responsible for maintaining the integrity of the cell envelope. Whether this up-regulation is linked to an increase in protein turnover associated with physical damage to the cell membrane, and consequently, an increased demand for newly synthesised proteins, or for translocation support proteins contributing to stabilising the secreted protein, remains to be determined once this pathway is better understood.

7.2.4 Independent adaptation to hyperosmotic and cold stresses induced activation of genes involved in cell division of *L. monocytogenes*.

Bacterial population growth relies on a complex matrix of cell division proteins the correct alignment of which is crucial for generating viable daughter cells. It is not surprising that such a finely tuned cellular process is quite vulnerable to environmental stress that can, if not corrected, disrupt the balance of cell septation and, thus, compromise the overall process. Gene sets associated with cell division and the septal ring were up-regulated in *L. monocytogenes* cells adapted to cold and hyperosmotic environments (Fig. 7.5).

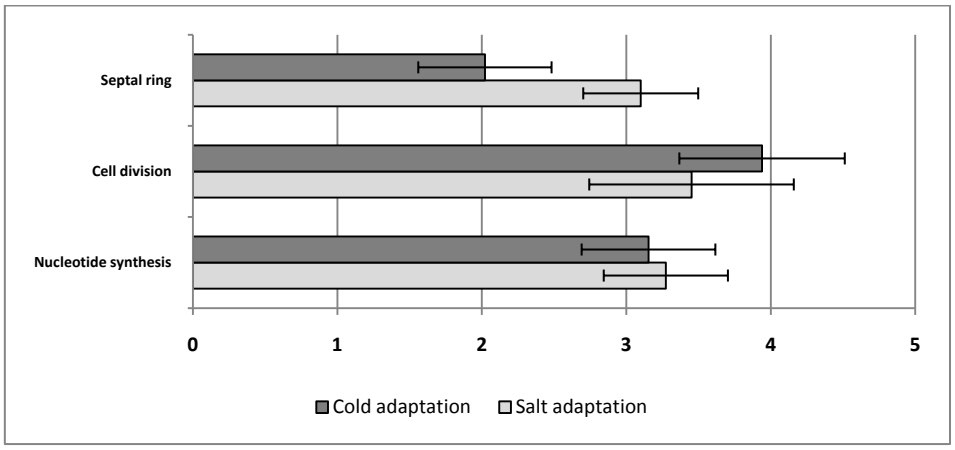


Figure 7.5 Average *T-value* scores for gene sets associated with cell division.

Low temperature appeared to influence the function of the cell division machinery, possibly through altering folding and, consequently, the structure of cell division proteins (5.3.1.3). High ionic strength induced by NaCl appears to interfere with the assembly of cell division machinery, perhaps also

through compromising the correct folding of the proteins (3.3.2.5). Interestingly, hyperosmotic conditions resulted in a higher increased transcription of septal ring proteins compared to low temperature (Fig. 7.5), suggesting that assembly and/or stability of the septal ring structure is vulnerable to the surrounding ionic composition.

7.2.5 Adaptation to hyperosmotic and cold stresses induced suppression of genes involved in carbohydrate metabolism of *L. monocytogenes*.

Stress adaptation in *L. monocytogenes* seems to be accompanied by a decrease in overall metabolic turnover, characterised by the evident slowing of growth. This was reflected in overall down-regulation of gene sets associated with carbohydrate transport and utilization observed in cold (5.3.1.13.1) and osmo-adapted cells (3.3.2.1.6 and 3.3.2.9).

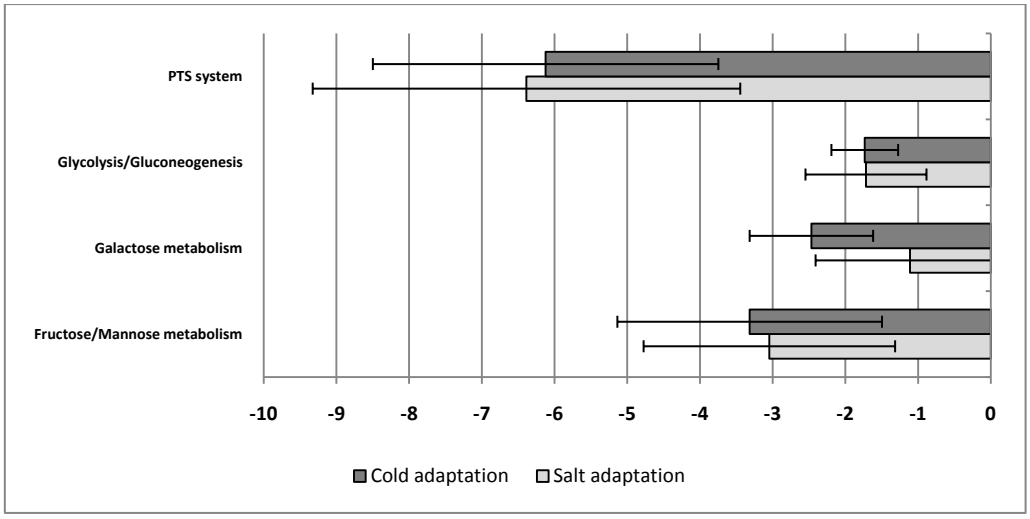


Figure 7.6 Average *T-value* scores for gene sets associated with carbohydrate metabolism.

Gene sets associated with PTSs were strongly suppressed in *L. monocytogenes* cells adapted to cold and salt stresses (Fig. 7.6). This corresponds to suppression of 3.2% of total *L. monocytogenes* genes, in turn restricting wastage of ATP on nonessential metabolic processes such as up-take of sugars, utilisation of which is suppressed due to overall metabolic slowdown.

7.2.6 Adaptation to hyperosmotic and cold stresses induced suppression of genes associated with flagella and other external membrane proteins.

Flagella motility is a highly advantageous but energetically demanding survival mechanism utilised by bacteria in extracellular environments (Kamp & Higgins, 2009). Adaptation to cold and salt stresses lead to suppression of gene sets associated with flagella assembly (refer to sections 5.3.1.14, and 3.3.2.8) with average *T-values* of -4.89 (±1.72) and -4.50 (±1.05), respectively. Chemotaxis was also suppressed (Fig. 7.7).

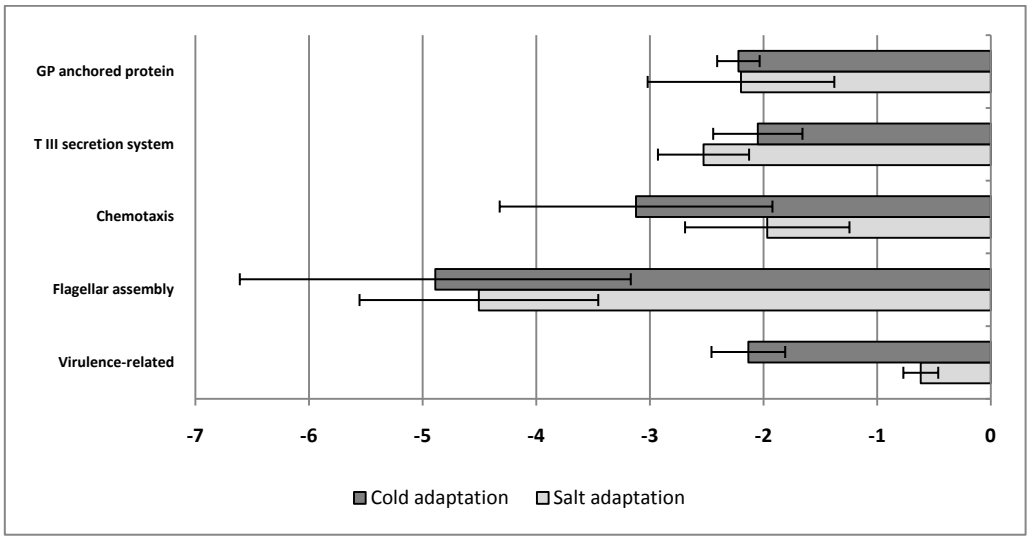


Figure 7.7 Average *T-value* scores for gene sets associated with flagella and other external cell proteins.

It appears that motility at 4°C and at high osmolarity is, most likely suppressed due to energy conservation in the stressed cells (discussed in detail in chapter 6).

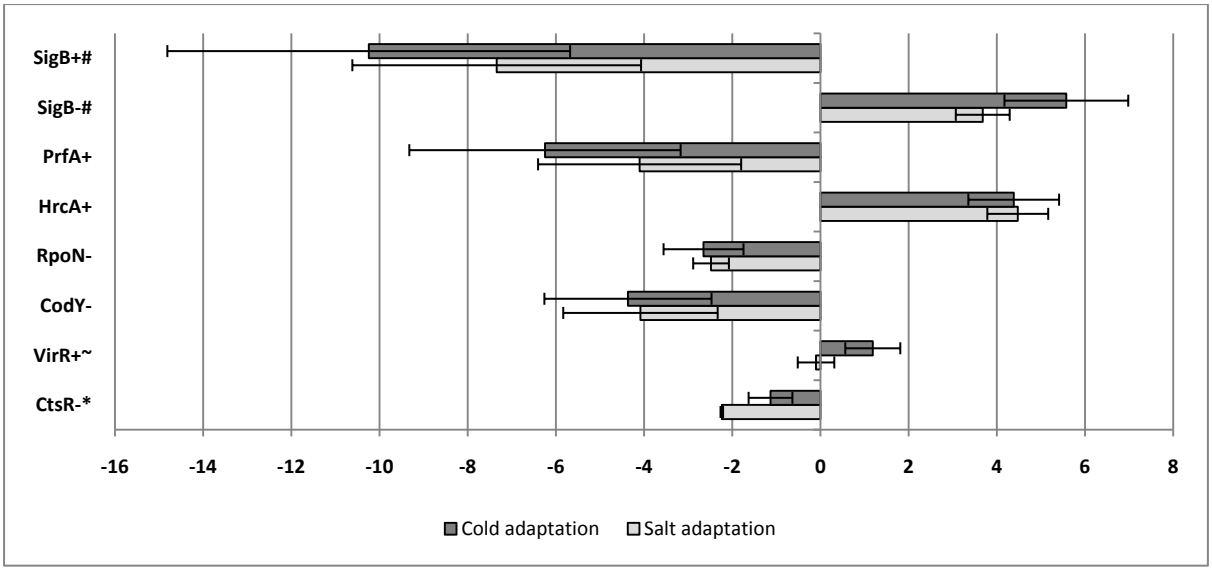
Flagella and flagellum-mediated motility are integral to the virulence of *L. monocytogenes* (O'Neil & Marquis, 2006). It was perhaps not surprising, then, that virulence related gene sets also showed suppression in stress adapted cells. Interestingly, cold temperature induced a stronger inhibition of virulence genes compared to hyperosmotic stress; suggesting that cold temperature is a stronger trigger for virulence suppression. This may possibly be due to inherent recognition of low temperature as environmental factor far removed from those encountered within a mammalian host, i.e. a manifestation of a saprophytic versus a parasitic lifestyle.

Overall, adaptation of *L. monocytogenes* to both stresses was associated with decreases in the expression of cell surface proteins, flagella, and other virulence-related external proteins e.g., internalins. The benefit in stripping of surface proteins in stressed cells is unclear, however might be influenced by the thickening and restructuring change of bacterial envelope associated with the acclimatisation to environmental stresses and could be a general feature of the species in its saprophytic mode.

7.2.7 Regulation of transcription during adaptation to hyperosmotic and cold stresses of *L. monocytogenes*.

Survival of *L. monocytogenes* under non-host-associated environmental stress conditions such as osmotic and cold stresses has been attributed to tightly regulated gene transcription. Regulation of gene expression in *L. monocytogenes* consists of a complex network of interactions between different transcriptional regulators and alternative sigma factors which is thus-far not fully comprehended (Chaturongakul *et al.*, 2008).The complexities of these interactions which contribute to fine-tuning gene expression under stress conditions is becoming more apparent with transcriptomic studies, although this information is still very preliminary.

L. monocytogenes adapted to environmental stress failed to show activation of the SigB regulon, evident by the strong down regulation of genes positively influenced by this general-stress master regulator in either cold (5.3.1.16) or osmotically-challenged cells (3.3.2.11) with average *T-values* of -10.24 (±4.57) and -7.34 (±3.27) respectively (Fig. 7.8). Proteomic (Abram *et al.*, 2008), transriptomic (Chaturongakul & Boor, 2006; Gray *et al.*, 2006; Raengpradub *et al.*, 2008) and phenotypic characterisation of SigB-null mutants had provided sufficient evidence that this alternative sigma factor categorically contributes to the ability of *L. monocytogenes* to survive and proliferate under various stress conditions encountered in non-host-associated environments. It is interesting, therefore, that SigB plays a less passive role in cells adapted to a given stress, it appears that active regulation of gene expression in such cells is left to other members of the regulatory network.



Analysis based on genes exhibiting increased expression in wild type EGDe and 10403 strains compared to corresponding σ^B null mutants (Hain *et al.*, 2008; Raengpradub *et al.*, 2008) .
~ regulon first established by (Mandin *et al.*, 2005)
* regulon described in (Hu *et al.*, 2007b)
+ indicate +ve regulation by a given regulon, - indicate –ve regulation by a given regulon.

Figure 7.8 *T-value* scores for major regulons of *L. monocytogenes*.

Synergistically PrfA regulon also showed repression in both cold and salt adapted cells, which is in agreement with the observed overall down-regulation of virulence genes in these cells and the heavy overlap between the SigB and of PrfA regulons. Interestingly, response regulator VirR, which positively controls transcription of additional virulence associated genes showed no significant change in transcription in salt adapted cells and only slight activation in cold adapted *L. monocytogenes*. VirR controls surface component modifications (Mandin *et al.*, 2005) most of which are also under multiple control by other regulators.

Genes suppressed by CtsR overall showed down-regulation in cells adapted to both stress factors; suggesting at least an indirect involvement of this response regulator in adaptation of this organism to both environmental stresses. Genes indirectly influenced by HrcA were de-repressed in *L. monocytogenes* adapted to either hyperosmotic stress or low temperature (Fig. 7.8). The majority of up-regulated proteins activated by HrcA include ribosomal proteins which showed an overall activation in cells adapted to both stresses (sections 3.3.2.11 and 5.3.1.16). The exact involvement of these repressors in stress response of *L. monocytogenes* is unclear particularly since regulation of genes by these regulators is intertwined with that of the SigB regulon (Hu *et al.*, 2007a; Hu *et al.*, 2007b).

RpoN regulon appeared to be involved at least indirectly, in regulation of transcription in osmotically and low temperature-adapted *L. monocytogenes* cells, with genes directly suppressed by RpoN being overall down-regulated. RpoN regulation contributes mostly to carbohydrate metabolism in *L. monocytogenes* EGD-e, by directly influencing PTS (Arous *et al.*, 2004), which was overall suppressed in cells adapted to both stresses.

Adaptation to both stresses induced the CodY regulon (Fig. 7.8). This negative regulator suppresses genes associated with carbohydrate metabolism and transport, motility and chemotaxis (Bennett *et al.*, 2007), which showed significant down regulation in *L. monocytogenes* adapted to hyperosmotic and also cold stresses (section 3.3.2.11 and 5.3.1.16). By comparison, CodY becomes de-repressed in *L. monocytogenes* when exposed to sudden hydrostatic pressure processing (Bowman *et al.*, 2008) and to a lesser extent in hyperosmotically shocked *L. monocytogenes* ATCC19115 strains (section 4.3.1.12).

7.3 General overview of a genetic response to stress in *L. monocytogenes* revealed by individual gene expression.

Analysis of gene expression in cells adapted to cold and hyperosmotic stress revealed significant strain specificity in terms of individual gene expression profiles though general patterns of expression were largely similar. These differences could contribute to the different growth rates observed under the same stress conditions.

Osmoadaptation was most notable in clinical strains ATCC 19115 with 404 genes showing ≥ two-fold increase in expression. By comparison ScottA and the animal isolate 70-1700 only increased expression of half the number of genes. Given that ScottA is quite halotolerant it is possible that it has a more efficient means of osmoadaptation while 70-1700 on the other hand may have a weaker capacity to osmoadapt as discussed in Chapter 3 (Fig. 7.9). Interestingly, of all significantly up-regulated genes observed in any of the three strains adapted to hyperosmotic stress, only 53 of those were found to be significantly increased in expression in all three strains (Fig. 7.9). Microarray analysis produces quite noisy data thus it is likely this number perhaps is an underestimate of the true number of co-responsive genes in these strains.

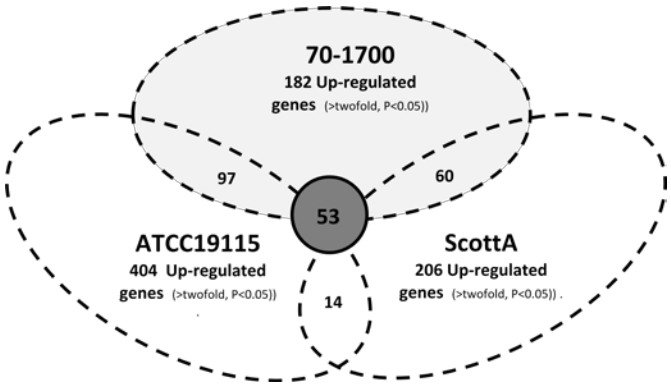


Figure 7.9 Venn diagram of significantly up-regulated genes observed in three strains of *L. monocytogenes* adapted to hyperosmotic stress.

By comparison cold adaptation induced activation of an approximately similar number of genes between the three strains tested (n=310 to 436, Fig. 7.10). A much larger number of these responsive genes behaved similarly between the strains suggesting a by-and-large more homogenous response.

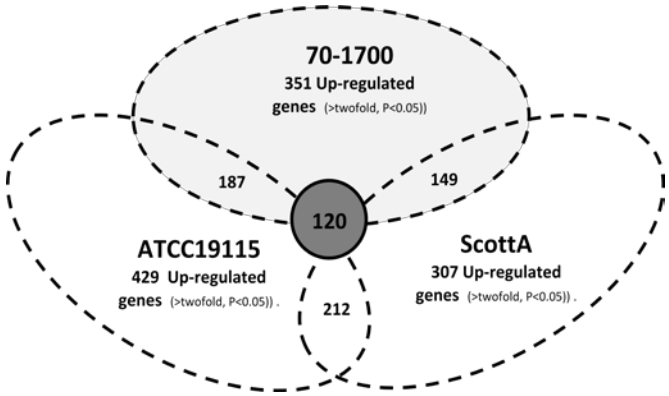


Figure 7.10 Venn diagram of significantly up-regulated genes observed in three strains of *L. monocytogenes* adapted to low temperature stress.

The magnitude of strain specificity was highlighted even further when responses to both environmental stresses were considered together. Strain ATCC19115 revealed 150 significantly up-regulated genes in cells adapted to both hyperosmotic stress and cold stress, 82 up-regulated genes were matching in response to both stresses in strain 70-1700, whereas ScottA showed up-regulation of 77 analogous genes (Fig. 7.11). Among these homologous genes only 22 were found to be significantly up-regulated in all three strains adapted to hyperosmotic stress and cold temperature (Table 7.1).

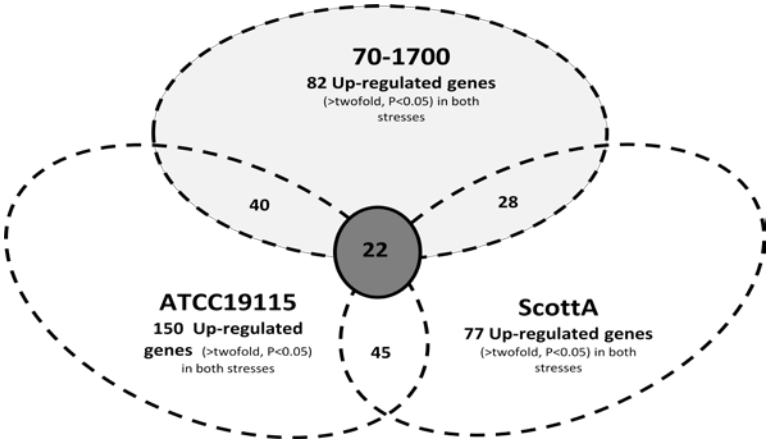


Figure 7.11 Venn diagram of significantly up-regulated genes similarly expressed following adaptation to hyperosmotic stress and cold temperature in *L. monocytogenes* strains.

Among these were a number of ribosomal proteins (section 7.2.1), Tig (*tig*), and peptidyl-prolyl cis-trans isomerise (cyclophili; *ppiB*) (Table 7.1). Tig has been shown to assist in the low temperature survival of the psychrophilic bacterium *Psychromonas arctica* (Lee *et al.*, 2009) as well as the survival of a soil bacterium *Sinorhizobium meliloti* under hyperosmotic conditions induced by NaCl (Miller-Williams *et al.*, 2006). Interestingly expression of both trigger factor and cyclophilin are essential for growth under starvation conditions in *B. subtilis* (Göthel *et al.*, 1998). Potentially a significant suppression of carbohydrate and amino acid metabolism observed in *L. monocytogenes* grown under hyperosmotic and low temperature conditions, mimics pseudo-starvation in these cells.

A recently characterised gene, *lmo2555* encodes UDP- glucose specific glycosyltransferase, LafA, responsible for Glc- diacylglycerol (DAG) synthesis a glycolipid which anchors the cell wall polymer to the bacterial membrane (Webb *et al.*, 2009).Deletion of *lmo2555* led to a complete absence of glycerolipids in *L. monocytogenes* strain 10403S (Webb *et al.*, 2009).

Table 7. 1 List of genes significantly up-regulated in all three strains of *L. monocytogenes* adapted to hyperosmotic stress and cold temperature.

Gene	Gene homolog	ATCC19115		ScottA		70-1700		Predicted or known function
		NaCl	4°C	NaCl	4°C	NaCl	4°C	
lmo0198	*gcaD*	**2.1** ***	**2.35** ***	**1.20** ***	**1.12** **	**1.34** ***	**1.15** ***	glucosamine-1-phosphate N-acetyltransferase / UDP-N-acetylglucosamine pyrophosphorylase
lmo0227		**3.2** ***	**1.88** ***	**2.68** ***	**1.31** ***	**1.32** **	**1.64** ***	putative tRNA-dihydrouridine
lmo0248	*rplK*	**3.36*****	**2.25** ***	**1.49** ***	**2.54** **	**1.79** ***	**3.16** ***	ribosomal protein L11
lmo0249	*rplA*	**3.37** ***	**1.71** **	**1.90** ***	**2.01** **	**1.08** **	**2.97** ***	ribosomal protein L1
lmo1267	*Tig*	**1.82** ***	**2.21** **	**1.10** **	**1.85** *	**1.59** ***	**1.01** *	trigger factor (prolyl isomerase)

Gene	Gene homolog	ATCC19115		ScottA		70-1700		Predicted or known function
		NaCl	4°C	NaCl	4°C	NaCl	4°C	
lmo1431		**2.67** ***	**3.06** ***	**2.02** ***	**2.10** ***	**1.37** ***	**1.97** ***	ABC transporter, ATP-binding protein
lmo1596	*rpsD*	**3.21** ***	**1.32** ***	**1.03** ***	**1.73** *	**1.48** ***	**1.99** ***	ribosomal protein S4
lmo1707		**1.95** ***	**1.56** **	**1.10** ***	**1.81** ***	**1.08** ***	**1.26** ***	unknown protein
lmo1784	*rpmI*	**2.39** ***	**1.32** ***	**1.80** ***	**1.66** **	**1.38** ***	**2.32** ***	ribosomal protein L35
lmo1816	*rpmB*	**3.27** ***	**4.34** ***	**3.01** ***	**3.58** **	**1.84** ***	**4.11** ***	ribosomal protein L28
lmo2048		**2.70** ***	**1.15** **	**2.59** ***	**1.75** ***	**1.64** ***	**2.09** ***	similar to uncharacterized conserved proteins
lmo2056		**1.43** **	**1.47** ***	**1.66** ***	**1.48** ***	**1.44** ***	**1.51** ***	similar to uncharacterized conserved proteins
lmo2192	*oppE*	**2.11** ***	**1.18** *	**2.26** ***	**1.15** *	**1.63** ***	**1.85** ***	similar to oligopeptide ABC transporter, ATP binding protein
lmo2194	*oppC*	**1.44** **	**1.82** *	**1.46** ***	**1.31** *	**1.43** ***	**1.74** ***	similar to oligopeptide ABC transporter, permease protein
lmo2195	*oppB*	**1.62** **	**1.63** ***	**1.38** ***	**1.71** **	**1.17** ***	**1.72** ***	similar to oligopeptide ABC transporter, permease protein
lmo2223		**2.41** ***	**2.28** **	**2.74** **	**1.89** *	**1.37** ***	**1.10** ***	similar to uncharacterized conserved proteins
lmo2376	*ppiB*	**1.92** **	**1.96** ***	**1.27** ***	**1.25** **	**1.39** ***	**1.20** ***	similar to peptidyl-prolyl cis-trans isomerase
lmo2428		**2.02** ***	**1.64** ***	**1.08** ***	**2.12** ***	**1.24** ***	**2.47** ***	similar to FtsK/RodA/SpoIIIE and related proteins
lmo2479		**1.44** *	**1.13** *	**1.14** ***	**1.27** ***	**1.47** ***	**1.31****	similar to uncharacterized conserved proteins
lmo2522		**2.11** **	**6.55** ***	**3.90** ***	**4.40** ***	**3.52** ***	**4.30** ***	similar to uncharacterized conserved proteins
lmo2548	*rpmE*	**2.59** ***	**2.49** ***	**1.95** ***	**3.32** **	**1.91** ***	**3.69** ***	ribosomal protein L31
lmo2555		**1.68** ***	**2.55** ***	**1.15** ***	**1.68** **	**1.13** ***	**1.09** ***	similar to glycosyltransferases

*=significance of Log ratio for each gene, where * P<0.05, ** P<0.01, *** P<0.001.

Activation of five genes encoding proteins of unknown function was observed in all three strains adapted to both stresses (Table 7.1). One of these was *lmo2522*and as described above must play a significant role in *L. monocytogenes* adaptation to the adverse conditions examined in this study. While the exact function of *lmo1431* product remains unknown, mutant of this gene in *L. monocytogenes* was found to have increased sensitivity to salt and pH stress (Gardan *et al.*, 2003a). A gene encoding an uncharacterised stress protein, *lmo2048* also showed up-regulation in *L. monocytogenes* in *vivo* (Camejo *et al.*, 2009; Van de Velde *et al.*, 2009).

Suppression of genes whose expression is non-essential for immediate survival under adverse conditions is an important mechanism of stress adaptation which allows re distribution and conservation of intracellular energy. A large number of genes were found to be significantly down-regulated in *L. monocytogenes* cells adapted to hyperosmotic stress (Fig. 7.12); only 25 of these were found to be similarly expressed in all strains adapted to hyperosmotic growth conditions. This was also the case for cells adapted to low temperature stress, with 35 genes being similarly down-regulated in all strains (Fig. 7.13).

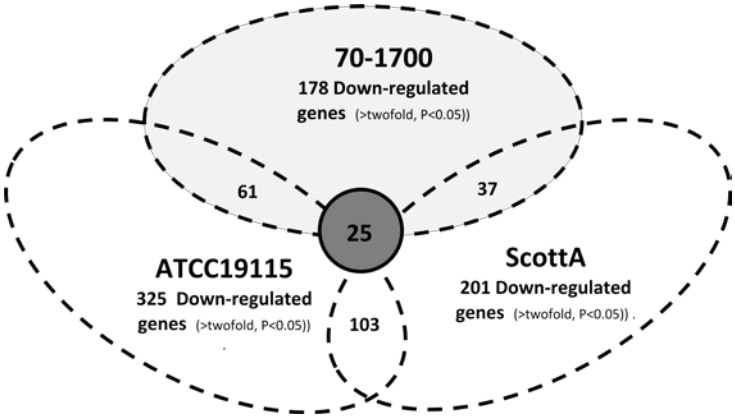


Figure 7.12 Venn diagram of significantly down-regulated genes observed in three strains of *L. monocytogenes* adapted to hyperosmotic stress.

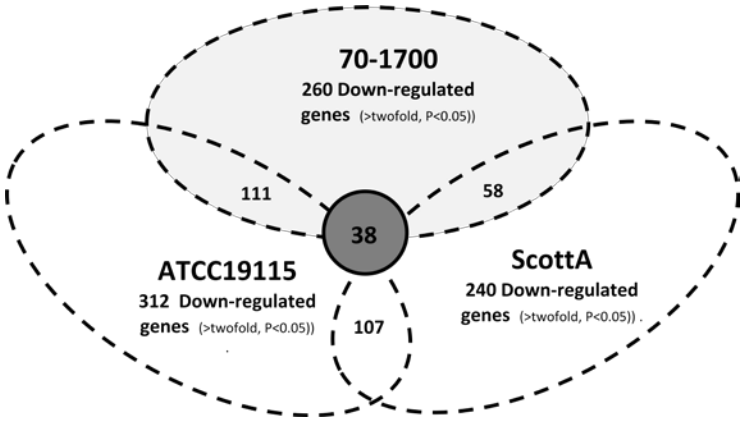


Figure 7.13 Venn diagram of significantly down-regulated genes observed in three strains of *L. monocytogenes* adapted to low temperature stress.

Comparison of down-regulated genes observed in all strains following adaptation to hyperosmotic stress and low temperature showed little similarity in transcription expression with only seven genes being equally repressed (Fig. 7.14). Amongst these, two genes *lmo2684* and *lmo2685* encode PTS transporters (Table 7.2); lmo0445 encoding a transcriptional regulator and also glutamate decarboxylase encoding gene *gadB*.

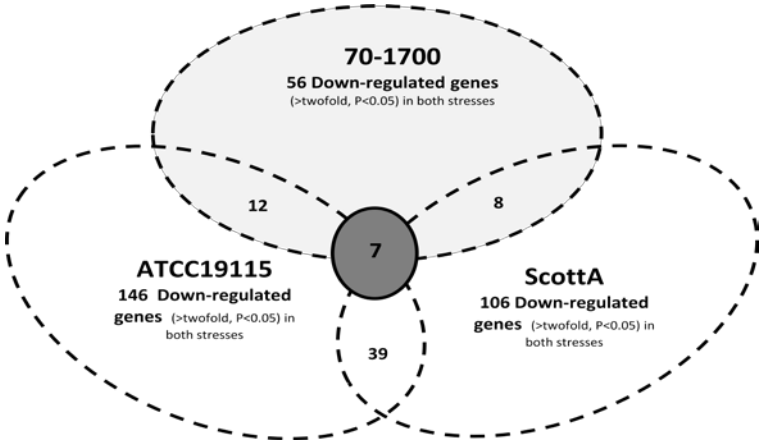


Figure 7.14 Venn diagram of significantly down-regulated genes similarly expressed following adaptation to hyperosmotic stress and cold temperature in *L. monocytogenes* strains.

Table 7. 2 List of genes significantly up-regulated in all three strains of L. monocytogenes adapted to hyperosmotic stress and cold temperature.

Gene	Gene homolog	ATCC19115		ScottA		70-1700		Predicted or known function
		NaCl	4°C	NaCl	4°C	NaCl	4°C	
lmo0265		**-3.11****	**-2.03****	**-2.28*****	**-3.49****	**-1.91*****	**-3.34*****	putative succinyl-diaminopimelate desuccinylase
lmo0445		**-2.67****	**-1.22***	**-1.29*****	**-1.10****	**-1.44*****	**-1.09*****	similar to transcriptional regulators/antiterminators
lmo0497		**-1.47****	**-1.17****	**-1.71***	**-1.54*****	**-1.57*****	**-1.38*****	similar to glycosyltransferases
lmo2363	gadB	**-3.97*****	**-4.26*****	**-5.11*****	**-4.69*****	**-1.05***	**-2.63****	glutamate decarboxylase
lmo2684		**-9.46*****	**-4.14*****	**-3.09****	**-1.11****	**-1.58****	**-2.85*****	similar to PTS system, cellobiose-specific IIC component
lmo2685		**-8.68*****	**-6.00*****	**-2.01*****	**-1.42****	**-1.23*****	**-2.86*****	similar to PTS system, cellobiose-specific IIA component

*=significance of Log ratio for each gene, where * P<0.05, ** P<0.01, *** P<0.001.

Overall, analysis of expression of analogous genes in *L. monocytogenes* strains that were adapted to cold and hyperosmotic stress revealed significant strain specificity in terms of individual gene expression profile.

7.4 Overview of a strain-specific gene activation in response to stress in *L. monocytogenes.*

A relatively small overlap in gene expression was observed between individual strains in response to either hyperosmotic or low temperature induced stress, suggesting that strain specific components dominate in the adaptive response of this organism to adverse stress conditions. It must be noted this complement of strain-specific responses only covers the *L. monocytogenes* "metagenome". In other words genes found in virtually all strains were examined here since they are complementary to

the probes used in the microarray analysis. This study did not extend to investigate strain-specific genes that also may have a role to play in specific responses to stress conditions.

Strain ATCC19115 is a serotype 4b (lineage I) strain of clinical origin and failed to grow at 12.5% (w/v) NaCl (section 2.3.1). It had a relatively average growth rate at 4°C (section 2.3.2). Hyperosmotic adaptation to 10.0% (w/v) NaCl induced significant up-regulation >two-fold of 109 genes which were not expressed in either strain ScottA or 70-1700 (Table D.1, Appendix D). Among the highly activated genes were the 23-fold activated *lmo2349* encoding an amino acid transporter protein, *lmo0189* encoding a protein homologous to Veg in *B. subtilis*, *svpA* (*lmo2185*) encoding a cell surface protein involved in iron transport; a protein translocase subunit encoding gene *secE* (*lmo0245*) and *cspL* which encodes a cold shock protein. Up-regulation of 66 unique genes was observed for the same strain adapted to low temperature environment (Table D.2, Appendix D). These included a large proportion (47.0%) of genes encoding proteins of unknown function such as *lmo1432* and *lmo2504*. Other highly activated genes included *lmo0949* encoding as yet uncharacterised transporter protein, *deaD* (*lmo0866*) encoding an ATP-dependent RNA helicase, and *lmo1647* encoding an enzyme involved in glycerophospholipid metabolism. Only two genes unique to ATCC19115 were expressed during adaptation to both stresses: *lmo0581* (putative SAM-dependent methyltransferase) and *lmo1186* (putative ethanolamine periplasmic transport protein).

Another serotype 4b strain ScottA, often studied as model strain in food-related stress studies, was found in this study to be relatively salt tolerant (section 2.3.1) and to have an average growth rate at 4°C. Adaptation to 12.0% (w/v) NaCl stress in this strain induced activation of 28 genes uniquely expressed in this strain only (Table D.3, Appendix D).These included *lmo2226* and *lmo815* both encoding proteins of unknown function, as well as *lmo2230* encoding a protein involved in signal transduction. Adaptation to cold temperature activated 50 uniquely expressed genes in this strain (Table D.4, Appendix D). These included *lmo1254* encoding a protein involved in trehalose metabolism; *lmo2186* encoding an iron transporter; *lmo0027* encoding a PTS transporter; *eutA* (*lmo1174*), encoding ethanolamine lyase reactivating factor, and *thrB* (*lmo2545*) encoding homoserine kinase. None of these genes were similarly expressed in adaptation to both stresses.

Strain 70-1700 is a serotype 4e strain of animal origin with a low salt tolerance and an average growth rate at 4°C. This strain induced activation of 54 uniquely expressed genes following adaptation to 8.0% NaCl (Table D.5, Appendix D). Expression of these was relatively weak with the highest of 4-fold up-regulation observed for *pduM* (lmo1162). Other genes included lmo2112 encoding a putative DNA binding protein and *apt* (*lmo1524*) encoding a protein involved in purine metabolism. Cold growth induced activation of 65 genes not expressed in either ATCC19115 or ScottA exposed to the same growth conditions (Table D.6 Appendix D). A large proportion of these (27.0%) included ribosomal proteins such as those encoded by *lmo2616*, *lmo2618-lmo2618* and *lmo2628*. Five genes uniquely up-regulated only in strain 70-1700 expressed following adaptation to both stresses included *lmo1452*, *lmo1095*, *lmo2111*, *lmo2207* and *lmo2540* encoding mainly uncharacterized proteins.

Strain specific gene expression responses constituted a large proportion of overall up-regulated genes in *L. monocytogenes* isolates. Strain specificity should therefore be considered when examining a particular response in this organism.

7.5 Summary and Conclusions.

Overall adaptation to hyperosmotic and low temperature growth conditions in three *L. monocytogenes* strains were found to have many parallels, especially when examined using a statistically-based ontological approach. Evidence was presented for strong activation of genes associated with protein synthesis, in particular those coding proteins of the ribosome and involved directly in transcription. Genes associated with DNA maintenance; modification to cell envelope, and cell division were also up-regulated. This suggests that adaptation to these adverse conditions engages similar mechanisms to cope with the induced stress.

Analysis of individual gene expression patterns revealed a significant proportion of differentially expressed genes amongst the three strains examined. Overall there were a significant proportion of strain-specific genes expressed following adaptation to either of the two environmental stresses studied. This strongly emphasises the importance of the influence strain-specific variability in *L. monocytogenes*.

Overall Conclusion and Perspectives:

This study is the first to assess the genetic bases of stress adaptation in multiple strains of *L. monocytogenes,* significantly contributing to the understanding of stress physiology in this organism.

Hyperosmotic and cold stress factors, though quite different in terms of physiochemical stress on bacterial cells, revealed many parallels in terms of gene expression in three strains of *L. monocytogenes* studied. The results suggest that a broadly similar genetic regulatory mechanism could be operating in response to cold and hyperosmotic stresses.

Results presented in this study raised some significant questions in particular in regards to compatible solute up-take in *L. monocytogenes* in response to environmental stress.
There was a strong evidence of strain-specific preference for compatible solutes in stress adapted cells. Assessing compatible solute composition in the cytosol of these cells by HPLC may potentially provide clarification to this observation.

There was an indication of enhanced peptide up-take suggested by up-regulation of the *opp* operon in cells adapted to both stresses. The importance of this operon in tolerance to both stresses was also independently established in a null mutant strain of *L. monocytogenes*, which produced the longest generation times when exposed to either 12.5% (w/v) NaCl or 4°C. There also appeared to be a strong activation of *betL* in osmo-adapted cells, previously thought to be involved only in initial stages of hyperosmotic stress response. Expression of this gene (and other genes) in osmo-adapted cells would have to be confirmed by q-PCR or proteomics, before the significance of this observation can be established. Up-regulation of *gad* genes in ATCC19115 strain shocked with 10.0% (w/v) NaCl suggested activation of GAD-system potentially counteracting the acidification of bacterial cytoplasm. Prior to this study the GAD-system has not been linked to hyperosmotic stress. Once again this observation would have to be confirmed by other means.

Two-dimensional tandem mass spectrometry-based proteomics assessment of *L. monocytogenes* in response to hyperosmotic and cold-stresses is currently being undertaken to investigate the significance of results presented in this thesis. This data will determine whether the transcriptomic data reliably couples to protein abundance changes and may provide additional insights into adaptation of *L. monocytogenes* to salt and cold stress.

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Appendices

A.1 Strain specific genes for four sequenced strains of L. monocytogenes available on KEGG.

A.1.1 Comparison of whole genome sequences available for *L. monocytogenes* revealed strain specific genes.

Table A.4 A list of genes with differential presence in various genomes derived from whole genome sequences available to date on KEEG database.

EGD-e[#]	F2635[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
(lmo0066)†	LMOf2365_0077	x[x]	x		similar to uncharacterised conserved protein, contains an ADP-ribosylation domain
(lmo0066)	LMOf2365_0082	x	x		similar to uncharacterized conserved protein; putative transposase; partially homologous to lmo0066
(lmo0072)	x	Lm4b_00079	x		unknown protein; partially homologous to lmo0072
(lmo0142)	x	(Lm4b_00151b)	LMHCC_1156		unknown protein; homology with lmo0142
(lmo0143)	LMOf2365_0166	x	x		unknown protein
(lmo0148)	x	(Lm4b_00149)	LMHCC_2488		unknown protein; homologous to lmo0148
(lmo0263)	**LMOf2365_0282**	**Lm4b_00290**	x	*inlD*	internalin D; homologous to lmo0263
(lmo0338)	LMOf2365_0360	x	LMHCC_2296		Unknown protein
(lmo0379)	x	x	LMHCC_2777		unknown protein; homology to lmo0379
(lmo0460)	x	Lm4b_02042	(LMHCC_2168)		putative membrane lipoptotein
(lmo0462)	x	Lm4b_02040	(LMHCC_2166)		unknown protein
(lmo0671)	x	(Lm4b_00698)	LMHCC_0236		putative secreted protein; homology to lmo0671
(lmo1117)	**LMOf2365_1111**	**Lm4b_01110**	x		similar to glyoxalase/bleomycin resistance protein/dioxygenase superfamily proteins
(lmo2310)	x	x	LMHCC_2580		putative phage protein GP35; homologous to lmo2310
(lmo2312)	x	x	LMHCC_1394		unknown protein; homology to lmo2312
lmo0017	**LMOf2365_0020**	**Lm4b_00020**	x	*capA*	similar to *B. subtilis* poly-gamma-glutamate biosynthesis enzyme CapA
lmo0036	**LMOf2365_0045**	**Lm4b_00045**	x	*argF*	ornithine/putrescine carbamoyltransferase
lmo0037	**LMOf2365_0046**	**Lm4b_00046**	x	*gadC*	putative amino acid/amine antiporter
lmo0038	**LMOf2365_0047**	**Lm4b_00047**	x	*aguA*	putative multifunctional agmatine deiminase/ornithine carbamoyltransferase/ putrescine carbamoyltransferase/ carbamate kinase
lmo0039	**LMOf2365_0048**	**Lm4b_00048**	x	*arcC*	carbamate kinase
lmo0040	**LMOf2365_0049**	**Lm4b_00049**	x	*aguA*	putative multifunctional agmatine deiminase/ornithine carbamoyltransferase/ putrescine carbamoyltransferase/ carbamate kinase
lmo0062	LMOf2365_0073	x	x		unknown protein
lmo0063	LMOf2365_0074	x	x	*esxB*	putative ESAT-6-like secretion system protein; weakly similar to S. aureus EsxB
lmo0064	LMOf2365_0075	x	x		unknown protein
lmo0065	LMOf2365_0076	x	x		unknown protein
lmo0066	LMOf2365_0084	x	x		unknown protein; partially homologous to lmo0066
lmo0068	LMOf2365_0085	x	x		unknown protein
lmo0069	LMOf2365_0086	x	x		unknown protein
lmo0070	LMOf2365_0087	x	x		putative membrane protein
lmo0071	LMOf2365_0088	x	x		unknown protein
lmo0072	**LMOf2365_0089**	**Lm4b_00074**	x		unknown protein
lmo0073	LMOf2365_0090	x	x		unknown protein

EGD-e[#]	F2635[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
lmo0074	LMOf2365_0091	x	x		unknown protein
lmo0079	LMOf2365_0096	x	x		unknown protein
lmo0082	**LMOf2365_0099**	**Lm4b_00085**	x		unknown protein
lmo0117	**LMOf2365_0135**	**Lm4b_00121**	x	*lmaB*	similar to Antigen B protein
lmo0118	**LMOf2365_0136**	**Lm4b_00122**	x	*lmaA*	predicted secreted protein; putative phage tail protein; similar to Antigen A protein
lmo0119	**LMOf2365_0137**	**Lm4b_00123**	x		similar to phage proteins
lmo0120	**LMOf2365_0138**	**Lm4b_00124**	x		similar to phage proteins
lmo0121	**LMOf2365_0139**	**Lm4b_00125**	x		putative bacteriophage tail tape measure proteins
lmo0122	**LMOf2365_0140**	**Lm4b_00126**	x		similar to protein gp17 from Bacteriophage A118
lmo0140	**LMOf2365_0513**	**Lm4b_00144**	x		unknown protein
lmo0142	x	Lm4b_00151b	(LMHCC_1156)		unknown protein
lmo0148	x	Lm4b_00149	LMHCC_2489		unknown protein
lmo0160	LMOf2365_0175	x	LMHCC_2479		putative peptidoglycan bound protein (LPXTG motif); putative collagen adhesion protein
lmo0215	LMOf2365_0226	x	LMHCC_2428	*ltrB*	similar to polysaccharide export/biosynthesis protein; homolog to S. aureus low temperature requirement protein B
lmo0252	x	x	LMHCC_2387		putative transcriptional regulator, CopY family; similar to beta-lactamase repressor protein
lmo0253	x	x	LMHCC_2386		putative membrane protein with peptidase activity; similar to beta-lactamase class C antirepressor proteins; putative beta-lactamase
lmo0254	x	x	LMHCC_2385		unknown protein
lmo0294	**LMOf2365_0315**	**Lm4b_00322**	x		putative transcriptional regulator, LysR family
lmo0295	**LMOf2365_0316**	**Lm4b_00323**	x	*nfsA*	similar to NADPH/FMN-dependent flavin reductases and oxygen-insensitive nitroreductases
lmo0302	x	Lm4b_00330			unknown protein
lmo0306	x	x	LMHCC_2325		unknown protein
lmo0307	x	x	LMHCC_2324		unknown protein with lipase/esterase domain
lmo0309	x	x	LMHCC_2322		similar to uncharacterized conserved proteins
lmo0334	**LMOf2365_0351**	**Lm4b_00351**	x		Unknown protein
lmo0339	x	x	LMHCC_2294	*ppa*	similar to inorganic pyrophosphatase
lmo0357	x	Lm4b_00376	LMHCC_2275	*frvA*	putative PTS system, fructose-specific IIA component
lmo0358	x	Lm4b_00377	LMHCC_2274	*fruA*	putative PTS system, fructose-specific IIBC component
lmo0361	x	Lm4b_00380	x	*tatC*	twin-arginine translocation system protein
lmo0362	x	Lm4b_00381	x	*tatA*	twin-arginine translocation system protein
lmo0365	x	Lm4b_00384	x	*efeU*	putative high-affinity iron transporter
lmo0366	x	Lm4b_00385	x	*efeO*	similar to periplasmic lipoprotein involved in high affinity iron transport
lmo0367	x	Lm4b_00386	x	*efeB*	putative iron-dependent peroxidase; putative cryptic ferrous ion transporter
lmo0378	x	Lm4b_00397	x		unknown protein
lmo0395	LMOf2365_0407	x	LMHCC_2241		similar to acetyltransferases; similar to blasticidin S-acetyltransferase
lmo0398	x	Lm4b_00422	LMHCC_2238	*mngA*	putative PTS system, fructose/mannose-like-specific IIA component
lmo0399	x	Lm4b_00423	LMHCC_2237	*frwB*	putative PTS system, fructose-like-specific IIB component
lmo0400	x	Lm4b_00424	LMHCC_2236	*frwC*	putative PTS system, fructose-like-specific IIC component
lmo0418	**LMOf2365_0437**	**Lm4b_00441**	x		unknown protein
lmo0421	x	x	LMHCC_2218	*rodA*	similar to FtsK/RodA/SpoIIIE and related proteins
lmo0422	x	x	LMHCC_2217	*lstR*	lineage-specific thermal regulator protein; similar to transcriptional regulator (PadR family)
lmo0423	x	x	LMHCC_2216	*rpoE*	RNA polymerase, sigma-24 (sigma C) subunit, ECF subfamily
lmo0438	**LMOf2365_0475**	**Lm4b_00465**	x		unknown protein; putative phage protein
lmo0474	**LMOf2365_0502**	**Lm4b_00493**	x		unknown protein
lmo0475	**LMOf2365_0503**	**Lm4b_00494**	x		unknown protein
lmo0477	**(LMOf2365_0506)**	**(Lm4b_00498)**	x		putative secreted protein
lmo0478	**LMOf2365_0506**	**(Lm4b_00498)**	x		putative secreted protein
lmo0479	x	Lm4b_00498	x		putative secreted protein
lmo0492	**LMOf2365_0522**	**Lm4b_00515**	x		putative transcriptional regulator, LysR family

EGD-e[#]	F2635[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
lmo0493	**LMOf2365_0523**	**Lm4b_00516**	x		similar to predicted hydrolases, CocE/NonD family
lmo0525	LMOf2365_0554	x	LMHCC_2107		similar to uncharacterized conserved proteins
lmo0614	**LMOf2365_0643**	**Lm4b_00640**	x		similar to acetyltransferases
lmo0630	LMOf2365_0659	x	x		putative PTS system operon regulator/IIA subunit
lmo0631	LMOf2365_0660	x	x	*frvA*	putative PTS system, fructose-like-specific IIA component
lmo0632	LMOf2365_0661	x	x	*frwC*	putative PTS system, fructose-like-specific IIC component
lmo0633	LMOf2365_0662	x	x	*frwB*	putative PTS system, fructose-like-specific IIB component
lmo0634	LMOf2365_0663	x	x	*kbaZ*	putative D-tagatose-6-phosphate kinase
lmo0671	x	Lm4b_00698	LMHCC_0236		putative secreted and/or lipoprotein
lmo0726	LMOf2365_0762	x	LMHCC_1897		unknown protein
lmo0746	x	x	LMHCC_1882		unknown protein
lmo0747	x	x	LMHCC_1881		unknown protein
lmo0748	x	x	LMHCC_1880		unknown protein
lmo0749	x	x	LMHCC_1879		putative transcriptional regulators
lmo0750	x	x	LMHCC_1878		unknown protein
lmo0751	x	x	LMHCC_1877		unknown protein
lmo0765	x	Lm4b_00781	x		similar to uncharacterized conserved proteins
lmo0766	x	Lm4b_00782	x	*ycjO*	putative sugar uptake ABC transporter, permease protein
lmo0767	x	Lm4b_00783	x	*ycjP*	putative sugar uptake ABC transporter, permease protein
lmo0768	x	Lm4b_00784	x	*ycjN*	putative sugar uptake ABC transporter, substrate binding protein
lmo0769	x	Lm4b_00785	x		putative alpha-1,6-mannase
lmo0770	x	Lm4b_00786	x		putative transcriptional regulator, LacI family
lmo0780	x	x	LMHCC_1855		unknown protein
lmo0783	x	Lm4b_00798	LMHCC_1851	*manX*	putative PTS system, mannose-specific IIB component
lmo0784	x	Lm4b_00799	LMHCC_1850	*manX*	putative PTS system, mannose-specific IIA component
lmo0868	**LMOf2365_0887**	**Lm4b_00886**	x		unknown protein
lmo0869	**LMOf2365_0888**	**Lm4b_00887**	x		similar to uncharacterized conserved proteins
lmo0870	**LMOf2365_0889**	**Lm4b_00888**	x		similar to amidohydrolases
lmo0888	x	Lm4b_00906	LMHCC_1738	*chpA*	similar to PemK-like autoregulated growth inhibitor protein
lmo0934	x	Lm4b_00954	LMHCC_1688	*yjeS*	uncharacterized Fe-S protein
lmo1030	**LMOf2365_1051**	**Lm4b_01050**	x		putative transcriptional regulator, LacI family
lmo1031	**LMOf2365_1052**	**Lm4b_01051**	x		putative L-fucose isomerase
lmo1032	**LMOf2365_1053**	**Lm4b_01052**	x	*tktA*	transketolase, thiamine binding
lmo1033	**LMOf2365_1054**	**Lm4b_01053**	x	*dxs*	putative 1-deoxy-D-xylulose-5-phosphate synthase (C-terminal region)
lmo1034	**LMOf2365_1055**	**Lm4b_01054**	x	*glpK*	glycerol kinase
lmo1035	**LMOf2365_1056**	**Lm4b_01055**	x	*bglF*	putative PTS system, beta-glucosides-specific IIABC component
lmo1036	**LMOf2365_1057**	**Lm4b_01056**	x	*ppk*	putative polyphosphate kinase
lmo1085	x	x	LMHCC_1548	*tagB*	putative CDP-glycerol:poly(glycerophosphate) glycerophosphotransferase; similar to S. aureus TarF and TarK
lmo1097	x	Lm4b_00546	LMHCC_1412		putative conjugative transposon/phage protein
lmo1098	x	x	LMHCC_1339		similar to TN916 ORF8
lmo1099	x	x	LMHCC_1340		similar to a protein encoded by TN916 (putative RNA polymerase specialized subunit sigma subunit, sigma24 homolog)
lmo1116	**LMOf2365_1120**	**Lm4b_01118**	x		similar to transcription regulator, AraC family
lmo1117	**LMOf2365_1121**	**Lm4b_01119**	x		similar to glyoxalase/bleomycin resistance protein/dioxygenase superfamily proteins
lmo1120	LMOf2365_1126	x	x		unknown protein
lmo1121	LMOf2365_1127	x	x		unknown protein
lmo1122	LMOf2365_1128	x	x		unknown protein
lmo1123	LMOf2365_1129	x	x		unknown protein
lmo1124	LMOf2365_1130	x	x		unknown protein
lmo1125	x	x	LMHCC_1528		unknown protein
lmo1127	**LMOf2365_1133**	**Lm4b_01129**	x		similar to uncharacterized conserved proteins

EGD-e[#]	F2635[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
lmo1139	x	x	LMHCC_1512		unknown protein
lmo1213	**LMOf2365_1222**	**Lm4b_01218**	x		putative transcriptional regulator, PadR family
lmo1214	**LMOf2365_1223**	**Lm4b_01219**	x		unknown protein
lmo1289	LMOf2365_1307	x	LMHCC_1285		putative peptidoglycan bound protein
lmo1307	x	x	LMHCC_1263		similar to uncharacterized conserved proteins
lmo1335	**LMOf2365_1352**	**Lm4b_01343**	x	*rpmG*	ribosomal protein L33
lmo1450	**LMOf2365_1469**	**Lm4b_01460**	x	*deaD*	similar to ATP-dependent RNA helicase
lmo1617	x	Lm4b_01628	LMHCC_0946	*emrB*	putative multidrug MFS efflux proteins
lmo1640	x	Lm4b_01651	x		unknown protein
lmo1655	**LMOf2365_1676**	**Lm4b_01665**			similar to uncharacterized conserved proteins; similar to VanZ proteins
lmo1656	**LMOf2365_1677**	**Lm4b_01666**			unknown protein
lmo1717	x	Lm4b_01731	LMHCC_0845		similar to uncharacterized conserved proteins
lmo1723	**LMOf2365_1747**	**Lm4b_01737**	x		putative permease
lmo1724	**LMOf2365_1748**	**Lm4b_01738**	x	*ybhF*	putative effux ABC transporter, ATP-binding protein
lmo1725	**LMOf2365_1749**	**Lm4b_01739**	x		putative transcriptional regulator, GntR family
lmo1980	x	Lm4b_01992	LMHCC_0580		unknown protein
lmo2047	**LMOf2365_2079**	**Lm4b_02068**	x	*rpmF*	ribosomal protein L32
lmo2084	x	Lm4b_02105	LMHCC_0473		putative aminoglycoside phosphotransferase
lmo2135	x	Lm4b_02159	LMHCC_0410	*fruA*	putative PTS system, fructose-specific IIC component
lmo2136	x	Lm4b_02160	LMHCC_0409	*frwB*	putative PTS system, fructose-specific IIB component
lmo2137	x	Lm4b_02161	LMHCC_0408	*mngA*	putative PTS system, fructose-specific IIA component
lmo2157	**LMOf2365_2189**	**Lm4b_02182**	x	*sepA/yjcS*	similar to predicted alkyl sulfatases and related hydrolases; similar to E. coli YjcS
lmo2257	**Lmof2365_2290**	**Lm4b_02284**	x		unknown protein
lmo2394	Lmof2365_2368	x	x		unknown protein
lmo2395	Lmof2365_2369	x	x		unknown protein
lmo2407	**Lmof2365_2381**	**Lm4b_02379**	x		unknown protein
lmo2420	Lmof2365_2391	x	LMHCC_0182		Unknown protein
lmo2463	x	Lm4b_02432	LMHCC_0137	*mdtC*	putative multidrug efflux transporter protein
lmo2470	**Lmof2365_2443**	**Lm4b_02439**	x		leucine rich repeat domain protein; similar to internalin
lmo2671	**Lmof2365_2651**	**Lm4b_02644**	x		glyoxalase/bleomycin resistance/dioxygenase superfamily
lmo2672	**Lmof2365_2652**	**Lm4b_02645**	x		similar to transcription regulator, AraC family
lmo2771	**Lmof2365_2761**	**Lm4b_02746**	x	*bglA*	putative cryptic beta-glucosidase/phospho-beta-glucosidase
lmo2772	**Lmof2365_2762**	**Lm4b_02747**	x	*bglF*	putative PTS system, beta-glucosides-specific IIABC component
lmo2773	**Lmof2365_2763**	**Lm4b_02748**	x	*bglG*	beta-glucoside-specific PTS system operon transcriptional terminator
lmo2776	**Lmof2365_2767**	**Lm4b_02751**	x		putative bacteriocin
lmo2805	Lmof2365_2797	x	LMHCC_2717		unknown protein
lmo2806	x	Lm4b_02781			unknown protein
lmo2807	x	x	LMHCC_2716		unknown protein
lmo2809	x	Lm4b_02782	x		unknown protein
lmo2821	Lmof2365_2812	Lm4b_02794	x	*inlJ*	internalin J
lmo2856	Lmof2365_2846	x	LMHCC_2665	*rpmH*	ribosomal protein L34

[#] Strain EGD-e (serotype 1/2a); KEGG code: *lmo*; number of nucleotides 2944528; number of protein genes 2846, first *L. monocytogenes* genome sequenced (Glaser et al.,2001).

[*] F2365 (serotype 4b); KEGG code: *lmf*; number of nucleotides 2905187; number of protein genes 2821 (Nelson et al., 2004).

[¥] Strain Clip81459 (serotype 4b); KEGG code: *lmc*; number of nucleotides 2912690; number of protein genes 2766.

[Ω] Strain HCC23 (serotype 4a) KEGG code: *lmh*; number of nucleotides 2976212; number of protein genes 2974

[x] Indicates corresponding no corresponding protein ortholog occurs in the given genome.

†A similar protein ortholog is present but similarity is comparatively low (usually less than 60% amino acid identity).

A.1.2 Comparison of whole genome sequences available for *L. monocytogenes* revealed EGD-e specific genes.

Table A.2 A list of EGD-e strain specific genes derived from whole genome sequences available to date on KEEG database.

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
lmo0067	x[x]	x	x	*yegU*	putative ADP-ribosylglycohydrolase
lmo0080	x	x	x		unknown protein
lmo0081	x	x	x		unknown protein
lmo0143	x	x	x		unknown protein
lmo0147	x	x	x		unknown protein
lmo0149	x	x	x		unknown protein
lmo0150	x	x	x		unknown protein
lmo0151	x	x	x		unknown protein
lmo0171	x	x	x		putative peptidoglycan bound protein
lmo0174	x	x	x		putative transposase
lmo0255	x	x	x		putative lipoprotein
lmo0262	x	x	x	*inlG*	internalin G
lmo0304	*x*	x	x		unknown protein
lmo0310	x	x	x		unknown protein
lmo0311	x	x	x		Unknown protein
lmo0312	x	x	x		Unknown protein
lmo0313	x	x	x	*tatD*	putative Mg-dependent deoxyribonuclease
lmo0329	x	x	x		putative transposase
lmo0330	x	x	x		putative transposase/integrase
lmo0332	x	x	x		Unknown protein
lmo0341	x	x	x		Unknown protein
lmo0379	x	x	x		unknown protein
lmo0380	x	x	x		unknown protein
lmo0435	x	x	x	*bapL*	biofilm associated peptidoglycan bound protein (LPXTG motif); putative adhesin
lmo0444	x	x	x		putative membrane protein
lmo0445	x	x	x		similar to transcriptional regulators/antiterminators
lmo0446	x	x	x		putative penicillin V acylase
lmo0447	x	x	x	*gadB'*	similar to glutamate decarboxylase
lmo0448	x	x	x	*gadC'*	glutamate:gamma-aminobutyric acid antiporter
lmo0465	x	x	x		unknown protein
lmo0466	x	x	x		unknown protein
lmo0467	x	x	x		unknown protein
lmo0468	x	x	x		unknown protein
lmo0469	x	x	x		unknown protein
lmo0470	x	x	x		contains DNA methylase domain
lmo0471	x	x	x		unknown protein
lmo0472	x	x	x		unknown protein
lmo0497	x	x	x	*yibD*	similar to glycosyl transferases
lmo0638	x	x	x		unknown protein
lmo0734	x	x	x	*rbsR*	putative transcriptional regulator, LacI family
lmo0735	x	x	x	*alsE*	putative allulose-6-phosphate epimerase
lmo0736	x	x	x	*rpiB*	ribose 5-phosphate isomerase B
lmo0737	x	x	x	*ydcP*	similar to uncharacterized conserved proteins; similar to predicted peptidase
lmo0738	x	x	x	*bglF*	putative PTS system, beta-glucosides-specific IIABC component
lmo0739	x	x	x	*bglA*	similar to cryptic 6-phospho-beta-glucosidase
lmo0801	x	x	x		putative peptidoglycan bound protein (LPXTG motif)
lmo0815	x	x	x		putative transcriptional regulator, MarR family
lmo0816	x	x	x	*paiA*	similar to B. subtilis polyamine pool homeostasis protein PaiA; putative N-spermidine/spermine acetyltransferases

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
lmo0827	x	x	x		putative transposase
lmo0828	x	x	x		putative integrase/transposase
lmo0924	x	x	x		similar to ABC transporter, ATP-binding protein (C-terminal part)
lmo0933	x	x	x		putative glycosyl transferase; putative dolichol mannosyltransferase
lmo0940	x	x	x		unknown protein
lmo1060	x	x	x	*cusR*	putative two-component response regulator
lmo1061	x	x	x	*baeS*	putative two-component sensor histidine kinase
lmo1062	x	x	x	*ybbP*	putative metabolite uptake ABC transporter, permease protein
lmo1063	x	x	x	*ybbA*	putative metabolite uptake ABC transporter, ATP-binding protein
lmo1077	x	x	x	*tagB*	putative CDP-glycerol:poly(glycerophosphate) glycerophosphotransferase; similar to S. aureus TarX
lmo1079	x	x	x		putative glucosyl transferase and membrane protein
lmo1080	x	x	x		putative glucosyl transferase
lmo1081	x	x	x	*rfbA*	glucose-1-phosphate thymidylyltransferase
lmo1082	x	x	x	*rfbC*	dTDP-4-dehydrorhamnose 3,5-epimerase
lmo1083	x	x	x	*rfbB*	dTDP-glucose 4,6-dehydratase
lmo1084	x	x	x	*rfbD*	dTDP-4-dehydrorhamnose reductase
lmo1090	x	x	x		putative glyosyl glycerol:poly(glycerophosphate) glycerophospho- transferase; similar to S. aureus TarX
lmo1091	x	x	x		similar to glycosyltransferases
lmo1100	x	x	x	*zntA*	putative Zn/Cd/Co/Pb efflux ATPase protein
lmo1101	x	x	x	*lspA*	putative prolipoprotein signal peptidase II
lmo1102	x	x	x	*arsR*	similar to predicted transcriptional regulator AsrR; similar to cadmium efflux accessory protein
lmo1103	x	x	x		similar to TN916 ORF13
lmo1104	x	x	x		similar to TN916 ORF14 (putative cell wall-associated hydrolase/invasion-associated protein)
lmo1105	x	x	x		similar to TN916 ORF15 (putative transmembrane transporter protein)
lmo1106	x	x	x		similar to TN916 ORF16 (similar to type IV secretory pathway, VirB4 components, predicted ATPase)
lmo1107	x	x	x		similar to TN916 ORF17
lmo1108	x	x	x		similar to TN916 ORF18 (putative antirestriction protein)
lmo1109	x	x	x		similar to TN916 ORF19
lmo1110	x	x	x		unknown protein
lmo1111	x	x	x		similar to TN916 ORF20 (similar to putative phage replication protein RstA)
lmo1112	x	x	x		similar to TN916 ORF21 (similar to DNA segregation ATPase FtsK/SpoIIIE and related proteins)
lmo1113	x	x	x		similar to TN916 ORF22
lmo1114	x	x	x		similar to TN916 ORF23
lmo1115	x	x	x		putative peptidoglycan bound protein
lmo1118	x	x	x		unknown protein
lmo1119	x	x	x	*dam*	putative adenine-specific DNA methyltransferase; similar to prophage proteins
lmo1188	x	x	x		unknown protein
lmo1648	x	x	x		unknown protein
lmo1659	x	x	x		unknown protein
lmo1876	x	x	x		similar to formyl-tetrahydrofolate synthetase C-terminal part
lmo1968	x	x	x		putative creatininase
lmo1969	x	x	x	*eda*	2-dehydro-3-deoxyphosphogluconate aldolase / 4-hydroxy-2-oxoglutarate aldolase
lmo1970	x	x	x	*php*	similar to phosphotriesterases and predicted metal-dependent hydrolases
lmo1971	x	x	x	*ulaA*	putative PTS system, pentitol-specific IIA component
lmo1972	x	x	x	*ulaB*	putative PTS system, pentitol-specific IIB component
lmo1973	x	x	x	*ulaC*	putative PTS system, pentitol-specific IIC component
lmo1974	x	x	x		similar to transcription regulator, GntR family
lmo2026	x	x	x		putative peptidoglycan bound protein
lmo2027	x	x	x		putative peptidoglycan bound protein
lmo2271	x	x	x		similar to phage protein
lmo2272	x	x	x		unknown protein
lmo2273	x	x	x		similar to protein gp30 [Bacteriophage A118]
lmo2274	x	x	x		similar to protein gp29 [Bacteriophage A118]

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
lmo2275	x	x	x		similar to protein gp28 [Bacteriophage A118]
lmo2276	x	x	x		unknown protein
lmo2277	x	x	x		similar to phage protein
lmo2278	x	x	x		similar to protein gp25 [Bacteriophage A118]; putative L-alanoyl-D-glutamate peptidase (endolysin)
lmo2279	x	x	x		holin protein; similar to protein gp24 [Bacteriophage A118]
lmo2280	x	x	x		similar to protein gp23 [Bacteriophage A118]
lmo2281	x	x	x		similar to protein gp22 [Bacteriophage A118]
lmo2282	x	x	x		similar to protein gp21 [Bacteriophage A118]
lmo2283	x	x	x		similar to protein gp20 [Bacteriophage A118]
lmo2284	x	x	x		similar to protein gp19 [Bacteriophage A118]
lmo2285	x	x	x		similar to protein gp18 [Bacteriophage A118]
lmo2286	x	x	x		similar to protein gp17 [Bacteriophage A118]
lmo2287	x	x	x		putative tape-measure; similar to protein gp16 [Bacteriophage A118]
lmo2288	x	x	x		similar to protein gp15 [Bacteriophage A118]
lmo2289	x	x	x		similar to protein gp14 [Bacteriophage A118]
lmo2290	x	x	x		similar to acterial surface proteins containing Ig-like domains; similar to protein gp13 [Bacteriophage A118]
lmo2291	x	x	x		putative major tail shaft protein; similar to protein gp12 [Bacteriophage A118]
lmo2292	x	x	x		similar to protein gp11 [Bacteriophage A118]
lmo2293	x	x	x		similar to protein gp10 [Bacteriophage A118]
lmo2294	x	x	x		predicted integral membrane protein; similar to protein gp9 [Bacteriophage A118]
lmo2295	x	x	x		similar to protein gp8 [Bacteriophage A118]
lmo2296	x	x	x		putative phage coat protein
lmo2297	x	x	x		putative phage scaffolding protein
lmo2298	x	x	x		putative minor capsid protein; similar to protein gp4 [Bacteriophage A118]
lmo2299	x	x	x		similar to minor capsid protein; putative portal protein
lmo2300	x	x	x		similar to putative terminase large subunit
lmo2301	x	x	x		similar to putative terminase small subunit
lmo2302	x	x	x		unknown protein
lmo2303	x	x	x		similar to protein gp66 [Bacteriophage A118]
lmo2304	x	x	x		similar to protein gp65 [Bacteriophage A118]
lmo2305	x	x	x		similar to bacteriophage tail assembly protein
lmo2306	x	x	x		uncharacterised conserved protein
lmo2307	x	x	x		unknown protein
lmo2308	x	x	x	*ssb*	single-stranded DNA-binding protein
lmo2309	x	x	x		Unknown protein
lmo2310	x	x	x		Unknown protein
lmo2311	x	x	x		Unknown protein
lmo2312	x	x	x		uncharacterised conserved protein
lmo2313	x	x	x		similar to a bacteriophage protein
lmo2314	x	x	x		unknown protein
lmo2315	x	x	x		similar to protein gp51 [Bacteriophage A118]
lmo2316	x	x	x	*dam*	similar to site-specific DNA methylases
lmo2317	x	x	x		similar to putative primosome component and related proteins; similar to protein gp49 [Bacteriophage A118]
lmo2318	x	x	x		similar to DNA single-strand annealing proteins
lmo2319	x	x	x		similar to phage protein
lmo2320	x	x	x		unknown protein
lmo2321	x	x	x		similar to protein gp45 [Bacteriophage A118]
lmo2322	x	x	x		similar to protein gp44 [Bacteriophage A118]
lmo2323	x	x	x		similar to protein gp43 [Bacteriophage A118]
lmo2324	x	x	x		similar to phage anti-repressor protein
lmo2325	x	x	x		unknown protein
lmo2326	x	x	x		similar to protein gp41 [Bacteriophage A118]
lmo2327	x	x	x		unknown protein
lmo2328	x	x	x		similar to predicted transcriptional regulators
lmo2329	x	x	x		similar to a putativephage repressor protein

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
lmo2330	x	x	x		similar to protein gp33 [Bacteriophage A118]
lmo2331	x	x	x		similar to protein gp32 [Bacteriophage A118]
lmo2332	x	x	x	*int*	putative integrase
lmo2333	x	x	x		unknown protein
lmo2364	x	x	x		putative transcriptional regulator
lmo2408	x	x	x		putative transcriptional regulator
lmo2409	x	x	x		unknown protein
lmo2410	x	x	x		unknown protein
lmo2550	x	x	x	*yfdH*	putative bactoprenol glucosyl transferase
lmo2576	x	x	x		peptidoglycan bound protein (LPXTG motif)
lmo2686	x	x	x		unknown protein
lmo2711	x	x	x		unknown protein
lmo2753	x	x	x		unknown protein
lmo2786	x	x	x	*yegU/bvrC*	ADP-ribosylglycohydrolase
lmo2787	x	x	x	*bglF/bvrB*	putative PTS system, beta-glucosides-specific IIABC component
lmo2788	x	x	x	*bglG/bvrA*	beta-glucoside-specific PTS system operon transcriptional antiterminator
lmo2841	x	x	x	*ycjM*	putative sucrose phosphorylase

[#] Strain EGD-e (serotype 1/2a); KEGG code: *lmo*; number of nucleotides 2944528; number of protein genes 2846, first *L. monocytogenes* genome sequenced (Glaser et al.,2001).

[*] Strain ATCC19115 also known as F2365 (serotype 4b); KEGG code: *lmf*; number of nucleotides 2905187; number of protein genes 2821 (Nelson et al., 2004).

[¥] Strain Clip81459 (serotype 4b); KEGG code: *lmc*; number of nucleotides 2912690; number of protein genes 2766.

[Ω] Strain HCC23 (serotype 4a) KEGG code: *lmh*; number of nucleotides 2976212; number of protein genes 2974

[x] Indicates corresponding no corresponding protein ortholog occurs in the given genome.

A.1 Comparison of whole genome sequences available for L. monocytogenes revealed a number of genes absent from the EGD-e genome but present in genomes of serotype 4b and 4a strains.

Table A.3 A list of genes absent from EGD-e genome derived from whole genome sequences available to date on KEEG database.

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
x[x]	LMOf2365_0954	Lm4b_00952	LMHCC_1689		putative dolichyl phosphate-mannose protein mannosyltransferase
x	LMOf2365_1094	Lm4b_01093	LMHCC_1554		unknown protein with sialidase like domain
x	LMOf2365_1095	Lm4b_01094	LMHCC_1552		putative glycosyl transferase, group 2 family protein
x	LMOf2365_1096	Lm4b_01095	LMHCC_1551		unknown protein with possible glycosyltransferase domain
x	LMOf2365_1102	Lm4b_01101	LMHCC_1545		putative glycosyl transferase, group 2 family protein
x	LMOf2365_1103	Lm4b_01102	LMHCC_1544	*tagF*	putative CDP-glycerol glycerophosphotransferase
x	x	Lm4b_02144	x		putative polyglycerol phosphate assembly and export protein
x	Lmof2365_2741	Lm4b_02728	x		putative glycosyltransferase, family 2 protein
x	x	x	LMHCC_0471		putative serine/threonine-specific protein kinase
x	x	x	LMHCC_0985		putative NADH-dependent FMN reductase/iron sulfur flavoprotein
x	x	(Lm4b_00349)	LMHCC_0014		putative peptidoglycan bound surface protein; weak homology to lmo0171
x	LMOf2365_0289	Lm4b_00297	LMHCC_2364		peptidoglycan bound protein (LPXTG motif)
x	LMOf2365_0413	Lm4b_00415	x		putative peptidoglycan bound protein

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
x	LMOf2365_0450	Lm4b_00454	LMHCC_2204		large putative peptidoglycan bound protein (LPXTG motif)
x	LMOf2365_0693	Lm4b_00685	LMHCC_1971		putative peptidogylcan bound protein (LPXTG motif)
x	LMOf2365_0694	Lm4b_00687b	x		putative peptidogylcan bound protein (LPXTG motif)
x	LMOf2365_0805	Lm4b_00804	x		putative peptioglycan boun cell surface protein
x	LMOf2365_1254	Lm4b_01248	LMHCC_1335		putative peptidoglycan bound cell surface protein
x	LMOf2365_1974	Lm4b_01963	LMHCC_2534		putative peptidoglycan bound cell surface protein; weak homology to lmo0842
x	Lmof2365_2638	Lm4b_02631	x		putative peptidoglycan bound protein (LPXTG motif); weak homology to lmo2396
x	x	x	LMHCC_2033		putative cell surface protein
x	x	x	LMHCC_1819		putative peptidogylcan bound protein (LPXTG motif); weak homology to lmo0842
x	x	x	LMHCC_2776		YD repeat containing protein; putative cell wall associated protein
x	x	x	LMHCC_2775		putative cell wall associated protein
x	LMOf2365_1900	Lm4b_01887	x		putative serine protease
x	x	x	LMHCC_0648		putative D-amino acid dehydrogenase, small subunit
x	Lmof2365_2679	Lm4b_02673	x		putative dATP pyrophosphohydrolase
x	LMOf2365_0627	Lm4b_00624	LMHCC_2031	*bioY*	putative biotin synthase
x	LMOf2365_0325	x			putative type II restriction endonuclease
x	LMOf2365_0327	x			putative cytosine-specific DNA methylase
x	LMOf2365_0328	x			putative HNH-like endonuclease
x	x	x	LMHCC_2321		putative type II restriction endonuclease
x	x	x	LMHCC_2320		putative cytosine-specific DNA methylase
x	LMOf2365_1119	Lm4b_01117	x		putative amino terminal protease
x	x	x	LMHCC_2845		putative Clp protease/DnaK/DnaJ chaperone, ATP binding subunit
x	x	Lm4b_00543	x	*hsdR*	putative type 1 restriction enzyme, endonuclease subunit
x	x	Lm4b_00544	x	*hsdM*	putative type 1 restriction enzyme, modification subunit
x	x	Lm4b_00545b	x	*hsdS*	putative type 1 restriction enzyme, specificity subunit
x	x	Lm4b_00546	x		putative bacteriophage integrase
x	x	Lm4b_00547b	x	*hsdS*	putative type 1 restriction enzyme, specificity subunit
x	x	Lm4b_00679	x		putative type II DNA modification methyltransferase
x	x	Lm4b_00680	x		putative phage encoded two-component histidine kinase
x	x	Lm4b_00681	x		putative phage encoded two-component response regulator
x	LMOf2365_1252	Lm4b_01246	x		putative phage protein GP1
x	LMOf2365_1253	Lm4b_01247	LMHCC_1336		putative phage protein GP2
x	Lmof2365_2798	x	x		putative abortive infection bacteriophage resistance protein
x	Lmof2365_2799	x	x		putative phage associated transcriptional regulator
x	x	x	LMHCC_2599		phage integrase
x	x	x	LMHCC_2598		unknown protein
x	x	x	LMHCC_2597		putative phage associated repressor
x	x	x	LMHCC_2596		putative phage associated regulator, XRE family
x	x	x	LMHCC_2595		similar to prophage CP4-57 regulatory protein
x	x	x	LMHCC_2594		unknown protein
x	x	x	LMHCC_2593		putative phage protein GP32
x	x	x	LMHCC_2592		putative phage protein GP52
x	x	x	LMHCC_2591		unknown protein
x	x	x	LMHCC_2590		putative phage protein GP45
x	x	x	LMHCC_2589		unknown protein
x	x	x	LMHCC_2588		similar to conserved phage associated protein
x	x	x	LMHCC_2587		similar to conserved phage associated protein
x	x	x	LMHCC_2586		putative phage DNA-directed DNA polymerase
x	x	x	LMHCC_2585		unknown protein
x	x	x	LMHCC_2584		unknown protein
x	x	x	LMHCC_2583		putative phage protein

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
x	x	x	LMHCC_2582		unknown protein
x	x	x	LMHCC_2581		unknown protein
x	x	x	LMHCC_2579		unknown protein
x	x	x	LMHCC_2578		unknown protein
x	x	x	LMHCC_2577		unknown protein
x	x	x	LMHCC_2576		similar to virulence-associated E domain protein
x	x	x	LMHCC_2575		putative phage protein with VRR_NUC domain
x	x	x	LMHCC_2574		putative phage DNA helicase-like protein
x	x	x	LMHCC_2573		putative MazG nucleotide pyrophosphohydrolase
x	x	x	LMHCC_2572		putative phage protein
x	x	x	LMHCC_2571		unknown protein
x	x	x	LMHCC_2570		putative phage terminase, small subunit
x	x	x	LMHCC_2569		putative phage terminase, large subunit
x	x	x	LMHCC_2568		putative phage portal protein
x	x	x	LMHCC_2567		putative phage ClpP-like serine protease
x	x	x	LMHCC_2566		putative phage major capsid protein
x	x	x	LMHCC_2565		putative phage protein GP6
x	x	x	LMHCC_2564		putative phage protein GP9
x	x	x	LMHCC_2563		putative phage major tail protein
x	x	x	LMHCC_2562		putative phage protein
x	x	x	LMHCC_2561		putative phage protein GP11
x	x	x	LMHCC_2560		putative prophage tail tape measure protein
x	x	x	LMHCC_2559		putative phage protein; similar to staphylocoagulase precusor; similar to phage tail protein
x	x	x	LMHCC_2558		unknown protein
x	x	x	LMHCC_2557		putative phage protein; some homology to altronate oxidoreductase
x	x	x	LMHCC_2556		putative phage protein GP20
x	x	x	LMHCC_2555		putative phage protein GP22
x	x	x	LMHCC_2554		unknown protein
x	x	x	LMHCC_2553		putative phage holin
x	x	x	LMHCC_2552		unknown protein
x	x	x	LMHCC_2551		putative prophage lysin/N-acetylmuramoyl-L-alanine amidase
x	x	x	LMHCC_2121	*cas*	putative CRISPR-associated protein, Cas6
x	x	x	LMHCC_2120		putative CRISPR-associated cxxc_cxxc protein, Cst1
x	x	x	LMHCC_2119	*cst*	putative CRISPR-associated regulatory protein, DevR family
x	x	x	LMHCC_2118		putative CRISPR-associated protein, Cas5
x	x	x	LMHCC_2117		putative CRISPR-associated helicase, Cas3
x	x	x	LMHCC_2116		putative CRISPR-associated protein, Cas1
x	x	x	LMHCC_2115		putative CRISPR-associated protein, Cas2
x	x	x	LMHCC_1412		putative phage integrase
x	x	x	LMHCC_1411		putative phage protein GP35
x	x	x	LMHCC_1410		putative phage protein
x	x	x	LMHCC_1409		putative phage protein GP37
x	x	x	LMHCC_1408		unknown protein
x	x	x	LMHCC_1407		putative phage-associated transcriptional regulator
x	x	x	LMHCC_1406		unknown protein
x	x	x	LMHCC_1405		unknown protein
x	x	x	LMHCC_1404		unknown protein
x	x	x	LMHCC_1403		unknown protein
x	x	x	LMHCC_1402		putative cytosine-specific DNA methyltransferase
x	x	x	LMHCC_1401		unknown protein
x	x	x	LMHCC_1400		unknown protein
x	x	x	LMHCC_1399		unknown protein

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
x	x	x	LMHCC_1398		unknown protein
x	x	x	LMHCC_1397		unknown protein
x	x	x	LMHCC_1396		unknown protein
x	x	x	LMHCC_1395		similar to conserved hypothetical protein, PhnA-like
x	x	x	LMHCC_1393		unknown protein
x	x	x	LMHCC_1392		putative phage protein GP37
x	x	x	LMHCC_1391		unknown protein
x	x	x	LMHCC_1390		unknown protein
x	x	x	LMHCC_1389		unknown protein
x	x	x	LMHCC_1388		putative Siphovirus protein GP157
x	x	x	LMHCC_1387		putative phage nucleotide-binding protein
x	x	x	LMHCC_1386		putative phage helicase
x	x	x	LMHCC_1385		putative phage protein GP59
x	x	x	LMHCC_1384		putative phage primase, P4 family
x	x	x	LMHCC_1383		putative phage protein
x	x	x	LMHCC_1382		unknown protein
x	x	x	LMHCC_1381		putative phage protein GP62
x	x	x	LMHCC_1380		putative phage protein GP53
x	x	x	LMHCC_1379		unknown protein
x	x	x	LMHCC_1378		putative phage protein GP65
x	x	x	LMHCC_1377		putative phage protein GP1
x	x	x	LMHCC_1376		putative phage terminase, large subunit
x	x	x	LMHCC_1375		putative phage portal protein
x	x	x	LMHCC_1374		putative phage associated Clp protease
x	x	x	LMHCC_1373		putative phage major capsid protein
x	x	x	LMHCC_1372		unknown protein
x	x	x	LMHCC_1371		putative phage protein GP7
x	x	x	LMHCC_1370		putative phage protein GP8
x	x	x	LMHCC_1369		putative phage protein GP9
x	x	x	LMHCC_1368		putative phage protein GP10
x	x	x	LMHCC_1367		putative phage tail shaft protein
x	x	x	LMHCC_1366		unknown protein
x	x	x	LMHCC_1365		unknown protein
x	x	x	LMHCC_1364		putative phage tail tape measure protein
x	x	x	LMHCC_1363		putative phage tail-associated protein
x	x	x	LMHCC_1362		putative phage protein GP15
x	x	x	LMHCC_1361		putative phage protein GP16
x	x	x	LMHCC_1360		unknown protein
x	x	x	LMHCC_1359		putative phage protein GP18
x	x	x	LMHCC_1358		putative phage protein GP19
x	x	x	LMHCC_1357		putative phage protein GP20
x	x	x	LMHCC_1356		putative phage protein GP17
x	x	x	LMHCC_1355		putative phage protein GP22
x	x	x	LMHCC_1354		putative phage protein GP23
x	x	x	LMHCC_1353		putative phage holin
x	x	x	LMHCC_1352		putative phage-associated L-alanoyl-D-glutamate peptidase
x	x	x	LMHCC_1351		unknown protein
x	x	x	LMHCC_1350		unknown protein
x	x	x	LMHCC_1349		unknown protein
x	x	x	LMHCC_1348		putative phage protein GP28
x	x	x	LMHCC_1347		putative phage protein GP29
x	x	x	LMHCC_1346		putative phage protein GP30
x	x	x	LMHCC_1345		unknown protein

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
x	x	x	LMHCC_1344		unknown protein
x	x	x	LMHCC_1343		putative ankyrin repeat domain protein
x	x	x	LMHCC_1342		putative cell wall surface protein, CnaB domain
x	x	x	LMHCC_1341		putative cell wall surface protein, CnaB domain
x	x	x	LMHCC_1340		putative RNA polymerase sigma-24 subunit, ECF subfamily
x	x	x	LMHCC_1339		putative conjugative transposon/phage protein
x	x	x	LMHCC_1338		unknown protein
x	x	x	LMHCC_1337		unknown protein
x	x	x	LMHCC_0776		putative prophage protein
x	x	x	LMHCC_0775		putative super infection exclusion protein
x	x	x	LMHCC_0472		putative phage integrase/recombinase
x	x	x	LMHCC_0466		putative transcriptional regulator, phage repressor
x	x	x	LMHCC_3004		unknown protein; phage protein GP26
x	x	x	LMHCC_3003		putative phage lysin
x	x	x	LMHCC_3002		putative phage holin
x	x	x	LMHCC_3001		putative phage GP18 protein
x	x	x	LMHCC_3000		putative phage GP17-1 protein
x	x	x	LMHCC_2999		unknown protein
x	x	x	LMHCC_2998		unknown protein
x	x	x	LMHCC_2997		putative phage GP20 protein
x	x	x	LMHCC_2996		putative phage GP19 protein
x	x	x	LMHCC_2995		putative phage GP18 protein
x	x	x	LMHCC_2994		putative phage GP17 protein
x	x	x	LMHCC_2993		putative tape-measure protein
x	x	x	LMHCC_2992		putative phage GP15 protein
x	x	x	LMHCC_2991		putative phage GP14 protein
x	x	x	LMHCC_2990		putative phage GP13 protein
x	x	x	LMHCC_2989		putative phage major tail shaft protein
x	x	x	LMHCC_2988		putative phage GP11 protein
x	x	x	LMHCC_2987		putative phage GP10 protein
x	x	x	LMHCC_2986		putative phage GP9 protein
x	x	x	LMHCC_2985		putative phage GP8 protein
x	x	x	LMHCC_2984		putative phage coat protein
x	x	x	LMHCC_2983		putative phage scaffolding protein
x	x	x	LMHCC_2982		putative phage GP4 protein
x	x	x	LMHCC_2981		putative phage portal protein
x	x	x	LMHCC_2980		putative phage terminase, large subunit
x	x	x	LMHCC_2979		putative phage terminase, small subunit
x	x	x	LMHCC_2978		putative phage protein
x	x	x	LMHCC_2977		putative phage GP66 protein
x	x	x	LMHCC_2976		putative phage GP65 protein
x	x	x	LMHCC_2975		unknown protein
x	x	x	LMHCC_2974		putative phage GP59 protein
x	x	x	LMHCC_2973		unknown protein
x	x	x	LMHCC_2972		unknown protein
x	x	x	LMHCC_2971		putative HNH endonuclease family protein
x	x	x	LMHCC_2970		putative single-strand DNA-binding protein
x	x	x	LMHCC_2969		putative phage GP59 protein
x	x	x	LMHCC_2968		putative phage GP69 protein
x	x	x	LMHCC_2967		unknown protein
x	x	x	LMHCC_2966		unknown protein
x	x	x	LMHCC_2965		unknown protein
x	x	x	LMHCC_2964		unknown protein

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
x	x	x	LMHCC_2963		unknown protein
x	x	x	LMHCC_2962		unknown protein
x	x	x	LMHCC_2961		unknown protein
x	x	x	LMHCC_2960		unknown protein
x	x	x	LMHCC_2959		unknown protein
x	x	x	LMHCC_2958		putative DNA (cytosine-5)-methyltransferase
x	x	x	LMHCC_2957		unknown protein
x	x	x	LMHCC_2956		unknown protein
x	x	x	LMHCC_2955		putative phage GP49 protein
x	x	x	LMHCC_2954		putative phage recombination protein
x	x	x	LMHCC_2953		putative phage GP47 protein
x	x	x	LMHCC_2952		putative phage GP45 protein
x	x	x	LMHCC_2951		unknown protein
x	x	x	LMHCC_2950		putative phage GP43 protein
x	x	x	LMHCC_2949		putative phage antrepressor protein
x	x	x	LMHCC_2948		unknown protein
x	x	x	LMHCC_2947		unknown protein
x	x	x	LMHCC_2946		unknown protein
x	x	x	LMHCC_2945		putative phage GP37 protein
x	x	x	LMHCC_2944		putative phage-associated transcriptional regulator, XRE family protein
x	x	x	LMHCC_2943		putative phage GP33 protein
x	x	x	LMHCC_2942		unknown protein
x	x	x	LMHCC_2941		putative phage GP15 protein
x	x	x	LMHCC_2940		putative phage GP32 lipoprotein
x	x	x	LMHCC_2939		putative phage integrase
x	x	x	LMHCC_2700		putative Abi-like protein
x	x	x	LMHCC_1822		putative resolvase/DNA invertase
x	x	x	LMHCC_1821		putative resolvase/DNA invertase
x	x	x	LMHCC_0981		putative site-specific recombinase TnpX
x	x	x	LMHCC_0980		putative site-specific DNA recombinase
x	x	x	LMHCC_0470		putative site specific recombinase
x	LMOf2365_1116	Lm4b_01114	x	*sagB*	streptolysin associated protein SagB-like
x	LMOf2365_1117	Lm4b_01115	x	*sagC*	streptolysin associated protein SagC-like
x	LMOf2365_1118	Lm4b_01116	x	*sagD*	streptolysin associated protein SagD-like
x	LMOf2365_0118	Lm4b_00104	LMHCC_2527		pyruvyl-transferase domain protein
x	x	Lm4b_01121	x		putative ATP-binding protein
x	LMOf2365_1142	Lm4b_01140	LMHCC_1517		putative acetyltransferase
x	LMOf2365_2058	Lm4b_02047	x		putative methyltransferase
x	x	x	LMHCC_2352		putative acyltransferase
x	x	x	LMHCC_1818		putative short-chain dehydrogenase/reductase
x	x	x	LMHCC_0984		putative short-chain dehydrogenase/reductase
x	LMOf2365_0457	(Lm4b_00457)	x		unknown protein
x	LMOf2365_0010	Lm4b_00010	LMHCC_2654		unknown protein
x	LMOf2365_0014	Lm4b_00014	x		unknown protein
x	LMOf2365_0032	Lm4b_00032	x		unknown protein
x	LMOf2365_0034	Lm4b_00034b	x		unknown protein
x	LMOf2365_0037	Lm4b_00037	x		unknown protein
x	x	Lm4b_00071b	x		unknown protein
x	x	Lm4b_00072b	x		unknown protein
x	x	Lm4b_00073	x		unknown protein
x	x	Lm4b_00075	x		unknown protein
x	x	Lm4b_00076b	x		unknown protein
x	x	Lm4b_00077	x		unknown protein

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
x	x	Lm4b_00078	x		unknown protein
x	LMOf2365_0272	Lm4b_00280	LMHCC_2374		unknown protein
x	LMOf2365_0278	Lm4b_00285	x		unknown protein
x	LMOf2365_0278	Lm4b_00286	x		putative secreted protein
x	LMOf2365_0355	Lm4b_00355	x		Unknown protein
x	LMOf2365_0357	Lm4b_00356	x		Unknown protein
x	LMOf2365_0358	Lm4b_00357	LMHCC_2297		Unknown protein
x	LMOf2365_0415	Lm4b_00417	x		putative membrane fusion protein; weak homology to lmo0193
x	LMOf2365_0418	Lm4b_00420	x		unknown protein
x	LMOf2365_0451	Lm4b_00455	x		unknown protein
x	LMOf2365_0458	Lm4b_00457	x		unknown protein
x	LMOf2365_0459	Lm4b_00458	x		unknown protein
x	LMOf2365_0466	Lm4b_00459	LMHCC_2199		unknown protein
x	LMOf2365_0468	Lm4b_00460	LMHCC_2198		heat repeat domain protein
x	LMOf2365_0481	Lm4b_00471	LMHCC_2181		unknown protein
x	LMOf2365_0492	Lm4b_00483	x		unknown protein
x	LMOf2365_0501	Lm4b_00492	x		unknown protein
x	x	Lm4b_00496	x		unknown protein
x	x	Lm4b_00497	x		unknown protein
x	x	Lm4b_00499	x		unknown protein
x	LMOf2365_0513	Lm4b_00506	LMHCC_2156		similar to conserved hypothetical protein
x	LMOf2365_0514	Lm4b_00507	x		unknown protein
x	LMOf2365_0667	Lm4b_00659	x		unknown protein
x	LMOf2365_0693.1	Lm4b_00686	x		unknown protein
x	x	Lm4b_00699	x		unknown protein
x	LMOf2365_0834	Lm4b_00833b	x		unknown protein
x	LMOf2365_0835	Lm4b_00834	x		unknown protein
x	LMOf2365_0885	Lm4b_00884	x		unknown protein
x	LMOf2365_0919	Lm4b_00918	LMHCC_1726		similar to conserved hypothetical protein
x	LMOf2365_0920	Lm4b_00919	LMHCC_1725		unknown protein
x	LMOf2365_1098	Lm4b_01097	x		unknown protein
x	LMOf2365_1115	Lm4b_01113	x		unknown protein
x	x	Lm4b_01120	x		unknown protein
x	x	Lm4b_01122	(LMHCC_1535)		unknown protein
x	x	Lm4b_01123b	LMHCC_1535		unknown protein
x	x	Lm4b_01124	LMHCC_1534		unknown protein
x	LMOf2365_1131	Lm4b_01125	LMHCC_1531		unknown protein
x	x	Lm4b_01127	LMHCC_1529		unknown protein
x	x	Lm4b_01139	x		unknown protein
x	LMOf2365_1143	Lm4b_01141b	LMHCC_1516		unknown protein
x	x	Lm4b_01669	LMHCC_0906		unknown protein; weak homology to lmo0116
x	LMOf2365_2138	Lm4b_02127	LMHCC_0441		Unknown protein
x	x	Lm4b_02326	x		similar to conserved hypothetical proteins
x	Lmof2365_2347	Lm4b_02346	LMHCC_0223		unknown protein
x	Lmof2365_2348	Lm4b_02347	x		unknown protein
x	Lmof2365_2361	Lm4b_02360	x		unknown protein
x	Lmof2365_2626	Lm4b_02619b	x		unknown protein
x	Lmof2365_2629	Lm4b_02622b	x		unknown protein
x	Lmof2365_2630	Lm4b_02623	x		putative membrane protein
x	Lmof2365_2631	Lm4b_02624	x		unknown protein
x	Lmof2365_2740	Lm4b_02727	x		unknown protein
x	x	Lm4b_02754	x		unknown protein
x	Lmof2365_2792	Lm4b_02776b	LMHCC_2722		unknown protein

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
x	Lmof2365_2796	Lm4b_02780	LMHCC_2718		putative membrane protein
x	LMOf2365_0035	x	x		unknown protein
x	LMOf2365_0064	x	LMHCC_2615		unknown protein
x	LMOf2365_0066	x	x		unknown protein
x	LMOf2365_0078	x	x		unknown protein
x	LMOf2365_0079	x	x		unknown protein
x	LMOf2365_0080	x	x		unknown protein
x	LMOf2365_0081	x	x		unknown protein
x	LMOf2365_0083	x	x		unknown protein; similar to B. subtilis YxxD
x	LMOf2365_0097	x	x		unknown protein
x	LMOf2365_0098	x	x		unknown protein
x	LMOf2365_0163	*x*	x		unknown protein
x	LMOf2365_0165	x	x		unknown protein
x	LMOf2365_0235	x	LMHCC_2419		unknown protein
x	LMOf2365_0273	x	x		unknown protein
x	LMOf2365_0313	x	x		unknown protein
x	LMOf2365_0323	x			unknown protein
x	LMOf2365_0348	x	x		Unknown protein
x	LMOf2365_0356	x	x		Unknown protein
x	LMOf2365_0381	x	LMHCC_2272		Unknown protein
x	LMOf2365_0409	x	x		unknown protein
x	LMOf2365_0410	x	x		unknown protein
x	LMOf2365_0411	x	x		unknown protein
x	LMOf2365_0412	x	x		unknown protein
x	LMOf2365_0449	x	LMHCC_2209		unknown protein
x	LMOf2365_0452	x	x		unknown protein
x	LMOf2365_0453	x	x		unknown protein
x	LMOf2365_0454	x	x		unknown protein
x	LMOf2365_0455	x	x		unknown protein
x	LMOf2365_0456	x	x		unknown protein
x	LMOf2365_0460	x	x		unknown protein
x	LMOf2365_0461	x	x		unknown protein
x	LMOf2365_0462	x	x		unknown protein
x	LMOf2365_0463	x	x		unknown protein
x	LMOf2365_0464	x	x		unknown protein
x	LMOf2365_0465	x	x		unknown protein
x	LMOf2365_0467	x	x		unknown protein
x	LMOf2365_0469	x	LMHCC_2196		unknown protein
x	LMOf2365_0470	x	x		unknown protein
x	LMOf2365_0500	x	x		unknown protein
x	LMOf2365_0668	x	x		unknown protein
x	LMOf2365_0687	x	x		unknown protein
x	LMOf2365_0703	x	LMHCC_1961		unknown protein
x	LMOf2365_0707	x	x		unknown protein
x	LMOf2365_0883	x	x		unknown protein
x	LMOf2365_0925	x	x		unknown protein
x	LMOf2365_0995	x	(LMHCC_1648)		unknown protein; fragment of DltA protein
x	LMOf2365_1112	x	x		unknown protein
x	LMOf2365_1122	x	x		unknown protein
x	LMOf2365_1123	x	x		unknown protein
x	LMOf2365_1125	x	x		unknown protein; weak homology to lmo0336
x	LMOf2365_1158	x	x		unknown protein
x	LMOf2365_1179	x	x		unknown protein

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
x	LMOf2365_1180	x	x		unknown protein
x	LMOf2365_1251	x	x		unknown protein
x	LMOf2365_1266	x	x		unknown protein
x	LMOf2365_1268	x	x		unknown protein
x	LMOf2365_1269	x	(LMHCC_1321)		unknown protein; fragment of adjacent gene
x	LMOf2365_1285	x	x		unknown protein
x	LMOf2365_1392	x	LMHCC_1196		unknown protein
x	LMOf2365_1394	x	x		unknown protein
x	LMOf2365_1568	x	x		unknown protein
x	LMOf2365_1571	x	x		unknown protein
x	LMOf2365_1581	x	x		unknown protein
x	LMOf2365_1675	x	x		unknown protein
x	LMOf2365_1737	x	x		unknown protein
x	LMOf2365_1811	x	x		unknown protein
x	LMOf2365_1813	x	x		unknown protein
x	LMOf2365_1833	x	LMHCC_0752		unknown protein
x	LMOf2365_2001	x	x		unknown protein
x	LMOf2365_2028	x	x		unknown protein
x	LMOf2365_2039	x	x		unknown protein
x	Lmof2365_2332	x	x		unknown protein
x	Lmof2365_2336	x	x		unknown protein
x	Lmof2365_2408	x	x		unknown protein
x	Lmof2365_2539	x	x		unknown protein
x	Lmof2365_2562	x	x		unknown protein
x	Lmof2365_2624	x	x		unknown protein
x	Lmof2365_2691	x	x		unknown protein
x	Lmof2365_2701	x	x		unknown protein
x	Lmof2365_2702	x	x		unknown protein
x	Lmof2365_2703	x	x		unknown protein
x	Lmof2365_2704	x	x		unknown protein
x	Lmof2365_2705	x	x		unknown protein
x	Lmof2365_2706	x	x		unknown protein
x	Lmof2365_2707	x	x		putative membrane protein
x	Lmof2365_2747	x	x		unknown protein
x	Lmof2365_2750	x	LMHCC_2763		unknown protein
x	Lmof2365_2764	x	x		unknown protein
x	Lmof2365_2791	x	x		unknown protein
x	x	x	LMHCC_2607		putative lipoprotein
x	x	x	LMHCC_2606		unknown protein
x	x	x	LMHCC_2605		putative lipoprotein
x	x	x	LMHCC_2604		unknown protein
x	x	x	LMHCC_2550		unknown protein
x	x	x	LMHCC_2549		unknown protein
x	x	x	LMHCC_2548		unknown protein
x	x	x	LMHCC_2547		TM2 domain protein
x	x	x	LMHCC_2388		unknown protein
x	x	x	LMHCC_2383		similar to conserved hypothetical protein
x	x	x	LMHCC_2381		unknown protein
x	x	x	LMHCC_2351		unknown protein
x	x	x	LMHCC_2350		unknown protein
x	x	x	LMHCC_2337		unknown protein
x	x	x	LMHCC_2336		unknown protein
x	x	x	LMHCC_2323		unknown protein

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
x	x	x	LMHCC_2301		Unknown protein
x	x	x	LMHCC_2295		Unknown protein
x	x	x	LMHCC_2276		Unknown protein
x	x	x	LMHCC_2205		unknown protein
x	x	x	LMHCC_2203		unknown protein
x	x	x	LMHCC_2202		unknown protein
x	x	x	LMHCC_2201		unknown protein
x	x	x	LMHCC_2200		unknown protein
x	x	x	LMHCC_2197		unknown protein
x	x	x	LMHCC_2193		unknown protein
x	x	x	LMHCC_2192		unknown protein
x	x	x	LMHCC_2189		unknown protein
x	x	x	LMHCC_2188		similar to conserved hypothetical protein
x	x	x	LMHCC_2187		unknown protein
x	x	x	LMHCC_2170		unknown protein
x	x	x	LMHCC_2155		unknown protein
x	x	x	LMHCC_2154		unknown protein
x	x	x	LMHCC_2153		unknown protein
x	x	x	LMHCC_2129		unknown protein
x	x	x	LMHCC_2100		unknown protein
x	x	x	LMHCC_2038		unknown protein
x	x	x	LMHCC_1997		unknown protein
x	x	x	LMHCC_1996		unknown protein
x	x	x	LMHCC_1969		unknown protein
x	x	x	LMHCC_1958		unknown protein
x	x	x	LMHCC_1957		unknown protein
x	x	x	LMHCC_1956		unknown protein
x	x	x	LMHCC_1906		unknown protein
x	x	x	LMHCC_1854		unknown protein
x	x	x	LMHCC_1849		unknown protein
x	x	x	LMHCC_1823		unknown protein
x	x	x	LMHCC_1820		unknown protein
x	x	x	LMHCC_1812		unknown protein
x	x	x	LMHCC_1796		unknown protein
x	x	x	LMHCC_1780		unknown protein
x	x	x	LMHCC_1779		unknown protein
x	x	x	LMHCC_1760		unknown protein
x	x	x	LMHCC_1759		unknown protein
x	x	x	LMHCC_1758		unknown protein
x	x	x	LMHCC_1757		unknown protein
x	x	x	LMHCC_1756		unknown protein
x	x	x	LMHCC_1724		unknown protein
x	x	x	LMHCC_1550		unknown protein
x	x	x	LMHCC_1536		unknown protein
x	x	x	LMHCC_1533		unknown protein
x	x	x	LMHCC_1532		unknown protein
x	x	x	LMHCC_1530		unknown protein
x	x	x	LMHCC_1518		unknown protein
x	x	x	LMHCC_1283		unknown protein
x	x	x	LMHCC_1273		unknown protein
x	x	x	LMHCC_0982		unknown protein
x	x	x	LMHCC_0938		unknown protein
x	x	x	LMHCC_0909		unknown protein; weak homology to lmo0475

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
x	x	x	LMHCC_0861		unknown protein
x	x	x	LMHCC_0814		unknown protein
x	x	x	LMHCC_0774		unknown protein
x	x	x	LMHCC_0773		putative lipoprotein
x	x	x	LMHCC_0772		unknown protein
x	x	x	LMHCC_0591		unknown protein
x	x	x	LMHCC_0578		unknown protein
x	x	x	LMHCC_0525		unknown protein
x	x	x	LMHCC_0513		unknown protein
x	x	x	LMHCC_0495		Unknown protein
x	x	x	LMHCC_0494		putative lipoprotein
x	x	x	LMHCC_0483		unknown protein
x	x	x	LMHCC_0469		unknown protein
x	x	x	LMHCC_0468		unknown protein
x	x	x	LMHCC_0467		unknown protein
x	x	x	LMHCC_0446		Unknown protein
x	x	x	LMHCC_0445		Unknown protein
x	x	x	LMHCC_0358		unknown protein
x	x	x	LMHCC_0243		unknown protein
x	x	x	LMHCC_0242		unknown protein
x	x	x	LMHCC_0238		unknown protein
x	x	x	LMHCC_0237		unknown protein
x	x	x	LMHCC_0235		unknown protein; weak homology to lmo1264
x	x	x	LMHCC_0159		putative lipoprotein
x	x	x	LMHCC_0133		unknown protein
x	x	x	LMHCC_0061		unknown protein
x	x	x	LMHCC_0024		unknown protein
x	x	x	LMHCC_0012		unknown protein
x	x	x	LMHCC_2844		unknown protein
x	x	x	LMHCC_2821		unknown protein
x	x	x	LMHCC_2779		unknown protein
x	x	x	LMHCC_2778		unknown protein
x	x	x	LMHCC_2774		unknown protein
x	x	x	LMHCC_2772		unknown protein
x	x	x	LMHCC_2766		unknown protein
x	x	x	LMHCC_2749		unknown protein
x	x	x	LMHCC_2748		unknown protein
x	x	x	LMHCC_2747		unknown protein
x	x	x	LMHCC_2746		unknown protein
x	x	x	LMHCC_2744		weak similarity to spore coat protein
x	x	x	LMHCC_2715		unknown protein
x	x	x	LMHCC_2714		unknown protein
x	x	x	LMHCC_2713		unknown protein
x	x	x	LMHCC_2709		unknown protein
x	LMOf2365_1681	Lm4b_01670	x		putative PTS system, sucrose-specific IIBC component
x	x	Lm4b_02327b	x		putative PTS system, cellobiose/beta-glucoside-specific IIA component
x	x	Lm4b_02328	x		putative PTS system, cellobiose/beta-glucoside-specific IIB component
x	x	Lm4b_02329	x		putative PTS system, cellobiose/beta-glucoside-specific IIC component
x	x	Lm4b_00033	x		putative ABC-type antibiotic efflux transporter, ATP binding protein
x	LMOf2365_0036	Lm4b_00036	x		putative ABC-type antibiotic efflux transporter, permease

EGD-e[#]	ATCC 19115[*]	Clip 81459[¥]	HCC23[Ω]	Gene	Function
x	LMOf2365_0267	Lm4b_00275	LMHCC_2379		putative maltose/maltodextrin uptake ABC transporter, substrate binding protein
x	LMOf2365_0268	Lm4b_00276	LMHCC_2378		putative maltose/maltodextrin uptake ABC transporter, permease
x	LMOf2365_0269	Lm4b_00277b	LMHCC_2377		putative maltose/maltodextrin uptake ABC transporter, permease
x	LMOf2365_0279	Lm4b_00287	x		putative ABC-type transporter, ATP binding protein
x	LMOf2365_0280	Lm4b_00288	x		putative ABC-type transporter, permease/ATP binding protein
x	LMOf2365_0416	Lm4b_00418	x		putative ABC-type transporter protein, ATP-binding protein
x	LMOf2365_0417	Lm4b_00419b	x		putative ABC-type transporter protein, permease
x	LMOf2365_1113	Lm4b_01111	x		similar to ABC transporter, ATP-binding protein
x	LMOf2365_1114	Lm4b_01112	x		similar to ABC transporter, permease
x	Lmof2365_2627	Lm4b_02620	x		putative ABC-type antibiotic efflux transporter, ATP binding protein
x	Lmof2365_2628	Lm4b_02621b	x		putative ABC-type transporter, permease
x	LMOf2365_0270	Lm4b_00278	LMHCC_2376		putative oligo-1,6-glucosidase
x	LMOf2365_0271	Lm4b_00279	LMHCC_2375	*gtfA*	sucrose phosphorylase
x	LMOf2365_1682	Lm4b_01671	x		putative N-acetylmuramic acid 6-phosphate etherase
x	x	Lm4b_02324	x		putative maltose 6-phosphate glucosidase
x	LMOf2365_0266	Lm4b_00274	LMHCC_2380		transcriptional regulator, LacI/DegA family
x	LMOf2365_0414	Lm4b_00416	x		transcriptional regulator, GntR family; weak homology to lmo0772
x	LMOf2365_1683	Lm4b_01672	x		putative transcriptional regulator, RpiR family
x	LMOf2365_2059	Lm4b_02048	x		putative transcriptional regulator, TetR family
x	x	Lm4b_02325	x		putative transcriptional regulator, BglG family
x	LMOf2365_0326	x			putative transcriptional regulator
x	x	x	LMHCC_1817		putative transcriptional regulator, MerR family
x	x	x	LMHCC_0983		putative transcriptional regulator, MerR family
x	x	x	LMHCC_0463		unknown protein
x	LMOf2365_0854	Lm4b_00853	LMHCC_1792		putative ABC transporter, ATP-binding/permease protein
x	Lmof2365_2416	Lm4b_02412	LMHCC_0157		putative cell surface protein
x	LMOf2365_0331	x	x		putative M48 family peptidase; similar to heat shock protein HtpX

[#] Strain EGD-e (serotype 1/2a); KEGG code: *lmo*; number of nucleotides 2944528; number of protein genes 2846, first *L. monocytogenes* genome sequenced (Glaser et al.,2001).

[*] Strain ATCC19115 also known as F2365 (serotype 4b); KEGG code: *lmf*; number of nucleotides 2905187; number of protein genes 2821 (Nelson et al., 2004).

[¥] Strain Clip81459 (serotype 4b); KEGG code: *lmc*; number of nucleotides 2912690; number of protein genes 2766.

[Ω] Strain HCC23 (serotype 4a) KEGG code: *lmh*; number of nucleotides 2976212; number of protein genes 2974

[x] Indicates corresponding no corresponding protein ortholog occurs in the given genome.

B.1 Materials and Equipment

B.1.1 Materials

B.1.1.1 Bacterial strains

L. monocytogenes strains used throughout the course of this study are described in Table A.1. Strains were stored at -80°C in brain heart infusion broth (BHIB) containing 15% (v/v) sterile glycerol.

Table B.5 *Listeria monocytogenes* strains used in the study.

Strain	Source	Lineage	Serotype
64-1495[‡], 64-2389[‡], 64-1495[‡], 64-2389[‡], 70-0421[‡], 70-2058[‡], 79-2048[‡], 79-2360[‡], 80-0910[‡], 80-2901[‡], 80-4762[‡], 80-4904[‡], 83-2099[‡], Joyce[‡]	ovine	II	1/2a
62-2853[‡], 66-0755[‡], 87-1599[‡]	bovine	II	1/2a
ATCC 10403[$], ATCC 19111[$]	clinical	II	1/2a
67-1759[‡], 68-2528[‡], 80-3354[‡], 80-3453[‡], 87-2555[‡]	avian	II	1/2a
72-0039[‡]	equine	II	1/2a
80-2942[‡], 86-3009[‡], 87-0041[‡], 90-0053[‡]	caprine	II	1/2a
FW03/0033[¥], FW03/0034[¥], FW03/0036[¥], FW04/0024[¥], FW04/0025[¥], FW04/0026[¥], FW03/0037[¥], 102-195-s-1-154[f], 102-265-s-3-352[f], 114-830-s-7-62[f]	food/factory	II	1/2a
Liver[‡], L1	unknown	II	1/2a
ATCC 19112[$], ATCC 7644[$], FW04/0018[¥], LO28	clinical	II	1/2c
FW04/0023[¥], Siliken 204231/1, 102-195-s-1-242[f], 102-195-s-1-367[f], 102-409-s-3-431[f]	food/factory	II	1/2c
FW03/0032[¥], 102-265-s-3-745[f]	food/factory	II	3a
FW04/0017[¥], FW04/0019[¥]	clinical	I	1/2b
FW04/0022[¥], 114-997-s-7-63[f]	food/factory	I	1/2b
71-0934[‡]	wallaby	I	1/2b
83-1617[‡]	ovine	I	1/2b
78-1098[‡]	avian	I	1/2b
L2	unknown	I	1/2b
102-231-s-7-566[f]	food/factory	I	3b
73-1801[‡]	bovine	I	3b
102-195-s-1-60[f]	food/factory	I	3c
63-5635[‡], 70-3167[‡], 77-2294[‡], 79-0869[‡], 79-1828[‡], 80-4798[‡], 83-0159[‡]	ovine	I	4b
69-0577[‡], 69-1363[‡], 70-0378[‡], 78-2183[‡], 84-1886[‡]	bovine	I	4b
71-3227[‡], ATCC 19115[$], FW04/0020[¥], FW04/0021[¥], ScottA	clinical	I	4b
74-2395[‡]	avian	I	4b
79-2336/3[‡]	equine	I	4b
84-2026[‡]	caprine	I	4b
92-0305[‡]	unknown	I	4b
70-1700[‡], 83-1804[‡], 85-2389[‡]	ovine	I	4e
80-2437[‡]	avian	I	4e
89-1931[‡]	canine	I	4e
102-231-s-7-232[f], 102-241-s-1-349[f]	food/factory	I	4e
78-2755[‡], 85-0010[‡]	ovine	III	4b
69-1793[‡], 69-1793/2[‡], 73-0336[‡], 76-1854[‡]	bovine	III	4b
86-0071[‡]	avian	III	4b
FW03/0035[¥]	food/factory	III	4b
64-0738[‡], 70-0249[‡]	ovine	III	4c
62-4693[‡], 68-2169[‡]	bovine	III	4a
71-0563[‡], 77-4745[‡], 78-0712[‡], 78-3565[‡], 78-3636[‡], 79-0430[‡], 79-	ovine	III	4a

Strain	Source	Lineage	Serotype
1994[‡], 79-2759[‡], 79-3194[‡], 80-0619[‡], 80-2880[‡], 80-3749[‡], 83-1885[‡], 83-2795[‡], 85-0658[‡]			
87-0707[‡]	avian	III	4a
ATCC 19114[$]	ruminant	III	4a
85-0567[‡]	unknown	III	4a
76-2120/1[‡]	ovine		7
Listeria innocua ATCC 33090[$]	bovine		6a
S20423[§], S20425[§], S20432[§], FRRW2343, FRRW2345, L522	food/factory		N/A
00P23755[ħ], 01P27654[ħ], 02P37678[ħ], 02P638[ħ], 02P79545[ħ], 02P82412[ħ], 03P136964[ħ], 05P24111[ħ], 91B3450[ħ], 97P107857[ħ], 98P130512[ħ], 98P1437[ħ], 98P40537[ħ], 99P154255[ħ], 99P63881[ħ]	clinical		N/A
67-1786[‡]	ovine		N/A
74-0990[‡]	bovine		N/A
79-2336[‡], 79-2336/1[‡], 79-2336/16[‡]	equine		N/A
LM412, LM412N, LMNR30, LO28 OPPA-	unknown		N/A

[¥] *L. monocytogenes* isolates obtained from the Medical Microbiological Diagnostic Unit, University of Melbourne, Victoria, Australia

[ƒ] *L. monocytogenes* factory isolates obtained from Dr. John Holah, Campden & Chorleywood Research Association, Chipping Campden, Gloucestershire, UK.

[§] *L. monocytogenes* isolates from smoked salmon fillet samples (C. Bittencourt, University of Tasmania)

[‡] *L. monocytogenes* animal derived isolates (most strains are designated by a year followed by four digit strain code) obtained by the Department of Primary Industries within Tasmania between 1962 and 1990.

[ħ] *L. monocytogenes* clinical isolates (strains are designated by a year followed by pathology identification code) obtained from the Microbiology Department, Royal Hobart Hospital, Tasmania.

[$] *L. monocytogenes* strains purchased from the American Type Culture Collection (ATCC), Manassas, USA

B.1.1.2 Bacteriological Media

The bacteriological media used throughout the course of this study were prepared and stored in accordance with manufacturer's instructions. All solutions were steralised by autoclaving at 121°C for 15 minutes unless otherwise specified.

1.5% Brain Heart Infusion Agar (BHIA)

Brain Heart Infusion broth (Oxoid CM0225B)	37g
Bacteriological Agar (Oxoid LP0011)	15g
dH2O	1L

0.2% Brain Heart Infusion Agar with 0.05g/L of 2,3,5-triphenyltetrazolium chloride (BHIA-TTC)

Brain Heart Infusion broth (Oxoid CM0225B)	37g
Bacteriological Agar (Oxoid LP0011)	2g
dH2O	1L
TTC (Sigma-Aldrich)	0.05g

0.3% Brain Heart Infusion Agar with 0.05g/L of 2,3,5-triphenyltetrazolium chloride (BHIA-TTC)

Brain Heart Infusion broth (Oxoid CM0225B)	37g
Bacteriological Agar (Oxoid LP0011)	3g
dH2O	1L
TTC (Sigma-Aldrich)	0.05g

Brain Heart Infusion Broth (BHIB)

Brain Heart Infusion broth (Oxoid CM0225B)	37g
dH2O	1L

Brain Heart Infusion Broth (BHIB) + 15% glycerol

Brain Heart Infusion broth (Oxoid CM0225B)	37g
dH2O	1L
Glycerol (Sigma G5516)	150mL

B.1.1.3 Reagents

Agarose I (Amresco AM0710, supplied by Astral Scientific, Australia)

Bromophenol blue (Sigma-Aldrich B5525)

Diethyl pyrocarbonate (DEPC) (Sigma-Aldrich D5758)

EDTA (Sigma-Aldrich E6758)

Ethanol (Sigma-Aldrich E702-3)

Ethidium bromide (Sigma-Aldrich E767)

Formaldehyde (Sigma-Aldrich F1635 and F9037)

Formamide (Sigma-Aldrich F7508)

Glycerol (Sigma-Aldrich G5516)

Lysozyme (Sigma-Aldrich L6876)

β -Mercaptoethanol (Sigma-Aldrich M3148)

MOPS (3-(N-Morpholino)propanesulfonic acid) (Sigma-Aldrich M5162)

Proteinase K (Sigma-Aldrich P6556)

RNAeasy Midi RNA Extraction Kit (Qiagen 75144)

RNAprotect Bacteria Reagent (Qiagen 76506)

Sodium acetate (Sigma-Aldrich S2889)

B.1.2 Equipment

Autoclave

Pressure cooker RY-150 from Rinnai (Australia) used with countdown timing systems.

Centrifuges

Universal 16A, Imbros, Australia;
Microcentrifuge 5417R, Eppendorf, Germany.
Sorvall SUPER T21, Kendo, USA.
Hettich Zentrifugen EBA12, HD Scientific, Australia.

Electrophoresis cells

Horizon 58 Life Technologies Horizontal Gel Electrophoresis Apparatus from Gibco BRL, USA.

Freezer (-80°C)

Ultra-low temperature freezer MDF-U50V from Sanyo, Japan.

Microplate reader

Benchmark from BioRad Laboratories, USA.

Bead Beater

1247W CELL DIS-BioSpec Mini Bead Beater

Platform mixer

Ratek Model OM6 from Adelab Scientific, Australia.

Power pack

Power/Pac Model 300, BioRad, USA.

Shaking incubator

TN3, Advantec, Toyi Roshi Int..

Spectrophotometer

SmartSpec™ 3000 from BioRad, USA.

Temperature data logger

Tinytag® from Hastings Data Loggers, Australia.

UV transilluminator with camera

UV transilluminator from UVP, USA with an EDAS 290 camera from Kodak, Australia.

Water bath

SWB20D from Ratex Instruments, Australia used with a refrigeration unit (Model RC2).

C.1 Growth parameters of Listeria monocytogenes isolates

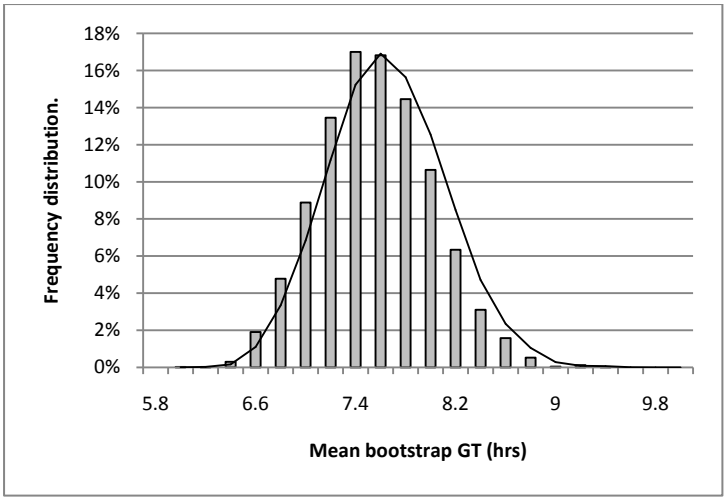
C.1.1 Growth parameters of isolates at 2.14M NaCl

Table C.1 Estimated generation time values for *L. monocytogenes* strains able to grow in brain-heart infusion broth supplemented with 12.0% sodium chloride.

Strain reference	Salt GT (hrs)	95% confidence lower	95% confidence upper	Serotype	Lineage	Strain origin
69-1363	5.4	0.8	1.1	4b	I	animal
70-0378	5.7	0.6	0.7	4b	I	animal
77-2294	5.7	0.8	1.2	4b	I	animal
84-1886	5.9	0.7	1.1	4b	I	animal
FW04/0020	5.9	1.0	1.5	4b	I	clinical
FW04/0021	5.9	0.6	0.8	4b	I	clinical
74-2395	6.1	0.8	0.9	4b	I	animal
71-3227	6.2	0.7	0.9	4b	I	clinical
92-0305	6.5	0.6	0.7	4b	I	unknown
85-2389	6.5	0.9	1.3	4e	I	animal
79-1828	6.6	0.6	0.6	4b	I	animal
ScottA	6.7	0.6	0.7	4b	I	clinical
70-3167	6.7	0.6	0.7	4b	I	animal
69-0577	6.9	0.7	0.9	4b	I	animal
79-0869	6.9	0.9	1.1	4b	I	animal
80-2437	7.0	0.5	0.6	4e	I	animal
84-2026	7.2	0.6	0.7	4b	I	animal
83-0159	7.4	0.4	0.5	4b	I	animal
80-4798	7.5	0.7	1	4b	I	animal
83-1804	7.6	0.9	1.2	4e	I	animal
63-5635	7.9	0.6	0.8	4b	I	animal
71-0934	8.7	0.7	0.8	1/2b	I	animal
79-2336/3	9.2	0.6	0.6	4b	I	animal
83-1617	9.6	1.0	1.1	1/2b	I	animal
L2	10.1	1.1	1.5	1/2b	I	unknown
FW04/0019	10.7	1.3	1.7	1/2b	I	clinical
FW04/0022	11.1	1.3	1.6	1/2b	I	food/factory
FW04/0017	11.2	1.5	2	1/2b	I	clinical
72-0039	6.1	0.5	0.7	1/2a	II	animal
FW04/0037	6.2	0.6	0.7	1/2a	II	food/factory
80-2942	6.3	0.8	1	1/2a	II	animal
80-2901	6.5	0.5	0.5	1/2a	II	animal
L1	6.5	0.4	0.5	1/2a	II	unknown
79-2360	6.8	0.7	0.8	1/2a	II	animal
80-4762	6.8	0.6	0.7	1/2a	II	animal
79-2048	6.9	0.4	0.3	1/2a	II	animal
87-1599	6.9	0.7	0.9	1/2a	II	animal
FW04/0023	6.9	0.7	0.9	1/2c	II	food/factory
87-2555	7.2	1.2	1.8	1/2a	II	animal
FW03/0033	7.4	0.8	1.1	1/2a	II	food/factory
80-4904	7.5	0.7	1.0	1/2a	II	animal
ATCC 10403	7.6	0.5	0.7	1/2a	II	clinical

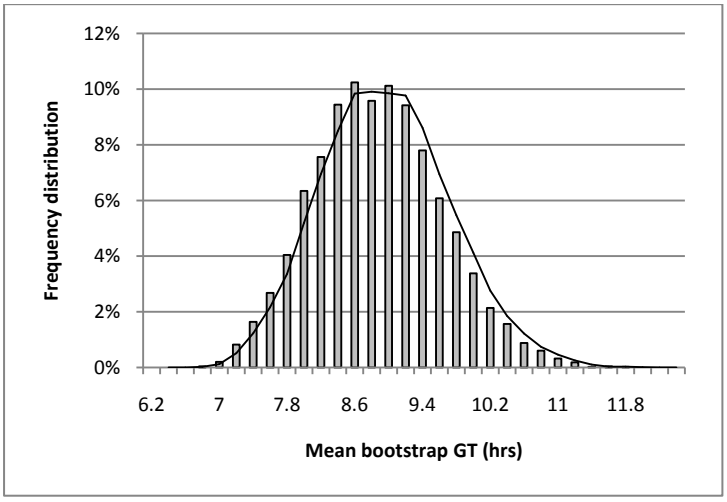
Strain reference	Salt GT (hrs)	95% confidence lower	95% confidence upper	Serotype	Lineage	Strain origin
87-0041	7.6	0.7	0.9	1/2a	II	animal
67-1759	8.0	0.5	0.5	1/2a	II	animal
FW03/0036	8.3	0.4	0.4	1/2a	II	food/factory
FW03/0032	8.5	0.9	1.0	3a	II	food/factory
66-0755	9.0	0.3	0.3	1/2a	II	animal
FW04/0024	9.0	0.6	0.6	1/2a	II	food/factory
FW03/0034	9.4	1.5	2.1	1/2a	II	food/factory
68-2528	10.1	0.8	0.9	1/2a	II	animal
S204231_1	10.5	1.2	1.5	1/2c	II	food/factory
80-3354	11.0	1.2	1.7	1/2a	II	animal
Joyce	11.5	0.7	0.8	1/2a	II	animal
FW04/0026	12.0	0.8	0.9	1/2a	II	food/factory
80-0910	12.2	1.4	1.6	1/2a	II	animal
FW04-0018	12.5	0.7	0.8	1/2c	II	clinical
LO28	14.1	1.2	1.4	1/2c	II	clinical
70-0421	14.2	1.9	2.6	1/2a	II	animal
FW03/0035	5.3	0.4	0.4	4b	III	food/factory
69-1793	6.2	0.8	1.1	4b	III	animal
68-2169	6.2	0.6	0.6	4a	III	animal
85-0658	6.2	0.6	0.8	4a	III	animal
79-3194	6.4	0.7	0.7	4a	III	animal
86-0071	6.4	0.6	0.7	4b	III	animal
80-2880	6.5	0.4	0.5	4a	III	animal
73-0336	6.5	0.8	1.1	4b	III	animal
70-0249	6.5	0.8	1.2	4c	III	animal
79-0430	6.6	0.6	0.7	4a	III	animal
87-0707	6.6	0.3	0.4	4a	III	animal
85-0010	6.7	0.5	0.6	4b	III	animal
83-2795	6.9	0.5	0.7	4a	III	animal
79-2759	7.0	0.6	0.7	4a	III	animal
80-3749	7.0	0.5	0.5	4a	III	animal
62-4693	7.1	0.6	0.9	4a	III	animal
77-4745	7.1	0.7	0.7	4a	III	animal
78-3636	7.1	0.6	0.9	4a	III	animal
80-0619	7.3	0.6	0.7	4a	III	animal
79-1994	7.6	1	1.2	4a	III	animal
78-0712	7.8	0.7	0.9	4a	III	animal
85-0567	7.8	0.5	0.6	4a	III	animal
83-1885	8.0	0.5	0.5	4a	III	animal
78-3565	9.0	1	1.3	4a	III	animal
67-1786	15.5	1.3	1.6	NA	NA	animal
76-2120/1	15.7	1.5	2.0	7	NA	animal
LO28 OPPA-	17.9	1.5	1.8	NA	NA	unknown

C.1.2 Population analysis of isolates at 2.14M NaCl



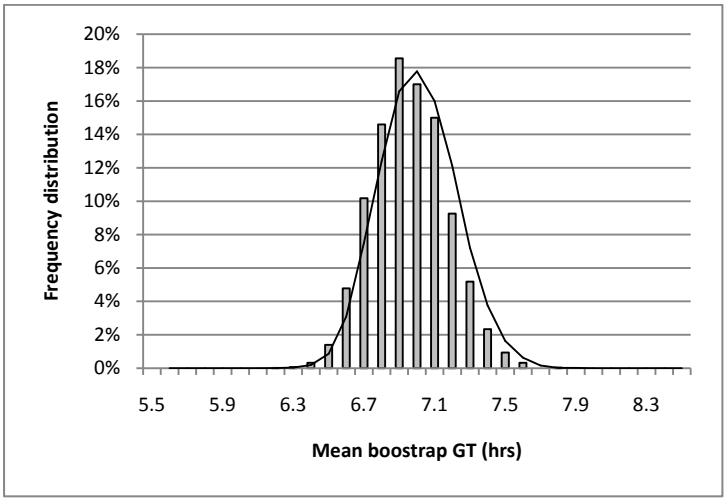
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	6.7
Upper bound	8.2

Figure C.3 Population analysis of *L. monocytogenes* lineage I growth rate estimates at 12.5% NaCl.



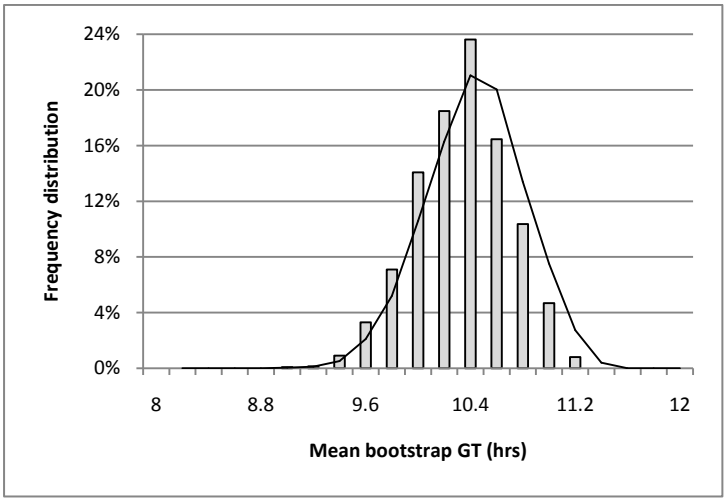
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	7.6
Upper bound	10.1

Figure C.4 Population analysis of *L. monocytogenes* lineage II growth rate estimates at 12.5% NaCl.



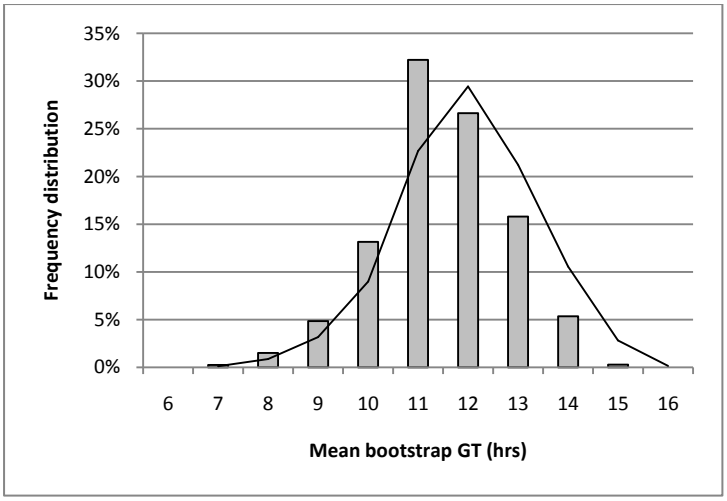
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	6.6
Upper bound	7.3

Figure C.5 Population analysis of *L. monocytogenes* lineage III growth rate estimates at 12.5% NaCl.



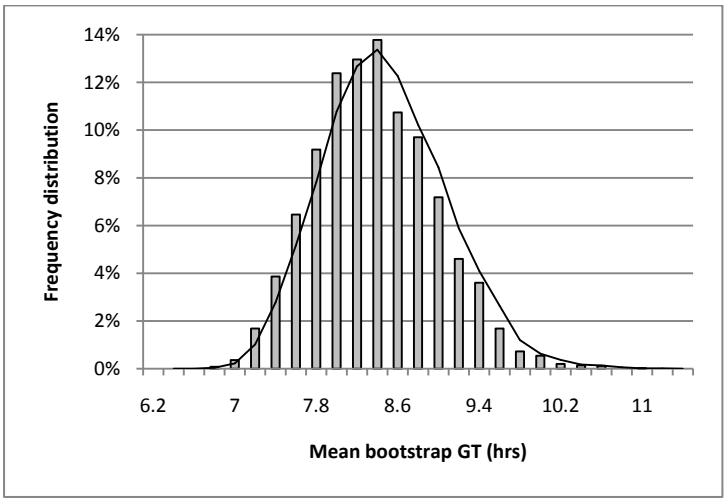
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	9.6
Upper bound	10.8

Figure C.6 Population analysis of *L. monocytogenes* serotype 1/2b growth rate estimates at 12.5% NaCl.



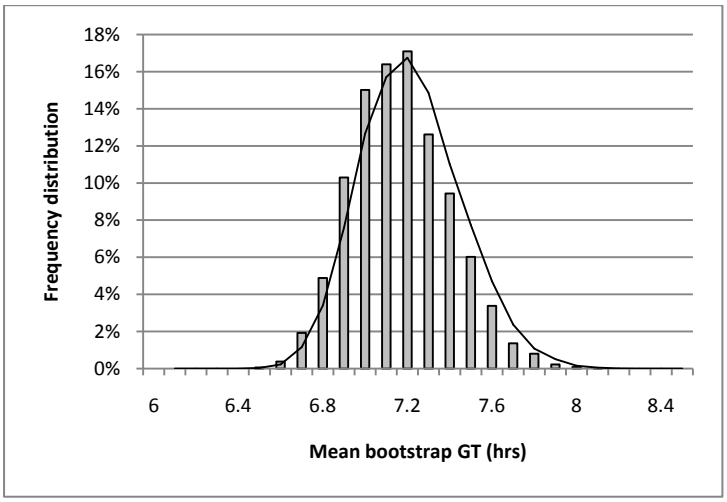
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	8.7
Upper bound	13.2

Figure C.7 Population analysis of *L. monocytogenes* serotype 1/2c growth rate estimates at 12.5% NaCl.



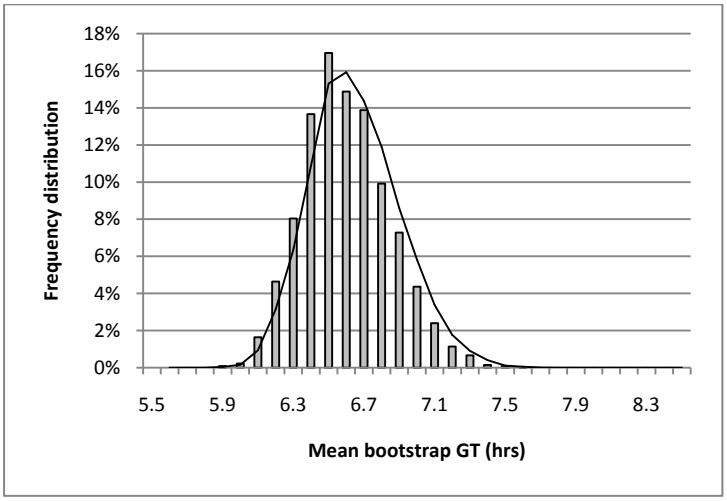
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	7.4
Upper bound	9.3

Figure C.8 Population analysis of *L. monocytogenes* serotype 1/2a growth rate estimates at 12.5% NaCl.



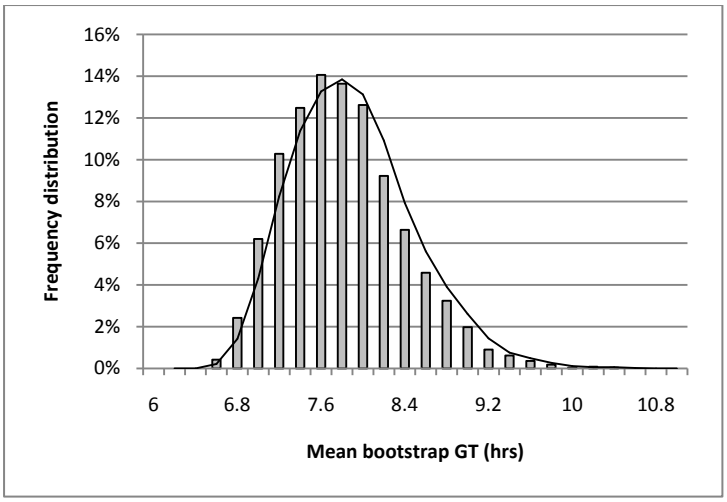
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	6.8
Upper bound	7.5

Figure C.9 Population analysis of *L. monocytogenes* serotype 4a growth rate estimates at 12.5% NaCl.



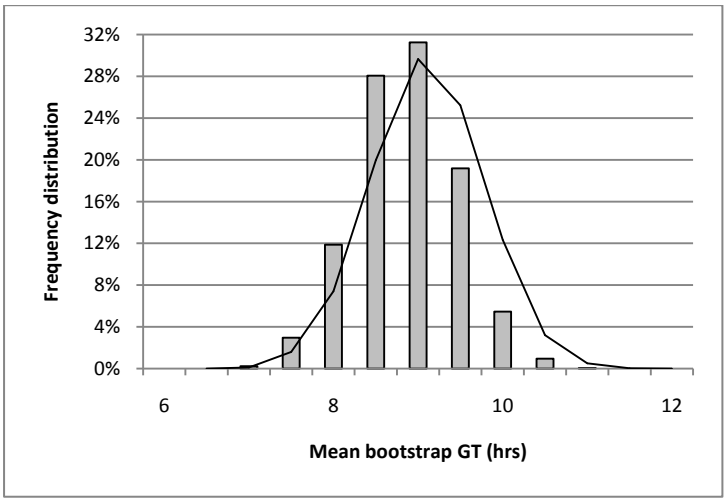
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	6.2
Upper bound	7.0

Figure C.10 Population analysis of *L. monocytogenes* serotype 4b growth rate estimates at 12.5% NaCl.



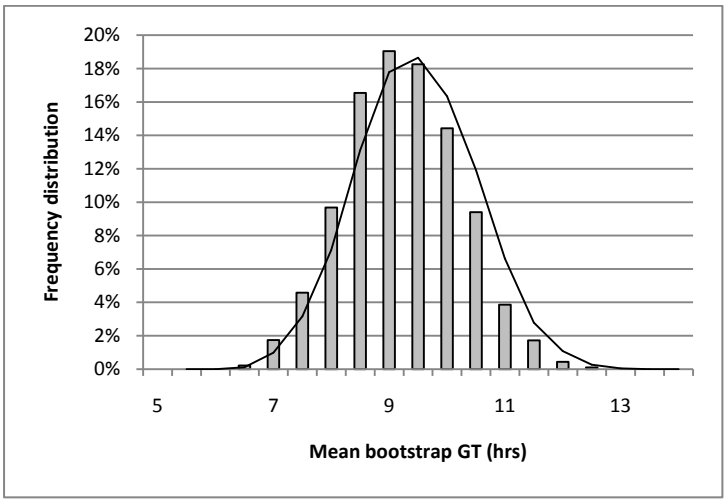
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	6.9
Upper bound	8.7

Figure C.11 Population analysis of *L. monocytogenes* animal isolates growth rate estimates at 12.5% NaCl.



Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	7.6
Upper bound	9.5

Figure C.12 Population analysis of *L. monocytogenes* food isolates growth rate estimates at 12.5% NaCl.



Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	7.4
Upper bound	10.7

Figure C.13 Population analysis of *L. monocytogenes* clinical isolates growth rate estimates at 12.5% NaCl.

C.1.3 Growth parameters of isolates at 4°C

Table C.2 Estimated generation time values of *L. monocytogenes* strains in brain-heart infusion broth at 4°C.

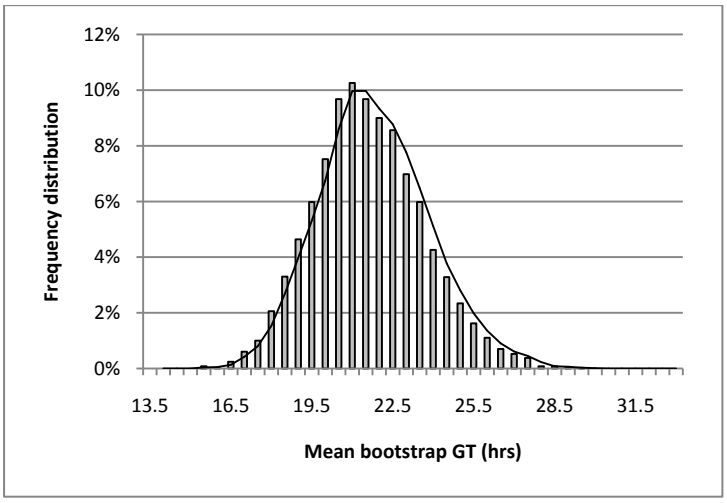
Strain reference	Average GT (hrs)	Standard error	Serotype	Lineage	Strain origin
76-1854	9.90	0.55	4b	III	animal
79-2336/3	10.20	0.55	4b	I	animal
79-2336/1	10.65	0.40	4b	I	animal
73-1801	12.23	0.55	3b	I	animal
79-2336/16	12.66	0.42	4b	I	animal
FW04/0037	13.17	0.50	1/2a	II	food/factory
79-1828	13.84	1.55	4b	I	animal
78-3636	13.94	1.30	4a	III	animal
FW03/0036	14.11	0.56	1/2a	II	food/factory
86-3009	14.27	0.55	1/2a	II	animal
87-0041	14.40	0.60	1/2a	II	animal
87-0707	14.47	1.12	4a	III	animal
87-1599	14.75	0.57	1/2a	II	animal
89-1931	15.11	0.70	4e	I	animal
78-2183	15.12	0.45	4b	I	animal
86-0071	15.17	0.47	4b	III	animal
FW04/0020	15.32	1.21	4b	I	clinical
77-4745	15.35	0.76	4a	III	animal
FW04/0024	15.37	1.39	1/2a	II	food/factory
FW04/0021	15.45	1.29	4b	I	clinical
FW03/0032	15.46	0.38	3a	II	food/factory
80-4904	15.55	0.36	1/2a	II	animal
85-0567	15.73	1.02	4a		unknown
72-0039	15.99	0.15	1/2a	II	animal
83-2099	16.12	0.43	1/2a	II	animal
84-2026	16.15	1.98	4b	I	animal
80-3354	16.41	1.05	1/2a	II	animal
80-2942	16.68	0.40	1/2a	II	animal
83-1617	16.71	0.38	1/2b	I	animal
78-1098	16.76	0.96	1/2b	II	animal
77-2294	16.80	0.40	4b	I	animal
87-2555	16.82	0.75	1/2a	II	animal
83-1804	16.89	2.19	4e	I	animal
71-0563	16.95	0.60	4a	III	animal
79-2360	16.97	0.49	1/2a	II	animal
80-0619	16.97	1.25	4a	III	animal
98P1437	17.08	1.27	NA	NA	clinical
102-265-s-3-745	17.19	1.37	3a	II	food/factory
83-1885	17.43	2.09	4a	III	animal

Strain reference	Average GT (hrs)	Standard error	Serotype	Lineage	Strain origin
FW03/0033	17.56	0.99	1/2a	II	food/factory
102-265-s-3-352	17.67	0.94	1/2a	II	food/factory
74-2395	17.97	0.44	4b	I	animal
80-4798	18.16	0.45	4b	I	animal
79-2048	18.20	1.63	1/2a	II	animal
102-195-s-1-154	18.22	0.38	1/2a	II	food/factory
67-1759	18.29	1.25	1/2a	II	animal
71-0934	18.41	1.20	1/2b	I	animal
79-2759	18.58	1.11	4a	III	animal
68-2169	18.60	1.22	4a	III	animal
L2	18.69	0.22	1/2b	I	unknown
84-1886	18.71	1.17	4b	I	animal
85-0010	18.86	0.89	4b	III	animal
83-0159	18.90	1.03	4b	I	animal
ATCC 7644	18.91	0.39	1/2c	II	clinical
L1	19.09	1.34	1/2a		unknown
ATCC 19112	19.10	1.48	1/2c	II	clinical
76-2120/1	19.15	0.59	7	NA	animal
FW04/0017	19.21	0.37	1/2b	I	clinical
74-0490	19.24	0.77	NA	NA	animal
ATCC 10403	19.48	2.36	1/2a	II	clinical
83-2795	19.49	2.58	4a	III	animal
66-0755	19.60	1.29	1/2a	II	animal
78-0712	19.62	1.14	4a	III	animal
SILIKEN 204231/1	19.63	1.90	1/2c	II	food/factory
85-2389	19.67	0.84	4e	I	animal
102-231-s-7-232	19.71	0.81	4e	I	food/factory
90-0053	19.79	1.15	1/2a	II	animal
79-3194	19.95	1.67	4a	III	animal
80-2437	19.96	0.39	4e	I	animal
78-2755	20.06	0.78	4b	III	animal
FRRW 2343	20.49	1.05	NA	NA	food/factory
85-0658	20.74	0.89	4a	III	animal
79-0869	20.77	1.07	4b	I	animal
114-830-s-7-62	20.77	1.26	1/2a	II	food/factory
79-2336	20.84	2.65	NA	NA	animal
78-3565	20.90	1.99	4a	III	animal
79-0430	21.21	1.32	4a	III	animal
62-4693	21.27	0.81	4a	III	animal
LM NR30	21.30	0.62	NA	NA	unknown
FW04/0022	21.32	0.71	1/2b	I	food/factory
20423	21.49	1.03	NA	NA	food/factory
102-195-s-1-60	21.61	1.02	3c	I	food/factory
ATCC 19114	21.62	1.14	4a	III	animal

Strain reference	Average GT (hrs)	Standard error	Serotype	Lineage	Strain origin
102-409-s-3-431	21.72	0.91	1/2c	II	food/factory
80-3354	21.77	0.18	4e	I	animal
69-0577	21.95	1.12	4b	I	animal
LM 412	21.95	0.80	NA	NA	unknown
FRRW 2345	22.06	0.66	NA	NA	food/factory
102-241-s-1-349	22.09	1.02	4e	I	food/factory
80-4762	22.32	0.40	1/2a	II	animal
92-0305	22.88	2.43	4b	I	unknown
102-195-s-1-367	23.21	1.05	1/2c	II	food/factory
FW04/0025	23.25	2.53	1/2a	II	food/factory
80-3453	23.31	1.31	1/2a	II	animal
64-0738	23.38	1.69	4c	III	animal
FW04/0018	23.51	0.55	1/2c	II	clinical
LM 412N	23.61	1.87	NA	NA	unknown
20425	23.67	2.50	NA	NA	food/factory
FW04/0026	23.67	1.40	1/2a	II	food/factory
114-997-s-7-63	23.75	1.48	1/2b	I	food/factory
ATCC 19115	23.86	1.19	4b	I	clinical
80-2880	24.02	0.53	4a	III	animal
L522	24.14	1.36	NA	NA	food/factory
FW04/0023	24.39	0.89	1/2c	II	food/factory
80-3749	24.56	3.39	4a	III	animal
80-0910	24.58	1.07	1/2a	II	animal
71-3227	24.58	0.69	4b	I	clinical
20432	24.75	2.13	NA	NA	food/factory
70-0249	24.88	2.04	4c	III	animal
70-0421	25.21	2.91	1/2a	II	animal
68-2528	25.29	1.16	1/2a	II	animal
80-2901	25.61	1.42	1/2a	II	animal
79-1994	26.26	0.84	4a	III	animal
LO28	26.26	1.33	1/2c	II	clinical
70-3167	26.26	2.71	4b	I	animal
97P107857	26.40	2.92	NA	NA	clinical
64-1495	26.56	1.02	1/2a	II	animal
102-231-s-7-566	26.94	1.13	3b	I	food/factory
69-1363	27.50	1.61	4b	I	animal
99P63881	27.51	0.63	NA	NA	clinical
70-0378	27.61	2.40	4b	I	animal
02P82412	27.64	1.47	NA	NA	clinical
ATCC 33090	27.87	1.33	6a	NA	animal
64-2389	28.73	2.80	1/2a	II	animal
FW03/0034	29.52	0.96	1/2a	II	food/factory
FW04/0019	29.66	1.35	1/2b	I	clinical
Joyce	29.69	2.17	1/2a	II	animal

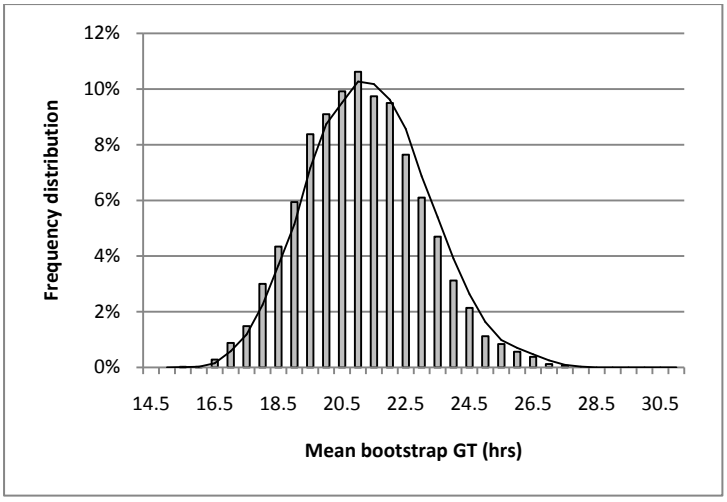
Strain reference	Average GT (hrs)	Standard error	Serotype	Lineage	Strain origin
02P79545	29.73	2.43	NA	NA	clinical
03P136964	30.07	1.00	NA	NA	clinical
00P23755	30.15	1.94	NA	NA	clinical
FW03/0035	30.18	3.38	4b	III	food/factory
98P130512	30.80	2.05	NA	NA	clinical
69-1793	31.35	1.33	4b	III	animal
ATCC 19111	31.42	2.34	1/2a	II	clinical
62-2853	31.72	0.10	1/2a	II	animal
02P00638	32.25	4.09	NA	NA	clinical
ScottA	32.28	1.34	4b	I	clinical
70-1700	32.66	2.44	4e	I	animal
01P27654	32.82	2.54	NA	NA	clinical
70-2058	34.99	1.74	1/2a	II	animal
99P154255	35.19	0.84	NA	NA	clinical
102-195-s-1-242	36.13	2.31	1/2c	II	food/factory
91B3450	37.60	2.42	NA	NA	clinical
02P37678	38.50	0.92	NA	NA	clinical
05P24111	40.64	1.43	NA	NA	clinical
63-5635	40.72	1.41	4b	I	animal
67-1786	42.02	0.47	NA	NA	animal
73-0336	47.08	1.24	4b	III	animal
LO28 OPPA-	60.45	5.17	NA	NA	unknown

C.1.4 Population analysis of isolates at 4°C



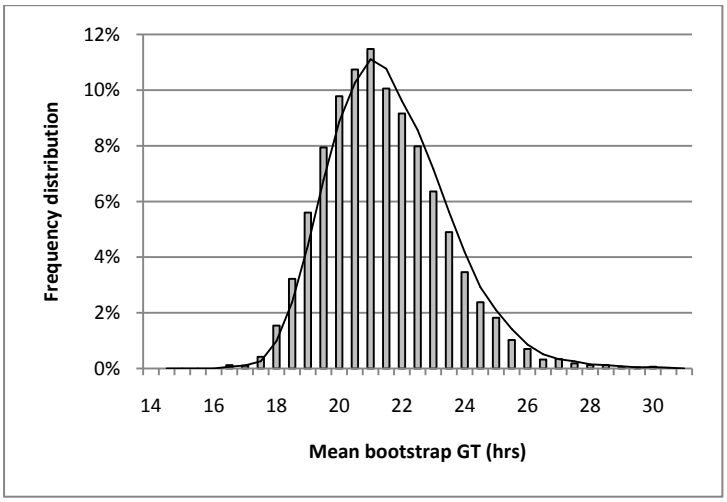
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	18.2
Upper bound	24.8

Figure C.14 Population analysis of *L. monocytogenes* lineage I growth rate estimates at 4°C.



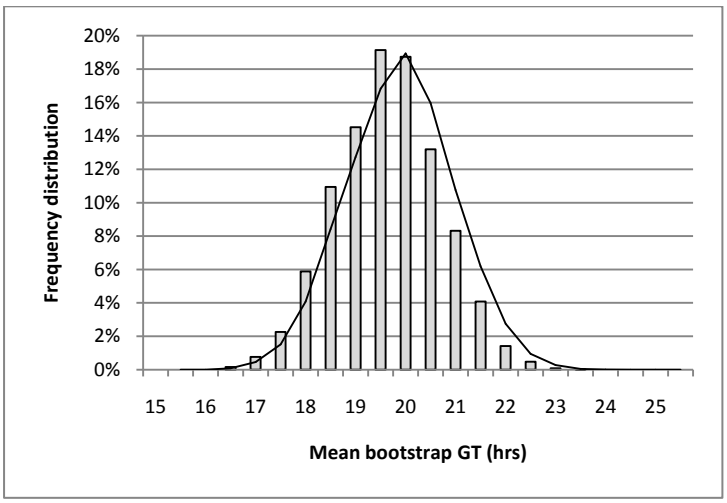
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	17.9
Upper bound	24.0

Figure C.15 Population analysis of *L. monocytogenes* lineage II growth rate estimates at 4°C.



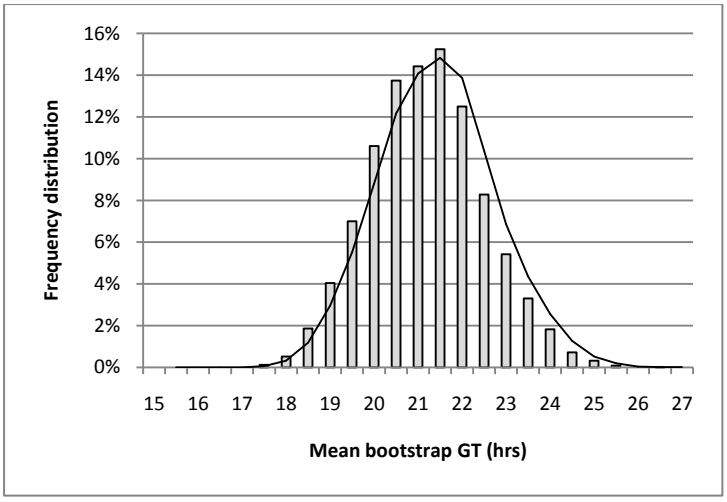
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	18.4
Upper bound	24.3

Figure C.16 Population analysis of *L. monocytogenes* lineage III growth rate estimates at 4°C.



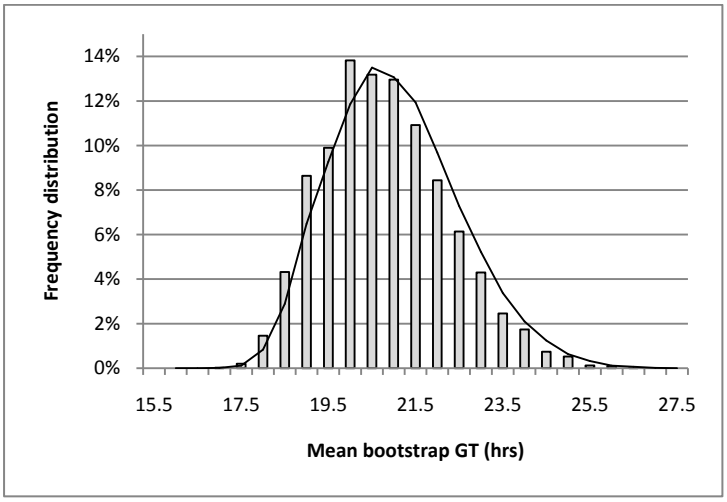
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	17.6
Upper bound	21.1

Figure C.17 Population analysis of *L. monocytogenes* serotype 4a growth rate estimates at 4°C.



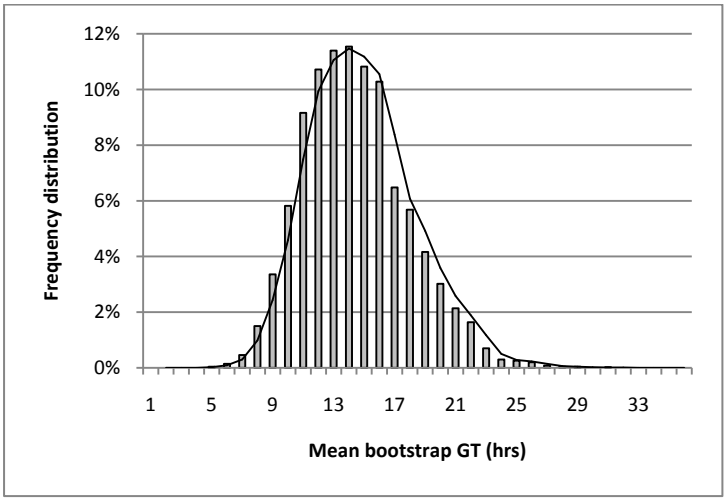
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	18.8
Upper bound	23.2

Figure C.18 Population analysis of *L. monocytogenes* serotype 1/2a growth rate estimates at 4°C.



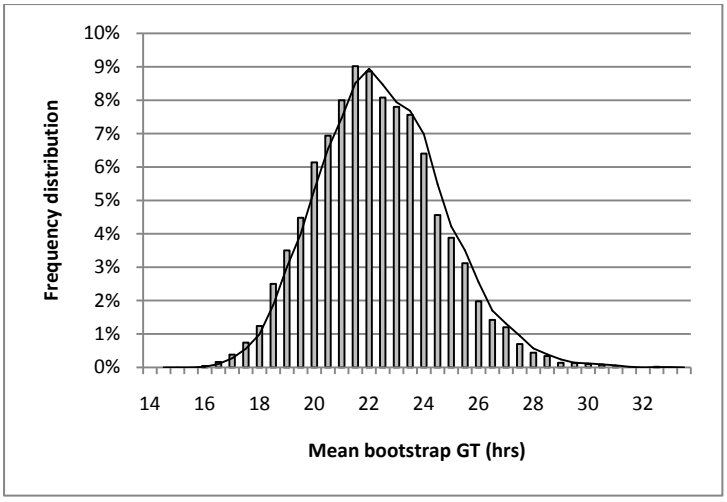
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	18.4
Upper bound	23.0

Figure C.19 Population analysis of *L. monocytogenes* serotype 1/2b growth rate estimates at 4°C.



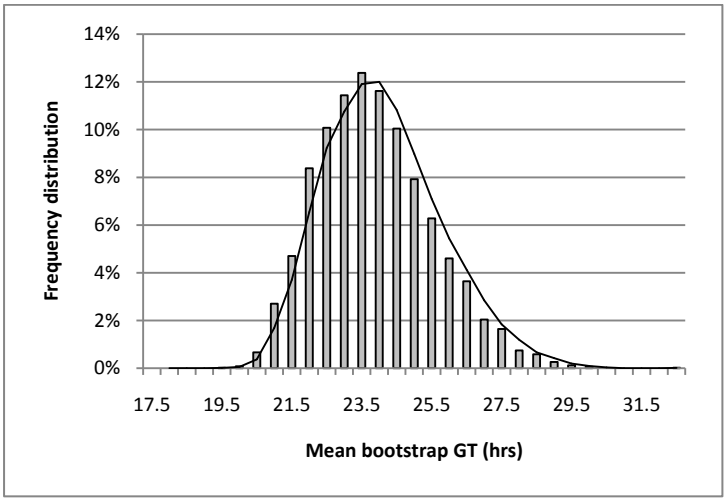
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	18.5
Upper bound	24.0

Figure C.20 Population analysis of *L. monocytogenes* serotype 4e growth rate estimates at 4°C.



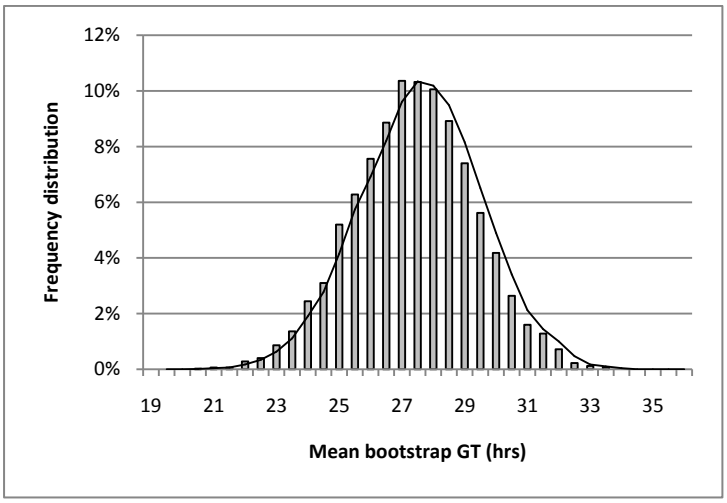
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	18.6
Upper bound	25.9

Figure C.21 Population analysis of *L. monocytogenes* serotype 4b growth rate estimates at 4°C.



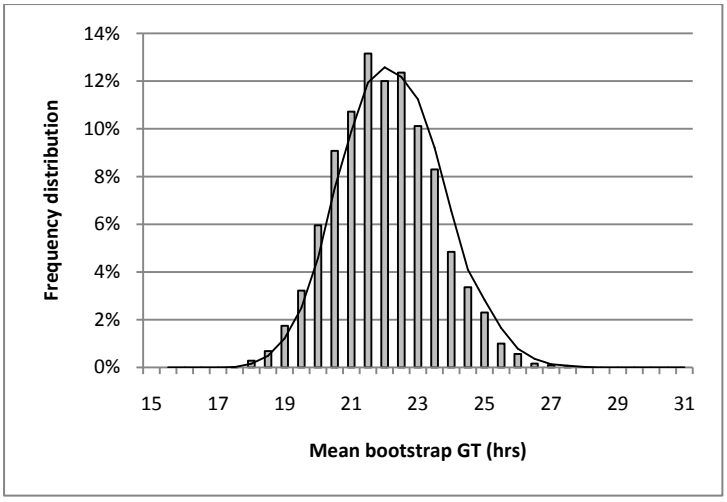
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	21.2
Upper bound	26.6

Figure C.22 Population analysis of *L. monocytogenes* serotype 1/2c growth rate estimates at 4°C.



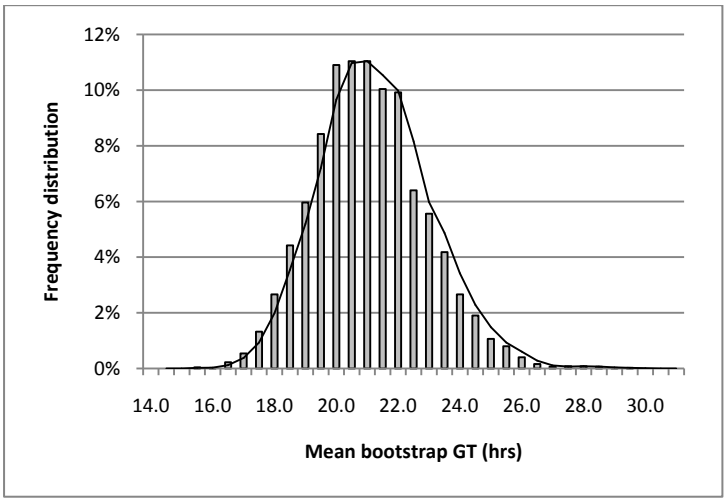
Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	23.9
Upper bound	30.4

Figure C.23 Population analysis of *L. monocytogenes* clinical isolates growth rate estimates at 4°C.



Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	19.4
Upper bound	24.3

Figure C.24 Population analysis of *L. monocytogenes* food isolates growth rate estimates at 4°C.



Confidence interval of the mean bootstrap	
Total sample	5000
Alpha	0.05
Confidence level	90
Lower bound	18.1
Upper bound	24.0

Figure C.25.0 Population analysis of *L. monocytogenes* animal isolates growth rate estimates at 4°C.

D.1 Strain-specific genes expressed in strain ATCC19115, ScottA and 70-1700 adapted to either hypeosmotic or cold stress.

D.1.1 Strain-specific gene expression for strain ATCC19115

D.1.1.1 Genes significantly expressed in ATCC19115 adapted to 10.0% (w/v) NaCl.

Table D.1 Genes significantly up-regulated >twofold in ATCC19115 only at 10.0%NaCl.

Gene*	Gene Homolog€	ATCC19115 NaCl		Predicted or known function€
		LR#	P¥	
lmo0007	*gyrA*	**1.43**	0.043	DNA gyrase, A subunit
lmo0013	*qoxA*	**1.97**	0.012	AA3-600 quinol oxidase subunit II
lmo0055	*purA*	**1.26**	0.037	adenylosuccinate synthetase
lmo0099		**1.76**	0.008	putative regulatory protein (similar to L. casei ManO protein)
lmo0141		**1.29**	0.021	unknown protein
lmo0153		**1.47**	0.046	similar to high affinity zinc ion ABC transporter, substrate binding protein
lmo0155		**1.78**	0.001	similar to high affinity zinc ion ABC transporter, permease protein
lmo0159		**2.44**	0.004	putative peptidoglycan bound protein (LPXTG motif)
lmo0162	*holB*	**2.38**	2.1E-04	DNA polymerase III delta' subunit
lmo0163		**1.27**	0.006	similar to uncharacterized conserved proteins
lmo0185		**1.69**	0.001	putative Mg-dependent deoxyribonuclease
lmo0189		**3.31**	7.4E-05	similar to uncharacterized conserved proteins; similar to B. subtilis Veg protein
lmo0191		**1.70**	0.002	similar to a putative phospho-beta-glucosidase
lmo0203	*mpl*	**1.37**	0.026	Zinc metalloprotease (elastase) propeptide
lmo0228	*lysS*	**1.27**	0.010	lysyl-tRNA synthetase (class II)
lmo0245	*secE*	**2.67**	0.004	preprotein translocase SecE subunit
lmo0247		**1.31**	0.014	unknown protein
lmo0264	*inlE*	**1.28**	0.047	internalin E
lmo0335		**1.34**	0.010	Unknown protein
lmo0371		**1.25**	0.005	putative transcription regulator, GntR family
lmo0455		**1.52**	0.003	similar to a regulator of polyketide synthase expression
lmo0484		**1.49**	0.047	similar to uncharacterized enzymes involved in biosynthesis of extracellular polysaccharides
lmo0581		**1.35**	0.020	similar to predicted SAM-dependent methyltransferases
lmo0663		**2.13**	2.5E-04	similar to predicted hydrolases of the HAD superfamily
lmo0665		**1.38**	0.023	unknown protein
lmo0777		**1.35**	0.004	unknown protein
lmo0821		**1.30**	0.028	putative lipoprotein
lmo0847		**1.76**	0.007	similar to polar amino acid ABC transporter, fused substrate binding and permease protein
lmo0931		**1.37**	0.002	similar to lipoate-protein ligase
lmo0957		**1.42**	0.041	glucosamine-6 phosphate isomerase
lmo1186	*eutH*	**1.40**	0.004	similar to ethanolamine utilisation EutH protein (putative periplasmic transport protein)
lmo1199	*cbiH*	**2.20**	0.001	precorrin-3B C17-methyltransferase
lmo1233	*trxA*	**2.09**	0.039	thioredoxin
lmo1237	*racE*	**1.45**	0.003	glutamate racemase
lmo1282		**1.83**	0.043	unknown protein

Gene*	Gene Homolog€	ATCC19115 NaCl		Predicted or known function€
		LR#	P¥	
lmo1364	*cspL*	**2.85**	3.4E-04	similar to cold shock protein (beta-ribbon, CspA family)
lmo1435		**1.55**	0.004	dihydrodipicolinate synthase
lmo1481	*holA*	**1.80**	0.018	DNA polymerase III, delta subunit
lmo1496	*greA*	**1.28**	0.012	transcription elongation factor
lmo1511		**1.45**	0.006	similar to lysophospholipase family proteins and other enzymes with an alpha/beta hydrolase fold
lmo1518		**1.59**	0.002	unknown protein
lmo1671		**1.37**	0.044	similar to zinc ion ABC transporter, substrate binding protein
lmo1673	*menB*	**1.61**	0.029	naphthoate synthase
lmo1698		**2.06**	3.2E-04	ribosomal-protein-alanine N-acetyltransferase
lmo1703		**1.38**	0.015	putative 23S rRNA (uracil-5-)-methyltransferase
lmo1709		**1.60**	0.019	similar to methionyl aminopeptidase
lmo1721		**1.26**	0.036	putative transcriptional regulator
lmo1758		**1.35**	0.004	similar to DNA ligase (NAD+)
lmo1769	*purQ*	**1.42**	0.040	phosphoribosylformylglycinamidine synthase, synthetase domain
lmo1805	*rncS*	**1.27**	0.035	similar to ribonuclease III
lmo1887		**1.31**	0.047	putative N6-adenine-specific DNA methylase
lmo1891	*recU*	**1.31**	0.014	similar to DNA repair and homologous recombination protein
lmo1901	*panC*	**2.42**	0.003	pantoate--beta-alanine ligase
lmo1946		**1.77**	0.006	similar to acyl-CoA hydrolase family proteins
lmo1950		**2.10**	0.001	segregation and condensation protein B
lmo1951		**1.65**	0.001	segregation and condensation protein A
lmo1958	*fhuB*	**1.45**	0.013	similar to ferrichrome ABC transporter, permease protein
lmo1975		**1.79**	0.006	DNA polymerase IV
lmo1976		**1.61**	0.001	similar to short-chain dehydrogenases of various substrate specificities
lmo1986	*ilvC*	**1.48**	0.005	ketol-acid reductoisomerase
lmo1988	*leuB*	**1.43**	0.003	3-isopropylmalate dehydrogenase
lmo2013		**1.66**	0.010	similar to uncharacterized conserved proteins
lmo2043		**1.28**	0.007	similar to uncharacterized conserved proteins
lmo2049		**1.35**	0.003	similar to predicted nucleotidyltransferases
lmo2058	*ctaA*	**1.29**	0.010	similar to heme O oxygenase
lmo2080		**2.07**	3.4E-04	putative lipoprotein
lmo2089		**1.65**	0.012	similar to esterase/lipase family proteins
lmo2137		**2.04**	0.001	similar to PTS system, fructose-specific IIA component
lmo2139		**1.68**	0.001	similar to sodium ion ABC transporter, ATP binding protein
lmo2166		**2.19**	0.010	similar to cyclic nucleotide-binding proteins (Crp-like)
lmo2167		**1.52**	0.004	similar to Zn-dependent hydrolases
lmo2180		**1.89**	0.001	similar to uncharacterized conserved proteins
lmo2182		**1.41**	0.017	similar to ferrichrome ABC transporter, ATP binding protein
lmo2184		**1.78**	4.2E-04	similar to ferrichrome ABC transporter, substrate binding protein
lmo2185	*svpA*	**3.20**	2.2E-05	cell surface protein p64; iron transport-associated domain
lmo2200		**1.31**	0.015	putative transcriptional regulator, MarR family
lmo2201		**2.66**	0.003	3-oxoacyl-[acyl-carrier-protein] synthase I/II
lmo2234		**1.49**	0.012	similar to predicted phosphosugar isomerases
lmo2243	*add*	**1.51**	0.002	putative adenosine deaminase

Gene*	Gene Homolog€	ATCC19115 NaCl		Predicted or known function€
		LR#	P¥	
lmo2244		**1.28**	0.021	similar to 23S RNA-specific pseudouridine synthase D
lmo2346		**2.29**	0.001	similar to polar amino acid ABC transporter, ATP-binding protein
lmo2349		**4.54**	2.8E-06	similar to polar amino acid ABC transporter, fused substrate binding and permease protein
lmo2350		**1.26**	0.037	similar to acetyltransferase (GNAT) family proteins
lmo2352		**1.58**	0.002	putative transcriptional regulator, LysR family
lmo2367	*pgi*	**2.01**	0.001	glucose-6-phosphate isomerase
lmo2371		**1.64**	0.001	similar to putative ABC transporter, permease protein
lmo2389		**1.64**	0.021	NADH dehydrogenase
lmo2424		**1.31**	0.035	thioredoxin domain-containing protein
lmo2427		**1.57**	0.005	similar to FtsK/RodA/SpoIIIE and related proteins
lmo2429		**1.43**	0.002	similar to ferrichrome ABC transporter, ATP binding protein
lmo2447		**1.85**	0.001	similar to transcription regulator
lmo2464		**1.64**	0.003	putative transcriptional regulator, TetR/AcrR family
lmo2472		**1.38**	0.041	similar to uncharacterized conserved proteins
lmo2476		**1.59**	0.029	aldose 1-epimerase
lmo2477	*galE*	**1.81**	0.014	UDP-glucose 4-epimerase
lmo2500	*phoR*	**1.30**	0.010	two-component phosphate sensor histidine kinase
lmo2530	*atpG*	**1.90**	0.002	F0F1-type ATP synthase, gamma subunit
lmo2544		**1.50**	0.001	thymidine kinase
lmo2556	*fbaA*	**2.41**	9.4E-05	fructose-bisphosphate aldolase
lmo2565		**1.65**	0.008	similar to HD superfamily phosphohydrolases
lmo2607	*rpsK*	**1.36**	0.003	ribosomal protein S11
lmo2630	*rplW*	**2.05**	0.002	ribosomal protein L23
lmo2704	*dnaX*	**1.34**	0.012	DNA polymerase III gamma and tau subunit
lmo2714		**2.18**	0.041	peptidoglycan bound protein (LPXTG motif)
lmo2735		**2.03**	0.007	sucrose phosphorylase
lmo2751		**1.28**	0.021	similar to ABC transporter, ATP-binding protein
lmo2769		**1.73**	0.008	similar to ABC transporter, ATP-binding protein
lmo2810	*gidA*	**1.27**	0.005	similar to glucose inhibited division protein A
lmo2834		**1.44**	0.018	similar to predicted dehydrogenases and related proteins

* Gene nomenclature used as per *L. monocytogenes* EGD-e genome. Genes mentioned in Appendix II and III were excluded from the analysis accordingly.
€ Gene homologs and predicted functions were obtained collectively from variety of sources including circulating literature and web based databases.
LR: log ratio. Genes were considered significantly up-regulated with LR >1.25 which is equivocal of twofold up-regulation.
¥ Genes with P value >0.05 were generally excluded from this table.

D.1.1.2 Genes significantly expressed in ATCC19115 adapted to 4°C.

Table D.2 Genes significantly up-regulated >twofold in ATCC19115 only following adaptation to cold.

Gene[*]	Gene homolog[€]	ATCC19115 4°C		Predicted or known function[€]
		LR[#]	P[¥]	
lmo0052		**1.45**	0.001	predicted signal transduction protein (contains modified GGDEF/DHH domains)
lmo0069		**1.52**	0.019	unknown protein
lmo0190	*ispE*	**1.20**	0.018	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
lmo0192	*purR*	**1.45**	0.001	purine operon repressor
lmo0224	*sul*	**1.30**	0.004	dihydropteroate synthase
lmo0226	*folK*	**1.59**	0.002	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase
lmo0289		**1.46**	0.001	similar to uncharacterized conserved proteins
lmo0292	*htrA*	**1.20**	0.015	similar to heat-shock protein htrA serine protease
lmo0369		**1.25**	0.001	similar to uncharacterized conserved proteins
lmo0481		**1.39**	0.001	similar to streptococcal myosin-cross-reactive antigen; membrane protein
lmo0482		**1.57**	3.1E-04	similar to predicted Fe-S-cluster redox enzymes
lmo0581		**1.23**	0.006	similar to predicted SAM-dependent methyltransferases
lmo0597		**1.97**	5.1E-05	similar to cAMP-binding proteins, Crp family
lmo0772		**1.55**	0.001	putative transcription regulator, GntR family
lmo0799		**1.49**	0.004	similar to blue-light photoreceptor
lmo0851		**1.44**	3.8E-04	unknown protein
lmo0866	*deaD*	**2.38**	1.7E-05	similar to ATP-dependent RNA helicase
lmo0899		**1.23**	0.007	similar to SprT homologs (predicted to have roles in transcription elongation).
lmo0907		**1.35**	0.007	similar to putative fructose-2,6-bisphosphatase
lmo0949		**2.48**	1.2E-05	similar to predicted permeases
lmo0951		**1.36**	0.001	similar to uncharacterized conserved proteins
lmo0966		**1.29**	0.001	similar to uncharacterized conserved proteins
lmo1186	*eutH*	**1.22**	0.001	similar to ethanolamine utilisation EutH protein (putative periplasmic transport protein)
lmo1238		**1.53**	0.002	similar to ribonuclease PH
lmo1352		**1.35**	3.9E-04	unknown protein
lmo1384		**2.00**	5.3E-05	similar to predicted sulfurtransferases
lmo1399		**1.73**	0.002	similar to predicted HD superfamily hydrolases
lmo1426	*opuCC*	**1.33**	0.001	glycine betaine/choline/proline ABC transporter, substrate binding protein
lmo1429		**1.37**	0.002	predicted membrane protein
lmo1430		**1.61**	2.9E-04	similar to uncharacterized conserved proteins
lmo1432		**2.93**	1.4E-05	unknown protein
lmo1437		**1.65**	4.3E-04	aspartate-semialdehyde dehydrogenase
lmo1449		**1.73**	4.4E-04	similar to endonuclease IV
lmo1451		**1.72**	2.1E-04	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
lmo1466		**1.26**	0.042	similar to predicted membrane-associated HD superfamily hydrolases
lmo1469	*rpsU*	**1.35**	0.013	ribosomal protein S21
lmo1521		**1.34**	0.001	similar to autolysin (N-acetylmuramoyl-L-alanine amidase)
lmo1523	*relA*	**1.47**	0.001	GTP pyrophosphokinase
lmo1526		**1.59**	0.001	putative integral membrane protein

Gene*	Gene homolog€	ATCC19115 4°C		Predicted or known function€
		LR#	P¥	
lmo1647		**2.14**	1.3E-04	putative 1-acylglycerol-3-phosphate O-acyltransferase
lmo1649		**1.74**	0.007	unknown protein
lmo1670		**1.62**	1.4E-04	similar to uncharacterized conserved proteins
lmo1745	*virR*	**1.49**	2.0E-04	putative two-component response regulator
lmo1746		**1.51**	0.001	similar to ABC transporter, permease protein
lmo1747		**1.67**	1.4E-04	similar to ABC transporter, ATP-binding protein
lmo1866		**1.29**	0.002	similar to uncharacterized conserved proteins
lmo1914		**1.39**	0.026	similar to predicted sensor kinases
lmo1941		**2.16**	3.3E-05	similar to uncharacterized conserved proteins
lmo2031		**1.75**	0.028	similar to predicted enzyme with a TIM-barrel fold
lmo2202		**1.96**	9.8E-05	3-oxoacyl-[acyl-carrier-protein] synthase III
lmo2204		**2.30**	0.018	unknown protein
lmo2209		**1.66**	1.4E-04	similar to predicted acetyltransferases
lmo2337		**1.44**	0.010	similar to fructose operon transcriptional repressor
lmo2403		**1.23**	0.005	similar to 5'-nucleotidase/2',3'-cyclic phosphodiesterase and related esterases
lmo2456	*pgm*	**1.46**	0.022	phosphoglyceromutase
lmo2458	*pgk*	**1.68**	0.040	phosphoglycerate kinase
lmo2485		**1.69**	3.5E-04	similar to putative stress-responsive transcriptional regulator PspC
lmo2503		**1.47**	0.002	similar to cardiolipin synthase
lmo2504		**2.65**	1.0E-04	similar to membrane-bound metallopeptidases
lmo2508		**1.84**	1.6E-04	similar to uncharacterized conserved proteins
lmo2514		**1.91**	7.3E-05	similar to uncharacterized conserved proteins
lmo2515	*degU*	**1.48**	1.8E-04	similar to B. subtilis two-component response regulator DegU
lmo2531	*atpA*	**1.71**	0.008	F0F1-type ATP synthase, alpha subunit
lmo2567		**1.99**	0.004	unknown protein
lmo2568		**2.19**	0.001	unknown protein
lmo2703		**1.26**	0.012	similar to uncharacterized conserved proteins

* Gene nomenclature used as per *L. monocytogenes* EGD-e genome. Genes mentioned in Appendix II and III were excluded from the analysis accordingly.
€ Gene homologs and predicted functions were obtained collectively from variety of sources including circulating literature and web based databases.
LR: log ratio. Genes were considered significantly up-regulated with LR >1.20 which is equivocal of twofold up-regulation.
¥ Genes with P value >0.05 were generally excluded from this table.

D.1.2 Strain-specific gene expression for strain ScottA

D.1.2.1 Genes significantly expressed in ScottA adapted to 12.0% (w/v) NaCl.

Table D.3 Genes up-regulated >two-fold in strain ScottA only adapted to 12.0% NaCl.

Gene*	Gene homolog€	ScottA NaCl		Predicted or known function€
		LR#	P¥	
lmo0018		**1.23**	0.002	beta-glucosidase
lmo0116	*lmaC*	**1.55**	0.004	similar to Antigen C protein; similar to protein gp66 from Bacteriophage A118
lmo0126		**1.29**	0.003	similar to phage proteins
lmo0128		**1.24**	0.001	similar to a holin protein from Bacteriophage phi-105
lmo0129		**1.30**	0.001	similar to autolysin (N-acetylmuramoyl-L-alanine amidase)
lmo0764		**1.45**	0.003	similar to lipoate-protein ligase
lmo1183		**1.31**	0.003	unknown protein
lmo1255		**1.49**	0.001	similar to PTS system, trehalose-specific IIBC component
lmo1266		**1.29**	0.011	unknown protein
lmo1299	*glnA*	**1.39**	0.005	glutamine synthetase
lmo1320	*polC*	**1.25**	0.004	DNA polymerase III alpha subunit
lmo1338		**1.09**	0.016	unknown protein
lmo1356	*accB*	**1.15**	0.004	acetyl-CoA carboxylase biotin carboxyl carrier protein
lmo1495		**1.18**	0.045	similar to uncharacterized conserved proteins
lmo1755	*gatA*	**1.24**	0.002	aspartyl/glutamyl-tRNA amidotransferase A subunit
lmo1766	*purN*	**1.33**	0.012	phosphoribosylglycinamide formyltransferase
lmo1772	*purC*	**1.11**	0.001	phosphoribosylaminoimidazole succinocarboxamide synthetase
lmo1815		**3.25**	0.002	similar to uncharacterized conserved proteins
lmo2038	*murE*	**1.43**	0.001	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase
lmo2042		**1.14**	0.008	similar to uncharacterized conserved proteins
lmo2127		**1.24**	0.002	CAAX amino terminal protease family protein
lmo2226		**3.95**	0.003	putative membrane protein
lmo2230		**2.34**	0.004	putative protein-tyrosine-phosphatase
lmo2369		**1.27**	0.003	similar to B. subtilis general stress protein 13 containing a ribosomal S1 protein domain
lmo2430		**1.34**	0.006	similar to ferrichrome ABC transporter, permease protein
lmo2505	*spl*	**1.66**	0.002	putative cell wall-associated hydrolases (invasion-associated proteins); peptidoglycan lytic protein P45
lmo2597		**1.54**	0.001	ribosomal protein L13
lmo2690		**1.28**	0.008	putative transcriptional regulator, TetR/AcrR family

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€ Gene homologs and predicted functions were obtained collectively from variety of sources including circulating literature and web based databases.
LR: log ratio. Genes were considered significantly up-regulated with LR >1 which is equivocal of twofold up-regulation.
¥ Genes with P value >0.05 were generally excluded from this table.

D.1.2.2 Genes significantly expressed in ScottA adapted to 4°C.

Table D.4 Genes significantly up-regulated >twofold in ScottA only at cold temperature.

Gene[*]	Gene homolog[€]	ScottA 4°C		Predicted or known function[€]
		LR[#]	P[¥]	
lmo0027		**1.74**	9.5E-05	similar to PTS system, beta-glucosides-specific IIABC component
lmo0111		**1.15**	0.004	predicted signal transduction protein (contains a membrane domain, an EAL and a GGDEF domain)
lmo0144		**1.60**	4.8E-05	unknown protein
lmo0168	*abrB*	**1.14**	3.2E-04	pleiotrophic regulator of transition state genes
lmo0272		**1.09**	0.001	similar to predicted hydrolases of the HAD superfamily
lmo0273		**1.69**	9.3E-05	similar to acetyltransferase (GNAT) family proteins
lmo0278		**1.08**	0.001	similar to sugar ABC transporter, ATP-binding protein
lmo0319		**1.44**	4.0E-04	6-phospho-beta-glucosidase
lmo0391		**1.12**	0.001	unknown protein
lmo0485		**1.27**	0.003	similar to nitroreductase-like family protein
lmo0487		**1.47**	7.0E-05	NUDIX domain containing protein
lmo0932		**1.29**	2.4E-04	similar to uncharacterized conserved proteins
lmo1174	*eutA*	**1.70**	8.9E-05	similar to ethanolamine utilization protein EutA, possible chaperonin protecting lyase from inhibition
lmo1176	*eutC*	**1.20**	3.3E-04	similar to ethanolamine ammonia lyase light subunit
lmo1199	*cbiH*	**1.15**	0.001	precorrin-3B C17-methyltransferase
lmo1254		**2.00**	1.4E-05	similar to alpha,alpha-phosphotrehalase
lmo1518		**1.30**	4.9E-04	unknown protein
lmo1572	*accA*	**1.07**	0.001	acetyl-CoA carboxylase carboxyl transferase subunit alpha
lmo1592	*thiI*	**1.48**	0.002	thiamine biosynthesis ATP pyrophosphatase
lmo1604		**1.35**	0.001	putative peroxiredoxin
lmo1665		**1.23**	0.007	unknown protein
lmo1705		**1.62**	1.3E-04	similar to deoxyguanosine/doxyadenosine kinase
lmo1750		**1.52**	1.4E-04	similar to uncharacterized conserved proteins
lmo1796		**1.10**	0.001	predicted RNA-binding protein
lmo1860		**1.62**	0.004	similar to peptidyl methionine sulfoxide reductase
lmo1879	*cspD*	**1.68**	2.0E-04	similar to cold shock protein (beta-ribbon, CspA family)
lmo1893		**1.14**	3.5E-04	unknown protein
lmo1901	*panC*	**1.51**	2.4E-04	pantoate--beta-alanine ligase
lmo1945		**1.10**	4.1E-04	predicted membrane protein
lmo1946		**1.19**	0.008	similar to acyl-CoA hydrolase family proteins
lmo1949		**1.22**	0.001	similar to 23S RNA-specific pseudouridine synthase B
lmo1953	*pnp*	**1.17**	2.2E-04	purine nucleoside phosphorylase
lmo1989	*leuC*	**1.08**	4.3E-04	3-isopropylmalate dehydratase large subunit
lmo1991	*ilvA*	**1.19**	2.8E-04	threonine dehydratase
lmo2062		**1.40**	4.0E-04	similar to copper export proteins
lmo2065		**1.59**	7.3E-05	Unknown protein
lmo2149		**1.31**	2.9E-04	similar to predicted pyrophosphatases
lmo2180		**1.29**	4.4E-04	similar to uncharacterized conserved proteins
lmo2185	*svpA*	**1.67**	1.1E-03	cell surface protein p64; iron transport-associated domain

Gene*	Gene homolog€	ScottA 4°C		Predicted or known function€
		LR#	P¥	
lmo2186		**1.98**	8.6E-05	peptidoglycan bound protein; iron transport-associated domain
lmo2258		**1.19**	0.001	unknown protein
lmo2302		**1.06**	4.5E-04	unknown protein
lmo2469		**1.19**	3.1E-04	putative amino acid permease
lmo2524	*fabZ*	**1.20**	0.044	similar to hydroxymyristoyl-(acyl carrier protein) dehydratase
lmo2545	*thrB*	**1.69**	1.1E-04	homoserine kinase
lmo2689		**1.45**	0.032	similar to Mg2+ transport ATPase
lmo2709		**1.40**	0.011	unknown protein
lmo2766		**1.07**	0.001	similar to transcriptional regulator, RpiR-like
lmo2825	*serC*	**1.24**	0.001	phosphoserine aminotransferase
lmo2835		**1.43**	0.046	similar to sugar phosphate isomerases/epimerases

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€ Gene homologs and predicted functions were obtained collectively from variety of sources including circulating literature and web based databases.
LR: log ratio. Genes were considered significantly up-regulated with LR >1 which is equivocal of twofold up-regulation.
¥ Genes with P value >0.05 were generally excluded from this table.

D.1.3 Strain-specific gene expression for strain 70-1700.

D.1.3.1 Genes significantly expressed in 70-1700 adapted to 8.0% (w/v) NaCl.

Table D.5 Genes significantly up-regulated >twofold in 70-1700 only at 8.0% NaCl.

Gene*	Gene homolog€	70-1700 NaCl		Predicted or known function€
		LR#	P¥	
lmo0006	*gyrB*	**1.15**	0.001	DNA gyrase, B subunit
lmo0016	*qoxD*	**1.25**	0.001	AA3-600 quinol oxidase subunit IV
lmo0293		**1.74**	0.013	similar to uncharacterized conserved proteins
lmo0670		**1.47**	0.004	unknown protein
lmo0913		**1.36**	2.5E-05	succinate-semialdehyde dehydrogenase (NADP+)
lmo1152	*pduB*	**1.22**	2.0E-04	similar to carboxysome shell protein; similar to S. enterica PduB/EutL protein
lmo1158	*pduK*	**1.39**	2.0E-05	similar to carboxysome shell protein; similar to S. enterica PduK protein
lmo1161	*eutJ*	**1.15**	3.5E-04	similar to ethanolamine utilization protein EutJ
lmo1162	*pduM*	**1.85**	1.2E-05	similar to S. enterica PduM protein
lmo1248		**1.28**	1.1E-04	putative 7,8-dihydro-8-oxoguanine triphosphatase
lmo1288	*luxS*	**1.12**	0.003	autoinducer-2 production protein luxS
lmo1425	*opuCD*	**1.31**	0.001	glycine betaine/choline/proline ABC transporter, permease protein
lmo1428	*opuCA*	**1.09**	0.001	glycine betaine/choline/proline ABC transporter, ATP-binding protein
lmo1430		**1.13**	1.7E-04	similar to uncharacterized conserved proteins
lmo1432		**1.10**	0.002	unknown protein
lmo1440		**1.16**	0.011	similar to uncharacterized conserved proteins
lmo1452		**1.15**	0.004	similar to uncharacterized conserved proteins

Gene*	Gene homolog€	70-1700 NaCl		Predicted or known function€
		LR#	P¥	
lmo1521		**1.22**	0.002	similar to autolysin (N-acetylmuramoyl-L-alanine amidase)
lmo1524	*apt*	**1.79**	0.006	adenine phosphoribosyltransferase
lmo1644		**1.31**	0.001	putative DNA/RNA helicase, Snf2 family
lmo1668		**1.36**	0.003	unknown protein
lmo1684		**1.39**	0.023	phosphoglycerate dehydrogenase and related dehydrogenase family
lmo1786	*inlC*	**1.20**	0.001	internalin C
lmo1999	*glmS*	**1.39**	2.6E-04	glucosamine--fructose-6-phosphate aminotransferase (isomerizing), putative
lmo2002		**1.50**	2.0E-05	similar to PTS system, mannose-specific IIB component
lmo2006	*alsS*	**1.61**	1.0E-04	alpha-acetolactate synthase
lmo2007		**1.17**	0.008	similar to mutiple sugar ABC transporter, substrate binding protein
lmo2008		**1.58**	2.3E-04	similar to mutiple sugar ABC transporter, permease protein
lmo2094		**1.17**	9.6E-05	putative; L-fuculose-phosphate aldolase
lmo2095		**1.56**	2.9E-04	similar to 1-phosphofructokinase
lmo2096		**1.19**	0.003	similar to PTS system, galacitol-specific IIC component
lmo2102		**1.37**	6.9E-05	predicted glutamine amidotransferase involved in pyridoxine biosynthesis
lmo2111		**1.22**	0.001	similar to NADPH-dependent flavin reductases and oxygen-insensitive nitroreductases
lmo2112		**1.82**	1.9E-05	putative DNA-binding protein
lmo2199		**1.31**	0.005	similar to predicted redox protein, regulator of disulfide bond formation
lmo2206	*clpB*	**1.33**	0.002	Clp endopeptidase ATP-binding subunit
lmo2207		**1.66**	5.0E-05	similar to putative integral membrane protein that interacts with FtsH
lmo2455	*eno*	**1.20**	0.013	enolase
lmo2482	*lgt*	**1.38**	3.8E-04	prolipoprotein diacylglyceryl transferase
lmo2488	*uvrA*	**1.11**	4.1E-04	excinuclease ATPase subunit
lmo2491		**1.19**	0.003	similar to predicted hydrolases of HD superfamily
lmo2528	*atpC*	**1.19**	0.022	F0F1-type ATP synthase, epsilon subunit
lmo2540		**1.20**	0.022	putative protein-tyrosine-phosphatase
lmo2580		**1.22**	0.003	similar to ABC transporter, ATP-binding protein
lmo2682	*kdpA*	**1.37**	0.001	high affinity K+-transporting ATPase, a chain
lmo2692		**1.17**	0.004	unknown protein
lmo2696		**1.30**	1.9E-04	similar to dihydroxyacetone kinase, C-terminal domain
lmo2715	*cydD*	**1.27**	0.001	ABC transporter involved in cytochrome bd biosynthesis, fused ATP-binding and permease proteins
lmo2733		**1.48**	0.002	similar to PTS system, fructose-specific IIABC component
lmo2745		**1.40**	0.001	similar to ABC transporter, ATP-binding protein
lmo2761		**1.59**	0.010	beta-glucosidase
lmo2767		**1.29**	0.010	similar to uncharacterized conserved proteins
lmo2848		**1.10**	0.011	L-rhamnose isomerase

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€ Gene homologs and predicted functions were obtained collectively from variety of sources including circulating literature and web based databases.

LR: log ratio. Genes were considered significantly up-regulated with LR >1 which is equivocal of twofold up-regulation.

¥ Genes with P value >0.05 were generally excluded from this table.

D.1.3.2 Genes significantly expressed in 70-1700 adapted to 4°C.

Table D.6 Genes significantly up-regulated in strain 70-1700 only adapted to cold

Gene*	Gene homolog€	70-1700 4°C		Predicted or known function€
		LR#	P¥	
lmo0049	*agrD*	**1.97**	1.1E-05	putative autoinducing peptide
lmo0223	*cysK*	**1.45**	4.8E-04	cysteine synthase
lmo0611	*acpD*	**1.53**	1.0E-04	similar to acyl carrier protein phosphodiesterases
lmo0810	*0*	**1.44**	2.4E-05	similar to polyamine ABC transporter, substrate binding protein
lmo0823	*0*	**1.57**	1.4E-04	similar to aldo/keto reductases, related to diketogulonate reductase
lmo0846	*0*	**1.25**	0.001	excinuclease ABC, C subunit
lmo0893	*rsbV*	**1.82**	5.4E-06	anti-anti-sigma factor (antagonist of RsbW)
lmo0894	*rsbW*	**1.49**	1.8E-05	sigma-B activity negative regulator
lmo0903	*0*	**1.21**	0.001	similar to predicted redox protein, regulator of disulfide bond formation
lmo0974	*dltA*	**1.28**	0.001	D-alanine-D-alanyl carrier protein ligase
lmo0991	*0*	**1.58**	2.4E-04	similar to membrane protein TerC
lmo1002	*ptsH*	**2.08**	4.0E-06	phosphocarrier protein HPr
lmo1016	*gbuC*	**1.47**	4.0E-05	similar to glycine betaine/proline ABC transporter, substrate binding protein
lmo1028	*0*	**1.28**	2.0E-04	similar to predicted hydrolases of the HAD superfamily
lmo1299	*glnA*	**1.45**	2.5E-04	glutamine synthetase
lmo1390	*0*	**1.27**	1.5E-04	similar to sugar ABC transporter, permease protein
lmo1452	*0*	**1.35**	5.1E-05	similar to uncharacterized conserved proteins
lmo1497	*udk*	**1.25**	0.001	uridine kinase
lmo1515	*0*	**1.42**	0.012	similar to predicted transcriptional regulators
lmo1570	*pykA*	**1.31**	1.5E-04	pyruvate kinase
lmo1735	*gltC*	**1.23**	0.013	transcription activator of glutamate synthase operon
lmo1815	*0*	**1.77**	0.001	similar to uncharacterized conserved proteins
lmo1909	*0*	**1.25**	4.9E-04	similar to uncharacterized conserved proteins
lmo2011	*yesM*	**1.39**	3.8E-04	two-component sensor histidine kinase
lmo2045	*0*	**1.58**	1.5E-05	unknown protein
lmo2091	*argH*	**1.48**	3.0E-05	argininosuccinate lyase
lmo2093	*0*	**1.21**	3.3E-04	unknown protein
lmo2095	*0*	**1.93**	8.4E-06	similar to 1-phosphofructokinase
lmo2103	*pta*	**1.66**	6.2E-06	phosphate acetyltransferase
lmo2111	*0*	**1.52**	2.9E-04	similar to NADPH-dependent flavin reductases and oxygen-insensitive nitroreductases
lmo2114	*0*	**1.32**	3.1E-05	similar to ABC transporter, ATP-binding protein
lmo2193	*oppD*	**1.19**	8.5E-05	similar to oligopeptide ABC transporter, ATP binding protein
lmo2207	*0*	**1.56**	9.9E-05	similar to putative integral membrane protein that interacts with FtsH
lmo2231	*0*	**1.34**	1.9E-04	similar to predicted Co/Zn/Cd cation transporters
lmo2254	*0*	**1.26**	2.9E-05	similar to permeases
lmo2342	*0*	**1.38**	0.019	similar to 16S RNA-specific pseudouridine synthase A
lmo2371	*0*	**1.25**	0.001	similar to putative ABC transporter, permease protein
lmo2540	*0*	**1.83**	0.003	putative protein-tyrosine-phosphatase
lmo2554	*0*	**1.62**	6.2E-05	similar to glycosyltransferases

Gene*	Gene homolog€	70-1700 4°C		Predicted or known function€
		LR#	P¥	
lmo2561	*argS*	**1.34**	1.5E-04	arginyl-tRNA synthetase
lmo2609	*rpmJ*	**1.27**	6.7E-05	ribosomal protein L36
lmo2615	*rpsE*	**1.72**	3.4E-04	ribosomal protein S5
lmo2616	*rplR*	**2.21**	3.7E-06	ribosomal protein L18
lmo2617	*rplF*	**1.76**	1.8E-05	ribosomal protein L6
lmo2618	*rpsH*	**2.38**	3.6E-06	ribosomal protein S8
lmo2619	*rpsN*	**2.10**	7.3E-05	ribosomal protein S14
lmo2620	*rplE*	**2.29**	2.6E-06	ribosomal protein L5
lmo2621	*rplX*	**2.18**	6.5E-06	ribosomal protein L24
lmo2622	*rplN*	**2.23**	4.7E-06	ribosomal protein L14
lmo2623	*rpsQ*	**2.07**	8.0E-05	ribosomal protein S17
lmo2624	*rpmC*	**1.88**	5.3E-05	ribosomal protein L29
lmo2625	*rplP*	**1.60**	1.2E-04	ribosomal protein L16
lmo2627	*rplV*	**2.01**	1.3E-04	ribosomal protein L22
lmo2628	*rpsS*	**2.40**	1.5E-05	ribosomal protein S19
lmo2629	*rplB*	**1.67**	3.0E-05	ribosomal protein L2
lmo2631	*rplD*	**1.81**	1.5E-04	ribosomal protein L4
lmo2632	*rplC*	**1.55**	1.3E-04	ribosomal protein L3
lmo2657	*0*	**1.31**	6.1E-05	similar to dGTP triphosphohydrolase
lmo2683	*0*	**1.57**	3.7E-04	similar to PTS system, cellobiose-specific IIB component
lmo2707	*0*	**1.66**	2.3E-04	unknown protein
lmo2717	*cydB*	**1.21**	2.0E-04	cytochrome D ubiquinol oxidase subunit II
lmo2773	*0*	**1.68**	0.018	beta-glucoside-specific PTS system operon regulator
lmo2810	*gidA*	**1.61**	9.2E-06	similar to glucose inhibited division protein A
lmo2854	*yidC*	**1.67**	2.0E-05	preprotein translocase YidC subunit

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