Listeria monocytogenes - Understanding the interaction of pathogen and host physiology during intracellular growth

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the School of Chemical Engineering and Analytical Science

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Abbreviations

AIDS  acquired immune deficiency syndrome
Amp  ampicillin
AKG  α ketoglutarate
APC  antigen presenting cell
Arp 2/3  actin related proteins 2/3
ATP  adenosine triphosphate
BHI  brain heart infusion
bp  base pair
CCR  carbon catabolite repression
CFU  colony forming unit
CNS  central nervous system
CRE  carbon repression element
DPBS  Dulbecco’s phosphate buffered saline
DC’s  dendritic cells
DMEM  Dulbecco’s modified Eagles medium
EI  electrospray ionisation
ER  endoplasmic reticulum
Erm  erythromycin
EVH1/2  Ena/Vasp homology domain ½
ESI  electrospray ionisation
FBS  foetal bovine serum
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>GC-MS</td>
<td>gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GGC</td>
<td>glutamine glutamate cycle</td>
</tr>
<tr>
<td>Gln</td>
<td>glutamine</td>
</tr>
<tr>
<td>Glu</td>
<td>glutamate</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>hpt</td>
<td>hexose phosphate transporter</td>
</tr>
<tr>
<td>INF</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>InlA/B/C</td>
<td>internalin A/B/C</td>
</tr>
<tr>
<td>IT</td>
<td>ion trap</td>
</tr>
<tr>
<td>Kan</td>
<td>kanamycin</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LB medium</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>LLO</td>
<td>listeriolysin O</td>
</tr>
<tr>
<td>LPXTG</td>
<td>leucine proline X threonine guanine</td>
</tr>
<tr>
<td>LLR</td>
<td>leucine rich repeat</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix assisted laser desorption ionisation</td>
</tr>
<tr>
<td>MCS</td>
<td>multiple cloning site</td>
</tr>
<tr>
<td>MHS</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NKC</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC-PLC</td>
<td>broad range phospholipases C</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>phosphoinositide</td>
</tr>
<tr>
<td>PEA</td>
<td>phosphoinositide ethanolamine</td>
</tr>
<tr>
<td>PEST</td>
<td>proline, glutamine, serine and threonine rich motif</td>
</tr>
<tr>
<td>PHS</td>
<td>phosphorylated hexose sugars</td>
</tr>
<tr>
<td>PIP</td>
<td>phosphoinositide / phosphoinositol phosphate</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>plcA/plcB</td>
<td>phospholipase A/B</td>
</tr>
<tr>
<td>PrfA</td>
<td>positive regulatory factor A</td>
</tr>
<tr>
<td>PTS</td>
<td>phosphoenolpyruvate sugar phosphotransferase system</td>
</tr>
<tr>
<td>QMA</td>
<td>quadrupole mass spectrometry</td>
</tr>
<tr>
<td>QQQ</td>
<td>triple quadrupole tandem mass spec</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDM</td>
<td>site directed mutagenesis</td>
</tr>
<tr>
<td>T3SS</td>
<td>type 3 secretion system</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TLR</td>
<td>toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TOF</td>
<td>time of flight</td>
</tr>
<tr>
<td>TRIS</td>
<td>trisma base/ tromethamine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>TSB</td>
<td>tryptone soya broth</td>
</tr>
<tr>
<td>VASP</td>
<td>vasodilator stimulated phosphoprotein</td>
</tr>
<tr>
<td>VCA</td>
<td>verprolin homology, cofilin homology and acidic</td>
</tr>
<tr>
<td>WAVE</td>
<td>WASP family verprolin homologous protein</td>
</tr>
</tbody>
</table>
Preface

I graduated from the University of Leeds, with a 2:1 in BSc Biology, where I developed a good understanding of a broad range of biological principles. I then went on to complete a MRes in Bioinformatics at the University of York, where I developed an interest in intracellular parasitism, with a final project on lateral gene transfer in *Leishmania*. Finally, I enrolled onto the Doctoral Training Centre at the University of Manchester to study Systems Biology and chose to study the intracellular parasite *Listeria monocytogenes* as a PhD subject.
Acknowledgements

Bismillah ar-rahman ar-rahim. Firstly, I would like to sincerely thank my supervisor Professor Ian Roberts for all the help during the project. It has been an extremely enjoyable and thoroughly rewarding experience, working under the guidance of Professor Roberts.

A big thank you to Professor Roy Goodacre and Rick Dunn for their help with the metabolomics based work of the project.

I would like to thank Dr David Corbett and Stephanie Schuler for help with all laboratory techniques, especially cloning and molecular biology. I would also like to thank Julie Wang for training in cell culture and infection methodology and constant help throughout the project.

A big thank you to all members of the C1202 laboratory: Marie Goldrick, Thomas Pointon, Warren Flood, Ashley Houlden, Adrian Jervis, Jane Jia, Jonathan Butler, Eva Haas, Deenah Osman and Alison Wood.

I would like to give my sincerest thanks to my parents, Mohammed Shafa Hussain and Shahnaz Akhtar, to whom I am entirely grateful and without their support and guidance this would not have been possible.

Finally, I would like to thank God for everything that I have been fortunate enough to receive.
Abstract

Listeria monocytogenes (*L. monocytogenes*) are Gram-positive, facultatively anaerobic and intracellular bacilli, occupying a wide range of ecological niches and are responsible for a number of serious infections in man. Primarily transmitted to humans through contaminated food stocks, *L. monocytogenes* invade mammalian cells in a phagosome, escaping and growing in the cell cytoplasm. Currently, there is a great deal of information about pathogenesis of *L. monocytogenes*, however, much less is known about the physiology of the bacteria. In particular, very little is known about the physiology during intracellular growth and even less about host cell physiology and changes in response to infection. The focus of this research was to address these issues using a multidisciplinary approach, utilising multiple biological techniques.

The catabolic metabolism of *L. monocytogenes* was elucidated using mutagenesis and protein purification studies. The results are not completely conclusive; however, it was shown that unlike in *Escherichia coli*, *L. monocytogenes* may not be dependent on fermentation enzymes Ldh and Pflb during anaerobic growth. Instead anaerobic respiration is hypothesised, utilising a putative fumarate reductase with fumarate as a terminal electron acceptor. The putative fumarate reductase gene was purified and confirmed to have enzymatic activity.

External and internal metabolism of HeLa cells, and the effect of *L. monocytogenes* infection was elucidated by mass spectrometry. The external metabolomic studies proved inconclusive. The internal metabolomic studies show that a number of key amino acids are being sequestered by *L. monocytogenes* during the course of an infection. Also, the studies show that a large number of carbon compounds are being sequestered by *L. monocytogenes*, pointing to a complex carbon metabolism for *L. monocytogenes* during intracellular growth.

A targeted analysis of the nitrogen metabolism of *L. monocytogenes* has shown that *L. monocytogenes* may utilise a number of nitrogen compounds with glutamine and glutamate being particularly important. The ability to synthesise glutamine *de novo* is shown to be essential for normal intracellular growth.
Chapter 1
Introduction
1.1) *Listeria monocytogenes* biology and background

*Listeria monocytogenes* (*L.monocytogenes*) are Gram-positive, facultatively anaerobic and intracellular bacilli. They belong to the genus *Listeria* along with another five species: *L.welshimeri, L.ivanovii, L.innocua, L.seeligeri,* and *L.grayi*. They are characterised by low percentage of guanine/cytosine bases in their genome and are also closely related to *Bacillus, Staphylococcus, Streptococcus* and *Clostridium* species.

*L.monocytogenes* is ubiquitous in the environment and has been isolated from varied sources such as soil, silage, sewage, water and faeces of healthy animals (Lyautey *et al.*, 2007). However, its primary environment is considered to be soil, where it lives as a saprophyte feeding off dead and decaying plant matter (Freitag *et al.*, 2009). Although *L.monocytogenes* is non-spore forming, it is extremely hardy, surviving adverse conditions and even growing in environments specifically designed to inhibit bacterial growth, for example, in the presence of heavy metal ions, high salt concentrations, and in extremes of pH (Freitag *et al.*, 2009).

1.2) *Listeria monocytogenes* disease

Of the six species in the *Listeria* genus only *L. monocytogenes* and *L.ivanovii* are considered pathogenic. *L.monocytogenes* is a potentially fatal pathogen to humans, whereas human *L.ivanovii* infections are highly unlikely with most infections restricted to ungulates such as sheep and cattle (Santagada *et al.*, 2004). Although only an opportunistic pathogen, *L.monocytogenes* is now a leading source of concern for the food industry, largely due to its extraordinary growth behaviour. Where most bacteria have their growth significantly limited by dropping temperatures below 4°C, *L.monocytogenes* can survive temperatures well below freezing and can proliferate at temperatures from 0.4°C to 45°C (Chan and Wiedmann, 2009). This range includes the temperature food is refrigerated at, thus exacerbating the threat of storing and eventual consumption of *L.monocytogenes* contaminated food (Ramaswamy *et al.*, 2007). As *L.monocytogenes* is readily killed by heat treating the main sources of
L. monocytogenes transmission include unpasteurised dairy products, smoked fish and post processed food stocks (Dalton et al., 1997).

L. monocytogenes is the causative agent of a potentially severe infection known as listeriosis. Although listeriosis is relatively rare and annual incidences are decreasing, it is still one of the most deadly food borne pathogens, with around one third of all clinical manifestations resulting in morbidity (Schuppler and Loessner, 2010). These rates exceed those from other bacteria such as Salmonella and Clostridium, making listeriosis the leading cause of morbidity due to food related infection (Mead et al., 2000).

1.2.1) Adult listeriosis

L. monocytogenes pathophysiology in humans is intrinsically linked to their ability to cross three important barriers in their host. These are the intestinal barrier, blood brain barrier and foetal-placental barrier (Lecuit et al., 2004). The passing of the intestinal barrier is perhaps of most significance as food initiated transmission account for 99% of listeriosis infections (Mead et al., 2000).

Adult infection is characterised by septicaemia and meningitis. Listerosis induced meningitis is often exacerbated by encephalitis which is very unusual for a bacterial infection (Ramaswamy et al., 2007). Listerosis affecting the central nervous system accounts for around 55-75% of cases whereas septicaemia accounts for 15-20% with non-typical infections making up the remainder (Vazquez-Boland et al., 2001). The infection is most prevalent in immune-compromised individuals, for example, transplant patients, elderly, individuals with impaired cell mediated immunity and those suffering from AIDS. The severity of the infection is highly correlated with mode of infection. Intravenous inoculations of L. monocytogenes in mice have a direct dose dependent lethality. This is contrasted by oral doses which are much less efficient due to difficulties associated with traversing the intestinal barrier (Lecuit, 2005). In contrast to the severe acute infection of individuals with impaired cell mediated immunity, infection of healthy adults can lead to self-limiting febrile gastroenteritis with at
least seven different outbreaks linked to *L. monocytogenes* (Ooi and Lorber, 2005).

If ingested, the incubation period for *L. monocytogenes* can vary from hours, in case of *L. monocytogenes* gastroenteritis, to over 80 days in the case of listerial bacteraemia (Ooi and Lorber, 2005).

Initial infection through the oral route leads to the subsequent invasion of intestinal epithelial cells. From here *L. monocytogenes* will spread to neighbouring enterocytes and leukocytes and are transferred to the liver by the portal vein (Melton-Witt *et al.*, 2012). Alternatively, *L. monocytogenes* will infect the Preyers patch, a set of lymphoid tissues found at the lowest portion of the small intestine and disseminate into the mesenteric lymph node (Pentecost *et al.*, 2006). The mesenteric lymph node represents a considerable barrier which *L. monocytogenes* have to breach as only around 1 in $10^3$ to 1 in $10^4$ bacterial cells manage to spread further (Melton-Witt *et al.*, 2012). The bacteria then reach the brain through haematogenous dissemination and cause the meningo-encephalopathic pathology associated with acute listeriosis (Toledo-Arana *et al.*, 2009).

### 1.2.2) Neonatal listeriosis

Neonatal listeriosis poses a significant danger for the unborn foetus. If *L. monocytogenes* is ingested it can reach the foetus through foetal-placental infection. Such an infection can be difficult to detect as the mother may be asymptomatic, with only common flu like symptoms. During this the neonate may suffer from septicaemia followed by abortion/still birth. In some cases direct vaginal transmission can cause late onset neonatal listeriosis. Infections have also been recorded after caesarean deliveries (Kessler and Dajani, 1990).
1.3) Host immune response against *Listeria monocytogenes* infection

Immunity can be either innate or adaptive. Innate responses mediate the first line of defence of an organism in a non-specific manner. Adaptive immunity is a specialised response, where the organism recognises the pathogen encountered allowing for a progressively robust response in postliminary infections. *L. monocytogenes* infections lead to the activation of both immune responses which must be overcome by the pathogen for a successful infection.

1.3.1) Innate response to *Listeria monocytogenes* infection

On the entry of *L. monocytogenes*, cascades of innate responses are activated to control the advancement of infection, which is a precursor to the specialised adaptive response.

Neutrophils are an essential response in the control of an infection due to their antibacterial properties. Neutrophils have been shown to aggregate in the liver forming micro abscesses within 16 hours of *L. monocytogenes* infection (Rogers et al., 1996). The infection also results in a program of inflammation with apoptosis, the release of neutrophil chemo-attractants, and increased adhesion for neutrophils (Rogers et al., 1996). Mice deficient in neutrophil production, exhibit highly severe infections, leading to early morbidity with large bacteria burdens in liver parenchyma cells (Rogers et al., 1996).

Macrophages, especially Kupfer cells, lead to the uptake and removal of *L. monocytogenes* at the infection site. In response to infection macrophages increase production of two cytokines - tumour necrosis factor alpha (TNF-α), and interleukin 12 (IL12). These two cytokines cooperatively induce production of interferon gamma (INF-γ) in natural killer cells (NKC), which leads to subsequent full activation of macrophages (Zenewicz and Shen, 2007b). As is common in neutrophils generation of reactive oxygen and nitrogen intermediates is important for macrophage-dependent termination of *L. monocytogenes* (Zenewicz and Shen, 2007b).
Unfortunately, the innate immunity mechanisms may not be sufficient for complete bacterial dismissal. This means that adaptive immunity is essential for long term protection from *L. monocytogenes* infection (see Fig. 1.1).

### 1.3.2) Adaptive immune response to *Listeria monocytogenes*

The *L. monocytogenes*-specific adaptive immune response succeeds the innate response. Dendritic cells, which are activated by toll like receptors (TLRs), initiate a signalling cascade, stimulating a T-cell response. Mice which are incapable of producing dendritic cells are incapable of generating a T-cell response when challenged with infection (Jung *et al.*, 2002). CD4 and CD8 T-cells are essential for a full clearance of bacterial infection, and rendering sterilising immunity. T-cells recognise and respond to antigens from *L. monocytogenes* which have been degraded by host proteasome and loaded onto major histocompatibility complex (MHC) proteins and presented by antigen presenting cells (APC) (Zenewicz and Shen, 2007b). APCs such as macrophages gain listerial antigens by either direct phagocytosis of *L. monocytogenes* or phagocytosis of other cells (alive or dead) containing *L. monocytogenes*, a process known as ‘cross presentation’ (Heath and Carbone, 2001). Both cross presentation and direct presentation of bacterial antigens are thought to be important for T-cell priming during a primary response to *L. monocytogenes* infection (Goldfine and Shen, 2007). MHC class 1 proteins present foreign antigens from within infected cells to CD8 T-cells and are associated with the endogenous presentation pathway. MHC class 2 proteins present external antigens to CD4 T-cells and are associated with the exogenous presentation pathway (Szalay *et al.*, 1994).

T-cells confer immunity by essentially removing the protective intracellular niche of *L. monocytogenes*. T-cells can directly lyse infected cells or they can secrete IFN-γ to activate macrophages, which then deal with the liberated *L. monocytogenes*.

Regulatory T-cells are a rank of T-cells which suppress the activity of other T-cells by secreting suppressive cytokines (Kursar *et al.*, 2002).
Neutrophils can phagocytise *L. monocytogenes* and generate reactive nitrogen and oxygen species. They also secrete IL-12 to amplify the anti-bacterial response. Macrophages also engulf *L. monocytogenes* and secrete cytokines IL-1, TNF-α, and IL-12, which stimulate natural killer cells to produce IFN-γ. This causes the full activation of macrophages. Infected cells release cytokines, which induce dendritic cells to also produce IFN-γ, which again increases the bactericidal properties of macrophages and neutrophils. Dendritic cells can also prime CD8⁺ T cells to proliferate and to differentiate into cytotoxic CD8⁺ T cells. Regulatory T-cells play a role in controlling CD8⁺ T-cell proliferation in a second challenge with *L. monocytogenes*.

(Adapted from Stavru et al., 2011)
important in down regulating the role of T-cells and although not necessarily important during primary *L. monocytogenes* infection, they have been shown to play a role limiting the expansion of memory CD8 T-cells during secondary infection with *L. monocytogenes* (Zenewicz and Shen, 2007b).

B-cell mediated immunity is considered to play a minor role against *L. monocytogenes*. This is largely due to the fact that *L. monocytogenes* is an intracellular parasite and the majority of the bacteria live and spread with limited contact with the extracellular environment. Thus, *L. monocytogenes* specific antibodies would be of limited use in halting *L. monocytogenes* cell to cell spread. However, some studies have shown that B-cells can reinforce a T-cell dependent response against *L. monocytogenes* through mechanisms other than antibody formation (Stavru *et al.*, 2011) Also, antibodies against *L. monocytogenes* virulence factors like listeriolysin O (LLO) can be used to target and stop their function, subsequently trapping *L. monocytogenes* in the primary phagosome (Edelson and Unanue, 2001).

1.3.3) *Listeria monocytogenes* invasion and evasion of immune system

Most bacteria are killed soon after being engulfed by macrophages, by maturation of phagosome through lysosomal interaction and production of key oxygen and nitrogen reactive species. However, *L. monocytogenes* has evolved to take advantage of this intracellular niche and actively proliferate within them. By taking advantage of the bactericidal mechanisms of macrophages and escaping the phagosome before maturation, around 10% of phagocytised *L. monocytogenes* are able to escape destruction (de Chastellier and Berche, 1994). This is achieved by utilising *L. monocytogenes* virulence factors: LLO and two phospholipases (see section 1.6), which are specifically involved in phagosomal escape. *L. monocytogenes* also actively invades a number of non-professional cells such as hepatocytes. These cells have a decreased capacity for antigen presentation compared to macrophages and also have attenuated antimicrobial activities, allowing *L. monocytogenes* to both evade T-cell mediated immunity and also the harsh environmental conditions associated with macrophage cells (Zenewicz and Shen, 2007a).
1.4) *Listeria monocytogenes* pathogenesis

*L. monocytogenes* pathogenesis is intrinsically linked to its extraordinary life cycle and its ability to survive intracellularly. The life cycle of *L. monocytogenes* can be split into the following steps:

1) Internalisation: Primary contact of bacteria and host leading to invasion.

2) Escape from primary phagosome: *L. monocytogenes* are internalised in a primary phagosome, in order to replicate they need to escape this confinement.

3) Intracellular growth: After escaping the primary phagosome, *L. monocytogenes* actively multiply in host cytoplasm.

4) Movement and spreading to adjacent cells: *L. monocytogenes* polymerise host cell actin to spread to neighbouring cells.

5) Escape from secondary phagosome: Lateral transmission of *L. monocytogenes* requires escape from a double membrane secondary phagosome.

Correct operation of these steps is vital for full *L. monocytogenes* virulence and defects at any point can lead to high attenuation. Each of these steps consists of complex interactions of multiple molecules (Fig. 1.2). These processes represent essential topics of discussion, especially for the discovery and targeting of anti-listerial strategies. The following sections will focus on these events and the molecular determinants that are involved.
Fig. 1.2 Diagram of *L. monocytogenes* invasion and life cycle in non-phagocytic cells

a) *L. monocytogenes* induces its uptake by binding of receptors of host cell to bacterial surface proteins
b) *L. monocytogenes* is internalised by membrane phagosome
c) Membrane disrupted by phospholipases and listeriolysin O
d) *L. monocytogenes* now divide in cytoplasm utilising host resources and polymerise host cell actin for motility
e) *L. monocytogenes* pass into neighbouring cell
f) Entry into the next cell is associated with a double membrane, which is lysed and the cycle can be repeated

(Adapted from Hamon *et al.*, 2006)
1.5) *Listeria monocytogenes* invasion

*L. monocytogenes* is capable of promoting its own uptake into non-phagocytic cells such as epithelial cells, endothelial cells and fibroblasts. Alternatively, *L. monocytogenes* can internalise passively by being engulfed by professional phagocytes (Hamon *et al.*, 2006). The ability of *L. monocytogenes* to infect and spread from cell to cell has been the focus of much attention as it is the primary mechanism allowing *L. monocytogenes* to avoid host immune responses.

Invading bacteria usually belong to one of two major paradigms in invasion, known as ‘zipper’ and ‘trigger’, which are well reviewed (Cossart and Sansonetti, 2004). The ‘trigger’ mechanism utilised by a number of bacteria including *Salmonella*, *Helicobacter* and *Shigella*, uses a type III or type IV secretion system (T3SS/T4SS) in order to inject specific bacterial proteins into the host cytosol, which subsequently leads to actin cytoskeletal rearrangements and bacterial internalisation (da Silva *et al.*, 2012). The ‘zipper’ mechanism is utilised by *L. monocytogenes* and is so called because of the close association with bacterial surface proteins and cell surface molecules (see Fig. 1.3) (Cossart *et al.*, 2003).

The entry procedure can be separated into three steps. Contact and adherence, phagocytic cup formation and finally, cup closure and retraction (Hamon *et al.*, 2006). The mechanism for *L. monocytogenes* entry involves proteins implicated in cell adhesion and activation of the cytoskeletal machinery, and is an excellent example of how an invasive bacterial species can manipulate host cell machinery to its own advantage.

There are a number of *L. monocytogenes* surface proteins that are implicitly involved in the entry into cells. Collectively, these proteins are known as internalins and they are of primary importance to the entry procedure.
Fig. 1.3 Schematic highlighting the machinery involved in the zipper mechanism induced by *L. monocytogenes* for entry into non phagocytic cells

Internalin A (InlA) and/or Internalin B (InlB) form direct interaction with the host cell E-cadherin and the Met receptor on the cell surface. Interaction of the Internalin proteins with their specific binding receptors results in activation of downstream signalling pathways, which in turn cause a remodelling of host cell cytoskeleton. This leads to the depolymerisation of the actin and subsequent tight envelopment of the bacteria by the host cells plasma membrane. InlB may also utilise β1 and β3 integrin receptors, which can induce clathrin-dependent endocytosis, this machinery can be used to invade non-phagocytic mammalian cells. (Adapted from da Silva *et al.*, 2012)
1.5.1) Internalin family

Internals are members of a multigene family which are distinguished by the presence of leucine rich repeats in their N-terminal domains, which interact with their respective host surface receptors (Hamon et al., 2006). There are 25 internalin-like proteins which have been identified in \textit{L. monocytogenes}, a number higher than any other known genome from Gram-positive bacteria (Bierne et al., 2007). These can be broadly separated into three families based on structural differences of specific bacterial surface binding domains: LPXTG, GW/WXL and secreted internals (Bierne et al., 2007).

The ‘LPXTG’ internals make up 19 of the 25 known \textit{L. monocytogenes} internals which can be seen in Fig. 1.4. They possess a region which covalently anchors the protein to the peptidoglycan of the bacterial cell wall. The region consists of a Leucine-Proline-X-Threonine-Glycine motif, a hydrophobic domain of around 20 amino acids and a positive residue tail (Bierne et al., 2007). The LPXTG region is the substrate of sortaseA (SrtA), a membrane anchored transpeptidase, mediating LPXTG, peptidoglycan anchoring. InlA is processed by the SrtA protein, and four other internals (InlG, InlH, Lmo0610 and Lmo0327) are thought to be substrates of SrtA. The remaining LPTXG internals are yet to be fully characterised (Bierne et al., 2007).

InlB and a functionally anonymous protein Lmo0549 belong to the second group of GW/WxL internals. These are characterised by a GW (InlB) and WxL (Lmo0549) domain in their C-terminus, which mediates cell surface association (Bierne et al., 2007).

Finally, the secretory internals are smallest of the internals and are non-anchoring proteins which are secreted extracellularly. Of this group Internalin C (InlC) is strongly transcribed in the cytoplasm of invaded phagocytic cells, suggesting that it may play a role in \textit{L. monocytogenes} infection post internalisation (Engelbrecht et al., 1996) (see Fig. 1.4).

Of all the internals, Internalin A (InlA) and Internalin B (InlB) are the best characterised.
Fig. 1.4 Schematic for the organisation of the internalin family of *L. monocytogenes*

The schematic highlights the domain organisation of *L. monocytogenes* internalins. Homologous regions are colour coded as indicated in the legend. Numbers within each individual domain indicate the number of repeats. The three main families of internalins are shown- LPXTG internalins (I), GW or WxL internalins (II) and Secreted internalins (III). (Adapted from Bierne et al., 2007)
1.5.2) Internalin A (InlA)

InlA, also known as Internalin is essential for *L. monocytogenes* entry into the human intestinal epithelial cell line Caco-2 (Gaillard *et al.*, 1991). In contrast to this, it is non-essential for invasion of the HeLa cancer cell line (Auriemma *et al.*, 2010). InlA plays a fundamental role in the invasion of *L. monocytogenes* into many cellular species. It induces entry by binding host cell adhesion molecule E-cadherin, which is predominantly localised at the basolateral surface of enterocytes and is briefly exposed at the surface of extrusion of apoptic cells (Ireton, 2007).

E-cadherin, belonging to the cadherin superfamily, is a transmembrane glycoprotein found on the host cell surface allowing calcium dependent cell to cell adhesion of epithelial cells (Mengaud *et al.*, 1996). Being a transmembrane protein, the ectodomain mediates homophillic ligations and cell to cell adhesion, whereas the highly conserved intracellular domain is associated with the actin cytoskeleton of the cell and its signalling pathways (Yap and Kovacs, 2003).

Binding of InlA to host cell E-cadherin leads to subsequent binding of β-catenins to the cytosolic domain of E-cadherin forming a complex. α catenin forms a low affinity interaction between β catenin in the complex and also F-actin, which functions as a molecular ‘switch’ controlling cytoskeletal organisation (Drees *et al.*, 2005).

There is a further protein complex involved in the coordination of actin remodelling and *L. monocytogenes* invasion, known as actin related proteins 2 and 3 (Arp2/3). Arp2/3 are highly conserved actin nucleators and are recruited at the bacterial entry site (Kovacs *et al.*, 2002). With the formation of the InlA/E-cadherin complex, Rac1 belonging to the Rho family of small GTPases, is activated at the bacterial/host contact juncture. Rac1 stimulates Arp2/3 to actuate actin agglomeration required for internalising *L. monocytogenes* (da Silva *et al.*, 2012).

These complex sequence of events lead to the depolymerisation of the actin and subsequent tight envelopment of the bacteria by the host cells plasma membrane.
(Cossart et al., 2003). The invading cell enters the host cell within this phagosomal compartment. It has been shown that \textit{L. monocytogenes} may only require InIA for entry to a number of cell species, as the expression of this protein in \textit{L. innocua}, allows this otherwise non-invasive bacteria, the ability to invade Caco-2 cells (Gaillard et al., 1991).

It has been well documented that wild type \textit{L. monocytogenes} is unable to infect murine cells via the oral tract (Sasakawa, 2009, Lecuit, 2005). Although murine E-cadherin is very similar to the human protein it is not a receptor for InIA. This has been attributed to a single residue substitution at position 16 located in an exposed loop in murine E-cadherin (Lecuit et al., 1999). This single residue has proven critical in the interaction of InIA and the E-cadherin protein. Replacing the proline residue with glutamine in the human E-cadherin, results in abrogation of interaction with InIA. Substituting glutamine for proline in murine E-cadherin causes complete gain in function (Lecuit et al., 1999).

As murine infections represent a versatile and biologically relevant model of human invasion, having \textit{L. monocytogenes} capable of infecting murine cells is highly significant and very desirable. This issue was resolved by modifying the genetic information for \textit{L. monocytogenes} InIA protein. Two single substitutions in InIA increase binding affinity by four orders of magnitude and extend binding specificity to include formerly incompatible murine E-cadherin, thus allowing infection of mice through oral route (Wollert et al., 2007).
1.5.3) Internalin B (InlB)

InlB is the other primary invasion protein, which interacts with the outer surface of the bacteria membrane facilitated by an interaction with lipotechoic acid (Jonquieres et al., 1999). InlB binds to hepatocyte growth factor (HGF) receptor also known as Receptor Tyrosine Kinase (RTK) Met (Shen et al., 2000). This allows *L. monocytogenes* to actively invade hepatocytes in the liver. Binding of InlB to Met, triggers downstream signalling pathways leading to the subsequent actin cytoskeletal integration required of internalising of *L. monocytogenes*.

Met receptor consists of an extracellular α chain and also a β chain which spans the membrane into the cytoplasm. Binding of InlB to the extracellular domain of Met causes the rapid tyrosine phosphorylation via the classical phosphatidylinositol 3 kinase pathway (PI3K) (Shen et al., 2000). Other proteins implicitly involved include Gab1 and CrkII. Gab1 couples Met to CrkII, which in turn promote recruitment of PI3K, promoting actin polymerisation (Ireton, 2007). Experiments with cells deficient in Met highlight that it is essential for InlB mediated entry of *L. monocytogenes* into a range of mammalian cells including fibroblasts and epithelial cells (Shen et al., 2000).

Whilst InlA is incapable of interacting with murine cell E-cadherin, InlB does not respond to guinea pig and rabbit cell surface proteins (Khelef et al., 2006). Human Met gene transfection results in InlB mediated response, which suggest that the animal Met receptor is unrecognisable to InlB (Khelef et al., 2006).
1.6) *Listeria monocytogenes* escape from phagosome

By observing avirulent mutants of *L. monocytogenes* it has been established that the lysis of the phagosome is essential to the virulence of *L. monocytogenes*. When internalised *L. monocytogenes* are in a membrane bound vacuole. In order to continue its lifecycle *L. monocytogenes* must escape this and gain free access to cytoplasmic nutritional sources and replicate. This is mediated by a number of essential virulence factors including listeriolysin O, and two phospholipases.

1.6.1) Listeriolysin O (LLO)

There is a large body of evidence which confirms that LLO is the primary virulence factor associated with *L. monocytogenes* infection, in particular escape from the primary phagosome (Kayal and Charbit, 2006).

LLO is a 58 kDa protein belonging to a family of cholesterol dependent cytolysins which are typified by streptolysin (Kayal and Charbit, 2006). It is encoded by the *hly* gene and is regulated by PrfA, a central temperature sensitive regulator of virulence genes (see section 1.8) (Scortti et al., 2007). LLO is responsible for escape from the primary phagosome formed by initial internalisation event, also the double membrane secondary phagosome associated with cell to cell infection (see Fig. 1.2) (Schnupf and Portnoy, 2007). LLO is activated by thiol reducing agents and is inactivated by the binding of cholesterol (Cossart et al., 1989).

LLO knock out mutants are avirulent in mice and lack the ability to escape the primary phagosome (Cossart et al., 1989). When the *hly* gene is re-complemented into the genome the virulence of the bacterium is also restored (Cossart et al., 1989). In mammalian cell lines other virulence factors can mediate phagosomal escape, which will be discussed further. By incorporating the LLO gene into naturally non-pathogenic bacteria, the capability to escape from vacuoles and grow in host cytoplasm is conferred. These studies highlight aspects of both LLO function and its importance to *L. monocytogenes* pathogenicity (Bielecki et al., 1990).
LLO is controlled on multiple levels, from transcription to eventual degradation. Transcriptional control of hly gene encoding LLO is under the control of the PrfA protein, which will be discussed in greater detail in section 1.8. Production of the hly gene is up-regulated under conditions that increase the activity of PrfA, including temperatures exceeding 37 °C and nutrient limitation (de las Heras et al., 2011).

The selectivity of LLO is tied with the acidic pH activation mentioned earlier. LLO is around 10-fold attenuated in the slightly basic cytoplasm as opposed to its optimal acidic pH of 5.5 found in phagosomes (Glomski et al., 2002). By comparatively examining LLO with a pH insensitive orthologue - perfringolysin O (PFO), a single residue - leucine 461 was found to be responsible for this attenuation at neutral pH. Replacing this residue conferred PFO levels of activity for LLO at neutral pH, confirming the loss pH dependency (Glomski et al., 2002). This is an important regulatory mechanism for LLO ensuring its activity is limited to where it is required.

A N-terminal proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST) like motif has been found in LLO and has been shown to be associated with virulence and responsible for the restriction of the LLO (Decatur and Portnoy, 2000). PEST like sequences are usually used as degradation tags for proteins in eukaryotes. However, it must be stated that in L.monocytogenes the exact mechanism for reducing LLO activity is still under debate (Dramsi and Cossart, 2002). Mutants expressing LLO in which the PEST sequence has been deleted cause the degradation of host cell membrane, possibly from the aggregation of LLO in the host cytosol (Decatur and Portnoy, 2000). Other evidence also suggests that the role of the PEST motif may be cell dependent, with PEST deleted mutants being fully haemolytic with impaired virulence in some cells, whilst being incapable of escaping the primary phagosome in other cells (Schnupf and Portnoy, 2007).

This multi-faceted regulation of LLO allows it to be selectively active within the acidic phagosome, thus upon release from the phagosome the LLO activity is reduced in the slightly more basic cytoplasm stopping any potential damage to the host cell plasma membrane (Schnupf and Portnoy 2007).
There is a large body of evidence implicating LLO with number of cellular functions not involved with its primary function. LLO is able to induce and facilitate signalling reactions (Hamon, Bierne et al. 2006). It is thought that along with the phospholipases, LLO generates calcium dependent lipid mediated signalling, which causes physiological changes in the host cell that favour *L. monocytogenes* (Kayal and Charbit, 2006). LLO has a substantial stimulatory effect on NFkB, phosphatidylinositol, calcium and protein-kinase-C signalling pathways (Hamon *et al.*, 2006). In the host cell, proteins encoded by the NFkB are implicitly involved in intracellular signalling, adhesion and various defence mechanisms (Gilmore, 2006). LLO has been shown to activate the host cells NFkB pathway in a number of cells including epithelial, endothelial and macrophage cells (Gilmore, 2006). As the NFkB pathway is involved in so many defence related mechanisms it is clear that any invading pathogen will have a substantial advantage if it can interfere with this pathway. LLO has been attributed to the activation of endothelial cell adhesion molecules. It has been demonstrated that *L. monocytogenes* which express LLO act as a proinflammatory stimulus inducing NFkB pathway (Kayal *et al.*, 1999). This effect has also been seen with free circulating LLO and purified LLO (Kayal *et al.*, 1999). The changes that occur may help *L. monocytogenes* during the infection process by enhancing the interactions between infected cells, which in turn makes it easier for horizontal transmission (Kayal *et al.*, 1999).

LLO has also been shown to play a role in other biological functions of *L. monocytogenes*, including internalisation and host cell interaction (Vazquez-Boland *et al.*, 2001). Rather interestingly, LLO also mediates interference of cellular physiology at early timepoints by disruption of mitochondrial dynamics and function (Stavru and Cossart, 2011). Interfering with mitochondria can help *L. monocytogenes* to preserve their replication niche by either inhibiting cellular death of host cells or killing agents which are inhibitory to bacterial dissemination. The multiple modes of regulation and activities of LLO in the cytoplasm are summarised in Fig. 1.5.
Fig. 1.5 Regulation and activities of LLO during infection and intracellular growth of *L. monocytogenes*

1) Entry into host cell  
2) Delays phagosome maturation  
3) Disruption of phagosomal membrane  
4) Limited activity in host cell  
5) Ubiquitin mediated degradation by host proteasomes  
6) Using the N-end rule pathway  
7) Presentation of MHC class I molecules on the cell surface  
8) Denaturation and degradation of LLO in the neutral pH of cell cytosol  
9) Inactivation of LLO monomers by putative bacterial factors  
10) Mediates disruption of secondary phagosome after secondary infection  
11) LLO pores lead to stimulation of phosphoinositide metabolism and may translocate PI-PLC across plasma and phagosomal membrane  
12) LLO monomers, oligomers and pores have been shown to cause numerous cellular responses in a variety of cells.  
(Adapted from Schnupf and Portnoy, 2007)
1.6.2) Phospholipases

Along with the LLO there are also two phospholipases C proteins which work together to break down intracellular vacuoles: PI-PLC and PC-PLC (Freitag et al., 2009). PI-PLC is encoded by the plcA gene and the PC-PLC by plcB.

PI-PLC is highly specific for phosphoinositol and glycosyl-PI-anchored proteins. It also has a slightly acidic active pH of around 5.5, which is suitable for the acidic phagosomal compartment where it is utilised.

PC-PLC hydrolyses a wide range of phospholipids, including spingomyelin, with a broader active pH of around 5.5 – 8 (Geoffroy et al., 1991). Its primary function seems to be disruption of the secondary phagosome with plcB deficient mutants incapable of plaque formation (Smith et al., 1995) The enzyme is synthesised inactive and maturation only occurs in the extracellular medium to avoid damage to bacterial membranes and phospholipids.

The two phospholipases are thought to be functionally collusive with LLO. The proposed mechanism for phagosomal escape states that pores formed by LLO in the membrane facilitate the access of phospholipases to their target phospholipids. These phospholipases then cause the dissolution of the plasma membrane (Schnupf and Portnoy, 2007). L.monocytogenes mutants lacking plcA and plcB have elucidated potential functions. Single mutations of these genes show virulence attenuation of 2-fold and 20-fold respectively. Mutants lacking both genes are 500-fold attenuated, with compromised ability to escape phagosome, supporting collaborative functionality of these virulence factors (Smith et al., 1995). Also, in the absence of LLO, PC-PLC is capable of primary phagosome lysis in many mammalian cell lines such as HeLa, dendritic cells and drosophila S2 cells (Mansfield et al., 2003). These findings confirm that these proteins work synergistically with LLO and that PC-PLC plays a larger role, with PI-PLC being less important.
1.7) *Listeria monocytogenes* cell to cell infection

The ability of *L. monocytogenes* to spread from cell to cell plays a critical role in the pathogen’s ability to avoid host immune responses. *L. monocytogenes* is able to infect neighbouring cells without entering intercellular space (Tilney and Portnoy, 1989). Tilney and Portnoy (1989), observing the infection of macrophage-like cell lines using electron microscopy, noticed that the bacteria move through the host cytoplasm into protrusions that extend into neighbouring cells. These protrusions are internalised by the neighbouring cell, along with the bacteria, which are now present in a double membrane. The movement and formation of protrusions has been attributed to the ability of *L. monocytogenes* to interact with the host cell cytoskeletal system.

1.7.1) ActA protein

The ActA protein was first shown to have a central role in *L. monocytogenes* motility when actA mutants could enter the host cytoplasm but were incapable of moving, just accumulating as micro-colonies in the cell, unable to spread to neighbouring cells (Kocks *et al.*, 1992).

ActA is responsible for the catalysis of actin filaments and is the product of a single actA gene (Cossart and Bierne, 2001). It functions by mimicking the activity of the eukaryotic WASP proteins, which facilitate the nucleation of actin, an event essential for the polymerisation of actin (Boujemaa-Paterski *et al.*, 2001).

ActA is a 639 amino acid, dimerised protein which can be divided into three distinct domains (Smith *et al.*, 1996). The N terminus which is associated with F actin assembly and bacterial motility contains a VCA (vrprolin homology, cofilin homology region and acidic) region. This region activates another key player in the polymerisation of actin, the Arp2/3 complex (Welch *et al.*, 1997). The central region of ActA contains proline repeats and the C terminus contains a transmembrane region connecting the protein to the bacterial cell wall (Vazquez-Boland *et al.*, 2001). Deletion analyses have revealed that the N terminus is essential for the function of the protein, whereas the central proline rich repeat
enhances the rate of motility by interacting with the focal adhesion protein vasodilator stimulated phosphoprotein (VASP) and related protein Mena (Frishknecht and Way, 2001).

It has been demonstrated that another protein complex, Arp2/3, is also required to initiate actin polymerisation at the bacterial surface (Fig. 1.6) (Welch et al., 1997). The ActA protein functions in a very similar way to the Wiskott Aldrick Syndrome Protein (WASP) (Boujemaa-Paterski et al., 2001). WASP is a multidomain, actin nucleating factor that associate and activates the Arp2/3 complex, through a VCA region. ActA proteins also contain a VCA region in their N terminus domain allowing them to interact with the Arp2/3 complex in a similar way. Subsequently Arp2/3 facilitates the polymerisation of actin (Boujemaa-Paterski et al., 2001).

The Arp2/3 complex alone is enough to form actin polymerisation at the surface of *L. monocytogenes*, however; it cannot induce actin based motility, suggesting that the involvement of other factors is also needed (Welch et al., 1997). Actin polymerisation in *L. monocytogenes* requires a number of additional proteins, which are highlighted in Fig. 1.6. These include VASP, which works in conjunction with the actin binding protein, profilin, which has a conserved structure similar to ActA (Cossart and Bierne, 2001). Profilin binds to monomeric ADP actin converting it to the readily polymerising ATP actin.

ActA binds the EVH1 (Ena/VASP homology) domain of VASP. This subsequently leads to the binding of profilin and a C terminal EVH2 region VASP binding F actin (Bachmann et al., 1999). The VASP protein is found extensively at sites of active actin polymerisation, for example at the surface of the bacterium (Laurent et al., 1999). Although functionally important, evidence suggests that Ena/VASP is nonessential, as mutations which terminate the association of VASP and ActA leads to a reduction not termination of motility (Laurent et al., 1999).
Fig. 1.6 Schematic diagram of proteins involved in actin based motility of L. monocytogenes

Essential factors for actin based motility. ActA are usually seen as dimers with its distribution at the surface of the bacteria. The motion of the bacteria is indicated by the arrow. Actin is utilized from the host cell, whilst other essential protein interactions are also shown during the assembly of the actin cloud.

(Adapted from da Silva et al., 2012 and Cossart and Bierne, 2001)
The Capping protein CapZ works in conjunction with cofilin, in order to ensure that the initiation of actin polymerisation is localised to the surface of the bacteria. It also ensures that older actin filaments are not polymerised (Cossart and Bierne, 2001). Finally, a number of cross-linking proteins involved in the formation of the comet tail that is found in many electron microscopic images trailing motile \textit{L. monocytogenes}. Of these proteins Actinin has been shown to be essential to comet tail formation (see Fig. 1.6) (Dabiri \textit{et al.}, 1990).

Apart from the essential role ActA plays in motility and virulence, ActA may also play a crucial defensive role for \textit{L. monocytogenes} by helping the bacterium evade autophagy. The recruitment of Actin, Arp2/3 and Ena/VASP may help to camouflage the bacteria thus mediating evasion from autophagy recognition (Yoshikawa \textit{et al.}, 2009).

\textbf{1.8) \textit{Listeria monocytogenes} regulation of virulence genes}

Observing the life cycle of \textit{L. monocytogenes} highlights two distinct conventions it may assume. In the environment it lives as a saprophyte and when the opportunity arises it becomes a pathogen. Almost all of the \textit{L. monocytogenes} genes that are associated with invasion, primary phagosomal escape and direct cell to cell transmission are regulated by the PrfA protein. PrfA is a 233 amino acid long, member of the Carp/Cap-Far family of transcription factors (Sheehan \textit{et al.}, 1995). The main purpose of PrfA regulation is to avoid unnecessary expression of virulence genes when living in the environment. The following section will focus on some of the knowledge currently available on the PrfA regulon.

Six of the main virulence factors playing essential role in \textit{L. monocytogenes} infection, \textit{prfA}, \textit{plcA}, \textit{plcB}, \textit{hly}, \textit{mpl}, \textit{actAB} and \textit{hpt} are under the control of the PrfA protein (Fig. 1.7). The expression of the PrfA protein is controlled on multiple levels, including transcriptional and translational (Scortti \textit{et al.}, 2007).

Comparisons of whole genome arrays of wild type \textit{L. monocytogenes} with \textit{prfA} mutants has shown that there are three groups of genes that are differentially
Fig. 1.7 Schematic of the core PrfA regulon of *L. monocytogenes*

The PrfA regulon consists of the pathogenicity island LIPI-1 encoding LLO (*hly*), ActA, PlcA, PlcB, Mpl and PrfA. Also included are three additional chromosomal loci: *inlAB* operon, *inlC* monocistronic gene and *hpt* gene encoding the hexose phosphate transporter. Promoters (P) are highlighted along with PrfA ‘box’ (squares) and transcripts (broken lines).

(Adapted from de las Heras *et al.*, 2011)

regulated by PrfA. 12 genes are shown to be upregulated in the first group. Eight genes are down regulated in the second group. Finally, a third group which
comprising of 53 genes, which are also up-regulated. Only two of these genes are preceded by a putative PrfA box, which is a 14bp ‘box’ of dyad symmetry used as a binding site for the PrfA protein (Milohanic et al., 2003). The majority of the genes regulated by PrfA are for transport and stress related functions. Other proteins have yet to have their functionality defined.

Transcriptional control of prfA gene is dependent on three promoter elements prfAP1, prfAP2 and plcA. The prfAP1 and prfAP2 promoters are located upstream of the prfA initiation codon. They are required as initiators of PrfA, generating the initial levels of protein, which is then reinforced by activation of the plcA promoter. There is negative feedback of PrfA on the prfAP1 and prfAP2 promoters whereas the plcA promoter is upregulated (de las Heras et al., 2011).

The expression of PrfA protein is temperature dependent. It is silent at 30°C and the virulence genes under its regulation are maximally expressed at 37°C (Sheehan et al., 1995). The mechanism of thermoregulation has been attributed to an 5’- untranslated mRNA of the prfAP1 promoter, which precedes the prfA gene (Freitag and Portnoy, 1994). At 30°C this region forms a secondary structure over the ribosome binding site, stopping translation of all downstream genes. At 37°C the UTR becomes unstable and unable to form the inhibitory secondary structure over the ribosome site (Johansson et al., 2002). This mechanism subsequently controls all PrfA dependent virulence genes at the homeostatic temperature of the host cell.

Finally an important post translational expressional control has also been attributed to the phosphorylation of specific sites on phosphotransferase systems (PTS) known as carbon catabolite repression (CCR).

1.9) Listeria monocytogenes metabolism
1.9.1) Intracellular growth strategies for bacteria

When observing growth of intracellular bacteria there are two predominant behavioural patterns that emerge. First there are those bacteria which simply exist within their newfound protective environment with little replication, for example some streptococci. Secondly, there are those which actively exploit their newly acquired environment, thus using eukaryotic cells as a predominant growth niche (Joseph and Goebel, 2007).

Bacteria that actively replicate intracellularly have adopted two distinct strategies to do so. Some invade and multiply in membrane bound vacuoles, whereas others facilitate their release from the primary phagosome and replicate directly in the intracellular cytoplasm.

Most intracellular bacterial species including, human pathogens *Mycobacterium, Salmonella* and *Legionella*, reside in the phagosome (Ray *et al.*, 2009). Seemingly, this is a strange strategy to adopt when so much of the nutrient required for bacterial growth are located in the cytosol. However, it is a far more complex situation with phagosomal growth conferring other advantages, such as avoiding host inflammasome and killing responses (Beuzón *et al.*, 2002). It also means the phagosome residing bacteria to be more selective with the nutritional uptake. Thus the bacteria can avoid draining the supply of the host too much and thus killing the host prematurely (Joseph and Goebel, 2007).

Unlike these bacteria, *L.monocytogenes* belongs to the smaller group that actively escape the primary phagosome to replicate in the host cytosol. Through the coordinated activity of LLO, PlcA and PlcB, *L.monocytogenes* disrupts and lyses the vacuole membrane, allowing escape into host’s cytoplasm. Once free in the cytosol it is required that *L.monocytogenes* adopt an alternative strategy for sustained growth. *L.monocytogenes* have their metabolism specifically synchronised to that of the host in order to maximise replication efficiency. It is clear that bacteria multiplying in the cytosol undergo extensive metabolic adaptations allowing them to do so. This is highlighted by research using microinjection to forcibly introduce non-cytosolic bacteria to the cytosol, leading to a significant reduction in the bacterial growth rate (Goetz *et al.*, 2001).
Many of the techniques used to study *L. monocytogenes* metabolism are based around knockout mutagenesis and comparative gene expression profiling. By making knockout mutants and observing differential intracellular growth as a result, it can be established which pathways may be of importance to *L. monocytogenes*. Alternatively, by using comparative gene expression profiles of *L. monocytogenes* cells grown in media with intracellular profiles, the genetic changes that take place under these conditions can be evaluated. More recent studies using 13C-isotopologue perturbation complement the other techniques allowing an initiatory, yet rather incomplete understanding of the growth characteristics of *L. monocytogenes*.

The majority of the metabolic pathways in *L. monocytogenes* are very closely related to those found in *Bacillus subtilis* (*B. subtilis*) (Joseph and Goebel, 2007). However, along with the similarities there are some key differences that play an important role in the relationship of *L. monocytogenes* and its host cell. Unfortunately, very little is currently known about the metabolic interactions of host and parasite once *L. monocytogenes* is growing intracellularly. What is known is that once *L. monocytogenes* has invaded it will obtain most of its nutrients from the host cell. These include hexose phosphate sugars and amino acids for which *L. monocytogenes* are auxotrophic. The following section of the report will focus on some of the most important aspects of *L. monocytogenes* growth and metabolism, especially intracellular growth.

**1.9.2) Laboratory growth of *Listeria monocytogenes***

For most experimental purposes *L. monocytogenes* is grown in Brain Heart Infusion (BHI) or Tryptone Soya Broth (TSB), which are complex media. Most of the molecular studies of virulence factors and gene expression were conducted with *L. monocytogenes* grown in these media. There are also a number of minimal media that support *L. monocytogenes* growth, which contain amongst other ingredients, a mix of amino acids, vitamins and cofactors essential for *L. monocytogenes* growth (Tsai and Hodgson, 2003, Phan-Thanh, 1997, Friedman and Roessler, 1961).
There are a number of limitations using complex media for metabolic experimentation, with the primary concern being that the medium contains undefined nutrients and carbon sources. This is a major limitation for metabolomic analysis purposes as changes in specific or trace amounts of metabolites become very difficult to detect. L. monocytogenes is able to grow in a number of minimal media compositions, albeit relatively poorly (Tsai and Hodgson, 2003, Premaratne et al., 1991). However, our own unpublished work has revealed a further concern, which is that minimal medium does not seem to be able to support growth of L. monocytogenes under strict anaerobic conditions (see section 4.1). This puts limitations on the study of L. monocytogenes metabolism under anaerobic conditions. Our own unpublished work has shown that minimal medium is incapable of supporting sequential L. monocytogenes growth under anaerobic conditions. Any growth that is seen may be a supplementary effect of serial subculturing or inoculating minimal medium with cultures grown in complex medium. This suggests that there are vital nutrients missing from the minimal medium or there is toxicity to the cells under anaerobic conditions which is not present under aerobic conditions (see results Chapter 4).

1.9.3) Listeria monocytogenes anabolic pathways

Amino acids are essential for a vast array of anabolic cellular processes for both the host and invading L. monocytogenes. By using systematic deletion of amino acids from the medium it has been established that only cysteine and methionine are essential for L. monocytogenes growth (Tsai and Hodgson, 2003). These growth requirements can be explained by the absence of genes required for their biosynthesis. L. monocytogenes also do not possess sulphate and nitrate reductases, so there is a dependency on reduced nitrogen and sulphate sources, which can be gained from these amino acids (Tsai and Hodgson, 2003).

Studies have shown that L. monocytogenes have some requirement for branched chain amino acids (BCAA) valine, isoleucine and leucine (Goldfine and Shen 2007). The addition of BCAA to a given medium has been shown to enhance the growth rate of L. monocytogenes (Joseph and Goebel, 2007). Due to the fact that
BCAA are synthesised through several metabolic intermediates, such as oxaloacetate, the general availability of them is good indicator of overall cell health (Goldfine and Shen 2007). *L.monocytogenes* biosynthesis of BCAA has been shown even in the presence of supplementary BCAA, which has been linked to the presence of glucose and catabolite repression of BCAA transport (Goldfine and Shen, 2007, Eisenreich *et al.*, 2006).

Other amino acids such as aromatic amino acids are also shown to be synergistically important. Knockout mutants incapable of synthesising individual aromatic amino acids have shown little or no effect in growth rate in comparison to wild type. However, mutants auxotrophic for all three aromatic amino acids (phenylalanine, tryptophan and tyrosine) are significantly impaired in terms of growth and virulence (Marquis *et al.*, 1993). *aro* mutants containing deletions in a common branch of biosynthesis pathway leading to aromatic compounds also show attenuated growth and virulence (Stritzker *et al.*, 2004). This is possibly because *aro* mutants eventually sequestering menaquinone, which is the only quinone produced by *L.monocytogenes*.

Carbon isotopologue studies into intracellular metabolism have shown that 50-100% of *L.monocytogenes* amino acids are provided by the host cell (Eylert *et al.*, 2008). Only three non-essential amino acids alanine, asparagine and glutamate, were shown to be synthesised *de novo*, whereas all essential ones were acquired from the host. This is also supported by comparative transcriptomics of intracellular and extracellular *L.monocytogenes*, showing an up regulation of genes associated with essential amino acids including BCAA and aromatic amino acids but not nonessential ones (Joseph *et al.*, 2006). This suggests that the majority of the nonessential amino acids are provided by the host cell, or alternatively, the host cell supply of essential amino acids is inadequate, forcing *L.monocytogenes* to upregulate genes involved in their synthesis (Joseph *et al.*, 2006).
1.9.4) *Listeria monocytogenes* catabolic pathways

*Listeria monocytogenes* is able to grow using aerobic and anaerobic metabolism. The *Listeria monocytogenes* genome has the steps for menaquinone biosynthesis but not coenzyme Q10 (ubiquinone) which is required as an electron acceptor in the electron transport chain (Goldfine and Shen 2007). Menaquinone is used extensively as a cofactor in the electron transport chain. The synthesis of menaquinone is dependent on a precursor branch of the aromatic amino acids pathway. Mutants which are unable to synthesize menaquinone undergo anaerobic respiration, even under aerobic conditions (Stritzker et al., 2004). Genomic analysis has revealed *Listeria monocytogenes* possesses enzymes required for glycolysis and the pentose phosphate pathway (Glaser et al., 2001). It also has a complete set of enzymes from the respiratory chain and the enzymes for fermentation pathway are also present. *Listeria monocytogenes* has an incomplete tricarboxylic acid (TCA) cycle as it does not possess alpha–ketoglutarate dehydrogenase, which converts alpha-ketoglutarate into succinylCoA (see Fig. 1.8) (Glaser et al., 2001). As a result of this incomplete cycle, *Listeria monocytogenes* is incapable of regenerating oxaloacetate through the TCA cycle from citrate. Carbon isotopologue perturbation has revealed that oxaloacetate is mostly produced from the carboxylation of pyruvate which is regulated by the pyruvate carboxylase enzyme (Eylert et al., 2008). It was also established that oxaloacetate is an essential precursor for the synthesis of Asp, Thr and Glu (Eylert et al., 2008). These findings suggest that pyruvate carboxylase (Pyc) plays an essential role in *Listeria monocytogenes* metabolism and this is confirmed by a *pyc* knockout mutant exhibiting attenuated growth in complex medium with inability to grow in defined medium. The mutant is also unable to replicate intracellularly in mammalian cells and shows highly reduced virulence in a mice sepsis model (Schar et al., 2010).
Fig. 1.8 Overview of *L. monocytogenes* TCA cycle
Schematic of the enzymatic steps involved in *L. monocytogenes* TCA cycle. *L. monocytogenes* does not possess alpha ketoglutarate dehydrogenase (red cross). This loss, causes an incomplete TCA cycle. Thus, *L. monocytogenes* cannot regenerate oxaloacetate through the TCA cycle and that most of the oxaloacetate must be formed from the carboxylation of pyruvate by pyruvate carboxylase.
(Adapted from http://www.pharmabase.org)
1.9.5) *Listeria monocytogenes* metabolic end products

The metabolic end products of *L. monocytogenes* from aerobic growth show the production of around 10 acids along with acetoin (Daneshvar *et al.*, 1989). More quantitative studies showing percentage carbon recoveries for end products show 28% lactate, 23% acetate and 26% acetoin for aerobic growth (Romick *et al.*, 1996). For anaerobic growth the lactate produced is increased substantially to 79%, the acetate production reduced to 2%. Other anaerobic end products include formate (5.4%), ethanol (7.8%) and carbon dioxide (2.3%) (Romick *et al.*, 1996). These results elucidate that a mixed acid fermentation is being utilised by *L. monocytogenes*, and that acetoin and lactate are good indicators for determining aerobic or anaerobic growth.

1.9.6) *Listeria monocytogenes* carbon metabolism

The uptake of sugars and sugar alcohols in *L. monocytogenes* is almost entirely dependent on the phosphotransferase system (PTS) (Mertins *et al.*, 2007). Genomic studies of *L. monocytogenes* have revealed more than 40 PTS related genes (Glaser *et al.*, 2001). Many of these genes are specifically associated with the uptake of single sugars, for example, four (*lmo0096–0098, lmo0781–0784*, and *lmo1997–2002*) are specific for mannose and seven (*lmo0034, lmo0901, lmo1095, lmo2683–2685, lmo2708, lmo2762, lmo2763, lmo2765, lmo2780, lmo2782, and lmo2783*) for cellobiose (Goldfine and Shen, 2007).

It has been shown that many of these PTS genes are upregulated when *L. monocytogenes* is growing intracellularly (Glaser *et al.*, 2001). The importance of these genes for intracellular growth is confirmed by PTS knockout mutants having substantially impaired growth rates in epithelial cells (Joseph *et al.*, 2006). Further experiments have shown that PTS sugars, which include glucose, fructose and mannose, are the preferred carbon sources for *L. monocytogenes* when grown in minimal medium (Tsai and Hodgson, 2003).

Non PTS dependent glucose uptake has been proposed for *L. monocytogenes*, however, a *ptsH* mutant which cannot utilise PTS dependent systems has an inability to grow in minimal medium using glucose as a carbon source (Mertins *et
This suggests that PTS transport is also almost entirely responsible for glucose transport in *L. monocytogenes* (Goldfine and Shen, 2007).

The genes for glycolysis are all present in *L. monocytogenes* and are among the highly expressed genes when growing in complex medium such as TSB (Goldfine and Shen 2007). A strong preference for PTS sugars when grown in complex medium has been shown, with glucose catabolised first. However, there is a reduction in the expression profiles of the glycolysis genes when *L. monocytogenes* is grown in minimal medium (Joseph et al., 2006). Along with this reduction there is an increased expression of the pentose phosphate pathway (PPP) genes. This effect is also seen in intracellular growth where comparative genomic studies reveal that the PPP is the major catabolic pathway for gluconeogenesis when carbon sources other than glucose are utilized (Joseph et al., 2006). Also, when growing intracellularly, PPP not glycolysis is the main source of carbon metabolism, perhaps a product of limited availability of PTS sugars in the host cytosol (Joseph et al., 2006).

PTS mutants (*ptsH*) have been shown to grow in complex medium at a limited rate, which points to the utilisation of alternate carbon compounds (Mertins et al., 2007). However, complex media, such as TSB, which is a tryptic digest of soya bean, are very difficult to define as they contain a rich source of carbon compounds. These can also vary from batch to batch, making it difficult to determine exactly which carbon sources are utilised by *L. monocytogenes*. Previous work has established that the carbon catabolism of *L. monocytogenes* is rather limited, with *L. monocytogenes* unable to utilize many carbon compounds such as maltose, sucrose and lactose (Tsai and Hodgson, 2003). *L. monocytogenes* is also unable to use caseamino acids as a source of carbon (Tsai and Hodgson, 2003). These results are further supported by the genomic data which also suggest that *L. monocytogenes* do not possess the necessary amino acid catabolism pathways (Joseph and Goebel, 2007). Some bacteria such *Mycobacterium tuberculosis* have the ability to utilise acetyl–CoA as a carbon source by using the anapleurotic glycolytic shunt pathway. This pathway increases the production of malate or oxaloacetate by utilising acetyl-CoA. The glyoxylate shunt genes are missing from *L. monocytogenes* genome, thus
rendering this pathway unusable. This also means *L. monocytogenes* is incapable of utilising fatty acids as a carbon source (Eisenreich *et al.*, 2006).

*L. monocytogenes* is capable of utilising phosphorylated hexose sugars (PHS) from the host, such as glucose 6 phosphate which are transported into the cell by Hpt transporter, which is under the control of the PrfA virulence regulator. Similarly to other genes under the control of PrfA, the *hpt* gene is highly upregulated upon entry into the host cell cytoplasm (Camejo *et al.*, 2009). The ability of *L. monocytogenes* to use PHS is limited to pathogenic *Listeria*, with non-pathogenic species incapable of utilising these sugars (Chico-Calero *et al.*, 2002). As the PHS are readily available in the host cell cytosol the ability to use such sugars may have important implications for *L. monocytogenes* pathogenicity. The importance of the *hpt* transporter to *L. monocytogenes* intracellular survival is confirmed by mutants lacking the gene, resulting in significantly impaired cytosolic proliferation (Chico-Calero *et al.*, 2002).

1.9.7) Carbon catabolite repression of *prfA*

A large body of evidence suggest that a number of carbon sources, including glucose and fructose, have an inhibitory effect on the *prfA* virulence regulator, known as carbon catabolite repression (CCR) (Milenbachs *et al.*, 1997). The CCR is a regulatory mechanism for bacteria, allowing them to alter the expression of genes associated with secondary carbon sources when the primary carbon sources are available (Gorke and Stulke, 2008). This allows *L. monocytogenes* to grow optimally in the presence of a complex variety of carbon sources using preferential carbon sources when they are available.

The precise mechanism for this effect and the extent to which particular sugars contribute is still unclear. Orthologous genes for most of the regulatory components of CCR in *B. subtilis* are also found in *L. monocytogenes*, suggesting a similar mechanism of control (Glaser *et al.*, 2001). The largest inhibitory effect has been shown by cellobiose, a plant derived carbohydrate. Cellobiose inhibition has been discovered at concentrations as low as 1 mM (Gilbreth *et al.*, 2004).
In Gram-positive bacteria such as *B. subtilis*, CCR is associated with catabolite control protein A (CcpA), a member of the LacI/GalR family of regulator proteins (Behari and Youngman, 1998). CcpA works in conjunction with its cofactor HPr, a member of the PTS pathway, phosphorylated at Ser position 46, by a specific ATP dependent Hpr-kinase (HprK) (Reizer *et al.*, 1998). This phosphorylation process is stimulated by glycolytic intermediates (Goldfine and Shen, 2007).

Together with CcpA, HPr binds to CRE (carbon repression elements) sites of CCR controlled genes leading to their repression. This is supported by *ccpA* and *hprK* mutants exhibiting the upregulation of CCR genes (Deutscher *et al.*, 1995). Typical CcpA dependent genes are involved in glycolysis (*gapA* operon), BCAA synthesis and also glutamate synthesis (*gltA/B*) (Blencke *et al.*, 2003). When cells are grown in the presence of glucose, CcpA also directly represses the *citZCH* operon and also regulates *citB* by inhibiting citrate synthesis and keeping CcpC, its major transcriptional regulator, activated (Mittal *et al.*, 2009).

In *L. monocytogenes*, direct CCR interaction of CppA with PrfA is unlikely as *hly*, encoding a major virulence factor LLO, in a *ccpA* mutant is still down regulated in the presence of sugars (Behari and Youngman, 1998). Alternatively, PrfA integration of components of the PTS mediated transport system has been suggested, with overexpression of PrfA leading to significant growth inhibition of *L. monocytogenes* in glucose containing medium (Marr *et al.*, 2006).

1.9.8) *Listeria monocytogenes* nitrogen metabolism

*L. monocytogenes* is capable of utilizing nitrogen from a number of sources including glutamine, arginine and possibly ethanolamine (Joseph and Goebel, 2007).

Glutamine seems to be the preferential and optimal nitrogen source for most bacteria, with it being converted to glutamate (Susan, 1999). However, in some environments the availability of glutamine may be limited, especially when *L. monocytogenes* is growing intracellularly. In this situation *L. monocytogenes* may adopt alternative nitrogen sources such as ammonium, so as not to
sequester valuable host nutrients (Tsai and Hodgson, 2003). Ammonium can be incorporated into glutamine and subsequently glutamate, by the enzyme glutamate synthase with 2-oxoglutarate as an additional substrate (Joseph and Goebel, 2007). Glutamate is the primary amino group donor for many amino acids and glutamine is the direct nitrogen donor for amino acids, purines pyrimidines and other key compounds (Tyler, 1978).

Glutamine can only be synthesized by using the glutamine synthetase enzyme (GS), whereas glutamate can be produced through multiple routes: from ammonia and 2-oxoglutarate, catalyzed by glutamate dehydrogenase (GDH), conversion of glutamine to glutamate by glutamate synthase (GOGAT) or a direct transamination event involving an amino group from another amino acid and 2-oxoglutarate. The GS/GOGAT cycle described above is overviewed in Fig. 1.9. Glutamate can also be synthesised through the Roc pathway via the degradation of arginine (Belitsky and Sonenshein, 2004).

In *B. subtilis* the ammonium is internalized by diffusion at high concentrations or active transportation using the NrgA transporter encoded by the *nrgA/B* operon (Detsch and Stulke, 2003). The product of the second gene in this operon, the NrgB protein, is a member of the PII family of regulatory proteins and has been shown to be localized at the cell membranes where it is associated with NrgA. As NrgB is required for full expression of the *nrgA/B* operon, it may translate the information of intracellular ammonium levels by modifying downstream regulatory factors, which are involved with the expression of GS and GOGAT (Detsch and Stulke, 2003). Transcriptional profiling has revealed that the operon controlling the production of NrgA/B in *L. monocytogenes* is upregulated during intracellular growth (Detsch and Stulke, 2003). This suggests that host cell ammonium may be a major intracellular nitrogen source for *L. monocytogenes* (Joseph *et al.*, 2006).
Fig. 1.9 Overview of *L. monocytogenes* glutamine/glutamate assimilation cycle

Schematic of glutamate/glutamine assimilation in *L. monocytogenes* is shown. In many bacteria, glutamine as nitrogen source is combined with α-ketoglutarate and converted to glutamate by glutamate synthase (GOGAT). Glutamate is the principal nitrogen donor to other amino acids in subsequent transamination reactions (amino transferase) (AT) and to nucleotides. Glutamate is also a product in the glutamate dehydrogenase (GDH) reaction, which converts glutamate into α-ketoglutarate. With the consumption of ammonia and ATP, glutamate can be converted to glutamine by the glutamine synthetase enzyme (GS).
Although the exact mechanism of ammonium regulation is yet to be elucidated in *Listeria*, orthologous genes that are involved in *B. subtilis* ammonium regulation have been found, suggesting the mechanisms of control may also be shared. Although the exact mechanism of ammonium regulation is yet to be elucidated in *Listeria*, orthologous genes that are involved in *B. subtilis* ammonium regulation have been found, suggesting the mechanisms of control may also be shared (Joseph and Goebel, 2007). In *Bacillus*, three proteins - GlnR, Tundra and CodY are thought to control gene expression in response to nitrogen availability (Sonenshein *et al.*, 2002). GlnR regulates the expression of *genera* operon shared (Joseph and Goebel, 2007). In *Bacillus*, three proteins – GlnR, TrnA and CodY encoding GS, which it represses under conditions of nitrogen excess (Doroshchuk *et al.*, 2006). TrnA is thought to act as both a repressor and an activator of transcription. TrnA binds allosterically to GS forming an inactive complex. This in turn stops TrnA from binding and activating its specific operators (Doroshchuk *et al.*, 2006).

*L. monocytogenes* genome contains orthologues of *glnR* and *codY*; however an orthologue of *tnrA* has not been identified. This hints to a potentially different mechanism of nitrogen regulation in *L. monocytogenes* (Joseph *et al.*, 2006).

Amino acids such as arginine, which can be utilized by arginine deaminase, could also be potential nitrogen sources. Arginine could be degraded into citrulline and ammonia by arginine deaminase supported by the upregulation of *arpj*, a specific arginine transporter in intracellularly replicating *L. monocytogenes* (Klarsfeld *et al.*, 1994). Citrulline itself could be degraded into a further ammonia molecule and ornithine.

*L. monocytogenes* possess the gene *lmo1742* a encoding a potential adenine deaminase. Adenine along with H₂O is inter-converted by adenine deaminase enzyme to hypoxanthine and ammonia. *lmo1742* has also been shown to be upregulated in intracellularly replicating *L. monocytogenes*, suggesting adenine could also be a limited nitrogen source utilized by *L. monocytogenes* (Camejo *et al.*, 2009).
1.9.9) Essential vitamins and cofactors

There are a number of vitamins and cofactors that *L. monocytogenes* is unable to synthesise, meaning that *L. monocytogenes* depend entirely on its host for their supply. Genomic analysis of *L. monocytogenes* has highlighted a deficiency of several important vitamin biosynthetic pathways. These include pathways for biotin, lipoic acid, riboflavin and thiamine, leading to the absolute requirement of these vitamins for *L. monocytogenes* growth (Goldfine and Shen, 2007).

The absence of any of these nutrients from the growth medium results in impaired growth (Tsai and Hodgson, 2003). The absence of lipoic acid and riboflavin stops growth completely, making them absolutely essential for *L. monocytogenes* growth.

Lipoic acid is a cofactor required for the pyruvate dehydrogenase enzyme (Pdh) complex, which catalyses the formation of acetyl CoA from pyruvate, playing a vital role in the aerobic metabolisms of most organisms (Ramaswamy et al., 2007). *L. monocytogenes* possesses two lipoate ligases which are essential for intracellular growth. A mutant lacking the lipoate ligase protein Lp1A1 showed an inability to grow in the host cell cytosol along with impaired virulence in animals by a factor of 300 (O'Riordan et al., 2003). Lp1A ligates exogenous lipoic acid to the E2 subunit of Pdh to produce E2-lipoamide, an essential mediator of aerobic metabolism. These findings indicate that the abortive growth is due to loss of Pdh activity and that host lipoic acid may be essential for intracellular growth of *L. monocytogenes* (O'Riordan et al., 2003).
1.10) Introduction to Systems Biology

The aim of this section is to provide an introduction to Systems Biology and modelling with the aim to discuss the rational and motivational forces behind such an approach. As this is a Systems Biology study, it is important to discuss the merits and potential difficulties associated with tackling complex biological problems in this way.

For many years now investigations in biology have largely followed classical reductionism. This is the idea that the whole can be understood by the ‘sum of its parts’ (Kitano, 2002). It is now evident that using this approach is not sufficient in understanding the functions of many aspects of biology. For example, the reductionism approach would attempt to understand the function of a cell by knowing its genes. This is like trying to understand how a car or plane functions by listing its components. Although the reductionist approach will provide a catalogue of components which is a vital initial step, it does not further our understanding of the underlying complexity of the engineered ‘system’. However, there has been a progressive shift in the way in which biologists approach their problems with the advent of Systems Biology. Leroy Hood and others co-founded the Systems Biology concept, an approach that focuses on the components of a system, with an emphasis on the emergent properties that arise as a result of component interaction. This holistic approach essentially changes the notion of what scientists look for in biology. Kitano (2002) provides an interesting analogy for the role of Systems Biology. According to Kitano, previous approaches in biology (reductionism) produce static road maps, whereas what we really seek to know are the traffic patterns, why such traffic patterns emerge, and how we can control them. Having a systems level understanding into biological systems requires various different properties. Firstly, we must know the structure of a system. These include the network of gene interactions and biochemical pathways. A good understanding of how a system behaves over time and under various conditions (Kitano, 2002). This can be obtained through metabolic, sensitivity and dynamic analysis. Also required, are methods which control the state of the cell and strategies to construct and modify biological systems based on simulation (Aloy and Russell, 2006).
Having given a short insight into some of the aspects of system biology I will now evaluate an important aspect of Systems Biology which is modelling/simulation. What are the benefits and limitations of modelling in the understanding of bio-systems?

Biological systems are massively complex. They comprise of many interacting subunits to produce a whole that is in constant harmony. Many of these sub systems also show chaotic behaviour, where small perturbations within the system can lead to dramatic changes that affect the whole of the resultant system. The ability to model these changes and to be able to predict an outcome is of great interest and benefit to the world of biology. Firstly, it allows viable predictions to be made regarding a system. This can save many companies and scientist time and money when deciding which avenue to take with a specific problem, which may be more cost effective. In other words simulation and modelling can be used to aid the intuition of scientists. While an assumption regarding an experiment may be simple, the consequences of the experiment may not be. Simulation allows scientists to anticipate such consequences. Simulation also allows specific tasks to be undertaken by the model. The scientist is in control of all variables which may be harder to control under the classical wet lab experiments. This may allow the scientist to gain more accurate and reliable results.

The disadvantages that arise when using simulations of biosystems are predominantly based on technological and technical limitations. Importantly, a model is only as good as the validation of the model. In many cases parameters of the model are unknown and can only be validated by experimental design. For example, when constructing an enzymatic pathway, parameters such as maximum velocity of the enzymes (amongst others) are required. These parameters need to be gained from experimental study and although can be predicted, need careful validation to ensure the model is biologically relevant.

The complexities of models are limited to the processing power of the machines used and more parameters need to be included as our understanding of biosystems expands. It is extremely challenging to simulate all aspects of a complicated biosystem. Even simple cells have hundreds of processes, each
involving different biological entities all interacting with each other. Trying to simulate all these processes accurately is very difficult. This also carries over to the fact that individual cells interact with each other, and groups of cells interact with other groups of cells. This leads to huge complexity that must be carried over to the model. In some cases this may mean that the modelling of the system is more costly than actual experiments which may negate any potential advantage obtained from modelling the system. These problems can be overcome in some ways using parallel computing methods, the use of multiple computers and their processors to carry out the computation needed (Vrugt et al., 2006).

It must be emphasised that modelling should not be seen as a replacement or even an alternative for experimental study. Models cannot be used until they are extensively validated against experimental results, testing biological accuracy. Instead, they should be seen as a tool to be used in conjunction with classical biology, to guide and provide extra focus to experimental work. They are also a tool to help a researcher to think analytically about a problem and to ask the right questions. This makes cellular modelling a cyclical study where the experimental lab results are used to validate and improve the model and the model can then be used to answer biological questions and further guide experimental study.

1.11) Listeria monocytogenes modelling approaches

Modelling of L. monocytogenes metabolism is a methodology which is still rather underdeveloped. However, there have been a number of studies which have focused on various aspects of L. monocytogenes cellular metabolism, for example, intracellular growth and host pathogen interaction (Schauer et al., 2010, Eisenreich et al., 2006).

There are a number of different modelling techniques, which can be used for modelling metabolic pathways in a given organism. Some of these require quite complicated experiments to determine enzymatic parameters such as Vmax, Km and Kcat (Bruggeman et al., 2005). Alternatively, one can make a model based on mass action kinetics, which are used to describe the dynamics of chemical reactions and reaction networks (Chellaboina et al., 2009).
Flux balance analysis (FBA) utilises mass action kinetics and calculates the flow of metabolites through a pathway, and from this, one can determine biological parameters such as, growth rate or the fate of biologically interesting metabolites (Orth et al., 2010).

Elementary mode analysis (EMA) has been utilised to model the in vitro metabolism of L. monocytogenes (Schauer et al., 2010). The methodology allows for analysis of multiple pathways, which are deconvoluted to their most simple form, whilst retaining a steady state (Papin et al., 2004). All pathways, which can be reduced and cannot be split further, are used for EMA analysis. A further analysis derived from subsets of EMA can be analysed by extreme pathway analysis (EPA). The EPA analysis can describe a complex system using linear combination of fewer EPA pathways (Fuchs et al., 2012).

Models can be built to incorporate a huge amount of data and it is possible to build genome scale models reaching thousands of pathways (Fuchs et al., 2012). Random mutant screening allows the identification of those mutants with a desired phenotype. From the mutant library key pathways essential to their survival are identified. The list of key enzymes reoccurring in the mutants can then be calculated and ranked, which can lead to identification of the most important pathways for growth (Fuchs et al., 2012).

There have been some interesting results gleaned from EMA. These include highlighting the crucial role of glycerol and purine metabolism. Also highlighted is the utilisation of fucose and the synthesis of glutathione, aspartate, serine and the BCAA during intracellular proliferation (Schauer et al., 2010). Also highlighted is the degradation of glucose predominantly through PPP as opposed to glycolysis, which is an observation supported by transcriptomic studies, which show the up regulation and down regulation of the PPP and glycolysis pathways respectively in intracellularly growing L. monocytogenes (Joseph et al., 2006).

1.12) Introduction to metabolomics

The completion of the first genome for a free living bacterial organism Haemophilus influenzae in 1995 represented a watershed moment for the field of biology.
(Fleischmann et al., 1995). For the first time there was access to a hitherto unprecedented amount of information regarding the genetic makeup of a living organism. This ushered in the ‘omics’ era, with transcriptomics and proteomics following soon after allowing an expansive assessment of cellular function at the transcriptional and protein level (see Fig. 1.10). These advances were essential precursors for metabolomics (Tang, 2011). Obviously the complexity of biological systems is vast, with many components, from RNA to proteins to metabolites. When trying to evaluate individual connections between these components the complexity increases significantly.

Metabolomics represents a key technique for Systems Biology with an emphasis on discovering emergent properties in a living system. Metabolomics is the study of the total set of metabolites that are in a given organism. The metabolites in question can be anything which the system is composed of or is utilising, for example, food, drugs and toxins. However, the exact constitution of a metabolite is debated with several different opinions regarding molecular cut off point, concentration and origin (Wishart et al., 2007).

The major applications of metabolomics research, which are currently being utilised are the identification and quantification of metabolite concentrations in a given sample (Fiehn, 2002). Metabolomics is of great interest to the medical community also. This is due to the fact that metabolite measurements are much less invasive, and also allow the early diagnosis of some major diseases such as cardiovascular conditions, diabetes and cancer. Much is made of the discovery of “biomarkers” for such purposes and their potentially lifesaving implications (Lewis et al., 2008, Kim et al., 2008). Finally a major area of research in the field of metabolomics is the integration of metabolomic data network models (Cottret et al., 2010).
Fig. 1.10 Omics research - a biological perspective

There are a vast number of components that make up a biological system, including, RNA, proteins and metabolites. Some of these components are even modified dependent on the requirements of the organism, for example, proteins are modified post translation. The smallest of the molecules are the end point of complex cellular functions within cells, tissues and organisms. This means that metabolic functions of a cell are the most proximal identifiers of cell. These represent a snapshot of the biological status of a cell, tissue or organ, which is closest to the phenotype. This can be exploited by using metabolites as an indicator of a disease state, the progression of a disease and even the results from a drug treatment. Metabolomics is the use of multiple tools to analyse the metabolome of a given organism or sample.

(Adapted from Lewis et al., 2008)
There are a number of key advantages for taking a metabolomics approach for system analysis. When taking samples for metabolomics analysis you are essentially taking a snap shot of the metabolic state of the system and this is the closest representation of the phenotype, showing exactly what is happening rather than what could be happening (Dettmer et al, 2007). The interaction of a cell’s genome and its environment is directly related to the metabolites that are present and their abundances. This provides a non-discriminatory assessment of the cellular state. This is something which is not always possible when concentrating solely on the transcriptome or the proteome. The ‘high throughput’ nature of the methodology also means that large sample sizes can be handled relatively quickly, increasing the ability to perform large scale analyses leading to more robust results.

Another advantage of metabolomics is that in comparison to the number of genes or proteins, there are far fewer metabolites, which, in theory at least, makes their subsequent analysis less complex. For example, the genome of *Saccharomyces cerevisiae* contains over 6000 known genes, yet it only contains 584 metabolites (Mewes et al., 1997, Förster et al., 2003). This is almost 10 times less, highlighting the difference in complexity for their subsequent analysis.

Although metabolites may in theory present a less complex proposition for global studies of organism, metabolomics is not without limitations. The comprehensive coverage of total metabolites is still largely incomplete, meaning that no metabolomic instrument measures or identifies all metabolites (Verpoorte et al., 2008). Also, significant modifications to metabolites need to be made before they can be analysed by some techniques, for example GC-MS (see section 1.12.4).

Combining the knowledge that has been obtained from the “omics” research represents a significant Systems Biology challenge and whilst sequencing of the human genome means there is a fully defined set of genetic data, the human metabolome is far from fully defined and requires further work and possible advancements in technology. The analytical techniques that are being used are always improving and are essential in reaching this goal.
Metabolomics studies are usually divided into two sub experimental categories, which are internal and external metabolomics.

Internal metabolomics is concerned with the intracellular metabolites, i.e. those metabolites contained within the cells. Measurement of the internal metabolome is a representation of the internal metabolic fluxes and enzymatic activities (Tang, 2011). These metabolites would include products of catabolic and anabolic pathways and include carbon sources and nitrogen sources. Intracellular metabolites are also known to contribute in signal transduction pathways interacting with enzymes and subsequently gene expression, for example, the metabolite glutamine is known to repress key components of the TCA cycle in _L.monocytogenes_ (Mittal et al., 2009).

Extracellular metabolome studies, also known as metabolite ‘footprinting’, focus on the excretion and uptake of metabolites from the secondary environment of the cells. As such, it is of great interest to the medical community. For example, metabolomes of blood, lymph fluid, urine and faecal matter are all examples applicable to an extracellular metabolome study (Wishart et al., 2006). The metabolites will also include secondary metabolites, such as growth factors, stress or communicative signals for cell to cell signalling and represent the primary information, which would be detected by a neighbouring cell (Poli et al., 2004). As such, an external metabolome study can provide valuable information and also new insight into the state of a living system, which would not always be elucidated from an intracellular study.

1.13) Metabolomics instrumentation and techniques

This section will discuss the pathway of metabolome analysis from chromatography to mass spectrometry and also key techniques that are currently used in the field of metabolomics.

The basic principles of Mass Spectrometry (MS) that are still used today were first established by J.J Thomson in 1913 with a MS system operating under vacuum pressure (Dunn, 2011). MS has since become an essential tool for metabolomics analysis. The main application of MS is as a probing tool,
identifying and quantifying constituents in a given sample. It is an extremely powerful technique with the ability to determine metals in water as low in number as PPQ (parts per quadrillion) (Soni et al., 1995).

MS requires the ionisation of a sample, usually to cations by the loss of electrons. The charged ions are then accelerated to the same velocity and are then deflected. The deflection of the ions is dependent on two criterions. The first is the mass of the ion; the lighter the ion the greater the deflection. The second is the charge of the ion, which depends on the number of electrons lost during ionisation - the greater the charge the greater the deflection of the ion. Finally, the ion signal is sent to a data system for analysis. Since the charge of the ion is a known quantity the mass/charge ratio (m/z ratio) is used to determine ion mass (Gross, 2004). For example, a molecule with a molecular weight of 1000, coupled to a single proton will be detected with an m/z ratio of 1001. A molecule of 1000 coupled to 2 protons will be detected with a m/z of 501 (mass of molecule + mass of proton/charge) (Dunn, 2011).

1.13.1) Pre-separation of sample

There are a number of considerations that need to be made before subjecting a sample to MS. Whilst one could potentially perform MS on an analytical sample that has not been separated by chromatography there are a number of good reasons not to do this. Most chemicals that are of interest in a mixture usually occur as isomers, which are better analysed by MS separately as they cannot be differentiated (Dunn, 2011). Also, ion suppression can lead to difficulties detecting particular metabolites which are either in small quantities or poorly ionised, chromatographic separation can aid this (Gross, 2004). Taking in these considerations, MS is usually coupled with a chromatography to separate out analyte into macromolecules, which are then sorted and analysed by the MS.

1.13.2) Chromatography

Chromatography is a collated term for a number of separation techniques used to separate a mixture of compounds into individual constitutive components. The separation is based on the movement of the compounds which are solubilised,
known as the ‘mobile phase’, this is then carried through a structure known as the ‘stationary phase’, and subsequently separated based on the partition coefficient of the analyte (Ettre, 1993).

1.13.3) Gas chromatography (GC)

Gas chromatography (GC) is a technique for separating closely related compounds or solutes in a given sample. The principle method of GC has been implemented for over 50 years and in its basic form is to pass a known amount of sample through a very long, thin silica based tube, known as a capillary column. This is done in conjunction with a carrier gas, usually helium or nitrogen. The gas represents the mobile phase and the stationary phase consists of either a solid or immobilised liquid which is coated on the internal wall of the capillary column (Dunn, 2011). Samples are vaporised before entering the capillary tube, along the length of the capillary the compounds are separated by their viscosity and in more advanced systems polarity.

1.13.4) Configurations of MS

In its simplest configuration a MS analyser consists of 4 components. These are highlighted in Fig. 1.11, and are: introduction source, ionisation source, mass analyser and detection system. This equipment can vary in size from the desktop oriented machinery to very large setups such as fourier transform ion cyclotron resonance mass analysers (Dunn, 2011).

1.13.5) Introduction source

The aim of the introduction source system is to introduce a sample in the correct format, which means ionised and in most cases intact. As mentioned previously, in some cases the sample is introduced directly and in other the sample is separated (for reasons discussed previously). In Systems Biology analyses the sample is usually separated as these are usually complex, while in simple studies looking at pure, single biochemical species the sample is usually introduced
Fig. 1.11 Simplified schematic of mass spectrometer (MS) analysis
The four most important steps of a MS procedure are shown. These are introduction source, ionisation source, mass analyser and detection system. There are a host of different techniques and methodologies for each step, each with particular disadvantages and advantages. This equipment can vary in size from the desktop oriented machinery to very large setups such as, fourier transform ion cyclotron resonance mass analysers. Usually, the MS set up is connected to a computer allowing data analysis of the results.
(Adapted from Dunn 2011)
directly, for example, matrix assisted laser desorption ionisation (MALDI) is usually applied for pure protein analysis.

1.13.6) Ionisation source

Ionisation source in MS has a single objective, to create a 'charged' chemical species (ion), which is subjected to mass analysis. There are a number of different ways in which this can be achieved depending on the species itself and the specific ionisation technique used. Ionisation can either be hard or soft. Soft ionisation means the production of ions without breaking chemical bonds, comparatively hard ionisation results in fragmentation of the original ions. There are a number of different ionisation techniques, which will be discussed briefly.

1.13.7) Matrix assisted laser desorption ionisation (MALDI)

MALDI (Downard and Chemistry, 2004) is an ionisation technique which is commonly used for purified proteins or analysis of single species. MALDI was first used as a technique to identify biopolymers and was then extended to synthetic polymers (Kruger and Karas, 2002). It predominately deals well with products which decompose/destruct or change in response to heat and non-volatile compounds. There are number of desirable qualities required of the matrix. The matrix should form a crystal base when dry, absorb strongly in the UV range, be composed of low molecular weight, and correct volatility (Yates, 1998). The analyte sample is usually mixed with this matrix in small quantities and then dried to form a co-crystal structure of matrix and analyte (Steen and Mann, 2004).

When this matrix and analyte mixture is bombarded with a UV pulsed nitrogen laser of specific wavelength, the energy is absorbed by the matrix, which converts the heat into excitation energy leading to sublimation and ionization of molecules (see Fig. 1.12). The presence of the matrix avoids excess energy reaching the analyte of interest preserving it from decomposition. Ionised compounds are then transferred to the mass analyser.
Fig. 1.12 Schematic highlighting principles of MALDI
Diagram depicting the components and theory behind the MALDI technique for ionisation of a sample. The analyte ions are usually mixed into a readily volatile, crystallised matrix base. The matrix/analyte mixture is bombarded with a pulsed nitrogen laser leading to the sublimation and ionisation of molecules from both the matrix and the analyte. These ionised molecules are then transferred to a mass analyser.
(Adapted from http://prescottbiochem09.wikispaces.com)
1.13.8) Electrospray ionisation (ESI)

Electrospray ionisation (ESI) First developed by Fenn and Tanaka in the 1970’s, unlike MALDI allows for the analysis of large volatile molecules directly from liquid phase. ESI is a soft ionisation technique producing ions with little defragmentation of the original sample compound. It is particularly suited to the mass determination of biomolecules and sequencing of nucleotides and especially large macromolecules. The basic principles of ESI involve passing a solubolised analyte through a highly charged needle. The needle tip from which the liquid emerges is charged with high voltage, usually 2-3 kilovolts (Fenn et al., 1989). The resulting field at the tip of the needle transfers charge to the emerging liquid (Gaskell, 1997). As solvent evaporation occurs, the droplet shrinks until it reaches the point that the surface tension can no longer sustain the charge (the Rayleigh limit) at which point a "Coulombic explosion" occurs and the droplet is torn apart, producing smaller droplets (Gaskell, 1997). As these charged droplets progress they are diminished by evaporation, a process which is aided by the introduction of a drying gas, usually warm nitrogen (Gaskell, 1997). Eventually, charged analyte ions with all of the solvent removed are passed through a sampling cone then onto intermediate and high vacuum where the MS analyser is held. For an overview of ESI machinery see Fig. 1.13.

The choice of solvent is critical in ESI as it has a direct effect on the observed spectrum. Non-polar solvents should not be used for ESI. The source of the entering liquid is usually from HPLC system. The ESI equipment is usually coupled directly to this, which has several inherent advantages such as minimal processing of the sample.
Fig. 1.13 Principles of electrospray ionisation
Diagram of the components and theory behind electrospray ionisation for the ionisation of a sample. The needle tip at the end of the capillary is usually charged, which is transferred to the solubilised analyte. As the solvent is passed through the needle small droplets are produced. Droplets undergo solvent evaporation and shrink until they reach the point at which the surface tension can no longer sustain the charge (the Rayleigh limit). At this point, a "Coulombic explosion" occurs and the droplet is torn apart, producing smaller droplets. The small droplets can repeat the process eventually producing charged analyte molecules. These are transferred to a suitable mass analyser.
(Adapted from http://www.magnet.fsu.edu/education/tutorials/tools/ionization_esi.html)
1.13.9) Electron impact ionisation (EI)

EI is usually applied to gasses, which can be directly introduced by thermal heating of the samples, or they can be introduced directly from a GC system (Dunn, 2011). This limits EI analysis to compounds which are volatile or can be made volatile, which are usually molecules of low molecular weight such as metabolites. It also means that this system is unsuitable for the analysis of proteins (Gross, 2004). The most frequent use of EI is coupled to GC-MS, where the eluent of the GC is introduced then bombarded with an electron beam which results in the production of a positively charged ion. These are then introduced to the mass analyser. EI is considered a ‘hard’ ionisation technique as the high energies involved in ionisation of the sample, breakdown the ions into fragments by covalent bond fission (Gross, 2004). These ‘fragment’ ions have a specific m/z ratio based on the chemical structure of the original ion, and these fragmentation are also reproducible. This means the ‘fragmentation pattern’ is routinely used to identify particular metabolites.

1.13.10) Mass analysers (MA)

The main purpose of the mass analyser is to separate ions based on their m/z ratios. Most mass analysers (MA) separate ions in an electrical field, however, some initial MA’s used magnetic fields. As with the ionisation sources there are a number of different methodologies that can be utilised, each with their own idiosyncrasies. These are quadrupole, quadrupole ion trap, time of flight and fourier transform ion cyclotron resonance. The following section will briefly discuss each method.

1.13.11) Quadrupole mass analyser (QMA)

Quadrupole mass analysers (QMA) have the advantage of being the cheapest of the MA’s to produce and run. They are also relatively simple and robust; however, this is countered by their low accuracy with limited low mass resolution. The QMA system is made using four identical metal cylinders/rods assembled parallel and symmetrically (see Fig 1.14). Two of these rods are applied with a direct current (DC) whilst the other two are applied with an alternating radio
frequency (RF) (El-Aneed et al., 2009). With the RF frequency activated a large number of molecules can pass through the QMA, when the DC current is also applied, this causes a complex oscillatory trajectory for the ions through the QMA. Ions which do not carry the correct m/z ratio will have the wrong trajectory and will collide with the QMA rods. Only ions with the correct m/z ratio for the DC/RF voltage applied will traverse safely to the detector. By altering the DC and RF voltages a mass range can be scanned (Dunn, 2011).

Triple quadrupole tandem mass analysers (QQQ) overcome the major limitation of QMA which is the low instrument sensitivity, where not all ions of different m/z ratios reach the MA. QQQ provides greater selectivity, better signal to noise ratio, allowing more reliable identifications and better signal to noise ratios. This allows quantitation when reaching the lower limits of mass and also a wider linear range. QQQ is also a more robust method with better reproducibility (Gross, 2004). QQQ mass analysers use a ‘single ion monitoring’ (SIM) technique where continuous detection of a single m/z charge can be performed (Gross, 2004). The QQQ consists of three quadrupoles (Q1-Q3) in tandem, Q1 and Q3 function as mass analysers. Q2 is special as it functions under vacuum conditions, is non-mass filtering and is designed as a collision cell, where ion fragmentation occurs. These fragments are then evaluated in Q3 by scanning the entire m/z range. By evaluating fragmentation patterns structural elucidation of original ions can be made. For example, ion ‘x’ consisting of ‘abc’ can be filtered from other ions at Q1, fragmented at Q2, allowing determination of ‘a’, ‘b’ and ‘c’ in Q3.
Fig. 1.14 Schematic of quadrupole mass analyser
A quadrupole mass analyser consists of four parallel rods that have a fixed DC and an alternating RF voltage applied to them. Ions from an applicable ionisation source traverse the centre of the quadrupoles. Only ions of a particular m/z for a set voltage can traverse the quadrupole safely to the detector. By varying the voltage applied, a mass range scan can be performed.
(Adapted from http://www.chm.bris.ac.uk/ms/images/quad-schematic2.gif)
1.13.12) Ion trap mass analyser (IT)

Ion traps or quadrupole ion traps, contain incoming ions and store them in an orbital motion, then eject ions to the detector. Only ions which are given the correct input of energy are allowed to escape the trap. The greater the m/z the greater the voltage needed for the ion to escape. This is an essential difference to QMA and QQQ systems which provide a continuous ‘stream’ of ions (Cooks and Kaiser, 1990). In terms of sensitivity and resolution the ion trap represents performance comparable to the other Q methods, however, does not possess the performance of a TOF system. This is partially resolved by the ability to perform tandem MS (MS/MS) (Dunn, 2011).

1.13.13) Time of flight mass analyser (TOF)

Time of flight (TOF) mass spectrometry has in recent years become an essential instrument for biological analysis, especially when it has been combined with ionisation sources such as MALDI or ESI (Simoa et al 2008). TOF in principle is one of the simpler MA techniques and works by elucidating m/z ration based upon flight time of ion through the instrument. The ions are initially accelerated to the same velocity then introduced into the TOF instrument, usually directly from the ionisation source such as MALDI. They are then separated by m/z as they pass through, with ions carrying a smaller m/s travelling faster (Sacau et al 2003).

TOF mass analysers have some advantages over other mass analysers. TOF can analyse a very broad mass range and can acquire multiple spectra per second, with very high acquisition rates (Dunn, 2011). As all of the ions are detected simultaneously TOF mass analysers are not thought of as scanning instruments and are usually coupled to high performance chromatography systems like GS. As such TOF is a highly suited technique for complex samples in metabolomics and proteomic studies.
Chapter 2
Aims and objectives
2.1) Aims and objectives

Currently there is limited knowledge about the physiology of the *L. monocytogenes*. In particular, very little is known about the physiology during intracellular growth and even less is known about host cell physiology and changes in response to infection. The focus of this study will be to address these issues using a multidisciplinary approach with a view to produce a testable model of host/parasite nitrogen exchange.

The key questions which were addressed are as follows:

1) What is the state of *L. monocytogenes* during intracellular growth? In particular, is the pathogen growing aerobically or anaerobically? This will be answered by using a combination of cell growth curves, mutagenesis and protein work.

2) Following infection, during intracellular growth, what pathways are induced or repressed, and what pathways are used by *L. monocytogenes* for both catabolism and anabolism? The intracellular and external metabolome was examined separately. The majority of this work was done using metabolomic analyses. Another aspect of this question was to establish how host cell metabolism is affected by the growth of *L. monocytogenes* in the cell cytoplasm? This is a key issue that we have very little knowledge about and something that needs to be addressed. Not enough is known about the changes that occur in the host cell as a response to bacterial infection. By using metabolomic analysis the aim was to analyse metabolites that are differentially utilised as a result of infection. Initially the study focused on the external metabolome of HeLa cells and looked at the changes that are occurring in the metabolome as a result of *L. monocytogenes* infection. This essentially meant evaluation of uptake and secretion into a defined medium, by HeLa cells as a result of *L. monocytogenes* infection.
3) Produced knock out mutants in key enzymes of the glutamine/glutamate cycle (GGC) and established their importance for intracellular growth using gentamicin killing assays. Using quantified metabolomics with a focus on the nitrogen metabolism of *L. monocytogenes* it was established what nitrogenous compounds are being utilised by *L. monocytogenes*. This was a targeted approach giving quantifiable concentrations of nitrogenous compounds. The data provided information on what nitrogen sources are being utilised by *L. monocytogenes*, whilst also indicating which sources are preferentially utilised.
Chapter 3
Materials and methods
3.1) General materials and methods

3.1.1) Chemicals, solutions, primers and reagents

All chemicals, solutions, primers and reagents were purchased from Sigma Aldrich and Gibco Invitrogen, unless specifically stated.

3.1.2) Bacterial growth methodology

*L. monocytogenes*, were routinely grown in 3% tryptone soya broth (TSB) medium at 37\(^\circ\)C, with shaking at 200 rpm, unless stated otherwise. TSB was prepared to manufacturer’s instructions by adding 30 g into 1 L of distilled water.

*E. coli* cultures were grown using Luria-Bertani (LB) broth. When appropriate, the cultures were supplemented with antibiotics. Ampicillin was used at a final concentration of 100 \(\mu\)g/ml and erythromycin was used at 300 \(\mu\)g/ml.

LB broth contained: 0.5% weight/volume (w/v) yeast extract, 1% w/v NaCl and 1% w/v tryptone.

All media were autoclaved at 121 \(^\circ\)C, 15 lb/in\(^2\), for 20 minutes before use and if producing agar plates, 1.5 % (w/v) bacteriological agar was added to the above formula, and poured into plates under sterile conditions.

3.1.3) *L. monocytogenes* media growth curves

*L. monocytogenes* strain EGDe InIA was grown in TSB, glucose defined medium (GDM) medium or MD10 medium (see Table 3.1 and Table 3.2) under aerobic and anaerobic conditions. Media were used to grow EGDe InIA *L. monocytogenes* and mutant strains at 37\(^\circ\)C with shaking at 200 rpm. If measuring anaerobic growth, bacteria were grown in either an anaerobic chamber or sterile syringes at 37\(^\circ\)C with no shaking. Growth was measured using a spectrophotometer at \(\text{OD}_{600}\) after blanking with the relevant medium sample. Alternatively the growth curves were produced by pipetting 300 \(\mu\)l samples and controls into a 96 well plates. Plates were analysed using ‘BioTek Powerwave XS2’ microplate reader and the appropriate software.
3.1.4) Media compositions

Tables 3.1-3.3 show the composition of media which were used throughout the study. Any changes made to these stocks will be specifically implied.

3.1.5) Bacterial strains used in this study

The bacterial strains used in this study are listed in Table 3.4

3.2) Molecular biology techniques

3.2.1) Polymerase chain reaction (PCR)

PCR was performed with a Touchdown Thermal Cycler from Hybai. Each PCR reaction was performed in a sterile 0.5 ml micro centrifuge tube containing a final volume of 50 µl. The reaction mixture contained 5 µl of 1 mM appropriate forward and reverse primer, 5 µl of 10x PCR buffer (Roche molecular biochemicals), 5 µl of 2,5 mM dNTPs (Bioline), 1 µl polymerase (Pfu/Pwo/Taq from Roche). The PCR reactions were carried out using an initial denaturing step of 95°C for 30 seconds, a primer specific T_m for 30 seconds and 72°C elongation step for a length dependent on product size. These steps were repeated for 30 cycles followed by final cycle of 72°C for 5 minutes. These steps are subject to modifications depending on the primers purchased and success of reaction. All primers were ordered online from Sigma Aldrich in Cambridge. The sequences and identifications of the primers used during the course of the study are shown in Table 3.5.

3.2.2) Colony PCR

Colony PCR was performed in a similar way to a PCR; however the template DNA was replaced with a single colony of bacteria suspended in 20 µl of water. A reference agar plate was used to streak colonies from the PCR reaction, allowing retrieval of positively identified clones.
<table>
<thead>
<tr>
<th>Medium Component</th>
<th>100X concentration g/L</th>
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<tbody>
<tr>
<td><strong>Solution 1</strong></td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>200</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>15</td>
</tr>
<tr>
<td>MnSO₄·4H₂O</td>
<td>2</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>1</td>
</tr>
<tr>
<td><strong>Solution 2</strong></td>
<td></td>
</tr>
<tr>
<td>Solution 3 from MD10</td>
<td>As for MD10 medium</td>
</tr>
<tr>
<td><strong>Solution 3</strong></td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2</td>
</tr>
<tr>
<td>Leucine</td>
<td>2</td>
</tr>
<tr>
<td>Tyrosine</td>
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</tr>
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</tr>
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<td>Tryptophan</td>
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</tr>
<tr>
<td>Methionine</td>
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</tr>
<tr>
<td>Phenylalanine</td>
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</tr>
<tr>
<td>Histidine</td>
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</tr>
<tr>
<td>Valine</td>
<td>3</td>
</tr>
<tr>
<td>Glutamic Acid</td>
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</tr>
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<td>Alanine</td>
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</tr>
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<td>Arginine</td>
<td>4</td>
</tr>
<tr>
<td>Lysine</td>
<td>5</td>
</tr>
<tr>
<td>Proline</td>
<td>5</td>
</tr>
<tr>
<td>Threonine</td>
<td>2</td>
</tr>
<tr>
<td>Cysteine</td>
<td>2</td>
</tr>
<tr>
<td><strong>Solution 4</strong></td>
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</tr>
<tr>
<td>Guanine</td>
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</tr>
<tr>
<td>Uracil</td>
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</tr>
<tr>
<td>Xanthine</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Solution 5</strong></td>
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</tr>
<tr>
<td>K₂HPO₄</td>
<td>456</td>
</tr>
</tbody>
</table>

**Table 3.1 Glucose defined medium constituents**

Table shows constituents for GDM. Stock solutions were at 100 times concentration and were diluted into sterile water. Solution 2 was filter sterilised (0.2 µm filter) as vitamins and cofactors were unstable at high temperatures. Lipoic acid was added at final concentration of 0.5 mM (Mcfeeters and Chen 1986).
Table 3.2 MD10 medium constituents

MD10 medium was prepared as above. Solution 1 was prepared in 930 ml of milli-Q water and then autoclaved. Solution 3 was then prepared as described above. Solution 2 was then made and supplemented with 10 ml of Solution 3 which has been filter sterilised (0.2 µm filter) (Trivett, 1971).
<table>
<thead>
<tr>
<th>Component</th>
<th>g/L</th>
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<tbody>
<tr>
<td>CaCl₂</td>
<td>0.2000</td>
</tr>
<tr>
<td>Fe(NO₃)₃·9H₂O</td>
<td>0.0001</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.0976</td>
</tr>
<tr>
<td>KCl</td>
<td>0.4000</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>3.7000</td>
</tr>
<tr>
<td>NaCl</td>
<td>6.4000</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>0.1090</td>
</tr>
<tr>
<td>Arginine</td>
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</tr>
<tr>
<td>Cysteine</td>
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</tr>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Leucine</td>
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</tr>
<tr>
<td>Lysine</td>
<td>0.1460</td>
</tr>
<tr>
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<td>0.0300</td>
</tr>
<tr>
<td>Phenylalanine</td>
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</tr>
<tr>
<td>Serine</td>
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<tr>
<td>Threonine</td>
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</tr>
<tr>
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<td>Tyrosine</td>
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<td>Myo-inositol</td>
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<tr>
<td>Niacinamide</td>
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<td>D-pantothenic acid</td>
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<td>Pyrodoxil</td>
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<tr>
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<tr>
<td>Pyruvic acid</td>
<td>0.1100</td>
</tr>
</tbody>
</table>

**Table 3.3 Dulbecco’s modified eagles medium composition (Sigma D6429)**

Dulbecco’s modified eagles medium composition is shown above. The cell culture work was done using this medium. Some experiments required slight modifications to the medium and these are highlighted accordingly in their respective sections.
<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E.coli DH5α</em></td>
<td>High competency cells used for wide array of microbiology techniques and used extensively for cloning</td>
<td>(Hanahan, 1983)</td>
</tr>
<tr>
<td><em>E.coli BL21(AI)</em></td>
<td>Cells used for expression of protein. Expression induced using 1mM IPTG and 0.2 % arabinose after growing to an OD$_{600}$ 0.4</td>
<td>Invitrogen</td>
</tr>
<tr>
<td><em>L.monocytogenes-EGDe</em></td>
<td>Wild type serotype 1/2a</td>
<td>(Glaser <em>et al.</em>, 2001)</td>
</tr>
<tr>
<td><em>L.monocytogenes EGDe InlA</em></td>
<td><em>L.monocytogenes</em> strain with mutation in <em>EGDe</em> InlA gene. Able to recognise murine E-cadherin.</td>
<td>(Wollert <em>et al.</em>, 2007)</td>
</tr>
<tr>
<td><em>L.monocytogenes Δpflb</em></td>
<td>Pyruvate formate lyase mutant. Mutant incapable of a key Acetyl-coA formation, a key fermentation step</td>
<td>This study</td>
</tr>
<tr>
<td><em>L.monocytogenes Δldh</em></td>
<td>Lactate dehydrogenase mutant. Mutant incapable of converting pyruvate to Lactate. A key fermentation step</td>
<td>This study</td>
</tr>
<tr>
<td><em>L.monocytogenes ΔldhΔpflb</em></td>
<td>A Double mutant of Pyruvate formate lyase and Lactate Dehydrogenase. Mutant theoretically incapable of fermentation growth.</td>
<td>This study</td>
</tr>
<tr>
<td><em>L.monocytogenes ΔglnA</em></td>
<td>Glutamine synthetase mutant. Mutant incapable of synthesising glutamine de-novo.</td>
<td>This study</td>
</tr>
<tr>
<td><em>L.monocytogenes ΔgltAB</em></td>
<td>Glutamate synthase mutant. Mutant incapable of converting glutamine to glutamate.</td>
<td>This study</td>
</tr>
<tr>
<td><em>L.monocytogenes Δgdh</em></td>
<td>Glutamate dehydrogenase mutant. Mutant incapable of converting α-ketoglutarate to glutamate.</td>
<td>This study</td>
</tr>
<tr>
<td><em>L.monocytogenes ΔgldΔgltAB</em></td>
<td>Glutamate dehydrogenase and Glutamate synthase double mutant. Mutant auxotrophic for glutamate.</td>
<td>This study</td>
</tr>
<tr>
<td><em>L.monocytogenes ΔargD</em></td>
<td>Arginine deaminase mutant. Mutant incapable of interconverting arginine to citrulline. Potentially important in nitrogen regulation.</td>
<td>This study</td>
</tr>
<tr>
<td><em>L.monocytogenes ΔadeD</em></td>
<td>Adenine deaminase mutant. Mutant incapable of interconverting adenine to hypoxanthine. Potentially important in nitrogen regulation.</td>
<td>This study</td>
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<td>Primer</td>
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<td>Lmo0078F1</td>
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<td>Lmo0078XF</td>
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<tr>
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</tr>
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</tr>
<tr>
<td>M13 R</td>
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<td>N/A</td>
</tr>
</tbody>
</table>
3.2.3) DNA sequencing

DNA sequencing reactions were performed using BigDye (PE Biosystems) sequencing kit, following manufacturer’s instructions. Each reaction was 20 µl including: 300 ng of plasmid DNA, 3.2 µl of forward and reverse primer which has been diluted to a concentration of 1 pm/µl, 3 µl of sequencing buffer (10x) and finally 2 µl of sequencing dye. After the sequencing reaction a PCR purification and DNA precipitation was performed. The procedure involved transferring the sequencing reaction mix to larger micro centrifuge tube, then adding 5 µl of EDTA at 125 mM, 60 µl of 100% v/v ethanol. This mixture was left at room temperature for 15 minutes then centrifuged at 16000 g for 30 minutes. Next, 60 µl of ice cold 70% v/v ethanol was added and the mixture was centrifuged for a further 30 minutes at 16000 g. After the final centrifugation, the supernatant was carefully removed, ensuring DNA pellet is not displaced and the micro centrifuge tubes were dried.

3.2.4) Mini prep extraction of plasmid DNA from *E.coli*

For high purity plasmid DNA from bacterial cultures, the QIAquick miniprep kit (Qiagen) was used. The procedure was followed in accordance with the manufacturer’s instructions. 5 ml of bacterial overnight culture was pelleted by centrifugation at 3000 g for 10 minutes. The pellet was suspended in an RNAse containing buffer P1. P2 buffer was used to lyse the cells and this was neutralised with buffer. The sample was then extremely gently mixed to avoid contamination with chromosomal DNA. The mixture was centrifuged to remove all lysates at 16000 g for 10 minutes. The supernatant was removed and placed into spin columns and DNA was bound to this column membrane then washed with ethanol, which was subsequently removed entirely. Finally the DNA was eluted in either 30 µl or 50 µl of elution buffer (EB).

3.2.5) Restriction enzymatic digestions

All restriction enzymes used in the study were purchased from Roche. These were used in accordance with manufacturer’s instructions. These include enzyme specific digestion buffers, temperatures and length. A typical digestion would be
incubated at 37°C for at least 3 hours. Enzymes were denatured using heat treatment at 65°C for around 20 minutes. The digested DNA was subsequently purified using a Qiagen QIAquick PCR purification kit.

3.2.6) Ligation reactions

Vector DNA and fragment ligation involved the digestion of the DNA with the appropriate enzymes as described above. The concentration of the DNA was measured and the vector and the fragments were mixed in a 3:1 molar ratio. Ligations were usually performed in 10 µl volumes, with 1 µl (5 Units / µl) ligase and 1 µl ligation buffer (10x). The ligation was performed at room temperature for 3 hours or refrigerated overnight.

3.2.7) PCR/DNA purification

For preparation of high purity DNA following PCR, ligation or digestion a QIAquick (Qiagen) PCR purification kit was used, following manufacturer’s instructions. The purification involved binding the DNA with 5X volume of binding buffer PB. This mixture was applied to the provided columns and the DNA was bound to the column membrane. DNA was then washed with ethanol containing buffer, ethanol removed and then eluted using elution buffer EB in either 50 µl or 30 µl depending on final concentration required. If more concentrated DNA was required, special MiniElute (Qiagen) columns allowed elution in a 10 µl volume.

3.2.8) DNA concentration quantification

DNA was routinely quantified using a NanoDrop ND1000 machine. 1µl of water was loaded to calibrate the machine, followed by 1 µl blank (usually the solution DNA has been eluted into) and finally 1 µl of the sample to be quantified.

3.2.9) Agarose gel electrophoresis

Agarose gel electrophoresis was carried out according to the guidelines of Sambrook and Russel (2001). The DNA sample was prepared by mixing with 10x DNA buffer, which helps to keep the sample in the wells and also facilitates the
migration of the sample through the gel. Samples mixed with buffer were applied to the wells and then run at 110 V in TAE buffer with ethidium bromide. This was done for an appropriate period of time, allowing good separation of the samples. A marker ladder was routinely added to identify band size (Sambrook and Russell, 2001).

3.2.10) Agarose gel purification

When separation of a DNA sample was necessary a DNA extraction was performed. The sample was run on an agarose gel as was described in section 3.2.9. The required sample band was cut from the gel under UV light. It was then extracted using a gel extraction kit (Qiagen). The gel sample was fully dissolved at 65°C in buffer QG and then applied to the column provided. Sample was bound to the column membrane by centrifugation at 16000 g for 1 minute. The column was washed with ethanol buffer PE to remove any residual agarose and other electrophoresis contaminants. Samples were eluted in 30 µl elution buffer (EB).

3.2.11) Visualisation of agarose gels

Gels were visualised using a UVI-tec UV docking system. Ultra violet light was used at the appropriate intensity to provide a clear picture, which was then manipulated using the system as desired.
3.3) Competent bacterial cell preparation

3.3.1) Competent *E.coli* cell preparations

An 2ml overnight culture of *E.coli* DH5α was inoculated into 200 ml of fresh LB medium supplemented with 30 mM MgCl$_2$. Cells were grown to mid log phase (OD$_{600}$ 0.4 - 0.6). The flask was then put on ice to slow growth, and cells were then transferred to 50 ml falcon tubes and centrifuged in a pre-cooled centrifuge at 3000 g, 4 °C for 15 minutes. The supernatant was carefully discarded and the remaining pellet was re-suspended in 12.5 ml of cold TFBI solution (see below). The cells were then incubated on ice for 10 minutes and centrifuged down again at 3000 g for 15 minutes. The TFBI supernatant was carefully discarded and pellet was re-suspended in 500 µl of cold TFBII solution (see below). Cells were ready to be used and were transferred to pre cooled micro centrifuge tube and snap frozen in liquid nitrogen.

TFBI solution – 294.5 mg potassium acetate, 121 mg rubidium chloride, 220 mg calcium chloride and 1 g manganese chloride were dissolved in 65 ml milli-Q water. 15 ml glycerol at a pH of 5.8 with 0.2 M acetic acid was added. The solution was made up 100 ml with sterile water then filter sterilised (0.2 µm filter) into two 50 ml falcon tubes.

TFBII solution – 209 mg MOPS, 1.65 g calcium chloride and 121 mg rubidium chloride were dissolved in 65 ml milli-Q water. 15 ml glycerol was added then the solution is pH t0 5.8 with 0.2 M acetic acid. The solution was made up 100 ml with sterile water, then filter sterilised (0.2 µm filter) into two 50 ml falcon tubes.

3.3.2) Competent *L.monocytogenes* cell preparations

An overnight 5 ml culture of *L.monocytogenes* was inoculated at 1:100 into 200 ml flask of TSB /sucrose (20% w/v) and was grown with shaking at 37°C for around 4 hours or to OD$_{600}$ of 0.2. 100 µl of 20 mg/ml penicillinG was added to the flask and was returned to incubator with shaking for additional 2 hours. The cells were transferred to 50 ml falcon tubes and centrifuged at 3000 g, 4 °C for 20 minutes. The supernatant was discarded and cell pellet was re-suspended in 25
ml cold HEPES sucrose solution (1 mM HEPES, 0.5 M sucrose, pH 7.0). The supernatant was discarded and cells were suspended in 25 ml fresh HEPES sucrose solution. This step was repeated once further and the final pellet was re-suspended in 250 µl of HEPES sucrose solution. Cells were ready to use and were used within a week.

3.3.3) Transformation of competent cells

3.3.3.1) Transformation of competent *E.coli* cells

An aliquot of competent cells (100 µl) was thawed on ice and around 50 ng of plasmid DNA was added to it. A second control aliquot was thawed. The cells were incubated on ice for 30 minutes and then heat shocked at 42°C for 60 seconds. After heat shock cells were returned to ice for 5 minutes and then 800 µl of sterile LB broth was added. Finally, cells were incubated at 37°C for 60 minutes and the mixture was plated onto agar plates supplemented with appropriate antibiotics. Plates were incubated overnight; ensuring control plates were clear and colony PCR was used to check any transformants.

3.3.3.2) Transformation of competent *L.monocytogenes* cells

To a cold electroporation cuvette (0.2 mm gap) 1 µg DNA (approx. 50 ng) was added (in a volume up to 10 µl). To this, 100 µl of competent *L.monocytogenes* cells were added. A second cuvette with just cells was prepared as a negative control. Cells were then electroporated using Gene pulser (BioRad), at 200 Ω, 25 µF and 2.5 kV. Immediately after electroporation 1ml of TSB was added cuvettes which were transferred to a 37°C incubator for 3 hours. Finally, the cells were plated onto TSB erythromycin plates and incubated for 48 hours at 37°C. Plates were parafilm covered to avoid drying and PCR was performed to establish positive transformants.

3.4) Knockout mutagenesis of wild type *L.monocytogenes* using homologous recombination

The following describes the protocol for generating a knockout mutant of *L.monocytogenes*. After PCR amplification of two flanking regions of the required
gene ~ 600 bp in length, were ligated, such that most of the gene was missing (See Fig. 3.1). Ideally, after fusing the two fragments together, there should be a few codons from the beginning of gene and the end of that were in the same reading frame as each other, creating an ‘in-frame deletion’ (see Fig. 3.1). When replacing the genes on the L.monocytogenes chromosome with the construct, a short, non-functional polypeptide will be generated instead of the functional gene. This should minimise the impact on downstream or upstream genes or regulatory regions. This was particularly important if for example, the first gene in a large operon to avoid interfere with the expression of the other genes in that operon. The plasmid into which the fragments were cloned was pAUL-A. See Fig. 3.2 for details and advantages for using pAUL-A vector (Chakraborty et al., 1992).

In order to insert the amplified DNA fragments into this plasmid, restriction enzyme sites were engineered into the primers (example shown in Fig. 3.1). Both the plasmid and the PCR amplified fragments were digested with the appropriate restriction enzyme.

Once the fragments were cloned into pAUL-A, the sequence was checked using plasmid specific primers M13-F and M13-R (see Table 3.4). If the sequence was correct the plasmid was transformed into L.monocytogenes (see section 3.3.3.2). Successful transformants were re-streaked onto erythromycin plates and incubated at 42°C. A few colonies were picked and re-streaked a second time at 42°C on erythromycin. At this point the plasmid should have stably integrated into the chromosome. This was checked by PCR (plasmid and chromosome specific primers).
Fig. 3.1 Schematic of a mutagenesis by homologous recombination

1) Primers were designed to amplify flanking regions approximately 600 bp, up and down stream of target gene with appropriate restriction sites
2) Flanking regions PCR amplified
3) Flanking regions digested with appropriate enzymes
4) Ligation step 1 – the flanking regions were fused together by restriction site C
5) Ligation step 2 – Flanking regions were inserted into digested pAUL-A
   Steps 4 and 5 can be done in tandem
6) Vector transformed into L.monocytogenes and mutation was selected for in presence of erythromycin
7) Sequential sub-culturing in absence of erythromycin – resulting in either the wild type gene or dysfunctional mutant. Mutant confirmation by colony PCR using primers up and down-stream of ‘P1F’ and ‘P2R’
**Fig. 3.2 Vector map of pAUL-A Vector**

- Replicates in *E. coli* and in *L. monocytogenes*
- Replication in Listeria was temperature sensitive (≤30°C)
- Confers erythromycin resistance (300 µg/ml in *E. coli*, and 5 µg/ml in *L. monocytogenes*) (Em<sup>R</sup>)
- lacZ promoter (lacZ)
- Carries a multiple cloning site from plasmid pUC19 (MCS)
- Cloning site was sequenced using M13F and M13R

(Adapted from Chakraborty et al., 1992)
Two individual colonies were grown in liquid at 30°C in the absence of selection, and were passaged ~7 times (these were grown to saturation and back-diluted 1:100). The last culture was serially diluted, and plated onto TSB in the absence of selection at 42°C. Well-isolated individual colonies were taken and patched onto TSB plates supplemented with 300 µg/ml and then duplicated onto TSB plates in the absence of antibiotic. Plates were then incubated at 42°C. Patches showing growth on TSB but not erythromycin were double-recombinants and were either the mutant or have reverted to wild type. Double colonies were re-streaked at 37°C and were screened by PCR using chromosome-specific primers that will be able to differentiate between wild type/mutant.

3.4.1) T tagged cloning

To avoid problems with restriction enzyme digestion of PCR products, PCR products were directly cloned following ‘A’ tailed into a commercially available T tailed pGEM T easy vector (Promega). A tailed the insert was automatically achieved by incubating the insert with a small quantity of Taq polymerase. This essentially adds an extra ‘A’ residue either end of the insert. The insert was then ligated into an appropriate vector such as pGEM T Easy Vector system (Promega). The insert was removed from the vector using appropriate restriction enzymes. The fragment was gel extracted and was guaranteed to be cut at both ends. This was then be cloned into the original vector.

3.5) Mammalian cell culture and metabolomic analysis

3.5.1) Preparation of L.monocytogenes EGDe InIA cells for infection

An overnight culture of EGDe InIA L.monocytogenes grown in TSB was set up. The Overnight culture was used to inoculate 100ml of TSB. Cells were then grown at 37°C until an OD$_{600}$ of 0.6 has been reached. Cells were then transferred to two 50 ml falcon tubes (cooled) and were centrifuged at 3000 g for 15 minutes at 4°C. Supernatant was discarded and then cells were re-suspended in PBS to wash them. Cells were centrifuged down again under the same conditions. This washing process was repeated further two times. After final centrifugation, cells were re-suspended in 15% v/v glycerol PBS (around 2 ml)
cells were then aliquoted into micro centrifuge tube (100 µl) and snap frozen in liquid nitrogen. Cell concentration was calculated by viable count. Cells were ready to use.

3.5.2) Viable cell counts

Viable counts were performed using the Miles and Misra technique (Miles et al., 1938). Serial dilutions of cells were plated out in three drops of a known volume, which were allowed to soak on the agar plate and dry. Agar plates were incubated overnight at 37ºC. Colonies were counted the following day and were multiplied by the dilution factor and also the volume of the drops to give colony forming units (CFU) per ml.

3.5.3) Maintenance of HeLa cells

Human cervical cancer cell line HeLa was routinely grown in Dulbecco’s modified Eagles medium as described in Table 3.3 (DMEM purchased from Gibco Invitrogen or Sigma Aldrich). DMEM was supplemented with 10 % v/v foetal bovine serum (FBS Invitrogen) and 4 mM L-glutamine (Gln) (Invitrogen) to make ‘complete medium’. The cells were grown in a microaerophilic chamber under 5% CO2 at 37ºC. Cells were grown until 80 % confluent and were then passaged. The cultures to be passaged were washed with Dulbecco’s Phosphate Buffered Saline (DPBS) and were trypsinised to detach cells using 0.05 % v/v trypsin EDTA for around 5 minutes. After trypsinisation the cells were quenched using an equal amount of ‘complete medium’. Usually cells were passaged at 1/5 to 1/20 ratios depending on new flask size and also time the cells were required. Trypsinisation was kept to a minimum as this selects for trypsin resistant cells and causes the passage count to increase too quickly. This volume was transferred to a new flask with fresh complete medium, thus completing the passaging process. HeLa cells were carried for a total of 20 passages from the original stock cells.
3.5.4) Freezing for storage and revival of HeLa frozen stock

HeLa cells were stored in liquid nitrogen, in freezing agent, which contains filter sterilized 80% v/v FBS and 20% v/v DMSO. Cells were trypsinised as described in section 3.5.3 but quenched with ice cold DMEM (no FBS/Gln). Cells were pelleted by centrifugation at 3000 g at 4 ºC. Pellet was resuspended in small amount of ice cold FBS. To this the prepared cryopreservant was added (80 % v/v FBS and 20% v/v DMSO) at a final concentration of 10 % v/v DMSO. Cells were aliquoted and slow frozen in a Styrofoam box with isopropanol -80 ºC overnight. Samples were then transferred to liquid nitrogen.

To thaw, cells were almost defrosted at 37 ºC. Cells were then transferred to a prepared flask with complete medium. Cells were added drop wise and were simultaneously mixed with gentle shaking. The following day cells are washed with warm PBS and fresh medium was added to remove residual cryopreservant.

3.5.5) Intracellular survival assay of *L. monocytogenes* in HeLa

Intracellular infection of *L. monocytogenes* for the purpose of growth analyses was done to the following protocol. Firstly the *L. monocytogenes* stocks for the infection were prepared as was discussed previously in section 3.5.1. HeLa cells were seeded into 6 well plates (Greiner) at 1.5x10^5 cells per well. The seeding density triples overnight and this was checked accurately by trypsinising and counting a random sample the following day.

Prior to the infection cells were washed twice with PBS. Thawed aliquots of *L. monocytogenes* (cell count known) were pipetted into DMEM at the desired Multiplicity of Infection (MOI). This was the number of bacteria per mammalian cells. So an MOI of 10 equals10 times the number of *L. monocytogenes* to mammalian cells. The bacteria containing medium was then added to the HeLa cells, and incubated in a microaerophilic chamber at 37 ºC for two hours, allowing uptake of bacteria. Following incubation for two hours, cells were washed three times with PBS and the medium was replaced with DMEM with10 µg/ml gentamicin (Invitrogen). This killed any extracellular bacteria and also prevented reinfection of HeLa cells. The experiment was started with a T0 timepoint taken.
At each time point, medium was removed; cells were washed three times with PBS and lysed with sterile 1 ml of ice-cold 0.5% v/v triton X-100 for at least 5 minutes. Lysates were collected and a viable count was performed. Each timepoint consisted of viable counts from three well replicates.

3.5.6) Infection of HeLa cells extracellular metabolomic studies

A confluent flask of HeLa cells was prepared and the following was the infection protocol for metabolomic studies.

Cells were trypsinised as described in section 3.5.3, with 7ml of TE and 13 ml of DMEM+FBS medium. The amount of cells that were needed for the experiment was calculated by transferring 10 µl of the trypsinised cells to a counting chamber. Medium was added to flaks until cells were at a concentration of 7.5x10^4 cells/ml. When at this concentration 2 ml of this cell suspension was put into each well of a 6 well plate. These plates were left overnight in a micro-aerophilic chamber at 30°C. One well from the 6 well plates was trypsinised and cell number was counted. A micro centrifuge tube of L.monocytogenes (EGDe InLA) cells for infection was thawed on ice and the appropriate amount of cells was added to DMEM. Cells were calculated depending on MOI required. For the purpose of the metabolomic analyses done in this study an MOI of 10 was used. The L.monocytogenes containing medium was then incubated at 35°C along with PBS and DMEM for around 15-20 minutes. Wells were washed with PBS twice. 2 ml of DMEM containing L.monocytogenes (at the appropriate MOI) was added to each well and incubated for 2 hours. This was the incubation time when L.monocytogenes will invade HeLa cells. During this time DMEM containing 10 µg/ml gentamicin was prepared and pre warmed to 35°C. After 2 hours, cells were washed twice with PBS and 2 ml of gentamicin containing DMEM was added to each well. This step ensures that any L.monocytogenes that have not infected HeLa cells or were remaining in the medium were killed and inactive. The T0 time point was taken by taking 1 ml of medium from well and filter sterilizing to remove any cells and then snap freezing in liquid nitrogen. This was repeated at each desired time point. The same infection methodology can be applied to larger T162 flasks. Snap frozen samples were then stored at -80°C until they were to be analysed by GCMS.
3.5.7) Infection of HeLa cells for intracellular metabolomic studies

To analyse the intracellular metabolome of HeLa and the effect of *L. monocytogenes* infection, cells were seeded as in the extracellular methodology described earlier. The cells were seeded in T162 flasks. Cells were infected with an MOI of 10 and cells were harvested at T10 (10 hour) timepoint. Cells were washed 3 times with PBS then extracted in 3 ml of -65°C 100 % v/v methanol. Cells were harvested by scraping and these samples were freeze thawed by liquid nitrogen and thawing on dry ice 3 times. Cell debris was removed by centrifugation at 16000 *g* for 10 minutes at 0°C, followed by collection of supernatant. Samples were stored at -80°C until analysis with GCMS.

3.6) Gas chromatography mass spectrometry (GS-MS)

Metabolomic by GC-MS was collaborated with Warrick Dunn, University of Manchester and CADET (centre for advanced discovery and experimental Therapeutics). The analysis was conducted using the following protocol.

A two stage chemical derivitisation was performed for all samples. Derivitisation allows the sample to be chemically modified to a form, which allows better separation, introduction into the MS machinery and improved sensitivity and response (Halket *et al.*, 2005). 50 μl of a 20 mg/ml solution of O-methoxylamine in pyridine solution was added to all dried samples followed by vortex mixing for one minute and heating at 60 °C for 30 minutes. 50 μl of N-acetyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was added after 30 minutes samples were then vortex mixed for one minute and then heated to 60 °C for 30 minutes. 20 μl of a retention index solution (0.5 mg/ml C_{10}, C_{12}, C_{15}, C_{19}, C_{22} n-alkanes dissolved in pyridine) was added after the derivatisation solution had cooled to room temperature and the solution was centrifuged (13,000 *g*, 15 minutes). 90 μl of each derivatised solution was transferred to glass vials containing 300 μl inserts and sealed with septum containing caps.

Samples were then randomly analysed within 48 hours after derivitisation. An Agilent 6890 gas chromatograph and 7673 auto sampler (Agilent Technologies,
Cheadle, UK) coupled to a LECO Pegasus III electron impact-time of flight mass spectrometer (LECO, Stockport, UK) was used for the analysis. 1 µl of derivatised solution was injected into the GC injector operating at 250°C applying a carrier gas (helium) at a flow rate of 1.0 ml/minute.

Chromatographic separations were performed on a DB-50 GC column (30 metres, 0.25 mm i.d, 0.25 µm film thickness; Supelco, Gillingham, UK) was a temperature program of 70 °C, hold for 4.5 minutes followed by a 20 °C/minute temperature ramp to 300 °C and held for 4.5 minutes. The effluent was introduced via a transfer line in to the mass spectrometer.

Data acquisition was performed in the m/z range of 50-600 at an acquisition rate of 15 Hz and a detector voltage of 1650 V. The transfer line and source temperatures used were 250 °C and 230 °C, respectively. Instruments were controlled and data acquisition was automated applying Chromato software v2.15 (LECO, Stockport, UK). Metabolite responses were normalised to an internal standard present in all samples at the same concentration (0.24mg/mL succinic d₄ acid). Metabolite peaks were then analysed for identification.

To provide putative or definitive identification of metabolite peaks, the mass spectrum and retention index associated with each metabolite peak was searched against mass spectral libraries including an in-house library - Manchester Metabolomics Database, metabolite-specific library from the Golm metabolome database or the commercially available NIST08 library. For cases where the mass spectrum (at least 70% match) and retention index (a match of +/- 10) for the metabolite peak matched to metabolites present in the Manchester Metabolomics Database then the metabolite identification was reported as definitive (Metabolomics Standards Initiative level 1 (4)). For cases where the mass spectrum (match>70%) only for the metabolite peak matched to metabolites present in the Golm Metabolome Database or NIST08 libraries then the metabolite identification was reported as putative.
3.6.1) Data analysis and MatLab

Data for the metabolomic studies was analysed using MatLab version R2009a and also Microsoft Excel 2010. The data analysis methods used principle component analysis (PCA), Students T Test, discriminate function analysis (DFA), box whisker plot analysis and analysis of variance (ANOVA).

Doubling times for all growth curves were calculated using online tool located at http://www.doubling-time.com/compute.php (Roth V. 2006)

Wilcoxon rank sum tests were conducted with the online tool located at http://elegans.som.vcu.edu/~leon/stats/utest.html.

3.7) Protein purification of Lmo0355 fumarate reductase

The initial steps in the purification of \textit{Lmo0355} involved cloning the gene into a his-tag vector, such as pQE-30 (Qiagen) or pET24 (Novagen). The gene was first PCR amplified using forwards/reverse primers flanking the \textit{Lmo0355} gene (see Table 3.1), using \textit{L.monocytogenes} colony for a template. The primers have appropriate restriction sites engineered into them at the 5’ and 3’ extremes. The gene was amplified using a high fidelity polymerase such as Pfu (Roche) to avoid base errors. Once amplified, the fragment was purified, digested with appropriate restriction enzymes and then ligated into the appropriate his-tag vector, which also had been digested with corresponding restriction enzymes. Once insert was correctly cloned into the vector, the insert was sequenced to ensure perfect gene sequence and then transformed into a suitable expression bacteria such as \textit{E.coli} BL21(AI). Bacterial starter cultures of the transformed BL21(AI) were grown overnight in LB supplemented with the appropriate antibiotics at the appropriate temperature. Overnight cultures were diluted 1:100 into fresh sterile LB broth and grown at 37°C, 200 rpm shaking, until an OD$_{600}$ 0.4 – 0.5 was reached. Protein expression was then induced by adding 0.2 % v/v 1mM arabinose and 1 mM IPTG to the medium and incubating the cultures for 3 hours at 37 °C, 200 rpm shaking. Cells were collected by centrifugation at 3000 g for 20 minutes. The cell pellet was stored at -20 °C.
3.8) French press cell lysis

Cell pellets after were resuspended in an appropriate volume of ice-cold French press buffer (20 mM Tris, 150 mM NaCl, 20% v/v glycerol pH 8.0) supplemented with protease inhibitor cocktail (Sigma). Cell suspensions were passed twice through a pre-cooled French pressure cell press (Thermo Electron Corporation) at 9000 psi. The cell debris was removed by centrifugation at 10000 g for 10 minutes. Samples were stored on ice and were used immediately for purification to avoid degradation of protein.

3.9) Nickel column protein purification of histidine-tagged proteins using imidazole

Histidine-tagged proteins were extracted from cellular lysates as follows. French press cell lysates were mixed with TritonX100 to a final concentration of 0.2% v/v on a rocker for 30 minutes in the cold room at 4ºC. 1 ml His-Trap FF nickel columns (GE Healthcare) were washed with 5 column volumes of equilibration buffer (50 mM Tris, 500 mM NaCl, 10 mM imidazole pH 8.0). The load was then gradually passed through the column and the effluent was collected (designated flow through). The columns were washed with 5 column volumes of wash A (50 mM Tris, 500 mM NaCl, 10 mM imidazole pH 8.0), 5 column volumes of wash B (50 mM Tris, 500 mM NaCl, 50 mM imidazole pH 8.0), and then wash C (50 mM Tris, 500 mM NaCl, 100 mM imidazole pH 8.0). Wash C samples were collected in 0.5ml fractions. The remaining protein was eluted with elution buffer (50 mM Tris, 500 mM NaCl, 250 mM imidazole pH 8.0) and collected in 0.5 ml fractions at 1 ml/min flow rate (designated E1 – E10). Purification samples were stored on ice until further use. To increase protein concentration the protein solution was either dialyzed or run through a spin column.

Samples were then analysed by Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS PAGE) and Western blot (WB) analysis.
3.10) SDS-PAGE

Proteins were separated by SDS PAGE with the Mini Protean 3 Electrophoresis System (Bio-Rad). Table 3.6 shows the composition of stacking and resolving gels. A resolving gel with a defined polyacrylamide concentration according to the size of the proteins to be examined was used. Higher polyacrylamide concentrations were used to separate smaller proteins.

The resolving gel was topped with a layer of isopropanol, which was removed after complete polymerisation of the gel. This helped provide a uniform gel whilst minimising bubbles. The stacking gel was then poured on top of the resolving gel and allowed to set. Protein samples were resuspended in 10x SDS-loading buffer (100 mM Tris pH 6.8, 2% v/v SDS, 20% v/v glycerol, 0.02% v/v bromophenol blue, 0.7% v/v β-mercaptoethanol) and were boiled for 5 minutes. Pre-stained molecular weight markers (Bio-Rad) were run alongside the samples to provide a reference of sample size. Samples were electrophoresed in SDS-PAGE running buffer (25 mM Tris base, 192 mM glycine, 0.1% w/v SDS, pH 6.8) at 80V through the stacking layer of the gel and then for 180 V until good separation was achieved alternatively the gel could be run 120 V all the way through to completion.

Gels were visualized by staining with Coomassie staining solution (0.8 g L⁻¹ Coomassie Brilliant Blue R-250, 40% v/v methanol, 10% v/v acetic acid, 50 v/v sterile distilled water)
<table>
<thead>
<tr>
<th>Component</th>
<th>Stacking gel</th>
<th>Resolving gels</th>
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<tbody>
<tr>
<td></td>
<td>5% stacking gel</td>
<td>8% resolving gel</td>
</tr>
<tr>
<td></td>
<td>2 gels, 2.5 ml/gel</td>
<td>2 gels, 5 ml/gel</td>
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<tr>
<td>1.0 M Tris, pH 6.8</td>
<td>0.63</td>
<td>/</td>
</tr>
<tr>
<td>1.5 M Tris, pH 8.8</td>
<td>/</td>
<td>2.5</td>
</tr>
<tr>
<td>30% v/v polyacrylamide</td>
<td>0.83</td>
<td>2.7</td>
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<tr>
<td>Sterile distilled water</td>
<td>3.4</td>
<td>4.6</td>
</tr>
<tr>
<td>10% w/v sodium dodecyl sulphate (SDS)</td>
<td>0.05</td>
<td>0.1</td>
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<tr>
<td>10% w/v ammonium persulfate (APS)</td>
<td>0.05</td>
<td>0.1</td>
</tr>
<tr>
<td>N,N,N,N'-tetramethylenediamine (TEMED)</td>
<td>0.005</td>
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**Table 3.6 Compositions of SDS PAGE gels**

Table highlights the necessary components of a SDS page gel. TEMED and Acrylamide were only added shortly before pouring the gel. Resolving gels are poured first, allowed to set and the stacking gel was added after with the gel combs in order to provide sample wells. The % resolving gel used was dependent on the size of the protein analysed with smaller % gels used for larger proteins. Once set the gels were ready to use.
3.11) Western blot

For the purpose of Western blotting, proteins separated by SDS-PAGE (section 3.12) were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Westram S, Whatman). Prior to transfer, the membrane was pre-soaked in methanol for at least 1 minute and then soaked in western transfer buffer (48 mM Tris, 40 mM glycine, 20% v/v methanol) (2.9g Tris, 1.5g glycine, 100 ml methanol and make to 500 ml with water) for at least 10 minutes. The SDS-PAGE gel and 4 pieces of Whatman 3MM paper were soaked in Western transfer buffer for at least 20 minutes. The following layers were then placed onto a Semi-Dry Transfer Cell (Bio-Rad) (from bottom to top): two pieces of Whatman 3 MM paper, the PVDF membrane, the SDS-PAGE gel, and two pieces of Whatman 3 MM paper. Proteins were transferred at 15 V for 20 minutes.

3.11.1) Visualisation of Western blot by Licor IRDyes

The membrane was prepared for visualisation by the following steps: the membrane was blocked by incubating in PBS with 5 % milk overnight and rocking at 4 °C (or 1 hour with rocking at RT). The membrane was washed in washing buffer (PBS, 0.1% tween 20, 5% milk) for 10 minutes, three times. Membrane was sealed in a bag and incubated with 6 ml of washing buffer with 1:1000 penta-his antibody for 1 hour at room temperature, with rocking. Alternatively, this step was performed with rocking overnight. Membrane was washed 3 timed in washing buffer for 10 minutes. Membrane was sealed in bag and incubated with 6ml of PBS-Licor blocking buffer (1:1) supplemented with appropriate secondary antibody (1:2000 IRDye 650 anti-mouse IgG). This was done for 1 hour at room temperature, with rocking, in darkness. Membrane was washed 3 times again in washing buffer for 10 minutes in darkness. Tween was removed by washing in PBS three times in darkness. The membrane was ready for visualisation using Odyssey scanner/software. One visualised the membranes were stored in darkness in PBS at 4 °C.
3.12) Methyl viologen assay of purified protein

The kinetic properties of potential fumarate reductase lm00355 were established by modifying a protocol used by Turner (1996). The fumarate dependent reoxidation of reduced methyl viologen was monitored at 600 nm. The assay buffer contained 0.45 M NaCl, 0.05 M HCl, and 0.2 mM methyl viologen at pH 7. Experiment was initiated by adding 0.6 mg of protein to the assay. The assay was performed under complete anaerobic conditions using a glove box and the spectrophotometer kinetic system CARY was used to determine initial rates at different concentrations.
Chapter 4
Anaerobic growth of *L. monocytogenes* in defined media
4.1) Introduction

For standard laboratory purposes *L. monocytogenes* is usually grown in nutritionally rich media, such as, tryptone soya broth (TSB) or bovine brain heart infusion (BHI) (Phan-Thanh, 1997). These media support growth of *L. monocytogenes* for a variety of different experimental purposes. However, these media have a critical flaw for the purposes of metabolomics or any study, which requires the composition of the medium to be defined. These complex medium contain a mix of nutrients that are not entirely known and also non-reproducible from batch to batch. For the purposes of metabolomics and for ascertaining changes in specific or trace metabolites this is not adequate. This necessitates a medium which is reproducible across experiments and is completely defined.

There are a number of synthetic minimal media, which have been produced for *L. monocytogenes* growth (Phan-Thanh, 1997, Tsai and Hodgson, 2003, McFeeters and Chen, 1986). These support a limited growth of *L. monocytogenes* in comparison to complex media, but can be reproduced from one experiment to the next. The focus of this study was to determine the growth rates of *L. monocytogenes* in a GDM (Romick *et al.*, 1996) and MD10 minimal medium (Trivett, 1971) and establish which medium is better for growth of *L. monocytogenes*. These particular media were chosen because MD10 was routinely used in our laboratory as a defined medium and GDM was documented to be capable of supporting anaerobic growth of *L. monocytogenes*. The study was of also of particular interest due to the prospective metabolomics which would be conducted in this study. See Table 3.1 – Table 3.3 for media compositions.

The main objective was to test the growth rates for *L. monocytogenes* strain EGDe InIA in GDM and MD10 minimal media and establish their ability to grow *L. monocytogenes* in reference to a complex medium like TSB. A particular emphasis was placed on the ability to grow *L. monocytogenes* anaerobically.
4.2) Results

Fig. 4.1 shows the growth yield, represented by end point optical density at wavelength 600 nm (OD\textsubscript{600}) values of \textit{L. monocytogenes} grown under aerobic conditions. The samples were inoculated 1:100 (v/v) from an overnight culture of \textit{L. monocytogenes} grown in TSB and their OD\textsubscript{600} was measured at 10 hours post inoculation at one hour intervals. Samples were grown at 37 °C with shaking at 200 rpm. The growth yield for EGDe InlA \textit{L. monocytogenes} in GDM was higher than those from MD10 medium. However, the growth yield for GDM is lower in comparison to the complex medium TSB (Fig. 4.1).

Initially the GDM was prepared following the protocol directed by Romick \textit{et al.}, (1996). However, after numerous unsuccessful attempts to grow EGDe InlA \textit{L. monocytogenes}, the decision was taken to modify the medium. The vitamin solution was considered to be the likely source of the problem as the quoted concentrations of vitamins and cofactors seemed extremely low in comparison to those found in other minimal media. The GDM vitamin solution was replaced with the vitamin solution used in MD10 medium (Table 3.2). Once this change had been made the resultant growth yields for GDM were higher and exceeded those gained from growth in MD10 (see Fig. 4.1).

Although it was established that GDM and MD10 media were capable of supporting \textit{L. monocytogenes} growth under aerobic conditions, another aspect of this study was to find a minimal medium that supports anaerobic growth of \textit{L. monocytogenes}. Studies in the past have shown that some minimal media are able to support anaerobic growth (McFeeters and Chen, 1986, Romick \textit{et al.}, 1996). However, this could not be replicated with the EGDe InlA \textit{L. monocytogenes} strain used in our laboratory.

Fig. 4.2 shows the overnight anaerobic (10 hours post inoculation) growth yields of modified GDM in comparison to TSB. The results highlight that the GDM cannot support comparative growth of \textit{L. monocytogenes} with significantly lower OD\textsubscript{600} values in comparison to those grown in TSB.
Fig. 4.1 Minimal media aerobic growth yield for EGDe InIA L. monocytogenes
The growth curves for three different media (TSB, MD10 and GDM) grown under identical conditions are shown. The experiment was conducted in replicates of 6, which were then averaged. Samples were grown at 37°C with shaking at 200 rpm in sterile flasks. The samples were inoculated 1/100 v/v from an overnight culture (EGDe InIA L. monocytogenes) and the next morning readings were taken at one hour intervals. The readings were taken until bacterial growth reached their stationary phase. The growth rates obtained from the GDM follow a similar trend to those from MD10 but the bacteria reach a lower OD$_{600}$. Both minimal media show attenuated growth in comparison to TSB complex medium.
Anaerobic growth OD₆₀₀ values for GDM and DMEM are shown in comparison to TSB. Each value represents the average of 6 replicates. Samples were grown at 37°C in sterile syringes. The bars 1 and 2 show the direct comparison of TSB and GDM. The bars 3 - 5 represent GDM supplemented with varying amounts of TSB medium. Bar 6 represents sequentially subcultured GDM. GDM is unable to support sequential anaerobic growth. The addition of salt to GDM does not endow GDM the ability to support anaerobic growth either (9). DMEM medium was also incapable of supporting anaerobic growth of *L. monocytogenes* growth (7) even with the additional supplementation of lipoic acid and biotin (8).
In order to ascertain whether agar versions of the minimal media may be able to support growth better than liquid cultures, agar variants of the GDM and MD10 were produced and streaked with *L. monocytogenes* EGDe InlA. These were then grown in anaerobic chambers overnight. It was found that the solid agar version of minimal media exhibited growth for *L. monocytogenes*. The initial streaking was performed from TSB medium agar onto GDM agar. Re-streaking a second round from GDM agar onto GDM agar showed significantly less growth, with a third round of re-streaking showing no growth. This essentially highlighted that there was transfer of a vital nutrient/nutrients from TSB that allows *L. monocytogenes* to grow for a limited time anaerobically.

This finding was investigated further by growing *L. monocytogenes* in liquid GDM which had been supplemented with sterile TSB at different concentrations (1:100, 1:50 and 1:20). The results show that the amount of anaerobic growth seen was directly correlated with the amount of TSB supplementation (Fig. 4.2 bars 3-5). When inoculating GDM to GDM, to test sequential growth, almost no growth was observed (bar 6). This highlighted that there was a potential carryover of nutrient/s from TSB which facilitated anaerobic growth. The previous studies, which have indicated that minimal media are capable of anaerobic growth of *L. monocytogenes*, may have also been inoculating from complex medium.

DMEM was routinely used for tissue culturing work (see Table 3.3) (Sigma). This is a defined medium with known concentrations of metabolites and nutrients. To ascertain whether this medium could support *L. monocytogenes* growth, a number of experiments were done using this medium. The results show that the medium was not capable to support *L. monocytogenes* any significant growth (Fig. 4.2, bar 7). The addition of lipoic acid and biotin, which were notable omissions from DMEM, also did not increase growth yield (Fig. 4.2, bar 8).

Sodium chloride (NaCl) was added to the GDM to establish if this could anaerobic growth. This decision was taken as NaCl was found in TSB and other complex media which do support anaerobic growth of *L. monocytogenes* and it was an obvious omission from the minimal medium. NaCl is present in TSB at a concentration of 80 mM, hence, GDM was supplemented with 80 mM NaCl and inoculated 1:100 (v/v) with EGDe InlA *L. monocytogenes*. The results showed that
NaCl was not the missing nutrient facilitating anaerobic growth. Supplementing GDM with a similar concentration of NaCl found in TSB did not confer the ability for GDM to support anaerobic growth of *L. monocytogenes* (Fig 4.1, bar 9). This indicated that other nutrient or combinations of nutrients are facilitating the ability for *L. monocytogenes* to grow anaerobically in TSB.

4.3) Discussion

The minimal media studies which were undertaken are of significant importance as having a minimal medium which can support *L. monocytogenes* growth under both anaerobic and aerobic conditions would prove to be advantageous for the metabolomic aspects of the project.

Complex media such as TSB are largely undefined in terms of their carbon sources and their nutrients. This makes metabolic profiling between two samples very difficult as complex media can vary from one batch to the next. It also means that studies into specific or trace metabolites utilised become almost impossible as it cannot be determined exactly what was in the medium to begin with. This makes obtaining a minimal medium capable of supporting *L. monocytogenes* growth highly advantageous. The two minimal media that have been utilised were MD10 and GDM.

As mentioned earlier the, GDM had to be modified significantly from the original protocol mentioned in Mcfeeters and Chen (1986), as it did not support any growth of *L. monocytogenes*. Upon examining the makeup of the medium, the decision was taken to replace the vitamin and cofactor solution and replace them with the vitamin solution of MD10. The original vitamin solution had what seemed to be inadequate concentrations of essential vitamins and cofactors such as lipoate, thiamine, riboflavin and biotin. These are all essential for optimal *L. monocytogenes* growth as highlighted in a number of studies (Tsai and Hodgson, 2003, O'Riordan *et al.*, 2003). By replacing this vitamin solution with the vitamin solution in MD10 medium, GDM exhibited growth rates, which exceed those of MD10. A possible explanation for this was that GDM contains a number of components not found in MD10. These include the amino acids tryptophan
and histidine, which have been shown to be markedly stimulatory or even essential in some strains of *L. monocytogenes* (Phan-Thanh, 1997, Friedman and Roessler, 1961)

Although there are a number of studies which have documented minimal media supporting growth of *L. monocytogenes* under anaerobic conditions (Tsai and Hodgson, 2003), it was something that could not be replicated in our laboratory with the EGDe InlA *L. monocytogenes* strain. The results from our studies suggest that the anaerobic growth documented in these studies could possibly be a result of inoculating the media with bacteria grown in complex medium such as TSB. As such a possible ‘carry over’ of essential nutrients was predicted. Alternatively, the growth could be specific to the strain of *L. monocytogenes* used in those particular studies.

This ‘carry over’ effect first noticed when *L. monocytogenes* was streaked onto minimal agar. If the initial colony was from a TSB plate, the minimal medium agar showed growth of *L. monocytogenes*. However, subsequent re-streaking from minimal agar to minimal agar showed no growth. Consequently, this led to further work looking at the effect of supplementing minimal medium with sterile TSB. This showed there was a definite TSB dependent growth increase (See Fig. 4.2 bars 3-5). These results point to one of two possibilities. The more likely scenario was that the minimal media lack vital nutrient/nutrients that are available in TSB conferring the ability to support anaerobic *L. monocytogenes* growth. The other, less likely, possibility was that the minimal media contain a component that was toxic to *L. monocytogenes* under anaerobic conditions but not under aerobic conditions as these media support aerobic growth.

Another medium that was tested for support of anaerobic growth was DMEM. DMEM was routinely used in our laboratory for mammalian tissue culturing (see Table 3.3. for medium composition). Although not strictly a minimal medium, DMEM is defined and would go some way to alleviating the difficulties associated with complex medium and subsequent metabolomics.

Unfortunately, the results indicate that DMEM was also incapable of supporting significant *L. monocytogenes* growth either aerobic or anaerobic. This was initially
thought to be a lack of essential lipoic acid and biotin. However, supplementing DMEM with lipoic acid and biotin at concentrations found in MD10 still resulted in no growth of *L. monocytogenes*. Anaerobic growth yields at 10 hours are displayed in Fig. 4.2.

A DMEM specific complication was that the medium contained a pH indicator. This made DMEM unstable causing it to react and change colour when exposed to the air and any subsequent pH change as a result of the *L. monocytogenes* inoculum. This made standardising measurements of the medium difficult and OD$_{600}$ reading for growth could not be completely accurate. However, even taking this into consideration, DMEM show very little growth, hence the discrepancies in OD$_{600}$ values due to the pH indicator are of no consequence.

### 4.4) Conclusions and future work

To conclude the results from this study, GDM, as was described by Romick *et al.*, (1992) needed substantial adaptation to support growth of our strain EGDe InlA *L. monocytogenes*, largely due to inadequate concentrations of essential nutrients. Once these were added back into medium at suitable amounts, the resultant growth rate of the GDM exceeded that of MD10. As such, the modified GDM was a better minimal medium to use for any subsequent study.

It still proved impossible to grow EGDe InlA *L. monocytogenes* anaerobically in any defined media with glucose as a carbon source. True anaerobic growth in this study was defined as growth achieved through sequential sub culturing from minimal medium to minimal medium. This includes DMEM and modified versions of it. Studies, which have suggested the contrary, may be experiencing growth as a result of inoculating from complex medium or alternatively growth, specific to the strain used.

All of the anaerobic experiments conducted in this study used glucose as a carbon source. Further experimentation regarding the role of different carbohydrate sources during anaerobic growth could help to establish a minimal medium capable of anaerobic growth of *L. monocytogenes* EGDe InlA.
Chapter 5
Insights into the catabolic metabolism of
*L. monocytogenes*
5.1) Introduction

*L. monocytogenes* is able to grow under both aerobic and anaerobic conditions (Vazquez-Boland *et al.*, 2001). It is currently accepted that *L. monocytogenes* undergo aerobic respiration during intracellular growth, although this view is supported with limited experimental validation from intracellularly grown *L. monocytogenes* (Joseph and Goebel, 2007).

During glycolysis a single molecule of glucose is degraded to pyruvate, the process of which generates 2 molecules of NADH. Pyruvate is further degraded to acetyl co A, which is fed into the TCA cycle. To maintain the glycolytic flux it is essential to regenerate and replenish the cellular pool of NAD+. Most bacteria in the presence of oxygen regenerate their NAD+ pool through the electron transport chain utilising oxygen as a terminal electron acceptor. Alternatively, bacteria may use fermentation. Fermentation reactions are used for substrate level phosphorylation and are usually utilised in the absence of key electron acceptors support respiration (Tseng *et al.*, 1996).

Fermentation pathways vary among microorganisms, also in their key enzymes and end products. For example, lactic acid fermentation, ethanol fermentation, butric acid fermentation and butanol-acetone fermentation. Although there are many forms, all fermentation pathways operate to form ATP and NAD+ (Clark, 1989).

*E. coli* can utilise a sugar based mixed acid fermentation that generates end products such as lactate, acetate and ethanol. During anaerobic conditions, conversion of one molecule of pyruvate to lactate requires one molecule of NADH, which is oxidised via the enzyme lactate dehydrogenase (Ldh). Ethanol formation converts two molecules of NADH to NAD+ and requires acetyl-CoA. One route of acetyl-CoA production in bacteria under anaerobic conditions is from the conversion of pyruvate, and requires the enzyme pyruvate formate lyaseB (PflB) (Zhu and Shimizu, 2004).

Previous studies have shown that *L. monocytogenes* may utilise a mixed acid fermentation, when growing under anaerobic conditions (Romick *et al.*, 1996)
Taking this into consideration, a targeted approach using site directed mutagenesis was taken to elucidate key properties of \textit{L. monocytogenes} growth in terms of respiration and especially growth under anaerobic conditions.

In \textit{E. coli} removal of both \textit{ldh} and also \textit{pflb} prevents anaerobic growth on a range of sugars (Singh \textit{et al.}, 2009). Glycolysis is conserved across many bacterial species (Masters \textit{et al.}, 1987). Also genomic analysis reveals \textit{L. monocytogenes} possesses homologous genes. Thus the assumption was made that mutation of the \textit{L. monocytogenes ldh} gene would render the bacterium incapable of fermentative growth (see Fig. 5.1).

\textbf{5.2) Results}

\textbf{5.2.1) Mutagenesis for the analysis of anaerobic growth}

The first gene that was knocked out by homologous recombination was the proposed lactate dehydrogenase (\textit{ldh}) gene the \textit{lmo0078}.

In EGDe \textit{L. monocytogenes} there are a number of genes listed as L-lactate dehydrogenase. \textit{lmo0078}, \textit{lmo1057}, \textit{lmo1534} and \textit{lmo0078} (annotated as \textit{ldh}) are all listed as lactate dehydrogenases.

D-Lactate dehydrogenases possess a “G-X-G-X-G” motif near residues 150-160, which are common to most NAD-linked dehydrogenases (Bunch \textit{et al.}, 1997). These motifs are also highly homologous across most species and the motif was identified in the \textit{lmo0078} gene. The open reading frame for \textit{lmo0078} was most similar to phosphoglycerate dehydrogenase but it had a 46% similarity and 61% identity to \textit{Pyrococcus abyssi} “probable lactate dehydrogenase” (Corbett unpublished, 2012). This was targeted as the most probable D-lactate dehydrogenase, so this was the gene targeted for homologous recombination mutagenesis.
Fig. 5.1 Schematic diagram of *L. monocytogenes* fermentation pathway for knockout mutagenesis

The schematic highlighting the important fermentation pathways associated with the knockout mutagenesis. The crosses show the steps that were removed with homologous recombination mutant generation. *ldh* which converts pyruvate to lactate in *L. monocytogenes* and was an important pathway for NAD+ regeneration under anaerobic growth. If *L. monocytogenes* utilises this enzyme to grow anaerobically it was predicted that removing this pathway was incapable of anaerobic growth. The *ldh* mutant was capable of anaerobic growth removing pyruvate formate lyase B (*pflb*) also gives little attenuation. Hence, the double mutant knocking out both genes was produced. (Adapted from Agyei and Danquah, 2012)
The Δldh mutant was tested for its ability to grow anaerobically, as described in section 3.1.3. The experiments revealed little attenuation of growth for the Δldh mutant, in comparison to the wild type (WT) (Fig. 5.2).

The second mutant produced by homologous recombination was pyruvate formate lyase B (lmo1406) (Δpflb). As with the lactate dehydrogenase there are a number of annotated pyruvate formate lyases listed for L. monocytogenes pflA, pflB and pflC. The pflB gene was targeted due to previous work by Singh et al (2009) producing a fermentation deficiency in E.coli by removing this gene in tandem with ldh.

The Δpflb mutant was also capable of anaerobic growth, suggesting that formate and acetate production through PflB was not essential for anaerobic growth (see Fig. 5.2).

Since individual mutants, Δpflb and Δldh showed little attenuation of anaerobic growth, a Δldh Δpflb double mutant removing both genes was produced. This was predicted to be incapable of anaerobic growth by both, acetate auxotrophy and inability to regenerate the NAD+ pool. The double mutant also exhibited very little attenuation of growth in comparison to the WT (See Fig. 5.2).

Assuming that the correct genes were targeted, these results indicate that L. monocytogenes do not rely on mixed acid fermentation as is the case in E.coli. Instead, anaerobic respiration utilising an alternative electron transport is hypothesised.

5.2.2) lmo0355 putative fumarate reductase purification and assay

Fumarate reductases are enzymes catalysing the reversible reaction of fumarate to succinate in the electron transport chain. Fumarate could be utilised by L. monocytogenes as an alternative electron acceptor for anaerobic respiration. This could explain the lack of phenotype detected in the fermentation mutants.
Fig. 5.2 Analysis of anaerobic growth of fermentation mutants against wild type in TSB medium

The diagram shows growth curves from four strains of *L. monocytogenes*, wild type (WT), Δ*pflB*, Δ*ldh* and Δ*ldh*Δ*pflB* double mutant. All strains were grown anaerobically in TSB medium and the experiment was conducted in triplicates. The growth of the mutants was very similar to the WT, with a small attenuation for Δ*ldh* and Δ*ldh*Δ*pflB*. 
Bioinformatical analysis revealed a putative *L. monocytogenes* fumarate reductase gene, the *lmo0355* encoding a 54 kDa protein (discussed in greater detail in section 5.3). The protein was his-tagged by cloning the gene into a pET-24 vector, which incorporates a C-terminal his-tag. The expressed protein was confirmed by Western blot analysis, shown in Fig. 5.3.

Protein production was then induced from *E. coli* BL21(AI) cultures with and purified from whole cell lysates (see section 3.9). Coomassie stained SDS PAGE gels for the induction, elution, and final purified protein are shown in Fig. 5.3, Fig. 5.4 and Fig. 5.5.

The purified protein was assayed under complete anaerobic conditions by indirect measurement of activity, measuring oxidation of reduced methyl viologen (MV) dye as a change in absorbance.

### 5.2.3) Enzymatic assay of purified *lmo0355* putative fumarate reductase

The enzymatic properties of *lmo0355* putative fumarate reductase were measured using a protocol modified from (Turner et al., 1999). The assay was dependent on the oxidation of methyl viologen (MV) dye which was measured as a direct change in absorbance. When reduced with sodium dithionite (SD) the MV changes from colourless/clear to blue. The initial absorbance reading for the assay can be adjusted by adding SD. As the assay was extremely sensitive to even trace amounts of oxygen, a number of control experiments were performed to ensure that any change of absorbance was as a direct result of enzymatic activity and not residual oxygen interfering with the assay.

These results are shown in Fig. 5.6, where the controls consist of assay buffer only, the enzyme and the assay buffer, and finally the substrate and the assay buffer. All three of the controls show a nominal level of change in absorbance. This indicates that the assay was stable and that on their own, neither the enzyme nor the substrate lead to any significant change in absorbance. When the assay was set up with both substrate and the enzyme there was a marked increase in percentage absorbance change, highlighted by Fig. 5.6. The level of
absorbance change was directly proportional to the amount of enzyme which was added to the assay, which supports an enzymatic dependent change. This control experiment was a 5 minute assay. A 30 minute assay was also performed and showed that the assay is still stable over a longer time (data not shown).

Having established that the assay can be performed without contamination from residual oxygen, the assay was then performed with a set enzyme concentration of 0.6 mg/ml and adding fumarate at increasing concentrations. This provided a Michaelis Menton plot from which important enzymatic parameters such as maximum velocity ($V_{\text{max}}$) and $K_m$ (substrate concentration at 0.5 $V_{\text{max}}$) were elucidated (see Fig. 5.7).
Fig. 5.3 Western blot of *lmo0355* putative fumarate reductase expressed from pET-24 vector

Overnight cultures of BL21 (AI) expressing *lmo0355* were diluted 1:100 into fresh sterile LB broth and grown at 37°C, 200 rpm shaking, until an OD$_{600}$ 0.4 – 0.5 was reached. Protein expression was induced by adding 0.2% v/v 1 mM arabinose and 1 mM IPTG to the medium and incubating the cultures for 3 hours at 37°C, 200 rpm shaking. Whole cell lysates boiled for 5 minutes prior to loading; samples were loaded at 1:10 ratio with SDS buffer; loaded at 10 µl/well into a 10% gel.

The induced and uninduced samples of the 54 kDa *lmo0355* putative fumarate reductase are shown, along with the positive control. The positive control is his-tagged ‘K5 lyase’ a 55 kDa bacteriophage protein. There is a clear increase in the production of the *lmo0355* in the post induced sample.
Fig. 5.4 Coomassie blue gel showing purified *lmo0355* putative fumarate reductase elution fractions

Coomassie stained SDS PAGE gel (10%) showing purified *lmo0355* elution samples. Whole cell lysates were boiled for 5 minutes prior to loading; samples loaded at 1:10 ratio with SDS buffer and loaded at 10µl/well.

*lmo0355* putative fumarate reductase elution fractions are shown (E1-E9) at the correct 54 kDa size. These were collected as 0.5 ml fractions during elution step of protein purification (see section 3.9). Elution fractions E5-E9 were desalted, pooled and concentrated for protein assay.
Fig. 5.5 Coomassie blue gel showing purified *lm0355* putative fumarate reductase from elution fractions pooled and concentrated

Coomassie stained SDS PAGE gel (10%) showing purified and concentrated *lm0355* putative fumarate reductase. Whole cells boiled for 5 minutes prior to loading: Samples loaded at 1:10 ratio with SDS buffer and loaded at 10 µl/well.

Protein was purified at 1.8 mg/ml. The band shows the purity of the enzyme used. Extra bands were analysed by mass spectrometry and were revealed to be *lm0355* putative fumarate reductase. These were possibly breakdown products of the protein.
Fig. 5.6 Percentage absorbance change in a 5 minute assay for activity of purified *lmo0355* putative fumarate reductase

Chart showing the absorbance change in a 5 minute assay for controls and enzyme samples. The controls show a very basal level in absorbance change, which indicates that individually the substrate (fumarate) and the enzyme do not affect the assay. The assays in which substrate and enzyme are combined show a greatly increased change in absorbance, which also seems to be proportional to the amount of enzyme added suggesting enzymatic activity of putative fumarate reductase *lmo0355*. 

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[133]
Fig. 5.7 Michaelis-Menton kinetics for putative fumarate reductase *lmo0355*

The enzymatic properties of putative fumarate reductase *lmo0355* are shown in a Michaelis-Menton plot. The initial velocities and parameters were calculated using Cary kinetic system as described in section 3.14. The $V_{max}$, which is the maximum velocity of the enzyme, was established to be 0.2 mM/min/mg. The Km, which represents the substrate concentration at half Vmax was ascertained to be 27.5 µM.
5.3) Discussion

Anaerobic growth can occur using alternative electron acceptors, such as nitrate and fumarate, a process known as anaerobic respiration. Alternatively, fermentation may be used with substrate level phosphorylation providing sugars and other organic compounds such as lactate, formate or acetate (Unden and Bongaerts, 1997).

A key issue in anaerobic growth is the recycling of reduced NADH. NAD+ is constantly consumed during glycolysis, with the processing of each glucose molecule reducing a net of 2 NAD+ molecules (Clark, 1989). Glycolysis would eventually stop once all NAD+ is consumed. This makes the regeneration of the NAD+ pool essential. Lactic acid fermentation is one method to do this by converting pyruvate to lactate using Ldh. In *E.coli* Ldh is the primary mechanism for regenerating the NAD+ pool. Thus, it was concluded that this mutation in *E.coli*, as opposed to acetate auxotrophy (caused by *pflb* deletion) was responsible for the inability of anaerobic growth in *E.coli* (Singh *et al.*, 2009).

Previous work by Romick (1996) and also unpublished results from our own laboratory (Corbett unpublished, 2012), quantifying metabolic end products of *L.monocytophages*, grown under aerobic and anaerobic conditions show that when grown aerobically in the presence of glucose, *L.monocytophages* secrete a large amount of acetoin. When grown under anaerobic conditions a large amount of lactate is secreted along with formate and ethanol (Romick *et al.*, 1996). These findings indicate that under anaerobic growth *L.monocytophages* utilises mixed acid fermentation. This provides an opportunity to target the fermentation pathways of *L.monocytophages* to produce an anaerobic growth deficiency. This could allow us to shed light on some aspects of *L.monocytophages* metabolism.

The *L.monocytophages Δldh* mutant produced in this study was tested for its ability to grow anaerobically and the results are shown in Fig 5.2. The mutant has a growth rate very similar to the WT.
Fig. 5.8 Schematic diagram of L.monocytogenes respiration and fermentation pathways for knockout mutagenesis

The schematic highlights the important fermentation and electron transport chain pathways. The steps that were removed with homologous recombination mutant generation are represented by red crosses. Pathways not found in L.monocytogenes are represented by a blue cross. In E.coli knocking out ldh renders it unable to grow anaerobically due to inability to restore redox balance. The decision was taken to knock out ldh and pflb in L.monocytogenes. These double mutants were still capable of growing anaerobically, suggesting an alternative mechanism to restore redox balance. L.monocytogenes do not possess ubiquinone and nitrate reductases, so these cannot be used. Cytochrome c and quinone oxidase knock out mutants still show ability to grow anaerobically. This leaves fumarate reductase as a potential mechanism for restoring redox balance for L.monocytogenes using fumarate as a terminal electron acceptor in anaerobic respiration.

(Adapted from Singh et al., 2009)
Pflb is responsible for the inter-conversion of pyruvate and coenzyme-A into formate and acetyl-CoA (See Fig. 5.1). Acetyl-CoA is subsequently converted to acetate. In *E.coli*, cells produce acetate as extracellular overflow product, known as ‘overflow metabolism’ (de Mey *et al.*, 2007). Removing *pflb* gene could have a number of effects including accumulation of pyruvate and shortage of acetyl-CoA. Acetyl-CoA could be produced by the enzyme acetyl kinase, however this reaction is carried out at the expense of ATP and may not be a viable long term strategy for the cell (Brown *et al.*, 1977).

The Δ*pflb* mutant produced in our laboratory proved to show little attenuation in comparison to the WT. Taking the fact that the individual mutants did exhibit any attenuation when growing anaerobically, the decision was made to remove both the *ldh* and also the *pflb* gene producing a double mutant.

The double mutant was expected to have an absolute deficiency in anaerobic growth as it was thought that the mutant would be both unable to regenerate its NAD+ pool and be auxotrophic for acetate. The double mutant was also capable of anaerobic growth; however, this was with a slight attenuation in comparison to the WT (see Fig. 5.2).

These series of mutagenesis experiments contradicted our initial hypothesis that *L.monocytophages* shares a simple reliance on fermentative pathway during anaerobic growth, as is the case in *E.coli*. These results seem to be at odds with the findings of Romick (1996), which show a significant increase of lactate for anaerobically growing *L.monocytophages*, a clear hallmark of fermentative growth. There a number of considerations that need to be made in light of their findings and ours.

Although *L.monocytophages* may preferentially grow anaerobically using fermentation as was highlighted by Romick (1999), the fact remains that *L.monocytophages* may not be dependent on this pathway as in *E.coli*. It may be the case that *L.monocytophages* preferentially shift their metabolism to other forms negating the need for fermentation. For example, intracellular profiling of *L.monocytophages* has shown a down regulation of cytochrome complex and components of the oxidative phosphorylation system and also glycolysis. Instead,
there is a strong upregulation of the pentose phosphate pathway, indicating that it may be a major catabolic pathway when glycolysis is no longer preferable (Joseph et al., 2006). This highlights that other pathways can be utilised by *L. monocytogenes*, when necessity dictates.

Another important consideration that should be made when discussing the fermentation mutant results is that *L. monocytogenes*, unlike *E. coli*, may be capable of utilising alternative fermentation pathways to mixed acid fermentation, to facilitate anaerobic growth. *B. subtilis* may be capable of utilising butanediol fermentation as well as mixed acid fermentation (Nakano and Zuber, 1998). Butanediol fermentation required two molecules of pyruvate and oxidises two molecules of NADH, producing CO$_2$, ethanol and butanediol. It is possible that a similar capability is utilised by *L. monoctyogenes*.

As discussed previously, in glycolysis, the limiting factor during anaerobic growth seems to be the restoration of the redox balance in the cell. There are alternative terminal electron acceptors that could be utilised for this purpose in the electron transport chain, a process known as anaerobic respiration.

Cytochrome oxidases represent key players in the electron transport chain and belong to the cytochrome oxidase family (Haddock and Jones, 1977). Other important steps include menaquinone and fumarate reductase. Cytochrome oxidases are membrane-bound heme proteins, which mediate redox reactions, and cytochrome c (*cydA/B*) and quinol oxidases (*qoxA/B*) are the two main types.

Previous work has established that *L. monocytogenes* grown aerobically produce a large amount of acetoin, a hallmark of aerobic growth (Romick et al., 1996). Unpublished work from our laboratory has also confirmed this finding (Corbett unpublished, 2012). Quite interestingly, *cydA/B* mutants produced in our laboratory secrete very small quantities of acetoin even under aerobic conditions (Corbett unpublished, 2012). This suggests that these mutants may not be growing under normal aerobic metabolism, even in the presence of oxygen. In addition these mutants show little attenuation when grown anaerobically.
aro mutants with deletions in a common branch of biosynthesis pathway leading to aromatic compounds show attenuated growth and virulence (Stritzker et al., 2004). This was possibly due to aro mutants eventually sequestering menaquinone, which is the only quinone produced by L. monocytogenes (Goldfine and Shen, 2007). Rather interestingly, aro mutants, auxotrophic for menaquinone, seem to undergo anaerobic respiration, even under aerobic conditions (Stritzker et al., 2004). Menaquinone is used in aerobic respiration where it acts as an electron mediator between enzyme complexes in the electron transfer chain; it eventually transfers two electrons to a suitable oxidant (Bentley and Meganathan, 1982). These results suggest that without menaquinone L. monocytogenes may not be able to fully utilise the electron transport chain and subsequently be forced into anaerobic metabolism.

L. monocytogenes do not possess nitrate reductases so nitrate was unlikely to be used as terminal electron acceptor (Vazquez-Boland et al., 2001). Furthermore, L. monocytogenes respiratory chain does not include the step for ubiquinone so ubiquinone dependent NADH dehydrogenase cannot be used. This leaves fumarate reductase as a potential approach for restoring redox balance by using fumarate as a terminal electron acceptor in anaerobic respiration (see Fig. 5.11).

In bacteria there are two types of enzyme complexes which are responsible for the reversible reaction of fumarate to succinate. Under aerobic conditions succinate dehydrogenase is utilised, converting succinate to fumarate. Under anaerobic conditions fumarate reductase is utilised, converting fumarate to succinate (Iverson et al., 1999). These proteins are also capable of catalysing their respective reverse reactions (Leys et al., 1999).

Membrane-bound fumarate reductase proteins consist of 4 subunits, FrdA,B,C and D. FrdA is the site of catalysis and is the NAD/flavoprotein subunit. FrdB is the iron sulphur subunit and FrdC,D are the membrane anchor subunits (Van Hellemond and Tielens, 1995).

Bioinformatical analysis revealed that L. monocytogenes do not possess FrdBCD subunits and have no obvious succinate dehydrogenase. A gene encoding a potential fumarate reductase has been identified, which was the lmo0355. This
protein was analogous to the FrdA subunit and is a soluble flavoprotein characterised as a fumarate reductase. \textit{lmo0355} putative fumarate reductase consists of four subunits including a covalently bound FAD prosthetic group, which is possibly the catalytic site of the protein. It also contains a lipoprotein membrane anchor site.

There are a number of lactic acid bacteria (LAB) which are capable of aerobic respiration as they contain rudimentary electron transport chain machinery. LAB have some idiosyncrasies in their aerobic respiration as they require exogenous heme and many are deficient in a number of menaquinone genes (Rezaiki \textit{et al.}, 2008). \textit{Enterococcus faecalis}, which is an intestinal commensal of humans and animals terminate their electron transport chains with a heme dependent cytochrome BD and fumarate reductase A subunit (EF\_2556) (Huycke \textit{et al.}, 2001). This enzyme is constitutively expressed and contains a flavin dependent active site and is mediated by menaquinone as an electron donor (Vanhellemont and Tielens, 1994). \textit{lmo0355} putative fumarate reductase shares 67 \% identity to EF\_2556 and 83 \% positivity.

\textit{lmo0355} putative fumarate reductase also shares some similarity to the \textit{Shewanella putrefaciens} fumarate reductase flavocytochrome c, with 40 \% identical residues to the protein (Leys \textit{et al.}, 1999). This \textit{Shewanella} fumarate reductase is unusual, being a soluble periplasmic protein, with major structural differences from the membrane bound complex found in \textit{E.coli} (Turner \textit{et al.}, 1999). To test if \textit{lmo0355} putative fumarate reductase was indeed functional, the protein was purified and assayed for enzymatic activity.

The \textit{lmo0355} gene was initially cloned into a pQE-30 his-tag expression vector. This expression system produced an N-terminus his-tag on the protein. The protein was not expressed correctly and could not be detected by Western blot analysis. This was possibly due to post translational processing of \textit{lmo0355} putative fumarate reductase leading to a cleaving of the his-tag from the protein. As the membrane anchor of the protein was located at the N-terminus, it is also possible that the his-tag interfered with the folding of the protein, which could be responsible for the issues faced during protein purification (data not shown).
After numerous failed attempts the decision was taken to clone the protein into pET-24 vector system which created a C terminus his-tag. The protein was now expressed correctly and detected by both SDS PAGE and Western blot analysis. The protein was purified by passing through a nickel column. The purified protein was assayed for enzymatic activity using methyl viologen dye, under completely anaerobic conditions (Turner et al., 1999).

Methylviologen dye becomes coloured when reduced by sodium dithionite under anaerobic conditions. This was used to assay *lmo0355* putative fumarate reductase. The assay was a measurement of the oxidation of reduced methyl viologen, as a result of enzymatic activity resulting in a direct absorbance change (see. Fig. 5.9). As the assay was very sensitive to any oxygen it was important to do the experiment under absolute anaerobic conditions and also have robust controls in place for all the constituents of the assay. These are depicted in Fig. 5.6 and show very little change in absorbance. The assay begins when the substrate (fumarate) is added to the enzyme, buffer solution and under these conditions there was a substantial change in the rate of the reaction (Fig. 5.6). Fig. 5.6 shows the percentage change in absorbance during a 5 minute assay. The controls show minimum level of absorbance change.

The enzymatic assays show a greatly increased absorbance change and as this was only seen in the presence of enzyme and substrate, it strongly suggests that *lmo0355* putative fumarate reductase does indeed possess enzymatic activity. Further evidence of the activity was provided by the absorbance changing proportionally to the amount of enzyme added to the assay (depicted in Fig. 5.6). The assay was repeated a third time with spectrophotometer readings for 30 minutes. This was to ensure that the assay controls are stable over a longer period of time (data not shown).

The final set of experiments were done by assaying the protein with increasing concentrations of substrate shown in Fig 5.7. This allowed the production of a Michaelis Menton plot, from which essential parameters of the protein can be calculated. The Vmax, which is the maximum velocity of the enzyme, was established to be 0.2 mM/min/mg protein.
Fig. 5.9 Assay of fumarate reductase using methyl viologen dye
The assay used to determine \emph{lmo0355} activity was dependent on the oxidation of methyl viologen, which has been reduced by the addition of sodium dithionite to give an absorbance reading close to 1. Fumarate reductase converts fumarate to succinate where its enzymatic site contains a FAD molecule. The rate at which fumarate was converted to succinate was directly related to the oxidation of methyl viologen, this was seen as a drop in absorbance, which can be measured.
This is the maximum velocity of enzyme in the presence of substrate concentration at saturation (Briggs and Haldane, 1925). The Michaelis constant (Km) is the substrate concentration at which the reaction rate was half Vmax. This was ascertained to be 27.5 µM fumarate. The Km represents the binding affinity of an enzyme for its substrate. Thus, the lower the Km value the higher the enzymes binding affinity (Briggs and Haldane, 1925).

The Km values from this study are comparable to the menaquinol fumarate reductase from *E.coli*, which has measured Km values of 20 µM and 30 µM for fumarate reduction (Maklashina *et al.*, 2006).

*lm0355* putative fumarate reductase is homologous to the individual subunit A of the FrdABCD complex, which are typical for fumarate reductases in many bacteria (Iverson *et al.*, 1999). LAB species contain homologues of FrdA subunit and are soluble fumarate reductases with catalytic activity. This provides evidence that individual FrdA subunit in the absence of the other major subunits could play a role in anaerobic growth in these bacteria. Our studies show that a putative FrdA subunit, *lm0355* from *L.monocytogenes* is capable of enzymatic activity. Thus, it is hypothesised that *lm0355* subunit may play a role in *L.monocytogenes* anaerobic respiration.

### 5.4) Conclusions

This study attempted to provide an insight into the catabolic metabolism of *L.monocytogenes* with an emphasis on anaerobic growth. It has been shown in this study that, unlike in *E.coli*, *L.monocytogenes* was not dependent on fermentation enzymes Ldh and Pflb for anaerobic growth. It is very important to emphasise that this conclusion is based on the assumption that the correct genes were targeted during the mutagenesis work.

As discussed previously, in EGDe *L.monocytogenes* there are a number of genes listed as L-lactate dehydrogenase. *lm0078, lm01057, lm01534* and *lm0078* (annotated as ldh) are all listed as lactate dehydrogenases. Although the gene encoding the most probable d-lactate dehydrogenase, the *lm0078* was removed, it is still possible the remaining putatively defined genes could be
encoding lactate dehydrogenases too. This could provide an alternative explanation for lack of a discernible phenotype.

The same could also be true for the Δpflb mutant, which was targeted for acetate auxotrophy. There are other putatively identified pfl genes (pflA and pflC) in the EGDe L.monocytogenes genome. Again, these genes could potentially be utilised by L.monocytogenes during anaerobic growth.

Alternatively L.monocytogenes may be utilising a different fermentation pathway, for example, the butanediol fermentation, which can be utilised by B. subtilis.

Mutagenesis work from our laboratory removing a number of essential genes in the electron transport chain, have demonstrated that the actual metabolism under oxygen limited growth was potentially very complex, including the possible utilisation of alternative electron acceptors such as fumarate and possibly using other catabolic pathways such as the pentose phosphate system (Corbett unpublished, 2012).

A potential fumarate reductase, lmo0355 was isolated, purified and assayed for activity. This was similar to FrdA subunit of the FrdABCD complex of a classical fumarate reductases found in a number of bacteria (Iverson et al., 1999). It was shown that this individual subunit does indeed possess enzymatic activity, with the ability to convert fumarate to succinate. This supports the hypothesis that this protein could play an important role in L.monocytopgenes anaerobic metabolism, where it would potentially be utilised with fumarate as the final terminal electron acceptor.

5.5) Future work

In terms of future work the most obvious issue that needs attention are the alternative ldh and pfl genes. These alternative genes also need to be evaluated for their possible importance during anaerobic growth. The genes could be targeted based on their transcription profiles, possibly using quantitative real time polymerase chain reaction (QRTPCR) from cells grown anaerobically. This could
identify, which genes are being utilised for anaerobic growth and thus identify a more targeted approach to the study.

Follow on experiments for the potential fumarate reductase could involve resolving the crystal structure for the protein.
Chapter 6
Metabolomic analysis of external HeLa cell metabolome and the effects of 
\textit{L.monocytogenes} infection
6.1) Introduction

Metabolomics, represent an excellent tool for the global analysis of a cell’s metabolic state. The following study was designed to examine the external metabolome of HeLa cells and the effect of infection with *L. monocytogenes*. Currently, the metabolism of *L. monocytogenes* is an area of research, in which a great deal of expansion is required. This is especially the case when considering the metabolic changes which occur to the host as a result of infection.

The focus of this study was on the external metabolism of HeLa cells and how *L. monocytogenes* infection changes this, a process known as metabolomic footprinting (Kaderbhai *et al.*, 2003). This is of great interest as the secretome can, in theory at least, provide a plethora of data, which can elucidate the important aspects of cellular metabolism. Similar methodology has been successfully used in a number of studies, for example, to differentiate between wild type *E. coli* and *E. coli* tryptophan mutants based on their metabolomic footprints (Kaderbhai *et al.*, 2003).

This study should be viewed as a proof of principle study to establish if it is possible to discriminate between HeLa cell footprint and the footprint of HeLa cells, which have been infected with *L. monocytogenes*. Correspondingly, the aim is to perform a global metabolite analysis to identify key metabolites, which are differentially measured as a result of the infection. The effects seen in the extracellular environment will directly reflect the activities of the HeLa cells. For example, most of the nutrition required for cell metabolism will be consumed from the extracellular milieu. Likewise, metabolomic end products are secreted into the extracellular environment. Also, metabolites associated with cellular stress could be secreted and detected by the methodology, which in itself could be very interesting (Devantier *et al.*, 2005). Thus, by analysing the extracellular environment, important clues to the intracellular metabolic state of the host cell could be gained, and how infection with *L. monocytogenes* changes this.
6.2) Results

The infection was conducted for a total of 10 hours, including a 2 hour incubation period, after which bacteria were removed and non-internalised bacteria killed by replacing DMEM with DMEM supplemented with 10 µg/ml gentamicin. Medium was sampled for uninfected controls and infected samples at T10, filter sterilised and analysed by GC-MS (see section 3.5 for more detail). The results from this study were mixed in terms of the information which could be ascertained from them. The following section will focus on the most important aspects of these results.

The GC-MS data from the first experiment were analysed using multivariate principal component analysis (PCA). The main objective of PCA is to reduce the dimensionality of a given dataset by explaining variance and covariance by a small number of linear combinations of these variables. Most of the variability in the data can be accounted for through a small number of principal components, which can be visualised (Dytham, 2011). The experiment contained a total of 4 groups with 6 samples in each group. The results are shown in Fig. 6.1. There was excellent separation of the control HeLa (C) and the infected HeLa (I) samples along principal component (PC) 3. There were also two controls in the experiment, which were medium (M) and medium containing only L. monocytogenes at the same cell count used in the HeLa infected samples (L). These were well separated from the HeLa samples along principal component 1. Another point to note is that there is very little separation of the medium and the L. monocytogenes controls.

The loadings plot (Fig. 6.2) shows those metabolites which most contribute to the separation of infected and uninfected profiles observed in the PCA plot (Fig. 6.1). The metabolites are shown as peaks and drops along the X axis of the graph. The most prominent metabolites were identified. In some cases identification was not possible, for reasons which will be discussed later.

The PCA analyses from consequent experiments are shown in Fig. 6.3. Experiment 2 was a 20 sample study (10 control vs. 10 infected) and experiment 3 was a 24 sample study (12 control vs. 12 infected).
Fig. 6.1 Principal component analysis (PCA) (1 vs. 3) HeLa metabolic footprint: Experiment 1

The PCA plot for principal components 1 vs.3 is shown. From experiment 1 the four groups are L (L. monocytogenes control), M (Medium control), IHL (Infected HeLa cells) and H... (HeLa cells). The plot shows that along principal component 1 there is clear separation of the HeLa cells from the controls. Along principal component 3 there is very little separation of L. monocytogenes and medium controls. This shows that any changes to the medium have very little contribution from extracellular L. monocytogenes external metabolome. Along the same component there is also a clear separation of the infected and uninfected profiles (highlighted with circles).
Fig. 6.2 Principal component analysis (PCA) loadings plot for Principal component 1 vs. 3. Experiment 1

The loadings plot highlights the specific metabolites that contribute to the separations that are seen in the PCA plot principal component 2 (Fig. 6.1). The 166 identified metabolites are along the X-axis and their contributions are shown as peaks and drops. The metabolites providing the most prominent peaks and drops are identified. The largest peak is for metabolite putrecine and other prominent metabolites include lactic acid, glycine and phosphate.
Fig. 6.3 Principal component analysis (PCA) HeLa metabolic footprint Experiment 2 and 3

PCA plots are shown for experiment 2 and 3. The uninfected HeLa controls are in red (C.) and the infected HeLa cells are blue (I.). Medium controls are shown in green (M.) The two PCA plots do not show clear separation of the groups as seen in experiment 1 (Fig. 6.1). This makes the results inconclusive, which is supported by univariate analysis which contains far less statistically differential metabolites between infected and uninfected HeLa groups (Table 6.1).
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Table 6.1 Fold change in significant metabolites: infected vs. uninfected

Table showing the fold change in metabolites from infected to uninfected. The results are shown as separate experiments along with significance P values. Any metabolite with a value greater than 1 shows an infection related secretion of that particular metabolite into the medium. Any value less than 1 represents an uptake of that metabolite from the medium.
The PCA plots in Fig. 6.3 highlight the experimental variation between the samples, which has been an issue throughout this study. The implications of this will be discussed in greater detail later.

The univariate analyses for significantly changing metabolites in all three experiments are shown in Table 6.1 This table shows the fold change in metabolites from uninfected and infected samples. Also shown is the statistical significance of the change, given as a P-value.

The results from subsequent experiments do not show consistency with the initial study. A large number of the metabolites which are significantly changing in the infected profiles are unidentifiable due to the inability to match retention times and peak heights to known metabolites in GC-MS database.

6.3) Discussion

The main objective of this study was to determine whether infection of HeLa cells with *L. monocytogenes* changes the metabolomic profile of their external environment. That is, is there a direct infection related change in the metabolic profiles of HeLa external metabolome?

The raw metabolomic peak values from the GC-MS data were analysed by principal component analysis (PCA). The first and most important question raised by this study is, does the infection of HeLa cells with *L. monocytogenes* causes a change in the metabolic profile of the external environment. The results in Fig. 6.1 show that there is excellent separation of the infected (I) and uninfected control profiles (C) of the HeLa cells. The control samples of *L. monocytogenes* (L) and medium (M) are well separated from the HeLa samples. Importantly, the L and M controls did not separate from each other, showing that there is very little effect on the metabolomic profile from non-internalised *L. monocytogenes*. The multivariate analysis shown in Fig. 6.1 provides evidence that there is indeed a change to the external profile of HeLa cells.

The loadings plot (Fig. 6.2) show those metabolites that most contribute to the separation of infected and uninfected profiles observed in the PCA plot (Fig. 6.1).
This information could be used to identify important metabolites that may not necessarily be found using univariate analyses. Other prominent metabolites identified by the loadings plot include glutamine, lactic acid, phosphate and cysteine.

The multivariate analysis of PCA provides a global view of the changes occurring to the metabolic profile. Univariate analyses, such as paired T-tests, determine changes in individual metabolites as a result of the infection. These are shown in Table 6.1.

The univariate analysis from the first experiment revealed some interesting findings. All of the metabolites reported in Table 6.1 are significantly different between infected and uninfected HeLa cells. Table 6.1 also shows the fold change of individual metabolites as a result of *L. monocytogenes* infection. Any number greater than 1 is a reduction of that metabolite in the medium, indicating an uptake and any number less than 1 is an increase in the medium suggesting secretion.

There are a number of metabolites which, according to this study are secreted into the medium. Tryptophan is significantly increased in the infected samples, with a fold change of 1.5, which represents a 50% increase. Interestingly most of the amino acids, which are significantly changing seem to be secreted into the medium. These include aromatic amino acids tryptophan and phenylalanine, which mammalian cells are unable to synthesise (Herrmann and Weaver, 1999). It is not clear why these metabolites should be increasing in the external environment. It is also counter intuitive, as one would expect an increase in demand for these metabolites as a result of infection due to the requirements of the parasite (Joseph and Goebel, 2007).

The initial study also reveals that lactic acid is secreted into the medium with a positive fold change of 1.18 in infected HeLa cells. As lactic acid is a metabolic end product of fermentation (Kim and Gadd, 2008), this could provide evidence of increased cellular anaerobiosis as a result of *L. monocytogenes* infection. HeLa cells are cancer cells and are likely to undergo anaerobiosis of metabolism known as the Warburg effect, and the results suggest this effect is increasing as
a result of *L. monocytogenes* infection (Vander Heiden *et al.*, 2009). Alternatively, this could be a by-product of fermentation metabolism of the internalised *L. monocytogenes*.

According to the initial study, glutamine is taken up from the medium and in rather large quantities, with a fold change of 0.6. Glutamine is known to play an critical role in a large number of essential cellular functions, including being a key compound for ammonium assimilation in mammalian cells (Newsholme *et al.*, 2003). Also, glutamine is known to play a prominent role in cellular stress and may be taken up as a stress response by the host cell (Oehler *et al.*, 2002, Matés *et al.*, 2002). Another reason for the increasing host cell requirement for glutamine could be the role which glutamine plays in the nitrogen metabolism of *L. monocytogenes*; this will be discussed in further detail in Chapter 8.

Other interesting amino acid uptakes include those of cysteine and methionine in the second experiment (Table 6.1). *L. monocytogenes*, strain EGDe has an absolute requirement for cysteine (Tsai and Hodgson, 2003) and other strains have been shown to have a requirement for both cysteine and methionine (Goldfine and Shen, 2007). *L. monocytogenes* lack sulphate and nitrate reductases, necessitating reduced nitrogen and sulphur sources, which readily explains their growth requirement for cysteine and methionine (Goldfine and Shen, 2007). The uptake of these metabolites in infected HeLa cells could represent *L. monocytogenes* consumption of host cysteine and methionine supply.

The results from the initial experiments led to further work on the HeLa footprint under infected and uninfected conditions. These results are shown in Fig. 6.3 and Table 6.1. The subsequent experiments were not as conclusive as our initial study for reasons which will be discussed later. The extracellular metabolome experiment was repeated 3 times and each time the significant matches for the metabolites were different (Table 6.1). Moreover, the particular metabolites detected were different between experiments. Table 6.1 shows the total number of metabolites detected in each study and the number of significantly different metabolites from infected HeLa cells. It also shows the number of metabolites which could not be identified. Unidentified metabolites are well established
limitations of metabolomics studies (Scalbert et al., 2009). Metabolite identification is dependent on matching retention times and peak heights to those found in databases. Sometimes this is not always possible, leading to many unknowns from a typical mass spectrometry data set (Brown et al., 2009, Bowen and Northen, 2010). This is highlighted by Table 6.2. From the first experiment there were 116 unidentified metabolites out of a total of 165, 34 out of 80 in the second and 38 out of 121 in the third experiment. Clearly, this is less than ideal, as a large number of these unknown metabolites are changing quite significantly as a direct result of the \textit{L. monocytogenes} infection and it would be very interesting to identify these.

Another issue with the methodology and subsequently results interpretation is the degree of identification for the metabolites. For example, a large number of known metabolites from these experiments are unidentified sugars. This means that the metabolites have been identified as a sugar, but cannot be accurately differentiated further due to mass and retention times being too similar.

PCA analysis of experiments 2 and 3 does not show clear separation of infected and uninfected groups (Fig. 6.3), which could be achieved in the first experiment (Fig. 6.1). This is supported by Table 6.1, which shows that in experiment 2 and 3 there are far fewer metabolites which change significantly. This lack of reproducibility is a concern for establishing accurate and robust conclusions based on the results. This is possibly due to the extreme sensitivity of the GC-MS and the fact that this methodology is very sensitive to experimental variation.

An alternative way to view the lack of reproducibility is that the external metabolome of the HeLa cells is not actually changing significantly and consistently as a result of infection with \textit{L. monocytogenes}. This is highlighted by the fact that most of the metabolites found in this study do not change significantly. For example, in experiment 1, of the 165 metabolite matches, only 69 were significantly different, and most of these were unidentified and were not expected to be core metabolites. Experiment 2 identified 80 metabolites and only 3 metabolites were significantly different.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total metabolites detected</td>
<td>165</td>
<td>80</td>
<td>121</td>
</tr>
<tr>
<td>Identified metabolites, which were significantly different between uninfected and infected</td>
<td>61</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>Unidentified metabolites, which were significantly different between uninfected and infected</td>
<td>37</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Total Unidentified metabolites</td>
<td>116</td>
<td>34</td>
<td>38</td>
</tr>
</tbody>
</table>

Table 6.2 Metabolite identification from intracellular metabolome experiments
Total number of metabolites identified from the extracellular metabolome experiments are shown along with those metabolites that were statistically significant. From the statistically significant metabolites, the number of unidentified metabolites are also shown.
These results may look anomalous at first glance; however, it is possible that they are showing an accurate picture of the external metabolome. Being an intracellular parasite, it is in the interest of *L. monocytogenes* not to make drastic changes to the host cell metabolism. Instead, the changes are thought to be more subtle, with *L. monocytogenes* metabolism being synchronised with that of the host (Joseph and Goebel, 2007). The lack of consistent, robust changes to the external metabolome identified in this study could indicate that this is indeed the case.

6.4) Future work

The metabolomics methodology which has been discussed in this section needs to be developed further. Once the protocol has been developed further, there are a number of different interesting studies that can be performed.

A particularly interesting route the study could take is tracking the change in metabolites over time. The study, which has been described above was an ‘end point’ study, essentially looking at the effects on the external metabolome, post infection. A timecourse study could give a more complete picture of the changes that are occurring during an infection, and could provide data on when these changes occur. Although, it must be emphasised that this should only be attempted once a robust protocol has been developed.

Preliminary work was undertaken on a timecourse version of the experiment in this study. Unfortunately, the timecourse experiments were not successful with large variability in the results making tracking the change in metabolites over the course of the infection very difficult. More attempts to develop this protocol in the future could bring some very interesting results.

6.5) Conclusions

Overall, the extracellular metabolism study of HeLa cells reveals some interesting changes on the metabolism as a result of *L. monocytogenes* infection. However, due to the experimental variability, the validity of these is difficult to express. It is
possible that the extracellular metabolome of HeLa cells are not actually affected significantly or consistently as a result of infection, explaining the experimental variability encountered in this study.
Chapter 7
Metabolomic analysis of effects of
*L. monocytogenes* infection on the
intracellular metabolome of HeLa cells
7.1) Introduction

Although the pathogenesis of \textit{L. monocytogenes} is well characterised and has been extensively reviewed (Ramaswamy \textit{et al.}, 2007, Freitag \textit{et al.}, 2009, Joseph and Goebel, 2007), very little is known about the physiology of \textit{L. monocytogenes} during intracellular growth, and even less is known about how the host cell physiology changes in response to infection. It is considered that the cytoplasmic environment is nutritionally challenging and an environment to which \textit{L. monocytogenes} need to adapt prior to the onset of replication (Bennett \textit{et al.}, 2007). However, the exact carbon and nitrogen sources which are utilised by \textit{L. monocytogenes} are unknown, as are the exact modifications to host cell physiology during infection.

The metabolomic study discussed in the previous chapter focused on the extracellular metabolome (footprint) of cells, i.e. what they take up from and secrete into the medium and how the infection at 10 hours changes this (see section 6.1). For this study the focus was shifted to the intracellular metabolome (fingerprint) of HeLa cells and effects of \textit{L. monocytogenes} infection. This can be achieved by metabolomic analysis of infected and uninfected mammalian cells by gas chromatography mass spectrometry (GC-MS). Metabolomic analyses represent a direct reflection of the physiological state of the cell by evaluating metabolites, which are small organic compounds of low molecular weight (Looser \textit{et al.}, 2005). As such metabolomics provides a highly relevant approach to understanding key biological processes that occur in a cell (Goodacre \textit{et al.}, 2004).

Comparative metabolomics approach has been used to study external stresses on cells, for example, the effects of different temperatures on the metabolomes of \textit{L. monocytogenes} (Singh \textit{et al.}, 2011). However, to the best of our knowledge, there have been very few studies looking at the metabolome of host cells and the effect of infection with \textit{L. monocytogenes}. The evaluation method of GC-MS is exquisitely sensitive and this study aimed to fulfil a number of objectives. Firstly, to ascertain if infection of HeLa cells with \textit{L. monocytogenes} has any change on their intracellular metabolic profiles. Secondly, to establish clues to the specific
metabolic changes that are occurring in the host cell and thirdly to predict key metabolites utilised by *L. monocytogenes* during intracellular growth.

### 7.2) Results

While the previous study focused on the extracellular metabolome, the focus of this study was the intracellular metabolome of HeLa cells and how *L. monocytogenes* infection affects it. The infection was conducted for a total of 10 hours at a MOI 10. This MOI was based on previous experiments conducted in our laboratory and was a number which allowed a sufficient bacterial load without inducing significant HeLa cell lysis at 8 hours, which is the time of metabolite extraction. This included a 2 hour period in which HeLa cells were exposed to bacterial infection, after which bacteria were removed and non-internalised bacteria were killed by replacing DMEM with DMEM 10 µg/ml gentamicin. Where previously the extracellular medium was sampled and analysed by GC-MS, in this study cells were lysed using 100 % methanol, cell debris was removed and supernatant sampled for metabolomic analysis.

The data from the GC-MS analysis were initially analysed using multivariate statistics, where multiple statistical variables are analysed simultaneously. This form of statistical analyses provides a global ‘picture’ of the dataset and can provide a summary of a large dataset. The data were analysed by principal component analysis (PCA). The PCA plots for all the experiments shown in Fig. 7.1. The PCA plots highlight that there is clear separation of infected profiles from the uninfected profiles.

The first objective from this study was to ascertain whether at 10 hours post infection with *L. monocytogenes* there is an effect on the metabolic profile of HeLa cells. The evidence from the initial multivariate analyses indicated a marked change in the intracellular metabolome of HeLa cells as a result of the infection (Fig. 7.1).

The heatmap shown in Fig. 7.2 was also adopted for the analysis of all identified metabolites. All identified metabolite datasets were combined to make a single heatmap. Heatmap analysis is a clustering tool which amalgamates metabolites
and samples which are behaving in a similar fashion. Like the PCA analysis, the heatmap shows clear separation of infected and uninfected profiles (X axis). The metabolites which are distributed in a similar pattern are clustered (Y axis). These metabolites include a large number of amino acids, which are reduced as a result of the infection.

The metabolomic data were also analysed using univariate analyses, which analyse changes in individual metabolites. These results are highlighted in Table 7.1, which lists all the known metabolites identified in this study, their fold changes and their P values. There were a total of 53 metabolites which were identified in the study and 26 of these changed significantly as a result of the infection.

As with the metabolic footprint study, there were a large number of unidentified metabolites. Unidentified metabolites are a well-established limitation for metabolomic based studies (Scalbert et al., 2009). These are summarised in Table 7.2. From all the experiments conducted a total of 231 metabolites were detected and of these 102 remained unidentified. This number does not include unidentified sugars. From the unidentified sugars group, a total 11 are changing significantly as a result of the infection. The most significant of these are shown as box and whisker plots in Fig. 7.3. The data shows there was a significant reduction in a range of sugars as a result of \textit{L. monocytogenes} infection.

The implications of these results will be discussed in greater detail in section 7.3.

\textbf{7.3) Discussion}

\textbf{7.3.1) Multivariate analysis}

The GC-MS data were first analysed by PCA. The PCA plots for all the experiments in this study are shown in Fig 7.1. The PCA plots indicate that there is clear separation of infected profiles from the uninfected profiles. This provides evidence that there is a marked change in the intracellular metabolome of HeLa cells as a result of \textit{L. monocytogenes} infection.
Fig. 7.1 PCA for intracellular metabolism GC-MS data; experiments 1, 2 and 3

Principal component analysis (PCA) plots of all experiments conducted for Chapter 7. The PCA plots show that there is separation of the control (Red ‘Cont.’) and infected (Blue ‘Inf.’) samples. Although separation of control and infected groups is seen, no tight clustering within the groups themselves is seen. This highlights the experimental variation between samples from the same group.
**Fig. 7.2 Heatmap for correlated GC-MS data of HeLa intracellular metabolites**

The heatmap is a visual representation of metabolite peak heights from the GC-MS data. The data set combines all defined metabolites from a sample set of 10 control vs. 10 infected are shown. These were then analysed by heatmap analysis and clustered into groups based on similarity. Red boxes represent low values, yellow boxes are high values and are on a graded scale. The infected and uninfected groups separate from each other, which is indicative of the change in metabolite profiles as a result of *L. monocytogenes* infection. The metabolites were also clustered, so that those metabolites behaving in a similar way are closer together. The heat map clusters most of the amino acids including isoleucine, cysteine and glycine, which were being reduced in the infected profiles. Also clustered are a number of metabolites which were increased in the infected profiles including aspartic acid, citric acid and octadecanoic acid.
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Fold Change</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Increasing in Infected HeLa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.6103</td>
<td>1.22E-07</td>
</tr>
<tr>
<td>Norleucine</td>
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</tr>
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</tr>
<tr>
<td>Tryptophan</td>
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<td>5.53E-05</td>
</tr>
<tr>
<td>Myo-inositol</td>
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<td>7.5E-05</td>
</tr>
<tr>
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<td>0.000104</td>
</tr>
<tr>
<td>Leucine</td>
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<td>0.001046</td>
</tr>
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<td>Citric acid</td>
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<tr>
<td>Pyrophosphate</td>
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<td>Maleic acid</td>
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<td>Perine</td>
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<td>Dodecanoic acid</td>
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<td>0.100830</td>
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<td>Octadecanoic acid</td>
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</tr>
<tr>
<td>Urea</td>
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</tr>
<tr>
<td>AMP</td>
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</tr>
<tr>
<td><strong>Decreasing in Infected HeLa</strong></td>
<td></td>
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<tr>
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<td>Isoleucine</td>
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</tr>
<tr>
<td>2-Pyrroolidone-5-carboxylic acid</td>
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</tr>
<tr>
<td>Cysteine</td>
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</tr>
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<td>Valine</td>
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<td>0.062167</td>
</tr>
<tr>
<td>Pyroxidine</td>
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<td>0.072056</td>
</tr>
<tr>
<td>Metabolite</td>
<td>Fold Change</td>
<td>P-value</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>-------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Alanine</td>
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<td>Oxoglutarate</td>
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</tr>
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</tr>
<tr>
<td>Pyrimidinone</td>
<td>1.0032</td>
<td>0.985919</td>
</tr>
</tbody>
</table>

Table 7.1 Defined metabolites which change between uninfected and infected from all GC-MS experiments in Chapter 7

Table highlighting all metabolites identified from the intracellular GC-MS metabolome from 3 experiments consisting of a total of 23 control and 23 uninfected samples. Fold change values were calculated from the averages of all replicates. Any fold change values higher than 1 represent a positive change and any value greater than 1 represent a negative change. Also shown are the P values for the change with all significant values highlighted (red). There are a total of 26 metabolites which have been recorded as changing significantly between infected and uninfected samples. All unidentified metabolites including unidentified sugars have been omitted from the table.
Fig. 7.3 Box whisker plots for undefined sugars from intracellular metabolome experiments
Box whisker plots for 6 of the most significantly changing unknown sugar metabolites from a total of 18 are shown. Their metabolite identifications are also shown. The elution time and fragmentation patterns for these metabolites are very similar, so they cannot be distinguished further. The box plots clearly show that these metabolites are reduced in HeLa cells infected with L.monocytogenes. This suggests that these sugars are utilised by L.monocytogenes during intracellular growth, indicating a rather complex carbon metabolism for intracellularly growing L.monocytogenes. These metabolites should be prioritised for identification in any future study.
The results from the PCA analysis are supported by the heatmap analyses (Fig. 7.2). Heatmaps cluster groups and variables, which are behaving in a similar manner. The heatmap also clusters the control and infected profiles, again showing that the profiles from the two groups are significantly different. The metabolites are also clustered, so that those metabolites behaving in a similar way are closer together. The heat map clusters most of the amino acids, including isoleucine, cysteine and glycine, which are being reduced in the infected profiles. Also clustered are a number of metabolites which were increased in the infected profiles including aspartic acid and citric acid.

There is a large body of evidence confirming significant changes to host cell physiology by intracellular parasites. These include modulation of host apoptosis response (Gao and Abu Kwaik, 2000), modulation of vesicular trafficking (Meresse et al., 1999) and immune response modulation (Woolard and Frelinger, 2008). Alternative studies looking at transcriptional analysis of macrophages infected with *M. tuberculosis* revealed a total of 463 differentially expressed genes in host cells, many of which were associated with metabolic pathways (Xu et al., 2003). These studies emphasise the large scale changes which host cells undergo during infection with a pathogen. The change in the infected profile of HeLa cells observed in this study could be indicative of a combination of such host cell modulations by the parasite, culminating in significant physiological adaptations by the host. Alternatively, the changes could be seen as a direct effect of *L. monocytogenes* nutritional requirements, in other words, changes observed reflect sequestering of particular metabolites in the host by *L. monocytogenes*.

An important control in these studies was to ensure that there is very little effect on the intracellular metabolic profiles simply caused by the extracellular metabolome of *L. monocytogenes* in the infected samples. A comparative mass spectrum is shown in Fig. 7.4. This shows the spectra of metabolites extracted from a confluent T75 HeLa flask in direct comparison to *L. monocytogenes* at a cell count of $8 \times 10^7$. This is an overestimate of the number of *L. monocytogenes* cells expected at 24 hours from an infection of a T75 HeLa flask. As such, it represents upper limits of bacterial load during an infection in and will provide a suitable control.
In order to confirm that measurements from the GC-MS analysis was directly based on the intracellular HeLa cell metabolome, a set of control experiments were conducted. The mass spectra shown above confirms that in comparison to the metabolites extracted from a T75 flask of HeLa cells (green), the *L.monocytogenes* sample (red), which represents the extracellular metabolome of *L.monocytogenes*, has very few prominent metabolite peaks. Thus, the results from the HeLa cells override any background data from the *L.monocytogenes* added to the infected samples.
The results show that there are very few peaks in the *L.monocytogenes* sample for unidentified metabolites. The mass spectrum for *L.monocytogenes* (red spectrum) only has 4 unidentified peaks compared to the much higher intensity spectrum for the HeLa control (green spectrum).

Based on the results from this control experiment, the assumption was made that the external metabolome of *L.monocytogenes* in the infected samples did not affect the intracellular metabolome of the infected cell. Hence, any measurements in the metabolic profile are from the host metabolism not the constituents of *L.monocytogenes*.

### 7.3.2) Univariate analysis

Univariate analyses are a less complex form of statistics and are carried out on single variables. Where the PCA analysis earlier allowed data analyses on a ‘global’ scale and examine changes across all metabolites, univariate analysis allow comparisons between individual metabolites. Table 7.1 shows identified metabolites from the metabolome studies along with their fold changes and significance values. The metabolites are from a number of important metabolic pathways, which will be discussed below in greater detail, with respect to intracellularly growing *L.monocytogenes*.

### 7.3.3) Fatty acid metabolism

The study showed that here were a number of metabolites associated with fatty acid metabolism which are changing as a result of *L.monocytogenes* infection. These include hexadecanoic acid, dodecanoic acid and octadecanoic acid, all of which are increased in the infected profiles. Triglycerides can be broken down into fatty acids along with glycerol and can be converted to glucose, via gluconeogenesis pathway (Masoro, 1977); however, their increase suggests an infection related hyperlipidemia. It has been shown that Infection and inflammation induce the acute phase response (APR), leading to multiple alterations in lipid and lipoprotein metabolism (Khovidhunkit *et al.*, 2004). This includes significantly increased levels of triglycerides as a result of bacterial
infections (Gallin et al., 1969, Sammalkorpi et al., 1988). The increased levels detected in the analyses could be such an infection related response.

There is a significant increase in citric acid with a fold change of 0.5487. Citric acid, whilst being an important intermediate in the citric acid cycle (TCA), can also be broken down into acetyl-CoA for fatty acid synthesis (Masoro, 1977). High levels of citric acid could indicate excess acetyl-CoA, which could subsequently be converted with malonyl-CoA to fatty acids (Coffee, 2004). The excess fatty acid readings from this study could be a reflection of this process.

7.3.4) Carbon metabolism

This study has generated a number of very interesting results with regards to the carbon metabolism of intracellular L.monocytogenes. Although in vitro L.monocytogenes is capable of utilising a number of carbon compounds, the exact compounds utilised during intracellular growth is unclear.

Based on genomic data, L.monocytogenes is capable of utilising glucose through glycolysis and the pentose phosphate pathway (Joseph and Goebel, 2007). However, transcriptional data has shown a strong intracellular down regulation for glycolysis genes whilst pentose phosphate pathway genes are upregulated (Joseph et al., 2006). Also upregulated is the Hpt glucose transporter, suggesting intracellular levels of glucose are actually quite low. Finally, a L.monocytogenes mutant impaired in glucose uptake replicates as efficiently as the wild type strain (Stoll and Goebel, 2010). All these data suggest that glucose is not an essential carbon source for L.monocytogenes. The GC-MS analysis from this study supports this hypothesis, detecting an insignificant change in glucose levels as result of the infection (Table 7.1).

Some studies have suggested that glycerol may be an important intracellular carbon source for L.monocytogenes (Eisenreich et al., 2006). However, these data do not necessarily support this, with glycerol change due to infection, being statistically insignificant (Table 7.1).
From the total metabolites detected, there are 27 unidentified sugars, of which 18 are significantly changing (Table 7.2). The reasons for the inability to define them further will be discussed in greater detail in section 7.4. 7 of the most significantly changing unidentified sugars are shown in in Fig. 7.4 as box whisker plots. Box and whisker plots represent a graphical representation of the numeric data providing a number of summaries. These include lower quartile range, median, upper quartile range and finally the extreme values (smallest and largest) (Govearts et al., 1998). Fig. 7.3 shows that there is a large reduction in the infected profiles for all the unidentified sugars. Indeed, out of the 18 metabolites which were identified as sugars and were statistically significant, 16 were reduced in the infected profiles. These results suggest that the carbon metabolism of intracellular *L.monocytogenes* may be complex. Also, it is unlikely that *L.monocytogenes* has an absolute preference for any one particular carbon source. Instead, the bacterium is likely to utilise a number of different carbon compounds, which seems to be a more prudent strategy for an intracellular parasite growing in a nutritionally compromised environment (Bennett et al., 2007).

### 7.3.5) Amino acid metabolism

Various studies have shed light on the potential amino acid metabolism of *L.monocytogenes* growing intracellularly. $^{13}$C isotopologue profiling has revealed that significant fractions of the amino acids utilised by *L.monocytogenes* are provided by the host (Eylert et al., 2008). This is supported by this metabolomic study, which showed that the most of the significantly reduced metabolites in the infected profiles are amino acids (Table 7.1). Bacterial $^{13}$C enrichments of glutamate, proline, valine, isoleucine and leucine showed that only minor portions of these amino acids are synthesised *de novo* by *L.monocytogenes*, thus they are provided by the host. The metabolomic data also supports this, with Isoleucine, valine, glutamate and glutamine decreased in the infected metabolic profiles, suggesting an uptake of these metabolites from the intracellular milieu (Table 7.1). Isoleucine is amongst the most significantly reduced amino acids with a fold change of over 3.
Table 7.2 Total unidentified metabolites from intracellular metabolome study

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<table>
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</tr>
</thead>
<tbody>
<tr>
<td>Total unidentified metabolites</td>
<td>102</td>
</tr>
<tr>
<td>Statistically significant unidentified metabolites</td>
<td>28</td>
</tr>
<tr>
<td>Unidentified sugars</td>
<td>27</td>
</tr>
<tr>
<td>Statistically significant unidentified sugars</td>
<td>18</td>
</tr>
</tbody>
</table>

The total numbers of metabolites from the intracellular metabolomic studies are shown above. There are a total of 102 unidentifiable metabolites and 28 of these could be of interest, as they were changing significantly as a result of *L. monocytogenes* infection. This highlights a major limitation of GC-MS based profiling. Along with the unidentifiable metabolites there were also 27 unidentified sugars, of which 18 were significant. These metabolites should be prioritised for identification in any future study.
PrfA is the global regulator protein for a number of virulence genes and is significantly increased during intracellular growth (Freitag et al., 2009) (See section 1.8). There is evidence to suggest that increasing concentrations of PrfA, leads to consumption of Isoleucine as a carbon source (Eisenreich et al., 2006). This is possibly a compensatory measure in response to reduced glucose levels associated with high PrfA concentration.

Although $^{13}$C studies have shown that isoleucine is one of the few amino acids which can be synthesised de novo, it has also been identified as important component in minimal medium for *L. monocytogenes* growth as it can be degraded to acetyl-CoA (Eylert et al., 2008, Eisenreich et al., 2006). Due to the incomplete TCA cycle of *L. monocytogenes* this alternative use for isoleucine could prove to be a metabolic requirement (Eisenreich et al., 2006). The very strong reduction of isoleucine in infected profiles could be indicative of the increased demand for isoleucine from parasite sequestering due to its metabolic multi-functionality.

There was a reduction in methionine and cysteine as a result of the infection. Cysteine is an essential amino acid for *L. monocytogenes* growth in a number of minimal media (Tsai and Hodgson, 2003, Premaratne et al., 1991). Analysis of the EGDe *L. monocytogenes* genome revealed that cysteine could be produced by processing sulphide by the putative o-acetylserine lyase (*lmo0595*) and putative cysteine synthaseA (*lmo0223*). However, previous studies have highlighted that EGDe *L. monocytogenes* do not possess genes for reduction of sulphate to sulphide, thus explaining the requirement for cysteine (Tsai and Hodgson, 2003). EGDe *L. monocytogenes* possesses an alternative pathway for cysteine production from thiosulfate condensed with o-acetylserine to form sulfocysteine, which is reduced to cysteine. However, thiosulphate cannot replace cysteine in minimal media (Tsai and Hodgson, 2003). The missing steps in key cysteine biosynthesis pathways explain the significant reduction of cysteine in the infected profiles (Goldfine and Shen, 2007).

Methionine has proven essential for the growth of strain 10403 *L. monocytogenes*; however, EGDe *L. monocytogenes* used in this study can
grow without methionine in minimal media (Tsai and Hodgson, 2003). This does not readily explain the strong reduction in methionine associated with *L. monocytogenes* infection (Table 7.1). A point of interest is that methionine is an essential amino acid for mammalian cells, whereas cysteine is not, and the reverse being true for *L. monocytogenes*. As such, it is possible that the strong reduction in methionine is a direct result of sequestering of host cell cysteine by *L. monocytogenes*, thus, leaving methionine as the primary sulphur containing amino acid available for the host cell.

A result which is rather difficult to explain is the significant increase in intracellular tryptophan. It is unclear as to why *L. monocytogenes* infection would lead to a significant increase in this amino acid. This is a result which is also observed in the metabolomic footprint study discussed in the previous chapter (Chapter 6 - Table 6.1). The results show that tryptophan was one of the most significantly increased metabolites in the extracellular medium as a result of infection. This specifies that infected HeLa cells are producing tryptophan at a significantly excessive rate and that the cells need to secrete large quantities into the surrounding medium. Why infection with *L. monocytogenes* would initiate this change is not entirely clear. There is evidence that T-cells deploy a nutrition deprivation defence mechanism against invading cells by sequestering key nutrients (Radtke and O'Riordan, 2006). Tryptophan catabolism by indoleamine 2,3-dioxygenase directly starves the parasite for tryptophan (Thomas *et al.*, 1993). This evidence contradicts the results from this study. However, it is important to emphasise that the tryptophan sequestering effect documented by Thomas *et al.* (1993) is not observed in HeLa cells but macrophages, which may have significantly different physiology and metabolic makeup to the HeLa cells used in this study (Cairns *et al.*, 2011).

There was a significant reduction in the amino acids glutamine and glutamate (Table 7.1). These amino acids play an essential role in the nitrogen metabolism and also in other aspects in the general metabolism of *L. monocytogenes*. The nitrogen metabolism will be discussed in detail in Chapter 8.
To summarise, the data provide evidence that *L. monocytogenes* utilise many amino acids directly from the host cell, an observation also confirmed through previous studies using $^{13}$C isotopologue profiling (Eylert *et al.*, 2008, Eisenreich *et al.*, 2006).

### 7.3.6) Polyamine metabolism

The polyamine - spermine is increasing significantly in infected samples with a fold change of 0.08. The actual function of spermine in cells is not entirely elucidated; however they have implications in a number of physiological processes including toxin activity, protection from oxidative stress and acid stress. They are also thought to be important bacterial growth factors, suggesting that this is a metabolite directly linked to the intracellular parasite (Shah and Swiatlo, 2008).

### 7.4) Experimental limitations

Although the metabolomic study has provided a plethora of very interesting information regarding the metabolism of *L. monocytogenes* and its host, there are a few caveats to this information and some limitations that need to be considered when examining the data. The following section will focus on the most important of these.

An extremely important consideration is that this series of metabolomic experiments was performed with HeLa cells, a human epithelial cancer cell line, routinely used for tissue culture work (Scherer, 1954). Although these cells may represent an accurate model for a mammalian *L. monocytogenes* infection, they have inherent disadvantages.

There are some significant metabolic changes to cancer cells which are not present in normal healthy cells. Unlike normal cells which preferentially rely on oxidative phosphorylation to generate energy, most cancer cells elevate glycolysis (Gatenby and Gillies, 2004). Most cancer cells also overexpress hypoxia inducible factor 1 α (HIF1 α), which leads to increased transcription of glycolytic enzymes and glucose transporters (Maxwell *et al.*, 2001). This is a
phenomenon known as the ‘Warburg effect’ and can lead to an anaerobosis of the cellular metabolism accompanied by the increased conversion of pyruvate to lactate and the reduction in the activity of the TCA cycle (Fig. 7.5) (Vander Heiden et al., 2009, Denkert et al., 2008). Another idiosyncratic aspect of cancer cell metabolism is their increased requirement for glutamine, which will be discussed in much greater detail in the context of the nitrogen metabolism in the next chapter (Wise and Thompson, 2010, Meng et al., 2010).

It is not clear which exact effects these changes would have on the metabolism of invading L. monocytogenes and the adaptations this would lead to. However, the carbon and energy metabolism encountered by L. monocytogenes in these ‘model host’ cells may be significantly different from that encountered during invasion of actual target cells, and it has been suggested that increased metabolic activity in cancer cells may lead to increased pathogen growth (Eisenreich et al., 2006).

One of the most conspicuous limitations of MS as an analytical tool is the number of unidentified metabolites in a given analysis. This is the case in this study, with a significant number of metabolites remaining unidentified. In fact, from the experiments conducted there are 102 metabolites which remain unidentified (Table 7.2), a number representing half of all the metabolite peaks recorded. Of these, 28 changed significantly as a result of infection and could be of great interest.

Metabolite identification is dependent on matching retention times and peak heights to those found in database. Sometimes this is not always possible, leading to many unknowns from a typical GC-MS data set (Brown et al., 2009, Bowen and Northen, 2010). There are a number of reasons why extracted peaks are left unidentified. Firstly, there is the signal to noise ratio, which, if too high, can lead to mismatching during comparison to a reference library. Secondly, some metabolites share very similar mass/charge ratios and have similar retention times. This means that deconvolution of multiple compounds is unsuccessful, leading to an uncertain or limited identification (Tsugawa et al., 2011). This is something which is seen in this study for the identification of sugars (Table 7.2).
Fig. 7.5 Metabolic adaptions of the cancer cell and the Warburg effect
Schematic highlighting the metabolic adaptations of a cancer cell. In the presence of oxygen normal cells will metabolise glucose to pyruvate via glycolysis, the pyruvate is then oxidised to CO₂ during oxidative phosphorylation. Without oxygen this cannot take place, cells can redirect pyruvate to generate lactate by anaerobic glycolysis. The Warburg effect leads to most of glucose being converted to lactate regardless of the availability of oxygen. Diagram adapted from (Vander Heiden et al., 2009)
There are a large number of sugars from this study which cannot be completely resolved. Finally, and most importantly, is the rather inconvenient fact that no reference library is complete (Tsugawa et al., 2011). If one would like to identify a metabolite known to be in a sample, one can run the sample against a standard of the desired metabolite. However, if there are a large number of compounds which need identification this process becomes prohibitively expensive, before even considering synthesis of non-commercially available standards (Tsugawa et al., 2011).

Metabolite identification issues also extend to reproducibility across multiple experiments. It was found that the metabolites identified across multiple experiments were not conserved. That is, metabolites found in one experiment were not always identified in another. This made it very difficult to simultaneously analyse data from multiple experiments. This was something which was encountered whilst taking all reasonable measures to reduce biological variation.

Biological variation across samples is an issue of concern during this study. This is something which is highlighted in the multivariate analysis of the samples, which show limited clustering within control and infected groups (Fig. 7.1). The GC-MS analysis is such an exquisitely powerful and sensitive technique that, when coupled with cell culturing techniques, which are difficult to standardise completely, variability can be an issue (Molloy et al., 2003). All reasonable measures were taken to keep this variability to a minimum. These include performing experiments on HeLa cells at the same passage number, keeping the same batch number for the DMEM used in an experiment and ensuring that HeLa T75 flasks and experiments are conducted for the same time (Chapter 3, Methods).

There are inherent limitations to the GC-MS methodology, which have been discussed in detail in section 1.13.4. Briefly, only compounds which are volatile enough and thermally stable are amenable to GC-MS analysis. Other methods, such as liquid chromatography mass spectrometry (LC-MS), would be more suitable for particular compounds such as lipids (Looser et al., 2005). Although this is an issue to consider, this is not really something that can be avoided
altogether as each MS technique has inherent advantages and disadvantages. Future studies could focus on alternative methods for example, LC-MS to identify compounds missing from this analysis, which is more suited to the identification of lipids (Gross, 2004).

A particular limitation in this study is that of failed samples. These are samples which have become corrupted, possibly during metabolite extraction. These samples lead to poor data from the subsequent GC-MS analysis. This was an issue with the sample and not a GC-MS issue. These samples did not give reliable readings during the GC-MS analysis and thus had to be removed from the study. This had the effect of reducing the number of control and infected samples used for the data analysis. A lot of effort was taken to ensure all samples were treated the same during the extraction procedure and since faulty samples could only be identified during GC-MS analysis, it is still unclear what the exact cause of the failed samples was.

7.5) Conclusions

The analyses of the GC-MS data confirm that there was a significant change in the metabolome of HeLa cells as a direct result from infection with L. monocytogenes.

Analyses of individual metabolites confirm that there was a reduction in a number of amino acids in infected profiles, which confirms the hypothesis that most of the amino acids utilised by L. monocytogenes are provided by the host cell (Eylert et al., 2008). These include large reductions in amino acids which have been confirmed as important to L. monocytogenes metabolism, for example, glutamine, methionine and cysteine.

Analysis of the carbon metabolism revealed large number unidentified sugars, which are significantly reduced in the infected profiles. This indicates that the carbon metabolism of intracellular L. monocytogenes may be rather complex, with the utilisation of a large number of carbon compounds. The exact carbon compounds cannot be fully identified due to limitations previously discussed.
The results highlight that this methodology can provide some excellent and exciting data with regards to the intracellular metabolism of parasite and the changes that occur to the host. The methodology has proven as a useful tool to provide an insight into overall aspects of host metabolism and the effect of *L. monocytogenes* infection. The methodology could be adapted for more detailed analyses using isotopically labelled metabolites of interest, which would provide a good follow on study from this work (Eylert *et al.*, 2008, Eisenreich *et al.*, 2006)
Chapter 8
Analysis of the nitrogen metabolism of *L. monocytogenes* during intracellular growth
8.1) Introduction

The previous chapter discussed the intracellular metabolism of *L. monocytogenes* and its effect on the host. It was a non-specific approach which attempted to identify global metabolite data for the host cell and how the metabolome changes as a result of infection. This was an approach which provided a comprehensive analysis of all measurable compounds in the sample. Although this approach was highly suited to the aims and objectives of the previous study, i.e. identifying biomarkers of metabolite change due to infection, the results were qualitative and not quantitative, so actual concentrations of the compounds were not determined, in contrast, relative abundance was given with respect to an internal standard.

The following study was conducted to elucidate the intracellular nitrogen metabolism of *L. monocytogenes*. This was a targeted approach where predefined specific metabolites were scanned and measured against a calibration curve from varying concentrations of said target metabolite. This approach increases accuracy of the detection whilst providing actual quantifiable values (Looser *et al.*, 2005, El-Aneed *et al.*, 2009).

Currently, it is thought that *L. monocytogenes* is capable of utilising nitrogen from a number of different sources including glutamine, arginine and, potentially, ethanolamine (Joseph and Goebel, 2007). Glutamine seems to be the preferential and optimal nitrogen source for most bacteria, where it is converted to glutamate, an essential donor of nitrogen in amino acid and nucleotide metabolism (Susan, 1999).

The host cell must provide most of the nitrogenous compounds required by *L. monocytogenes* for intracellular growth (Joseph and Goebel, 2007). This allowed for a targeted approach using GC-MS, measuring the abundance of the nitrogenous compounds in the host cell and establishing how infection of *L. monocytogenes* changes this.

As glutamine and glutamate are considered primary nitrogen sources for intracellularly growing *L. monocytogenes*, key knockout mutants in glutamine
and glutamate cycle (GGC) were produced. These mutants were subjected to growth assays to highlight any phenotypic changes in growth patterns \textit{in vitro} and also \textit{in vivo}.

\subsection*{8.2) Results}

The experiment conducted was the GC-MS quantification analysis of the potential nitrogenous compounds described earlier. The infection of HeLa cells was conducted using a similar methodology to the intracellular metabolite profiling, which was discussed in the previous chapter. The HeLa cells were infected for a total of 10 hours with an MOI of 10. After 10 hours the HeLa cells were lysed and extracted using pure methanol, and extractions were freeze thawed and then analysed by GC-MS. For a detailed description please see section 3.5.7.

The results are presented in the form of box whisker plots in Fig. 8.1. The Box whisker plots provide an overview of the GC-MS data range for each group, control and infected. The data clearly show that there is a reduction in the infected profiles for most of the nitrogenous compounds. In addition Table 8.1 highlights the fold changes of metabolites from the GC-MS data and shows their statistical significance. A Wilcoxon rank sum test was used to calculate statistical significance. Fold change values were calculated using average values from the control and infected profiles. Values for the control were divided by the values for the infected. Any number greater than 1 represents a negative fold change, i.e. the metabolite is reduced in the infected profiles. Any number less than 1 represented an increase of the metabolite in the infected profiles.

Adenine, ethanolamine and ammonia were not detected in the samples, so these are not shown in the data presented here. There is a negative fold change for arginine; however, this is not statistically significant. The other nitrogenous sources present significant changes, which are highlighted by Fig. 8.1 and Table 8.1. From the nitrogenous compounds citrulline represents the largest fold difference (1.9) between uninfected and infected profiles.
<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Fold Change</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>1.147</td>
<td>0.214</td>
</tr>
<tr>
<td>Citrulline</td>
<td>1.956</td>
<td>0.024</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.274</td>
<td>0.006</td>
</tr>
<tr>
<td>Glutamine</td>
<td>1.269</td>
<td>0.014</td>
</tr>
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</table>

Table 8.1 Fold change for nitrogenous compounds
Fold changes using Wilcoxon rank sum test for measured nitrogenous compounds are shown in the table. Fold change values were calculated using average values from 12 control and 12 infected profiles. Values for the control were divided by the values for the infected. Any value greater than 1 is a negative fold change, with the metabolite reduced in the infected profiles. Any number less than 1 represents a positive fold change with the metabolite increased in infected profiles. P values are also shown for the metabolites with significant reductions highlighted in red.
Fig. 8.1 Box and whisker plots for quantification of potential nitrogen sources for *L. monocytogenes*

Box and whisker plots represent a graphical representation of the numeric data providing a number of summaries. These include lower quartile range, median, upper quartile range and finally the extreme values (smallest and largest). Results show the spread of the data between 12 samples infected and 12 uninfected samples. All of the metabolites are reduced in the infected samples. Apart from arginine all metabolite reductions are significant. The reductions in these metabolites suggest that these sources are being sequestered by the intracellular *L. monocytogenes*. 
Glutamine and glutamate represent the most statistically significant changes. The importance of these changes and their implications will be discussed in greater detail in the following section.

As discussed previously, glutamine has been proposed as an essential nitrogen source for intracellular bacteria (Merrick and Edwards, 1995). To test the importance of the glutamine/glutamate cycle (GGC) to intracellularly growing *L. monocytogenes*, a series of knockout mutants were produced. These are presented in Fig. 8.2 and consist of glutamate dehydrogenase (GDH), which converts α-ketoglutarate to glutamine, glutamine synthetase (GS), which in converts glutamate to glutamine, glutamate synthase (GOGAT), which converts glutamine to glutamate, and finally, a GOGAT and GDH double mutant (Fig. 8.2). The mutants were produced using homologous recombination and the methodology is discussed in detail in section 3.4. Confirmations of the knockout mutations are depicted in Fig. 8.3.

*in vitro* experiments were first conducted in TSB medium, which is a tryptic digest of soya bean and was used for laboratory growth of *L. monocytogenes* (Goldfine and Shen, 2007). The second sets of *in vitro* experiments were conducted in GDM, the composition of which is described in Table 3.3.

The growth experiments for GGC mutants against the wild type (WT) in TSB medium are shown in Fig. 8.4. The results show that growth rate of ΔgltAB (blue) was very similar to the WT (yellow), with a doubling time of 42 minutes and 40 minutes respectively. There was a slight reduction in final OD\textsubscript{600} for Δgdh (red) and also a slightly longer doubling time of 48 minutes. However, the most profound difference was in growth of the ΔglnA mutant. The doubling time for ΔglnA was 70 minutes, which was substantially slower than the WT. The results clearly show that losing the ability to synthesize glutamine *de novo* (or other possible downstream event) significantly impaired *L. monocytogenes* growth even in a complex medium like TSB.
Fig. 8.2 Glutamine/glutamate assimilation pathway and *L. monocytogenes* mutants produced

Glutamine as nitrogen source is combined with α ketoglutarate and converted to glutamate by glutamate synthase (GOGAT). Glutamate is the principal nitrogen donor to other amino acids in subsequent transamination reactions (AT) and to nucleotides. Glutamate is also a product in the glutamate dehydrogenase (GDH) reaction which inter-converts glutamate into α ketoglutarate. With the consumption of ammonia and ATP, glutamate can be converted to glutamine by the glutamine synthetase enzyme (GS). Knockout mutants produced are highlighted in red and include GDH (Δgdh), GS (ΔglnA), GOGAT (ΔgltAB) and a GDH/GOGAT (Δgdh/ΔgltAB) double mutant.
1) **Glutamine synthetase (GS)**

![Agarose gel electrophoresis images from colony PCR of nitrogen mutants, to confirm gene deletion by homologous recombination. All screening primers used are shown in Table 3.5.]

<table>
<thead>
<tr>
<th></th>
<th>Mutant</th>
<th>Wild type</th>
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<tbody>
<tr>
<td>Size bp</td>
<td>1000</td>
<td>1500</td>
</tr>
<tr>
<td></td>
<td>2000</td>
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<td>3000</td>
<td>3213 bp</td>
</tr>
<tr>
<td></td>
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</table>

2) **Glutamate dehydrogenase (GDH)**

![Agarose gel electrophoresis images from colony PCR of nitrogen mutants, to confirm gene deletion by homologous recombination. All screening primers used are shown in Table 3.5.]

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<thead>
<tr>
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<th>Mutant</th>
<th>Wild type</th>
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<tbody>
<tr>
<td>Size bp</td>
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<td></td>
<td>2000</td>
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<td></td>
<td>3000</td>
<td>3325 bp</td>
</tr>
<tr>
<td></td>
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<td>1975 bp</td>
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</table>

3) **Glutamate synthase (GOGAT)**

![Agarose gel electrophoresis images from colony PCR of nitrogen mutants, to confirm gene deletion by homologous recombination. All screening primers used are shown in Table 3.5.]

<table>
<thead>
<tr>
<th></th>
<th>Mutant</th>
<th>Wild type</th>
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<tbody>
<tr>
<td>Size bp</td>
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<td></td>
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<td></td>
<td>3000</td>
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<td></td>
<td></td>
<td>6278 bp</td>
</tr>
<tr>
<td></td>
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**Fig. 8.4 Analysis of GGC mutant growth against WT in TSB medium**

GGC mutants grown in tryptone soy broth (TSB), a complex tryptic digest of soya bean. The experiment represents average values from 6 replicates. There is clear attenuation of the ΔglnA mutant lacking the enzyme GS even in a complex medium like TSB (green). This highlights that the ability to process glutamine through the GGC is essential for normal *L. monocytogenes* growth.

**Doubling time**

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<table>
<thead>
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<tbody>
<tr>
<td>WT</td>
<td>42 minutes</td>
</tr>
<tr>
<td>ΔgltAB</td>
<td>40 minutes</td>
</tr>
<tr>
<td>Δgdh</td>
<td>48 minutes</td>
</tr>
<tr>
<td>ΔglnA</td>
<td>70 minutes</td>
</tr>
</tbody>
</table>
A second TSB growth experiment was conducted, where the ΔglnA was grown in TSB supplemented with 5 mM glutamine. The results for this experiment are shown in Fig. 8.5. By supplementing TSB with 5 mM glutamine an increase of OD_{600} values for ΔglnA was observed (green). The doubling time is also reduced by 21 minutes. Although, there was a reduction in doubling time in comparison to ΔglnA grown in just TSB (red), WT (yellow) levels of growth were still not restored.

A GDM experiment (Fig. 8.6) was designed to test the mutant’s ability to grow with limited nitrogen sources. The minimal medium contained only arginine and ferric ammonium citrate as potential nitrogen sources and the experiment was designed to test the importance of free glutamine for *L. monocytogenes* growth. The results show that all strains struggle to grow in this medium; however, the ΔglnA was particularly impaired, exhibiting very little growth (green).

Supplementing GDM with glutamine at a concentration of 5 mM did result in growth for ΔglnA mutant (Fig. 8.7). Observing the graph reveals that there is a two phase growth. Initially the growth of the ΔglnA mutant is similar to the WT, however at around T4, the growth rate slows. This is possibly a result of glutamine levels becoming limited.

To accompany the *in vitro* experiments a number of *in vivo* experiments were also conducted using gentamicin killing/protection assay. The GGC mutants were tested for their ability to invade and proliferate in mammalian HeLa cells. The growths of these mutants were tested in direct comparison to a WT control.

The Δgdh mutant exhibited no attenuation for intracellular growth, which are supported by no significant change at any timepoint. The mutant also had a very similar doubling time during 2 hours to 8 hours post infection, which were 50 minutes and 52 minutes for the WT and mutant respectively (Fig. 8.8).

The intracellular growth of the ΔgltAB was attenuated. Although, the reduction was statistically significant at 4 hours, 8 hours and 24 hours the growth trend is very similar to the WT (Fig. 8.9).
Fig. 8.5 Analysis $\Delta glnA$ mutant against WT in TSB medium - after the addition of 5 mM glutamine

The effect of 5 mM Gln supplementation was examined and extra Gln does increase the rate of growth for the $\Delta glnA$ mutant (Green) in comparison to $\Delta glnA$ without Gln supplementation (Red). The results shown are the average of 6 replicates. Both mutants are still significantly impaired in comparison to the WT (Yellow).

Doubling time

<p>| | |</p>
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>24 minutes</td>
</tr>
<tr>
<td>$\Delta glnA$</td>
<td>69 minutes</td>
</tr>
<tr>
<td>$\Delta glnA + Gln$</td>
<td>46 minutes</td>
</tr>
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</table>
GGC mutants were tested for their ability to grow in GDM. The results shown are the average of 6 replicates. The medium was modified to contain only arginine and ammonium ferric citrate as nitrogen sources. It must be noted that all strains grow poorly in this medium compared to TSB. The ΔglnA mutant is completely incapable of growth in this medium (Green).

**Doubling time**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>92 minutes</td>
</tr>
<tr>
<td>ΔglnA</td>
<td>N/A</td>
</tr>
<tr>
<td>ΔgltAB</td>
<td>84 minutes</td>
</tr>
<tr>
<td>Δgdh</td>
<td>90 minutes</td>
</tr>
</tbody>
</table>
Fig. 8.7 Analysis of GGC mutant growth WT type in GDM - after the addition of 5 mM glutamine
The minimal medium GDM was supplemented with 5 mM glutamine. The results are averages of 6 replicates. The results show that there is a slight increase in growth for the ΔglnA mutant, however the rate is still significantly lower than the other GGC mutants and also the WT. The addition of glutamine presents a dual growth phase. The initial rate up to 4 hours is similar to the WT, however the rate seems to drop after 4 hours. This is possibly due to the additional glutamine being sequestered.

**Doubling time**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>= 90 minutes</td>
</tr>
<tr>
<td>ΔglnA</td>
<td>= 195 minutes</td>
</tr>
<tr>
<td>ΔgltAB</td>
<td>= 96 minutes</td>
</tr>
<tr>
<td>Δgdh</td>
<td>= 96 minutes</td>
</tr>
</tbody>
</table>
Fig. 8.8 Gentamicin killing assay for Δgdh mutant vs. WT
The result for intracellular proliferation is shown for WT against Δgdh mutant. The results represent the average of 9 replicates. It is clear that the Δgdh mutation does not possess an intracellular phenotype. The phenotype for the mutant is very similar for that of the WT with very similar doubling times from 2 hours post infection and 8 hours post infection (see below). Also there is no significant difference between the two CFU counts at any time point. These results indicate that GDH does not play an important role in the intracellular metabolism of L. monocytogenes.

Doubling time 2hrs – 8hrs
WT = 50 minutes  
Δgdh = 52 minutes

<table>
<thead>
<tr>
<th>Time Points</th>
<th>WT vs. Δgdh</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>0.464</td>
</tr>
<tr>
<td>T2</td>
<td>0.737</td>
</tr>
<tr>
<td>T4</td>
<td>0.061</td>
</tr>
<tr>
<td>T8</td>
<td>0.366</td>
</tr>
<tr>
<td>T24</td>
<td>0.658</td>
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</tbody>
</table>
The analysis of doubling times reveals that the $\Delta gltAB$ mutant had a doubling time of 63 minutes from 2 hours to 8 hours post infection compared to 55 minutes for the WT (Fig. 8.9). The results suggest that the $\Delta gltAB$ gene is not essential for intracellular growth. The results are similar for the $\Delta gdh/\Delta gltAB$ double mutant which has a very similar growth trend and doubling time (Fig. 8.10).

The $\Delta glnA$ mutant was significantly attenuated for intracellular growth (Fig. 8.11). The WT recovers quickly after the addition of gentamicin from 2 hours post infection to 8 hours post infection with a doubling time of 50 minutes. The $\Delta glnA$ mutant struggles to grow in comparison to the WT, having a doubling time of 155 minutes. Interestingly $\Delta glnA$ mutant exhibits a almost linear growth rate during the course of the assay and post 8 hours growth is similar to that of the WT, albeit at a lower cell density. This suggests that the $\Delta glnA$ mutant is particularly impaired during initial periods of growth after infection of HeLa cells.

The lack of growth from $\Delta glnA$ was highlighted by the control experiment shown in Fig 8.10. The objective of this experiment was to provide a reference for the attenuation of the $\Delta glnA$ mutant. A direct comparison of the $\Delta glnA$ mutant and a $\Delta hly/plcB$ (LLO and PC-PLB) mutant, which was completely avirulent, was also conducted, and the results are shown in Fig. 8.10. The $\Delta glnA$ mutant, although attenuated, did continue to grow during the course of the assay as shown by the 24 hour timepoint in Fig. 8.10.

Two control experiments were conducted to establish whether supplementation of the DMEM medium with 10 mM glutamine restored WT phenotype. The DMEM was supplemented with 10 mM glutamine. The results are shown in Fig. 8.10 and indicate that the additional glutamine in the medium did indeed lead to an increase in growth for the $\Delta glnA$ mutant (red). The doubling time for the $\Delta glnA$ mutant is 141 minutes which was reduced to 90 minutes with the addition of glutamine. However, supplementing with glutamine did not restore WT levels of growth, with the $\Delta glnA$ mutant still significantly attenuated in comparison to the 55 minutes doubling time of the WT. The implications of these results will be discussed in the following section.
Fig. 8.9 Gentamicin killing assay for ΔgltAB (GOGAT) vs. WT

The growth of the ΔgltAB single mutant lacking the GOGAT gene is shown. The results are the average of 9 replicates. The ΔgltAB mutant exhibits a small attenuation in growth in comparison to the WT. The mutant is significantly attenuated 4 ours post infection, however the growth follows a similar trend to the WT. This suggests that glutamate production through the GS/GOGAT cycle is not essential for intracellular survival. This result could also indicate that glutamate was produced through alternative routes, such as, the roc pathway, thus, abrogating the effect of losing the gltAB gene.

Doubling time 2hrs – 8hrs

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>ΔgltAB</th>
<th>ΔgdhΔgltAB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>55 min</td>
<td>63 min</td>
<td>60 minutes</td>
</tr>
</tbody>
</table>

| Students T test P Values for time points, WT vs. ΔgltAB |
|-------------|-----------|-----------|-----------|-----------|-----------|-----------|
| T0          | 0.679     | 0.894     | 0.023     | 0.043     | 0.006     |
Fig. 8.10 Gentamicin killing assay for Δgdh/ΔgltAB (GDH/GOGAT) vs. WT
The intracellular growth of the Δgdh/ΔgltAB double mutant is shown. Δgdh/ΔgltAB mutant is incapable of producing glutamate through the GS/GOGAT cycle. The results are the average of 9 replicates. The Δgdh/ΔgltAB mutant has a very similar phenotype to the ΔgltAB single mutant. They also share very similar doubling times as seen below. The double mutant is also significantly attenuated after 4 hours post infection. Since the Δgdh single mutation has no discernible phenotype, and the growth trend is so similar to the ΔgltAB mutant, the attenuation seen in the double Δgdh/ΔgltAB mutant can be attributed to the loss of ΔgltAB.

**Doubling time 2hrs – 8hrs**

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<tbody>
<tr>
<td>WT</td>
<td>55 minutes</td>
</tr>
<tr>
<td>ΔgltAB</td>
<td>63 minutes</td>
</tr>
<tr>
<td>ΔgdhΔgltAB</td>
<td>60 minutes</td>
</tr>
</tbody>
</table>

**Students T test P Values for time points, WT vs. Δgdh/ΔgltAB**

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>T2</th>
<th>T4</th>
<th>T8</th>
<th>T24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.821</td>
<td>0.152</td>
<td>0.016</td>
<td>0.019</td>
<td>0.050</td>
</tr>
</tbody>
</table>
Fig. 8.11 Gentamicin killing assay for ΔglnA (GS) mutant

The growth of the ΔglnA mutant, which lacks the GS gene is shown. The results are the average of 9 replicates. The ΔglnA mutant is significantly defected for intracellular proliferation in comparison to the WT with a significantly longer doubling time 2 hours to 8 hours (see below). the growth of the ΔglnA mutant after 8 hours is similar to that of the WT, which suggests that the mutant is particularly impaired during the initial growth after infection. The GS enzyme represents the sole pathway for glutamine synthesis, losing this has a profound effect on the growth of L.monocytogenes.

Doubling time 2hrs – 8hrs

WT = 50 minutes
ΔglnA = 155 minutes

| Students T test P Values for time points, WT vs. ΔglnA |
|-----------------------------|-----------------------------|
| T0  | T2  | T4  | T8  | T24 |
| 0.121 | 0.168 | 0.000 | 0.000 | 0.000 |
Fig. 8.12 Gentamicin killing assay controls for ΔglnA (GS) mutant
The growths of the ΔglnA mutant controls are shown. The results are the average of 6 replicates. The first control supplemented the external medium with 10 mM Gln to test if WT phenotype could be restored. Gln supplementation leads to an increase in growth (A - Red), however the growth is still significantly lower than the WT. This is also reflected in the average doubling times for the strain, which show that the rate of growth has almost doubled with the addition of Gln yet is still significantly slower than the WT (See below). The second control was to establish a reference point for the attenuation of the ΔglnA mutant (B). The control assay was performed against a Δhly/plcB, which is incapable of escaping the primary phagosome, thus incapable of intracellular growth. Although the ΔglnA mutant is significantly impaired it does continue to grow over the 24 hr infection period.

**Doubling time 2hrs – 8hrs**
- WT = 55 minutes
- ΔglnA = 141 minutes
- ΔglnA + Gln = 90 minutes
8.3) Discussion

Our current knowledge on the nitrogen metabolism of *L. monocytogenes*, especially when growing intracellularly, is limited. Most information available is based on genomic and transcriptional data, where the presence of particular pathways or proteins can elucidate the ability of *L. monocytogenes* to utilise specific nitrogenous compounds (Goldfine and Shen, 2007).

It is currently accepted that *L. monocytogenes* is able to utilise a number of compounds for its nitrogen metabolism. Taking these potential nitrogen sources into consideration, a targeted approach using GC-MS, was taken, looking at ammonium, arginine, citrulline, ethanolamine, glutamine and glutamate. The primary objective of the study was to elucidate the intracellular nitrogen metabolism of *L. monocytogenes*. The study was conducted using the same methodology as the intracellular profiling discussed in the previous chapter. Whereas the analysis in the previous study was a global scan of all metabolites detected, in this study the focus was on previously mentioned nitrogenous compounds. These compounds were specifically targeted using GC-MS analysis giving a quantifiable numbers as opposed to relative abundance given in the global profiling analysis.

8.3.1) Univariate analysis of GC-MS data

The data from the GC-MS quantification was analysed using univariate analysis such as paired T-tests, Wilcoxon rank sum tests and box and whisker plots. Unlike the global metabolic profile study, which revealed a large dataset with a large number of variables, this more targeted approach only provided data for a relatively small number of variables; hence the multivariate analyses used in the previous study were not necessary.

The results from the box whisker analysis are shown in Fig. 8.1. The results show that all the detected nitrogenous compounds measured were reduced in the infected metabolic profiles. This data is supported by the fold change reported in Table 8.1.
Of the measured metabolites only arginine is not reduced in a statistically significant manner (Table 8.1). All of the remaining metabolites are significantly reduced, the strongest being glutamine and glutamate. This suggests that there is increased consumption of these metabolites as a result of the infection. As *L. monocytogenes* must obtain their nitrogenous compounds from the host, the assumption is that these compounds are being utilized by intracellular *L. monocytogenes*, thus explaining the reduction in infected profiles.

Glutamine is widely regarded as the primary source of nitrogen for many bacteria (Merrick and Edwards, 1995, Doroshchuk et al., 2006). That glutamine and glutamate are the most significantly changing metabolites in this study (Table 8.1) support the hypothesis these are amongst the key nitrogen compounds utilised by *L. monocytogenes* (Merrick and Edwards, 1995). Although the data show glutamine is being preferentially used, glutamine also plays an exceptionally important role in the metabolism of mammalian host cell, making it likely that the availability of glutamine becomes limited during intracellular growth (Newsholme *et al.*, 2003, Doroshchuk *et al.*, 2006). In such a situation *L. monocytogenes* may utilise alternative nitrogen sources such as ammonium (Tsai and Hodgson, 2003).

Ammonium is amongst the metabolites not shown, as it was not accurately detected in the samples. However, in the literature there is evidence demonstrating the importance of ammonium for intracellular *L. monocytogenes*, for example, upregulation of the NrgA/B operon encoding ammonia transporters during intracellular growth (Detsch and Stulke, 2003).

Although the exact mechanism of ammonium regulation is yet to be elucidated in *Listeria*, orthologous genes which are involved in *Bacillus* ammonium regulation have been found, suggesting the mechanisms of control may also be shared (Joseph and Goebel, 2007). The operon controlling the production of NrgA/B in *L. monocytogenes* is upregulated during intracellular growth (Detsch and Stulke, 2003). These data suggest ammonia could play an important role as a nitrogen source for *L. monocytogenes.*
Ammonium is incorporated into glutamine, and subsequently glutamate, by the enzyme glutamate synthase with 2-oxoglutarate as an additional substrate (Joseph and Goebel, 2007). In B. subtilis the ammonium is internalized by diffusion or active transportation using the NrgA transporter protein along with the NrgB protein. These translate the information of intracellular ammonium levels by modifying downstream regulatory factors, which are involved with the expression of glutamine synthetase and glutamate synthase (Detsch and Stulke, 2003). Unpublished work from our laboratory indicates that the ability to import ammonia may not be essential to L. monocytogenes, as ΔnrgA/B mutant did not show significant attenuation for intracellular growth (Goldrick 2012, unpublished).

An alternative source for intracellular ammonium could also include host arginine, which can be degraded into citrulline and ammonia by the enzyme arginine deaminase (Joseph and Goebel, 2007). The reduction for arginine in this study is not significant, whereas the change in citrulline is significant (Fig. 8.1, Table 8.1). This is a rather strange result as the two metabolites are linked. However, it could possibly be explained by the presence of arginine and absence of citrulline in the DMEM used during the course of the infection. Thus, depleted arginine can be uptaken from the medium, whilst citrulline cannot. Citrulline can be degraded to ammonia via the enzymes ornithine carbamoyl transferase and carbamoyl carboxy kinase encoded by the L. monocytogenes-specific arcBCD operon (lmo0036-0039). This could explain the reasons for its significant reduction in the infected profiles (Fig. 8.1 and Fig. 8.2) (Goldfine and Shen, 2007). These results indicate that although arginine could be utilised by L. monocytogenes, it is not amongst the preferential nitrogen sources for L. monocytogenes.

Ethanolamine is also hypothesized to be utilised as a nitrogen source (Goldfine and Shen, 2007, Joseph et al., 2006). Ethanolamine was not detected in the control or infected profiles. This is interesting as there are some theories suggesting that ethanolamine, liberated from phosphatidylethanolamine (PEA) in phospholipid membranes may provide an important carbon and nitrogen source during intracellular growth (Joseph et al., 2006). Ethanolamine is thought to be generated by the degradation of PEA, a substrate for the phospholipase
PlcB. Ethanolamine itself is then hydrolysed into ammonia and acetaldehyde by the enzyme ethanolamine ammonia lyase (Joseph et al., 2006). The gene encoding ethanolamine ammonia lyase, *eutBC*, is upregulated during intracellular growth, again supporting ethanolamine utilisation during intracellular growth (Joseph et al., 2006). However, exactly how much nitrogen can be liberated from phospholipid membranes and whether this quantity could provide a sustainable nitrogen source is still unclear. The inability to detect ethanolamine in the GC-MS analysis suggests that the quantity of ethanolamine present during an infection is not actually very high and unlikely to be a major nitrogen source. It is important to emphasise that this does not exclude that ethanolamine can be utilised by *L. monocytogenes* as a nitrogen source, only that quantities of it are not very high.

8.3.2) *In vivo* and *in vitro* growth analysis

From the GC-MS data it was clear that glutamine and glutamate are highly reduced in infected profiles. Thus, these were prioritised for further analysis, using mutagenesis, *in vitro* growth assay and also gentamicin killing assays. Gentamicin killing assays are designed to test the ability of pathogens to invade and grow in mammalian cells. The assay is based on the principle that some antibiotics are incapable of traversing the cell membrane of the mammalian host, thus any bacteria internalized are not killed and these can be assayed (Mandell, 1973). To test the importance of glutamine and glutamate to *L. monocytogenes* growth, a series of knockout mutants from the glutamine/glutamate cycle (GGC) were produced and tested for both *in vitro* and *in vivo* growth. These are overviewed in Fig. 8.2.

8.3.3) Glutamate dehydrogenase mutant (*Δgdh*)

The *L. monocytogenes Δgdh* mutant lacking a GDH does not possess a significant growth phenotype when grown in TSB medium (Fig. 8.4), minimal medium (Fig. 8.6) or intracellular growth (Fig. 8.8). GDH can interconvert α-ketoglutarate to glutamate, however, studies in *E.coli* have shown that GDH lacking mutants exhibit no growth phenotypes under normal conditions (Helling, 1994). There is some evidence supporting the idea that GDH may become
important under energy limiting conditions (Helling, 1998). Examination of the GGC pathway (Fig. 8.2) reveals that GDH does not require ATP to produce glutamate; hence, this may confer an advantage for its utilisation during energy limited growth. Under low ammonia conditions GOGAT and GS are responsible for glutamate production. GDH has a relatively low affinity for ammonium therefore thought to require very high concentrations of ammonium to convert $\alpha$-ketoglutarate to glutamate (Sakamoto et al., 1975).

The data from this study suggests that GDH is not a preferential route for glutamate synthesis in intracellular L. monocytogenes. This is highlighted by the gentamicin killing assay showing an intracellular doubling rate for the mutant which is almost identical to the WT (50 minutes and 52 minutes respectively) (Fig. 8.8).

**8.3.4) Glutamine synthetase mutant (\(\Delta glnA\)) / Glutamate synthase mutant (\(\Delta gltAB\))**

Glutamate is synthesized in bacteria using $\alpha$-ketoglutarate, in one of two pathways. The GDH enzyme has already been discussed above. The other pathway is the GOGAT/GS pathway, which forms glutamate through the amidation of glutamate to form glutamine (by GS), which is followed by the reductive transfer of the amide group to $\alpha$-ketoglutarate to give two glutamate molecules, representing a net gain of one (Helling, 1998).

A \(\Delta gltAB\) mutant deficient in the GOGAT enzyme had no phenotype when grown in TSB (Fig. 8.4) and in minimal medium (Fig. 8.6). The gentamicin killing assay revealed that this mutant is attenuated for intracellular growth, in comparison to the WT (Fig. 8.9). However, the growth trend is very similar to WT, and internalized \(\Delta gltAB\) L. monocytogenes are able to grow at a similar rate as highlighted by their similar doubling times (Fig. 8.9).

A glutamate auxotroph mutant was also constructed by removing genes for both GDH (\(gdh\)) and also genes for GOGAT (\(gltAB\)). The \(\Delta gdh/\Delta gltAB\) mutant had an intracellular phenotype very similar to the \(\Delta gltAB\) mutant (Fig. 8.10). That the
attenuation seen in the ΔgltAB and Δgdh/ΔgltAB double mutant was not more pronounced is surprising. The same mutation in other organisms, have a much more profound effects. For example, ΔgltBD (encoding GOGAT) deficient mutants in E. coli and Klebsiella aerogenes exhibit significantly poor growth on a range of nitrogen sources (Goss et al., 2001). This is also surprising when considering that the biosynthesis of glutamate intersects two major aspects of cellular metabolism, linking the carbon metabolism (α ketoglutarate from TCA cycle) to nitrogen assimilation. Nevertheless, this highlights the metabolic robustness of L. monocytogenes, an essential trait for an intracellular pathogen, which lives in an environment where nutrients are in limited supply (Freitag et al., 2009).

The ΔglnA mutant lacking glutamine synthetase (GS) was pointedly attenuated when grown in a complex medium, such as TSB (Fig. 8.4). It was also completely incapable of growth in defined minimal medium with limited nitrogen sources (Fig. 8.6). Supplementing the TSB and GDM with 5 mM glutamine resulted in an increase of growth (a doubling time reduction of 21 minutes); however the strain was still attenuated in comparison to the WT (Fig. 8.6/Fig. 8.7). Interestingly, there was a dual phase growth in the minimal medium experiment supplemented with glutamine (Fig. 8.7). The initial growth up to 4 hours is similar to the WT, however, after 4 hours, the growth slows, suggesting that the additional glutamine added to the medium was sequestered.

There was an equally profound reduction in intracellular growth for the ΔglnA mutant. Fig. 8.11 highlights that there is a significant attenuation in growth for the ΔglnA mutant. The growth for the mutant is severely attenuated during the initial phase of growth from 2 hours post infection, to 8 hours post infection.

To put the reduction into perspective, a control experiment was also done using a Δhly/plcB mutant (Julie Wang, Roberts Lab, unpublished data), which is deficient in the hly gene encoding for LLO and plcB, encoding a phospholipase PC-PLC (Fig. 8.12). The Δhly/plcB mutant was completely incapable of escaping the primary phagosome after internalisation, an event, which has been shown to be essential for the normal proliferation of L. monocytogenes (Cossart et al., 1989). These results show the growth, post 8 hours is similar to the WT,
albeit at a much lower cell density. Overall the mutant seems to grow at a linear and limited growth, unlike the WT, which reaches a high cell density after 8 hours.

Experiments which involved supplementation with glutamine resulted in increased growth for ΔglnA across all experiments performed. Fig. 8.12 highlights the difference in growth for the ΔglnA mutant and the WT. Also shown is the increase in growth that supplementing the medium with 10 mM glutamine brings. There is an increase in growth rate highlighted by a reduction in doubling time from 141 minutes to 90 minutes in the presence of glutamine. It is important to emphasise that, although supplementing the extracellular medium with 10 mM glutamine increases growth, the ΔglnA mutant is still attenuated in comparison to the WT (Fig. 8.12).

These results have interesting implications for the state of the metabolism in the ΔglnA mutant. ΔglnA mutants are incapable of synthesising glutamine and thus would be completely dependent on extracellular supply of glutamine. Losing the enzyme GS, caused significant disruption to the normal growth rate of _L._monocytogenes. Since supplementing with glutamine does not restore WT levels of growth for ΔglnA, it is likely that the ability to produce glutamine _de novo_ is very important for normal _L._monocytogenes growth. Alternatively, the results could indicate that glutamine uptake in the ΔglnA mutant is not sufficient to restore WT levels of growth.

That the attenuation of the ΔglnA mutant is very pronounced in comparison to the ΔgdhΔgltAb double mutant is unexpected. Examination of the GGC cycle would suggest an interdependency of the glutamate and glutamine metabolites as their synthesis are fundamentally linked. However, it is possible that the observed inconsistency between the two mutants could be explained by examining their synthesis pathways.

Glutamate can be synthesized via a range of metabolic pathways, many of which are indirectly dependent on glutamine, for example GS, GDH and GOGAT (Fig. 8.13) (Helling, 1994). Additionally, glutamate can be generated from alanine and also aspartate in combination with AKG using a transaminase
enzyme (Klose and Mekalanos, 1997). Examination of the *L. monocytogenes* genome reveals that it does possess a candidate aspartate transaminase *Imo01619* (data not shown).

In *B. subtilis* the Roc pathway can be utilised to produce glutamate (See Fig. 8.13) via arginine which is converted to ornithine and subsequently glutamate (Picossi et al., 2007). The alternative glutamate synthesis pathways could potentially be used for glutamate synthesis in the Δ*gdh*/Δ*glt* double mutant, which could partially explain the relatively limited reduction in growth.

The synthesis of glutamine is solely dependent on the activity of GS and this is the primary point of nitrogen assimilation in many bacteria (Merrick and Edwards, 1995). Glutamine plays an essential role in a number of varying metabolic pathways, including amino acids, amino sugars, and nucleotide synthesis. However the immediate product of glutamine is glutamate regulation of the cit*Z* gene encoding for a citrate synthetase, the rate limiting enzyme of the TCA cycle (Mittal et al., 2009).

Hence, considering the unique route of synthesis for glutamine, coupled with the multifaceted functionality of the amino acid, it is not surprising that loss of the only path of glutamine synthesis (Δ*glnA*), results in a significant growth defect in vitro and in vivo. Supplementing the growth medium with glutamine does not completely resolve the growth defect. This suggests that removal of GS is having a profound effect on the *L. monocytogenes* metabolism and it is possible that losing GS is also affecting *L. monocytogenes* glutamate pools as the two metabolites are inherently linked in the GGC.

In the intracellular growth assays of Δ*glnA*, the lack of phenotype restoration by glutamine supplementation could be explained by the 'second hand' nutrient uptake which is associated in intracellular parasitism.
Fig. 8.13 Simplified schematic for glutamine and glutamate synthesis in *B. subtilis*

Metabolic context for the synthesis of glutamine and glutamate is shown. GS is completely responsible for the synthesis of glutamine. GS/GOGAT cycle is the primary glutamate production pathway. The Roc pathway and amino transferases present alternative glutamate synthesis routes in *B. subtilis*; however, the genes in this pathway have not yet been identified in *L. monocytogenes.*

(Adapted from Picossi *et al.*, 2007)
In other words, *L. monocytogenes* is dependent on the intracellular environment for nutrients. Thus increasing the concentration of glutamine in the extracellular medium, does not necessarily equate to a sufficient supply of glutamine in the intracellular environment of the HeLa cells. This is especially true when taking into consideration the extraordinary metabolic necessities of the HeLa cell. HeLa cells are cancer cells and as such have a number of very peculiar metabolic demands. The most prominent of this is the Warburg effect, which leads to the anaerobiosis of cellular metabolism (Vander Heiden *et al.*, 2009). Another idiosyncratic aspect of cancer cell metabolism is their increased requirement for glutamine (Wise and Thompson, 2010, Meng *et al.*, 2010). The increased requirement for glutamine in cancer cells has been explained by the use of glutamine as an alternative carbon source and as an essential mitochondrial substrate (Reitzer *et al.*, 1979). Also, cancer cells channel α-ketoglutarate derived from glutaminolysis into the TCA cycle to replenish metabolic intermediates such as NADPH for redox control and fatty acid synthesis (Meng *et al.*, 2010).

These results indicate that the usable glutamine levels in the intracellular HeLa environment may not be very high. It also suggests that the host cell may be sequestering glutamine at an excessive rate thus explaining the significant reduction in the Δ*glnA* mutant, even after supplementing with external glutamine.

To conclude, the *in vitro* and *in vivo* analyses have revealed that glutamine plays an exceptional role in the metabolism of *L. monocytogenes*. Where glutamate auxotrophy causes a small attenuation, losing GS and the ability to synthesize glutamine *de novo* caused significantly impaired growth for *L. monocytogenes* in a range of different environment.

### 8.4) Experimental limitations and caveats

As the metabolite extraction procedure for the quantification study was identical to that of the metabolic profiling, the same experimental limitations are valid here. These were discussed in detail in Chapter 7.
8.5) Conclusions

The quantification of nitrogenous compounds has revealed some very interesting results, which indicate that glutamine is a preferential nitrogen source for intracellular *L. monocytogenes*. However, other nitrogenous compounds were also significantly reduced, which points to a complex, multifaceted utilisation of nitrogenous compounds.

The levels of ethanolamine were below the threshold of GC-MS detection, suggesting that levels of ethanolamine are not very high. This could be seen as evidence to contradict the theory which suggests that ethanolamine liberated from host cell membranes could be used as a nitrogen source.

The data from this study suggests that GDH is not a preferential route for glutamate synthesis in intracellular Glutamate auxotrophy causes a small attenuation, which indicates a compensatory effect from alternative pathways for glutamate synthesis in *L. monocytogenes*. The importance of glutamine for intracellular growth is emphasized by the result that losing GS and the ability to synthesize glutamine *de novo* causes significantly impaired growth for *L. monocytogenes* in a range of different growth environments.

8.6) Future work

The quantification analysis study revealed some very interesting findings and also proved to be a good proof of principle study for using metabolomics for a targeted analysis of nitrogenous compounds in an intracellular pathogen.

There are a number of different analyses which still need to be conducted to further the understanding of the intracellular nitrogen metabolism of *L. monocytogenes*.

The first process that would need to be completed to ensure accuracy and validity of the results is the re-complementation and analysis of the GGC mutant genes especially the ΔglnA mutant, which exhibits a strong phenotype. This is an important process to provide robust evidence that the phenotypic behavior of
the mutant is as a direct result of the mutagenesis of the particular gene in question. This is something I hoped to complete during the course of the study, but unfortunately, timing constraints did not allow this.

Along with the metabolomic quantification of WT genes, there was also metabolomic quantification of nitrogenous compounds for the GGC knockout mutants was performed. I hoped that this would provide an excellent comparative tool to examine the effects of the GGC mutant knockouts on the uptake of nitrogenous sources and establish which alternative nitrogen sources are utilised when primary sources such as glutamine cannot be synthesised. This experiment was completed, with 5 samples per mutant strain (5 control flasks vs. 5 infected flasks). The data for these experiments was not shown in this chapter as unfortunately, there were a number of issues with the GC-MS analysis of the GGC mutants.

The first and most pressing issue is that a large number of the samples failed. That is, the readings from these samples were either inaccurate or below the limits of detection. This is possibly as a result of some issue during the metabolite quenching and subsequent extraction. This meant that a data set of 5 control vs. 5 infected for a mutant was reduced and being a very small data set to begin with any subsequent analysis and conclusions would not prove to be very robust. To put this into perspective, the total number of WT *L. monocytogenes* infections for GC-MS analysis (across multiple experiments) was 35 infected flasks against 35 control flasks. This is a relatively large number of samples, which helped to increase the confidence and robustness of the conclusions drawn from their analysis. This is an area I would like to see readdressed, rather than analysing all the GGC mutant strains at once, any future study should initially focus on the Δ*glnA* mutant, which exhibits a strong phenotype. An emphasis should be placed on getting a good dataset, which can provide accurate and robust data.

As the GC-MS data is quantifiable, the data could potentially be used to build a metabolic model of the GGC pathway of *L. monocytogenes* nitrogen metabolism during intracellular growth. The hope for this study was to produce a model of *L. monocytogenes* GGS using complex pathway simulator COPASI (Hoops *et al.*, 2006).
and populate the model using metabolite concentrations from the GC-MS analysis. Since in silico mutations are produced relatively easily, for example by reducing the flux for a particular enzyme in the model, the model could then be tested using actual GC-MS data for change in metabolite pools in GGC mutants. Although a basic GGC model has been produced, this still requires considerable development and also requires experimental data from the GC-MS analysis of the GGC mutants, which was not achieved due to reasons already discussed.

An alternative methodology which could be adapted for this study is the $^{13}$C isotopic labeling of glutamine. Such an approach was already taken with the $^{13}$C glucose and has revealed some very interesting results (Eisenreich et al., 2006, Eylert et al., 2008). The isotopic labeling of glutamine, would not only provide excellent quantifiable data, but would also provide a tool to track the metabolic fate of the labeled glutamine through the host and also the pathogen. This experiment could also provide some excellent flux data to incorporate into a mathematic model of nitrogen assimilation in L.monocytogenes.

Finally, it would be very interesting to conduct some mouse model infections using the GGC mutants. Such a study will allow us to ascertain how successfully these mutants grow and disseminate in larger scale systems, such as a mouse model.
Chapter 9
Overall conclusions and future work
9.1) Conclusions

The work conducted during the course of this study aimed to research the metabolism of *L. monocytogenes* and the host cell during infection. This was targeted through a multi-disciplinary approach using wide-ranging biological methodologies. It was a broad study and there have been some very interesting results which have been gleaned regarding the metabolism of *L. monocytogenes*. In this section the most important of these results will be summarised and some future directions of the work will be discussed.

A large amount of work was conducted to gain an insight into the catabolic metabolism of *L. monocytogenes* with an emphasis on anaerobic growth (Chapter 5). It has been shown that, unlike *E. coli*, *L. monocytogenes* may not be dependent on fermentation enzymes Ldh and Pflb for anaerobic growth.

Based on the mutagenesis work conducted in this study and also unpublished work from our laboratory, the hypothesis was formulated that fumarate was serving as a terminal electron acceptor for anaerobically growing *L. monocytogenes*. To test this, a potential fumarate reductase, encoded by *lmo0355*, was isolated, purified and assayed for activity. This protein was similar to FrdA subunit of the FrdABCD complex of a classical fumarate reductases found in a number of bacteria (Iverson et al., 1999). It was shown that this individual subunit possess enzymatic activity, with the ability to convert fumarate to succinate. This supports the hypothesis that this protein could play an important role in *L. monocytogenes* anaerobic metabolism, where it would potentially be utilised with fumarate as the final terminal electron acceptor.

The extracellular metabolome experiments provided mixed results (Chapter 6). Some experiments did reveal interesting changes. However, as discussed in detail in section 6.3, there were a large number of factors which affected the validity of the results. The key issue is variability across different experiments. It is possible that the extracellular metabolome of HeLa cells are not actually affected significantly or consistently as a result of infection, explaining the experimental variability encountered in this study.
The analyses of the intracellular metabolome by GC-MS data confirm that there is a significant change in the metabolome of HeLa cells as a direct result from infection with \textit{L. monocytogenes} (Chapter 7). The results show that there are significant reductions in number of metabolites from a number of different aspects of metabolism including sugars and amino acids. In particular, analysis of the carbon metabolism revealed large numbers unidentified sugars, which were significantly reduced in the infected profiles. This indicates that the carbon metabolism of intracellular \textit{L. monocytogenes} may be complex, with the utilisation of a large number of carbon compounds.

Overall, the results support previous hypotheses which suggest that most of the amino acids utilised by \textit{L. monocytogenes} are provided by the host cell (Eylert \textit{et al.}, 2008). Also, the results show that there is a marked change in the intracellular physiology of a host cell after infection with \textit{L. monocytogenes}. Importantly, for future research, the results highlight that this methodology can provide some excellent and exciting data regards to the intracellular metabolism of parasite and the changes that occur to the host. The methodology has proven useful as a screening study, providing an insight into overall aspects of metabolism.

The quantification of nitrogenous compounds indicates that glutamine is a preferential nitrogen source for intracellular \textit{L. monocytogenes}. However, other nitrogenous compounds are also significantly reduced, which points to a complex, multifaceted utilisation of nitrogenous compounds (Section 8.3).

The data from the intracellular growth assays show that GDH is not a preferential route for glutamate synthesis and intracellular glutamate auxotrophy causes a small attenuation, which indicates a compensatory effect from alternative pathways for glutamate synthesis in \textit{L. monocytogenes}. The importance of glutamine for intracellular growth is emphasised by losing the enzyme glutamine synthetase (the only synthesis route for glutamine). Losing the ability to synthesize glutamine \textit{de novo} causes significantly impaired growth for \textit{L. monocytogenes}, \textit{in vivo} and \textit{in vitro}. 

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9.2) Future work

The most obvious issue from the catabolic metabolism experiments are the alternative \textit{ldh} and \textit{pfl} genes (Chapter 5). These alternative genes need to be evaluated for their possible importance during anaerobic growth. The genes could be targeted based on their transcription profiles, possibly using quantitative real time polymerase chain reaction (QRTPCR) from cells grown anaerobically. This could identify, which genes are being utilised for anaerobic growth and thus provide a more targeted approach to the study. Follow on experiments to further study the potential fumarate reductase (\textit{lmo0355}), could involve resolving the crystal structure for the protein.

For the extracellular metabolome study, any subsequent work should focus on developing the methodology. In this project the extracellular metabolome study represented many challenges including experimental variation. These need to be addressed before this aspect of the project can be moved forward. Once in place it would be particularly interesting to track the change in metabolites over time. The study which has been described above was an ‘end point’ study, essentially looking at the effects on the external metabolome, post infection. A timecourse study could give a more complete picture of the changes that occur during an infection, and provide data on the time these changes are occurring.

In terms of the intracellular metabolome a very interesting route for future work is carbon isotopologue profiling. A number of studies have already used carbon isotopologue profiling to provide an insight into the \textit{in vivo} metabolism of \textit{L.monocytogenes} (Eylert \textit{et al}., 2008). In principle, carbon isotopologue profiling can be applied to study the carbon metabolism of any biological system, including intracellular bacteria and their hosts. Currently, this method has been used to study intracellular changes within \textit{L.monocytogenes}. The method could be adapted to analyse changes in a wide variety of host systems, during and after infection with \textit{L.monocytogenes}.

A potentially gainful extension of the intracellular nitrogen metabolism work conducted (Chapter 8) would be to apply this methodology to isotopically labelled glutamine. This means that the uptake of glutamine from the
extracellular medium could be tracked through the host and through the bacteria. It would not only provide excellent data regarding the degradation and usage of glutamine in *L. monocytogenes*, but could also provide flux data to model.
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