Modulation of Phosphoinositide Metabolism
by Intracellular Pathogenic Bacteria

Listeria monocytogenes

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Abstract

Submitted by JIAHUI WANG for the Degree of Doctor of Philosophy in The University of Manchester and entitled ‘Modulation of Phosphoinositide Metabolism by Intracellular Pathogenic Bacteria Listeria monocytogenes’ in April, 2012.

Listeria monocytogenes is a Gram-positive facultative intracellular bacterium with a wide ecological niche and causes a number of diseases in human and animals. It invades mammalian host cells and escapes from the vacuoles prior to replication in the host cell cytoplasm and infecting adjacent cells via actin-based mobility. Phosphoinositide (PIP) metabolism is essential to mammalian cells in signal transduction, actin remodelling, endosome dynamics and membrane trafficking. Modulation of host PIP metabolism by bacteria PIP phosphatases is important for pathogenicity and virulence of many human pathogens.

In this study the function of two L. monocytogenes tyrosine and inositol phosphatases LipA and LipB were studied in vitro. The lipA and lipB deletion mutants generated in EGDe and InLA strains were not affected in invasion but were attenuated in intracellular growth in Caco-2 and Hela M cell lines but not in mouse macrophages. Deletion of lipA or lipB did not affect the actin polymerisation but caused reduced plaque number in the plaque assay. The turnover of five PIPs in Hela M cells during L. monocytogenes infection were studied by expression of fluorescent protein tagged domains that specifically recognizes individual PIPs. L. monocytogenes did not affect the metabolism of PI4P, PI(4,5)P2, PI(3,4,5)P3 but co-localised with PI3P at 1.5 hr post-infection and with PI(3,4)P2 at 6 hr to 24 hr post-infection.

The PI(3,4)P2 effector protein lamellipodin was discovered to be recruited to actin-associated L. monocytogenes at 4 hr to 24 hr post-infection in Hela M cells. This discovery leads to the hypothesis of a novel mechanism of lamellipodin-dependant cell-to-cell spread. The lipA mutant was found to be attenuated in PI(3,4)P2 recruitment and therefore hypothesized to participate in the proposed lamellipodin pathway by converting PI(3,5)P2 into PI5P, leading to the activation of PI3K and subsequent production of PI(3,4)P2. LipB showed partial localisation at the Golgi complex when over-expressed in Hela M cells, and it was assumed to act mainly as a protein-tyrosine phosphatase.

In summary, this study provides some evidence on L. monocytogenes modulating host PIP metabolism by the production of inositol phosphatases. It gives us a better understanding on the intracellular growth of this pathogenic bacterium, and on the interaction between host and parasite.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>Akt</td>
<td>serine-threonine kinase</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>actin-related proteins 2 and 3</td>
</tr>
<tr>
<td>BHI</td>
<td>brain-heart infusion</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>bsh</td>
<td>bile salt hydrolase</td>
</tr>
<tr>
<td>CCR</td>
<td>carbon catabolite repression</td>
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<tr>
<td>CFP</td>
<td>cyan fluorescent protein</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCs</td>
<td>dendritic cells</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>EEA1</td>
<td>early endosomal antigen-1</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ENTH/ANTH</td>
<td>Epsin N-terminal homology</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>Erm</td>
<td>erythromycin</td>
</tr>
<tr>
<td>EVH1/2</td>
<td>Ena/VASP homology domain 1 or 2</td>
</tr>
<tr>
<td>FAPP1</td>
<td>four-phosphate-adaptor protein</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>FERM</td>
<td>4.1 protein, ezrin, radixin and moesin</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FYVE</td>
<td>Fab1, YOTB, Vac1 (vesicle transport protein), and EEA1</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine disphosphate</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GW</td>
<td>dipeptide Gly-Trp</td>
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[15]
*hpt*  
hexose phosphate transporter  

**I(1,4,5)P₃**  
Inositol-1,4,5-triphosphate  

**IFN**  
interferon  

**IL**  
interleukin  

**InlA/InlB/InlC**  
internalin A/B/C  

**iNOS**  
inducible nitric oxide synthase  

**IR**  
Immunoglobulin-like fold  

**Kan**  
kanamycin  

**KO**  
knockout  

**LAMP-1**  
lysosome-associated membrane protein-1  

**LB medium**  
Luria-Bertani medium  

**LCV**  
*Legionella*-containing vacuole  

**LipA/LipB**  
*Listeria* phosphatase A/B  

**LLO**  
listeriolysin O  

**Lpd**  
lamellipodin  

**LPXTG**  
leucine-proline-X-threonine-glycine  

**LRR**  
leucine-rich repeat  

**MCV**  
*Mycobacterium*-containing vacuole  

**MCS**  
multiple cloning site  

**MDCK**  
Madin-Darby canine kidney cell  

**mEC1**  
murine E-cadherin  

**MEM**  
minimal essential medium  

**MHC**  
major histocompatibility complex  

**MLN**  
mesenteric lymph node  

**MOI**  
multiplicity of infection  

**mRFP**  
muated red fluorescent protein  

**MTM**  
myotubularin  

**MVB**  
multivesicular body  

**NEAA**  
non-essential amino acids  

**Neo**  
neomycin  

**NK cell**  
natural killer cell  

**NO**  
nitric oxide  

**ORF**  
open-reading frame  

**OSH**  
oxysterol binding protein homologues of  
*Saccharomyces cerevisiae* 

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>OSHP</td>
<td>oxysterol binding protein</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-D</td>
<td>Dulbecco's 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>PDK1</td>
<td>3-phosphoinositide-dependent protein kinase</td>
</tr>
<tr>
<td>PDZ</td>
<td>post synaptic density protein PSD95, <em>Drosophila</em> disc large tumor suppressor Dlg1, zonula occludens-1 protein z0-1</td>
</tr>
<tr>
<td>PEST</td>
<td>proline, glutamic acid, serine and threonine-rich motif</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PH domain</td>
<td>pleckstrin homology domain</td>
</tr>
<tr>
<td>PHD</td>
<td>plant homeo domain</td>
</tr>
<tr>
<td>PI</td>
<td>phosphoinositol</td>
</tr>
<tr>
<td>PI3P/PI4P/PI5P</td>
<td>phosphoinositol-3/4/5-monophosphate</td>
</tr>
<tr>
<td>PI(3,4)P2/PI(4,5)P2/PI(3,5)P2</td>
<td>phosphoinositol-3,4,5-triphosphate</td>
</tr>
<tr>
<td>PI3P/PI4P/PI5P</td>
<td>phosphoinositol-3,4,5-triphosphate</td>
</tr>
<tr>
<td>PIP</td>
<td>phosphoinositol phosphate, or phosphoinositide</td>
</tr>
<tr>
<td>PIP5K</td>
<td>phosphoinositol phosphate-5-kinase</td>
</tr>
<tr>
<td>PI3K/PI4K</td>
<td>phosphatidylinositol 3/4-kinase</td>
</tr>
<tr>
<td>PI-PLC</td>
<td>phosphatidylinositol-specific phospholipase C</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>PTB domain</td>
<td>phosphotyrosine-binding domain</td>
</tr>
<tr>
<td>PtK2</td>
<td><em>Potorous Tridactylis</em> kidney epithelial cell</td>
</tr>
<tr>
<td>PTS</td>
<td>phosphoenolpyruvate-sugar phosphotransferase system</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RNI</td>
<td>reactive nitrogen intermediates</td>
</tr>
<tr>
<td>PX</td>
<td>phagocytic oxidase Phox</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue</td>
</tr>
<tr>
<td>S-D</td>
<td>Shine-Dalgarno</td>
</tr>
<tr>
<td>SCV</td>
<td><em>Salmonella</em>-containing vacuole</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>SDM</td>
<td>site-directed mutagenesis</td>
</tr>
<tr>
<td>SHIP</td>
<td>Src homology 2 domain–containing inositol phosphatase</td>
</tr>
<tr>
<td>T3SS/T4SS</td>
<td>type III/IV secretion system</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
</tr>
<tr>
<td>TAPP1</td>
<td>tandem PH domain-containing protein 1</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TR</td>
<td>texas red</td>
</tr>
<tr>
<td>TRITC</td>
<td>tetramethylrhodamine-5, 6-isothiocyanate</td>
</tr>
<tr>
<td>TSB</td>
<td>tryptone soya broth</td>
</tr>
<tr>
<td>VAMP8</td>
<td>vesicle-associated membrane protein 8</td>
</tr>
<tr>
<td>VASP</td>
<td>vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>VCA</td>
<td>vrprolin homology, cofilin homology and acidic</td>
</tr>
<tr>
<td>WAVE</td>
<td>WASP-family verprolin-homologous protein</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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Preface

The author graduated from Shandong University in China in July 2006 with a first class honored degree of Bachelor of Engineering in Bioengineering. Then she took the course of MSc in Immunology and Immunogenetics in The University of Manchester in Sep 2006. In this course she did a five-month research project in Prof. Ian S. Roberts’ lab entitled ‘Intracellular survival of *Listeria monocytogenes* in macrophages’. After receiving the Master of Science degree, she was offered a place as a PhD student in Prof. Roberts’ group in 2008 to continue her study on the bacteria-host interaction for *L. monocytogenes*. The result of her PhD work is reported in this thesis.
Chapter 1 Introduction

1.1 *Listeria monocytogenes*—a food borne pathogen

*Listeria monocytogenes* is a Gram-positive, facultative, food-borne intracellular pathogenic bacterium and is one of the six species within the genus *Listeria* (Rocourt, 1999; Vazquez-Boland et al., 2001). *L. monocytogenes* inhabits a broad ecologic niche and has been isolated from a variety of food, vegetation, water, effluents, soil, and faecal matter from animals and humans (Gray and Killinger, 1966; Welshimer and Donker-Voet, 1971; Weis and Seeliger, 1975; Fleming et al., 1985; Fischetti et al., 2000). Furthermore, this bacterium is able to adapt and survive in a broad range of pH from 4.5 to 9.0, temperature between –1 °C to 45 °C, high salt concentrations (10% NaCl) and even long periods of drying and freezing with subsequent thawing (Junttila et al., 1988; Schuchat et al., 1991). In addition, *L. monocytogenes* is one of the most studied intracellular bacteria and is used as a model system to study the induction of phagocytosis and intracellular parasitism (Vazquez-Boland et al., 2001).

1.2 Taxonomy

The genus *Listeria* is closely related to other Gram-positive low G+C bacteria such as *Bacillus*, *Staphylococcus*, *Streptococcus*, *Clostridium* and *Enterococcus*, and currently consists six species: *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi* (Vazquez-Boland et al., 2001). Amongst them, *L. monocytogenes* and *L. ivanovii* are potentially pathogenic. *L. monocytogenes* was originally isolated in 1926 by Murray and colleagues after an outbreak of severe mononucleosis amongst rabbits and guinea pigs in an animal house (Murray et al., 1926). In contrast, *L. ivanovii* was identified in the 1960s from lambs with congenital listeriosis (Ivanov, 1962). *L. monocytogenes* is a major food-borne pathogen potentially lethal in humans and animals, while *L. ivanovii* infection in humans is very
uncommon but often infects ungulates such as sheep and cattle (Sergeant et al., 1991; Wesley, 1999). A third species, *L. seeligeri* is generally regarded as non-pathogenic, although it has been implicated to be responsible for human listeriosis in at least one case (Rocourt et al., 1986). The other three species are regarded as harmless saprophytes.

### 1.3 Diseases caused by *L. monocytogenes*

#### 1.3.1 Listeriosis in animals

*L. monocytogenes* is a widespread pathogen where infection has been reported in more than 40 species of wild and domesticated animals over six continents (Seeliger, 1961) and of major veterinary importance in cattle, sheep and goats, but in the United Kingdom it is mainly in sheep (Anon, 1983). Animals acquire the organism from the environment through grazing, amplified by faecal contamination of soil and vegetation. In New Zealand in the 1930s, Gill (1931) described circling disease, a rhombencephalitis of sheep that may affect flocks fed spoiled silage. *L. monocytogenes* has also been implicated as a cause of abortion and prematurity in ruminants. Intravenous and oral models of *L. monocytogenes* infection in rodents can duplicate the illness seen in the natural state in animals, including maternal sepsis and abortion (Lanmerding et al., 1992).

#### 1.3.2 Listeriosis in humans

In humans, *L. monocytogenes* can cause a rare but very severe listeriosis, with a mortality rate of 30% or higher, in spite of early antibiotic treatment (Rocourt and Brosch, 1992). Due to the ubiquitous occurrence of *L. monocytogenes* and its ability to grow at refrigeration temperature, it has caused a number of large food-borne outbreaks involving hundreds of individuals (Schwartz et al., 1989; Dalton et al., 1997; Schlech, 2000).
A major source of infection is unpasteurised dairy products (such as yoghurt, cheese, chocolate, and milk), smoked fish, sausages, salad, as well as other industrially produced ready-to-eat food (Dalton et al., 1997; Schlech, 2000; Vazquez-Boland et al., 2001).

*L. monocytogenes* is an opportunistic pathogen with the elderly, pregnant women, neonates and the foetus being most susceptible to infection (Vazquez-Boland et al., 2001). Susceptibility is also increased in immunosuppressed transplant patients, those with impaired cell-mediated immunity and debilitated adults with underlying diseases such as Acquired Immune Deficiency Syndrome (AIDS) (Vazquez-Boland et al., 2001). Other predispositions include alcoholism, alcoholic liver disease and replacement of joints or prosthetic heart valves (McLauchlin, 1997).

There are two forms of listeriosis in the human: prenatal listeriosis and listeriosis in adult patients. In both instances, the predominant clinical syndrome corresponds to disseminated infection or to local infection in the central nervous system (CNS) (Vazquez-Boland et al., 2001).

### 1.3.2.1 Foetomaternal and neonatal listeriosis

*L. monocytogenes* can proliferate symptomatically in the vagina and uterus. A woman infected by *L. monocytogenes* may be asymptomatic but commonly have flu-like or pyelonephritis symptoms before the early onset of labor. If the mother becomes symptomatic, it is usually in the third trimester. Symptoms include fever, myalgia, arthralgia and headache. Abortion, stillbirth and preterm labor are complications of gastro and urinary infection (Fischetti et al., 2000).

There are two types of neonatal listeriosis. The first type is the early-onset of neonatal listeriosis, which is characterised clinically by prematurity, sepsis at
birth, fever, a diffuse maculopapular cutaneous eruption and significant hepatic involvement with jaundice (Evans et al., 1985). The mortality rate of early-onset listeriosis is very high even with treatment and stillbirth is common in this setting. The other type, the late-onset neonatal listeriosis is acquired through vaginal transmission, although it also has been reported with caesarean deliveries. The symptoms include fever, irritability, bulging fontanelles and meningismus and they usually developed 1 to 2 weeks following delivery (Kessler and Dajani, 1990).

1.3.2.2 Listeriosis in adult

The Listerial infections most frequently reported in non-pregnant adults are that affecting the CNS (55-70% of the cases). Another frequent form of listeriosis, bacteraemia or septicaemia, is responsible for 15-50% of the cases. The rest 5-10% of the cases are related to other atypical clinical forms (Vazquez-Boland et al., 2001).

CNS infection: Meningitis is the most frequently recognised condition in L. monocytogenes infection, where there are two clinical presentations. The first is typical sub-acute bacterial meningitis characterised by fever, headache, and neck stiffness (Skogberg et al., 1992). During epidemics of food-borne listeriosis, Listeria meningitis can occur in apparently healthy individuals of all ages. In sporadic diseases, patients more commonly have obvious defects in cell-mediated immunity that predispose them to listeriosis. The second form of CNS listeriosis in adults is a rhombencephalitis that has features characteristic of the same illness in animals described as circling disease (Armstrong and Fung, 1993). Fever, headache, nausea and vomiting occur early, and signs of meningeal irritation are less commonly present. Subsequently, patients develop multiple cranial nerve abnormalities accompanied by cerebellar dysfunction, including ataxia (Fischetti et al., 2000).
Listeria sepsis: *Listeria* sepsis, or bacteraemia without CNS involvement, represents one-third of adult cases of invasive listeriosis. In non-pregnant adults, *Listeria* sepsis almost always occurs in patients with malignancy, organ transplant, or other immunocompromised states. The patients often appear severely ill with fever, nausea, vomiting and malaise (Doganay, 2003). The presentation is unspecific and is similar to other types of bacteria sepsis (Fischetti *et al.*, 2000; Doganay, 2003).

There are other clinical syndromes in adults such as endocarditis, gastroenteritis, pneumonia, pleuritis, hepatitis, peritonitis localised abscesses, arthritis and conjunctivitis, but the frequency of these infections is relatively low (Vazquez-Boland *et al.*, 2001).

1.4 Pathogenesis of infection and intracellular life cycle of *L. monocytogenes*

The pathogenesis of *L. monocytogenes* is closely related to its remarkable adaptation to intracellular survival in macrophages and other types of host cells. Its intracellular life cycle includes the following steps: i) internalisation of the bacteria via either phagocytosis by the host cell or bacteria-mediated invasion; ii) escape from the primary phagosome; iii) replication inside the cytoplasm; iv) spread to neighbouring cells by actin polymerisations. (Fig. 1.1)

1.4.1 Internalisation

The invasion of *L. monocytogenes* is either a passive process through conventional phagocytosis by phagocytic cells such as macrophage, or induced by its own invasion machinery. Entry into non-phagocytic cells is induced by binding to receptors on the host cell by the bacterial surface proteins collectively named internalins, of which internalin A and internalin B are the best characterised. This ligand-to-receptor binding induced entry is
described as a ‘zipper’ mechanism opposed to the ‘trigger’ mechanism employed by many other bacteria (e.g. *Salmonella enterica*) in which bacterial proteins are injected into the cytosol of the host cell via the type III or type IV secretion system (T3SS or T4SS) (Zhou *et al.*, 1999; reviewed by Cossart and Sansonetti, 2004).

**Figure 1.1 Different stages in the life cycle of *L. monocytogenes*.** Adapted from Tilney and Portnoy (1989). i) Entry of the bacteria into the host cell by passive or induced phagocytosis is mediated by the internalin family of proteins. ii) The lysis of the phagosome allows for the bacterium to escape into the cytosol. This is mediated through LLO, PI-PLC and PC-PLC. iii) Cytosolic replication can then occur. iv) Actin from the host cell is recruited by ActA allowing actin-based motility and the formation of pseudopods. These pseudopods are phagocytosed by neighbouring cells, forming double-membrane phagosomes. Again, LLO, PI-PLC and PC-PLC mediate *L. monocytogenes* to escape from the phagosomes, and the cell cycle reinitiates.
Both InlA and InlB are necessary and sufficient for the internalisation of *L. monocytogenes* into cell types such as hepatocytes, enterocytes, fibroblasts, epithelial cells and endothelial cells (Hamon *et al.*, 2006). Entry of *L. monocytogenes* into mammalian cells is a dynamic process involving actin polymerisation and membrane remodelling, and is a good example of how a bacterium can manipulate host-cell signalling and endocytic pathways to its own advantage (Webster, 2002; Hamon *et al.*, 2006). Entry is divided into three stages: contact and adherence, phagocytic cup formation and finally phagocytic cup closure and retraction (Cossart and Sansonetti, 2004).

### 1.4.1.1 Internalin A

InlA is a cell wall protein which belongs to the internalin family characterised by the presence of leucine-rich repeats (LLRs) that are involved in specific protein-protein interactions (Gaillard *et al.*, 1991). The receptor of InlA is the E-cadherin, which is a transmembrane protein belonging to the cadherin superfamily of cell-cell adhesion molecules located mainly at cell junctions and allow calcium-dependent adhesion between epithelial cells (Mengaud *et al.*, 1996). *L. monocytogenes* exploits the E-cadherin localisation at the intestinal barrier to invade enterocytes during the initial stages of host colonisation (Lecuit *et al.*, 2001). The extracellular domain of E-cadherin is normally involved in homophilic adherence with neighbouring epithelial cells, while the intracellular domain is linked to the actin cytoskeleton through intervening proteins and is critical for physiological processes such as cell signalling and differentiation (Steinberg and McNutt, 1999). InlA is covalently linked to the cell wall of *L. monocytogenes* by the distal LPXTG motif, which is characterised by the Leucine-Proline-X-Threonine-Glycine conserved sequence, and its LRR units interact with the first ectodomain of E-cadherin and induces the endocytosis of the bacteria (Schubert and Heinz, 2003).
During host cell invasion, *L. monocytogenes* hijacks the molecular machinery associated with the cytoplasmic tail of E-cadherin. Upon the binding of InlA to host E-cadherin, the cytosolic protein β-catenins bind to the intracellular domain of the E-cadherin, forming the InlA/E-cadherin/β-catenin complex (Lecuit *et al.*, 2000). The α-catenin associates with β-catenin in the complex as a monomer, but with F-actin as homodimers, acting as a molecular switch that controls cytoskeleton organisation depending on its local concentration (Drees *et al.*, 2005). Recruitment of α-catenin to the *L. monocytogenes* entry site is dependent on ARHGAP10, a Rho-GAP domain protein that interacts with the small GTP-binding protein Arf6 (Sousa *et al.*, 2005; Seveau *et al.*, 2007).

The signaling that directly orchestrates actin remodeling and *L. monocytogenes* engulfment into host cells involves the Arp2/3 (actin-related proteins 2 and 3) complex, a highly conserved actin nucleator, which is recruited at the bacterial entry site. Upon the binding of InlA to E-cadherin, The Rho family GTPase Rac1 is locally activated at the site of bacterial attachment and in turn stimulates Arp2/3 in a WAVE (WASP-family verprolin-homologous protein)/WASP (Wiskott-Aldrich syndrome protein)-independent way to drive actin assembly necessary for *L. monocytogenes* uptake (Fig. 1.2). Cortactin, which also is regulated by Rac1, has been identified to be a critical regulator of actin assembly, able to bind directly both Arp2/3 and F-actin. Moreover, the tyrosine kinase Src is possible to activate cortactin. Src-mediated phosphorylation of E-cadherin is also triggered by InlA during *L. monocytogenes* infections (Fig. 1.2). (Reviewed by da Silva *et al.*, 2012)

However, E-cadherin based internalisation of *L. monocytogenes* to murine intestinal epithelial cells could not occur due to a substitution of glutamate by proline at position 16 in murine E-cadherin (mEC1), leading to the inability of challenging murine with *L. monocytogenes* by the oral route in
laboratory (Lecuit et al., 1999; Lecuit et al., 2001). To resolve this issue, an EGDe::InlA\textsuperscript{m} strain was generated by Wollert and colleagues (2007) which contained two substitutions of amino acids that greatly improved the binding affinity of InlA for mEC1. This strain made it possible that murine models can be challenged via oral route, which can represent a system closely resembling the human situation during a Listeria l infection (Wollert et al., 2007).

Figure 1.2 Cytoskeletal rearrangements induced during L. monocytogenes invasion of host cells. (da Silva et al., 2012). Cytoskeletal rearrangements induced by the binding of InlA to E-cadherin involve the Arp2/3 complex activation that is regulated by the Rho GTPases such as Rac1 and Cdc42 in a WAVE (WASP-family verprolin-homologous protein)/WASP (Wiskott-Aldrich syndrome protein) independent way. The Rac1 regulated protein Cortactin binds both actin and Arp2/3 complex to regulate actin remodeling. The tyrosine kinase Src mediates activation of Cortactin as well as phosphorylation and ubiquitinisation of E-cadherin. The recognition of InlB to its ligand Met triggers the PI3K pathway via the recruitment of Gab1, which couples Met to the CrkII adaptor protein and PI3K IA. PI3K promotes actin polymerisation by produing PIP3. During this process Rac1 is also activated, leading to the activation of Arp2/3 complex and actin polymerisation. +: induction. Ub: ubiquitin. PIP3: PI(3,4,5)P\textsubscript{3}.

1.4.1.2 Internalin B

InlB is also a member of the internalin family and its coding gene \textit{inlB} is located in the same operon as \textit{inlA} (Gaillard et al., 1991). It is involved in the
invasion of *L. monocytogenes* into a broad range of cell lines including hepatocytes and nonepithelial cells (Dramsi *et al.*, 1995). As shown in Fig. 1.3, its N-terminal domain presents a signal sequence followed by seven LRR domains, one IR region, and one B repeat, which is poorly understood and not yet modelled (Bierne and Cossart, 2002). Its C-terminal GW region, a conserved tandem repeat beginning with the dipeptide Gly–Trp, mediates noncovalent anchorage to the bacterial cell wall (Bierne and Cossart, 2002).

The hepatocyte growth factor receptor Met, has been identified as the main receptor of InlB on target cells (Shen *et al.*, 2000). Initial engagement of Met by InlB elicits downstream signaling pathways that culminate in F-actin cytoskeletal remodeling needed for bacterial uptake.

InlB binds to Met through the concave surface of LRR region and triggers the classical phosphatidylinositol 3-kinase (PI3K) pathway, via recruitment of Gab1, Cbl and Shc on phosphotyrosine residues of the intracytoplasmic part of Met. Gab1, a multiple-site tyrosine-phosphorylated adaptor protein, couples Met to the adaptor protein CrkII and to p85 regulatory subunit of type IA PI3K (Ireton, 2007). CrkII and Gab1 promote membrane recruitment of PI3K. CrkII also contains two SH3 domains, one of which stimulates PI3K activity (Dokainish *et al.*, 2007). PI3K promotes actin polymerisation by generating PI(3,4,5)P3 to uncap barbed actin filaments (Fig. 1.2) (Hartwig *et al.*, 1995). During this process, small GTPases Rac1 and Cdc42 are also activated, which then activate WAVE and/or N-WASP, leading to the activation of Arp2/3 complex and the actin polymerisation of host cells (Bierne *et al.*, 2001; Seveau *et al.*, 2007). How the PI3K pathway activates Rac1 and Cdc42 is unknown. The activation of PI3K upon phagocytosis of *L. monocytogenes* also activates the anti-apoptotic Akt (serine-threonine kinase)/PKB (protein kinase B) pathway, as well as the transcription factor NF-κB (Mansell *et al.*, 2001).
Depending on the receptors expressed by host cells, InlA and InlB stimulate bacterial entry individually or in concert (Pentecost et al., 2010). It has been reported that InlA and InlB did not play an important role in the association with host cells, but affected the intracellular survival and entry of *L. monocytogenes* (Vadia et al., 2011).

### 1.4.1.3 Other proteins related to internalisation

Besides InlA and InlB, other members of the internalin repertoire play a role similar to that of InlA and InlB in the interaction with eukaryotic cell surface. In total, 25 members of the internalin family encoding putative internalins have been identified in *L. monocytogenes* (Fig. 1.3). These internalins could be divided into three groups, namely the LPXTG, the GW or WxL (a novel type of cell wall associated domain), and the secreted internalins. (Bierne et al., 2007)

The LPXTG internalins consists of nineteen members, including InlA, InlE, InlF, InlG, InlH, InlI, InlJ. They are characterised by the C-terminal sorting signal known to direct covalent anchorage to the peptidoglycan of Gram-positive bacteria, which contains the LPXTG motif, followed by a hydrophobic domain of about 20 amino acids and a tail of positively-charged residues (Bierne et al., 2007). The second group of internalins comprises two proteins, InlB and Lmo0549, a protein of unknown function, that display in their C-terminal part a GW or WxL domain respectively that directs non-covalent association to the cell surface (Bierne et al., 2007). The last group of internalins contains the smallest internalins, InlC, Lmo2445, Lmo2027 and Lmo2470, which all lack any known cell-wall anchor domain. Therefore these internalins are thought to be fully released into the extracellular medium (Bierne et al., 2007).
In addition to the role of internalins in the entry of *L. monocytogenes*, LLO is also reported as a critical invasion factor that perforates the host cell plasma membrane to activate *L. monocytogenes* internalisation into human hepatocyte HepG2 and Hela cells (Vadia *et al.*, 2011). LLO plays a critical role in bacterial entry, but not in bacterial association with host cells (Vadia *et al.*, 2011). *In vitro*, extracellular LLO perforates host cells at neutral pH, and induces the formation of internalisation vesicles that accommodate large particles (bacteria or 1 µm beads) via a cholesterol-, dynamin-, and F-actin-dependent, but clathrin-independent pathway (Vadia *et al.*, 2011).

Figure 1.3 Schematic representation of the structural features of the 25 members of the internalin family encoded within the *L. monocytogenes* EGDe genome. (Bierne *et al.*, 2007). Homologous regions are color coded as indicated in the legend. Numbers within different domains indicate the number of repeats.
1.4.2 Escape from phagosome

Following invasion, the internalised *L. monocytogenes* remains in a membrane-bound phagosome before escaping into and replicating in the cytoplasm. This escape is achieved by the pore-forming toxin listeriolysin O (LLO) in combination with two phospholipases phosphatidylinositol-specific phospholipase C (PI-PLC) and broad-range phospholipase C (PC-PLC), which enhance the degradation of the phagosome membrane (Vazquez-Boland *et al.*, 2001; Schnupf and Portnoy, 2007).

1.4.2.1 LLO

LLO is encoded by the *hly* gene. It is a pore-forming toxin belonging to a large family of thiol-activated cholesterol-dependent cytolysins that are secreted by numerous Gram-positive bacteria (Dramsi and Cossart, 2002). LLO is a key virulence factor essential for the pathogenicity of *L. monocytogenes*. It has a vital role not only in intracellular parasitism, but also in several other functions in the interaction of *L. monocytogenes* with the host cells (Vazquez-Boland *et al.*, 2001).

LLO mediates lysis of not only the primary phagosome formed after the uptake of extracellular bacteria, but also the double-membrane secondary vacuole formed upon cell-cell spreading (Gedde *et al.*, 2000). According to the study of Henry *et al.* (2006), LLO also plays an important role in delaying vacuole maturation and the fusion of *L. monocytogenes*-containing vacuoles with LAMP-1 (lysosome-associated membrane protein-1)-positive lysosomes. The delay in phagosome maturation is believed to be caused by the action of LLO in generating lesions in the phagosomal membrane that uncouple pH and calcium gradients essential for phagosomal maturation. LLO-dependent delayed vacuole maturation facilitates *L. monocytogenes* escape by increasing the duration of *L. monocytogenes* residence in penetrable
compartments. This escape before the fusion with lysosomes also prevents the contents of perforated lysosomes from spilling into the cytoplasm, thus maintaining the habitable cytosolic environment (Henry et al., 2006).

The compartmentalisation of LLO activity to the phagosome is important in preventing cytotoxicity to the host cell. It has become clearer that this compartmentalisation is achieved by the regulation of LLO activity on multiple levels including transcription, synthesis, protein activity and protein degradation (Schnupf and Portnoy, 2007).

At the transcriptional level, the hly gene is largely controlled by PrfA (positive regulatory factor A), the major transcriptional regulator of virulence genes in L. monocytogenes (the PrfA regulation will be discussed in detail in section 1.5) (Domann et al., 1993). In vitro hly is up-regulated under conditions that increase the expression and activity of PrfA, which include the 37 °C body temperature, nutrient limitation and intracellular growth (Schnupf and Portnoy, 2007).

Close to the N-terminus of LLO protein, there is a PEST-like motif, which is rich in proline (P), glutamic acid (E), serine (S) and threonine (T) (Rogers et al., 1986). PEST sequences are usually found in proteins with short half-lives in eukaryotic cells and can act as phosphorylation/degradation tags (Rechsteiner and Rogers, 1996). However, in L. monocytogenes, the PEST-like sequence is not necessary for proteasome-mediated degradation of intracellular LLO (Schnupf et al., 2006). Instead, the PEST-like region of LLO mediates cytotoxicity by influencing the amount of LLO that is synthesized or secreted by the bacteria when they are growing in the host cytosol through an unknown mechanism. This translational repression of LLO is considered a major regulatory mechanism that controls LLO activity during cytosolic growth (Schnupf and Portnoy, 2007).
The third level of LLO regulation is the pH-dependent loss of LLO activity. The optimal pH for LLO activity is 5.5, and at neutral pH the LLO activity is ten times lower (Glomski et al., 2002). The pH optimum of LLO could either prevent premature rupture of the acidifying vacuole and/or restrict the pore-forming activity of LLO during growth in the neutral pH of the cytosol. (Glomski et al., 2002)

Another post-translational regulation of LLO is the degradation of LLO by host proteasomes. Although host-mediated degradation of LLO during a wild-type *L. monocytogenes* infection is not a major mechanism that controls LLO activity, when bacterial regulatory mechanisms are compromised, host-mediated degradation contributes significantly to the compartmentalisation of LLO. Thus, *L. monocytogenes* may promotes the survival of host cells by utilizing host cell compartment-specific proteolysis as a back-up mechanism to prevent inappropriate pore-formation at the host plasma membrane (Schnupf et al., 2006).

In addition to its pore-forming ability, LLO has also showed signaling functions as activating NF-κB, mitogen-activated protein kinase, phosphatidylinositol, calcium and protein kinase C signaling pathways although these functions requires further investigation (Hamon et al., 2006; Kayal and Charbit, 2006).

**1.4.2.2 Phospholipase C**

Besides LLO, *L. monocytogenes* also secretes two phospholipase C enzyme to facilitate the lysis of intracellular vacuoles: PI-PLC and PC-PLC.

The first description of phospholipase production in *L. monocytogenes* dates back to 1962 in a opacity reaction in egg yolk agar that correlates with hemolytic activity (Fuzi and Pillis, 1962). This hemolytic activity was originally
considered to be due to the hemolysin itself but was later identified as phosphatase activities (Vazquez-Boland et al., 2001).

The PI-PLC is encoded by the *plcA* gene. It is similar to the PI-PLCs from *Bacillus thuringiensis*, *Bacillus cereus*, *Staphylococcus aureus*, and some eukaryotic PI-PLCs (Mengaud et al., 1991). PI-PLC is highly specific for phosphoinositol (PI) and glycosyl-PI-anchored protein and has the optimal pH between 5.5 and 6.5, suggesting its activity restriction in acidified phagocytic vesicles like LLO (Vazquez-Boland et al., 2001). However, it is much less active on glycosyl-PI-anchored proteins than PI-PLC from *B. cereus* and *B. thuringiensis* (Gandhi et al., 1993). In synergy with LLO, PI-PLC induces hydrolysis of PI and produces diacylglycerol (DAG) in macrophages, leading to mobilisation of a protein kinase, Cδ and the subsequent elevation of intracellular calcium levels (Goldfine et al., 2000; Wadsworth and Goldfine, 2002).

The PC-PLC is encoded by the *plcB* gene, which occupies the third position in the lecithinase operon. It is a secreted phosphatidylcholine cholinephospho-hydrolase which resembles the enzyme responsible for lecithinase activities in *B. cereus* and *Clostridium perfringens* (Vazquez-Boland et al., 2001). PC-PLC hydrolyzes a broad spectrum of phospholipids including sphingomyelin, and are active over a wide pH range (5.5 to 8.0) (Geoffroy et al., 1991). PC-PLC is synthesized as an inactive proenzyme that is matured in the extracellular medium consisting the metalloprotease encoded by *mpl* (Domann et al., 1991). This mechanism is necessary for *L. monocytogenes* to prevent bacterial membrane damage from the degradation of its own phospholipids. PC-PLC facilitates the disruption of the secondary phagosome, thus the *plcB* deletion strain is defective in plaque formation (Smith et al., 1995).
Both PI-PLC and PC-PLC are synergistic with LLO in lysis of primary and secondary phagosome vacuoles. In the \( \Delta plcA\Delta plcB \) double mutant, the ability of \textit{L. monocytogenes} to escape from the primary phagosome and to spread cell-to-cell is compromised to a greater extent than with either mutation alone, suggesting the two phospholipases have overlapping functions in the primary and secondary phagosome (Marquis \textit{et al.}, 1995; Smith \textit{et al.}, 1995). In the absence of LLO, PC-PLC can still promote lysis of primary vacuoles in many human epithelial lines such as Hela cell, immature human dendritic cells as well as \textit{Drosophila} S2 cells, while PI-PLC makes only a minor contribution (Marquis \textit{et al.}, 1995; Schnupf and Portnoy, 2007). Overall, PI-PLC plays a minor individual role in virulence but acts synergistically with PC-PLC and LLO to achieve the optimal levels of escape from primary and secondary phagosomes.

1.4.3 Cytosolic growth

Following the escape from the phagosomes, \textit{L. monocytogenes} starts to multiply in the host cell cytoplasm. The cytosol was thought to be a favourable environment for bacteria growth. The LLO expressing \textit{Bacillus subtilis} or \textit{Escherichia coli} precoated with LLO could both escape from vacuoles and survive in the cytoplasm of macrophage J774 cells or epithelial cells respectively (Bielecki \textit{et al.}, 1990; Monack and Theriot, 2001). However, although non-pathogenic bacteria introduced into the cytosol can grow under certain circumstances, in no case could they replicate to the same extent as cytosolic bacterial pathogens, implying that specific adaptations by these pathogens are important for optimal bacterial proliferation (O'Riordan and Portnoy, 2002). One example of this adaptation is the intracellular pathogen \textit{Shigella flexneri} is able to grow in the cytosol of macrophages and epithelial cells, and overcomes restricted levels of iron, magnesium, and phosphate by up-regulation of the \textit{sitABCD} system, the \textit{mgtA} gene, and genes of the \textit{phoBR} regulon (Lucchini \textit{et al.}, 2005).
In the case of *L. monocytogenes*, it is highly adapted to the cytoplasm of mammalian host cells, where it is able to multiply with a generation time comparable to that in rich medium (Fuchs *et al.*, 2012). Isotopolog profiling analysis demonstrated that glucose-6-phosphate and/or the C3-compounds resulting from glucose catabolism are utilized as the major carbon sources, and non-carbohydrate nutrients such as glycerol and amino acids are used as alternative carbon- and energy source by *L. monocytogenes* inside the host cells (Eylert *et al.*, 2008). *L. monocytogenes* can use nitrogen sources such as ethanolamine during replication in epithelial cells and the oxidative pentose phosphate pathway, but not glycolysis, is the predominant sugar metabolism pathway in the host environment (Joseph *et al.*, 2006). The central metabolism of *L. monocytogenes* is characterised by the incomplete tricarboxylic acid cycle due to a lack of 2-oxoglutarate dehydrogenase as demonstrated by genome analysis and 13C-labeling studies (Glaser *et al.*, 2001; Eisenreich *et al.*, 2006). Therefore, the precursor of Asp, oxaloacetate, is generated exclusively in *L. monocytogenes* via carboxylation of pyruvate by the ATP-dependent pyruvate carboxylase PycA (Schar *et al.*, 2010).

It has been shown that the up-regulation of the major virulence gene cluster, including *hly*, *plcA*, *mpl*, *actA*, *plcB* (see section 1.5) as well as the other known PrfA-regulated genes, *inlA*, *inlB*, *inlC*, and *hpt*, is the most prominent response of *L. monocytogenes* to the growth conditions of the host cell cytosol (Joseph *et al.*, 2006). The regulations by PrfA as well as other factors result in well-balanced metabolic flows within the host cell by the intracellular pathogen *L. monocytogenes* (Fuchs *et al.*, 2012).

### 1.4.4 Actin-based motility and cell-cell spread

Shortly after entering into the cytoplasm of host cells, *L. monocytogenes* induces the polymerisation of host actin filaments and uses the force generated by actin polymerisation to migrate around the cell and spread to
adjacent cells via the cell membrane (Dramsi and Cossart, 2002). The recruitment of, and polymerisation of actin was stimulated directly by the Listerial surface protein ActA, the product of a single actA gene, bypassing the receptor-mediated signal transduction pathways that induce actin filament formation during phagocytosis and chemotaxis (Cossart, 2000).

ActA is an envelope protein of 639-amino-acids that functions as a dimer with a transmembrane motif in its C-terminal region that anchors the molecule to the bacterial cell wall (Domann et al., 1992). Its N-terminal region plays an essential role in containing all the necessary information to initiate F-actin assembly and bacterial movement (Vazquez-Boland et al., 2001).

ActA functions by mimicking the activity of the eukaryotic WASP family of actin nucleating factors. ActA contains a VCA (villin homology, cofilin homology and acidic) region at the N-terminal, which is also present in the WASP proteins. The VCA domain activates the Arp2/3 complex, which is the key player responsible for actin polymerisation (Pizarro-Cerda and Cossart, 2006). The ability of ActA to bind to and activate the Arp2/3-complex is achieved primarily by binding to Arp2/3 at the cofilin homology region (Fig. 1.4a). The acidic region increases the efficiency of actin nucleation and the rate of motility and is thought to form a second Arp2/3-binding and/or activation site (Fig. 1.4a). The multimeric Arp2/3-complex is recruited and activated by both ActA and WASP-proteins (Lambrechts et al., 2008). In addition to activating Arp2/3, ActA also interacts with ATP–G-actin through the actin-binding region (Fig. 1.4a). However, this region is not essential for motility in infected cells (Skoble et al., 2000).

The central region of ActA has four proline-rich repeats that contain FPPPP or FPPIP motifs (Fig. 1.4a). These short proline-rich stretches mimic those of the host cell cytoskeletal proteins zyxin, vinculin and palladin, which are
associated with focal adhesions and/or stress fibers (Skoble et al., 2000). ActA binds to the EVH1 (Ena/VASP homology domain 1) domain of VASP (Vasodilator-stimulated phosphoprotein) protein through this polyproline region to control the geometry of the network formed by the Arp2/3 complex (Cossart and Bierne, 2001; Pizarro-Cerda and Cossart, 2006). VASP is a protein found in sites of active actin polymerisation and is a substrate for cGMP- or cAMP-dependent kinases. It can recruit profilin and provide polymerisation-competent actin monomers to the N-terminus of ActA. It also seems to interact with F-actin through its C-terminal EVH2 domain, thus providing a linkage of the bacterium to the tail (Fig. 1.4 b). (Cossart and Bierne, 2001)

The actin tail formation and steady state motility of L. monocytogenes rely on the polarized distribution of ActA. By a combination of non-polarized secretion and continuous cell wall growth from the septal region, ActA localises at one distal end and at part of the sides of the bacterium (Rafelski and Theriot, 2006). As a result, F-actin initially accumulates along the sides of the bacterium and slowly moves to the bacterial pole as a tail forms (Rafelski and Theriot, 2005).

The actA knockout mutant (ΔactA) of L. monocytogenes can escape from the vacuole but grow in the cytosol as microcolonies in the perinuclear area without moving intracellularly or spreading to neighbouring cells (Cameron et al., 2000; Vazquez-Boland et al., 2001).

Beside the essential role of ActA in intracellular movement of L. monocytogenes, it is also found to play a key role in the evasion of autophagy. The Arp2/3 complex, Ena/VASP and actin recruited on the surface of L. monocytogenes camouflage the bacteria against autophagy recognition (Yoshikawa et al., 2009). Therefore, the bacteria could avoid being killed by the host autophagy pathway.
Figure 1.4 a) Domain organisation of the ActA protein, its presentation on the bacterium, and the target sites for host cell proteins. (Lambrechts et al., 2008). The N-terminal part (residues 1–293) of bacterially presented ActA contains the following domains: A, acidic region; AB, actin-binding region, C, coflin homology region. The central region (residues 293–390) is the polyproline region (PLP). The transmembrane domain (TM) spans residues 614–639. b) Model of actin assembly induced by ActA. Adapted from Cossart and Bierne (2001). The ActA is represented as a dimer and its polar distribution at the bacterial surface is represented as a hatched area. The bacterium is moving from left to right as indicated by the arrow.
1.5 Organisation and regulation of *L. monocytogenes* virulence genes

Regulated expression of virulence genes appears to be crucial for pathogens that encounter multiple environments during their lifecycles. This may be particularly true for environmental pathogens such as *L. monocytogenes* which must adapt to life in the outside world as well as to life within host cells. Transcriptional regulation of gene expression represents an important mechanism for *L. monocytogenes* adaptation to new environments. Among all of the putative transcriptional regulators identified in the *L. monocytogenes* genome sequence, PrfA stands out for its essential role in regulating the expression of virulence gene products. (Miner et al., 2007)

Six of the virulence factors (*prfA, plcA, hly, mpl, actA* and *plcB*) playing essential roles in *L. monocytogenes* infection are encoded by a 9 kb virulence gene cluster localised on the chromosome of *L. monocytogenes* between the two house-keeping genes *ldh* and *prs*. They are under the control of a 27 kD protein PrfA, which is referred to as PrfA-dependent virulence gene cluster or *Listeria* pathogenicity island 1 (Chakraborty et al., 2000) (Figure 1.5.1). The LLO-encoding gene, *hly*, occupies the central position in this locus followed by the lecithinase operon, compromising the *mpl, actA* and *plcB* genes in the same transcriptional direction. These genes transcribed either as *mpl-actA-plcB* transcript under the control of the *mpl* promoter or as shorter transcripts, *mpl*-transcript and *actA-plcB* transcript. Upstream *hly* gene locates the *plcA-prfA* operon transcribed in the opposite orientation (Vazquez-Boland et al., 2001). Besides the PrfA-dependent virulence gene cluster, several other important virulence genes are also positively regulated by PrfA protein, which include the *inlA, inlB* and *inlC* internalins, hexose phosphate transporter (*hpt*) and bile salt hydrolase (*bsh*) (Miner et al., 2007).

Regulation of PrfA activity is composed of three levels: transcriptional, post-transcriptional and post-translational control.
Figure 1.5 The PrfA regulon in L. monocytogenes. (Miner et al., 2007) prfA: positive regulatory factor; plcA: phosphatidylinositol phospholipase C; hly: listeriolysin O; mpl: metalloprotease; actA: actin-polymerisation protein; plcB: broad-range phospholipase C (lecithinase); inlA, inlB: large cell wall associated internalins A and B; inlC: small secreted internalin; hpt: hexose phosphate transporter; bsh: bile salt hydrolase; and + indicates transcriptional induction. Thin arrows above the gene arrows indicate the different transcripts.
Transcriptional control of the prfA gene is mediated by three separate promoter elements: prfAP1, prfAP2 and plcA promoters. Promoter prfAP1 and prfAP2 are located directly upstream of the prfA translation initiation codon and are separated by approximately 80 nucleotides (Freitag et al. 1993). They are required to generate the initial levels of PrfA protein that can subsequently activate expression from the plcA promoter and increase overall levels of PrfA expression, and are essential for the bacteria to escape the initial phagosomes (Freitag and Portnoy 1994). The third promoter, plcA promoter, produces a bicistronic plcA-prfA transcript, as well as a monocistronic plcA transcript (Camilli et al. 1993; Freitag and Portnoy 1994). The bicistronic plcA-prfA transcript provides for an additional increase of PrfA within the cytosol and is required for spread of intracellular bacteria to adjacent host cells (Camilli et al. 1993). Both prfAP1 and prfAP2 are negatively influenced by PrfA, while the plcA promoter is up-regulated by it (Freitag et al. 1993). Although the down-regulation of prfA gene expression by the PrfA protein is not essential in the virulence of L. monocytogenes in a mouse model, it is assumed to play a role outside the host (Miner et al., 2007).

The post-transcriptional control of the PrfA-regulated gene expression lies in the temperature control of the transcription from the prfAP1 promoter. Temperature has been shown to significantly influence the expression of virulence factors in L. monocytogenes, and it appears that this is accomplished, at least in part, via transcripts directed by the prfAP1 promoter. At 30 °C or below, the 5’-UTR of the prfAP1-directed mRNA forms a secondary structure that masks the ribosome-binding region of prfA and inhibits prfA mRNA translation. This structure becomes unstable at higher temperatures thus enabling translation of prfA mRNA and for the production of PrfA at temperatures of 37°C or above. In contrast, the plcA and prfAP2 promoters do not appear subject to this thermoregulation, and therefore
are likely to contribute to the expression of some PrfA-dependent genes at temperatures at or below 30 °C. (Johansson et al. 2002)

The third control, post-translational regulation of PrfA, firstly involves the sugar-mediated repression of PrfA-dependent genes. PrfA-dependent gene expression was reduced in the presence of sugars transported through the phosphoenolpyruvate-sugar phosphotransferase system (PTS), such as glucose, fructose, mannose or cellobiose, but did not occur with non-PTS carbon sources such as glucose-6-phosphate or glycerol (Renzoni et al., 1997; de las Heras et al., 2011). This repression occurs through inhibition of PrfA activity via the carbon catabolism repression (CCR) network, in which the central PTS components Hpr and its kinase HprK may be involved through an unknown mechanism (Renzoni et al. 1997; Deutscher, 2008). The carbon source regulation of PrfA represents a metabolic mechanism of *L. monocytogenes* in responding to the environment (de las Heras et al., 2011). Recognition of PrfA as a member of the Crp/Fnr family of transcriptional regulators (Korner et al. 2003) provided further support for the supposition that a small molecule cofactor or some form of post-translational modification is required to convert PrfA to an active state (Vega et al. 1998). A series of mutations have been generated in multiple sites of the PrfA protein, many of which showed significant increase in the affinity of PrfA to the PrfA-regulated gene promoters, or produced increased levels of the PrfA-dependent gene products such as ActA, LLO, PlcA, PlcB, InlA, and InlB. (Shetron-Rama et al. 2003; Eiting et al. 2005)

Although the PrfA regulon is undeniably the most important and most recognised force in the regulation of the virulence genes in *L. monocytogenes*, other bacterial factors that contribute to virulence regulation distinct from PrfA have also been identified, which will not be discussed in detail here. Overall, these systems enabled *L. monocytogenes* to
be a robust organism which survives both as an intracellular pathogen and environmental bacteria.

1.6 Infection route of *L. monocytogenes* in humans

As a food-borne pathogen, *L. monocytogenes* infects the human through the ingestion of contaminated food. Animal experiments on mice and rats demonstrated that epithelial multiplication in the intestinal mucosa was not required by *L. monocytogenes* for establishing a systemic infection (Marco *et al.*, 1992; Pron *et al.*, 1998).

By penetrating into the intestinal epithelial cells at the tip of intestinal villi and spreading to adjacent enterocytes, *L. monocytogenes* was able to cause enteritis short time after ingesting contaminated food. The bottleneck for intestinal villus colonisation is 1 in $10^6$ bacteria (Melton-Witt *et al.*, 2012). Gross intestinal lesions may develop if the intestine was exposed to extensive pathogenic *L. monocytogenes*. (Vazquez-Boland *et al.*, 2001)

The *L. monocytogenes* shed back from the tips of infected intestinal villi into the lumen inside extruded enterocytes can reinfect the lamina propria macrophages, then disseminates in infected leukocytes and transported directly to the liver via the portal vein. Alternatively, *L. monocytogenes* infects the Preyer’s patch, disseminates into the mesenteric lymph node at the ratio of 1 out of $10^2$ to $10^3$ bacteria, enter the systemic blood circulation and is then carried to liver and spleen (Fig. 1.6) (Melton-Witt *et al.*, 2012). In liver, most of the bacteria are captured by the Kupffer cells that line the sinusoids and killed by the resident macrophages. The remaining bacteria start to multiply, primarily in hepatocytes (Vazquez-Boland *et al.*, 2001). In murine model of *L. monocytogenes* infection in spleen, the bacteria first localise within macrophages in the marginal zone between the T cell-rich white pulp and the B cell-rich red pulp (Conlan, 1996). These infected cells
Figure 1.6 Model of bacterial dissemination from intestinal villi to liver and mesenteric lymph nodes. (Melton-Witt et al., 2012). (arrow 1) Ingestion of *L. monocytogenes* leads to enterocyte invasion at the tip of intestinal villi. Once internalized, *L. monocytogenes* replicates and spreads to neighboring enterocytes via cell-to-cell spread. (arrow 2) The basement membrane under the intestinal epithelium prevents bacterial extension into the lamina propria. Bacteria are shed back from the tips of intestinal villi into the lumen inside extruded enterocytes. Subsequently, bacteria reinfect Peyer’s patches (PP) (arrow 3), lamina propria macrophages (arrow 4), and other enterocytes (arrow 5). *L. monocytogenes* could disseminate in infected leukocytes (dendritic cells and/or macrophages) via the portal vein directly to the liver (arrow 6) and along afferent lymphatic vessels to the mesenteric lymph nodes (MLN) (arrow 7). The bacteria disseminates further via the systemic circulation to liver and spleen.
Figure 1.7 Successive steps of human listeriosis. Reproduced from Lecuit (2007)
then migrate into the white pulp region and form the beginning of a focus of infection that expands as neighboring cells become infected by the intercellular spread of bacteria (Mandel and Cheers, 1980).

If not controlled properly by the immune system notably at the liver and spleen levels, *L. monocytogenes* infection may cause prolonged and asymptomatic bacteremia. It may then cross the blood-brain barrier, causing meningitis and encephalitis; or through the blood-placental barrier, result in generalised infections in neonates (Fig. 1.7) (Reviewed by Lecuit, 2007).

1.7 Immunity against *L. monocytogenes* infection and immune-invasion of *L. monocytogenes*

Upon infection, *L. monocytogenes* passes through the intestinal barrier, enters the blood stream and taken up by splenic and hepatic phagocytes, where the majority are killed within the phagosomes. However, a small percentage of the bacteria are able to escape destruction and invade the cytosol where the race between bacterial replication and the immune response begins.

1.7.1 Innate immunity to *L. monocytogenes* infection

Early after infection, a cascade of innate immune response serves to control the bacteria spread, allowing time for the antigen (Ag)-specific adaptive immune response to achieve sterilizing immunity. This involves a coordinated interaction between many cell types and the production of numerous cytokines (Messingham and Harty, 2007).

Early reduction of bacterial number is mediated by a cytokine-dependent inflammatory response, primarily interferon-γ (IFN-γ) and tumour necrosis factor (TNF), that results in recruitment of additional activated macrophages.
and neutrophils for bacterial destruction (Nickol and Bonventre 1977; Bancroft et al. 1991). IFN-γ and TNF are essential for primary defense against infection with *L. monocytogenes*.

Neutrophils play a key role in the early control of *L. monocytogenes* infection, appearing at sites of infection in liver within the first 24 hours (Rogers et al., 1996). Mice depleted of neutrophils succumbed to an early, lethal infection, with large bacteria-laden foci in the liver parenchyma (Rogers et al., 1996). *L. monocytogenes* infection of hepatocytes results in apoptosis of the hepatocytes and in the release of neutrophil chemoattractants. Neutrophils migrating into the liver removed apoptotic hepatocytes and destroyed the released bacteria (Rogers et al., 1996).

The activation of macrophages and natural killer (NK) cells also plays an important role (Fig. 1.8). *L. monocytogenes* in the liver are taken up by macrophages, leading to their production of TNF-α and interleukin-12 (IL-12). These two cytokines synergize to cause NK cell secretion of IFN-γ. In combination with TNF-α, IFN-γ leads to full macrophage activation (Edelson and Unanue, 2000). Activated macrophages display increased levels of class II MHC (major histocompatibility complex) molecules and become listericidal by producing nitric oxide (NO), which is detrimental to intracellular pathogens. Macrophage inducible nitric oxide synthase (iNOS) catalyzes two mono-oxygenase reactions, hydrolyzing L-Arginine and producing NO. Among the three isoforms of NO synthase, iNOS is the most prevalent isoform expressed in murine macrophages (from the *nos2* gene) (MacMicking et al., 1997). The combination of NO and superoxide can make the highly reactive product peroxynitrite. NO and the reactive nitrogen intermediates (RNI) that derive from it, including nitrite, nitrate and peroxynitrite, are all bactericidal and play a central role in the ability of the activated macrophage to kill ingested pathogens (Nathan, 1997; Shaughnessy and Swanson, 2007).
Beside activated macrophages, a population of dendritic cells (DC) is also found to be recruited to the site of infection which produces TNF and iNOS. These TNF- and iNOS-producing DCs are also essential for the control of bacterial growth \textit{in vivo} in mice (Serbina \textit{et al.}, 2003).

\textbf{Figure 1.8} \textbf{The activation of macrophages.} Adapted from Unanue \textit{et al.} (1997). A resident macrophage that takes up \textit{L. monocytogenes} produces a number of cytokines, some of which induce the NK cell to produce IFN-\(\gamma\) (induction is represented by +). Other cytokines, like IL-10, inhibit this process (inhibition is represented by -). The IFN-\(\gamma\) drives the macrophage to an activated state. The activated macrophages express high levels of MHC class II molecules (represented by two parallel lines) and co-stimulators, like intercellular adhesion molecule-1 and B7 molecules (represented by a wavy line). The activated macrophage is active in: reducing the growth of \textit{L. monocytogenes} in vacuoles (closed circle) or in the cytosol following vesicle lysis (broken circle); in presenting peptides to \textit{L. monocytogenes-specific} T-cells (marked as Th0); and in inducing the differentiation of the Th0 cell to Th1 (by its release of IL-12). NO" represents nitric oxide.
However, in the absence of the adaptive response, innate immune mechanisms are unable to effectively achieve bacterial clearance (Bancroft et al., 1991). Additionally, humoral immunity does not appear to play a significant role in the clearance of *L. monocytogenes* and providing protective immunity, as antibody responses are very weak (Edelson and Unanue, 2000). Thus, primary sterilizing immunity and long-term protective immunity to *L. monocytogenes* are almost entirely mediated by the T-cell mediated adaptive immunity (Messingham and Harty, 2007).

1.7.2 Adaptive immunity against *L. monocytogenes* infection

Ag-specific immune response is initiated through encounter of a naïve T-cell bearing a T-cell receptor specific for bacterial peptide/MHC complexes on the surface of an antigen-presenting cell (APC). The Ag-specific immune response against *L. monocytogenes* relies on distinct populations of T cells responding to the bacterial antigens presented by MHC Class Ia, MHC Class Ib, or MHC Class II molecules. Regardless of the MHC restrictions, DCs are the most potent activators of naïve CD4+ and CD8+ T cells. (Muraille et al., 2005)

APCs such as DCs and macrophages acquire *L. monocytogenes* antigens by either directly infected with *L. monocytogenes*, or through phagocytosis of other *L. monocytogenes*-containing live or dead cells, also called ‘cross-presentation’ (Heath and Carbone, 2001). Both cross-presentation of exogenous Listerial antigens and direct presentation of intracellular bacterial antigens are assumed to contribute to T-cell priming during a primary response to *L. monocytogenes* (Messingham and Harty, 2007).

MHC Class I restricted CD8+ T cells comprise the majority of T cells responding to *L. monocytogenes* infection due to the intracytoplasmic location of this pathogen. To be accessible for MHC Class I presentation in an
infected cell, a bacterial protein must be secreted into the cytoplasm for degradation by the proteasome. The degraded peptides must be transported to the Golgi complex via the transporter associated with antigen processing (TAP) and loaded onto nascent MHC Class Ia, or Class Ib molecules, processed by endogenous MHC Class I presentation pathway, leading to the presentation of typically 8–10 amino acids long Listerial peptide by MHC Class I molecules on the surface of APCs. (Pamer and Cresswell, 1998; Cresswell et al., 2005; Messingham and Harty, 2007)

In the case of *L. monocytogenes*, MHC Class Ia molecules recognise degraded peptides from the virulence factors such as LLO and viability factors such as p60, a constitutively secreted protein of *L. monocytogenes*. Four major *L. monocytogenes*-derived epitopes of CD8+ T cells presented by H2-Kd have been identified. Two of the peptides derive from p60. A third epitope derives from LLO and the forth from a secreted metalloprotease (Busch et al., 1999). MHC Class Ib molecules are more conserved, of which the most well defined non-classical MHC molecule in mice, H2-M3, is capable of presenting peptides that contain N-formyl methionine (f-Met) at the amino terminus, a property exclusive to bacterial and mitochondrial proteins (Pamer and Cresswell, 1998).

Although responses to *L. monocytogenes* are multiclonal, CD8+ T-cell populations specific for independent antigens undergo expansion with coordinate kinetics, indicating that T-cell responses are independent of the quantity or duration of *in vivo* antigen presentation (Busch et al., 1998).

In addition to the robust responses of MHC Class I restricted CD8+ T cells, infection with *L. monocytogenes* also induces strong activation of MHC Class II restricted CD4+ T cells, in which MHC Class II restricted antigens are acquired by APCs through phagocytosis of extracellular bacteria. Several MHC Class II restricted Listerial epitopes, derived primarily from LLO, p60
and some *L. monocytogenes* surface proteins have been identified (Geginat *et al.*, 2001). Although the precise role for CD4+ T cells in the control of *L. monocytogenes* infection are not totally clear, antigen recognition by CD4+ T cells stimulates the production of copious quantities of Th1 cytokines and IFN-γ that aid in bacterial clearance via activation of other cell types, including DCs and bactericidal macrophages (Hsieh *et al.*, 1993; Harty and Bevan, 1995).

In mice, protective immunity to *L. monocytogenes* is solely dependent on MHC Class Ia-restricted CD8+ T cells. Re-exposure to *L. monocytogenes* results in a rapid mobilisation of Ag-specific cells from memory CD8+ T-cell pool, resulting in efficient clearance of challenge doses that are lethal for naïve mice (Lalvani *et al.*, 1997). *L. monocytogenes*-specific memory CD8+ T cells have increased ability to respond to secondary infection, and are also less dependent on the co-stimulatory molecules required for priming antigen-specific naïve T cells (Iezzi *et al.*, 1998). MHC Class Ib-restricted primary effector cells can contribute to protective immunity to *L. monocytogenes* in the absence of an MHC Class Ia-restricted response. However the expansion of MHC Class Ib-restricted memory cell population is limited by the MHC Class Ia-restricted memory responses. (Reviewed by Messingham and Harty, 2007)

The mechanism of bacterial killing by CD8+ T cells remains unclear. Although protective immunity to *L. monocytogenes* in wild type mice is dependent on MHC Class Ia-restricted CD8+ T cells, examination of gene knockout mice has not identified a single effector mechanism that is absolutely required for protective immunity (Messingham and Harty, 2007). The production of TNF and INF-γ, as well as the cytolytic activity of CD8+ T cells are all dispensable for protective immunity. It is possible that the mechanisms of T-cell mediated protection are distinct in different tissues and multiple mechanisms exist (Pamer, 2004).
Ag-specific CD4+ T cells have not been as extensively studied in mice model and controversy remains (Messingham and Harty, 2007). *L. monocytogenes*-specific CD4+ T cells are capable of providing protective immunity to naïve mice independent of CD8+ T cells, although to a lesser extent. The CD4+ T cell response differs the CD8+ T cell response in that the number of memory CD8+ T-cells is stable, while *L. monocytogenes*-specific memory CD4+ T-cell numbers decline over time (Schiemann *et al.*, 2003). CD4+ T-cell mediated clearance of infected cells is largely dependent on Ag-specific cytokine-driven phagocyte recruitment and subsequent nonspecific target-cell killing (Messingham and Harty, 2007).

**1.7.3 Immune evasion of *L. monocytogenes***

As a pathogenic bacterium, *L. monocytogenes* has evolved many strategies to avoid the destruction by host immune system. The intracellular niche allows *L. monocytogenes* to evade some immune responses that otherwise are very effective against the bacteria. Generally, the strategies used by *L. monocytogenes* include: avoid macrophage mediated or B-cell mediated killing and reduce immune surveillance by residing in nonprofessional APCs (Zenewicz and Shen, 2007).

After being phagocytosed by macrophages, about 10% of *L. monocytogenes* could escape from the phagosomes mediated by LLO or PLCs before the fusion of phagosomes with lysosomal compartments, therefore avoid the destruction by reactive oxygen and nitrogen species or harsh pH conditions (de Chastellier and Berche, 1994).

Besides macrophages, *L. monocytogenes* can also invade many types of nonprofessional APCs, such as hepatocytes and enterocytes. These cells have limited antigen presentation capacities compared to macrophages. This
benefits the bacteria in evading T-cell mediated immunity (Zenewicz and Shen, 2007).

B-cell mediated immunity contributes very little in clearing *L. monocytogenes* infection. This is due to the intracellular nature of this bacterium, as B cells have very little opportunity to encounter extracellular Listeria or its antigen. Although some bacteria could be found by B cells during infection, the low amounts of antibodies that are induced are completely unable to confer protection during a rechallenge with *L. monocytogenes* (Mackaness, 1962). Since the majority of the bacteria remains intracellular or spread to neighboring cells intracellularly without encountering extracellular milieu, *L. monocytogenes*-specific antibodies are of limited use in controlling bacterial spread (Zenewicz and Shen, 2007).

In conclusion, *L. monocytogenes* has evolved to limit immune system recognition and responses to infection. Understanding the host-pathogen balance of this organism has provided and still is providing us a better understanding towards infection and immunity.

### 1.8 Phosphoinositide metabolism in eukaryotic cells

Phosphoinositols are glycerolipids composed of a hydrophobic membrane anchor DAG, and a D-myo-inositol head group attached to DAG via a di-ester phosphate at the D-1 position, leaving 5 free hydroxyls (Downes *et al*., 2005). They can be phosphorylated in the D-3, 4 and 5 positions to generate at least seven unique second-messenger molecules phosphoinositol phosphate (PIP, or phosphoinositide). PIPs are phosphorylated by kinases, dephosphorylated by phosphatases, or hydrolysed by phospholipases to exert their regulatory role (Fig. 1.9 a and b) (Hilbi, 2006). The localisation of these metabolic enzymes is tightly controlled to ensure the spatial restriction and steady-state level of specific PIP, allowing both temporal and spatial...
signalling regulation (Di Paolo and De Camilli, 2006). PIP metabolism plays a vital role in the regulation of receptor-mediated signal transduction, actin remodelling, membrane trafficking and endosome dynamics in eukaryotic cells (Hilbi, 2006).

The recruitment and activation of PIP-metabolic enzymes on specific membrane compartments or subcompartments are mediated or enabled by the activity of small GTPases from the Ras superfamily. These include proteins implicated in signalling (e.g. the Ras family), actin regulation (e.g. the Rho family), organelle and vesicular transport (e.g. the Arf, Arl and Rab families) (Behnia and Munro, 2005). PIPs can in turn regulate the nucleotide cycle of GTPases. They could also act as co-receptors to specific membrane compartments. These events can occur simultaneously on the same membrane compartment, forming complex feedback loops (Di Paolo and De Camilli, 2006).

The effectors of PIPs are peripheral membrane proteins that bind to the lipid via specific recognition folds, which include PH (pleckstrin homology), FYVE (Fab1, YOTB, Vac1, and EEA1), PX (found in phagocytic oxidase), A/ENTH (Epsin N-terminal homology), FERM (4.1 protein, ezrin, radixin and moesin) domains, C2 domain (a protein structural domain), GRAM domain (found in glucosyltransferases and myotubularins), PHD (plant homeo domain) domain, PTB (phosphotyrosine-binding) domain and PDZ (named from post synaptic density protein PSD95, Drosophila disc large tumor suppressor Dlg1 and zonula occludens-1 protein zo-1) domain (Downes et al., 2005; Lemmon, 2003; Di Paolo and De Camilli, 2006). Some effector proteins bind to PIP and small GTPase simultaneously, such as the EEA1 (early endosomal antigen-1), which promotes endocytosis by binding to PI3P and Rab5 on phagosome/early endosome (Simonsen et al., 1998). (Fig. 1.9 c)
a) **The structure of PI.** The numbers indicate the 5 positions on the D-myo-inositol ring that can be phosphorylated.

b) **Metabolic reactions leading to the generation of seven PIP species from PI.** Reactions indicated with dotted arrows have been shown *in vitro*, but their importance in living cells remains unclear. Solid arrows indicated reactions confirmed *in vivo*. Identified PIP metabolic enzymes in mammalian cells are marked on the arrows. DAG: diacylglycerol.

c) **PIP-binding modules, their binding preference and some examples of proteins that contain them.**
1.8.1 PIPs in plasma membrane dynamics

PI(4,5)P₂, which predominantly accumulates on the plasma membrane and to a lesser extent on Golgi apparatus, participates in almost all the events that occur at or involve the cell surface including endocytosis, phagocytosis, exocytosis, ion channels transport, cell adhesion and microtubule capture (Di Paolo and De Camilli, 2006).

PI(4,5)P₂, together with its product PI(3,4,5)P₃ formed by PI3K, have been proved to modulate endocytosis or phagocytosis by interacting with cytoskeletal effectors such as dynamin-2, WASP and Rho guanosine exchange factors. (Botelho et al., 2004; Pizarro-Cerda et al., 2007)

During phagocytosis and endocytosis, PI(4,5)P₂ accumulates at the phagocytic cups and projecting pseudopods (Odorizzi et al., 2000). PI(4,5)P₂ is mostly synthesized locally from the serial conversion of PI into PI4P by phosphoinositol-4-kinase (PI4K), and PI4P into PI(4,5)P₂ by type I phosphoinositol phosphate-5-kinase (PIP5K I) in cells. They are then delivered to the cell surface by membrane carriers derived from the Golgi complex and from recycling organelles, and can also be produced locally at the plasma membrane (Odorizzi et al., 2000). PI(4,5)P₂ is then converted into PI(3,4,5)P₃ by type I PI3K. After this, two different pathways occur between FcγR-mediated phagocytosis and CR3-mediated phagocytosis, which are distinguished by the Fcγ and CR3 phagocytic receptor on the surface of mammalian cells (Underhill and Ozinsky, 2002). (Fig. 1.10)

During FcγR-mediated phagosome formation, although PI4K and PI3K remain associated with the maturing phagosome, PIP5K detaches upon sealing, leading to the depletion of PI(4,5)P₂ and transient accumulation of PI(3,4,5)P₃, which stimulates actin nucleation (Hilbi, 2006; Bohdanowicz et al., 2010). On maturation of the phagosome, the catabolism of PI(3,4)P₂
(mediated at least in part by PLC) and PI(3,4,5)P₃ are essential for the shedding of actin and fusion of phagosomes with the endosome/lysosome compartment. This is due to the binding of PI(3,4,5)P₃ to the PH domain of GDP-GTP exchange factors for Rac. This process plays a major role in the remodelling of the actin cytoskeleton in its specific direction (Cantley, 2002; Botelho et al., 2004)

![Diagram](image)

**Figure 1.10 Diagrammatic representations of PIP metabolism and actin recruitment.** (Bohdanowicz et al., 2010) A) FcγR-mediated phagocytosis. B) CR3-mediated phagocytosis. PIPs are showed in gray while enzymes and other substances are showed in black.

In CR3-mediated phagosome formation, the appearance of PI(4,5)P₂, PI(3,4,5)P₃ and actin remodelling still occurs. However, different from the FcγR-mediated phagosome, PIP5K still remains, which allows de novo PI(4,5)P₂ and PI(3,4,5)P₃ synthesis and actin polymerisation (Bohdanowicz et al., 2010). During phagosome sealing, the 5’-phosphoinositide phosphatase...
Inpp5B likely accounts for the hydrolysis of \( \text{PI}(4,5)\text{P}_2 \) and \( \text{PI}(3,4,5)\text{P}_3 \). Thus in early endosomes, accumulation of \( \text{PI}(4,5)\text{P}_2 \) and \( \text{PI}(3,4,5)\text{P}_3 \) is observed possibly due to the depletion of Inpp5B by the presence of PI3P (Bohdanowicz et al., 2010).

Besides phagocytosis, \( \text{PI}(4,5)\text{P}_2 \) is also directly implicated in exocytosis due to its critical role in vesicle priming. The level of \( \text{PI}(4,5)\text{P}_2 \) in the plasma membrane is positively correlated with the size of the readily releasable pool of secretory vesicles (Milosevic et al., 2005). It also indirectly affects exocytosis through PLC-cleaved product DAG and Inositol-1,4,5-triphosphate \( \text{I}(1,4,5)\text{P}_3 \), in that DAG is a critical ligand for the priming factor and \( \text{I}(1,4,5)\text{P}_3 \) mediates calcium responses modelling/triggering secretion (Rhee et al., 2002; Di Paolo and De Camilli, 2006).

### 1.8.2 PIPs in endosomal dynamics and intracellular membrane traffic

The organelles of the secretory and endocytic pathways in eukaryotic cells have distinct functions, molecular composition, and luminal environment (Roth, 2004). Membrane traffic is the process by which membrane lipids, integral membrane proteins, and the soluble protein content of membrane organelles are moved from the endoplasmic reticulum (ER) where they are synthesized to the sites where they function, and the rates and direction of movement of membrane proteins and lipids are regulated in response to signals from the extracellular environment (Roth, 2004). PIPs play a crucial role in trafficking among the plasma membrane, the Golgi complex and the lysosomes (Di Paolo and De Camilli, 2006).

As \( \text{PI}(4,5)\text{P}_2 \), \( \text{PI}(3,4,5)\text{P}_3 \) are rarely found intracellularly, monophosphorylated PIs and \( \text{PI}(3,5)\text{P}_2 \) function largely in intracellular organelles, in particular, membrane traffic (Roth, 2004). Two primary PIPs of interest are PI3P and PI4P, of which the latter is the more abundant in cells (Downes et al., 2005).
In mammalian cells, PI3P is highly enriched on early endosomes and on the internal vesicles of multi-vesicular endosomes (Gillooly et al., 2000). This therefore suggests that it is a major determinant of early endosome membrane identity and participates in nearly all aspects of endosomal function (Di Paolo and De Camilli, 2006, Robinson and Dixon, 2006). The PI3P can recruit proteins containing FYVE, PX or PH domains. Some of its effector proteins, such as EEA1, cooperate with Rab5 GTPase to stimulate endosome fusion (Simonsen et al., 1998).

PI3P is mainly produced from PI by a Rab5 effector, the class III PI3K, a homologue of VPS34 in yeast cells. It is also generated during the endocytic pathway through the sequential dephosphorylation of PI(3,4,5)P₃ (Di Paolo and De Camilli, 2006). PI3P can also be phosphorylated by the Fab1p kinase in yeast or PIKfyve kinase in mammalian cells to generate PI(3,5)P₂, which is present on late endocytic compartments, multivesicular bodies and lysosomes (Weber et al., 2009).

A major function of PI(3,5)P₂ is to trigger budding of vesicles from late endosomes (Di Paolo and De Camilli, 2006). PI(3,5)P₂ is thought to be involved in osmotic stress responsiveness and is essential for the maintenance of vacuole size and homeostasis in yeast (Gary et al., 1998). It has been reported to be of importance in late endosomal trafficking in yeast as well (Shaw et al., 2003). The depletion of PI(3,5)P₂ by defecting Fab1p or PIKfyve kinase causes enlarged, poorly acidified vacuoles and lethality at elevated temperatures in both yeast and mammalian cells (Cooke et al., 1998; Sbrissa et al., 1999; Ikonomov et al., 2001).

Dephosphorylation of PI3P and PI(3,5)P₂ is carried out by members of the myotubularin and Sac I families. Myotubularin and its related proteins (MTMs) are members of a large family of phosphatases and pseudophosphatases that are highly conserved in eukaryotic cells from fungi.
to humans (Laporte et al., 1996). It comprises at least ten human members, six in *Drosophila melanogaster* and *Caenorhabditis elegans*, and one in yeast (Laporte et al., 1996). Over-expression of myotubularins in human and yeast results in reduced levels of PI3P and PI(3,5)P$_2$, with dramatically enlarged endosomal structures (Blondeau et al., 2000; Walker et al., 2001; Kim et al., 2002; Tsujita et al., 2004).

PI4P is the predominant PIP in the Golgi complex region. The clathrin adaptor protein AP1 binds to Golgi by docking on Golgi membrane PI4P through non-conventional PIP binding domains (Wang et al., 2003). The association of AP1 to the membrane is also dependent on small GTPase ARF1. PI4P acts as the precursor for synthesising PI(4,5)P$_2$. PI4P generated in the Golgi and Golgi-derived carriers is probably converted to PI(4,5)P$_2$ either *en route* to the plasma membrane or immediately after it reaches the plasma membrane (Wang et al., 2003). The spacial restriction of PI4P to the Golgi complex is ensured by the action of phosphatases, one of which is likely to be Src1, that may dephosphorylate PI4P when retrograde traffic occurs from the Golgi complex to ER (Guo et al., 1999).

PI(4,5)P$_2$ and PI(3,4,5)P$_3$ are also involved in the regulation of actin filament reassembly. PI(4,5)P$_2$ mainly regulates actin polymerisation at the plasma membrane or membrane microdomains by upregulating actin-assembly promoting proteins and downregulating actin-assembly inhibitory proteins (Hilpela et al., 2004). PI(3,4,5)P$_3$ promotes actin polymerisation mainly via the activation of the Rho family of small GTPases (Hilpela et al., 2004).

**1.8.3 PIPs in the nucleus**

PIPs and PIP-binding proteins have been described in non-membrane locations in the nucleus. PI4P and PI(4,5)P$_2$ have been identified in the murine cell nucleus, and nuclear PIP metabolism is regulated independently
from that present elsewhere in the cell (Cocco et al., 1987). PI(4,5)P₂ has been found to regulate chromatin-remodeling complexes. The hydrolysis products of PI(4,5)P₂ by PI-PLC, DAG and I(1,4,5)P₃, are also important secondary messengers in the nucleus. PIPs are suggested to play a role in regulation of nuclear functions such as transcription, pre-mRNA splicing and processing machinery (York, 2006).

1.8.4 PI3K signaling pathway

Class I PI3K is a lipid kinase that generates PI(3,4,5)P₃, while Class II PI3K synthesises PI(3,4)P₂ from PI4P. Both PI(3,4,5)P₃ and PI(3,4)P₂ bind to the PH domain of the 57 kD Akt (also named as PKB) and induce the Akt/PKB pathway, which is one of the most critical pathways in regulating cell survival (Song et al., 2005). The PI3K-Akt signaling pathway is activated by many types of cellular stimuli or toxic insults and regulates fundamental cellular functions such as transcription, translation, proliferation, growth, and survival (Datta et al., 1999).

Following the production of PI(3,4,5)P₃ by PI3K on the inner side of the plasma membrane, Akt/PKB translocates and binds to the phospholipids. The interaction of the Akt-PH domain with PI(3,4,5)P₃ is thought to provoke conformational changes in Akt/PKB, resulting in the exposure of its two main phosphorylation sites, Thr³⁰⁸ and Ser⁴⁷³ (Alessi et al., 1996). Phosphorylation of the Thr³⁰⁸ site by PDK1 (3-phosphoinositide-dependent protein kinase), and Ser⁴⁷³ site by a yet unknown enzyme, leads to the activation of Akt/PKB (Alessi et al., 1996; Ma et al., 2008).

PDK1 was first reported to be activated by both PI(3,4,5)P₃ and PI(3,4)P₂ (Alessi et al., 1997). Subsequently it was discovered that PI(3,4)P₂ levels correlates with the level of Akt/PKB phosphorylation at Ser⁴⁷³, while PI(3,4,5)P₃ levels correlates with phosphorylation at Thr³⁰⁸ (Ma et al., 2008).
Furthermore, the cytosolic enzyme activity of the Akt/PKB pathway is dependent upon the PI(3,4)P$_2$ level, while the membrane-associated enzyme activity is dependent upon the PI(3,4,5)P$_3$ level (Ma et al., 2008). The activated Akt/PKB is then translocated to the cytoplasm and nucleus where many of its substrates are located. The activated Akt/PKB phosphorylates substrates that promote cell survival and stimulate cell growth. (Delcommenne et al., 1998; Lynch et al., 1999)

Besides PI(3,4,5)P$_3$ and PI(3,4)P$_2$, PISp is also involved indirectly in Akt/PKB activation, because PISp could activate PI3K, which in turn leads to activation of the Akt/PKB pathway (Pendaries et al., 2006). Notably, the Akt/PKB activity is regulated by enzymes such as PTEN (phosphatase and tensin homologue) and SHIP (Src homology 2 domain–containing inositol phosphatase) 5-phosphatase. PTEN could convert PI(3,4,5)P$_3$ to PI(4,5)P$_2$ (Haas-Kogan et al., 1998) and SHIP produce PI(3,4)P$_2$ from PI(3,4,5)P$_3$ (Ma et al., 2008). The balance of PI(3,4,5)P$_3$ and PI(3,4)P$_2$ as well as multiple phosphatases and kinases forms a complex set of events that together impinge the downstream effectors of Akt/PKB pathway (Ma et al., 2008).

### 1.9 Modulation of host cell PIP metabolism by pathogenic bacteria

Intracellular pathogenic bacteria determine the interaction with eukaryotic host cells upon initial contact and throughout the encounter. Given the importance of PIP metabolism in eukaryotic cells, it is not surprising that intracellular pathogenic bacteria develop various strategies to interfere with host cell PIP metabolism to facilitate their entry into the host cells, intracellular survival and actin-based motility: i) produce PIP-binding proteins that use PIPs as membrane anchor; ii) introduce their own PIP-metabolizing enzymes that directly alter host PIP levels; iii) produce lipid and protein factors that activate/inactivate or recruit host cell PIP-metabolizing enzymes (Fig. 1.11). (Weber et al., 2009)
Figure 1.11 Pathogen trafficking pathways and host phosphoinositide metabolism. Adapted from Weber et al. (2009). *Listeria monocytogenes* surface protein InlB engages the host cell receptor Met resulting in the activation of PI3K I and production of PI(3,4,5)P3. PI4K Ila and IIb are recruited to the entry site, produce PI4P and, thus, facilitate the internalisation of the bacteria. *Brucella abortus* entry and replication in host cells are dependent on class I, II and III PI3Ks. The bacteria employ a T4SS to establish an ER-derived replicative vacuole. *Legionella pneumophila* effector proteins translocated by a T4SS anchor to the LCV membrane via PI4P (SidC, SidM) or PI3P (LidA, LpnE). PI4P is produced by PI4K IIIb and/or Dd5P4/OCRL1, the latter of which is present on LCVs. *Mycobacterium tuberculosis* inhibits endosomal maturation by removal of PI3P from the phagosome (indicated by the crosses). To this end, the PI phosphatases SapM and MptpB are secreted, and the PI analogue lipoarabinomannan (LAM) inhibits the activation of PI3P-producing PI3K III. The precursor phosphatidylinositol mannoside (PIM) promotes continuous interactions of MCVs with endosomes. *Shigella flexneri* translocates the PI polyphosphatase IpgD via a T3SS into the host cell cytoplasm. IpgD causes alterations in the cell morphology by hydrolysing PI(4,5)P2. This process specifically yields PI5P, which in turn activates PI3K I and cell survival pathways dependent on the Akt kinase. *Salmonella enterica* uses a T3SS to trigger uptake, thereby translocating the IpgD homologue SopB/SigD into host cells. SopB enhances bacterial uptake by removing PI(4,5)P2 from the entry site, activates Akt pathways. Dependent on SopB and the PI3K III VPS34, PI3P is produced, which promotes the formation of a replicative vacuole by recruiting the retromer component SNX1 (sorting nexin-1) and the v-SNARE VAMP8. Major arrows indicate vesicle trafficking pathways, minor black arrows represent the secretion of effectors and modulation of PI-metabolizing enzymes, and red arrows designate PI conversion by enzymatic reactions.
1.9.1 *Shigella flexneri*

Intracellular pathogenic bacteria *S. flexneri* modulates phagocytosis by macrophages and uptake by non-phagocytic cells (e.g. epithelial cells) using the ‘trigger’ mechanism and rapidly escape from the phagosome to the cytoplasm by injecting bacterial effectors directly into the host cell through a type III secretion system (Hilbi, 2006).

One of the secreted effectors is the virulence factor IpgD, which behaves as a PI4-phosphatase that specifically dephosphorylates PI(4,5)P₂ into PI5P. This dephosphorylation leads to the accumulation of PI5P in the eukaryotic cell (Marcus et al., 2001; Niebuhr et al., 2002). Like many other type III secreted proteins, IpgD is stored in the bacterial cytoplasm in association with a specific chaperone and is translocated into the host cytoplasm upon contact with epithelial cells (Niebuhr et al., 2000). At the site of entry, the breakdown of PI(4,5)P₂ into PI5P results in dramatic plasma membrane and cytoskeleton rearrangements by detaching PI(4,5)P₂-binding proteins, which promotes bacterial entry into the host cell. During the bacteria entry, the Class I PI3K was activated in a PI5P-dependent pathway, which activation of Akt/PKB pathway, leading to prolonged survival of the host cell and efficient replication of the bacteria (Pendaries et al., 2006).

1.9.2 *S. enterica*

*S. enterica* enters non-phagocytic host cell by the ‘trigger’ mechanism. Following uptake, the bacteria replicate in *Salmonella*-containing vacuoles (SCV) which communicate with the endosomal and recycling pathways (Steele-Mortimer, 2008). SCVs transiently accumulate EEA1, small GTPase Rab5 and the transferrin receptor, and later recruit Rab7, LAMP-1, and vacuolar protein ATPase (Steele-Mortimer, 2008). However, the maturation of SCV to a bactericidal compartment is impaired (Marcus et al., 2001).
The *S. enterica* T3SS effector SigD/SopB, is an IpgD homologue that functions in the invasion of target cells as well as in the maturation of SCVs (Marcus et al., 2001). It showed PIP and inositol polyphosphate phosphatase activities, which preferentially hydrolyses PI(3,4)P₂, PI(3,5)P₂ and PI(3,4,5)P₃, yielding PI3P. SigD/SopB could also convert PI(4,5)P₂ into PI5P as well as dephosphorylate inositol polyphosphate. (Marcus et al., 2001)

Upon encountering with non-phagocytic cells, SigD/SopB is injected into the host cell via the SP-1 T3SS. It has been proposed that SigD/SopB promotes bacteria entry by depleting PI(4,5)P₂ on the plasma membrane and generating PI3P through a complex and tightly controlled process. PI3P then binds VAMP8 (vesicle-associated membrane protein 8) and modulates actin dynamics to promote *S. enterica*-induced invasion (Terebiznik et al., 2002; Hernandez et al., 2004; Dai et al., 2007). SigD/SopB is also known to mediate the formation of spacious phagosomes following bacterial entry by maintaining high levels of PI3P in the membrane of SCVs (Hernandez et al., 2004). This thereby disturbs vesicular traffic and inhibits phagosome-lysosome fusion (Hernandez et al., 2004). In addition to controlling membrane dynamics, SigD/SopB also triggers Akt/PKB signalling pathway by activating PI3K through a yet unknown mechanism (Mallo et al., 2008).

1.9.3 *Mycobacterium tuberculosis*

Similar to *S. enterica*, the intracellular pathogen *M. tuberculosis* replicate in *Mycobacterium*-containing vacuoles (MCVs) following entry into host cells. MCVs are characterised by Rab5 but not Rab7, ATPase or lysosomal hydrolases (Russell et al., 2002). *M. tuberculosis* enters macrophages in a PI3K-dependent manner and arrests the bactericidal endocytic pathway via depletion of PI3P on MCVs (Vergne et al., 2005).
During infection, *M. tuberculosis* secretes a PI3-phosphatase SapM, which hydrolyses PI3P into PIs and therefore depletes PI3P from the MCV membrane (Weber *et al.*, 2009). Moreover, another secreted virulence protein MtptB contains an active site similar to eukaryotic lipid phosphatase such as PTEN. MtptB exhibits activity toward phosphotyrosine, phosphoserine and PI substrates. It readily dephosphorylates all three monophosphorylated PIPs (PI3P, PI4P and PI5P) as well as PI(3,5)P₂ *in vitro* (Beresford *et al.*, 2007). Taken together, SapB and MtptB act in concert to deplete PI3P from MCVs. As PI3P is essential for phagosome-lysosome fusion, the depletion of PI3P arrests the maturation of *Mycobacterium*-containing phagosome and facilitates its survival in macrophages. (Vergne *et al.*, 2005; Weber *et al.*, 2009)

### 1.9.4 *Legionella pneumophila*

*L. pneumophila* is a parasite in both environmental protozoa and alveolar macrophages (Fields *et al.*, 2002). It triggers its own uptake by phagocytes and replicates in *Legionella*-containing vacuoles (LCVs). LCVs avoid fusion with lysosomes, instead acquire small GTPases implicated in the endosomal or late secretary pathway, interact with early secretory vesicles and fuse with ER (Weber *et al.*, 2009).

*L. pneumophila* secretes virulence factor SidC/SdcA, which localises to the LCV membrane after translocation. SidC/SdcA and another virulence protein SidM are PI4P-binding effectors *in vitro* (Weber *et al.*, 2006). SidC/SdcA and SidM function in recruiting ER vesicles to LCVs and as a Rab1 guanine nucleotide exchange factor respectively (Ragaz *et al.*, 2008; Murata *et al.*, 2006). Another *L. pneumophila* PI-binding effector protein LidA preferentially binds to PI3P and PI4P, contributing to the recruitment of early secretory vesicles to LCVs (Brombacher *et al.*, 2009). In general, *L. pneumophila* exploits host monophosphorylated PIPs to anchor distinctive
effector proteins to the LCV membranes, which interferes with the fusion of LCVs to host endosomes and thus prevent lysis of the bacteria (Weber et al., 2009).

1.9.5 L. monocytogenes

Given the examples above, it is apparent that many intracellular pathogenic bacteria manipulate the host cell PIP metabolism in various ways to favor their survival. Therefore it is assumed that L. monocytogenes also shares these mechanisms.

The PIPs have been shown to be important for the intracellular survival of L. monocytogenes. The L. monocytogenes surface protein InlB promotes bacterial internalisation into host cells by stimulating host tyrosine phosphorylation, PI3K activity and rearrangements in the actin cytoskeleton (Pizarro-Cerda et al., 2007). Furthermore, PI3K is required for the intracellular motility of L. monocytogenes, possibly by converting PI(4,5)P_2 to PI(3,4,5)P_3 on the surface of L. monocytogenes in the host cell cytoplasm, as PI(3,4,5)P_3 seems to play a critical role in the actin-based intracellular movement and cell-cell spread in L. monocytogenes (Sidhu et al., 2005). It has also been discovered that the production of PI4P by type II PI4K α and β at the entry site is essential for InlB-mediated internalisation of the bacteria (Pizarro-Cerda et al., 2007). After internalisation of L. monocytogenes, PI3P is recruited to Listeriae-containing vacuoles and is an essential marker for Listeriae-containing vacuole identity. The Listeriae-containing vacuoles are also positive for Rab7 marker, but negative for Rab5 or LAMP-1, thus are delayed in fusion with the LAMP-1-positive lysosome (Henry et al., 2006; Shaughnessy and Swanson, 2007). However, there was no evidence for the manipulation and subversion of PIP metabolism by the production of PIP phosphatases in L. monocytogenes as in other intracellular bacteria, which is surprising.
1.10 LipA and LipB—two tyrosine and lipid phosphatases produced by *L. monocytogenes*

Bioinformatic analysis of the sequenced genome of *L. monocytogenes* EGDe, revealed the presence of two open-reading frames (ORFs) *lmo1800* and *lmo1935* that contained the P-loop active site (CXXGXDRT/A) typical of protein tyrosine phosphatases (PTP) and PI phosphatase (Beresford et al., 2007; Beresford et al., 2010; Kastner et al., 2011). This site is highly conserved and maintains close proximity between the catalytic cysteine and the arginine, which is important for phosphate binding (Kastner et al., 2011). (Fig. 1.12)

Preliminary work in our lab discovered the lipid phosphatase activity of Lmo1800 and Lmo1935 (Bennett and Roberts, unpublished data), which brought out interest into these proteins in that they may shed light on how the intracellular bacteria *L. monocytogenes* affect host PIP metabolism. A few recent studies further revealed some characteristics of these proteins, and Lmo1800 was given the name *Listeria* phosphatase A (LipA). In this study Lmo1935 was named as LipB.

![Figure 1.12 ClustalW (1.83) multiple protein sequence alignment of LipA, LipB in *L. monocytogenes* with the lipid phosphatase myotubularin in human (MTM1) and *Caenorhabditis elegans*. Shaded areas indicate the highly conserved sequence of P-loop active site typical of phosphoinositol phosphatases. Asterisks indicate the key amino acids for enzymatic activity.](image-url)
lipA (lmo1800) is located at positions 1872724 to 1873620 of the L. monocytogenes chromosome between ffh, encoding the signal recognition particle protein, and lmo1799, a gene encoding a putative LPXTG protein. This 298-amino-acid protein was once predicted to be a lipoprotein anchored to the cell surface due to the identification of a predicted lipid attachment sequence (Baumgartner et al., 2007). However, a later study suggested that LipA contains a functional 30-amino-acid N-terminal export signal for secretion to the extracellular environment (Kastner et al., 2011). The predicted protein structure of LipA shows strong homology to the M. tuberculosis phosphatase MptpB (Madhurantakam et al., 2005).

lipB (lmo1935) is located from position 2009006 to 2009986 of L. monocytogenes chromosome between hup, which is similar to non-specific DNA-binding protein HU, and gpsA, similar to NADPH-dependent glycerol-3-phosphate dehydrogenase. The sublocation of LipB is unknown although no signalling peptide was identified through bioinformatics analysis (Bennett and Roberts, unpublished data).

Enzymatic activity profile and substrate specificity of the putative phosphatase LipA and LipB have been studied. Both proteins showed protein phosphatase and lipid phosphatase activity. LipA and LipB specifically dephosphorylate phospho-tyrosine but not phospho-serine or phospho-threonine. LipA readily dephosphorylated PI3P, PI4P, PI5P, and PI(3,5)P2, while LipB use only the three monophosphates PI3P, PI4P and PI5P as substrates and has significantly lower lipid phosphatase activity than LipA. (Beresford et al., 2010; Kastner et al., 2011)

LipA showed no PrfA-dependent expression (Baumgartner et al., 2007). Recent studies showed that deletion of the lipA gene does not affect the growth and stress resistance of L. monocytogenes in vitro. Nor does it impair the replication of the bacteria in mouse J774 macrophage-like cell line or the
rectal epithelial cell line CMT-93, and L2 fibroblast cells (Kastner et al., 2011). In addition, LipA was not found to be required for cell-to-cell spread (Kastner et al., 2011). LipA is highly expressed in blood, while LipB is slightly down-regulated in human blood and no significant change in intestine. (Joseph et al., 2006; Kastner et al., 2011)

However, the functions of the two L. monocytogenes phosphatases LipA and LipB during the intracellular life cycle of L. monocytogenes are still largely a mystery.

1.11 Aim of the project

This study is focused on how L. monocytogenes affects host PIP metabolism by the production of phosphatases LipA and LipB.

My aims are to:

a) Understand the changes in host PIP metabolism during infection by L. monocytogenes;
b) Understand how host PIP metabolism is manipulated by L. monocytogenes during infection;
c) Characterise the roles of the two PIP phosphatases LipA and LipB in L. monocytogenes infection.

In conclusion, this study will generate a clearer picture of the manipulation of host cell PIP metabolism by L. monocytogenes and provide a better understanding of the intracellular survival of this pathogenic bacterium.
## Chapter 2 Methods and Materials

### 2.1 Bacteria strains, media and growth conditions

Bacteria strains used in this study were listed below in Table 2.1.

<table>
<thead>
<tr>
<th>Bacteria Strains</th>
<th>Features</th>
<th>Sources and References</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli DH5α</td>
<td>$\Phi 80$dlacZΔM1, recA1, endA1, gyrA96, thi-1, hsdR17, (rk-mk+), supE44, relA1, deoR, Δ(lacYZA ΔargF) U169, phoA</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>L. monocytogenes EGDe</td>
<td>wild type, serotype 1/2a</td>
<td>Glaser et al. (2001)</td>
</tr>
<tr>
<td>L. monocytogenes InlA</td>
<td>InlA$^{S192N-Y369S}$ mutation in the inlA gene to be able to recognise murine E-cadherin</td>
<td>Wollert et al. (2007)</td>
</tr>
<tr>
<td>L. monocytogenes EGDe::Δhly</td>
<td>hly deletion mutant of EGDe</td>
<td>Bennett and Roberts, unpublished data</td>
</tr>
<tr>
<td>L. monocytogenes EGDe::ΔlipA</td>
<td>lipA deletion mutant of EGDe</td>
<td>Bennett and Roberts, unpublished data</td>
</tr>
<tr>
<td>L. monocytogenes EGDe::ΔlipB</td>
<td>lipB deletion mutant of EGDe</td>
<td>Bennett and Roberts, unpublished data</td>
</tr>
<tr>
<td>L. monocytogenes EGDe::ΔlipAΔlipB</td>
<td>lipA and lipB double deletion mutant of EGDe</td>
<td>Bennett and Roberts, unpublished data</td>
</tr>
<tr>
<td>L. monocytogenes InlA::Δhly</td>
<td>hly deletion mutant of InlA</td>
<td>This study</td>
</tr>
<tr>
<td>L. monocytogenes InlA::ΔlipA</td>
<td>lipA deletion mutant of InlA</td>
<td>This study</td>
</tr>
<tr>
<td>L. monocytogenes InlA::ΔlipB</td>
<td>lipB deletion mutant of InlA</td>
<td>This study</td>
</tr>
<tr>
<td>L. monocytogenes InlA::ΔlipAΔlipB</td>
<td>lipA and lipB double deletion mutant of InlA</td>
<td>This study</td>
</tr>
<tr>
<td>L. monocytogenes InlA::ΔplcB</td>
<td>plcB deletion mutant of InlA</td>
<td>This study</td>
</tr>
<tr>
<td>L. monocytogenes InlA::ΔhlyΔplcB</td>
<td>hly and plcB double deletion mutant of InlA</td>
<td>This study</td>
</tr>
<tr>
<td>L. monocytogenes InlA::GFPuv</td>
<td>chromosomal fusion of single copy GFPuv sequence inserted downstream the rpoC gene in InlA strain</td>
<td>This study</td>
</tr>
</tbody>
</table>
L. monocytogenes
InlA::mCherry
chromosomal fusion of single copy
mCherry sequence inserted downstream
the rpoC gene in InlA strain
This study

<table>
<thead>
<tr>
<th>Bacteria strains, features and sources involved in this study.</th>
</tr>
</thead>
</table>

E. coli were routinely cultured in Luria-Bertani (LB) broth (containing 1% tryptone (w/v), 0.5% yeast extract (w/v) and 1% NaCl (w/v)) at 37 °C with shaking at 200 rpm or with additional 1.5% agarose on plate. Where appropriate, culture media were supplemented with ampicillin (Amp) at 100 μg/ml, erythromycin (Erm) at 300 μg/ml (Erm was dissolved in 70% Ethanol to the stock concentration of 30 mg/ml) or kanamycin (Kan) at 50 μg/ml.

L. monocytogenes were routinely cultured in 3% (w/v) typtone soya broth (TSB) at 37 °C with shaking at 200 rpm or with additional 1.5% agarose on plate. Where appropriate, cultures were supplemented with Erm at 5-20 μg/ml or Kan at 50 μg/ml.

2.2 Plasmids

The plasmids used in this study and their key features and sources are listed below in Table 2.2

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Features</th>
<th>Sources and References</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUNK1</td>
<td>E. coli-L. monocytogenes shuttle plasmid, Erm'</td>
<td>Pilgrim et al. (2003)</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>Cloning vector, Amp'</td>
<td>Promega</td>
</tr>
<tr>
<td>pEGFP-N2</td>
<td>Expression vector, P_{cmv1}, GFP_{mut1}, Kan', Neo', oriR pUC, oriR SV40</td>
<td>Clontech laboratories Inc.</td>
</tr>
<tr>
<td>pGFP_{uv}</td>
<td>Donor of GFP_{uv} gene, oriR pUC, Amp'</td>
<td>Clontech laboratories Inc.</td>
</tr>
<tr>
<td>pSG1729</td>
<td>Donor of GFP_{mut2} gene, amyE::spc(gfp_{mut2}), Amp'</td>
<td>Provided by Heath Murray</td>
</tr>
<tr>
<td>pNF8</td>
<td>Mob'(IncP), oriR pAMβI, oriR pUC, Pdlt-GFP_{mut1}, Erm'</td>
<td>Andersen et al. (2006)</td>
</tr>
<tr>
<td>pJEBAN6</td>
<td>Mob'(IncP), oriR pAMβI, oriR pUC,</td>
<td>Andersen et al.</td>
</tr>
<tr>
<td>Vector Name</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>---------------------------------------------------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>pBluescript-RpoND</td>
<td>Cloning vector, containing the 1.2 kb rpoC downstream region, Amp’</td>
<td>Corbett, unpublished data</td>
</tr>
<tr>
<td>pLSV1</td>
<td>Cloning vector, pUC18 derivative, ori (Ts), Erm’</td>
<td>Wuenscher et al. (1991)</td>
</tr>
<tr>
<td>pLSV1-lipA</td>
<td>pLSV1 shuttle vector containing 650 bp flanking each side of lipA, Erm’</td>
<td>Bennett and Roberts, unpublished data</td>
</tr>
<tr>
<td>pLSV1-hly</td>
<td>pLSV1 shuttle vector containing 650 bp flanking each side of hly, Erm’</td>
<td>Bennett and Roberts, unpublished data</td>
</tr>
<tr>
<td>pAULA</td>
<td>Cloning vector, pJDC9 derivative, ori (Ts), Erm’</td>
<td>Chakraborty et al. (1992)</td>
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<tr>
<td>pmCherry</td>
<td>Donor of mCherry gene, oriR pUC, Amp’</td>
<td>Clontech laboratories Inc.</td>
</tr>
<tr>
<td>pAULA-plcB</td>
<td>Cloning vector, containing 600bp flanking each side of plcB, Erm’</td>
<td>This study</td>
</tr>
<tr>
<td>pBlueScript-rpoND-GFP&lt;sub&gt;uv&lt;/sub&gt;</td>
<td>GFP&lt;sub&gt;uv&lt;/sub&gt; sequence insert into BamH I site of pBluescript-RpoND, Amp’</td>
<td>This study</td>
</tr>
<tr>
<td>pAULA-GFP&lt;sub&gt;uv&lt;/sub&gt;</td>
<td>pAULA vector containing rpoC-GFP&lt;sub&gt;uv&lt;/sub&gt; dropped from pBlueScript-RpoND-GFP&lt;sub&gt;uv&lt;/sub&gt;, Erm’</td>
<td>This study</td>
</tr>
<tr>
<td>pBlueScript-rpoND-mCherry</td>
<td>mCherry sequence insert into BamH I site of pBlueScript-RpoND, Amp’</td>
<td>This study</td>
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<tr>
<td>pAULA-mCherry</td>
<td>pAULA vector containing rpoC-mCherry dropped from pBSRpoND-mCherry, Erm’</td>
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<tr>
<td>pUNK1-LipA</td>
<td>Promoter and full coding sequence of lipA in pUNK1, Erm’</td>
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<tr>
<td>pUNK1-LipB</td>
<td>Promoter and full coding sequence of lipB in pUNK1, Erm’</td>
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<td>pUNK1-PrpoB-mCherry</td>
<td>rpoB promoter and mCherry in pUNK1, Erm’</td>
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<td>pUNK1-GFP&lt;sub&gt;mut2&lt;/sub&gt;</td>
<td>GFP&lt;sub&gt;mut2&lt;/sub&gt; inserted into pUNK1, Erm’</td>
<td>This study</td>
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<tr>
<td>pUNK1-PrpoB</td>
<td>rpoB promoter inserted into pUNK1, Erm’</td>
<td>Corbett and Roberts, unpublished data</td>
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<td>pAULA-lipB</td>
<td>pAULA containing 600bp flanking each side of lipB, Erm’</td>
<td>This study</td>
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<tr>
<td>pEGFP-LipA</td>
<td>Expression vector of LipA-EGFP in pEGFP-N2 backbone, Kan’</td>
<td>This study</td>
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<tr>
<td>pEGFP-LipA-</td>
<td>Single Cys to Ala substitution in the enzyme</td>
<td>This study</td>
</tr>
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</table>
Table 2.2 Plasmids, features and sources used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Feature</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>pEGFP-LipB</td>
<td>Expression vector of LipB-EGFP in pEGFP-N2 backbone, Kan'</td>
<td>This study</td>
</tr>
<tr>
<td>pEGFP-LipB-SDM</td>
<td>Single Cys to Ala substitution in the enzyme active site of LipB on pEGFP-LipB, Kan'</td>
<td>This study</td>
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<tr>
<td>pcDNA3.1(+)N-myc</td>
<td>myc sequence inserted before the MCS of pcDNA3.1(+) backbone, Pcmv, Amp'</td>
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<td>pcDNA3.1(+)myc-LipA</td>
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<td>This study</td>
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<td>pEGFP-Lpd</td>
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<td>Provided by Frank B. Gertler</td>
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<td>pEGFP-FENS/FYVE</td>
<td>Expression vector of EGFP-FENS/FYVE, Kan'</td>
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<td>Balla et al. (2005)</td>
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<td>pEGFP-PLCσ-PH</td>
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<td>Varnai and Balla (1998)</td>
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<td>pYFP-Akt-PH</td>
<td>Expression vector of YFP-Akt-PH, Kan'</td>
<td>Watton and Downward (1999)</td>
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<td>pEGFP-TAPP1-PH</td>
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<td>Kimber et al. (2002)</td>
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<td>pYFP-ARNO-PH</td>
<td>Expression vector of YFP-ARNO-PH, Kan'</td>
<td>Venkateswarlu et al. (1998)</td>
</tr>
</tbody>
</table>

2.3 DNA manipulation

2.3.1 Preparation of plasmid DNA from E. coli

High purity plasmid DNA was extracted from E. coli using the QIAprep Spin Miniprep Kit (Qiagen) in accordance with the manufacturer’s instructions. Briefly, 5 ml of a bacteria overnight culture with antibiotic selection was pelleted by centrifuging at 3660 xg for 5 min, resuspended in RNase A-containing buffer P1 provided, lysed with the NaOH/SDS-containing buffer P2 provided for less than 5 min, then neutralised and adjusted to high-salt...
binding conditions with the solution N3 provided. Vigorous stirring and vortexing must be avoided during lysis to avoid chromosomal DNA contamination. After centrifuging the lysates at 13000 rpm on a bench-top centrifuge (Eppendorf) for 10 min, the protein- and chromosomal DNA-free supernatant was transferred to spin columns provided and was centrifuged again at 13000 rpm for 1 min, during which the plasmid DNA bound to the column membrane. The elutant was removed, the column washed with ethanol-containing washing buffer provided and then removed of any trace of ethanol. Finally the plasmid DNA was eluted with 50 μl or 30 μl buffer EB which contains 10 mM Tris-Cl (pH 8.5).

2.3.2 Restriction endonuclease digestion of DNA

All restriction endonucleases and buffers were purchased from Roche Molecular Biochemicals unless otherwise stated and used according to the manufacturer’s instruction. Appropriate amount of DNA was mixed with the restriction enzyme and buffer and incubated at 37 °C (unless otherwise stated) for at least 2 hrs or at room temperature (RT) overnight. Endonucleases were inactivated by heating at 65 °C or 80 °C (according to different enzymes) for 20 min or removed by PCR purification procedure (section 2.3.3).

2.3.3 Purification of restriction digests and PCR products

For preparation of high purity DNA following restriction digests or PCR, the QIAquick PCR Purification Kit (Qiagen) was used in accordance with the manufacturer’s instructions. Briefly, the PCR products were mixed with five times the product volume of binding buffer provided. This mixture was applied to spin columns and centrifuged at 13000 rpm for 1 min, leaving the DNA fragments bound to the membrane in the columns. The columns were then washed with ethanol-containing washing buffer to remove enzymes, reaction buffers, unincorporated bases, and the desired fragments were
eluted with 50 μl or 30 μl buffer EB provided which contains 10 mM Tris-Cl (pH 8.5).

2.3.4 Ligation reactions

Vector DNA and desired fragments for ligation were digested with the appropriate endonuclease(s) as described above. The concentrations of vector DNA and the desired fragments were determined through agarose gel electrophoresis (see section 2.3.5) and mixed at 1:3 vector-insert molar ratio to a total volume in which every 10 μl contains 1 μl (400 unit) T4-ligase (New England Biolabs) and 1 μl 10X ligation buffer which contains 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP and 25 μg/ml bovine serum albumin. The mixture was incubated at 4 °C overnight or at RT for a minimum of 1 hr.

2.3.5 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out according to Sambrook and Russell (2001). DNA loading buffer (0.1% (w/v) bromophenol blue, 20 mM Tris, pH 8.0, 30% glycerol, 6X solution) was added to the sample to acquire a 1X solution. Samples were applied to the wells of a 0.5%-1.5% (w/v) agarose (Longza)-TAE (0.5 M Tris, 5.7% acetic acid, 10 mM EDTA, pH 8.0) gel depending on the size of the fragments and electrophoresed at 110 V in TAE buffer containing 5 μg/ml ethidium bromide till DNA fragments were visualised with an ultraviolet transilluminator.

2.3.6 Gel purification of DNA fragments

DNA fragments could be retrieved from agarose gel using QIAquick Gel Extraction Kit (Qiagen) according to the manufacture’s instruction. The desired DNA fragment was cut out from the agarose gel under UV light. It was then fully dissolved at 65 °C in the buffer QG provided before applying to the column provided. The column was centrifuged at 13000 rpm for 1 min,
leaving the DNA fragment bound to the membrane in the column. The column was washed with the buffer PE provided to remove the agarose, loading buffer, ethidium bromide and other impurities from DNA samples and the desired fragments were eluted with 30 μl buffer EB provided which contains 10 mM Tris-Cl (pH 8.5).

2.3.7 Polymerase chain reaction (PCR)

PCR was performed using a Touchdown Thermal Cycler (Hybai). Each 20-50 μl reaction contains 1 mM of appropriate forward and reverse primers each, 1/10 of the volume of 10X PCR buffer (Roche Molecular Biochemicals), 2.5 mM dNTPs (Bioline), 0.5-1 μl template DNA and 0.1-0.5μl Taq polymerase (Roche Molecular Biochemicals). Where the accuracy of PCR product was desired, 1 μl 1/8 dilution of Pwo (Roche) polymerase in PCR buffer as also included. PCRs were carried out under the following cycles: 95 °C for 30 sec, Tm °C for 30 sec and 72 °C for N min (where Tm is the lower melting temperature between the two primers minus 5 °C; N is the estimated length in kb of the fragment being amplified) for 30 cycles, followed by 1 cycle of 72 °C for 10 min. All primers were purchased from Sigma-Aldrich. The sequences and characterisations of the primers used in this study are listed below in Table 2.3

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Enzyme</th>
<th>Explanation</th>
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<tbody>
<tr>
<td>lipB-KO-1F</td>
<td>GACTGAATTCTATTTATACGAATGATGATGTG</td>
<td>EcoRI</td>
<td>Amplify the lipB-KO1 5’ sequence of lipB, for the construction of pAULA-lipB</td>
</tr>
<tr>
<td>lipB-KO-1R</td>
<td>ACTAGGATCCACGAGTAACTTCCATATTTTCC</td>
<td>BamHI</td>
<td>Amplify the lipB-KO1 3’ sequence of lipB, for the construction of pAULA-lipB</td>
</tr>
<tr>
<td>lipB-KO-2F</td>
<td>GTCAGGATCCATGTTAGAATAAAXTAGGCAA</td>
<td>BamHI</td>
<td>Amplify the lipB-KO2 5’ sequence of lipB, for the construction of pAULA-lipB</td>
</tr>
<tr>
<td>lipB-KO-2R</td>
<td>CACTAAGCTTTACGTACTTCAAGTTACC</td>
<td>HindIII</td>
<td>Amplify the lipB-KO2 3’ sequence of lipB, for the construction of pAULA-lipB</td>
</tr>
<tr>
<td>Primer (Gene)</td>
<td>Sequence (forward/reverse)</td>
<td>Restriction Enzyme</td>
<td>Description</td>
</tr>
<tr>
<td>---------------------</td>
<td>-----------------------------</td>
<td>-------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>GFP&lt;sub&gt;mut2&lt;/sub&gt;-F</td>
<td>ATCTTAAGATCTAAGTAGCTGATTAAATGGGAGGGAATA</td>
<td>Bgl II</td>
<td>Amplify the GFP&lt;sub&gt;mut2&lt;/sub&gt; coding sequence, contains the stop codon cassette and the rpoB S-D region</td>
</tr>
<tr>
<td>GFP&lt;sub&gt;mut2&lt;/sub&gt;-R</td>
<td>ATCTTAGAGCTCTTTAGAATTC</td>
<td>Sac I</td>
<td>Amplify the GFP&lt;sub&gt;mut2&lt;/sub&gt; coding sequence</td>
</tr>
<tr>
<td>pUNK1-F</td>
<td>AGAGTTGTAGCTCTTGTAGCG</td>
<td>-</td>
<td>Screen for inserts in the pUNK1 plasmid</td>
</tr>
<tr>
<td>pUNK1-R</td>
<td>TTCAGCAACATTTTAAACTGG</td>
<td>-</td>
<td>Screen for inserts in the pUNK1 plasmid</td>
</tr>
<tr>
<td>M13-F</td>
<td>TGTTAAAAGCAGGCGGAGT</td>
<td>-</td>
<td>Screen for inserts in the pAULA and pGEM-T Easy plasmids</td>
</tr>
<tr>
<td>M13-R</td>
<td>GAAACAGCTATGACCAGT</td>
<td>-</td>
<td>Screen for inserts in the pAULA and pGEM-T Easy plasmids</td>
</tr>
<tr>
<td>pmCherry-F</td>
<td>ATCTTAAGATCTAAGTAGCTGATTAAATGGGAGGGAATA</td>
<td>Bgl II</td>
<td>Amplify the mCherry coding sequence, contains the stop codon cassette and the rpoB S-D region</td>
</tr>
<tr>
<td>pmCherry-R</td>
<td>CATGAAAGATCTTTACCTTTACCTTAAGTC</td>
<td>Bgl II</td>
<td>Amplify the mCherry coding sequence, contains a TAA stop codon</td>
</tr>
<tr>
<td>plcB-KO-1F</td>
<td>AGATAAAGAATTCTCTGTAAAGTC</td>
<td>EcoR I</td>
<td>Amplify the plcB-KO1 5’ sequence of plcB, for the construction of pAULA-plcB</td>
</tr>
<tr>
<td>plcB-KO-1R</td>
<td>CGATAAAGAGCCACCTTTTTTTGATTTTCAT</td>
<td>BamH I</td>
<td>Amplify the plcB-KO1 3’ sequence of plcB, for the construction of pAULA-plcB</td>
</tr>
<tr>
<td>plcB-KO-2F</td>
<td>ATTCTAGGATCCACAATGAAATAAACAATATTAGG</td>
<td>BamH I</td>
<td>Amplify the plcB-KO2 5’ sequence of plcB, for the construction of pAULA-plcB</td>
</tr>
<tr>
<td>plcB-KO-2R</td>
<td>ATATCTAAGCTCTGGAGAATACATCTGAGG</td>
<td>Hind III</td>
<td>Amplify the plcB-KO2 3’ sequence of plcB, for the construction of pAULA-plcB</td>
</tr>
<tr>
<td>GFP&lt;sub&gt;uv&lt;/sub&gt;-F</td>
<td>ATCTTAAGATCTAAGTAGCTGATTAAATGGGAGGGAATA</td>
<td>Bgl II</td>
<td>Amplify the GFP&lt;sub&gt;uv&lt;/sub&gt; coding sequence, contains the stop codon cassette and the rpoB S-D region</td>
</tr>
<tr>
<td>GFP&lt;sub&gt;uv&lt;/sub&gt;-R</td>
<td>CATGAAAGATCTTTATTTTGTAGCTGC</td>
<td>Bgl II</td>
<td>Amplify the GFP&lt;sub&gt;uv&lt;/sub&gt; coding sequence</td>
</tr>
<tr>
<td>lipAcomp-F</td>
<td>CAGTCAGGATCCTAATTCAGA</td>
<td>BamH I</td>
<td>Amplify lipB coding sequence</td>
</tr>
<tr>
<td><strong>lipAcomp-R</strong></td>
<td>AGCCAAAAACCCATTC</td>
<td>with promoter region, for the construction of pUNK1-LipA</td>
<td></td>
</tr>
<tr>
<td><strong>lipBcomp-F</strong></td>
<td>CATTCACTGAGGGTTCTTCAGATGAGTGAATTCTTCTGAGCCTTAG</td>
<td>Amplify lipB coding sequence with promoter region, for the construction of pUNK1-LipB</td>
<td></td>
</tr>
<tr>
<td><strong>lipBcomp-R</strong></td>
<td>CTTACGCTAGATCTGAAGAACTCTAGGTTCGCTAGGAGCTTAG</td>
<td>Amplify lipA coding sequence with promoter region, for the construction of pUNK1-LipB</td>
<td></td>
</tr>
<tr>
<td><strong>lipAx-F</strong></td>
<td>GTGGCGCAAAGGCTCAAGCAG</td>
<td>- Screen for the lipA gene</td>
<td></td>
</tr>
<tr>
<td><strong>lipAx-R</strong></td>
<td>GAAGAAAGTTTCAATGGGCAACG</td>
<td>- Screen for the lipA gene</td>
<td></td>
</tr>
<tr>
<td><strong>Hlyx-F</strong></td>
<td>GTTGATTAGTGGGTGATCC</td>
<td>BamH I Screen for the hly gene</td>
<td></td>
</tr>
<tr>
<td><strong>Hlyx-R</strong></td>
<td>GATTAAGTTCTAAGCTTGCC</td>
<td>- Screen for the hly gene</td>
<td></td>
</tr>
<tr>
<td><strong>EGFP-N-SEQ</strong></td>
<td>CGTCGCCGTCAGCTCGACCAG</td>
<td>- Sequencing for inserts in the pEGFP-N2 plasmid</td>
<td></td>
</tr>
<tr>
<td><strong>lipAexpr-F</strong></td>
<td>CATTCACTGAGGGTTCTTCAGATGAGTGAATTCTTCTGAGCCTTAG</td>
<td>Amplify lipA coding sequence, for the construction of pEGFP-LipA</td>
<td></td>
</tr>
<tr>
<td><strong>lipAexpr-R</strong></td>
<td>CAGTCAGGATGGCAATTTTGAATAGAATGCTGCAAAAGC</td>
<td>BamH I Amplify lipA coding sequence, for the construction of pEGFP-LipA</td>
<td></td>
</tr>
<tr>
<td><strong>lipBexpr-F</strong></td>
<td>CATGCTGAAATTTCAATGGGCAACG</td>
<td>EcoR I Amplify lipB coding sequence, for the construction of pEGFP-LipB</td>
<td></td>
</tr>
<tr>
<td><strong>lipBexpr-R</strong></td>
<td>TTATATGATGCGGTTCTTAAGCTATTTTGG</td>
<td>BamH I Amplify lipB coding sequence, for the construction of pEGFP-LipB</td>
<td></td>
</tr>
<tr>
<td><strong>In1A-F</strong></td>
<td>GTGAGAAAAAACGATATGTATG</td>
<td>- Screen for the inlA gene</td>
<td></td>
</tr>
<tr>
<td><strong>In1A-R</strong></td>
<td>ATAGTCTCCGCTGTAGTTTTCG</td>
<td>- Screen for the inlA gene</td>
<td></td>
</tr>
<tr>
<td><strong>T7</strong></td>
<td>GTAATACGACTCATAAGG</td>
<td>- Screen for pBluescript-rpoND-GFPuv/mCherry</td>
<td></td>
</tr>
<tr>
<td><strong>T3</strong></td>
<td>AATTAACCTCATAAAGG</td>
<td>- Screen for pBluescript-rpoND-GFPuv/mCherry</td>
<td></td>
</tr>
<tr>
<td><strong>rpoC-Screen-F</strong></td>
<td>TATTAAGAATTTTGAAGC</td>
<td>- Screen for the insertion of GFPuv or mCherry into the L. monocytogenes chromosome</td>
<td></td>
</tr>
<tr>
<td><strong>rpoC-Screen-R</strong></td>
<td>GATAATTGTGCTGCTTTTGC</td>
<td>- Screen for the insertion of GFPuv or mCherry into the L. monocytogenes chromosome</td>
<td></td>
</tr>
<tr>
<td>Reaction</td>
<td>Primers</td>
<td>Restriction Enzymes</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>---------</td>
<td>--------------------</td>
<td>-------------</td>
</tr>
<tr>
<td><strong>pLSV1-Eco</strong></td>
<td>GTTTTCAGCTACGACGT</td>
<td>-</td>
<td>Screen for inserts in pLSV1</td>
</tr>
<tr>
<td><strong>pLSV1-Bam</strong></td>
<td>AATAAGCTTGCTAGAGGT</td>
<td>-</td>
<td>Screen for inserts in pLSV1</td>
</tr>
<tr>
<td><strong>PrpoB-F</strong></td>
<td>ATCGAACCGGGTTGCTAGAAAATATTGACATAGG</td>
<td>Sma I Xma CL</td>
<td>Amplify the <em>rpoB</em> promoter region</td>
</tr>
<tr>
<td><strong>PrpoB-R</strong></td>
<td>ATCGAACCGGGTTGCTAGAAAATATTGACATAGG</td>
<td>Sma I Xma CL</td>
<td>Amplify the <em>rpoB</em> promoter region</td>
</tr>
<tr>
<td><strong>LipA-SDM-F</strong></td>
<td>CTGTTCCTTCAGGTAGGCTGGAAAAGAC</td>
<td>-</td>
<td>Site-directed mutagenesis in pEGFP-LipA</td>
</tr>
<tr>
<td><strong>LipA-SDM-R</strong></td>
<td>CTGTTCCTTCAGGTAGGCTGGAAAAGAC</td>
<td>-</td>
<td>Site-directed mutagenesis in pEGFP-LipA</td>
</tr>
<tr>
<td><strong>LipB-SDM-F</strong></td>
<td>CCGGTGTTTTTCCAGCAGTTGAGAAAAGAC</td>
<td>-</td>
<td>Site-directed mutagenesis in pEGFP-LipB</td>
</tr>
<tr>
<td><strong>LipB-SDM-R</strong></td>
<td>CCGGTGTTTTTCCAGCAGTTGAGAAAAGAC</td>
<td>-</td>
<td>Site-directed mutagenesis in pEGFP-LipB</td>
</tr>
<tr>
<td><strong>myc-LipA-F</strong></td>
<td>CTAGCTGGATCCATGAAAAATTGGTAAAGTACAGG</td>
<td>BamH I</td>
<td>Amplify the coding sequence of LipA, for the construction of pcDNA3.1(+)‐myc-LipA</td>
</tr>
<tr>
<td><strong>myc-LipA-R</strong></td>
<td>CTAGCTGGATCCATGAAAAATTGGTAAAGTACAGG</td>
<td>BamH I</td>
<td>Amplify the coding sequence of LipA, for the construction of pcDNA3.1(+)‐myc-LipA</td>
</tr>
<tr>
<td><strong>myc-LipB-F</strong></td>
<td>CTAGCTGGATCCATGAAAAATTGGTAAAGTACAGG</td>
<td>BamH I</td>
<td>Amplify the coding sequence of LipB, for the construction of pcDNA3.1(+)‐myc-LipB</td>
</tr>
<tr>
<td><strong>myc-LipB-R</strong></td>
<td>CTAGCTGGATCCATGAAAAATTGGTAAAGTACAGG</td>
<td>BamH I</td>
<td>Amplify the coding sequence of LipB, for the construction of pcDNA3.1(+)‐myc-LipB</td>
</tr>
<tr>
<td><strong>lipA-KO-1F</strong></td>
<td>GACGGATCCCCCTGATTGAAAAAGCGCAAACCG</td>
<td>BamH I</td>
<td>Amplify the lipA-KO1 5’ sequence of <em>lipA</em>, for the construction of pLSV1-<em>lipA</em></td>
</tr>
<tr>
<td><strong>lipA-KO-1R</strong></td>
<td>GACGGATCCCCCTGATTGAAAAAGCGCAAACCG</td>
<td>BamH I</td>
<td>Amplify the lipA-KO1 3’ sequence of <em>lipA</em>, for the construction of pLSV1-<em>lipA</em></td>
</tr>
<tr>
<td><strong>lipA-KO-2F</strong></td>
<td>GACGGATCCCCCTGATTGAAAAAGCGCAAACCG</td>
<td>BamH I</td>
<td>Amplify the lipA-KO2 5’ sequence of <em>lipA</em>, for the construction of pLSV1-<em>lipA</em></td>
</tr>
<tr>
<td><strong>lipA-KO-2R</strong></td>
<td>GACGGATCCCCCTGATTGAAAAAGCGCAAACCG</td>
<td>BamH I</td>
<td>Amplify the lipA-KO2 3’ sequence of <em>lipA</em>, for the construction of pLSV1-<em>lipA</em></td>
</tr>
</tbody>
</table>
| **hly-KO-1F** | CAGTGGATCCAGTTAATCGTCTCTAATACAC | BamH I | Amplify the hly-KO1 5’ sequence of *hly*, for the
Table 2.3 List of primers used in this study. Underlines indicate the restriction enzyme digestion sites in the primers.

2.3.8 Colony PCR

Colony PCR was performed as standard PCR apart from using a single colony of *E. coli* or *L. monocytogenes* as the source of template instead of DNA fragments. Meanwhile, this colony was retrieved by streaking onto another plate. For colony PCR, the reaction needs an additional step of 5 min at 95 °C for one cycle at the beginning of the cycles to lyse the cells.

2.3.9 Quantification of DNA

The concentration of DNA was measured using the ND-1000 apparatus (NanoDrop).

2.3.10 DNA sequencing

Automated sequencing reactions were carried out using the BigDye version 1.1 terminator sequencing kit for cycle sequencing (PE Biosystems) according to the manufacturer’s instructions. For per 20 μl sequencing reaction, 300 ng of template DNA as mixed with 3.2 μl 1 pmol/μl custom forward or reverse
primer, 3 μl 5X Sequencing Buffer (PE Biosystems) and 2 μl Terminator Mix (PE Biosystems) and ddH2O. To precipitate DNA after sequencing reaction, in each PCR tube, 5 μl of 125 mmol EDTA and 60 μl 100% ethanol were added followed by 15 min incubation at RT. After 30 min of centrifugation at 13000 rpm in bench-top centrifuge (Eppendorf), the supernatant was carefully pipetted out, followed by addition of 60 μl 70% (v/v) ethanol and another 15 min centrifugation at 13000 rpm. Supernatant was removed again and the samples were left air dry at RT for 10 min. The nucleotide sequence of dried samples were analysed with ABI Prism 3100 Genetic Analyser. Nucleotides were aligned using the ClustalW website (http://www.ebi.ac.uk/Tools/clustalw/).

2.3.11 Cloning with the pGEM-T Easy vector system

DNA was cloned using the pGEM-T Easy vector system (Promega) according to the manufacturer’s instructions. Per reaction, 1 μl Taq polymerase amplified PCR product (~25 ng) was ligated with 1 μl 50ng/μl pGEM-T Easy vector using 1 μl T4 ligase (5 units) in a total volume of 10 μl, and the mixture was incubated at RT for 1 hr. Ligated plasmids were transformed into DH5α on LB/Amp/IPTG/X-gal plates (100 μg/ml Amp, 0.1 mM IPTG and 40 μg/mL X-gal). Successful clones were screened with M13-F/R primers by colony PCR from the white colonies.

2.4 Transformation of DNA into bacteria

2.4.1 Preparation of chemically competent E.coli

A fresh 2 ml of E. coli DH5α overnight culture of was diluted into 200 ml LB medium containing 30 mM MgCl₂ and grown to mid-log phase (OD₆₀₀=0.4-0.6). Cells were pelleted by centrifuging at 3660 ×g, 4 °C for 15 min and resuspended gently in 60 ml sterile solution A (10 mM MgCl₂, 50 mM CaCl₂ with10 mM MES). After 20 min incubation on ice, cells were
pelleted again for 5 min at 3660 xg, 4 °C and resuspended with 12 ml ice-old solution A with 50% (v/v) glycerol as a cryoprotectant. Cells were aliquoted into 200 μl in each eppendorf tube, frozen in liquid nitrogen and stored at -80 °C until needed. Frozen competent cells were thawed gently on ice prior to transformation.

2.4.2 Preparation of electrocompetent *L. monocytogenes*

A fresh 5 ml overnight culture of *L. monocytogenes* was diluted into 100 ml 20% (w/v) sucrose in TSB and grown at 37 °C for 3-4 hr till OD_{600}=0.2. One hundred microlitre 20 mg/ml Penicillin G was added and growth was allowed for another 1.5-2 hr. The cells were pelleted by 15 min centrifugation at 3660 xg, 4°C for 15 min, washed once with 50ml ice-cold Hepes-sucrose solution (1 mM Hepes, 0.5 M sucrose, pH 7.0) and twice with 25 ml Hepes-sucrose solution. Pelleted cells were finally resuspended in 500 μl Hepes-Sucrose solution. Cells were stored at 4 °C for less than one week until needed. (Park and Steward, 1990)

2.4.3 Transformation of plasmid DNA into chemically competent *E. coli*

A 50-100 μl volume of *E. coli* competent cells was mixed with no less than 100 ng plasmid and incubated on ice for 30 min. Cells were heat shocked at 42 °C for 45 sec and incubated on ice for 5 min, followed by the addition of 800 μl LB medium. Cells were incubated at 37 °C for 45 min and plated out on LB agar plates with appropriate antibiotics.

2.4.4 Transformation of plasmid DNA into electrocompetent *L. monocytogenes*

In ice-cold electroporation cuvettes (BioRad Laboratories), 100 μl *L. monocytogenes* competent cells were mixed with 1 μg plasmid DNA. Cells were electroporated using Gene Pulser apparatus (BioRad Laboratories) at field strength of 2.5 kV/cm, 200 Ω and 25 μF with a time constant of
approximately 3.47 ms. Fresh 1 ml of TSB was immediately added into the
cuvette and the cells were incubated for 3 hr at 30 °C without shaking before
plated out on TSB agar plates with appropriate antibiotics.

2.5 Site-directed mutagenesis

A pair of complemented mutagenic primers was designed containing the
desired substitution of nucleotides flanked by 15 bp unmodified sequence
on each side of the mutation site. Per 50 μl of reaction contains 5 μl 10X Pfu
Ultra buffer (Stratagene), 2 μl of 25 ng/μl template plasmid DNA, 12.5 μl of
10 ng/μl each primer, 2.5 μl 2.5 mM dNTPs, 1 μl Strategy Pfu Ultra
polymerase (Stratagene) and 14.5 μl ddH₂O. PCR was carried out under the
following cycles: 95 °C for 30 sec for 1 cycle, 30 cycles of 95 °C for 30 sec,
55 °C for 1 min and 68 °C for 7 min. The PCR product was digested with Dpn I
before transforming into E. coli DH5α and plated out on LB plate with
appropriate antibiotics. Successful mutated plasmids was purified from
cultured single colonies and verified by sequencing. A schematic picture of
this procedure is shown in Fig. 2.1

Figure 2.1 Schematic picture of site-directed mutagenesis. (Loening, 2005).
2.6 Mutagenesis in *L. monocytogenes* via induced homologous recombination

A plasmid was generated by amplifying from *L. monocytogenes* EGDe chromosomal DNA two 600-650 bp fragments flanking either end of the target gene, including the first and last six codons of the coding sequence. The resultant PCR products were digested with restriction enzymes, sites for which included in the primer sequences, and inserted into either pLSV1 or pAULA plasmid at the multiple cloning site (MCS). Plasmids pLSV1 and pAULA are *L. monocytogenes*-E.coli shuttle vectors carrying the temperature-sensitive gram-positive replication origin and Erm resistance. (Fig. 2.2 a)

As shown in Fig. 2.2 b, *L. monocytogenes* transformed with this plasmid were grown at 30 °C on TSB plates containing 5 μg/ml Erm. Single colonies were re-streaked onto TSB/Erm plates and incubated at 42 °C, which selects for colonies with the plasmid integrated into the chromosome through homologous recombination. Selected single colonies were inoculated in 100 ml TSB and incubated at 37 °C with shaking at 200 rpm. After reaching stationary stage, 1 ml culture was transferred into a new flask and the subculture repeated. This serial subculturing was repeated 6 times without Erm to remove the plasmid from cells and to allow time for the second recombination to take place. Finally, the culture media was diluted to $10^{-5}$ to $10^{-7}$ and plated out on TSB plates. Individual colonies were replica streaked onto TSB and TSB/Erm plates at 42 °C to select for non-Erm resistant strains which had lost the plasmid. Successful mutants were screened by colony PCR using screening primers binding outside the recombination region (Fig. 2.2 a).
Figure 2.2 a) Schematic picture of the generation of a knock-out plasmid. Two 600-650 bp sequences flanking the target gene amplified with KO1-F/R and KO2-F/R primers are cloned into pAULA or pLSV1 plasmid at their MCS. ori.: replication origin.

b) Schematic picture of mutagenesis by homologous recombination. ① and ② indicate the two possible outcomes of the second recombination.
2.7 Mammalian cell culture

2.7.1 Mammalian cell lines and culture conditions

Human cervical cancer cell line Hela M was routinely cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma) supplemented with 10% Foetal Bovine Serum (FBS, Invitrogen) and 2mM L-glutamine (Invitrogen) and grown at 37 °C with 5% CO₂. Cells were passaged when reached 70 to 80 % confluency. Sub-confluent cultures were washed with Dulbeccos’ Phosphate Buffered Saline (PBSD, Sigma) and trypsinised with 0.05% (w/v) trypsin/EDTA (1x) (Invitrogen) for 2-5 min until cells were detached. An equal amount of DMEM complete medium was added to quench the trypsin and the cell suspension was pipetted up and down gently to dissociate the cell clumps. An appropriate volume of cell suspension (1/10 to 1/20) was then transferred into new flasks or plates with addition of fresh media.

Human enterocyte-like cell line Caco-2 was routinely cultured in Minimum Essential Medium (MEM) supplemented with 10% FBS, 2mM L-glutamine and 1mM Non-essential Amino Acids (NEAA, Invitrogen) and grown with 5% CO₂ at 37 °C. Sub-confluent cultures of 60-70% confluency were washed with PBSD and trypsinised with 0.25% (w/v) trypsin/EDTA (1x) (Invitrogen) for 2-5 mins till the majority of the cells rounded up. An equal amount of complete medium was added to quench the trypsin and the flask was tapped by hands to help lifting up the cells. The cell suspension was transferred to a falcon tube and passed through a 0.8 mm syringe needle to break up cell clumps. An appropriate volume of cell suspension (1/3 to 1/5) was then transferred into new flasks or plates with addition of fresh medium.

Mouse macrophage cell line J774.A1 was routinely cultured in RPMI medium (Invitrogen) supplemented with 10% FBS and 2mM L-glutamine and grown under 5% CO₂ at 37 °C. Sub-confluent cultures of 70-80% confluency were washed with PBSD and scraped off in a small volume of complete medium.
An appropriate volume of cell suspension (1/3 to 1/5) was then transferred into new flasks or plates with addition of fresh medium.

Where visualisation of mammalian cells was required, sterile 1 cm diameter coverslips were placed at the bottom of the culture dish or 6-well plate before adding the cell suspension on top.

### 2.7.2 Freeze down and revival of mammalian cells

Mammalian cells to be preserved were grown in large quantity in T225 flasks to sub-confluency. Freezing agent was prepared by mixing 80% FBS with 20% DMSO (v/v), which was sterilised by passing through a 0.22 μm syringe filter, and kept on ice. Cells were trypsiniised as usual, quenched with sufficient ice-cold serum-free medium, and pelleted by centrifuging at 500 xg, 4 °C for 3 min. Cells were resuspended in a small volume of ice-cold FBS, before adding drop-wise the same volume of prepared FBS/DMSO to obtain a final concentration of 10% DMSO in FBS (v/v). Cell suspension was alliqoted to 1.5 ml and transferred to each ice-cold 2 ml cryovial and was slow frozen in a styrofoam box with isopropanol at -80 °C overnight. Frozen samples were transferred into liquid N₂ the next day for long-term storage.

To recover frozen stock of mammalian cells, the cryovial was quickly defrosted in 37 °C water bath till only a tiny piece of ice remained. Cells were transferred drop-wise into a flask containing warm complete medium while shaking gently to mix. Cells were washed with PBSD and fresh medium was added the next day in order to remove residual DMSO.

### 2.7.3 Transient transfection of plasmid DNA into Hela M cells

DNA was transfected into Hela M cells using Fugene 6 Transfection Reagent (Roche). For transfecting each well of the 6-well plate, 3 μl Fugene 6 transfection reagent was diluted with 97 μl serum-free DMEM medium in an
eppeendorf tube, which was flicked 10 times to mix before incubating at RT for 5 min. 1 μg of plasmid DNA was then added into the mixture, mixed by flicking and incubated at RT for 30 min. The mixture was added dropwise onto Hela M cell monolayer or cell suspensions (e.g. after passaging and before the cells adhere to the plate) while the plate was shaken gently to mix. Proteins produced from the plasmid were detectable in Hela M cells 24 hr post-transfection.

2.8 Intracellular survival assay of *L. monocytogenes*

Different strains of *L. monocytogenes* for infection were grown in TSB at 37 °C, 200 rpm to mid-log phase (OD_{600}=0.5), washed twice with phosphate buffered saline (PBS) and resuspended in PBS with 15% (v/v) glycerol as a cryoprotectant. Aliquots of this suspension were frozen in liquid nitrogen and stored at -80 °C. The number of bacteria per ml was determined by viable account using Miles and Misra method (Miles *et al.*, 1938).

Mammalian cell lines were seeded at 1.5×10^5 cells per well 24 hr before infection (48 hr for Caco-2 or J774.A1 cell) in 6-well tissue culture plates (Greiner) to obtain the final density of 5×10^5 cells per well on the day of infection. Prior to infection, the cells were washed twice with PBSD. Frozen *L. monocytogenes* aliquots were thawed on ice and the mammalian cell monolayers were infected with desired multiplicity of infection (MOI), which is the number of bacteria per mammalian cell (e.g. MOI 10 means 10 bacteria per cell). An additional step of centrifugation at 1000 xg for 10 min was required for Hela M cell infection but not for Caco-2 or J774.A1 cells. Following 2 hr incubation (1hr for Caco-2 cell, 45 min for J774.A1 cell) at 37 °C to allow uptake of the bacteria, the cells were washed three times with PBSD and overlaid with serum-free medium containing 10-50 μg/ml gentamicin (Invitrogen) to kill any extracellular bacteria as well as prevent re-infection. The time when gentamicin was added to the cell was counted
as T<sub>0</sub>. Incubation was allowed at 37 °C until desired time-point. At each time point, medium was removed from the wells and cells were washed three times with PBS and lysed with 1 ml sterile ice-cold 0.5 % (v/v) Triton X-100 in PBS for 3 min. The lysates were collected for viable count and the concentration of bacteria in each well was recorded as colony forming unit (CFU) per ml. At each time point, the concentration of each strain was the average of the counts from three wells.

### 2.9 Immunofluorescent staining and imaging

For visualisation of bacteria, 1 ml broth culture was pelleted by centrifuging at 13000 rpm for 5 min and resuspended in 50 µl PBS, which was left to air-dry on a glass slides and heat-fixed with flame. For visualising mammalian cells, cells were grown on coverslips placed at the bottom of culture dishes or plates. Bacteria on glass slides or mammalian cells on coverslips were fixed with 3% (w/v) paraformaldehyde (PFA) in PBS for 30 min at RT.

The PFA/PBS solution was prepared by dissolving 6 g PFA in 160 ml of PBS at 80 °C, followed by addition of 200 µl 0.1 M CaCl<sub>2</sub> and 200 µl 0.1 M MgCl<sub>2</sub> while the solution is warm, and adjusted to pH 7.4 with a final volume of 200 ml. Aliquots of the PFA/PBS solution was stored at -20 °C and can be kept at 4 °C for up to 1 week.

Fixed coverslips or slides were washed with PBS for 3 times with additional 10 mM glycine, pH 8.5 in the second wash to remove any trace of PFA. For labelling intracellular structures, samples were permeablised with 0.1% (v/v) Triton X-100 in PBS for 4 min at RT followed by 3 washes with PBS.

Specific primary and secondary antibodies were diluted depending on the requirement of each antibody in 0.5 mg/ml BSA in PBS right before labelling. Different primary antibodies or secondary antibodies could be mixed.
together providing no cross-reaction would occur. Samples were incubated
with primary antibodies at RT for 20 min, washed 3 times with PBS, followed
by incubation with secondary antibodies for 20 min and 3 times wash with
PBS. The slides were then mounted in mounting agent Mowiol-488 (Sigma)
which contained 10% (w/v) Mowiol-488, 25% (v/v) Glycerol in 0.1M Tris, pH
8.5.

For labelling DNA, 200 ng/ml DNA dye Hoechst 33342 (Sigma) or 1/100 DAPI
(Sigma) was included in the secondary staining. For actin staining, 1/300
FITC-phalloidin (Sigma) or 1/300 TRITC-phalloidin (Fluka) was included in the
secondary staining. *L. monocytogenes* was stained with 1/500 Rabbit-αListeria
(Abcam) and 1/200 Sheep-αRabbit-TexasRed (Abcam). Golgi
complex were stained with 1/500 Rabbit-αGolgin97 (Nobuhiro Nakamura,
Kyoto Sangyo University, Japan) or 1/100 Sheep-αGrasp65 (Martin Lowe,
University of Manchester) primary antibody and 1/200 Sheep-αRabbit-
TexasRed (Abcam) or 1/800 Donkey-αSheep-Alexa594 (Molecular Probes)
secondary antibody. Myc-tagged proteins were stained with 1/100
Mouse-αmyc primary antibody and 1/200 Donkey-αMouse-Alexa488
(Molecular Probes) secondary antibody. Lamellipodin was stained with 1/100
Rabbit-αLpd (Frank B. Gertler, The Koch Institute MIT, US) and 1/500
Sheep-αRabbit-Cy5 (Abcam).

Stained samples were visualised using wide-field fluorescent microscope.
Images were collected on an Olympus BX51 upright microscope using a
10x/0.30 Plan FLN objective and captured using a CoolSnap ES camera
(Photometrics). Specific band-pass filter sets for DAPI, FITC, TexasRed, Cy3
and Cy5 were used to prevent bleed-through from one channel to the next.
Images were then processed and analysed using ImageJ
2.10 Plaque assay

Following routine passage Hela M cells were seeded at 5 X 10^5 cells per well into 6-well plates 24-48 hr before infection to ensure 100% confluency with no visible gaps among the cells. Cell monolayers were infected with *L. monocytogenes* with MOI 0.05 as described before. Cells were washed with PBS and all liquid was removed from the wells 2 hr post-infection. Cells were overlaid with 2 ml sterile 37 °C melted 1% (w/v) SeaPlaque Agarose (Lonza) in DMEM (prepared by mixing melted 1% (w/v) SeaPlaque Agarose with the same volume of 2X DMEM, and then sterilised by passing through the 0.22 μm syringed filter) containing 10 μg/ml gentamicin. After the agarose became solid, another 1 ml of serum-free DMEM was added in each well to prevent the gel from drying. Visible plaques started to develop after 3 days. (Oaks *et al.*, 1985)

2.11 Quantification of fluorescence expression

The level of bacteria cells expressing fluorescence could be quantified using KC4 SynergyHT-1 reader (BIO-TEK) with software KC4 Kineticale for Windows, version #3.4. Briefly, a 5 ml bacteria overnight culture was centrifuged at 3660 xg at 4 °C, was washed twice with PBS and resuspended in 500 μl PBS. The samples were applied to the wells of 96-well plate in 100 μl volume and read under desired wavelength in triplicate.

2.12 Calculation of mean doubling time of *L. monocytogenes*

The mean doubling time of *L. monocytogenes* from t1 to t2 was calculated by the following formula:

$$Mean\ doubling\ time = \frac{\log 2}{\log \frac{Q2}{Q1}} \times (t2 - t1)$$

Q1 and Q2 represent the number of bacteria at t1 and t2 respectively.
Chapter 3 *L. monocytogenes* EGDe::ΔlipA and EGDe::ΔlipB mutants are attenuated in intracellular growth in Hela M cells *in vitro*

3.1 Introduction

*L. monocytogenes* produces two protein and inositol phosphatases LipA and LipB that may play important roles in the survival of the bacteria in host cells. However, the deletion of either or both genes did not seem to affect the growth of *L. monocytogenes* in TSB media (Bennett and Roberts, unpublished data). In order to find out whether the deletion of *lipA* or *lipB* introduces any phenotype during intracellular growth *in vitro*, knock out mutations ΔlipA, ΔlipB and Δhly were generated in strain EGDe (Bennett and Roberts, unpublished data), which would be used in intracellular survival assay in the human epithelial cell line Hela M cells.

3.2 Determination of the optimal conditions of intracellular survival assay in Hela M cells

In the literature, the infection of Hela M cells with *L. monocytogenes* was normally carried out with MOI ranged from 1 to 100, and the intracellular growth of the bacteria was followed for less than 8 hr (Francis and Thomas, 1996; Temoin *et al.*, 2008; Burkholder *et al.*, 2011). We were interested to determine the intracellular growth curve of *L. monocytogenes* over longer time periods. As such, it was necessary to determine the optimal MOI in the intracellular survival assay, where the bacteria load was large enough to be counted while not too large that all of the infected Hela M cells died prematurely. Therefore, MOI of 1, 10, 50 and 100 were used in Hela M cell infection with *L. monocytogenes* wild type strain EGDe. Results of the intracellular growth curves are showed in Fig. 3.1 and 3.2.
Figure 3.1 Intracellular survival assay of *L. monocytogenes* EGDe in Hela M cells at MOI 50 and 100. Gentamicin was added to a final 50 μg/ml concentration at T₀ and reduced to 10 μg/ml at T₅. The number of intracellular bacteria was shown as CFU/ml at each time point. Each data point was the average of two independent experiments. Error bars showed the standard error for each data.

Figure 3.2 Intracellular survival assay of *L. monocytogenes* EGDe in Hela M cells at MOI 1 and 10. Gentamicin was added to a final 50 μg/ml concentration at T₀ and reduced to 10 μg/ml at T₅. The number of intracellular bacteria was shown as CFU/ml at each time point.
As shown in Figure 3.1 and 3.2, at T₀ there was no gentamicin in the medium, therefore the T₀ bacteria count reflected the total number of cell-associated bacteria. After gentamicin was added, extracellular bacteria were killed within an hour which accounted for the sharp decrease in bacteria count at T₁. The difference between T₀ and T₁ reflected that less than 1% of *L. monocytogenes* internalised into Hela M cells within the 2 hr incubation time.

The number of intracellular bacteria increased rapidly until T₈, when replication rate slowed down, and reached stationary phase by T₂₄. From T₂₄ to T₄₈, there was a slight decrease in bacteria count, most likely due to the increased number of ruptured host cells caused by the over-loaded intracellular bacteria.

For MOI 1, 10 and 50, the number of cell-associated bacteria (T₀) and internalised bacteria (T₁) increased proportionally to the number of bacteria used for infection, while their growth rates remained roughly the same up to T₂₄. However, over MOI 50, the increase of MOI no longer led to increased number of internalised *L. monocytogenes*. This indicated that at MOI 50 the number of bacteria attached to the Hela M cell surface were at saturation during the 2 hr incubation period. Furthermore, after T₁ the bacteria infected at MOI 100 replicated at first at the same rate with those at MOI 50, then its growth became slower after T₈ and declined to a lower number than MOI 50 from T₂₄ to T₄₈. When checked under the light microscope, very few Hela M cell ruptured at T₈ even with MOI 100. By T₂₄, there were notably large numbers of bacteria inside the Hela M cells and in the culture media, which were assumed to be killed by gentamicin. The number of ruptured Hela M cells increased with the MOI, with massive cell death observed for MOI 100 at T₂₄ and T₄₈.
Therefore, exceeding MOI 50 in the intracellular survival assay in Hela M cells not only failed to increase the number of bacteria internalised into the host cell, but also placed extra burden on host cells which led to early cell death.

In addition, with MOI 1 the bacteria counts were very low at early time points, leading to bigger experimental errors especially during the viable count. Altogether, MOI of 10 to 50 \( L.\) monocytogenes per Hela M cell was chosen for the intracellular survival assay in this study.

### 3.3 \( L.\) monocytogenes EGDe, EGDe::\( \Delta \)lipA, EGDe::\( \Delta \)lipB mutants were attenuated in intracellular growth in Hela M cells

After establishing the intracellular growth curve of EGDe in Hela M cells, EGDe::\( \Delta \)lipA, EGDe::\( \Delta \)lipB and EGDe::\( \Delta \)hly mutants (kindly provided by Hayley Bennett) were studied in the intracellular survival assay to determine whether mutations in either \( \text{lipA} \) or \( \text{lipB} \) affect the intracellular growth of \( L.\) monocytogenes in Hela M cells. Strain EGDe was used as a positive control, EGDe::\( \Delta \)hly was used as negative control since it was defective in intracellular growth (Fig. 3.3).

The results showed that at T\(_0\) there were similar numbers of cell-associated bacteria for all four strains. By T\(_1\) there were the same numbers of intracellular EGDe, EGDe::\( \Delta \)lipA and EGDe::\( \Delta \)lipB but significantly more EGDe::\( \Delta \)hly (\( P=1.08 \times 10^{-5}, n=4 \)), suggesting no defect in invasion for both EGDe::\( \Delta \)lipA and EGDe::\( \Delta \)lipB, but increased invasion efficiency for EGDe::\( \Delta \)hly. All three mutants multiplied at a lower rate than EGDe (mean doubling time between T\(_1\) and T\(_8\): EGDe 58 min, EGDe::\( \Delta \)lipA 77 min, EGDe::\( \Delta \)lipB 80 min, EGDe::\( \Delta \)hly 215 min) and there were significantly less mutants than the EGDe by the end of the experiment (\( P=0.0366 \) for EGDe::\( \Delta \)lipA, \( P=0.0409 \) for EGDe::\( \Delta \)lipB, \( P=0.0102 \) for EGDe::\( \Delta \)hly at T\(_{24}\), n=4),

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indicating impaired intracellular growth for EGDe::ΔlipA, EGDe::ΔlipB and EGDe::Δhly mutants in Hela M cells.

Figure 3.3 Intracellular survival assay on Hela M cells with *L. monocytogenes* EGDe, EGDe::ΔlipA, EGDe::ΔlipB and EGDe::Δhly at MOI of 50. Gentamicin was added to a final 50 μg/ml concentration at T₀ and reduced to 10 μg/ml at T₅. At the indicated time points the number of intracellular bacteria was shown as CFU/ml. Each data point represents the average of four independent experiments. Error bars showed the standard error for each data.

3.4 Discussion

Previous research reported that a ΔlipA mutation had no detectable effect on *in vitro* intracellular growth in BHI (brain-heart infusion) media, in J774.A1 macrophage-like cell line, CMT-93 rectal epithelial cell line or L2 fibroblast cell line (Kastner *et al.*, 2011). Likewise no difference was observed in the uptake of the wild type or ΔlipA mutant into bone marrow-derived macrophages or CMT-93 cells, or in phagosomal escape in RAW 264.7 cells (Kastner *et al.*, 2011).
In this study, both EGDe::ΔlipA and EGDe::ΔlipB strains had the same growth rate in TSB media as the wild type strain EGDe with the doubling time of approximately 40 min during exponential phase (data not shown), which matches the published data (Kastner et al., 2011). However, although the uptake into human epithelial cell line Hela M cells was unaffected for both ΔlipA and ΔlipB mutants, there was a significant deficiency in intracellular growth for EGDe::ΔlipA and EGDe::ΔlipB strains. This is the first time that a phenotype was observed for ΔlipA and ΔlipB mutants in intracellular growth in vitro. The inconsistency of this discovery with previous observation might indicate that the phosphatases LipA and LipB play different roles among various host cell types in vitro. Significantly in the work of Kastner et al. (2011) the epithelial cell line used was a rectal cell line, which will be different to the Hela M cells that are cervical cancer cells. This difference in cell lines could well explain the differences between these two data sets.

The EGDe::Δhly mutant used to be used as a negative control in L. monocytogenes intracellular survival assays in Caco-2 cells, because it is unable to produce LLO and is compromised in its ability to escape from the phagosome (Gaillard et al., 1987). However, in the infection of Hela M cells, although EGDe::Δhly showed reduced intracellular growth, it was not attenuated as significantly as reported in certain murine derived cell lines (Portnoy et al., 1988). This was due to the fact that in Hela M cells the PC-PLC could mediate the lysis of primary and secondary phagosome membrane in the absence of LLO (Grundling et al., 2003). However, besides the pore-forming function, LLO probably also participates in other stages of infection, such as in activation of several intracellular signalling pathways, e.g. NF-κB, MAPK and protein kinase C pathways and Ca²⁺-dependent signalling (Hamon et al., 2006; Kayal and Charbit, 2006). Therefore, although the EGDe::Δhly mutant is able to escape from the phagosome, it is impaired in intracellular growth due to the absence of LLO, which matches our result that EGDe::Δhly was attenuated but was not completely defective in
intracellular growth. The EGDe::Δhly strain possibly contains unexpected mutations, which accounts for its increased internalisation rate, as this has not been reported previously.

In summary, we have established the optimal condition of MOI 10 to 50 used in the intracellular survival assay in Hela M cells infected with *L. monocytogenes*. The two EGDe::ΔlipA and EGDe::ΔlipB mutants showed significantly reduced intracellular growth in Hela M cells, indicating the important roles both phosphatases played during infection. Negative control strain EGDe::Δhly was not as attenuated as expected and could contain unexpected mutations. Therefore it would not be used in further study.
Chapter 4 *L. monocytogenes* InlA::ΔlipA and InlA::ΔlipB mutants are attenuated in intracellular growth *in vitro*

4.1 Introduction

The results from the last chapter showed that EGDe::ΔlipA and EGDe::ΔlipB mutants were attenuated in intracellular growth in Hela M cells. One of our next questions was whether the same defect could be observed *in vivo*, which would be performed in murine model. In the rest of my study, the EGDe::InlAm strain (hereafter refered to as the InlA strain) was used as wild type strain and all the mutations described in the following chapters were constructed in the InlA strain background so that these mutated strains could be used in animal studies. In this chapter, the ability of lipA and lipB deletion mutants to grow inside different cell types was studied, in order to characterise the roles of the two PIP phosphatases LipA and LipB in the intracellular growth of *L. monocytogenes*.

4.2 InlA strain was unaffected in intracellular growth in Hela M cells

The InlA strain and EGDe strain were used to infect Hela M cells in parallel to confirm that the mutation in *inlA* does not affect the intracellular growth of *L. monocytogenes* (Fig. 4.1). The result showed that the intracellular growth rate of EGDe and InlA in Hela M cells were similar (mean doubling time between T₁ and T₈: EGDe 94 min, InlA 103 min) and with the same number of detectable bacteria by T₂₄, indicating the mutation in the *inlA* gene does not affect the intracellular replication of the wild type strain EGDe *in vitro*. 
4.3 Generation of deletion mutations in the InlA strain

Mutation strains of InlA::ΔlipA, InlA::ΔlipB, InlA::ΔlipAΔlipB, InlA::ΔplcB, InlA::Δhly and InlA::ΔhlyΔplcB were generated with plasmid pLSV1-lipA, pAULA-lipB, pAULA-plcB and pLSV1-hly via induced homologous recombination.

The principle for this mutagenesis is first to construct a plasmid containing a cloned deletion derivative of the gene to be inactivated together with sequences upstream (5') and downstream (3') to the gene in either of the temperature sensitive suicidal vectors pLSV1 and pAULA. These plasmids only replicate below 34 °C such that when *L. monocytogenes* containing either of these plasmids are incubated at 42 °C in the presence of antibiotic selection, homologous recombination occurs between the plasmid and the chromosome, leading to the integration of the entire plasmid onto the chromosome.

Figure 4.1 Intracellular survival assay on Hela M cells with *L. monocytogenes* EGDe and InlA at MOI 50. Gentamicin was added to a final 50 μg/ml concentration at T₀ and reduced to 10 μg/ml at T₅. At the indicated time points the number of intracellular bacteria was shown as CFU/ml.
chromosome. By subsequently serially sub-culturing the stain at 30 °C in the absence of selection, the plasmid is re-excised by homologous recombination and lost, leaving either the original wild type or mutated gene on the chromosome (Pilgrim et al., 2003). Successful mutagenesis was established by colony PCR. Each mutated strain only contains the translational start, stop, and fewer than 15 codons of the ORF in question. A schematic demonstration of this process is shown in Fig. 2.2.

Each of the four different mutations were generated by amplifying 600-650 bp of the immediate 5’ and 3’ regions of the target genes by PCR with L. monocytogenes chromosomal DNA as template. The cloned fragment sizes were shown in Table 4.3, and primers used are shown in Table 2.3. The amplified PCR products were digested with the appropriate restriction enzymes (Table 2.3) and simultaneously ligated into pLSV1 or pAULA vector respectively. Successful clones of pLSV1-lipA and pLSV1-hly plasmids were screened and confirmed by DNA sequencing using primers pLSV1-Bam and pLSV1-Eco (Table 2.3) which bind outside the cloning region on the pLSV1 (Bennett and Roberts, unpublished data). Successful clones of pAULA-lipB and pAULA-plcB plasmids were screened using primers M13-F and M13-R (Table 2.3) which bind outside the cloning region on pAULA and confirmed by DNA sequencing with the same primers (Fig. 4.2).

Plasmids pLSV1-lipA, pAULA-lipB, pAULA-plcB, pLSV1-hly were transformed into InlA strain and InlA::ΔlipA, InlA::ΔlipB, InlA::ΔplcB and InlA::Δhly mutants generated as described. The double mutant InlA::ΔlipAΔlipB was generated by deleting lipB form InlA::ΔlipA with pAULA-lipB plasmid; InlA::ΔhlyΔplcB was generated by deleting plcB from InlA::Δhly with pAULA-plcB plasmid using the same procedure.

Successfully generated deletion mutants InlA::ΔlipA, InlA::ΔlipB, InlA::ΔlipAΔlipB and InlA::Δhly were confirmed by colony PCR using
screening primers lipAx-F/R, lipBx-F/R, hlyx-F/R (Table 2.3) which bind to 700-800bp upstream and downstream of each ORF (Fig. 4.3 c, d, e, f). Mutants InlA::ΔplcB and InlA::ΔhlyΔplcB was confirmed by colony PCR using primers hlyx-F/R, plcB-KO1-F and plcB-KO2-R (Table 2.3, Fig. 4.3 g, h).

Figure 4.2 Confirmation of pAULA-\textit{lipB} and pAULA-\textit{plcB} by colony PCR. a) Colony PCR of \textit{E. coli} DH5α containing pAULA-\textit{lipB} with M13-F/R primers. Predicted size: 1.2 kb. d) Colony PCR of \textit{E. coli} DH5α containing pAULA-\textit{plcB} with M13-F/R primers. Predicted size: 1.2 kb.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Target Gene</th>
<th>5' and 3' Fragments</th>
<th>Fragment sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLSV1-\textit{lipA}</td>
<td>\textit{lipA}</td>
<td>lipA-KO1, lipA-KO2</td>
<td>604bp, 603bp</td>
</tr>
<tr>
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<td>\textit{lipB}</td>
<td>lipB-KO1, lipB-KO2</td>
<td>602bp, 587bp</td>
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<tr>
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<td>\textit{hly}</td>
<td>hly-KO1, hly-KO2</td>
<td>641bp, 642bp</td>
</tr>
<tr>
<td>pAULA-\textit{plcB}</td>
<td>\textit{plcB}</td>
<td>plcB-KO1, plcB-KO2</td>
<td>616bp, 610bp</td>
</tr>
</tbody>
</table>

Table 4.1 The 5' and 3' fragments of the target genes cloned for the construction of the plasmids used in mutagenesis. KO1 indicated the 5' sequence, KO2 indicated the 3' sequence of the target gene.
Figure 4.3 c), d), e), f), g), h) Confirmation of the deletion mutations by colony PCR.

c) Colony PCR of InlA::\textit{ΔlipA} and wild type InlA with lipAx-F/R primers. Predicted size: 1.7 kb for InlA::\textit{ΔlipA}, 2.5 kb for InlA.
d) Colony PCR of InlA::\textit{ΔlipB} and InlA with lipBx-F/R primers. Predicted size: 1.6 kb for InlA::\textit{ΔlipB}, 2.5 kb for InlA.
e) Colony PCR of InlA::\textit{ΔlipAΔlipB} with lipAx-F/R and lipBx-F/R primers. Predicted size: 1.7 kb for product using lipAx primers, 1.6 kb for product using lipBx primers.
f) Colony PCR of InlA::\textit{Δhly} and InlA with hlyx-F/R primers. Predicted size: 1.4 kb for InlA::\textit{Δhly}, 3 kb for InlA.
g) Colony PCR of InlA::\textit{ΔplcB} and InlA with plcB-KO1-F/plcB-KO2-R primers. Predicted size: 1.2 kb for InlA::\textit{ΔplcB}, 2.2 kb for InlA.
h) Colony PCR of InlA::\textit{ΔhlyΔplcB} and InlA with plcB-KO1-F/plcB-KO2-R primers. Predicted size: 1.2 kb for InlA::\textit{ΔhlyΔplcB}, 2.1 kb for InlA. DNA marker HyperLadder I (Bioline) was used as an indication of the sizes of bands.
4.4 Deletion of *lipA* caused attenuation in intracellular growth of *L. monocytogenes* in Caco-2 cells

4.4.1 InLA::ΔlipA was attenuated in intracellular growth in Caco-2 cells

The intracellular growth of *L. monocytogenes* InLA::ΔlipA mutant was examined in the human enterocyte cell line Caco-2 cells in comparison to InLA strain as well as negative control strain InLA::Δhly (Fig. 4.4).

The result showed that all three strains internalised into Caco-2 cells as efficiently. Following uptake, the InLA strain multiplied at exponential rate and reached stationary phase by T_{8.5} (mean doubling time of 54 min between T_{1.5} and T_{8.5}). The bacteria number then remained steady till T_{24}. The control strain InLA::Δhly multiplied at a slower rate (mean doubling time of 96 min between T_{1.5} and T_{8.5}) and had a nearly 10-fold decrease in recovery by T_{24}. The cell counts of InLA::Δhly was significantly lower than InLA at T_{8.5} (P=0.0024) and T_{24} (P=0.0020), indicating a severe defect in intracellular growth in Caco-2 cells. InLA::ΔlipA also showed to be significantly attenuated in growth comparing to the InLA at T_{8.5} (P=0.0059, mean doubling time of 73 min between T_{1.5} and T_{8.5}) and T=24 (P=0.0062), but not as much as InLA::Δhly.
4.4.2 Plasmid complementation restored the intracellular growth of InlA::ΔlipA in Caco-2 cells

To confirm that the growth defect for InlA::ΔlipA was due to the deletion of lipA gene, the full coding sequence of lipA with promoter region was re-introduced into InlA::ΔlipA on pUNK1 plasmid to test whether the intracellular growth of InlA::ΔlipA could be restored.

The complementation plasmid pUNK1-LipA was generated by first amplifying the entire coding sequence of lipA and its promoter region, 146 bp 5’ to the ATG start codon, with lipAcomp-F/R primers (Table 2.3). The 1110 bp PCR fragment was digested with BamH I and Pst I prior to ligation into similarly digested L. monocytogenes-E. coli shuttle vector pUNK1. Successful clones of pUNK1-LipA were screen using pUNK1-F and pUNK1-R primers (Table 2.3, Fig. 4.5) and confirmed by sequencing with the same primers.
Both pUNK1-LipA and pUNK1 plasmids were transformed into wild type strain and pUNK1 was also transformed into InlA::ΔlipA mutant. Prior to examining their intracellular growth in vitro, the stability of pUNK1 plasmid within L. monocytogenes without antibody selection was tested, since during intracellular growth there would be no Erm selection to maintain the plasmid. An overnight culture of InlA(pUNK1) in TSB with 5 μg/ml Erm was inoculated 1/100 into fresh TSB media without Erm and incubated at 37 °C with shaking at 200 rpm. At regular intervals the concentrations of Erm-resistant L. monocytogenes and total L. monocytogenes were counted by performing viable count on TSB plates with and without Erm (Fig. 4.6). The two almost identical growth curves showed that even without the selective pressure of Erm, the bacteria retained the pUNK1 plasmid for at least 24 hr, indicating that pUNK1 plasmid was very stable inside L. monocytogenes.

The InlA strain and InlA::ΔlipA mutant with either pUNK1 or pUNK1-LipA plasmid were then tested in intracellular survival assay in Caco-2 cells (Fig. 4.7). The result demonstrated that compared to the significantly attenuated InlA::ΔlipA(pUNK1) strain (P=0.047 at T24 against InlA(pUNK1)), the complemented InlA::ΔlipA(pUNK1-LipA) strain multiplied at the same rate as InlA (mean doubling time between T1 and T8: InlA(pUNK1) 55 min, InlA::ΔlipA(pUNK1) 77 min, InlA::ΔlipA(pUNK1-LipA) 55 min) and restored to 86.7% of the final yield for InlA(pUNK1) (P=0.722).

This result confirmed that the defect of the lipA deletion mutant in intracellular growth in vitro was caused by the deletion of lipA and the loss of LipA function.
Figure 4.5 Confirmation of pUNK1-LipA plasmid by colony PCR with pUNK1-F/R primers. Predicted size: ~300 bp for pUNK1, 1.5 kb for pUNK1-LipA. DNA marker HyperLadder I was used as an indicator for the size of the bands.

Figure 4.6 pUNK1 plasmid stability test. At each time point the viable count of *L. monocytogenes* calculated from TSB plates with (+) or without (-) Erm were shown as CFU/ml.
4.5 lipA and lipB deletion mutants were attenuated in intracellular growth in Hela M cells

In the previous chapter, EGDe::ΔlipA and EGDe::ΔlipB were shown to be attenuated in intracellular growth in Hela M cells, the same experiments were repeated with InlA::ΔlipA, InlA::ΔlipB and InlA::ΔlipAΔlipB mutant strains.

4.5.1 InlA::ΔplcBΔhly was defect in intracellular growth Hela M cells

The escape of L. monocytogenes from phagosomes in Hela M cells is partially mediated by PC-PLC, which is encoded by plcB. Therefore, deletion of both hly and plcB leads to a complete defect for L. monocytogenes in escaping...
from the phagosome in Hela M cells and prevent it from replicating in the cytosol.

The intracellular growth of InlA::ΔplcB and InlA::ΔhlyΔplcB was tested in Hela M cells (Fig. 4.8). Compared to InlA, both mutants showed a significant defect in internalisation ($P=0.041$ between InlA::ΔplcB and InlA, $P=0.032$ between InlA::ΔhlyΔplcB and InlA at $T_1$), and the number of InlA::ΔhlyΔplcB was significantly lower than InlA::ΔplcB at $T_1$ ($P=0.0063$). A severe attenuation in intracellular growth for both mutants was observed as well ($P=0.0064$ between InlA::ΔlipB and InlA, $P=0.0061$ between InlA::ΔhlyΔplcB and InlA at $T_{24}$). The levels of InlA::ΔhlyΔplcB remained the same as $T_1$ all through the experiment, indicating no overall increase in bacteria.

![Figure 4.8 Intracellular survival assay on Hela M cells with *L. monocytogenes* InlA, InlA::ΔplcB and InlA::ΔhlyΔplcB at MOI 10. Gentamicin was added to a final 10 μg/ml concentration at $T_0$. At the indicated time points the number of intracellular bacteria was shown as CFU/ml. Each data point represents the average of two independent experiments. Error bars showed the standard error for each data.](image)
4.5.2 *lipA* and *lipB* deletion mutants were attenuated in intracellular growth in Hela M cell.

The intracellular growth of InlA::Δ*lipA*, InlA::Δ*lipB* and InlA::Δ*lipAΔlipB* was studied in intracellular survival assay in Hela M cells and compared to that of the wild type InlA strain (Fig. 4.9). None of the three mutants were impaired in the internalisation to Hela M cells, but all exhibited reduced growth rate throughout the experiment (mean doubling time between T₁ and T₈: InlA 53 min, InlA::Δ*lipA* 86 min, InlA::Δ*lipB* 82 min and InlA::Δ*lipAΔlipB* 124 min). The numbers of viable bacteria detected at T₂₄ for all three mutants were significantly lower than the wild type, with the double mutant most attenuated (*P*=0.0099 between InlA::Δ*lipA* and InlA, *P*=0.017 between InlA::Δ*lipB* and InlA, *P*=0.0035 between InlA::Δ*lipAΔlipB* and InlA). However, there is no significant difference among the three mutants.

![Figure 4.9 Intracellular survival assay on Hela M cells with *L. monocytogenes* InlA, InlA::Δ*plcA*, InlA::Δ*plcB* and InlA::Δ*lipAΔlipB* at MOI of 10. Gentamicin was added to a final 10 μg/ml concentration at T₀. At the indicated time points the number of intracellular bacteria was shown as CFU/ml. Each data point represents the average of two independent experiments. Error bars showed the standard error for each data.](image-url)
4.5.3 Plasmid complementation restored the virulence of InlA::ΔlipB in Hela M cells

To confirm that the deletion of lipB gene accounted for the intracellular growth defect of InlA::ΔlipB, the full coding sequence of lipB was re-introduced into InlA::ΔlipB by pUNK1 plasmid to test whether the virulence could be restored.

The complementation plasmid pUNK1-LipB was generated by first amplifying the entire lipB gene and its promoter region, 105 bp 5’ to the ATG start codon, with lipBcomp-F/R primers (Table 2.3). The 1140 bp PCR fragment was digested with Bgl II before ligation into BamHI I digested pUNK1. Successful clones were screened using pUNK1-F and pUNK1-R primers (Table 2.3, Fig. 4.10 a) and the orientation of LipB was confirmed by Pst I/Sca I double digestion (Fig. 4.10 b, c). The pUNK1-LipB plasmid was sequenced with pUNK1-F/R primers (Table 2.3). Both pUNK1-LipB and pUNK1 plasmids were transformed into InlA and pUNK1-LipB was transformed into InlA::ΔlipB. The transformed strains were used in intracellular survival assay in Hela M cells (Fig. 4.11). The result showed that in comparison to the significant difference between InlA::ΔlipB(pUNK1) and InlA(pUNK1) at T24 (P=0.0304), the complemented InlA::ΔlipB strain multiplied at similar rates as InlA (pUNK1) (mean doubling time between T1 and T6: InlA(pUNK1) 48 min, InlA::ΔlipB(pUNK1) 82 min, InlA::ΔlipB(pUNK1-LipB) 49 min) and restored to 81.5% of the final yield of InlA at T24 (P=0.525). This result confirmed that the defect in intracellular growth in vitro of the lipB deletion mutant was caused by loss of LipB function.
Figure 4.10  a) Confirmation of pUNK1-LipB by colony PCR with pUNK1-F/R primers. Predicted size: ~300 bp for pUNK1, 1.5 kb for pUNK1-LipB. b) Confirmation of pUNK1-LipB by *Pst*I/*Sac*I digestion. Predicted size: three bands of 5.6 kb, 630 bp and 370 bp for correct orientation of LipA; 5.3 kb, 770bp and 630 bp for opposite orientation. DNA marker HyperLadder I was used as an indication for band sizes. c) Schematic diagram of the *Pst*I and *Sac*I restriction site on pUNK1-LipB.

Figure 4.11 Complementation of *lipB* with pUNK1-LipB plasmid in Hela M cell at MOI 10. Gentamicin was added to a final 10 μg/ml concentration at T₀. At indicated time points the number of intracellular bacteria was shown as CFU/ml. Each data point represents the average of two independent experiments. Error bars showed the standard error for each data.
4.6 Deletion of lipA and lipB did not affect the intracellular survival of *L. monocytogenes* in J774.A1 cells

A third cell line mouse macrophage-like cell line J774.A1 was used as the host cell for the lipA and lipB mutants. Intracellular survival assay with InlA, InlA::ΔlipA, InlA::ΔlipB and InlA::ΔlipAΔlipB were performed in J774.A1 cells as described. A much lower MOI of 0.5 was used as macrophages were much more efficient in taking up the bacteria. The result (Fig. 4.12) showed no significant difference in either internalisation or any of the time points between the wild type and mutants, indicating no growth defect for all three mutants in J774.A1 cells.

**Figure 4.12** Intracellular survival assay on J774.A1 cells with *L. monocytogenes* InlA, InlA::ΔplcA, InlA::ΔplcB and InlA::ΔlipAΔlipB at MOI 0.5. Gentamicin was added to a final 10 μg/ml concentration at T₀. At the indicated time points the number of intracellular bacteria was shown as CFU/ml. Each data point represents the average of three independent experiments. Error bars showed the standard error for each data.
4.7 *lipA* and *lipB* deletion mutants were unaffected in intracellular mobility but were attenuated in forming plaques

Having observed the phenotype of impaired intracellular growth in epithelial cell lines *in vitro* for *lipA* and *lipB* mutants, the next step was to study whether LipA and LipB play a role in the intracellular mobility and cell-to-cell spread, which was revealed by phaloidin staining and plaque assay respectively.

4.7.1 InlA::ΔlipA and InlA::ΔlipB were unaffected in actin polymerisation and actin-based intracellular movement

The intracellular mobility of *L. monocytogenes* was studied by phaloidin staining, which recognises the host cell F-actin. Hela M cells were infected with InlA::ΔlipA, InlA::ΔlipB together with positive control InlA and negative control InlA::ΔhlyΔplcB. Phaloidin staining was performed as described (see section 2.9).

As shown in Fig. 4.13, phaloidin binds to host F-actin, revealing the Hela M cell cytoskeleton network. In InlA, InlA::ΔlipA and InlA::ΔlipB infected Hela M cells, actin was observed to be recruited to the surface of some of the bacteria, a small number of which formed comet-like actin tails (Fig. 4.13). Although the length of the tails varied among individual bacterium, no apparent difference was observed in the appearance of the tails formed by InlA, InlA::ΔlipA or InlA::ΔlipB mutants, revealing no deficiency in the recruitment or polymerisation of host actin filaments. No actin accumulation or tail formation was observed on the control strain InlA::ΔhlyΔplcB, which further confirmed that it is unable to escape from the phagosome (Fig. 4.13).
Both the number of actin-associated *L. monocytogenes* and those with actin tails were counted in randomly chosen Hela M cells which were infected with InlA, InlA::*ΔlipA*, InlA::*ΔlipB*, InlA::*ΔhlyΔplcB* and uninfected control. Hela M cells were infected with the above strains at MOI 50. Cells were fixed at 4.5 hr post-infection and stained with Hoechst 33342 DNA Dye (blue), FITC-Phalloidin (green), Rabbit-αListeria and Sheep-αRabbit-TexasRed (red) antibodies. Pictures were taken at 600x magnification. Arrows showed *L. monocytogenes* with comet-like actin tails; arrows with asterisk showed *L. monocytogenes* recruiting actin in the absence of tails.

Both the number of actin-associated *L. monocytogenes* and those with actin tails were counted in randomly chosen Hela M cells which were infected with InlA, InlA::*ΔlipA* and InlA::*ΔlipB* (Fig. 4.14). The result showed that: first of all, in *lipA* and *lipB* mutants, the number of intracellular bacteria per Hela M cell was a lot lower than the wild type, which fits the observation of reduced viability in the intracellular survival assay; secondly, the percentages of intracellular bacteria associated with actin or comet tails were not statistically different between either mutant and the wild type. At 4.5 hr post-infection, around 25% of intracellular bacteria were associated with actin, and slightly over 2% started actin-based movement.

In conclusion, the phalloidin staining suggested that LipA and LipB do not affect the bacteria’s intracellular mobility via the recruitment and induced polymerisation of host actin.
4.7.2 InlA::ΔlipA, InlA::ΔlipB and InlA::ΔlipAΔlipB formed reduced number of plaques in plaque assay in Hela M cells

Plaque assay is a standard method used to determine virus concentration in terms of infectious dose. It has also been used to study the cell to cell spread of intracellular bacteria, e.g. *S. flexneri* and *L. monocytogenes*, in host cells (Oaks *et al.*, 1985; Havell, 1986; Tilney and Portnoy, 1989).

Plaque assays were performed on Hela M cell monolayers infected with *L. monocytogenes* InlA, InlA::ΔlipA, InlA::ΔlipB, InlA::ΔlipAΔlipB, and InlA::ΔhlyΔplcB strains at MOI 0.1. White uniform pin-point sized plaques started to develop 2 days post infection and reached about 1.0 mm in diameter on day 3 (Fig. 4.15). The morphology of a single plaque was a clump of disrupted Hela M cells surrounded by the intact cell monolayer. If [120]
incubated for longer period, the centre of the plaque would become a clear area. The number of plaques in each well of 6-well plates was counted 3 days post infection in triplicate.

As shown in Fig. 4.16, there was a significantly reduced number of plaques formed in each well by InlA::ΔlipA (P=4.83 X 10^{-8}, n=3), InlA::ΔlipB (P=9.91 X 10^{-8}, n=3) and InlA::ΔlipAΔlipB (P=6.37 X 10^{-9}, n=3) while no plaques were detected in either the uninfected control cells or in cells infected with InlA::ΔhlyΔplcB. Furthermore, InlA::ΔlipAΔlipB formed significantly less plaques than InlA::ΔlipA (P=7.99 X 10^{-6}, n=3) and InlA::ΔlipB (P=0.00146, n=3) as well. The colour of the agarose changed from red to yellow as the plaque number increased, reflecting a reduced pH possibly caused by the growth of the bacteria. However, the sizes of plaques formed by all strains were essentially the same.

InlA::ΔlipA was also complemented with pUNK1-LipA in the plaque assay in Hela M cells (Fig. 4.17). The result showed that the complemented InlA::ΔlipA strain restored to 74.8% the number of plaques formed by InlA(pUNK1). And the difference between InlA(pUNK1) and InlA::ΔlipA(pUNK1-LipA) was not statistically significant (P=0.264, n=3), while the plaque number formed by InlA::ΔlipA(pUNK1) was significantly lower than both InlA(pUNK1) (P=0.0294, n=3) and InlA::ΔlipA(pUNK1-LipA) (P=0.00104, n=3). Therefore, the impaired ability of InlA::ΔlipA in forming plaques could be complemented by pUNK1-LipA.
Figure 4.15 Photos of the plaque assay on Hela M cells. Hela M cells were infected with InlA, InlA::ΔlipA, InlA::ΔhlyΔplcB, InlA::ΔlipB, InlA::ΔlipAΔlipB and uninfected control at MOI 0.1. Pictures were taken 3 days after infection.

Figure 4.16 Number of plaques formed by *L. monocytogenes* in plaque assay on Hela M cells. Hela M cells were infected with InlA, InlA::ΔlipA, InlA::ΔhlyΔplcB, InlA::ΔlipB, InlA::ΔlipAΔlipB and uninfected control at MOI 0.1. Each data represents the average plaque number in three wells 3 days post-infection. Error bars showed the standard error for each data.
4.8 InlA::ΔlipA, InlA::ΔlipB, InlA::ΔlipAΔlipB were not attenuated in internalisation into Hela M cells at low MOI

The internalisation ability of InlA::ΔlipA, InlA::ΔlipB and InlA::ΔlipAΔlipB mutants was tested in intracellular survival assay in Hela M cells, using a low MOI of 0.1 as in plaque assay. The result (Fig. 4.18) showed that at T₁, the numbers of intracellular bacteria of all three mutants were not significantly different from InlA, suggesting no attenuation in invasion in lipA or lipB mutants.
4.9 Discussion

4.9.1 The use of the InlA strain

Previously, the mouse model has been widely used in studying listeriosis and the immune response against intracellular bacteria. Mice are innately resistant to oral infection with *L. monocytogenes* due to a single amino acid difference between murine and human E-cadherin (Lecuit *et al.*, 2001). Thus in many experimental systems the infection of mice was performed either through intravenous or intraperitoneal infection of mice, or by generating transgenic mice producing human alongside murine E-cadherin in its enterocytes (Mackaness, 1962; Buchmeier and Schreiber, 1985; Lecuit *et al.*, 2001; D’Orazio *et al.*, 2006; Stockinger *et al.*, 2009).

An InlA strain has been engineered by Wollert *et al.* (2007) which contained two substitutions of Ser$_{192}$-to-Asn$_{192}$ and Tyr$_{369}$-to-Ser$_{369}$ in its InlA protein.
This greatly improved the binding affinity of InlA for mouse E-cadherin so that it could invade mouse gut epithelium, making the oral challenge of mice possible. This therefore can represent a system closely resembling the human situation during a *L. monocytogenes* infection. In this study all mutations were generated in the InlA strain background, so that all our *in vitro* studies of the two phosphatases LipA and LipB could be compared with the *in vivo* study in mouse model in the future.

Although the InlA strain was reported to increase the adhesion in Caco-2 cells (Wollert *et al.*, 2007), it showed no difference from the EGDe strain in its ability to internalise into and replicate inside Hela M cells in our study. This could be explained by the fact that the bacterial entry into Hela M cells is fully dependent on InlB, while in Caco-2 and the hepatocyte cell line HepG2 both InlA and InlB contribute to the entry of *L. monocytogenes*. (Gaillard *et al.* 1991; Dramsi *et al.*, 1995)

### 4.9.2 Infection of Caco-2, Hela M and J774.A1 cells by *L. monocytogenes*

In previous study, the ΔlipA mutant was reported not to be impaired in intracellular growth in mouse J774.A1 macrophage-like cell line, the rectal epithelial cell line CMT-93 or L2 fibroblast cells (Kastner *et al.*, 2011). However, in this study, although InlA::ΔlipA and InlA::ΔlipB showed no defect in J774.A1 cell line, they both exhibited reduced intracellular growth in Hela M cells and Caco-2 cells, which indicated that the role of LipA and LipB may be dependent on the host cell types.

Caco-2 cells were the first cell type used in the *in vitro* model of penetration and intracellular growth of *L. monocytogenes* (Gaillard *et al.*, 1987). It is suitable as a host cell for *L. monocytogenes* because it is very efficient in internalisation of the bacteria, and displays typical enterocyte differentiation under standard culture, such as the development of a brush border which
contains some enzymes that are normally located in the small intestine (Rousset, 1986). However, the challenges using Caco-2 cells are that they grow slower, tend to form clumps, and the size and shape of the cells were not uniform among the population.

Hela M cells are less efficient in taking up the bacteria, thus it benefits from an extra step of centrifugation in the infection stage and longer infection time. The main advantages of Hela M cells are their flat morphology, large cytoplasmic area and ease of culture. They also reveal distinct patterns of filamentous actin, a feature not observed with Caco-2 cells (Francis and Thomas, 1996).

Considering that InlA::ΔlipA showed attenuated phenotype in both cell types, and that Hela M cells were more suitable for imaging and studying the actin-based movement of L. monocytogenes, Hela M cells were eventually chosen as the main host cell type in this study.

4.9.3 InlA::ΔlipA, InlA::ΔlipB and InlA::ΔlipAΔlipB were attenuated in intracellular growth

The ΔlipA, ΔlipB and ΔlipAΔlipB mutations were generated in InlA strain and studied in intracellular survival assay. The same phenotype of unaffected internalisation and reduced intracellular growth was observed for InlA::ΔlipA and InlA::ΔlipB in Hela M cells, with approximately 1.5 times longer mean doubling times between T₁ and T₈ than InlA and significantly lower numbers of bacteria recovered at T₂₄, which was similar to EGDe::ΔlipA and EGDe::ΔlipB. InlA::ΔlipAΔlipB showed even more severe attenuation in the intracellular growth in Hela M cells, with 2.3 times longer mean doubling times between T₁ and T₈ than InlA and the lowest number of bacteria recovered at T₂₄. Plasmid complementation of lipA and lipB fully restored the
intracellular growth of both mutants. This further suggested that LipA and LipB are both required for the intracellular growth of *L. monocytogenes*.

In total, these results provided evidence for the important role of the two phosphatases LipA and LipB during the intracellular survival of *L. monocytogenes*.

**4.9.4 LipA and LipB were not required in actin polymerisation**

Phalloidin staining of *L. monocytogenes* infected Hela M cells showed that LipA and LipB were not involved in the recruitment of host actin or actin-based movement. Kastner *et al.* (2011) reported that LipA was not required for cell-to-cell spread in a plaque assay using L2 fibroblasts. In this study, plaque assay using Hela M cells infected with InlA::ΔlipA, InlA::ΔlipB and InlA::ΔlipAΔlipB mutants all showed significantly reduced numbers of plaques, with the double mutant produced significantly less plaques than each single mutant. However, this reduction may not reflect impaired cell-to-cell spread, as the plaques formed by the mutants were the same size as InlA.

Our first hypothesis was that the defect in forming plaques was due to their impaired internalisation in Hela M cells. This defect was not observed in the intracellular survival assay since 100 times more *L. monocytogenes* were used in the intracellular survival assay than in the plaque assay. As such any defect in invasion in either a *lipA* or *lipB* mutant might be masked by the large inoculum used in the gentamycin killing assay. However, when the intracellular survival assay was repeated with low MOI 0.1 no defect in invasion was detectable meaning that the reduced number of plaques cannot be explained by a reduction in invasion (Fig. 4.18).
A second explanation was that, the reduced plaque number might be a consequence of the growth defect of lipA and lipB mutants in Hela M cells. That is, the reduced number of intracellular bacteria lowered the chances that they spread to adjacent cells, as the intracellular movement of L. monocytogenes is stochastic.

Another possibility is that LipA and LipB might play some contributory role in the efficient escape from the double membrane vacuole following spread into an adjacent cell. The deletion of lipA and lipB could thus stop or slow down the bacteria from further multiplying and spreading, also leading to reduced average number of bacteria per cell. However, this hypothesis awaits further studying.
Chapter 5 Modulation of host PIP metabolism by *L. monocytogenes* in Hela M cells

5.1 Introduction

In previous chapters, the phenotypes of the *lipA* and *lipB* mutations were identified and the important role of LipA and LipB in intracellular grow of the bacteria established. A second aim of this study is to understand the changes in host PIP metabolism during infection by *L. monocytogenes* and how host PIP metabolism is manipulated by *L. monocytogenes* during infection. Previous work of Henry *et al.* (2006) has shown that PI3P is associated with *L. monocytogenes*-containing phagosomes. GFP- or mRFP-tagged Akt-PH and PLCδ-PH domains were also reported to co-localise with *L. monocytogenes* during the internalisation of the bacteria and during the actin polymerisation at 4 hr post-infection in PtK2 (*Potorous tridactylis* kidney epithelial) cells and MDCK (Madin-Darby canine kidney) cells (Sidhu *et al.*, 2005). Akt-PH domain recognising PI(4,5)P$_2$ and PI(3,4,5)P$_3$ was reported to be recuited to *L. monocytogenes* during actin polymerisation in these two cell lines as well (Sidhu *et al.*, 2005). In this study, a similar approach was used by transfecting the host Hela M cell so that it expresses fluorescently labelled probes that specifically recognise individual PIPs. These probes would be GFP or YFP (yellow fluorescent protein) fused with FENS/FYVE domain that recognises PI3P (Ridley *et al.*, 2001), with FAPP1-PH domain that recognises PI4P (Dowler *et al.*, 2000), PLCδ-PH domain that recognises PI(4,5)P$_2$ (Sidhu *et al.*, 2005), ARNO-PH domain that recognises PI(3,4,5)P$_3$ (Venkateswarlu *et al.*, 1998), TAPP1-PH domain that recognises PI(3,4)P2 (Dowler *et al.*, 2000), and Akt-PH domain that recognises both PI(3,4)P$_2$ and PI(3,4,5)P$_2$ (Sidhu *et al.*, 2005). Also, the presence of the *L. monocytogenes* could be visualised by constructing fluorescent *L. monocytogenes* strains or by antibody staining. This would give us a clearer picture of the PIP association and turnover.
during infection by *L. monocytogenes*. In addition, infection of the Hela M cells expressing these chimeric probes with the *lipA* and *lipB* mutants would help us understand the function of LipA and LipB in modulating the host PIP metabolism.

### 5.2 Visualisation of *L. monocytogenes*

In order to visualise *L. monocytogenes* by fluorescent microscopy, several strategies have been employed including i) inserting a copy of the fluorophore gene into *L. monocytogenes* chromosome that allows the expression of the fluorescent protein inside *L. monocytogenes* (Balestrino *et al.*, 2010), ii) expressing a fluorescent protein from plasmid inside *L. monocytogenes* (Andersen *et al.*, 2006) and iii) using *L. monocytogenes*-specific antibodies (Radtke *et al.*, 2011). All three methods were tested in this study.

#### 5.2.1 Chromosomal expression of fluorescent proteins in *L. monocytogenes*

**5.2.1.1 Generation of fluorescent *L. monocytogenes* strain InlA::GFP$_{uv}$ and InlA::mCherry**

Two fluorescent *L. monocytogenes* strains InlA::GFP$_{uv}$ and InlA::mCherry were constructed by inserting the coding sequences of the green fluorescent protein variant GFP$_{uv}$ or a red fluorescent protein mCherry preceded by the *rpoB* (β subunit of RNA polymerase gene) Shine-Dalgarno (S-D) region into the *L. monocytogenes* InlA chromosome downstream of the *rpoC* (β’ subunit of RNA polymerase) gene. Therefore the two proteins could be constitutively expressed from the chromosome under the control of the *rpoB* promoter. (Fig. 5.1)

The coding sequence of GFP$_{uv}$ or mCherry was PCR amplified from pGFP$_{uv}$ or pmCherry plasmid with GFP$_{uv}$-F/R or mCherry-F/R primers (Table 2.3),
generating the 776 bp GFP$_{uv}$ and 766 bp mCherry sequences. GFP$_{uv}$-F and mCherry-F primers both included a stop codon cassette, which contained stop codons in all three reading frame to ensure the termination of any translation upstream of GFP$_{uv}$ or mCherry, followed by a 25 bp $rpoB$ S-D region.

Two 600 bp adjacent sequences downstream of the $rpoC$ gene were cloned into plasmid pBluescript with the incorporation of Kpn I/Xba I restriction site at either ends and a BamH I site in between (Fig. 5.1). Bgl II digested GFP$_{uv}$ or mCherry sequences were ligated into the BamH I site as both enzymes shared the same sticky ends, forming pBluescript-rpoND-GFP$_{uv}$ and pBluescript-rpoND-mCherry respectively (Fig. 5.1). E. coli colonies containing pBluescript-rpoND-GFP$_{uv}$/mCherry showed bright green or red colour on plates and successful constructs were selected by colony PCR using T3 and T7 primers (Table 2.3, Fig. 5.2 a). The pBluescript-rpoND- GFP$_{uv}$/mCherry plasmid was then digested with Kpn I and Xba I and the 2 kb fragment containing either GFP$_{uv}$ or mCherry was then inserted into Kpn I and Xba I digested vector pAULA, forming pAULA-GFP$_{uv}$ and pAULA-mCherry (Fig. 5.1). Successful constructs were screened by colony PCR with M13-F/R primers (Table 2.3, Fig. 5.2 b) and confirmed by sequencing with the same primers. No visual detectable expression of either GFP or mCherry was observed in E. coli DH5α (data not shown).
Figure 5.1 Schematic diagram of the strategy used for constructing chromosomal fusion of mCherry or GFP<sub>uv</sub> in *L. monocytogenes*.
pAULA-GFP$_{uv}$ and pAULA-mCherry were then transformed into *L. monocytogenes* InlA strain, colonies grew at 30 °C had no visual detectable expression of GFP or mCherry. Chromosomal fusions were generated as described in Methods and materials (see section 2.6), resulting in the insertion of GFP$_{uv}$/mCherry downstream of *rpoC*. Successful fusions were confirmed by colony PCR using *rpoC*-screen-F/R (Table 2.3) which binds 200 bp up- and down-stream of the cloned *rpoC*-downstream-region, as well as GFP$_{uv}$-F/R and mCherry-F/R primers (Fig. 5.3). The integrity of GFP$_{uv}$ or mCherry sequence was verified by DNA sequencing.
Figure 5.3  a) Confirmation of InlA, InlA::GFPuv and InlA::mCherry by colony PCR. Colony PCR of InlA::GFPuv with rpoC-Screen primers (1, predicted sized 2.4 kb) and GFPuv primers (2, predicted size 780 bp). Colony PCR of InlA with rpoC-Screen primers (3, predicted size 1.6 kb). Colony PCR of InlA::mCherry with rpoC-Screen primers (4, predicted sized 2.4 kb) and mCherry primers (5, predicted size 770 bp); InlA with rpoC-Screen primers (6, predicted size 1.6 kb). DNA marker HyperLadder I (Bioline) was used as an indication of the sizes of bands. b) Schematic diagram of the primer binding sites at the GFPuv or mCherry insertion region on the *L. monocytogenes* chromosome. Green area represented the two *rpoC* downstream
5.2.1.2 InlA::GFPuv has the same growth rate in TSB and in Hela M cell as the InlA strain

The growth of *L. monocytogenes* InlA::GFPuv in TSB media was tested in comparison with that of the InlA strain (Fig. 5.4). The result showed that the growth curve of InlA::GFPuv in TSB was identical to that of InlA, indicating no defect in growth in TSB media was introduced by the insertion of GFPuv.

The ability of InlA::GFPuv in intracellular survival was also tested by intracellular survival assay in Hela M cells (Fig. 5.5). The result showed no significant difference between InlA::GFPuv and InlA at any time point, and the growth rates were identical (mean doubling time of 96 min for InlA, 100 min for InlA::GFPuv between T1 and T6), suggesting the insertion of GFPuv sequence causes no defect in intracellular growth *in vitro* either.

![Figure 5.4 Growth curve of *L. monocytogenes* InlA and InlA::GFPuv in TSB media.](image)

The InlA and InlA::GFPuv overnight cultures were diluted 1/100 into TSB media and incubated at 37 °C with shaking at 200 rpm. OD\textsubscript{600} was measured at the indicated time points.
5.2.1.3 Expression and visualisation of GFPuv and mCherry in L. monocytogenes

The fluorescence level of InlA::GFPuv from the overnight culture was quantified using KC4 plate reader. As shown in Table 5.1, there was a 5.5-fold increase in the level of GFP expression in InlA::GFPuv than InlA although the expression level was still relatively low.

InlA::GFPuv and InlA::mCherry were then examined under fluorescent microscope. The overnight cultures of InlA, InlA::GFPuv and InlA::mCherry were examined with different microscopes. InlA::GFPuv could not be seen with the widefield fluorescent microscopes in our facility as no matching filter set was available. Under the confocal microscope, InlA::GFPuv was seen as green rods (Fig. 5.6). InlA::mCherry was barely visible under the wide-field
fluorescent microscope. The expression level of mCherry protein was very low. Increasing the laser strength or extending exposure time would cause the sample to bleach in a few seconds. Therefore the InlA::mCherry was not suitable for visualising intracellular bacteria.

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</tr>
<tr>
<td>InlA</td>
<td>133</td>
<td>135</td>
<td>133</td>
<td>134</td>
<td>39</td>
</tr>
<tr>
<td>InlA::GFPuv (1)</td>
<td>310</td>
<td>299</td>
<td>304</td>
<td>304</td>
<td>209</td>
</tr>
<tr>
<td>InlA::GFPuv (2)</td>
<td>318</td>
<td>313</td>
<td>306</td>
<td>312</td>
<td>216</td>
</tr>
<tr>
<td>InlA::GFPuv (3)</td>
<td>313</td>
<td>311</td>
<td>311</td>
<td>312</td>
<td>216</td>
</tr>
</tbody>
</table>

**Table 5.1 The fluorescence level of InlA and InlA::GFPuv overnight cultures.** Samples were prepared in triplicate by resuspending the bacteria pellet from 1 ml of overnight culture (approximately $10^9$ bacteria) in 100 μl PBS. Excitation wavelength: 360±40 nm, Emission wavelength: 528±20 nm, Sensitivity 50. All readings were justified by subtracting the average Blank reading.

**Figure 5.6 Visualisation of InlA::GFPuv (left) and InlA (right) with confocal microscope.** Samples were prepared by smearing 50 μl overnight culture on the slides. Pictures were taken with Nikon C1 Upright 90i Confocal Microscope at 600x magnification plus confocal zoom. Settings: pinhole 5um, scan speed 400Hz unidirectional. Images for FITC were excited with the 488nm laser lines, Emission 515/30 nm. Scale bars showed 10 μm.
5.2.2 Plasmid expression of fluorescent proteins in *L. monocytogenes*

5.2.2.1 Construction of pUNK1-*PrpoB*-mCherry, pUNK1-*PrpoB*-GFP_{mut2} 

The 430 bp promoter region for *rpoB* gene was amplified from EGDe chromosomal DNA with Taq polymerase and PrpoB-F/R primers which contained the *Xma* CL restriction site at both ends (Table 2.3). Purified PCR product was then inserted into pGEM-T Easy vector as previously described (Fig. 5.7). Successful pGEM-*PrpoB* was screened by colony PCR using M13-F/R primers (Table 2.3). Successful clones had a 670 bp PCR product (data not shown).

The 770 bp full coding sequence of mCherry was amplified from pmCherry with mCherry-F/R primers containing *Bgl* II restriction site at both ends (Table 2.3). *Bgl* II digested mCherry was then inserted into *BamH* I digested pUNK1. Following overnight ligation the ligation mixture was digested again with *BamH* I to remove the self-ligated vector molecules. Successful clones of pUNK1-mCherry were screened by colony PCR with pUNK1-F/R primers (Table 2.3). Successful clones had a 1 kb product (Fig. 5.8 a). The correct orientation of mCherry was checked by *Nco I/EcoR* I double digestion (Fig. 5.7, 5.8 b).

The 430 kb *PrpoB* containing fragment was then released from pGEM-*PrpoB* by *Xma* CL digestion (Fig. 5.7) and then isolated via gel purification as previously described in section 2.3.6. Purified *PrpoB* was then inserted into *Xma* CL digested pUNK1-mCherry, forming pUNK1-*PrpoB*-mCherry. Successful clones were screened by colony PCR with pUNK1-F/R primers (Table 2.3) with successful clones having a 1.45 kb fragment (Fig. 5.8 c). Correct orientation the inserted *PrpoB* was confirmed by sequencing with pUNK1-F primer (Table 2.3).
Figure 5.7 Schematic diagram of the strategy used for constructing the pUNK1-PrpoB-mCherry plasmid.
The same strategy was used in constructing pUNK1-PrpoB-GFPmut2. Full coding sequence of GFPmut2, another derivative of GFP protein, was amplified from pSG1792 with GFP mut2-F/R primers (Table 2.3), producing an 848 bp PCR product. However, the construction of pUNK1-PrpoB-GFPmut2 was not successful. Strategies of inserting the PrpoB into pUNK1-GFPmut2 and inserting GFP mut2 into pUNK1-PrpoB plasmid (Corbett and Roberts, unpublished data) were applied, but both failed to generate the pUNK1-PrpoB-GFPmut2 plasmid despite numerous attempts.

5.2.2.2 Fluorescence level expressed from pUNK1-PrpoB-mCherry in E. coli and L. monocytogenes

The level of fluorescence of E. coli DH5α and L. monocytogenes InlA transformed with pUNK1-PrpoB-mCherry was quantified using the plate reader. The result showed that despite the large increase in the expression of detectable fluorescence in DH5α(pUNK1-PrpoB-mCherry), there was little detectable mCherry expressed in InlA(pUNK1-PrpoB-mCherry) (Table 5.2).
Fluorescence microscopy also confirmed that InlA(pUNK1-PrpoB-mCherry) was not observable (data not shown).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Blank</th>
<th>Sample</th>
<th>Average</th>
<th>Justified</th>
</tr>
</thead>
<tbody>
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<td>DH5α</td>
<td>52</td>
<td>46 48 49</td>
<td>47</td>
<td>0</td>
</tr>
<tr>
<td>DH5α(pUNK1-PrpoB-mCherry)</td>
<td>52</td>
<td>28694 29242 29039</td>
<td>28992</td>
<td>28945</td>
</tr>
<tr>
<td>InlA</td>
<td>55</td>
<td>66 67 69</td>
<td>67</td>
<td>14</td>
</tr>
<tr>
<td>InlA(pUNK1-PrpoB-mCherry)</td>
<td>54</td>
<td>128 124 103</td>
<td>118</td>
<td>65</td>
</tr>
</tbody>
</table>

Table 5.2 Fluorescence level of mCherry expressed from pUNK1-PrpoB-mCherry in E. coli and L. monocytogenes. Samples were prepared in triplicate by resuspending the bacterial pellet (~10^9) from 1 ml of overnight culture in 100 μl PBS. Excitation wavelength: 590±20 nm, Emission wavelength: 645±20 nm, Sensitivity 50. Average readings were justified by subtracting the average Blank reading.

5.2.2.3 Expression of fluorescent protein from pNF8 and pJEBAN6 in L. monocytogenes

In order to generate fluorescently labelled L. monocytogenes, two pAULA derived plasmids pNF8 and pJEBAN6 were used, from which the fluorescent protein GFP_{mut1} or DsRedExpress are expressed under the control of a constitutive promoter Pdlt (Andersen et al., 2006).

Introduction of either pNF8 or pJEBAN6 into E. coli DH5α resulted in colonies that appeared either bright green or pink in colour. However, L. monocytogenes containing these two plasmids were not obvious coloured. The fluorescence level of L. monocytogenes InlA transformed with pJEBAN6 or pNF8 was tested using the plate reader (Table 5.3). The result showed that InlA(pJEBAN6) had a 13-fold increase in the fluorescence level than InlA, and the fluorescence in InlA(pNF8) increased dramatically from 0 to 1368.

Intracellular survival assay with InlA, InlA(pJEBAN6) and InlA(pNF8) in Hela M cells were performed (Fig. 5.9). The growth curves of all three strains were
almost identical, which suggested that InlA containing either pJEBAN6 or pNF8 had no effect on the intracellular growth of the bacteria \textit{in vitro}.

Mid-log phase cultures ($\sim$OD$_{600}$=0.4) of InlA(pJEBAN6) and InlA(pNF8) grown in TSB media with 5 $\mu$g/mL Erm and fixed on glass slides, stained with DAPI DNA dye and checked under the fluorescent microscope (Fig. 5.10). The result showed that 73% of the InlA(pJEBAN6) and 99% of InlA(pNF8) were fluorescent, although the brightness varied among individual bacteria, indicating different expression level of GFP$_{\text{mut1}}$ or DsRedExpress among the population.

To increase the ratio of fluorescent bacteria in InlA(pJEBAN6), different concentrations of Erm in TSB media were tested in the subculture of InlA(pJEBAN6) (Table 5.4). The result suggested that increasing the Erm concentration to 20 $\mu$g/ml would significantly increase the ratio of plasmid-bearing bacteria in the population while not affect the growth rate, leading to enhanced visibility of the bacteria.

InlA(pJEBAN6) and InlA(pNF8) infected Hela M cells were fixed, stained and checked under fluorescence microscope (Fig. 5.11). The result showed that the luminance from intracellular \textit{L. monocytogenes} were not as bright as in TSB culture. Comparing the number of total \textit{L. monocytogenes} revealed by Hoechst 33342 DNA stain and the number of fluorescent bacteria, it is shown that the majority of the intracellular \textit{L. monocytogenes} successfully expressed GFP$_{\text{mut1}}$ or DsRedExpress from the plasmids.
Table 5.3 Fluorescence level of DsRedExpress or GFPmut1 expressed from Pjeban6 AND pNF8 in E. coli and L. monocytogenes. Samples were prepared in triplicate by resuspending the bacterial pellet (~10^9) from 1 ml of overnight culture in 100 μl PBS. Average readings were justified by subtracting the average Blank reading.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Blank</th>
<th>Sample</th>
<th>Average</th>
<th>Justified</th>
</tr>
</thead>
<tbody>
<tr>
<td>InlA</td>
<td>85</td>
<td>102</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>InlA(pJEBAN6)</td>
<td>85</td>
<td>279</td>
<td>274</td>
<td>278</td>
</tr>
</tbody>
</table>

Excitation wavelength: 360±40 nm, Emission wavelength: 460±40 nm, Sensitivity 40

<table>
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<th>Justified</th>
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</thead>
<tbody>
<tr>
<td>InlA</td>
<td>25</td>
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<td>24</td>
<td>24</td>
</tr>
<tr>
<td>InlA(pNF8)</td>
<td>26</td>
<td>1357</td>
<td>1393</td>
<td>1432</td>
</tr>
</tbody>
</table>

Excitation wavelength: 485±20 nm, Emission wavelength: 580±20 nm, Sensitivity 30

Table 5.4 Erm concentration affected the percentage of InlA containing pJEBAN6. Viable counts of overnight cultures of InlA(pJEAN6) grown in TSB with different concentration of Erm were performed on both TSB and TSB/Erm plates, suggesting the concentration of total bacteria and bacteria containing pJEBAN6 plasmid respectively.

<table>
<thead>
<tr>
<th>Concentration of Erm in overnight culture</th>
<th>CFU/ml on TSB plate</th>
<th>CFU/ml on TSB plate with 5 μg/ml Erm</th>
<th>Percentage of InlA(pJEBAN6) in the culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μg/ml</td>
<td>1.53 x 10^6</td>
<td>1.07 x 10^6</td>
<td>69.9%</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>1.48 x 10^6</td>
<td>1.2 x 10^6</td>
<td>81.1%</td>
</tr>
<tr>
<td>15 μg/ml</td>
<td>1.46 x 10^6</td>
<td>1.37 x 10^6</td>
<td>93.8%</td>
</tr>
<tr>
<td>20 μg/ml</td>
<td>1.43 x 10^6</td>
<td>1.43 x 10^6</td>
<td>100%</td>
</tr>
<tr>
<td>25 μg/ml</td>
<td>1.3 x 10^6</td>
<td>1.32 x 10^6</td>
<td>100%</td>
</tr>
</tbody>
</table>
Figure 5.9 Intracellular survival assay in Hela M cells with *L. monocytogenes* InlA, InlA(pJEBAN6) and InlA(pNF8) at MOI 10. Gentamicin was added to a final 10 μg/ml concentration at T₀. At each time point the number of intracellular bacteria was shown as CFU/ml. Each data point was the average of four independent experiments. Error bars showed the standard error for each data.

Figure 5.10 Visualisation of the mid-log phase InlA(pJEBAN6) and InlA(pNF8) by fluorescent microscope. Mid-log phased bacteria culture in TSB was heat fixed on glass slide, stained with DAPI for 20 min and visualised by fluorescent microscope at 600x magnification. DNA was shown in blue, InlA(pJEBAN6) was shown in red and InlA(pNF8) was shown in green. Insets showed amplified local areas from the picture.
One interesting observation was that as the *L. monocytogenes* grew in Hela M cells for longer, the brightness of each fluorescent bacteria and the percentage of fluorescent bacteria in the population increased significantly, indicating the accumulation of the fluorescent protein inside the bacteria. As shown in Fig. 5.12, at 24 hr post-infection, the fluorescence from individual bacteria was very strong. There was a 100% of fluorescent *L. monocytogenes* in total intracellular bacteria. Furthermore, the Golgi structure in the host cells was distorted, indicating the host cells were very ill from the huge number of intracellular pathogen.

Figure 5.11 Imaging of InlA(pJEBAN6) and InlA(pNF8) in Hela M cells 4 hr post-infection. Hela M cells infected with InlA(pJEBAN6) and InlA(pNF8) at MOI 10, fixed 4 hrs post-infection, stained with Hoechst 33342 DNA dye and checked under microscope at 600x magnification. DNA was shown in blue, InlA(pJEBAN6) was shown in red and InlA(NF8) was shown in green. Insets showed magnified local areas from the picture.
Figure 5.12 InlA(pJEBAN6) and InlA(pNF8) infected Hela M cells 24 hr post-infection. Hela M cells were infected with InlA(pJEBAN6) at MOI 10. Gentamicin was added to 10 μg/ml final concentration at 2 hr post-infection. Cells were fixed at 24 hrs post-infection, stained with Hoechst 33342 DNA dye, Rabbit-αGolgin97 and Goat-αRabbit-Alexa488 and checked under microscope at 600x magnification. DNA was shown in blue, InlA(pJEBAN6) was shown in red and Golgi was shown in green. Insets showed magnified local areas from the picture.
5.2.3 Antibody staining of *L. monocytogenes*

*L. monocytogenes* could also be visualised through staining with *L. monocytogenes*-specific antibody. As showed in Fig. 5.13, antibody stained bacteria appeared slightly larger than normal size as the antibodies bound to the surface of the bacteria. Staining of *L. monocytogenes* infected Hela M cells showed 100% recognition of the bacteria, although a low level of background and impurities exited.

![Staining of L. monocytogenes infected Hela M cell with anti-Listeria antibody.](image)

Figure 5.13 Staining of *L. monocytogenes* infected Hela M cell with anti-*Listeria* antibody. Hela M cells infected with InlA at MOI 10. Gentamicin was added to a final concentration of 10 μg/ml at 2 hr post-infection. Cells were fixed at 4.5 hr post-infection, stained with Hoechst 33342 DNA dye, Rabbit-αListeria, Goat-αRabbit-TexasRed and FITC-phalloidin and checked under microscope at 600x magnification. DNA was shown in blue, InlA was shown in red and host actin was shown in green. Insets showed two magnified bacteria in the picture.

5.3 Localisation of five PIPs in Hela M cells during *L. monocytogenes* infection

5.3.1 Localisation of five PIPs in Hela M cells during *L. monocytogenes* InlA infection

The distribution of five phosphoinositides in host Hela M cells during *L. monocytogenes* infection was studied, by transfecting Hela M cells with expression vectors to express EGFP or YFP tagged domains which specifically
recognise individual phosphoinositide (kindly provided by Prof. Martin Lowe, University of Manchester, Table 5.5). These transfected cells were then infected with *L. monocytogenes* InlA and the localisation of each PIP in infected or uninfected cells at different time points were compared using immunofluorescence microscopy.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Target PIP</th>
<th>Fluorophore</th>
</tr>
</thead>
<tbody>
<tr>
<td>FENS/FYVE</td>
<td>PI3P</td>
<td>EGFP</td>
</tr>
<tr>
<td>FAPP1/PH</td>
<td>PI4P</td>
<td>EGFP</td>
</tr>
<tr>
<td>PLCδ-PH</td>
<td>PI(4,5)P₂</td>
<td>EGFP</td>
</tr>
<tr>
<td>Akt-PH</td>
<td>PI(3,4,5)P₃+PI(3,4)P₂</td>
<td>YFP</td>
</tr>
<tr>
<td>TAPP1-PH</td>
<td>PI(3,4)P₂</td>
<td>EGFP</td>
</tr>
<tr>
<td>ARNO-PH</td>
<td>PI(3,4,5)P₃</td>
<td>YFP</td>
</tr>
</tbody>
</table>

Table 5.5 The six probes used in this study for the recognition of PIPs.

5.3.1.1 InlA recruited PI3P to its surface in early stage of infection

Hela M cells were transfected with the pEGFP-FENS/FYVE for 24 hr before infected with *L. monocytogenes* InlA at MOI 10, 50 or 100. Gentamicin was added to the media 2 hr post-infection to 10 μg/ml final concentration. Cells were fixed at 1.5, 2.5, 3.5, and 5.5 hr post-infection together with uninfected control. Cells and bacteria were stained and visualised with fluorescent microscope.

In the uninfected cells (Fig. 5.14 a), the expressed EGFP-FENS/FYVE probe which recognises PI3P aggregated in numerous small particle structures in the cytoplasm, presumably to be the early endosomes. At the 1.5 hr time point, PI3P was observed to accumulate on the surface of some of the bacteria (Fig. 5.14 b). The edge of these ‘pockets’ form by PI3P were ruffled, indicating that PI3P were more likely enriched on the bacteria-containing endosomes rather than on the surface of the bacteria. Comparing to the *L. 
monocytogenes not coated by PI3P, those bacteria recruiting PI3P were significantly less bright (Fig. 5.14 b). It was likely that the bright ones were those still outside or attached to the surface of the host cell while the dimmer ones were engulfed inside phagosomes thus were less well stained by the antibody. The co-localisation of PI3P with L. monocytogenes InlA disappeared at 2.5 hr and was not observed on later time points.

Figure 5.14 a) Hela M cells expressing EGFP-FENS/FYVE. Hela M cells were transfected with pEGFP-FENS/FYVE for 24 hr and stained with Hoechst 33342 DNA dye. b) EGFP-FENS/FYVE co-localised with L. monocytogenes InlA in Hela M cells at 1.5 hr post-infection. Hela M cells were transfected with pEGFP-FENS/FYVE for 24 hr, infected with InlA at MOI 100, fixed at 1.5 hr post-infection. DNA was stained with Hoechst 33342 DNA dye and appeared blue in DAPI channel. InlA stained with Rabbit-αListeria and Sheep-αRabbit-TexasRed and appeared red in TexasRed channel, EGFP-FENS/FYVE appeared green in FITC channel. Photos were taken at 600x magnification. Insets showed amplified local areas in the picture. Arrows pointed at two bacteria co-localised with EGFP-FENS/FYVE.
5.3.1.2 *L. monocytogenes* did not affect the localisation of PI4P

Hela M cells were transfected with pEGFP-FAPP1-PH 24 hr before infection with *L. monocytogenes* InlA at MOI 1, 50 and 100. Gentamicin was added to the media 2 hrs post-infection to 10 μg/ml final concentration. Cells were fixed at 1.5, 2.5, 3.5, and 6 hr post-infection together with uninfected control. Cells and bacteria were stained and visualised with fluorescent microscope.

In the uninfected control (Fig. 5.15 a), PI4P recognised by EGFP-FAPP1-PH accumulated mainly at some web-like structures next to one or both sides of the host cell nucleus, presumably to be the Golgi apparatus. Some network structures were also observed in the cytoplasm, possibly due to the presence of PI4P on the endoplasmic reticulum. However, in none of the time points was *L. monocytogenes* InlA observed to co-localise with or affect the distribution of PI4P in Hela M cell (Fig. 5.15 b).

5.3.1.3 *L. monocytogenes* did not affect the localisation of PI(4,5)P₂

Hela M cells were transfected with pEGFP-PLCδ-PH 24 hr before being infected with *L. monocytogenes* InlA at MOI 1 to 100. Gentamicin was added to the media 2 hrs post-infection to 10 μg/ml final concentration. Cells were fixed at 1.5, 2.5, 3.5, and 6 hr post-infection together with uninfected control. Cells and bacteria were stained and visualised with the fluorescent microscope.

In the uninfected control (Fig. 5.16 a), EGFP-PLCδ-PH, which specifically recognises PI(4,5)P₂, was observed to enrich primarily on the plasma membrane, as the margin of the cells were decorated with some worm-like structures which were thought to be the membrane ruffles. In some cells EGFP-PLCδ-PH was enriched next to the cell nucleus, which was assumed to be the Golgi apparatus. In none of the time points were *L. monocytogenes*
InlA observed to co-localise with PI(4,5)P₂ or affect the distribution of PI(4,5)P₂ in Hela M cell (Fig. 5.16 b).

5.3.1.4 *L. monocytogenes* did not affect the localisation of PI(3,4,5)P₃

Hela M cells were transfected with pYFP-ARNO-PH 24 hr before infected with *L. monocytogenes* InlA at MOI 1 to 100. Gentamicin was added to the media 2 hrs post-infection to 10 μg/ml final concentration. Cells were fixed at 1.5, 2.5, 3.5, and 6 hr post-infection together with uninfected control. Cells and bacteria were stained and visualised with the fluorescent microscope.

In the control cell (Fig. 5.17 a), YFP-ARNO-PH, which specifically recognises PI(3,4,5)P₃, was observed to be enriched on the host cell nucleus, and also on some microtubule-network-like structures. However, in none of the time points were *L. monocytogenes* InlA observed to co-localise with PI(3,4,5)P₃ or affect its distribution in Hela M cell (Fig. 5.17 b).
Figure 5.15 a) Hela M cells expressing EGFP-FAPP1-PH. Hela M cells were transfected with pEGFP-FAPP1-PH for 24 hr and stained with Hoechst 33342 DNA dye. b) Localisation of EGFP-FAPP1-PH in Hela M cells at 1.5 hr post-infection by L. monocytogenes InlA. Hela M cells were transfected with pEGFP-FAPP1-PH for 24 hr, infected with InlA at MOI 50, fixed at 3.5 hr post-infection. DNA was stained with Hoechst 33342 DNA dye which appeared blue. InlA was stained with Rabbit-αListeria and Sheep-αRabbit-TexasRed which appeared red and EGFP-FAPP1-PH appeared green. Photos were taken at 600x magnification.
Figure 5.16  

a) Hela M cells expressing EGFP-PLCδ-PH. Hela M cells were transfected with pEGFP-PLCδ-PH for 24 hr and stained with Hoechst 33342 DNA dye.

b) Localisation of EGFP-PLCδ-PH in Hela M cells at 2.5 hr post-infection by *L. monocytogenes* InlA. Hela M cells were transfected with pEGFP-FAPP1-PH for 24 hr, infected with InlA at MOI 10, fixed at 2.5 hr post-infection. DNA was stained with Hoechst 33342 DNA dye and appeared blue. InlA was stained with Rabbit-αListeria and Sheep-αRabbit-TexasRed which appeared red, and EGFP-PLCδ-PH appeared green. Photos were taken at 600x magnification.
Figure 5.3.1.3 a) Hela M cells expressing EGFP-PLCδ-PH. Hela M cells were transfected with pEGFP-PLCδ-PH for 24 hr and stained with Hoechst 33342 DNA dye. b) Localisation of EGFP-PLCδ-PH in Hela M cells at 2.5 hr post-infection by L. monocytogenes InlA. Hela M cells were transfected with pEGFP-FAPP1-PH for 24 hr, infected with InlA at MOI 10, fixed at 2.5 hr post-infection. DNA was stained with Hoechst 33342 DNA dye which appeared blue. InlA was stained with Rabbit-αListeria and Sheep-αRabbit-TexasRed which appeared red, and EGFP-PLCδ-PH appeared green. Photos were taken at 600x magnification.

Figure 5.17 a) Hela M cells expressing YFP-ARNO-PH. Hela M cells were transfected with pYFP-ARNO-PH for 24 hr and stained with Hoechst 33342 DNA dye. b) Localisation of YFP-ARNO-PH in Hela M cells at 3.5 hr post-infection by L. monocytogenes InlA. Hela M cells were transfected with pYFP-ARNO-PH for 24 hr, infected with InlA at MOI 100, fixed at 3.5 hr post-infection. DNA was stained with Hoechst 33342 DNA dye which appeared blue. InlA was stained with Rabbit-αListeria and Sheep-αRabbit-TexasRed which appeared red, and YFP-ARNO-PH appeared green. Photos were taken at 600x magnification.
5.3.1.5 *L. monocytogenes* InlA co-localised with PI(3,4)P$_2$ in late stages of infection

Hela M cells were transfected with pEGFP-TAPP1-PH 24 hr before infection with *L. monocytogenes* InlA at MOI 1 to 100. Gentamicin was added to the media 2 hr post-infection to 10 μg/ml final concentration. Cells were fixed at 1.5, 2.5, 3.5, 6 hr and 24 hr post-infection together with uninfected control. Cells and bacteria were stained and visualised with the fluorescent microscope.

In the uninfected control (Fig. 5.18 a), EGFP-TAPP1-PH, which specifically recognise PI(3,4)P$_2$, did not seem to accumulate in any cell structures or organelles but distributed evenly in the cytoplasm. However, in the InlA infected cells, PI(3,4)P$_2$ were recruited to the surface of the bacteria at 6 hr post-infection, and also form comet-tail-like structures behind some of the bacteria (Fig. 5.18 b). This association could still be observed at 24 hr post-infection (Fig. 5.18 c). Interestingly, the bacteria associated with PI(3,4)P$_2$ were less brightly stained than those unassociated ones, possibly due to the reduced the accessibility of anti-*Listeria* antibody to the bacteria as a result of PI(3,4)P$_2$ accumulation.

5.3.1.6 *L. monocytogenes* did not affect the localisation of YFP-Akt-PH

Hela M cells were transfected with pYFP-Akt-PH 24 hr before infected with *L. monocytogenes* InlA at MOI 1 to 100. Gentamicin was added to the media 2 hrs post-infection to 10 μg/ml final concentration. Cells were fixed at 1.5, 2.5, 3.5, and 6 hr post-infection together with uninfected control. Cells and bacteria were stained and visualised with the fluorescent microscope.

In the control cell (Fig. 5.19 a), YFP-Akt-PH, recognising both PI(3,4)P$_2$ and PI(3,4,5)P$_2$, were observed to be enriched mainly on the host cell nucleus
Figure 5.18 a) Hela M cells expressing EGFP-TAPP1-PH. Hela M cells were transfected with pEGFP-TAPP1-PH for 24 hr. b) EGFP-TAPP1-PH co-localised with *L. monocytogenes* InlA in Hela M cells at 6 hr post-infection. Transfected cells were infected with InlA at MOI 50 and fixed at 6 hr post-infection. c) EGFP-TAPP1-PH co-localised with *L. monocytogenes* InlA in Hela M cells at 24 hr post-infection. Transfected cells were infected with InlA at MOI 1 and fixed at 24 hr post-infection. Cells were stained with Hoechst 33342 DNA dye which appeared blue, InlA were stained with Rabbit-αListeria and Sheep-αRabbit-TexasRed which appeared red, EGFP-TAPP1-PH appeared green. Photos were taken at 600x magnification. Insets showed amplified local areas in the picture. Arrows pointed at bacteria co-localised with EGFP-TAPP1-PH.
Figure 5.19 a) Hela M cells expressing YFP-Akt-PH. Hela M cells were transfected with pYFP-Akt-PH for 24 hr. b) Localisation of YFP-Akt-PH in Hela M cells at 3.5 hr post-infection by *L. monocytogenes* InlA. Transfected Hela M cells were infected with InlA at MOI 100, fixed at 3.5 hr post-infection. DNA was stained with Hoechst 33342 DNA dye which appeared blue. InlA was stained with Rabbit-αListeria and Sheep-αRabbit-TexasRed which appeared red, and YFP-ARNO-PH appeared green. Photos were taken at 600x magnification.
with no specific localisation in the cytoplasm. However, although PI(3,4)P₂ was also recognised by Akt-PH domain, no co-localisation of YFP-Akt-PH with L. monocytogenes was observed at any of the time point as expected.

5.3.2 Localisation of five PIPs in Hela M cells during infection with L. monocytogenes lipA and lipB mutants

Having studied the localisation of five host cell PIPs during L. monocytogenes InlA infection, the next question was whether LipA and LipB play a role in the host PIP metabolism.

5.3.2.1 lipA and lipB mutants did not affect the localisation of PI3P, PI4P, PI(4,5)P₂ and PI(3,4,5)P₃

Hela M cells transfected with pEGFP-FENS/FYVE, pEGFP-FAPPI1-PH, pYFP-ARNO-PH, pYFP-Akt-PH and pEGFP-PLCδ-PH respectively were infected with InlA::ΔlipA, InlA::ΔlipB or InlA::ΔlipAΔlipB at the same conditions as used previously with InlA. Cells and bacteria were stained and visualised with fluorescent microscope. None of the mutants was found to affect the localisation of PI3P, PI4P, PI(4,5)P₂ or PI(3,4,5)P₃ at any time point as infected with InlA strain throughout the experiments (data not shown).

5.3.2.2 ΔlipA and ΔlipAΔlipB co-localised with PI(3,4)P₂ at a lower ratio than InlA

During the infection of pEGFP-TAPP1-PH transfected Hela M cells, InlA::ΔlipA, InlA::ΔlipB and InlA::ΔlipAΔlipB were all observed to co-localise with PI(3,4)P₂ at 6 hr and 24hr post-infection (Fig. 5.20).

The number of total intracellular bacteria in transfected cells and the PI(3,4)P₂ associated L. monocytogenes were counted in randomly chosen transfected Hela M cells for InlA, InlA::ΔlipA, InlA::ΔlipB, InlA::ΔlipAΔlipB and
InlA::ΔhlyΔplcB strains from slides fixed at 6 hr post-infection. The same infections were also performed on none-transfected Hela M cells, in which the cells were fixed and stained with phalloidin. The number of total intracellular bacteria and actin-binding bacteria was counted in randomly chosen Hela M cells. The percentage of PI(3,4)P₂- or actin-associated bacteria in total bacteria was calculated and compared in and Fig.5.21.

The result showed that approximately 50% of the intracellular bacteria were associated with actin at this time point. There was no statistically significant difference in the percentage of actin-binding L. monocytogenes between the InlA and any of the InlA::ΔlipA, InlA::ΔlipB or the InlA::ΔlipAΔlipB mutants (Fig. 5.21). This confirms our earlier data (see Fig. 4.14). There was 46% of the intracellular InlA co-localised with PI(3,4)P₂. However, significantly less InlA::ΔlipA (P=0.0175) and InlA::ΔlipAΔlipB (P=0.00132) were associated with PI(3,4)P₂ comparing to InlA, while InlA::ΔlipB showed no such defect (P=0.221) (Fig. 5.21). The negative control strain InlA::ΔhlyΔplcB did not co-localise with actin or PI(3,4)P₂ at all.
Figure 5.20 Localisation of PI(3,4)P₂ with InlA::ΔlipA, InlA::ΔlipB and InlA::ΔlipAΔlipB in Hela M cells. Hela M cells were transfected with pEGFP-TAPP1-PH for 24 hr and infected with InlA::ΔlipA, InlA::ΔlipB and InlA::ΔlipAΔlipB at MOI 50. Gentamicin was added at 2 hr to 10 µg/ml final concentration. Cells were fixed at 6 hr post-infection, stained with Hoechst 33342 DNA dye which appeared blue, with Rabbit-αListeria and Sheep-αRabbit-TexasRed which appeared red. EGFP-TAPP1-PH appeared green. Photos were taken at 600x magnification. Arrows pointed at bacteria co-localised with EGFP-TAPP1-PH.
Figure 5.21 Percentage of *L. monocytogenes* InlA and mutant strains co-localised with actin or PI(3,4)P2 in Hela M cells 6 hr post-infection. Hela M cells transfected with pEGFP-TAPP1-PH were infected with InlA and mutant strains at MOI 50. At the same time untransfected Hela M cells were also infected. Cells were fixed 6 hr post-infection, stained with Hoechst 33342 DNA dye, Rabbit-αListeria and Sheep-αRabbit-TexasRed. Untransfected cells were also stained with FITC-Phalloidin. The number of bacteria co-localised with actin or PI(3,4)P2 and total intracellular bacteria were counted from randomly chosen phallloidin stained cells, or from successfully transfected Hela cells. Each data represented the average of three independent experiments. Error bars showed the standard error for each data.
5.4 The localisation of LipA and LipB proteins in Hela M cells

The localisation of the two *L. monocytogenes* phosphatases LipA and LipB in Hela M cells was studied by visualisation of over-expressed LipA and LipB in Hela M cells.

5.4.1 Over-expression of LipA and LipB with C-terminal GFP tag

The 1075 bp *lipA* and the 1109 bp *lipB* fragments without stop codons were amplified by PCR from the *L. monocytogenes* EGDe chromosomal DNA with primers *lipAexpr* -F/R and *lipBexpr* -F/R (Table 2.3). The *lipA* fragment was digested with *Pst* I and *BamH* I and the *lipB* fragment digested with *EcoR* I and *BamH* I before inserting into similarly digested pEGFP-N2 plasmid in-frame with EGFP gene. Successful clones of pEGFP-LipA and pEGFP-LipB were screened with *lipAexpr* -F/R and *lipBexpr* -F/R primers (Table 2.3, Fig. 5.22) and confirmed by sequencing with EGFP-N2-SEQ primer (Table 2.3).

![Figure 5.22](image)

**Figure 5.22** a) Colony PCR of pEGFP-LipA with *lipAexpr* -F/R primers. Predicted size: 1.1 kb. b) Colony PCR of pEGFP-LipB with *lipBexpr* -F/R primers. Predicted size: 1.1 kb. DNA marker HyperLadder I (Bioline) was used as an indication of the sizes of bands.
In mammalian cells, these plasmids would over-express the LipA and LipB phosphatases with EGFP protein attached to their C-terminus. Hela M cells were transfected with pEGFP-N2, pEGFP-LipA or pEGFP-LipB for 24 hr and the cells were fixed and stained for fluorescent microscopy (Fig. 5.23).

The transfection of both pEGFP-N2 and pEGFP-LipA were very efficient, with approximately 50% of the cells expressing the fluorescent protein. Besides, the amount of fluorescent proteins expressed in each cell was very high. In the pEGFP-N2 transfected control cells, the expressed EGFP protein was enriched primarily on the Hela M cell nucleus and to a lesser extend distributed in the cytoplasm with no specific target organelle. Over-expressed LipA-EGFP protein showed an almost identical distribution pattern in Hela M cell as the control, indicating either no specific target organelle for LipA in Hela cells, or the localisation of LipA was in the nucleus and cytoplasm (Fig. 5.23). In contrast, only a very small proportion of pEGFP-LipB transfected Hela M cells were detected expressing the LipB-EGFP protein and the fluorescence level was very low in the transfected cell, indicating either a lower transfection efficiency of this plasmid, or a lower expression efficiency of LipB-EGFP from the plasmid. LipB-EGFP was mainly cytosolic with some enrichment close to the cell nucleus, presumably on the Golgi apparatus (Fig. 5.23).

To confirm the localisation of LipB-EGFP on the Golgi apparatus as well as identify whether LipB targets trans- or cis- side of Golgi, pEGFP-lipB transfected Hela M cells were stained with the trans-Golgi marker αGolgin97 or with αGrasp65, which tightly binds the cis-Golgi structure GM130. The result (Fig. 5.24) showed that both αGolgin97 and αGrasp65 antibodies co-localised with LipB-EGFP, confirming that LipB was enriched on Golgi apparatus. However, as LipB-EGFP was also diffused in the cytoplasm and caused a strong background, it was very hard to identify whether it was the cis- or trans- Golgi that LipB targeted.
Figure 5.23 Over-expression of EGFP, LipA-EGFP and LipB-EGFP in Hela M cells. The proteins were expressed from plasmids named on the left. Cells were fixed 24 hr post transfection. DNA was stained with Hoechst DNA dye which appeared blue. LipA-EGFP and LipB-EGFP were shown in green. Photos were taken at 600x magnification.
Figure 5.24 Co-localisation of LipB-EGFP with anti-Golgi markers in Hela M cells. Hela M cells were transfected with pEGFP-LipB for 24 hr. Cells were fixed and stained with Hoechst 33342 DNA dye and either Rabbit-\(\alpha\)Golgin97 with Goat-\(\alpha\)Rabbit-TexasRed or Sheep-\(\alpha\)Grasp65 with Mouse-\(\alpha\)Sheep-Alexa594. Photos were taken at 600x magnification. LipB-EGFP appeared green. Both Golgi markers appeared red.
5.4.2 Over-expression of inactivated LipA and LipB with C-terminal GFP tag

To further elucidate whether the localisation of LipB was due to its phosphatase activity, the phosphatase active site of LipB was neutralised by changing the key amino acid Cys\(^{215}\) (TGC) to Ala\(^{215}\) (GCA) in the P-loop phosphatase active site of LipB (HCTAGKDR between amino acids 212 and 217) via site-directed mutagenesis as previously described in section 2.5, producing pEGFP-LipB-SDM. This mutation would completely abolish the enzyme activity.

Hela M cells transfected with pEGFP-LipB-SDM expressed inactivated LipB-SDM-EGFP protein. The substitution of Cys\(^{215}\) to Ala\(^{215}\) did not seem to affect the transfection efficiency, the expression level or the localisation of LipB protein. As shown in Fig. 5.25, the over-expressed neutralised LipB-EGFP still co-localised with the Golgin97 in Hela M cells.

![Figure 5.25 Over-expression of neutralized LipB-EGFP in Hela M cells. Hela M cells were transfected with pEGFP-LipB-SDM. Cells were fixed 24 hr post-transfection and stained with Hoechst 33342 DNA dye, Rabbit-αGolgin97 and Sheep-αRabbit-TexasRed. DNA appeared blue, LipB-SDM-EGFP was shown in green, Golgin97 appeared red. Photos were taken at 600x magnification.](image-url)
Although LipA did not show specific target in Hela cells, its phosphatase active site was also neutralised by changing the key amino acid Cys$^{189}$ (TGT) to Ala$^{189}$ (GCA) by the same method, producing pEGFP-LipA-SDM. However, the neutralisation of LipA enzyme activity did not alter its location in Hela M cells, as no specific target was observed either (data not shown).

5.4.3 Over-expression of LipA and LipB with N-terminal myc tag

The full coding sequence of LipA and LipB were amplified by PCR from EGDe chromosomal DNA without the promoter region using myc-LipA-F/R and myc-LipB-F/R primers (Table 2.3). Both fragments were digested with EcoRI and BamHI and ligated with similarly digested pcDNA3.1(+)-N-myc plasmid. Successful clones were confirmed by sequencing with the T7 primer (Table 2.3).

In mammalian cells, these plasmids will over-express the LipA and LipB phosphatases with a myc tag (GAACAAAAGCTCATCTCAGAAGAGGATCTG, encoding myc tag EQKLISEEDL) at the N-terminus. Hela M cells were transfected with the empty vector pcDNA3.1(+) -N-myc, pcDNA3.1(+) -myc-LipA or pcDNA3.1(+) -myc-LipB for 24 hr before fixation, were stained with anti-myc antibody and visualised on microscope.

As showed in Fig. 5.26, Hela cells transfected with pcDNA3.1(+) -N-myc did not have any signal in FITC channel, as the empty vector only produced a very short myc epitope which could not be recognised by the anti-myc antibody. The expression of myc-LipA and myc-LipB were both very poor, leading to high background noise. Different from the C-terminal tagged LipA-EGFP, the N-terminal myc tagged LipA protein appeared soluble only in the cytoplasm but not in the cell nucleus, while the myc-LipB target the same location in cytoplasm, presumably Golgi, as the LipB-EGFP.
Figure 5.26 Over-expression of myc-LipA and myc-LipB in Hela M cells. Myc-LipA and myc-LipB were expressed from the plasmids named on the left. Cells were fixed 24 hr post-transfection and stained with Hoechst 33342 DNA dye which appeared blue, with Mouse-αmyc and Donkey-αMouse-Alexa488 which appeared green. Photos were taken at 600x magnification.
5.5 Lamellipodin co-localised with *L. monocytogenes* in Hela M cells

The localisation of an Ena/VASP-binding protein lamellipodin (Lpd), which also contains a PH domain that specifically binds to PI(3,4)P₂, during *L. monocytogenes* InlA infection was studied by either immunofluorescent staining or over-expression of EGFP tagged lamellipodin.

5.5.1 Staining of InlA(pNF8) infected Hela M cells with anti-Lpd antibody

Hela M cells were infected with *L. monocytogenes* InlA containing the pNF8 plasmid at MOI 10. Gentamicin was added to 10 μg/ml final concentration. Infected cells as well as uninfected control cells were fixed at 4 hr, 8 hr and 24 hr post-infection, stained with DAPI, TRITC-Phalloidin and anti-Lpd antibodies and visualised under fluorescent microscope.

In uninfected Hela M cells, the cellular level of lamellipodin was very low and lamellidin was not observed to accumulate at the margin of Hela M cells (Fig. 5.27). Lamellipodin was observed to co-localise with both the InlA strain and actin at 4 hr post-infection (Fig. 5.28). This co-localisation was most abundant at 8 hr (Fig. 5.29) and were still observable at 24 hr post-infection (Fig. 5.30). Lamellipodin only co-localised with *L. monocytogenes* that recruited actin, but not all actin-rich *L. monocytogenes* were associated with lamellipodin.
Figure 5.27 Anti-Lpd and phalloidin staining of Hela M cells. Hela M cells were fixed and stained with antibodies. Cell nucleus were stained with DAPI which appeared blue. Lamellipodin was stained with Rabbit-αLpd and Sheep-αRabbit-Cy5 which appeared cyan. Actin was stained with TRITC-Phalloidin which showed red. Green color showed the level of bleed-through from other channels to FITC channel. Pictures were taken at 600x magnification.

Figure 5.28 Co-localisation of *L. monocytogenes* InlA(pNF8) with lamellipodin and actin at 4 hr post-infection in Hela M cells. Hela M cells were infected with *L. monocytogenes* InlA(pNF8) at MOI 10 and fixed at 4 hr post-infection. DNA was stained with DAPI which appeared blue. Lamellipodin was stained with Rabbit-αLpd and Sheep-αRabbit-Cy5 which were colored cyan. Actin was stained with TRITC-Phalloidin which showed red. *L. monocytogenes* appeared green. Pictures were taken at 600x magnification. Insets showed amplified local areas in the picture. Arrows pointed at a bacterium co-localised with actin and lamellipodin.
Figure 5.29 Co-localisation of *L. monocytogenes* InlA(pNF8) with lamellipodin and actin at 8 hr post-infection in Hela M cells. Hela M cells were infected with *L. monocytogenes* InlA(pNF8) at MOI 10 and fixed at 8 hr post-infection. DNA was stained with DAPI which appeared blue. Lamellipodin was stained with Rabbit-αLpd and Sheep-αRabbit-Cy5 which was colored cyan. Actin was stained with TRITC-Phalloidin which showed red. *L. monocytogenes* appeared green. Pictures were taken at 600x magnification. Insets showed amplified local areas in the picture. Arrows pointed at bacteria co-localised with actin and lamellipodin.
Figure 5.30 Co-localisation of *L. monocytogenes* InlA(pNF8) with lamellipodin and actin at 8 hr post-infection in Hela M cells. Hela M cells were infected with *L. monocytogenes* InlA(pNF8) at MOI 10 and fixed at 24 hr post-infection. DNA was stained with DAPI which appeared blue. Lamellipodin was stained with Rabbit-αLpd and Sheep-αRabbit-Cy5 which was colored cyan. Actin was stained with TRITC-Phalloidin which showed red. *L. monocytogenes* appeared green. Pictures were taken at 600x magnification. Insets showed amplified local areas in the picture. Arrows pointed at a bacterium co-localised with actin and lamellipodin.
5.5.2 Over-expression of Lpd-EGFP in Hela M cells infected with *L. monocytogenes* *InlA(pJEBAN6)*

Hela M cells were transfected with pEGFP-Lpd 24 hr prior to infection with *L. monocytogenes* *InlA* containing the pJEBAN6 plasmid at MOI 10. Gentamicin was added to 10 μg/ml final concentration. Infected cells as well as uninfected control cells were fixed at 4 hr, 8 hr and 24 hr post-infection, stained with DAPI and anti-lamellipodin antibodies and visualised under fluorescent microscope.

In uninfected Hela M cells, Lpd-EGFP was over-expressed in the cytoplasm and did not target any specific organelle (Fig. 5.31). Lamellipodin was observed to co-localised with *L. monocytogenes* *InlA* at 4 hr and 8 hr post-infection (Fig. 5.32, 5.33, 5.34). This co-localisation could not be clearly observed at 24 hr due to the overwhelming number of intracellular baceteria (data not shown). Lpd-EGFP was observed to accumulate on the surface of the bacteria (Fig. 5.33 and 5.34), at one end of the bacteria (Fig. 5.32) or forming comet-tails behind the bacteria (Fig. 5.33 and 5.34), which very much resembled the recruitment of actin and actin polymerisation process during *L. monocytogenes* infection.
Figure 5.31 Over-expression of Lpd-EGFP in Hela M cells. Hela M cells were transfected with pEGFP-Lpd for 24 hr, fixed and stained with DAPI which showed blue. Lpd-EGFP appeared green. Picture was taken at 600x magnification.
Figure 5.32 Co-localisation of *L. monocytogenes* InlA(pJEBAN6) with Lpd-EGFP at 4 hr post-infection in Hela M cells. Hela M cells were transfected with pEGFP-Lpd for 24 hr, infected with InlA(pJEBAN6) at MOI 10, fixed at 4 hr post-infection and stained with DAPI which showed blue. Lpd-EGFP appeared green and InA appeared red in color. Picture was taken at 600x magnification. Insets showed amplified local area in the picture. Arrows pointed at the Lpd-EGFP accumulation at one end of the bacterium.
Figure 5.33 Co-localisation of *L. monocytogenes* InLA(pJEBAN6) with Lpd-EGFP at 8 hr post-infection in Hela M cells. Hela M cells were transfected with pEGFP-Lpd for 24 hr, infected with InLA(pJEBAN6) at MOI 10, fixed at 8 hr post-infection and stained with DAPI which showed blue. Lpd-EGFP appeared green and InA appeared red in color. Picture was taken at 600x magnification. Insets showed amplified local area in the picture. Arrows pointed at Lpd-EGFP forming a comet-tail behind a bacterium. Arrows with asterisk pointed at Lpd-EGFP co-localised with InLA.
Figure 5.34 Co-localisation of *L. monocytogenes* InlA(pJEBAN6) with Lpd-EGFP at 8 hr post-infection in Hela M cells. Hela M cells were transfected with pEGFP-Lpd for 24 hr, infected with InlA(pJEBAN6) at MOI 10, fixed at 8 hr post-infecton and stained with DAPI which showed blue. Lpd-EGFP appeared green and InA appeared red in color. Picture was taken at 600x magnification. Insets showed amplified local area in the picture. Arrows pointed at Lpd-EGFP forming a comet-tail behind a bacterium. Arrows with asterisk pointed at Lpd-EGFP co-localised with InlA.
5.6 Discussion

5.6.1 The visualisation of \textit{L. monocytogenes}

5.6.1.1 Comparison of the three methods used in visualisation of \textit{L. monocytogenes}

In this study, three methods were exploited in order to visualise \textit{L. monocytogenes}: chromosomal expression, plasmid expression and antibody staining. They each have their advantages and disadvantages in regard to the ease of use and efficiency.

The use of chromosomal fusions that result in the stable expression of a fluorescent protein provides live-time expression of fluorescence inside the bacteria. Single copy expression from the chromosome provides homogenous fluorescence among the bacteria population, thus allows quantitative studies to be performed. It is also suitable for both fixed slides and live imaging of the bacteria, as well as in flow cytometry. The main disadvantage for this method is the difficulty in construction of the strains.

Previously chromosomal integration of a single copy GFP sequence has been successfully constructed in bacteria and eukaryotic cells such as \textit{Zymomonas mobilis} (Delgado \textit{et al.}, 2002), \textit{Drosophila melanogaster} (Casso \textit{et al.}, 2000). In 2010, Balestrino and colleagues reported successful construction of integrative fluorescent plasmids for single copy constitutive chromosomal expression of GFP, CFP (cyan fluorescent protein) and YFP proteins in \textit{L. monocytogenes}. However, instead of inserting the fluorophore sequence itself into the chromosome via homologous recombination as I did, their study involved the integration of the whole pPL2 or pAT18 integrative vectors, carrying the \textit{Phyper} promoter and fluorophore sequences, into the chromosome (Balestrino \textit{et al}, 2010).
The second method of plasmid expression of fluorescent protein has been widely used as a reporter system for many bacteria such as *S. enterica Typhimurium*, *Streptococcus suis* and *Mycobacterium marinum* (Valdivia et al., 1996; Lun et al., 2004; Clark et al., 2009). Several studies have reported plasmid-expressed fluorescence labelling system in *L. monocytogenes* (Fortinea et al., 2000; Andersen et al., 2006; Balestrino et al., 2010). Plasmids that express fluorescent proteins are easier to construct than chromosomal fusion. It could also be used in live-cell imaging and allows real-time monitoring of bacteria. The shortcomings in this method are, firstly antibiotic selection is required in the maintenance of the plasmid over long period of time; secondly, the heterogeneity in the expression level in individual bacterium causes unequal fluorescence level among the population (Balestrino et al., 2010). Furthermore, the high copy expression of fluorescent protein might impose a metabolic burden to the bacteria and other genes on the plasmid might affect the virulence as well. For example, the invasiveness and SPI-1 gene expression of *S. enterica* was adversely affected by the GFP expressing vector pBR322 with chloramphenicol resistance (Clark et al., 2009).

The last method, antibody staining, allows a quick and efficient detect of the bacteria. By changing the secondary antibody, *L. monocytogenes* could be easily labelled with different fluorophores in order to be distinguished from other cell structures. The main drawback is that this method could only apply to fixed cells, thus not suitable in live imaging or flow cytometry.

**5.6.1.2 Analysis of the chromosomal and plasmid expression of mCherry**

In our study, the full coding sequence GFP_{uv} and mCherry with the 5′ untranslated region including the rpoB S-D sequence were inserted downstream of the rpoC gene. In the chromosomal fusion strain, GFP_{uv} and mCherry were co-transcribed with the house-keeping genes rpoB and rpoC,
which should maintain a constant level of transcription. The rpoB S-D region has been used successfully in initiating the translation of lacZ gene inserted to L. monocytogenes chromosome (Corbett et al., 2011, Corbett et al., 2012). The InlA::GFPuv strain could be visualised under confocal microscopes. Correct filter set was needed to visualise this strain in widefield snapshot fluorescent microscope. The fusion of mCherry into InlA chromosome was successful, but the expression level was too low for microscopy.

In my plasmid expression study, pUNK1-PrpoB-mCherry plasmid failed to express mCherry in L. monocytogenes to a visible level, while in E. coli DH5α, the expression level of mCherry was very high (Table 5.2). Although Balestrino et al. (2010) succeeded in chromosomal expression of GFP, CFP and YFP proteins, they were not able to generate a strain expressing red fluorescence in either chromosomal fusion or plasmid expression. Plasmids harbouring the DsRed, its variants mRFP1 or mCherry encoding genes and the Phyper promoter all failed to express fluorophore in L. monocytogenes, but easily expressed in E. coli, which mirrors our observation.

Through bioinformatic analysis, I found out that the codon usage of L. monocytogenes was not favourable for the translation of mCherry protein. The codons in mCherry sequence were analysed and compared to the codon usage in L. monocytogenes and E. coli (Table 5.6). The majority of the codons used in mCherry were not the most favourite codon in L. monocytogenes. In particular, the coding sequence for mCherry preferentially uses the codon CTG for the amino acid Leu, but this is the least utilized codon for Leu in L. monocytogenes accounting for 0.4% of the total codons in cDNA. Likewise, the codon CCC preferentially used for the amino acid Pro in mCherry was the least used codon in L. monocytogenes. Other amino acids such as Ser, Ala, His and Glu are all encoded by the least frequently used codons in L. monocytogenes. In contrast, in E. coli, the codon usage was largely in favour of the codons in mCherry, with the only exception of the Pro codon.

[180]
However, the unfavourable codon usage in *L. monocytogenes* was not the only reason for the low expression level of mCherry from pUNK1-PrpoB-mCherry or in the chromosome. Table 5.6 showed that the codon usage in DsRedExpress was almost identical to mCherry, but pJEABAN6 plasmid could express DsRedExpress protein to visible level. The pJEABAN6 backbone pAT18 and pUNK1 plasmids are both high copy number plasmid as well (Fortinea *et al.*, 2000). Therefore, it is most likely that the *rpoB* promoter in pUNK1-PrpoB-mCherry did not provide enough transcription as the *dlt* promoter in pJEABAN6.

Comparing the codons in mCherry and DsRedExpress to GFP$_{mut1}$ (Table 5.6), we can see that the codons in GFP$_{mut1}$ expressed from pNF8 plasmid is much more favourable for bacterial expression than the two mammalian codon optimised red fluorescent proteins. This matches our observation that the expression level from pNF8 was much higher than that from pJEABAN6 in *L. monocytogenes* (Table 5.3).

In summary, the chromosomal and plasmid expression of fluorescent proteins in *L. monocytogenes* were determined by both the transcription level and the codon usage. In our study, the *rpoB* promoter might not be strong enough to induce enough transcription of mCherry from the chromosome or from pUNK1-PrpoB-mCherry plasmid, nor was the mCherry codon optimised for bacteria expression, thus we were not able to visualise the InlA::mCherry or InlA(pUNK1-PrpoB-mCherry) strains with fluorescent microscope.
Table 5.6 Codon usage of mCherry, DsRedExpress and GFPmut1 by *L. monocytogenes* and *E. coli*. The numbers in bold indicate codons rarely used in the bacteria.
5.6.2 The distribution of PIPs during *L. monocytogenes* infection

PIPs play an important role in the reversible recruitment of proteins to restricted intracellular membranes, which allows the temporal and spatial regulation of complex cellular functions such as signal transduction, cytoskeleton architecture and membrane trafficking (Czech and Corvera, 1999; Weber *et al.*, 2009). The localisation of each PIP has been studied mainly via the expression of GFP fused with the PH domains that show a PIP-selective binding profile *in vitro* (Gillooly *et al.*, 2000; Balla and Varnai, 2009). In this study, I looked into the localisation of PI3P, PI4P, PI(4,5)P₂, PI(3,4)P₂ and PI(3,4,5)P₃ in Hela M cell line during the infection by *L. monocytogenes*.

### 5.6.2.1 PI4P, PI(4,5)P₂, PI(3,4,5)P₃

In mammalian cells, the plasma membrane is enriched primarily with PI4P, PI(4,5)P₂ and transiently with PI(3,4,5)P₃ during signal transduction and phagocytosis (Henry *et al.*, 2006). PI4P predominantly accumulates on the Golgi apparatus, secretory vesicles and also on ER (Blumental-Perry *et al.*, 2006).

In this study, the PI4P specific EGFP-FAPP1-PH probe as predicted was intensely accumulated on the Golgi apparatus and to a lesser extent on the ER as well. No enrichment on the plasma membrane was observed in Hela M cells. It needs to be stated that the PI4P exists in several discrete pools rather than on a single organelle in the cells (Roy and Levine, 2004). The FAPP1-PH, OSH1 (oxysterol binding protein homologues of *S. cerevisiae*)-PH and OSHP (oxysterol binding protein) domains recognise the Golgi pool of PI4P both in yeast and in mammalian cells (Levine and Munro, 2002; Roy and Levine, 2004). The PI4P-recognising OSH2-PH domain was reported to recognise both plasma membrane and Golgi pool of PI4P while another study found it only localised to the plasma membrane but did not show Golgi
localisation (Roy and Levine, 2004; Balla et al., 2009). There is not a single domain that has been identified to be able to detect the all PI4P pools in the cell (Balla et al., 2009). The InlA strain did not affect the localisation of PI4P in Hela M cells. Although PI4P is the substrate of both LipA and LipB, the infection of InlA::ΔlipA or InlA::ΔlipB showed no difference from the InlA in pEGFP-FAPP1-PH transfected cells.

PI(4,5)P₂, recognised by EGFP-PLCδ-PH probe, was observed on the plasma membrane and Golgi, which conforms previous observations (Di Paolo and De Camili, 2006). It has been reported that PLCδ-PH and Akt-PH domains simultaneously present on the surface of L. monocytogenes in PtK2 cells 4 hr post-infection due to their binding to PI(4,5)P₂ and PI(3,4,5)P₃. However, no co-localisation of PI(4,5)P₂ with the bacteria was observed in this study. It is possible that this co-localisation is dependent on cell types, or this was due to unidentified experimental artifact.

PI(3,4,5)P₃ could be recognised by both Akt-PH and ARNO-PH domains, between which the former is less specific as it recognises PI(3,4)P₂ as well. In Hela M cells, the YFP-ARNO-PH probe did not show specific localizaion and it had displayed nucleus localisation as well. The transient accumulation of PI(3,4,5)P₃ during the induced endocytosis of L. monocytogenes was not be observed due to the fixed time points selected. The InlA strain and mutants were not observed to recruit PI(3,4,5)P₃ at any time point.

However, our observation could not exclude the possibility of L. monocytogenes directly or indirectly altering the cellular levels of PI4P, PI(4,5)P₂ and PI(3,4,5)P₃. The transfection efficiency and the expression level of EGFP/YFP fusion probes were varied among the host cells. Fluorescent microscopy does not allow an accurate quantitative estimation of the PIP levels. Therefore just by eye observation we could not determine whether
the PIP level was affected by *L. monocytogenes* unless a dramatic change occurred.

### 5.6.2.2 PI3P

PI3P was found mainly on phagosomes, early endosomes and on the internal vesicles of multivesicular endosome in mammalian cells (Weber *et al.*, 2009). PI3P has been reported to be recruited to early endosomes containing or perforated by *L. monocytogenes* up to 30 min post-infection (Henry *et al.*, 2006).

In my study, the PI3P-rich *Listeriae*-containing phagosomes were observable at 1.5 hr post-infection, but disappeared by 2.5 hr. At 1.5 hr post-infection some of the bacteria were assumed to internalize into Hela M cells and still remained in the early endosomes, which were decorated by PI3P on the membrane. By 2.5 hr, most of the internalised bacteria have escaped from the phagosome, leading to the dissociation of PI3P with the bacteria.

### 5.6.2.3 PI(3,4)P2 and lamellipodin

PI(3,4)P2 has been reported to be detected at the phagocytic sites in macrophages (Horan *et al.*, 2007). My result showed that in Hela M cells PI(3,4)P2 was cytosolic with no specific compartmentalisation or plasma membrane association.

The most interesting observation was the association of PI(3,4)P2 with *L. monocytogenes* InlA and InlA::ΔlipA, InlA::ΔlipB, InlA::ΔlipAΔlipB mutants during the cytosolic growth stage of the bacteria. This was the first time a co-localisation of PI(3,4)P2 with intracellular pathogenic bacteria was directly observed. PI(3,4)P2 was enriched on the surface of the *L. monocytogenes* and also formed comet-tail-like structures, presumed to be where actin
polymerisation occurred. No association of PI(3,4)P₂ was observed with InlA::ΔhlyΔplcB strain, which was unable to escape from the phagosome, suggesting the recruitment of PI(3,4)P₂ occurred only after *L. monocytogenes* escaped from the phagosome. Phalloidin staining at the same time point further confirmed that by 6 hr post-infection many of the bacteria were outside the phagosome and replicated in the cytosol. As the PIPs are membrane anchored lipids, it is assumed that PI(3,4)P₂ was either produced at the surface of *L. monocytogenes* (e.g. through the dephosphorylation of PI(3,4,5)P₃ or phosphorylated from PI4P), or recruited to the bacteria by effector proteins not yet discovered on the surface of *L. monocytogenes*.

At 6 hr post-infection 46% of the total intracellular the InlA stain were associated with PI(3,4)P₂, which is the same ratio as actin-associated bacteria, leading to our assumption that the recruitment of PI(3,4)P₂ coincides with the actin polymerisation process. However, during the infection of pEGFP-TAPP1-PH transfected Hela M cells with the lipA and lipB single or double mutants, there were significant lower ratios of InlA::ΔlipA and InlA::ΔlipAΔlipB associated with PI(3,4)P₂, even though they were not affected in actin polymerisation. Therefore this accumulation PI(3,4)P₂ may be independent from actin polymerisation, but is restricted to actin-recruiting bacteria.

It has been reported that the recruitment of lamellipodin, a structural protein actin that has a much higher affinity to PI(3,4)P₂ than any other tested 3’-phosphorylated phosphoinositides (e.g. PI3P or PI(3,4,5)P₃), to the leading edge of the MDA-231 breast cancer cells was dependent on PI(3,4)P₂ (Bae *et al.*, 2010). Increasing the plasma membrane availability of PI(3,4)P₂ via the depletion of profilin 1 leads to increased cell mobility (Bae *et al.*, 2010). Lamellipodin was reported to be recruited to *Vaccinia Virus* and *Enteropathogenic E. coli* at their interface with the actin tail or actin pedestal, but was not recruited to *S. flexneri* or *L. monocytogenes* up to 4 hr
post-infection (Krause et al., 2004). However, my result of anti-Lpd antibody staining and overexpression of EGFP-tagged lamellipodin both showed that lamellipodin was accumulated on the surface of _L. monocytogenes_ InlA or forming comet tails behind the bacteria from 4 hr to 24 hr post-infection. Co-staining of infected Hela M cells with anti-Lpd antibody and phalloidin further showed co-localisation of lamellipodin with actin. Therefore, instead of the well-received actin-based mobility model that _L. monocytogenes_ uses the force generated by actin polymerisation to shoot into adjacent cells, we propose a novel model that the extrusion of _L. monocytogenes_ could be assisted by the recruitment of lamellipodin in a PI(3,4)P2-dependent manner. Lamellipodin directs _L. monocytogenes_ to the peripheral region of the host cell with the assistance of PI(3,4)P2, causing rearrangement of cytoskeleton and possibly the development of filopod at the protruding site of the bacteria, which leads to the projection of _L. monocytogenes_ and reduction in the resistance force against the actin-powered movement. This process was assumed to occur in concert with actin polymerisation, but the two processes were probably independent from each other. Therefore, ΔactA mutant is predicted to be unaffected in the recruitment of PI(3,4)P2.

We also hypothesized that the recruitment of lamellipodin was partially induced by the phosphatase LipA, which dephosphorylates PI3P, PI4P, PI5P and PI(3,5)P2. The dephosphorylation of cytosolic PI(3,5)P2 could produce PI5P, which leads to the activation of PI3K, producing PI(3,4)P2 and PI(3,4,5)P3, and the dephosphorylation of PI(3,4,5)P3 also produces PI(3,4)P2 (Fig. 5.35). This production of PI(3,4)P2 might occur quickly on the surface of _L. monocytogenes_ when LipA was secreted, which then recruits the PI(3,4)P2-binding lamellipodin. In ΔlipA and ΔlipAΔlipB mutants, there would be no production of PI5P on the surface of the bacteria, leading to reduced PI3K activation and less production of PI(3,4)P2 (Fig. 5.35). However, LipA can not be the only source of the PI(3,4)P2 produced, as in ΔlipA and ΔlipAΔlipB mutants PI(3,4)P2 was detected on the surface of some the bacteria as well.
It is likely that other unknown enzymes also contributes to elevating PI(3,4)P₂ level, in which LipA plays an regulatory role.

In future study, the localisation of lamellipodin during \textit{L. monocytogenes} \textit{ΔlipA} and \textit{ΔlipAΔlipB} infection should shed light on whether LipA plays a role in the hypothesized lamellipodin pathway. Secondly, the localisation of PI5P and PI(3,5)P₂ are to be studied during \textit{L. monocytogenes} InlA and mutants infection. It is predicted that at 6 hr post infection, PI5P would accumulate on the surface of \textit{L. monocytogenes} InlA, but not on \textit{ΔlipA} and \textit{ΔlipAΔlipB} mutants. No co-localisation of PI(3,5)P₂ with \textit{L. monocytogenes} should be observed. However, currently there is not a promer domain identified yet that binds specifically to PI5P or PI(3,5)P₂. Furthermore, it is predicted inhibition of the PI3K activity with PI3K inhibitor (e.g. LY294002) would stop the recruitment of PI(3,4,5)P₃ and lamellipodin.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5.35.png}
\caption{A hypothesized role of LipA during \textit{L. monocytogenes} infection. LipA mediated the production of PI5P from PI(3,5)P₂ on the surface of the bacteria, which activates the PI3K and leads to downstream production of lamellipodin-binding PI(3,4)P₂.}
\end{figure}
5.6.2.4 Akt-PH recognition of PI(3,4)P$_2$ and PI(3,4,5)P$_3$

One problem raised in this study was that although PI(3,4)P$_2$ co-localised with *L. monocytogenes*, this association was not observed in YFP-Akt-PH labelled Hela M cells, which should recognise both PI(3,4)P$_2$ and PI(3,4,5)P$_3$. Akt-PH was reported to be recruited to actin-rich tails of motile *L. monocytogenes* in MDCK cells and Ptk2 cells as well (Sidhu *et al.*, 2005). This was probably because: i) the association of Akt-PH with *L. monocytogenes* is dependent on cell types; ii) the Akt-PH domains preferentially bound to PI(3,4,5)P$_3$ over PI(3,4)P$_2$ in Hela M cells, resulting in the poor recognition of *L. monocytogenes*-associated PI(3,4)P$_2$; iii) the high-level expression of soluble Akt-PH domain in the cytoplasm of Hela M cells occupied the recognition sites of PI(3,4,5)P$_3$ and PI(3,4)P$_2$, which might affect the normal PIP metabolism by *L. monocytogenes*.

5.6.2.5 Discussion for the expression of EGFP/YFP tagged PIP-binding domains

The visualisation of PIPs inside mammalian cells was based on the premise that some protein molecules possess high affinity and specificity to the inositol headgroup of specific PIPs (Balla and Varnai, 2009). By expressing fluorescent protein tagged PIP-specific domains in mammalian cells, the changes in host PIP metabolism during *L. monocytogenes* infection could be followed. However, several issues need to be minded during this procedure.

a) In my results, FENS/FYVE, ARNO-PH AND Akt-PH all showed very prominent nuclear localisation. This was because all of these PIP-recognition domains could penetrate the nuclear membrane and enter the nucleus (Balla and Varnai, 2009). This nuclear localisation was not due to the PIP-binding activity of these domains, but possibly mediated by the numerous basic residues characteristic of these domains (Balla and Varnai,
2009). In contrast, PIP that is abundantly present and compartmentalised in the cells would prevent its recognition domain from entering the nucleus (Balla and Varnai, 2009). This was supported by our observation that the PI(4,5)P₂-binding PLCδ-PH domain showed no nuclear localisation, and the nuclear localisation for the PI(3,4)P₂-binging TAPP1-PH and PI4P binding FAPP1-PH domains were not very strong.

b) As the PIP-binding probes were over-expressed in Hela M cells, the high level of the expressed domains might compete with other PIP effector proteins and therefore inhibit the cellular responses regulated by the PIPs, and even causes cell morphology change (Balla and Varnai, 2009). The invasion and intracellular survival of *L. monocytogenes* utilises host PIPs such as PI4P and PI(3,4,5)P₃. Therefore the inhibition effect of these PIP-binding domains might cause negative effects the survival of the bacteria, although no apparent inhibition of *L. monocytogenes* growth was observed in the transfected cells.

c) In this study, fixed cell immunofluorescent microscopy was used to observe the localisations of PIPs at different stages of *L. monocytogenes* infection. However, this method could not quantify the PIP level on certain cellular compartment, nor could it follow the changes in within a short time span during *L. monocytogenes* infection. In the future, live cell imaging could be used to help solve these issues, by which the subtle changes in PIP level could be directly observed.

5.6.3 The localisation of LipA and LipB

In this study, LipA was overexpressed with either the C-terminal EGFP tag or the N-terminal myc tag. LipA-EGFP was primarily enriched on the Hela M cell nucleus, which was the same as the EGFP expressed on its own, while myc-LipA was cytosolic with no specific compartmentalisation. Bioinformatic
analysis also revealed no nucleus targeting sequence in the LipA protein. Therefore, this indicated that the enrichment of LipA was caused by the EGFP tag.

Similarly, the localisation of LipB was studied by the overexpression of LipB-EGFP or myc-LipB. In both cases LipB was detected in the cytoplasm with some enrichment on the Golgi apparatus. Whether this enrichment was on cis- or trans- side of the Golgi was not clear from staining with the trans-Golgi marker Golgin97 or the cis-Golgi marker Grasp65 due to the poor expression level of LipB-EGFP and myc-LipB. In mammalian cells, the Golgi apparatus functions in protein modification, vesicular transport as well as transportation of cells around the cells (Cooper, 2000). It also contains PIPs such as PI4P and PI(4,5)P_2. Therefore, it is likely that during L. monocytogenes infection, LipB is secreted into the host cell cytoplasm and is partially recruited to the Golgi apparatus, where it exerts its phosphatase function against phosphotyrosine and the three monophosphoinositides PI3P, PI4P and PI5P.

Neutralisation of the phosphatase activity of LipB by a substitution of Cys with Ala in the P-loop signature site did not alter the distribution of LipA in Hela M cells. This indicated that the possible recruitment of LipB to the Golgi apparatus was not dependent on its phosphatase activity but was probably directed by other signalling motifs in LipB.

Comparing the enzymatic activity of LipA and LipB, their protein phosphatase activities were at similar levels. However, the PIP phosphatase activity for LipA is 10 times higher than LipB (Beresford et al., 2010). This data, together with the observation that LipB but not LipA is enriched on the Golgi apparatus, leads to the hypothesis that in contrast to LipA, LipB function more as a protein phosphatase rather than lipid phosphatase in the host cell than LipA. This could also explain why ΔlipA mutant is affected in
the recruitment of PI(3,4)P₂ while ΔlipB mutant is not, as the LipA has a
greater impact on the host PIP metabolism than LipB due to its higher lipid
phosphatase activity.

However, in this study the tagged LipA and LipB were expressed abundantly
in the Hela M cells, therefore our observation might not reveal the precise
localisation of LipA and LipB during the *L. monocytogenes* infection, where
the production of both phosphatases would be considerably lower.
Developing LipA and LipB specific antibodies would provide a more accurate
and alternative approach in locating the two phosphatases.
Chapter 6 General discussion

This research aims at studying the modulation of host PIP metabolism during *L. monocytogenes* infection via the production of two newly discovered phosphatases LipA and LipB.

The roles that LipA and LipB played during the intracellular survival of *L. monocytogenes* were studied via intracellular survival assay with *lipA* and *lipB* knockout mutants in two human epithelial cell line Caco-2 and Hela M cells as well as mouse macrophage J774.A1 cell line. ΔlipA, ΔlipB and ΔlipAΔlipB mutants were unaffected in invasion but significantly attenuated in intracellular growth in either Caco-2 cells or Hela M cells. No growth defect of *lipA* or *lipB* mutant was observed in J774.A1 cells. Deletion of *lipA* or *lipB* did not affect the actin polymerisation. Cell-to-cell spread did not seem to be affected as the same sized plaques were formed by *lipA* mutant as InlA. However, reduced number of plaques formed by ΔlipA, ΔlipB and ΔlipAΔlipB mutants was observed in plaque assays.

The turnover of five PIPs in Hela M cells during *L. monocytogenes* infection as well as the roles that LipA and LipB played were studied. *L. monocytogenes* InlA and mutants did not seem to affect the localisation of PI4P, PI(4,5)P$_2$ and PI(3,4,5)P$_3$. PI3P were recruited to *L. monocytogenes* containing phagosomes as previously described. The most important finding was the first observation of the recruitment of PI(3,4)P$_2$ and one of its effector lamellipodin to the *L. monocytogenes*. This leads to the hypothesis of a new model of cell-to-cell spread, in which *L. monocytogenes* produces PI(3,4)P$_2$ on its surface to induce the recruitment of lamellipodin, which facilitates cytoskeleton rearrangement and formation of filopodia at the *L. monocytogenes* escaping site and assist its movement out of the cell (Fig. 6.1).
Overexpression of LipA in Hela M cells suggested no specific compartmentalisation of this secreted phosphatase in the cytoplasm. One of the functions of LipA was hypothesized to convert PI(3,5)P₂ into PI5P on the surface of *L. monocytogenes*, leading to the activation of PI3K, which then produces PI(4,5)P₂, which helps to recruit lamellipodin.

This hypothesis could also partially explain the reduced plaque number for *lipA* mutants. PI(4,5)P₂ accumulated on the surface of a smaller ratio of Δ*lipA* and Δ*lipAΔlipB* while no obvious reduction in the PI(3,4)P₂ level on individual bacteria as observed. Therefore the smaller proportion of PI(3,4)P₂-rich *lipA* mutants among the population leads to reduced proportion of...
lamellipodin-associated ΔlipA and ΔlipAΔlipB, and therefore reduced proportion of bacteria that efficiently spread to adjacent cells. While for those that do accumulate PI(3,4)P₂, no cell-to-cell defect occurs therefore the same sized plaques are formed as the wild type. Besides this, the overall intracellular growth defect and/or reduced ability to escape secondary vacuoles might contribute to the reduced plaque formation, resulting in a more severe reduction in plaque number (a 75% reduction in InlA::ΔlipA) than in PI(3,4)P₂-associated bacteria (a 36% reduction in InlA::ΔlipA).

On the other hand, the other phosphatase LipB did not seem to affect the localisation of any of the five PIPs studied in this project. Overexpression of LipB showed it partially localised at the Golgi complex, the organelle where protein modification and transportation mostly occurs and PI4P is most abundant. Its enzymatic activity profile also showed much less inositol phosphatase activities comparing to LipA. It is therefore hypothesized that LipB functions more as a protein phosphatase, while its lipid phosphatase activity contributes less in the intracellular survival of *L. monocytogenes*. Although LipB may not be involved in the proposed PI(3,4)P₂-lamellipodin interaction, the general growth defect and possible attenuation in double-layered phagosome escape might still account for the reduction in plaque number form by ΔlipB and ΔlipAΔlipB mutants. Despite our study of the function of LipB, it is still unknown whether LipB is a secreted protein. Its protein sequence does not contain any signalling sequence or membrane anchor. Considering that the inositol substrates of LipB are not present inside *L. monocytogenes*, it is likely that it is secreted rather than remain inside the bacteria, which still awaits validation.

In addition, the visualisation of *L. monocytogenes* has been studied with three different approaches. Chromosomal and plasmid expression of fluorescent proteins in *L. monocytogenes* was found to rely on both a strong promoter and optimised codon usage of the fluorescent protein. The GFP
derived proteins are generally better expressed in *L. monocytogenes* than the red fluorescent protein derivatives (e.g. mCherry and DsRed) due to better codon recognition by *L. monocytogenes*. The use of pJEBAN6 and pNF8 plasmid and anti-*Listeria* antibody provide specific recognition and flexibility in the visualisation of *L. monocytogenes*.

In summary, this research focused on the characterisation of the two *L. monocytogenes* protein and inositol phosphatases LipA and LipB, whether *L. monocytogenes* modulates the host phosphoinositide metabolism during infection, and how LipA and LipB participate in this modulation. Both *lipA* and *lipB* genes are required for the intracellular growth in Caco-2 and Hela M cells but not J774.A1 macrophages. They are not required for actin-based motility. LipA is hypothesized to induce the production of PI(3,4)P$_2$ on the surface of the bacteria by converting PI(3,5)P$_2$ into PI5P. LipB is considered to be secreted during infection and localises to the Golgi complex, where it exerts its role in the dephosphorylation of phosphotyrosine. A novel model of lamellipodin assisted cell-cell spread was also proposed based on the recruitment of lamellipodin to *L. monocytogenes*.

Future work needs to be carried out in revealing other roles of LipA and LipB during *L. monocytogenes* infection as well as testing the hypothesis proposed. The following work could be done to begin with:

a) Determination of whether LipB was a secreted protein. This could be done by plasmid expression of GFP-tagged LipB in *L. monocytogenes* and western blot on cytoplasm and membrane precipitation as previously described (Kastner *et al.*, 2011).

b) Studying the ability of *lipA* and *lipB* in escaping the secondary vacuoles.

c) Looking into the localisation of PI5P, PI(3,5)$_2$ during *L. monocytogenes* InlA and mutants infection.

d) Studying the localisation of lamellipodin during the infection of ΔlipA, ΔlipB, ΔhlyΔplcB and ΔactA
mutants, and whether other lamellipodin-modulation factors would affect the cell-to-cell spread of the bacteria.

Altogether, this study provides some evidence on \textit{L. monocytogenes} modulates host PIP metabolism by the production of two virulence factor phosphatases. This gives us a better understanding on the intracellular growth of this pathogenic bacterium, and on the interaction between host and parasite.
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