Genes involved in attachment of *Listeria monocytogenes* to abiotic surfaces

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

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September 2012

Abstract

This study investigated genes involved in attachment of *Listeria monocytogenes* to abiotic surfaces. *L. monocytogenes* has previously been shown to attach to a variety of different abiotic surfaces, including polystyrene, stainless steel and glass. Consequently biofilm formation is a significant problem in food processing industries. An existing attachment assay was modified to allow screening of transposon mutants for a deficiency in attachment of bacteria to wells of a microtitre plate. Using this assay, three mutants were identified that had previously been shown to have reduced attachment to stainless steel and glass. The location of insertion of the transposon into each of the mutants M113, B380 and M237 was mapped to the genes *lmo1226*, *lmo0401* and *lmo0501*, respectively. Deletion mutants were made in these genes and the gene *lmo0402* to better clarify the role of these genes in attachment. The gene *lmo1226* was identified as a drug exporter of the RND superfamily, responsible for the streptomycin resistance exhibited by *L. monocytogenes* strain 10403s. *Lmo1226* is also involved in attachment, virulence, and growth of *L. monocytogenes* in sodium chloride. The genes *lmo0401* and *lmo0501* were identified as an α-mannosidase and a BglG transcriptional antiterminator respectively, and were identified to be involved in attachment alongside another transcriptional antiterminator (*lmo0402*). The transcriptional antiterminators were shown to be involved with transcription of the Bgl operon which encodes genes required for phosphoenolpyruvate phosphate transferase systems in *L. monocytogenes*. The results from these experiments have helped to elucidate the processes involved in *L. monocytogenes* attachment to surfaces, and have identified targets for future antimicrobials against this bacterium.

Acknowledgements

Firstly I would like to thank my supervisor Professor Peter Andrew for all his help and guidance during this thesis, and also for giving me the opportunity to study for this PhD.

I would also like to thank Professor Ian Roberts and Dr Dave Corbett for providing me with the InlAm strain and for the guidance with the mutagenesis, and Dr Richard Haigh for providing me with the Caco-2 cells and for the help and advice with growing them provided by him and Dr Hannah Brewin. Thank you also to Dr Hasan Yesilkaya for his suggestion of the cloning kit.

Thanks to the members of lab 227 and the department of 3I, both past and present, especially Rana, Hannah, Claire, Eulyn, Mafalda, Mina, Sonia, Magda, and Nawal thanks for all the friendship, support and lots of laughs. Thanks especially to Emma who kept me from loosing my mind at the end, and proof read some of my thesis for me.

To my family, Mum, Dad, and Ali, thanks for your love and encouragement. I am very grateful for everything you have all done for me. Thanks also to my Nan who is an inspiration to me, and the rest of my family for your continued love and support. To my 'adopted sister' Helen, thanks for your friendship and support and cheering me up when it got too much. Would be lost without you mate! Thanks also to Chris' family for all their support. To everyone I have lost during this PhD, both my granddads, my auntie Ezz, and of course my good friend Simon, I miss you more than I could ever say and I wish you were all still with me.

And finally to Chris, this thesis would never have been completed if it wasn't for you. You gave me the energy and strength to finish, and supported me every step of the way. Thanks for taking such good care of me and calming me down when it got too much! I love you so much.

Abbreviations

ANOVA	Analysis of variance
bp	Base pairs
Caco-2	Human colon epithelial carcinoma cell line
cfu	Colony forming units
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
npH20	Nanopure water
NaCl	Sodium chloride
EPS	Exopolysaccharide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	β-D-1-thiogalactopyranoside
kb	Kilo base pairs
Kv	Kilovolt
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEP-PTs	Phosphoenol pyruvate phosphotransferase
qRTPCR	Quantitative reverse transcriptase PCR
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
μF	Microfaraday
UV	Ultraviolet
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

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Chapter 1 – Introduction

Listeria monocytogenes is a Gram-positive coccobacillus that is readily isolated from different environmental niches and a known pathogen of both humans and animals. *L. monocytogenes* causes an invasive infection termed listeriosis. Progression of listeriosis can vary greatly resulting in a gastrointestinal illness or in more severe cases encephalitis, septicaemia, or abortion in pregnant females in both animals and humans. *L. monocytogenes* is able to infect healthy individuals, but is predominately a pathogen of the elderly, immunocompromised or pregnant women, and is normally of food-borne origin. Studies at a genomic level into this organism are leading to a better understanding of how *L. monocytogenes* establishes infection in humans.

Despite this increase in understanding of the mechanisms involved in infection, the number of cases of listeriosis has risen markedly within the last decade, primarily thought to be due to changes in lifestyle. *L. monocytogenes* has become a significant problem within food processing factories such as cheese dairies and meat packaging factories due to the ability of this bacterium to adhere to surfaces and persist in extreme environments. It is well known that *L. monocytogenes* is able to adhere to surfaces and form a biofilm yet despite this there is still little understanding into the complex processes involved in biofilm formation by this organism, further study is still required in this area. This study will attempt to elucidate some of the key genes involved in the process of attachment of this organism to abiotic surfaces. This introduction will give a broad background into *L.*

monocytogenes and then go on to discuss biofilm formation and the factors involved in these processes.

1.1 *Listeria monocytogenes*: An overview

1.1.1 History

The organism *Listeria monocytogenes* was first isolated by Murray *et al*. in 1926, when it was identified as the causative agent of a septicaemic infection of rabbits and guinea pigs (Murray *et al.*, 1926). Investigations into the cause of death of these animals led to the isolation of a small Gram-positive coccobacillus which was identified as the cause of the infection. The isolated organism was originally termed *Bacterium monocytogenes* by Murray. *Bacterium* was thought to be chosen due to the doubt over the correct taxonomic placement of the bacteria at this time, and *monocytogenes* was chosen to reflect the significant monocytosis observed in the blood of the infected animals. A year after the discovery by Murray, Pirie described a Gram positive bacterium that he had isolated from the liver of wild gerbils in South Africa. Pirie termed this organism *Listerella hepatolytica*, named in honour of the renowned surgeon Joseph Lister (Pirie, 1940). Subsequently the bacterium isolated by Pirie was recognised to be the same as that isolated by Murray in 1926 and the organism was renamed as *Listeria monocytogenes,* as the genus *Listerella* had been previously used (Pirie, 1940). Retrospectively it is now thought that the organism termed *Bacillus hepatis* isolated from necrotic foci in a rabbit liver by Hulphers in 1911 was most likely also *L. monocytogenes* making this the first identifiable case of infection by this organism (Gray & Killinger, 1966).

For many years following its initial identification *L. monocytogenes* was thought to be an animal pathogen, as the majority of the first identifiable cases of listeriosis occurred in animals. The first confirmed isolation of listeriosis in sheep was made by Cors in Germany in 1925 (Gray & Killinger, 1966), this was followed by an outbreak of encephalitis of unknown cause in cattle in 1928, also in Germany, which is likely to have been caused by *L. monocytogenes*.

Although *L. monocytogenes* was initially thought to be a pathogen of animals sporadic incidences of human infection began to be reported. The first case of human listeriosis was reported in 1929 in Denmark (Gray & Killinger, 1966). The organism isolated in this infection was originally termed *L. monocytogenes var. hominis*, to indicate that this bacterium was thought to be a variant of the originally identified *L. monocytogenes*, the term *var hominis* was later dropped as more human cases were identified. Retrospectively it is now known that infections caused by *L. monocytogenes* have been prevalent for a long time. There are records of Gram-positive rods that are now considered to be *L. monocytogenes* being isolated from patients as early as 1891 in France and 1893 in Germany (Gray & Killinger, 1966), the first recorded *L. monocytogenes* culture was isolated from a patient with meningitis in France by Dumont and Cotoni in 1921 (Dumont & Cotoni, 1921).

Despite these initial cases *L. monocytogenes* infections still remained a rare occurrence up until the early 1980s when cases of listeriosis seemed to increase and a number of outbreaks were observed. Numbers of cases in the United States

at this time were reported to be approximately 7.4 cases per million population or about 1,850 cases per year with about 425 deaths (Ciesielski *et al.*, 1988; Gellin *et al.*, 1991). It was not until this point that the first case of listeriosis caused by the consumption of contaminated foods was reported. In 1981 an outbreak of listeriosis occurred in Canada which when investigated was linked back to a contaminated food source. This outbreak was reported to involve 41 cases, 18 of which resulted in mortality, with the majority of cases occuring in neonates or pregnant women. The cause of this outbreak was traced to some coleslaw which had been grown in contaminated manure (Schlech *et al.*, 1983). Since this outbreak there have been many incidents worldwide of foodborne infection with foods such as soft cheeses, dairy products, patés and uncooked meats being identified as the causative agent (Schlech *et al.*, 1983; Allerberger & Wagner, 2010). With numbers of cases rising in recent years combined with the high mortality rate of listeriosis infections this bacterium is still a major problem for the food processing industry.

1.1.2 Taxonomy and subtyping of *L. monocytogenes*

The taxonomic location of *Listeria monocytogenes* has undergone changes since its initial identification. Biochemical analysis of the *L. monocytogenes* genome has identified this organism as having a low G&C content placing the genus *Listeria* within the division *Firmicutes* alongside other low G&C Gram positive organisms such as *Bacillus spp.*, *Streptococcus spp., Staphylococcus spp.* and *Clostridium spp.* WIthin the genus, *L. monocytogenes* and *L. ivanovii* specifically exhibit a high level of homology to *Bacillus* (Glaser *et al.*, 2001). Within the division *Firmicutes* is the family *Listeriaceae*, which contains the genus *Listeria* and the genus

Brochothrix. Phylogenetic analysis of the 16S ribosomal DNA has identified that the closest relation to *Listeria* are members of the genus *Brochothrix* confirming the placement of them both within the same family.

The genus *Listeria* currently contains 10 species: *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri, L. murrayi, L. fleischmannii, L. marthii, L. roucourtiae,* and *L. grayi* (Graves *et al*., 2010; Leclercq *et* al, 2010; Bertsch *et al*., 2013) , these species have been confirmed as belonging to the genus *Listeria* based on DNA-DNA hybridisation studies (Rocort *et al*., 1982, Hartford and Sneath, 1993), and 16S ribosomal DNA analysis (Collins *et al.*, 1991). The DNA hybridisation study carried out by Hartford and Sneath identified that the closest relationship within the genus was between the species *L. monocytogenes* and *L. ivanovii*, which are the only two pathogenic members of this genus. This observation is supported by comparative genomic analysis (Schmid *et al*. 2005). Hartford and Sneath also identified that the species *L. grayii* was the least homologous of the genus (Harford & Sneath, 1993).

The subtying of strains of *L. monocytogenes* is of critical importance in epidemiology and allows identification of the cause of outbreaks of disease (Jadhav *et al*., 2012). Currently, within the species, *L. monocytogenes* can be further subtyped into groups by a variety of methods. The first of the methods used is the classification of *L. monocytogenes* into serovars based on the teichoic acid (O)and the flagella (H) antigens displayed on the surface of the cell. This technique however sometimes causes cross reactions with *Bacillus spp.* due to similarities in

the cell wall structure. Table 1.1 demonstrates the 13 *L. monocytogenes* serovars that are currently used and the respective O and H antigens used for typing. The method of sero-agglutination is expensive, as well as being time consuming, consequently it is not used as often to subtype strains (Palumbo *et al*., 2003, Doumith *et al*. 2004).

Serovar	O antigens	H antigens
1/2a	I, II, III	A, B
1/2b	I, II, III	A, B, C
1/2c	I, II, III	B, D
3a	II, III, IV, (XII)[a], (XIII)	A, B
3b	II, III, IV, (XII), (XIII)	A, B, C
3c	II, III, IV, (XII), (XIII)	B, D
4a	III, (V), VII, IX	A, B, C
4ab	III, V, VI, VII, IX, X	A, B, C
4b	III, V, VI	A, B, C
4c	III, V, VII	A, B, C
4d	III, (V), VI, VIII	A, B, C
4e	III, V, VI, (VIII), X	A, B, C
7	III, XII, XIII	A, B, C

Table 1.1 - List of somatic and flagella antigens used for typing *L. monocytogenes* and there subsequent serovar. [a] brackets denote an antigen that is not always detected. Table adapted from Bergeys Manual of Systematic Bacteriology (2011).

Several other methods are now employed for the detection and subtyping of *L. monocytogenes*, which are all based on molecular techniques. Strains are now classified by evolutionary lineage (Ward *et al*., 2004). Evolutionary lineage has been determined by the use of restriction fragment length polymorphism (RFLP) combined with phylogenetic analysis. When classified into evolutionary lineage originally three distinct lineages of *L. monocytogenes* were thought to exist (I, II, and III) (Ward *et al.*, 2004; Roberts *et al*. 2006), recently however the work of Ward *et al*. (2008) proposed a reclassification of a lineage III subgroup into a separate fourth lineage IV. Each of these four known lineages seems to have distinct genotypic and phenotypic characteristics which enable them to exist within their particular ecological niche. Lineage I and II are currently the best characterised of these evolutionary lines, with strains classified as Lineage I usually belonging to serovars 1/2b, 3b, 4b, 4d and 4e. Lineage II usually contains strains belonging to serovars 1/2a, 1/2c, 3a and 3c, although serovars may cross more than one lineage (Orsi *et al*., 2011). Lineage I strains are those strains that are most associated with human clinical cases of listeriosis and also appear to be more associated with horizontal gene transfer between species. Lineage II strains are most often isolated from contaminated food sources and within the farming environment (Orsi *et al*., 2011) and are less likely to be found in human outbreak cases, which is surprising as it could be assumed that food outbreaks would originate from environmental contamination of food. Bergholz *et al*. (2010) suggested that this inconsistency of lineages isolated from outbreaks was due to the ability of lineage II strains to survive in the high salt levels experienced during infection. Lineage II also appears to be more associated with either plasmids

conferring metal resistance or higher recombination rates. Some strains also show attenuated virulence due to premature stop codons in the *inlA* gene (Corsi *et al*., 2011). Severino *et al*. (2007) investigated the differences in gene expression in lineage I and II strains; they observed many differences between the lineages including the upregulation of motility associated genes in lineage II strains.

Lineage III strains are primarily thought to involve those isolated from cases of animal listeriosis, and approximately 1% of human cases (Ward *et al*. 2008). At this present date strains included in lineage IV are rarely isolated; however they include strains isolated from humans, animals and food.

A more recent approach to subtyping strains of *L. monocytogenes* was developed in 2004 and employs a multiplex PCR that further divides the four major serovars (1/2a, 1/2b 1/2c and 4b) into distinct PCR subgroups (Doumith *et al.*, 2004) . The method offers a quick and efficient alternative to the traditional serotyping and allows screening of many strains at once. The development of this method also allows further identification of relationships between serovars and has so far identified several new PCR serogroups.

1.1.3 Bacterium characteristics

The genus *Listeria* exhibits important characteristics which make the genus recognisable from other bacteria. Some of these characteristics help to exploit a wide range of ecological niches and survive within the environment. Many of the characteristics discussed below are transposable across the genus, however for

the purpose of this thesis each of the characteristics will be discussed in regards to *L. monocytogenes*.

L. monocytogenes is a Gram-positive cocco-bacillus measuring approximately 0.4-0.5 by 1-2μm in size (Bergeys manual, 2011). Cells of *L. monocytogenes* are normally observed by microscopy as individual cells but will sometimes form short chains, although longer filaments have also been observed, mainly in older cultures, or when exposed to stress, with filaments up to the length of >6μm. On clear agar, colonies of *L. monocytogenes* appear translucent and non-pigmented with a round, low convex shape with a smooth surface. Colonies are known to exhibit a blue-green sheen when observed on clear agar under oblique white light (Bergeys manual, 2011).

L. monocytogenes is known to be a facultative anaerobe which benefits this organism in both survival in the environment and during pathogenesis (Vail *et al*., 2012). *L. monocytogenes* is known to be able to exist in biofilms which are thought to be very oxygen depleted environments, so the ability of this organism to survive anaerobically will allow survival within the environment of a biofilm. Also as food processing factories often use modified atmosphere packaging or vacuum packaging, the food is often stored under conditions of low oxygen. The facultative nature of *L. monocytogenes* enables the bacterium to overcome this environment (Lungu *et al*., 2009). Currently most studies characterising *L. monocytogenes* have been carried out under aerobic conditions and there is limited information on the

mechanisms involved for survival and replication of this bacterium under conditions of low oxygen (Lungu *et al*., 2009).

Cells of *L. monocytogenes* display between four and six peritrichous flagella at temperatures less than 37°C (Peel *et al.*, 1988) which are responsible for its classic tumbling motility. Flagella are encoded by the gene *flaA* and expression of this gene has been shown to be temperature dependent (Way *et al*., 2004), with repression of expression at 37°C which appears to be essential for virulence. The absence of flagella at temperatures of 37°C implies that flagella are not required for infection in humans (Kathariou *et al*., 1995; Grundling *et al*., 2004).

L. monocytogenes can also survive in extreme salt conditions due to its ability to grow in sodium chloride (NaCl) and also the ability of *L. monocytogenes* to mount an osmotolerant response (OTR) (Faleiro *et al*., 2003). Strains of *L. monocytogenes* have been reported to grow in salt concentrations of more than 10% NaCl. This ability to grow in high salt concentrations allows them to survive in a wider range of environments and in the conditions found in food processing factories.

1.1.4 Environmental survival

L. monocytogenes can be isolated from a wide range of ecological niches and is known to survive in adverse conditions longer than any other non-spore forming species of bacterium (Ivanek *et al.*, 2006). The presence of *L. monocytogenes*

contamination in environment samples has been reported to be in as much as 7.5% in urban environment samples, and 1.4% of samples from rural environments (Sauders *et al.*, 2006). This section will discuss briefly the organisms' survival in environments such as soil, water, sewage and in animals. It will also discuss the prevalence of *L. monocytogenes* in food processing factories.

L. monocytogenes is known to be able to survive for long periods in soil. It was first reported that this organism could survive in soil by Welshimer (1960), who demonstrated that inoculation of *L. monocytogenes* into fertile soil resulted in the cells surviving for up to 295 days. Several other papers have subsequently reported the presence of *L. monocytogenes* in soil and plant samples (Weis *et al.*, 1975; Welshimer *et al*., 1971). A recent study by McLaughlin *et al*., (2011), identified that highly motile strains were more likely to survive in soil. The strain EGD-e has been reported to survive in soil for up to a year (Piveteau *et al*., 2011). Numbers of *L. monocytogenes* in soil have been reported as up to 170 cells/100g of soil (Watkins *et al.*, 1981).

Gene expression in cells surviving in soil has recently been subject to investigation (Piveteau *et al*., 2011), with expression being measured after 0, 15 min, 30 min and 18 hours of being in soil. It was observed that as the bacterium adapted to the soil a number of genes were upregulated, including, genes involved in the phosphoenolpyruvate - dependent phosphate transport systems, ABC transporters, and enzymes involved in catabolism, for example chitinases. As soil environments are thought to be scarce in nutrients it appears that genes involved in catabolism of

substrates seem to be upregulated. Genes involved in virulence of *L. monocytogenes* appeared to be down regulated (Piveteau *et al*., 2011).

L. monocytogenes is also known to be present in freshwater and seawater, with amounts of *L. monocytogenes* being reported as 70 cells/kg in seawater (Fenlon, 1985). *L. monocytogenes* has been shown to survive for extended periods in pond water, with survival being recorded of up to 63 days (Botzler *et al*., 1974). More recently a study by Hansen *et al*. (2006) looked to see if *L. monocytogenes* survived in freshwater environments outside of food processing environments and compared the survival of strains inside food processing aquatic environments with outside aquatic environments. The presence of this organism was found increasingly proportionate to the degree of human involvement in these aquatic environments. This implies that most *L. monocytogenes* strains found in aquatic environments is due to human intereference rather than any environmental factors. This is in direct contradiction to what would be expected as *L. monocytogenes* contamination would be expected in water due to the presence of this organism in the soil.

Although little published information is available on the presence of *L. monocytogenes* in sewage, high levels of this organism have been reported in river water close to sewage outlets (Fenlon *et al*., 1996). This study also reported numbers of up to 120 cfu/ml in effluent coming into the sewage plant, whereas effluent and anaerobically treated sludge had numbers of 21 and 1.1 cfu/ml of *L. monocytogenes*, respectively. A study by Wagner *et al*. (2009) looked at survival of

Listeria and other pathogens in a variety of different sewage conditions. They identified that organisms survived better in anaerobic conditions that aerobic ones, suggesting that *L. monocytogenes* survive anaerobically in sewage.

The high amounts of *L. monocytogenes* in sewage are directly attributable to the numbers in effluent produced by humans and animals (Ivanek *et al.*, 2006). This bacterium has been isolated from the faeces of a wide range of animal species and birds (Yoshida *et al*., 2000). Listeriosis appears to be a big problem due to the large amount of *L. monocytogenes* present in ruminants (cattle, sheep, and goats) which can lead to contaminated food sources (Fenlon, 1996). Although numbers of infected animals are high, numbers of animals showing acute infections are low, indicating many of the animals are asymptomatic.

The presence of *L. monocytogenes* in food processing factories, and consequently in food is an increasing problem. Numbers of *L. monocytogenes* in food have been reported to be high due to the food handling processes, rather than the contamination of the raw foods coming into the food processing plants (Ivanek *et al*., 2006). The current recommended concentration of *L. monocytogenes* presence in food is below 100cfu/g of food, this figure is enforced by the Food Standards Agency. The isolation of *L. monocytogenes* from food processing factories has been reported often and is a particular problem in cheese dairies (Delgard de silva *et al.,* 2001; Chambel *et al*., 2007). Studies have identified a wide range of serotypes prevalent, with the serotypes present varying weekly, sometimes even daily (Hu *et al*., 2006); this implies that no one serotype is persisting within the

environment and that the strains present are changing all the time. Within artisanal cheese dairies *L. monocytogenes* has been isolated from a wide range of surfaces such as drains, conveyor belts, sinks, and benches (Chambel, *et al*., 2007).

Listeria is essentially a saprophyte by nature, has been isolated from a range of plant matter including cabbage, cucumber, sprouts and mushrooms (Heisick *et al*., 1998; Strapp *et al*., 2003). Its ability to switch from surviving within a wide range of environments found in nature to becoming a pathogen has made it a model organism for investigating host-pathogen interactions (Xayarath & Freitag, 2012). Little is currrently known about the molecular determinants of this bacterium which allows plant colonisation to occur, however it is known that this organism is capable of forming biofilm on plant matter (Gorski *et al*., 2009). *L. monocytogenes* is also a well known plant saprophyte, and utilises characteristics such as flagella to colonise plants (Gorski *et al*., 2009). Goski *et al.* (2009) highlighted the importance of flagella in the colonisation of alfafa sprouts with *L. monoctyogenes*. This study found however that motility was not important, just the presence of flagella.

1.1.5 Listeriosis

L. monocytogenes is an invasive disease which can have several clinical manifestations in humans. This section will discuss the epidemiology of listeriosis, the pathology of infection, and briefly discuss the virulence factors used by this bacterium to cause infection.

1.1.5.1 Epidemiology of human listeriosis

Infection with *L. monocytogenes* causes a range of invasive infections collectively known as listeriosis. Listeriosis manifests in a variety of ways, ranging in severity from gastroenteritis, to meningitis, and termination of pregnancy in infected pregnant women. Gastroenteritis with no associated complications is the main manifestation in healthy patients (Vazquez-Boland *et al.*, 2001). Although incidence of listeriosis worldwide is generally low, varying between 0.1-11.3 per million in different countries (Gellin *et al.*, 1991), it can be particularly harmful for pregnant women, elderly people, and people who are immunocompromised. Consequently, due to the high rate of infection in immunocompromised people listeriosis has a mortality rate of 20-30%. *L. monocytogenes* is known to be able to cross blood barriers such as the placenta in pregnant women, which can lead to miscarriage, and the blood - brain barrier in the brain, resulting in meningitis (Vazquez-Boland *et al.*, 2001).

Since its initial discovery, cases of human listeriosis have remained relatively low, with incidence of infection by *L. monocytogenes* during the 90s as low as approximately 110 cases a year across England and Wales (Gillespie *et al*., 2010). Since then however, during the period of 2001-2008, reports of infection by this organism increased dramatically up to an average of 188 cases yearly. This increase in cases has also been observed in other European countries, as demonstrated in Figure 1.1 (Allberger and Wagner, 2009). The figure demonstrates the increase in L*. monocytogenes* cases in six European countries between the years 1999-2009. As can be seen from this figure, all countries show a significant

increase, with numbers of cases in Germany and the Netherlands showing the biggest increase by far.

To investigate a putative link between listeriosis and areas of social deprivation Gillepsie *et al*. (2010) carried out surveillance between 2001-2008 using epidemiological data available from the Health Protection Agency. Gillespie *et al*. identified that the prevalence of listeriosis was much higher in deprived areas, and that this increase was more associated with areas more likely to purchase convenience, or ready to eat (RTE), food. Gillespie *et al*. also hypothesised that changing attitudes towards nutrition seem to be partially responsible for the increasing rise of *L. monocytogenes* infections. An increasing tendency to use foods past their sell by dates, and the consumption of convenience foods with long refrigeration shelf lives would contribute to the rise in listeriosis infections.

A number of large outbreaks have occurred since the first description of a food-borne outbreak of listeriosis in 1981. These outbreaks have been associated with a wide variety of foods including cheeses, raw salads, fish, pates, milk, hot dogs and meat. Long incubation times of the bacterium make tracing the outbreak of the infection problematic. Outbreaks are difficult to trace and often result in the infection being widespread before the source is identified (Vazquez-Boland *et al.*, 2001).

Along with the increase in cases of listeriosis in recent years, a change in clinical manifestation has also been noted (Gillespie *et al*., 2006). There has been a

considerable increase in cases presenting as bacteraemia in older patients. Khatamzas *et al*. (2010) also noted that in a case study featuring two incidences of listeriosis, both of the cases presented as bacteraemia. Khatamzas *et al*. (201) also demonstrated that cases of bacteraemia in over 60s have increased dramatically since 2008. An increase in listeriosis in France has also been attributed to an increase in bacteraemia in the over 60s, with a particular prevalence in those with existing co-morbidities (Anses, 2009). The observed increase in cases of listeriosis within recent years however, could also be accounted for by improved knowledge of the general public and an increased awareness, which could lead to better reporting of infection. Techniques are improving all the time to allow faster diagnosis of infection so the increase in cases could in actual fact reflect an increase in diagnosis.

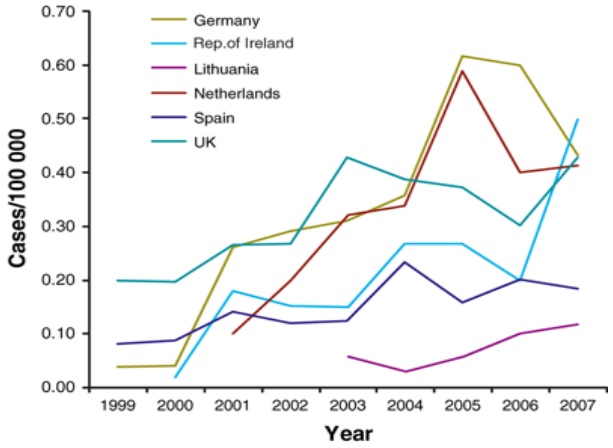

Figure 1.1 - Graph to demonstrate the increase in number of cases of listeriosis per 100,000 populations between the years 1999-2007 in six European countries. Figure modified from Allberger and Wagner (2009).

Many incidences of listeriosis have now been identified as having food-borne origin, due to the high amount of biofilms in food processing factories. It is likely that these outbreaks are caused by biofilm forming organisms, however it is not possible to say this for definite despite the presence of bacteria in food processing factories where these outbreaks originate. Most recent outbreaks of Listeriosis can be traced back to various food sources. A study by Gaul *et al*. (2013) traced the cause of an outbreak in 2010 in Texas back to contaminated diced celery originating from a food processing plant. In this food processing plant *L. monocytogenes* was isolated from a wide variety of surfaces including equipment and floors. This outbreak resulted in 10 cases of Listeriosis in immunocompromised patients, 5 cases of which resulted in fatality. A similar

outbreak was investigated by the Centre of Disease Control in 2011, when the cause of Listeriosis was traced back to contaminated cantaloupe melon (Prevention CfDCa, 2011). This was a much larger multistate outbreak, which was traced back to melon purchased from a farm in Colorado. This outbreak resulted in 84 cases, from 19 different states, fifteen of which resulted in fatality, and one in miscarriage. Again as this resulted from a food processing factory it is likely that biofilm forming organisms were involved.

1.1.5.2 Pathogenicity of Listeriosis

Since the discovery of the first identified virulence factor, hemolysin, for *Listeria monocytogenes* in the 1980s (Vazquez-Boland *et al*., 2001), many studies have been carried out into the pathogenesis and virulence factors in this organism. This section will give an overview of how *L. monocytogenes* causes infection in humans and highlight which virulence factors are involved. Figure 1.2 shows the infectious cycle of *L. monocytogenes*, which is summarised in the paragraph below.

The minimum dose required for infection by *L. monocytogenes* is still not known, but is thought to be at least 10^6 bacteria (Vazquez-Boland *et al.*, 2001). Upon entry into the host, the bacterium must first resist the highly acidic environment of the stomach. This environment explains the large dose of *L. monocytogenes* required to cause infection by this organism. After passing through the stomach and upon reaching the intestines *L. monocytogenes* must cross the intestinal barrier. The bacteria are thought to enter the intestinal epithelial cells (M-cells) at the Peyer's

patches found in the intestines (Vazquez-Boland *et al.*, 2001). Adhesion of *L. monocytogenes* to the intestinal epithelia has been identified to occur using the internalin InlA, which is known to bind to the human intestinal epithelial receptor, E-cadherin. Upon entering Peyer's patches *L. monocytogenes* encounter a large amount of phagocytic cells such as macrophages, monocytes and neutrophils. On encountering the phagocytic cells the bacteria are either taken up by phagocytic cells or must utilise InlA, InlB or ActA to invade non-phagocytic cells. On entering the host cell *L. monocytogenes* are engulfed in a phagocytic vacuole. The bacterium then escapes the phagosome, utilising a hemolytic toxin, Listeriolysin O. This toxin, coupled with phospholipases, destroys the membrane and allows escape into the cytoplasm. When in the cytoplasm the bacteria replicate (with a doubling time of approximately one hour, slower than observed *in vitro*), and are immediately surrounded by actin filaments. These filaments later rearrange to form an actin tail, which propel the bacteria across the cytoplasm in a random motion. Some bacteria eventually come into contact with the cell membrane at which point they produce protrusions into a neighbouring cells. These protrusions are then engulfed by a new vacuole and the process starts all over again.

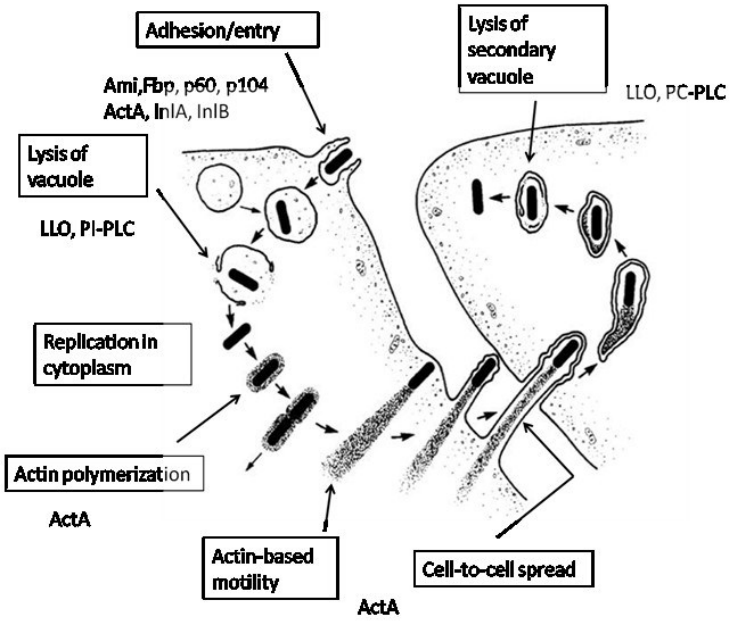

Figure 1.2 - Infectious cycle of *L. monocytogenes*, adapted from Pizarro-Cerda Javier *et al*. (2004).

1.1.5.3 Virulence factors

Listeriolysin O

Listeriolysin O (LLO) is a cholesterol dependent hemolysin of the same group as pneumolysin from *Streptococcus pneumoniae*, perfringolysin from *Clostridium perfringens* and Streptolysin O from *Streptococcus pyogenes* (Schnupf & Portnoy, 2007). Much research has been carried out into this toxin and now it is clear that LLO has many roles (Schnupf & Portnoy, 2007).The primary role is in escape of the bacterium from the phagolysosome, to do this the toxin must bind to the 3β-hydroxy group of the cholesterol molecule within the membrane and insert itself into the membrane binding monomers to form a pore (Dramsi and Cossart, 2002). Pores formed on the membrane of the phagolysosome will allow bacteria to escape into the host cell cytosol. Escape from the phagosome is not completely

LLO dependant, as it is enhanced by the phospholipases. However LLO negative mutants have been shown to be unable to escape from the phagosome so LLO must be crucial for escape (Schnupf & Portnoy, 2007). The activity of LLO is unique from the other toxins in this group, as it has an optimal pH of 5.5-6 (Dramsi and Cossart, 2002). As LLO requires this lower pH, it is suspected that the phagosome is first acidified before LLO forms pores. It is not yet known why this pH is needed however it could be that the LLO gene *hly* is only expressed in acidic conditions.

Listeriolysin S

A streptolysin S-like hemolytic and cytotoxic toxin has recently been identified in *L. monocytogenes* (Cotter *et al*. 2008). This group of toxins was originally identified in Group A *Streptococcus* and is thought to be involved with cytotoxicity, activation of inflammatory responses and resistance to polymorphonuclear neutrophil resistance. *In silico* analysis identified a gene cluster in *L. monocytogenes* encoding a similar toxin, this was later termed *Listeria* pathogenicity island 3 (or LIPI-3). The structure of the *L. monocytogenes* gene cluster is shown in Figure 1.3. *llsA* encodes the toxin which has been shown only to be expressed in conditions of oxidative stress (Cotter *et al*. 2008). This gene cluster is not found in all strains, but when it is present the isolate is hemolytic under the right conditions. Within the cluster are the genes *llsB*, *llsY* & *llsD* which are thought to encode the synthetase needed to produce the mature active form of the toxin, *llsG* & *llsH* which encode an ABC transporter spanning the bacterial membrane to allow the toxin to be

released from the cell, and *llsP*, a CAAX protease. *llsX* does not currently have any sequence similarity and its function is yet unknown (Cotter *et al*. 2008).

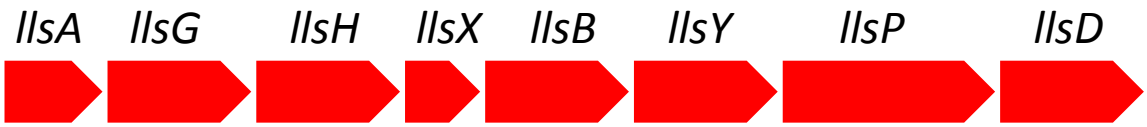

Figure 1.3 - The *lls* gene cluster

Internalin family

Listeria utilize a family of leucine rich proteins called the called Internalins which can interact with the host cell surface of non-phagocytic cells. Genes encoding the proteins InlA and InlB are contained within a gene cluster, originally termed LIPI-1, on the chromosome. Alongside this cluster, several other genes have been identified through *in silico* analysis to contain high sequence similarity to *inlA* and *inlB* (Bierne *et al*., 2007). InlA and InlB are currently the only internalins to be well characterised. The presence of an internalin was first reported by Gaillard *et al*. in 1991. Through screening of transposon mutants they identified a 5911bp region which contained three open reading frames (ORF A, *inlA* and i*nlB*). Cloning of these open reading frames identified *inlA* to be important in invasion of epithelial cells. InlA is a surface expressed protein of 744 amino acids long and is known to interact with the host receptor E-cadherin (Mengard *et al*. 1996). However this interaction is species specific as *Listeria* does not seem to interact with mouse E-cadherin but will interact with both the human and Guinea-pig form of this receptor. The gene *inlB* encodes a 630 amino acid surface expressed protein and is also

important for entry into host cells, by binding with different ligands such as glycosaminoglycans (GAG), gC1qr and the hepatocyte growth factor c-Met, which seems to indicate InlAB acts as a growth factor. However studies have indicated that *inlB* is only essential for entry into proliferative cells (Dramsi *et al*.,1995).

Regulation of virulence genes by *prfA*

Listeria contains a transcriptional regulatory factor *prfA* (Postive regulatory factor A). PrfA is a 237 residue protein, which is structurally related to the catabolite gene activator protein (cap). The main role of the PrfA protein is to activate transcription of promoters located on the genome with the consensus sequence TTAACANNTGTTAA (Vazquez-Boland *et al.*, 2001). PrfA is now known to regulate up to 145 genes, some more strongly than others (Heras *et al*. 2011). Nine genes however are considered part of the core virulence regulon that is actively transcribed by PrfA, six of which are in the cluster known as *Listeria* pathogenicity island 1 (LIPI-1). PrfA is responsible for directly regulating expression of the hemolysin, Listeriolysin O (encoded by *hly*), the operon *mpl-actA-plcB-orfX*, which can be transcribed either as one transcript by PrfA binding to the promoter of *mpl,* or as two short transcripts one starting at *mpl* and one starting at the promoter *actA* (Heras *et al*.2011). The other prfA regulated virulence genes include the *inlAB* operon, and the hexose phosphate transporter *hpt.* The other genes seem to be indirectly regulated to varying degrees, which is possibly due to the lack of the prfA box. These genes involve sugar transport systems, metabolic enzymes and genes involved in the stress response of *L. monocytogenes* (Milohanic *et al*., 2003).

actA

L. monocytogenes utilises the host cell machinery to enable motility within the cell using ActA. The protein ActA is a 639 amino acid protein which is responsible for recruitment of host cell actin by mimicking the eukaryotic protein WASP. It is composed of three distinct domains: (1) N-terminal domain, (2) central domain, containing proline rich repeats, and (3) C-terminal domain, containing a highly hydrophobic region (Vazquez-Boland *et al.*, 2001). ActA utilises the host cell protein VASP (vasodialator-stimulated phosphoprotein) to recruit actin from in the cell which binds directly to ActA and forms a network of polymerised actin filaments at one end of the bacterial cell. The actin filament extends and becomes branched, which exert a force on the bacterium driving it in a forward motion (Portnoy *et al*., 2002).

Phospholipases

Listeria spp. produce three different phospholipases two of which, PlcA and PlcB, are specific to *L. monocytogenes* (Vazquez-Boland *et al.*, 2001). The *plcA* gene encodes phosphatidylinositol (PI) specific phospholipase C. This enzyme has an optimum pH of between 5.5 and 6.5 like LLO, indicating it would be active in phagocytes (Vazquez-Boland *et al.*, 2001). The *plcB* gene sits within what is known as the lecthinase operon, along with the genes *mpl* and *actA*, and encodes a 264 amino acid propeptide, which is cleaved to give an active enzyme. Both phospholipases are thought to have a role in escape of the bacteria into the cytosol, as *hly* -ve mutants were observed to be able to escape and replicate within

the cytosol. *plcB* is also thought to be involved in the lysis of secondary vacuoles during cell-to-cell spread as well as escape from the primary phagosome whereas *plcA* does not seem to be involved in cell-to-cell spread of this organism (Vazquez-Boland *et al.*, 2001).

Metalloprotease

Listeria also contains a metalloprotease (*mpl*) which also aids escape from the vacuole. *mpl* is present only in pathogenic strains of *L. monocytogenes* and encodes an amino acid motif common only to zinc-dependent metalloproteases. The gene *mpl* is part of the lecthinase operon and is involved in the maturation of phospholipase C. Mpl is transported across the membrane as an inactive proprotein, where it is cleaved into its mature, active form. Mpl activity has been shown to be regulated by pH (Forster *et al.,* 2011) and is active at lower pH, as are the phospholipases.

1.2 Bacterial attachment to abiotic surfaces

Abiotic surfaces can be defined as surfaces not associated or derived from living organisms, this involves surfaces made out of materials such as polystyrene (as in a microtitre plate), stainless steel and glass; abiotic surfaces are abundant in food processing factories (Chambel,L. *et al.,* 2007). Examples of biotic surfaces would include cell culture, plant and vegetable matter or living tissue. The processes in attachment of bacteria to either of these surfaces have yet to be fully elucidated. For the purpose of this thesis the factors considered will mainly be ones involved in attachment of *L. monocytogenes* to abiotic surfaces. Bacterial attachment and subsequent biofilm formation is problematic in the food processing industry, in bacterial infections and on medical implants/devices. Due to the problems raised in killing bacteria contained within a biofilm a better understanding of the processes behind it is of high importance. This section will discuss biofilms and how they are formed, and the information known about *L. monocytogenes* biofilm formation and the current understanding of it at a molecular level. This section will also discuss current methods for investigating biofilm formation.

1.2.1 Biofilm formation

Many bacterial species are now known to be able to attach to a wide range of surfaces, yet the processes behind this are still unclear. The first recorded description of bacterial biofilms comes from a study by Henrici (1933) which identified the presence of biofilms formed by water-borne organisms. Henrici observed glass slides left in an aquarium for varying periods of time using microscopy. He noted microcolonies increasing in size over time and observed that

they were difficult to remove. This study also gave the first observation of EPS as Henrici noted that some of the cells appear to be surrounded by a sheath of 'gum' which attached the cells to the slide. This study paved the way for further investigations into microbial communities and EPS.

Palmer and White (1997) presented an early model for attachment, showing that biofilm formation is a continual process. This model starts with initial attachment occurring when bacteria come in to contact with a surface. Palmer and White suggested that no type of surface has yet to be discovered to which bacteria cannot adhere. Attachment is hypothesised to be non-specific, and possibly due to factors such as cell surface charge, or charge of the surface to which the bacterium is adhering. This study goes on to state that the attached cells then appear to enter a period of growth within a 3-dimensional structure and cells begin to form microcolonies. These microcolonies continue to grow and form macrocolonies and begin to produce 3-dimensional structures, which often resemble mushrooms or columns. These structures which eventually grow together and bridge the gaps between them. Exopolysaccharide (EPS) is thought to be produced by many different species of bacteria at this final stage in attachment process enabling the bacteria to stick firmly to the surface and helping to support the 3-dimensional structures created.

A more recent study has indicated that although this model still holds true the process is more complex (Monds & O'Toole, 2009). This model was first proposed following studies in the organism *Myxococcus xanthus* and can be seen in Figure

1.4.They propose that initial attachment to surfaces should be separated into reversible and irreversible stages. Initial attachment is then followed by growth and maturity of the biofilm as proposed by Palmer and White (1997). Monds and O'Toole (2009) also propose the addition of a fifth stage to the model. During this stage the bacteria disperse from the biofilm, and cells disperse elsewhere where they can attach to new surfaces and start the process over again.

Exopolysaccharide production is important in the biofilm formation of bacteria but the composition of EPS is still not fully characterised. However it is thought to be composed of polysaccharides, nucleic acids and proteins (Renier *et al*., 2011). The presence of extracellular DNA also appears to be an important component of bacterial biofilms, especially in early biofilms (<60 hours) as the addition of DNase to biofilms, causes them to dissipate (Whitchurch *et al*., 2002).

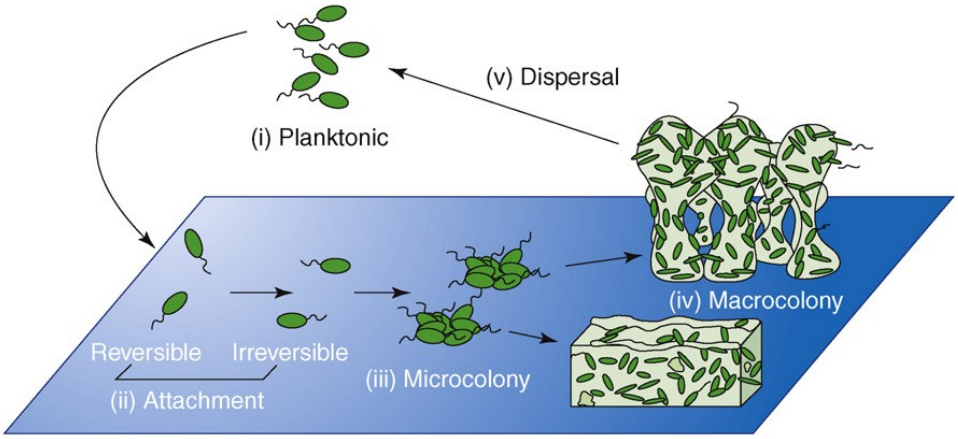

Figure 1.4 - Model of bacterial biofilm formation as proposed by Monds and O'Toole (Monds and O'Toole, 2009), (1) Planktonic bacteria (2) Attachment separated as two steps, reversible and irreversible (3) 3D growth and the formation of microcolonies (4) maturation of biofilm and formation of macrocolonies (5) further growth and dispersal of cells which can initiate new biofilms

1.2.2 Advantages of biofilm formation

Biofilms are mainly thought to be an advantage to cells and provide bacteria with an increased chance of survival. Advantages of living within a biofilm include increased resistance to UV light, disinfectants and antibiotics, and resistance to predators (Davey *et al*., 2000). Consequently, preventing biofilm formation, or disrupting the biofilm, is currently a new focus in the search for new therapeutic agents. This section will briefly summarise the benefits to bacteria of forming a biofilm.

Biofilms are thought to produce channels, to help in situations where nutrients are scarce, these channels are comparable to that of a circulatory system. By attaching

to the surface bacteria are thought to trap nutrients to to the surface, and then transported them through the channels produced in the biofilm. The channels are also thought to remove toxic products (Davey *et al*., 2000). Multispecies biofilms can also be highly advantageous to bacteria, as some nutrients may require more than one strain of bacteria to degrade the substrate before it can be utilised, for example, during anaerobic digestion, the degradation of organic matter into methane and carbon dioxide requires more than one species of bacteria.

One of the biggest advantages to bacteria in the formation of biofilms is the protective nature that biofilms provide to a wide range of environmental challenges. Biofilms seem to confer a survival advantage against bactericidal activity by a range of antimicrobials. Although the current mechanisms behind this increased resistance is not yet clear, the involvement of EPS has been implicated. It is thought that resistance to antibiotics and disinfectants is due to problems with diffusion into bacterial EPS. The EPS matrix was originally proposed to do this by acting as an ion exchanger, preventing diffusion of antimicrobials through the EPS (Davey *et al*., 2000). Other theories for the increase resistance exhibited by the bacteria include: (1) degradation of the microbiocide, (2) genotypic changes in the biofilm bound organisms resulting in antibiotic resistance, or (3) reduced growth rate of biofilm associated bacteria, and consequently reduced metabolic activity of the bacteria, resulting in less targets for the antimicrobials to act on (Batoni *et al*. 2011).

EPS is also identified as providing resistance to UV radiation and desiccation. The

presence of EPS produced by *Pseudomonas aeruginosa* biofilms has been proven to prevent DNA damage from UV radiation. Protection from dehydration has also been demonstrated as a result of EPS, as EPS producing strains of *Escherichia coli, Acinetobacter calcoaceticus*, and *Erwinia stewartii* have shown resistance to desiccation, unlike non-EPS producing variants of these organisms (Ophir & Gutnik, 2004).

Horizontal gene transfer is highly likely in organisms trapped in a biofilm. The high density of biofilms means that the cells are in closer contact to each other allowing transfer of genetic material. Transfer of genetic material could result in an increase in antimicrobial resistance and other advantageous phenotypes in bacteria contained in a biofilm. Extracellular DNA has been shown to be a necessary component of the EPS produced by *Pseudomonas spp* within a biofilm, indicating that horizontal gene transfer is likely (Whitchurch *et al*., 2002).

As well as conferring resistance to antimicrobial agents, disinfectants and UV radiation the production of biofilms on surfaces in food processing also confer more resistance of the bacteria to shear stress when exposed to a liquid flow (Teodosio *et al*. 2011). Teodósio et al demonstrated that an increase in flow changed the architecture of the biofilm to cope with the conditions.

1.2.3 *Listeria monocytogenes* biofilm formation

L. monocytogenes is found in high prevalence in food processing factories due to the ubiquitous nature of this organsim enabling it to survive the harsh environments

it encounters. The formation of biofilms helps with the survival of *L. monocytogenes* in these environments. This section will briefly summarise some of the information known about *L. monocytogenes* biofilm formation, discuss the current research into whether *L. monocytogenes* produces exopolysaccharide, and discuss the genes already known to be involved in *L. monocytogenes* biofilm formation.

L. monocytogenes biofilms are of high significance in the food processing industry with conveyor belts, drains, sinks and stainless steel surfaces often being found to be contaminated. This problem is especially high in artisanal cheese dairies found in Spain and Portugal. A recent study taking samples from cheese dairies in Portugal found *Listeria* spp. present in 6 dairies out of the 8 sampled (Chambel *et al*., 2007). Out of the 203 *Listeria* spp. isolates taken across the sites 85 of these were identified as *L. monocytogenes* by PCR. This study also looked at prevailing serotypes; 4b was the most prevalent in 52% of isolates, with an equal amount of serotypes 1/2b or 3b and 1/2a or 3a found.

L. monocytogenes is also found to form biofilms in other types of food processing factories, including those involved in meat processing. Midelet and Carpentier (2002) carried out a study simulating the conveyor belts found in meat processing environments, this study showed that *L. monocytogenes* attached more firmly to the polyvinylchloride than any other organism and was also the most resistant to disinfection.

L. monocytogenes serotype seems to be linked to the bacterium's ability to adhere to surfaces. Nilsson *et* al. (2011) studied 95 different strains of *L. monocytogenes* and looked at how different conditions affect the organism's ability to form biofilm. Their study concluded that strains belonging to serotype 1/2a were significantly better biofilm producers than any other serotype, this is supported by the work of Boruki *et al*. (2003) who identified those strains belonging to division II (i.e. serovar 1/2a and 1/2c) as the best biofilm producers. Nilson *et al*. (2011) also concluded that as no carbohydrate polymers were isolated during this study that *L. monocytogenes* must not produce a carbohydrate containing polymer. This is in contrast to the study by Boruki *et al*. (2003), who during their investigation into 80 strains of *L. monocytogenes* developed a microtitre plate method using staining with ruthenium red. Ruthenium red binds irreversible to carbohydrates, so the uptake of ruthenium red by *L. monocytogenes* indicates the presence of carbohydrates within the bacterial EPS.

Listeria monocytogenes biofilms have increasingly been identified as important in a clinical setting too, with reports of biofilms on prosthetics. Chrdle and Stárek (2011) reported the presence of a *L. monocytogenes* biofilm on a prosthetic hipjoint in combination with *Staphylococcus epidermidis*. *L. monocytogenes* biofilms have also been reported as a complication on ventriculoperitoneal shunts in young children (Le Monnier *et al*., 2011).

Most bacteria produce an extracellular matrix normally composing of a mixture of exopolysaccharides, DNA, proteins and other extracellular material, such as

teichoic acids (Renier *et al*., 2011).These components help to stabilise the 3D matrix, of the biofilm as well as providing protection for the cell. It is still widely disputed whether *L. monocytogenes* produces an exopolysaccharide composing of carbohydrates, such as that found in biofilms of other organisms such as *Pseudomonas spp.* and *Vibrio spp.* However electron microscopy has identified the presence of fibre like substances which have been proposed to be EPS, as demonstrated by Figure 1.5 (Boruki *et al*., 2003; Faadaee-Shohada *et al*., 2010). It has also been proposed however that these structures are just as a result of the dehydration of the cells from the fixing processes used for electron microscopy (Boruki *et al*., 2003). Midelet & Carpentier (2002) in their study looking at the transfer of various microorganisms onto beef also looked at the EPS forming ability of these microorganisms. They utilised an enzyme linked lectinsorbent assay (ELLA) to try and detect for EPS production by *L. monocytogenes* alongside other organisms using the peroxidase lectins ConA and WGA which bind sugars in EPS. They found that although *L. monocytogenes* showed a high level of attachment to a variety of surfaces, no sugars could be detected with the WGA lectin and although some could be detected with the conA lectin this was much lower than other organisms used in this study including *Pseudomonas putida*. This study implies that if *L. monocytogenes* does produce EPS containing sugars it must produce it in much lower levels.

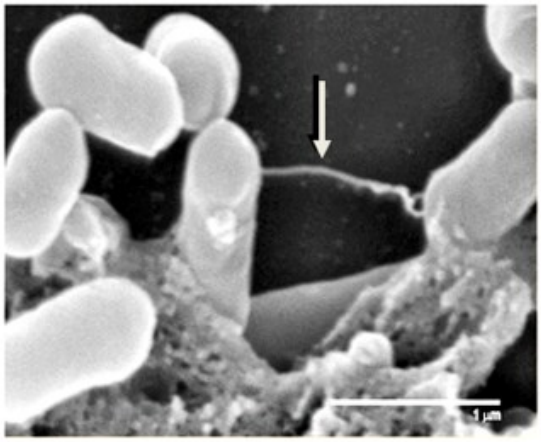

Figure 1.5 - Scanning electron microscope image of *L. monocytogenes* and the fibre like extracellular material (indicated by arrow) (Fadaee-Shohada *et al*., 2010).

Ruthenium red staining assays have been also been utilised to assay the levels of carbohydrate present in the extracellular matrix. Boruki *et al*., (2010) demonstrated a high level of ruthenium red binding in *L. monocytogenes* adhered to glass, which related to a high amount of crystal violet binding. However they concluded that these data are not conclusive as to the presence of *L. monocytogenes* EPS as ruthenium red could also be binding to surface attached carbohydrates, this could also be true of the peroxidase bound lectins utilised by Midelet and Carpentier (2002).

It has been shown that any extracellular matrix produced by *L. monocytogenes* however does contain extracellular DNA (eDNA) (Harmsen *et al*., 2010; Renier *et al*., 2011). Studies indicate that eDNA is important in the initial attachment of *Listeria* to surfaces which has been supported by the addition of DNaseI to cultures which lead to a significant reduction in attachment (Harmsen *et al*., 2010). The

length of the eDNA seems to play a crucial role in attachment, as low molecular weight DNA appears to inhibit attachment rather than promote it. Extracellular DNA has been shown to be an important component of EPs in other organisms such as *Ps. aeruginosa*.

1.2.3.1 Molecular determinants of *Listeria* biofilm formation

The sequencing of the *L. monocytogenes* genome has enabled *in silico* analysis which has enabled researchers to identify putative genes involved in biofilm formation. Using a combination of deletion mutants made in these identified genes and the creation of transposon libraries in *L. monocytogenes* several genes thought to be involved in the processes of formation of biofilm by *L. monocytogenes* have been identified. Genes identified include cell wall associated proteins, genes required for motility structures, as well as genes involves in cell transduction signal systems. Several examples of these will be discussed below.

Biofilm-associated protein (BapL)

The Biofilm associated protein (Bap) was first identified and characterised in S*taphylococcus aureus* it is a known surface expressed protein of 2276 base pairs long. The Bap protein of *S. aureus* was shown to be involved in primary adherence of this organism to polystyrene using a microtitre plate assay (Cucarella *et al*., 2004), the Bap protein was also identified to be involved with persistence of infection of mice with *S. aureus*. Jordan *et al*., (2008) used *In silico* analysis of the *L. monocytogenes* genome to look for protein sequences showing the typical

LPXTG motif associated with cell wall associated proteins. The *in silico* analysis lead to the identification of the gene *lmo0435*, the protein encoded by this gene showed some homology to that of the Bap protein identified in *S. aureus*. An insertion mutant was created within this gene, which when tested showed significantly reduced attachment to polystyrene in a microtitre plate assay (Jordan *et al*., 2008). However screening of *L. monocytogenes* isolates from cheese dairies across Spain and Portugal using PCR only identified the presence of this gene in 4 out of 17 dairy strains, indicating this gene is not essential for environmental survival. Unlike the Bap gene in S. aureus, an *In vivo* study using this insertion mutant concluded this gene was not essential for virulence in a mouse model of infection.

Flagella

Flagella have been identified in playing an important role in biofilm formation in many species (Renier *et al*., 2011). *L. monocytogenes* is known to have between 4 and 6 peritrichous flagella per bacterial cell and are known to be expressed only at temperatures lower than 30°C (Peel *et al*. 1988). Above this temperature transcription of the gene *flaA*, which is responsible for the production of the flagellin monomers that compose the flagella, is stopped and *Listeria* becomes non-motile. Regulation of the expression of flagella in *L. monocytogenes* is complex and involves several regulatory factors and is thought to be involved in both the initial attachment stage of biofilm formation and in later stages of dispersion of the biofilm. The involvement of flagella attachment of *L. monocytogenes* was initially

investigated by Vatanyoopaisarn *et al*., (2000). This study demonstrated that the non-flagellated *L. monocytogenes* mutant *fla2*, showed significantly less attachment to stainless steel, at temperatures of 22°C, than the flagellated wild type strain NCTC 7973. Differences in attachment between these two strains were not observed at 37°C, due to the flagella not being expressed at this temperature. The study by Vatanyoopaisarn *et al*. (2000) also demonstrated that the reduction in attachment in the *fla2* mutant was only present for the first four hours of the biofilm process, when measuring levels of attachment in *L. monocytogenes* left to adhere for 6 hours or longer there was no significant difference between the *fla2* mutant and the wild type strain, this confirms that flagella play an important role in the initial stages of attachment. The role of flagella in initial attachment of *L. monocytogenes* has been confirmed by several other studies. Todhanakasem and Young (2008) used flow cells to investigate levels of attachment of mutants of *L. monocytogenes* that either lacked flagella, or expressed non-motile flagella, they identified that both mutants showed reduced initial attachment. When biofilms of these mutants were left to develop they observed a production of a 'hyperbiofilm' of very high density, due to the lack of either the flagella, or the motility of flagella, preventing the bacteria from dispersing and spreading from the site of initial attachment and going on to produce a mature biofilm.

Cell - cell signalling

Cell - cell signalling is important in biofilm maturation. The most commonly employed of cell signalling methods is quorum sensing, which allows cells to co-

ordinate gene expression in environments that require a synchronized population response. *Listeria monocytogenes* has two characterised quorum sensing systems, the Agr system and the autoinducer-2 LuxS system (Miller *et al*., 2001; Garmyn *et al*., 2009). The Agr system is composed of an operon of four genes, *agrBCDA*, and acts as a two component system, with *agrB* and *agrD* encoding a transmembrane protein and a propeptide AgrD, and AgrC and AgrA encoding the sensor kinase and response regulator respectively. Agr system deletion mutants showed reduced attachment to glass, stainless steel and polystyrene with up to 62% less attachment to glass in comparison to the wild type shown in one study (Garmyn *et al*., 2009). It is not yet known why this system seems to affect attachment.

The luxS system was first identified in *Vibrio harveyi* (Bassler *et al*., 1997) and is found in both Gram positive and Gram negative organisms. In *L. monocytogenes* the genes *pfs* and *luxS* encode two enzymes, which catalyse the conversion of the toxic substance S-adenosyl homocytein (SAH) into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD), DPD later rearranges itself into the Auto Inducer 2 (AI-2) (Garmyn *et al*., 2009), which is thought to be a universal signal involved in bacterial cell communication. Two studies so far have identified that strains with mutations in *luxS* lead to an increase in the ability of *L. monocytogenes* to attach to glass and stainless steel (Challan Belval *et al*., 2006; Sela *et al*., 2006). Attachment of bacteria with these LuxS mutations results in a denser, thicker biofilm, indicating that the presence of the *luxS* gene in *L. monocytogenes* either represses the formation of biofilm or possibly allows for better dispersion of the

biofilm.

RelA and *hpt*

Using transposon mutagenesis the genes *relA* and *hpt* were identified to be important in surface attached growth (Taylor *et al*., 2002) when tested using a microtitre plate assay. Both these genes are involved in the mounting of a stringent response in starvation. The *relA* gene is involved in the synthesis of (p)ppGpp, and the *hpt* gene codes for a 6-oxopurine phosphoribosyltransferase which is involved in converting purines into the corresponding nucleotide. The study also identified that the level of expression of *relA* increased during surface attached growth, suggesting that a stringent response is mounted during biofilm formation.

Phosphoenolpyruvate phosphotransferase systems (PEP-PTS)

A study by Gorski *et al*. (2003) investigated genes in *L. monocytogenes* involved in attachment to radish tissue, using transposon mutants in an attachment assay at different temperatures. They identified five mutants that showed reduced attachment, four of which showed a reduction in motility. The mutant that was still motile was identified to be in a gene encoding a BglG transcriptional antiterminator which is located upstream from genes which code for a B-glucosides transport system, the phosphoenolpyruvate phosphorotransferase system (PEP-PTS), which is thought to be involved in dulcitol transport in *L. monocytogenes*. This mutant only showed reduced attachment at temperatures of 30°C and lower. PEP-PTS systems transport B-glucosides inside the bacterial cell in the presence of a high

amount of sugar, phosphorylating them in the process. Transcription of the PEP-PTS genes is thought to be regulated by the BglG transcriptional antiterminator (Raveh *et al*., 2009). *L. monocytogenes* contains 84 PEP-PTS associated genes, which is the highest number found within a single organism (Glasser, 2001). Currently the involvement of the PEP-PTS genes or their associated transcriptional antiterminators in unknown, however it has been hypothesised that the transport of sugars by these systems in *Streptococcus mutans* and *Pseudomonas aeruginosa* is involved in the production of carbohydrate containing exopolysaccharides during biofilm formation.

prfA

Recently the virulence regulator prfA has been identified as playing an important role in the regulation of biofilm formation in *L. monocytogenes* (Lemon *et al*., 2010). This transcriptional regulator is important in virulence by controlling the expression of key virulence factors. In the study by Lemon *et al*. (2010), a deletion mutation in the *L. monocytogenes* gene *prfA* resulted in a biofilm deficient mutant when tested for biofilm production on polystyrene and glass, this deficiency was shown to be after the initial attachment stage, during biofilm maturation, flagellum based motility was not affected, the explanation as to why this mutant produces less biofilm is not currently known. This reduction in biofilm formation observed in the mutant strain, was seen at all temperatures studied (~24°C, 30°C, and 37°C).

1.2.4 Methods for investigating biofilm formation

Currently a number of methods exist for both quantitative and qualitative analysis of biofilms in *L. monocytogenes*. Methods include viewing by electron microscopy, micriotitre assays and agar selection techniques. Variations in methods between studies can sometimes result in conflicting information. This section will discuss the positives and negatives of some of the methods currently utilised.

Microtitre plate assays are the most commonly used method for quantification of attachment of *L. monocytogenes* to plastic surfaces. These methods are either based on the organism binding to the surface of the well of the microtitre plate, or to a coupon placed at the bottom of the well. As there is no flow of liquid in these systems, or normally no replacement of liquid, environmental conditions will change throughout the time of the assay (nutrient deprivation, accumulation of toxic products, etc) (Coenye *et al*., 2010). There are considerable advantages to these systems in that they are cheap, only small volumes of reagents are required, and they can be used to process a number of samples at once. This method is often used to screen for mutants that are deficient in biofilm formation. Many variations occur in the methods of measuring biofilm formation in microtitre plate assays. Some assays are set up to measure surface attached growth as the method described in Taylor *et* al. (2002), where as some are to measure biofilm after a set period of time such as 6 hours in the case of Jordan *et al*. and some as much as 40 hours (Boruki *et al*., 2003). To quantitate the amount of adhered bacteria in these assays, cells are normally stained with 0.1% crystal violet solution which allows quantification of the number of attached cells, by measuring the

absorbance of crystal violet which has been taken up by the cells. This assay quantitates the entire amount of cells adhered to the surface rather than any specific part of the biofilm, such as the amount of EPS produced. These microtitre plate assays can also be utilised to examine the effect on biofilm formation of certain conditions such as specific incubation temperatures, different growth media, and O_2 concentrations (Coenye *et al*., 2010). The microtitre plate method also comes with some disadvantages however, as it does not allow for distinction of live and dead organisms, expecially in those with short incubation times, also these method do not allow for direct visualisation of the biofilm. To examine whether the bacteria in a biofilm are alive or dead as well as looking at surface attached growth it may be possible to utilise a live/dead stain which can be viewed under fluorescence microscopy. A variation on the microtitre plate method, the Biofilm ring test, has been used to measure biofilm in *L. monocytogenes* (Chavant *et al*., 2007). This method measures the immobilisation of paramagnetic beads, which are contained within the liquid culture medium.

As staining of the biofilm produced in microtitre plate assays only gives an indication of amount of adhered cells, a number of other stains have been utilised to attempt to quantify the amount of exopolymeric substances produced. The presence of EPS produced by *L. monocytogenes* is much debated, assays have utilised ruthenium red staining (Boruki *et al*., 2003), and congo red agar (Delgado da Silva *et al*., 2001) to attempt to detect the presence of any exopolymeric substances produced. These stains are thought to bind to any carbohydrates present, and therefore would indicate a presence of carbohydrate containing EPS.

However it is also feasible that this dye may also be binding to carbohydrates bound to the *L. monocytogenes* cell surface rather than secreted EPS.

Scanning electron microscopy (SEM) has been used in several studies to show *L. monocytogenes* attachment to a variety of different surfaces including stainless steel and PVC (Beresford, 2002; Broucki *et al*., 2003). This method has also been utilised to try and visualise the presence of any exopolymeric substances produced by *L. monocytogenes*. In this method pre-sterilised coupons are exposed to bacterial culture and incubated for the desired amount of time before being washed and fixed. SEM allows direct visualisation of the biofilm and allows observation of any EPS, however SEM does not allow for quantification of cells adhered to surface so cannot be used to accurately compare attachment between strains.

Flow displacement systems have also been utilised to assess biofilm formation in *L. monocytogenes* (Perni *et al*., 2007; Szlavik *et al*., 2012). These systems were used to test adherence of *L. monocytogenes* to stainless steel or glass surfaces, some of which had been coated with dairy or meat products, under a continuous flow system at low and high shear stress. These systems were also used to look at persistence of strains of *L. monocytogenes* (Szlavik *et al*., 2012). The study by Perni *et al*. (2007) was also utilised to compare strains that were from locations that had previously been exposed to liquid flow to strains that were from static locations. These systems give a good insight into biofilms in food processing factories as they have been exposed to shear stress, so are a good model for biofilm formation. However again quatification as to the amount of cells adhered is

not possible.

Biofilm production can also be studied at the transcriptomics levels. If genes within a particular organism are known already to be responsible for adhesion to surfaces quantification of expression of these genes can be performed by qRTPCR. Oliveria and Cunha (2010) utilised this method to look at adhesion of *Staphylococcus epidermidis* to surfaces. They measured the expression of the *ica* locus which encodes for the adhesion responsible for attachment. However again although this gives you a quantitative measurement for biofilm production, again this does not take into account biofilm architecture. Also the expression of genes involved in biofilm may also be subject to a range of other external factors.

Although all these methods can be utilised to study biofilms, ideally a combination of some of them would be utilised to give both a quantitative measurement as well as giving some insight into the structure of the biofilm.

1.3 Aims

The overall aim of this thesis was to identify genes involved in attachment of *Listeria monocytogenes* to abiotic surfaces to attempt to elucidate the process involved in attachment of this organism. To achieve this aim firstly an existing attachment assay was modified to give consistent results of attachment of *L. monocytogenes* to polystyrene microtitre plate. This method was then used for comparison of levels of attachment of previously constructed transposon mutants to their isogenic parent during the early attachment phase of biofilm formation to

plastic surfaces. Any mutants identified as surface attachment deficient (SAD) were selected, and deletion mutations in these genes made to confirm the involvement of these genes in attachment. Further phenotypic characterisation of these mutants was also carried out.

2. Materials and Methods

2.1 Materials

Unless otherwise stated all chemicals and media reagents were from Sigma Aldrich (UK) and Oxoid (Thermo-Fisher, UK) respectively. All primers for PCR and reverse transcriptase PCR were from Eurofins UK.

2.2 General methods

2.2.1 Bacterial strains and growth conditions

For all bacterial strains used in this study see Table 2.2.1. All strains of *Listeria monocytogenes* were routinely grown in Tryptone Soya Agar (TSA; 16g TSA added to 400ml dH_2O) or Tryptone soya broth (TSB; 12g TSB added to 400ml dH_2O) unless otherwise stated. For growth of strains containing the transposon Tn917, media were supplemented with 1μg/ml erythromycin and 25μg/ml lincomycin. All strains of *Escherichia coli* were grown in Luria Bertani Agar or broth (10 g Tryptone, 5g Yeast extract, 10 g NaCl to 1L of water. For Luria Bertani agar, 1.5% (w/v) agar was added). Media for growth of strains carrying plasmids were supplemented with antibiotics as described in Section 2.2.2. All media were prepared using distilled water and autoclaved at 121ºC at 15psi for 20 minutes. Frozen stocks were made of all bacterial strains for long term storage at -80ºC in 10% (v/v) glycerol (Fisher, UK) to act as a cryoprotectant.

Table 2.2.1 – List of bacterial strains used in this study and their source

Organism	Strain	Source
L. monocytogenes	EGD-e	Frozen stocks, Department of 3I, University of Leicester
L. monocytogenes	10403s	Frozen stocks, Department of 3I, University of Leicester
L. monocytogenes	M113	Transposon library created by Dr Mark Beresford
L. monocytogenes	M237	Transposon library created by Dr Mark Beresford
L. monocytogenes	B380	Transposon library created by Dr Mark Beresford
L. monocytogenes	C52	Dairy isolate from the LMTOOCHE project (Chambel *et al*. 2007)
L. monocytogenes	SA11	Dairy isolate from the LMTOOCHE project (Chambel *et al*.2007)
L. monocytogenes	C882	Dairy isolate from the LMTOOCHE project (Chambel *et al*.2007)
L. monocytogenes	G8	Dairy isolate from the LMTOOCHE project (Chambel *et al*.2007)
L. monocytogenes	T8	Dairy isolate from the LMTOOCHE project (Chambel *et al*.2007)
L. monocytogenes	SA264	Dairy isolate from the LMTOOCHE project (Chambel *et al*.2007)
L. monocytogenes	T46A	Dairy isolate from the LMTOOCHE project (Chambel *et al*.2007)
L. monocytogenes	SA641	Dairy isolate from the LMTOOCHE project (Chambel *et al*.2007)
L. monocytogenes	A9	Dairy isolate from the LMTOOCHE project (Chambel *et al*.2007)
L. monocytogenes	T582	Dairy isolate from the LMTOOCHE project (Chambel *et al*.2007)
L. monocytogenes	G222	Dairy isolate from the LMTOOCHE project (Chambel *et al*.2007)
L. monocytogenes	InlAm	Prof Ian Roberts, University of Manchester
L. monocytogenes	Δ*lmo0401*	Deletion mutant in gene *lmo0401* created in this study
L. monocytogenes	Δ*lmo0402*	Deletion mutant in gene *lmo0402* created in this study
L. monocytogenes	Δ*lmo0501*	Deletion mutant in gene *lmo0501* created in this study
L. monocytogenes	Δ*lmo1226-1*	Deletion mutant in part of the gene *lmo1226* created in this study
L. monocytogenes	Δ*lmo1226-2*	Deletion mutant of the whole gene *lmo1226* created in this study
L. monocytogenes	InlAm Δ*lmo1226-1*	Deletion mutant of part of the gene *lmo1226* created in this study made in an InlAm background
L. monocytogenes	InlAm Δ*lmo1226-2*	Deletion mutant of entire gene *lmo1226* created in this study made in an InlAm background
E. coli	XL-10 Gold	Ultracompetent cells from Stratagene

2.2.2 Plasmids

All plasmids used in this study are shown in Table 2.2.2. Extracted plasmids were stored at -20°C. Stock solutions of Erythromycin required for growth of strains carrying these plasmids was prepared in 100% ethanol and was filter sterilised using a 0.2µm acrodisc (Pall Scientific) before being added in the required concentration to autoclaved media. Erythromycin was added to all strains harbouring plasmids at a concentration of 300µg/ml in *E. coli* strains and 5µg/ml in *L. monocytogenes* strains.

Table 2.2.2 – List of all plasmids used or created within this study

Plasmid	Reference	Characteristics
pAULA	(Chakraborty *et al.* 1992)	Temperature sensitive vector used for recombination, erythromycin resistance cassette
pINF401	This study	pAULA plasmid with fragments of gene *lmo0401* inserted, see figure 5.2 for details
pINF402	This study	pAULA plasmid with fragments of gene *lmo0401* insert, see figure 5.2 for details
pINF501	This study	pAULA plasmid with fragments of gene *lmo0501* insert, see figure 5.2 for details
pINF1226-1	This study	pAULA plasmid with fragments of gene *lmo1226* insert , see figure 4.3 for details
pINF 1226-2	This study	pAULA plasmid with fragments of gene up and downstream of *lmo1226* insert , see figure 4.3 for details

2.2.3 Primers

All primers used were from Eurofins MWG Operon. Primers were diluted in nanopure H_2O to a stock concentration of 100pmol/µl, and to a working concentration of 20pmol/µl. Primer stocks were stored at -20°C. Details of primers used are set out in Table 2.2.3. All primers were designed for this study except those used for Arbitrary PCR. The primers Starpharb1, 2 and 3, 5.1, 5.2, 5.3, 3.1, 3.2 and 3.3 were used as described in Knobloch *et al*. (2003).

Table 2.2.3 – List of PCR primers used for this study

Primer	Sequence 5'-3'
Stapharb1	GGCCACGCGTCGACTAGTCANNNNNNNNNNGATAT
Stapharb2	GGCCACGCGTCGACTAGTCANNNNNNNNNNGATCA
Stapharb3	GGCCACGCGTCGACTAGTCA
5.1	ATCGATACAAATTCCTCGTAGG
5.2	AACCGTTACCTGTTTGTGCC
5.3	CCAATCACTCTCGGACAATAC
3.1	TTTAGTGGGAATTTGTACCCC
3.2	GGGAGCATATCACTTTTCTTGG
3.3	GAACGCCGTCTACTTACAAGC
M113C	GCGCAACAGAACTAGCAGCCAC
M113D	GAGCCCGTGTGCTTGACCTGG
M113E	TTGTGCTTCGCGACCGTGC
M113F	GCGATGCTGAAAGCGGGCTA
M13UR	CAGGAAACAGCTATGAC
401A	TGGGGTCACGGAAGGTGCGA
401B	GCGGTTAGGGCGTAGCGCAT
402A	TCCAAGGCAAGGAACGCCACT
402B	ATGCTTAACGAAGCTCCCACAATAAGT
501A	GCGCATTCTTAAGCACCGAACCC
501B	AGCGACGCCAGAAGTTCCGC
401_INF Fw1	CGGTACCCGGGGATCTTGCGCTACTGCCCGGAAGA
401_INF Rv1	TCGGTGTAGGTTCTGCCCGCGCGATTTGGTATTCCCGCGT
401_INF Fw2	ACGCGGGAATACCAAATCGCGCGGGCAGAACCTACACCGA
401_INF Rv2	CGACTCTAGAGGATCGCGCGTCTGGTGTCGCAA
402_INF Fw1	CGGTACCCGGGGATCGAAACACTATATGGCATGCTCCTT
402_INF Rv1	AGCGCTTGATGTGCTCCGTCAAAATCGTATCCGCCAAGTCTTC
402_INF Fw2	TGACGGAGCACATCAAGCGCTATTATCCG
402_INF Rv2	CGACTCTAGAGGATCACGGCGCGAACGGATTGT
501_INF Fw1	CGGTACCCGGGGATCGACGCATGTATCTGGATAACAG
501_INF Rv1	AACCCCGCTCGGACAGAGCACCACTCGCTTCCTGCTAACT
501_INF Fw2	TGCTCTGTCCGAGCGGGGTTACTTTTTCA
501_INF Rv2	CGACTCTAGAGGATCGAGTCGATTGACCCCTGCA
1226_INF FW1	CGGTACCCGGGGATCTGGCTTGCAGCAGCAGTCGT
1226_INF RV1	GGCACCTTGTCCAGCAGCGATGGGTTGAGACCGGCTCCCA
1226_INF FW2	TGGGAGCCGGTCTCAACCCATCGCTGCTGGACAAGGTGCC
1226_INF RV2	CGACTCTAGAGGATCGAGCGCGACGAGCACTAC
1226_INF2 fw1	CGGTACCCGGGGATCGGGCATTGCGGCTGGATTT
1226_INF2 Rv1	GCCACCAATTGCCTCGTCCGAATACGACTGCTGCTGCAAGCC
1226_INF2 fw2	GGCTTGCAGCAGCAGTCGTATTCGGACGAGGCAATTGGTGGC
1226_INF2 Rv2	CGACTCTAGAGGATCGCCCGTGTGCTTGACCTGGT

2.2.4 Attachment Assay

This method was used to measure the attachment of *L. monocytogenes* to abiotic surfaces and was adapted from Taylor *et* al. (2002). Bacterial cultures were streaked onto TSA from frozen stock and incubated overnight at the desired temperature. Colonies from the plate were inoculated into 10ml TSB and incubated, shaking overnight at 220rpm, at the desired temperature. Following incubation, the optical density of the culture was read on a spectrophotometer (Jenway) at 500nm, and the culture was diluted to an OD_{500nm} of 0.7 with fresh TSB. Next, 200µl of this culture was dispensed into each well of a 96-well flat bottomed polystyrene plate (Greiner, UK), and the plate was incubated, stationary, at the desired temperature for two hours. After incubation the supernatant was gently removed and the wells washed twice with 220µl Phosphate Buffered Saline (136mM NaCl, 2.68mM KCl, 10.14mM Na_2HPO_4, and 1.76mM KH_2PO_4, pH7.4). The wells were air-dried for 15 minutes next to a Bunsen burner. Cells were then heat fixed onto the bottom of the plate in a hot air oven set at 80ºC, for thirty minutes, rotating the plate half way through to ensure even heat distribution. Wells were subsequently stained with 220µl 0.1% (v/v) methyl crystal violet (TCS Biosciences) solution for one minute. The methyl crystal violet was then gently removed, ensuring the edges of the well were not touched with the pipette. Excess crystal violet was removed by gently washing wells three times with 220µl PBS. Stain was then recovered from adhered cells with 220µl 80:20 Ethanol:Acetone solution (Thermo Fisher) for 15 minutes. Absorbance was read at 595nm with a Biorad 680 Microplate Reader.

2.2.5 Counting of bacterial colonies

Bacterial colonies were counted to give colony forming units per millilitre using the Miles and Misra method (Miles and Misra, 1938). Serial dilutions were made by the addition of 200µl of bacterial culture to the first well of a row in a 96-well microtitre plate; 180µl of PBS was added to the remaining wells in that row. Next, 20µl of the bacterial culture was serially diluted across the plate (creating tenfold serial dilution) changing tips in between each dilution; finally 20µl was discarded from the last well. Agar plates were marked into sections and 50µl of each dilution was plated in each section as three drops, starting with the highest dilution. Plates were left to dry and then incubated inverted at the correct temperature.

2.2.6 Chromosomal DNA extraction

Chromosomal DNA was extracted using a modified version of the method by Dillard and Yother (1991). An overnight culture was harvested by centrifugation at 4000xg for 10 minutes. The pellet was re-suspended in 2.5ml TE buffer (10mM Tris pH8.0, 1mM EDTA), and 0.25ml of 10% (w/v) sodium dodecyl sulphate. After the pellet was resuspended, it was incubated at 65°C for 15 minutes followed by incubation at 4°C for one hour. Debris was removed by centrifugation at 11000xg for 10 minutes at 4°C and ethanol was added at twice the volume of the retrieved supernatant. This was then placed at 20°C overnight. DNA was retrieved by centrifugation at 1000xg for 45 minutes at 4°C and the pellet was dried in a vacuum chamber and resuspended in nanopure H_20. The resuspended DNA was

cleaned with a PCR purification kit (Qiagen, UK). DNA was viewed on a 1% (w/v) agarose gel (as described in 2.2.8), and was quantified using a nanodrop spectrophotometer (ThermoFisher).

2.2.7 Colony PCR

Colony PCR was carried out for screening of strains for genes or mutations. A sweep of colonies was inoculated into 100µl sterile nanopure H_2O and boiled for 10 minutes. The boiled colonies formed the template for the PCR. 5µl of this template was used in each reaction with 1µl of primer (100pmol/ml), 10µl of MyTaq Red PCR buffer (Bioline), sterile nanopure H_2O to a volume of 49.5µl, and 2.5 units of Taq polymerase. PCR reactions were carried out in a Biometra T1 gradient thermal cycler with the following cycle: Initial denaturation at 95°C for 2 minutes, followed by 30 cycles of 95°C for 30 seconds (denaturing), 60°C for 30 seconds (annealing; temperature depended on the melting temperature of the primers) and 72°C for 3 minutes (elongation; time depended on the size of the product), and finally anelongation stage of 72°C for 10 minutes.

2.2.8 Agarose gel electrophoresis

DNA was separated by agarose gel electrophoresis to allow visualisation and confirmation of band size in accordance with the method by Sambrook *et al*. (2000). Agarose (Bioline) was prepared at concentrations of 0.8, 1 and 1.5% (w/v) depending on the size of the DNA. Agarose was dissolved in TAE buffer (40mM

Tris acetate, 1mM EDTA, pH 8.0) and ethidium bromide added to a concentration of 0.2µg/ml. DNA was mixed in a 1:5 ratio with gel loading buffer (Fermentas), with 6µl loaded into each well, PCR products amplified using MyTaq Red polymerase (as described in section 2.2.7) already contained loading dye so were directly loaded into each gel. DNA ladders were used (1kb and 100bp, NEB) to quantitate the DNA band (prepared according to manufacturer's instructions). Electrophoresis was done at 80-100 volts for 45 minutes. DNA was visualised under UV light from a transilluminator, and photographed using a gel doc system (ImageQuant 100, GE Healthcare).

2.2.9 Extraction and digestion of plasmid DNA

A colony of *E. coli* containing pAUL-A plasmid (Figure 2.1) was inoculated into 10ml Luria Bertani broth and incubated at 37°C, overnight, shaking at 220rpm. Plasmid was extracted following the incubation using a Qiagen Maxi Prep kit according to kit instructions. Following the purification the resulting plasmid was eluted in 1.5ml nanopure H_20. Eluted pAUL-A plasmid was cleaned using Wizard S/V gel and PCR cleanup system (Promega), in accordance with the kit instructions and resuspended in a volume of 30µl of nanopure H_20. This was then digested with *BamH1* in the following reaction:

DNA	up to 10µg re-suspended in 30µl
BamH1	40 units
10x Buffer	5µl
100 x BSA	0.5µl
Water	up to a volume of 50µl

Digests were incubated at 37°C for one hour in a water bath, and then cleaned again with the Wizard S/V gel and PCR cleanup system. Cleaned digests were resuspended in 30µl nanopure H_20, and were run on an agarose gel to visualise the digest (see Section 2.2.7). Concentrations of cut plasmid were determined using a Nanodrop spectrophotometer (Thermo Fisher).

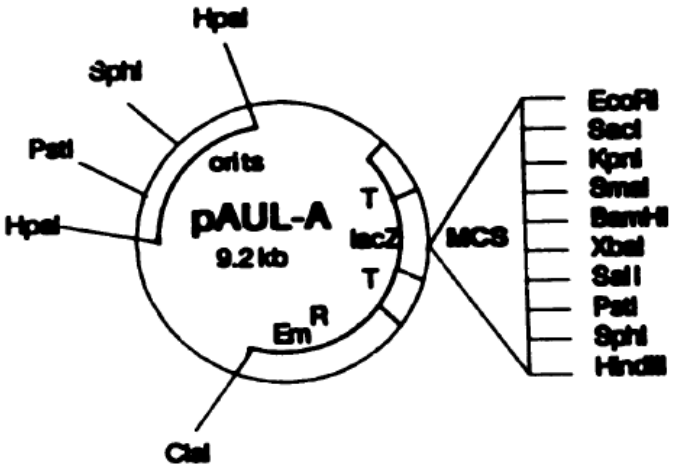

Figure 2.1 – Plasmid map of pAUL-A plasmid (Chakraborty *et al*., 1992)

2.2.10 Cloning into digested plasmid

Plasmids were constructed using an Infusion HD cloning vector kit (Clontech Laboratories, part of Takara Biotech). Primers were designed to amplify two separate products up and downstream of the part of the gene that was to be

deleted. Primers were designed for each PCR product with a 15 base pair overlapto the BamH1 restriction site (part of the multiple cloning site) of the plasmid, and a 15 bp overlap to the other PCR product (see Figure 2.2). The plasmid was digested using the restriction enzyme BamH1, as described in Section 2.2.8.

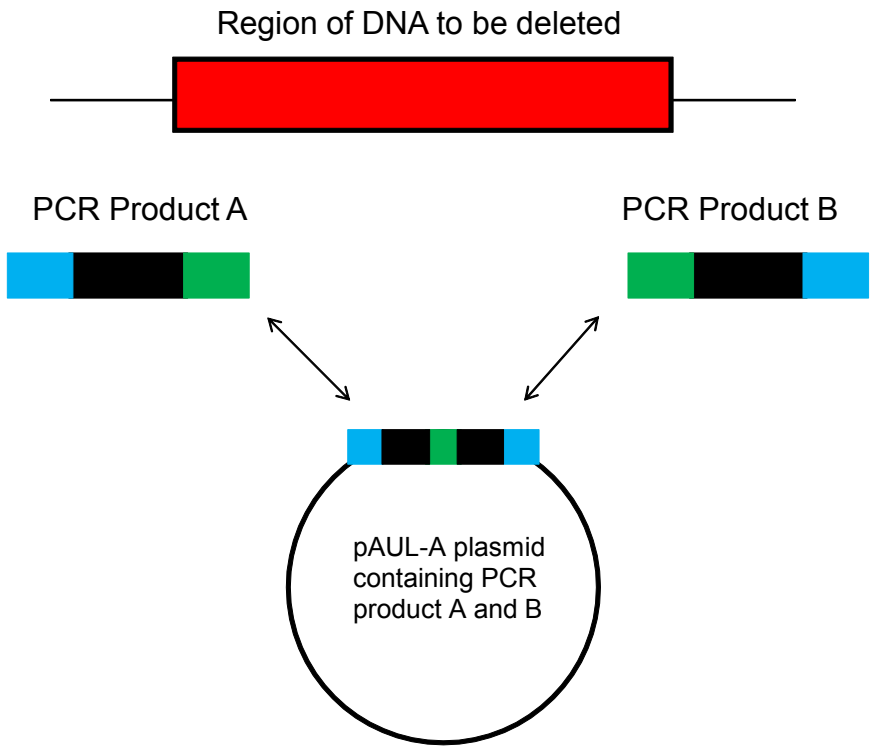

Figure 2.2– Figure depicting the cloning procedure into pre-digested plasmid. The blue regions indicate the ends that overlap with the *BamH1* restriction site and the green regions indicate the part of the PCR products that overlap each other.

PCR products were amplified with these primers using colony PCR as described previously in Section 2.2.7. PCR product sizes were confirmed by running them on an agarose gel as described in Section 2.2.8. PCR products were purified using the Wizard S/V gel and PCR cleanup system (Promega), in accordance with kit instructions. To clone the cleaned PCR products in to the plasmid ligation reactions were set up as follows:

Purified PCR fragments	50ng each
Digested plasmid	100ng
5X In-Fusion HD enzyme premix	2µl
nanopure H_2O	up to 10µl

Negative and positive controls were also set up substituting water for the PCR fragments for the negative control, and using the kit control insert and the kit pUC19 control plasmid for the positive control. Reactions were incubated at 50°C for 15 minutes, before being used for transformation into *E. coli* (see Section 2.2.11). Excess ligation reaction was stored at -20°C for future use.

2.2.11 Transformation of *E. coli* by heat shock

E.coli XL-10 gold cells (Stratagene) were transformed with derivatives of the pAUL-A plasmid in accordance with manufacturer's instructions. SOC medium (2g Tryptone, 0.5g Yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose in 100ml distilled H_2O) was pre-warmed at 42°C to facilitate cell recovery after transformation. Competent cells were defrosted slowly on ice,

mixing gently by flicking the tube. 15ml Falcon tubes (BD) were pre-chilled on ice before adding 50µl of cells to each tube. 2µl of plasmid was added to each Falcon tube and the tube was mixed and placed on ice for 30 minutes. Cells were then heat-shocked in a waterbath at 42°C for 30 seconds before being quickly submerged back on ice for two mins. Following the heat shock process 950µl of prewarmed SOC was added immediately to each tube, and cells were incubated with gentle shaking (150rpm) at 37°C for one hour. Varying volumes of cells were plated onto Luria agar containing 300µg/ml Erythromycin, 80µg/ml X-Gal (prepared in DMSO), 100µM IPTG (prepared in sterile nanopure H_2O) and spread onto the plate. Plates were incubated overnight at 37°C. Following overnight incubation transformants containing inserts were identified as white colonies on the plate.

2.2.12 Preparation of *L monocytogenes* cells for electroporation.

This method was modified from a protocol by Park and Stewart (Park & Stewart 1990). First, 0.5L of medium A (Brain heart Infusion broth, and 0.5M Sucrose) was inoculated with 10ml of a *Listeria* overnight culture. This culture was grown shaking at 37ºC until reaching an optical density (600nm) of 0.2. When reaching this optical density 10µg/ml Penicillin G was added and incubation continued until reaching an OD_{600nm} of 0.5-0.7. The bacterial cells were then harvested by centrifugation for 10 minutes at 3000xg, 4ºC. The pellet was washed with 250ml of Solution one (0.5M Sucrose and 1mM Hepes) and centrifuged under the same conditions. This washing step was then repeated with first 100ml then 30ml of Solution1. On completion of washing steps the pellet was resuspended in 500µl of Solution 1 and

kept on ice. The cells were aliquoted into 40µl volumes in microcentrifuge tubes and stored at –80ºC until use.

2.2.13Transformation of *L. monocytogenes* by electroporation

Aliquots of 40µl of electrocompetent cells were pipetted into prechilled sterile electroporation cuvettes. Next 100ng of plasmid DNA was added to the cells, mixed gently and incubated on ice for exactly one minute. The cuvette was then placed in the electroporator (Biorad) between the two electrodes. The parameters used for electroporation were 25µF, 12.5kV cm^{-1}, and 1000Ω. Immediately after electroporation 1ml of prewarmed SOC medium was added to the cuvette and the suspension was mixed by pipetting then transferred into a 15ml tube. The transformed cells were incubated at 30ºC for 3 hours with gentle shaking at 150rpm to allow recovery. After incubation 250µl volumes were pipetted onto Brain Heart Infusion agar (14.8g Brain heart infusion, 1.5% (w/v) Agar) containing erythromycin and these were incubated at 30ºC for 48 hours.

2.2.14 Recombination of plasmid into *L. monocytogenes*

Recombination of plasmid DNA in to the *L. monocytogenes* chromosome was carried out as described previously (Corbett *et al*., 2011). Colonies from the transformation were plated onto BHI agar and incubated at 42°C overnight, colonies that grew after overnight incubation were subcultured onto fresh BHI Agar and incubated overnight again at 42°C. Integration of the plasmid into the

chromosome was confirmed by colony PCR using a primer that annealed to chromosomal DNA up or downstream from the region being deleted, paired with M13 universal reverse or M13 universal forward, respectively. Colonies positive for chromosomal insertion of plasmid were inoculated into 10ml BHI broth and incubated at 30°C for 24hours. Cultures were further subcultured twice a day for three days diluting 1:100 into fresh BHI. Following sub-culturing strains were serially diluted and 100µl of the higher dilutions (10^{-5} to 10^{-7}) were plated onto BHI agar without erythromycin and incubated at 42°C overnight. Colonies were then replica plated onto BHI with and without Erythromycin and incubated at 30°C overnight. Colonies that were erythromycin-sensitive were selected for colony PCR (Section 2.2.7) to determine if a double crossover had taken place.

2.2.15 RNA extraction

RNA was extracted using the method of Mangan *et al*. (2002). Strains were grown in TSB to mid exponential phase (OD_{500nm} 0.7-0.8) before extracting RNA. To 200ml of bacterial culture, 800ml of GTC solution (5M guanidine thiocyanate, 0.5% (w/v) sodium n-lauryl sarcosine, 25mM tri-sodium citrate, 0.1M 2-mercaptoethanol and 0.5% (v/v) tween 80) was added and the solution was mixed rapidly by stirring. The suspension was centrifuged at 3000xg for 20 min, the supernatant was discarded and the pellets resuspended in 1ml GTC solution. Resuspended pellets were transferred to microcentrifuge tubes, and centrifuged at 13000 rpm for 20 seconds in a microcentrifuge. The resulting pellet was resuspended in 1.2ml Trizol and immediately transferred to a ribolyser blue matrix tube (qBiogene, UK) and

processed in a Ribolyser (Hybaid) for 45 seconds at a power setting of 6.5. The tube was then left at room temperature for five minutes. Next, 0.2ml of chloroform was added to the tube which was mixed by vortexing for 15 seconds, and then left at room temperature for a further three minutes. This was then centrifuged at 12000xg for 15 minutes at 4°C. After centrifugation the upper clear aqueous layer was transferred to a fresh microcentrifuge tube. This process with chloroform was repeated and on removal of the second clear aqueous layer, 0.5ml of Isopropanol was added. After incubation at room temperature for 15minutes, the RNA was centrifuged at 4°C at 12000xg for ten minutes, and the resulting pellet was resuspended in RNase free water. RNA cleanup was then performed using the Qiagen RNA extraction kit and RNA was visualised by agarose gel electrophoresis, and quantified using the nanodrop (ThermoFisher)

2.2.16 Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 software. For analysis of *in vitro* experiments one way ANOVA, with Banoffi post tests, or a student's T-test was used to determine the P-value. In vivo survival experiments were analysed using the Kaplan Meier survival curve analysis.

2.2.17 *In silico* analysis

In silico analysis was performed to identify the genes using Blastn at NCBI (http://www.ncbi.nlm.nih.gov/). NCBI was also used for looking analysing DNA sequence data and the *L. monocytogenes* genome, and BlastP was used for analysis of *L. monocytogenes* protein sequences. SPLIT4 (Juretic. 2002) was used for protein structure analysis and pSORTB to identify the location of the protein (Yu, 2010). TOPO2 software (Johns, SJ.) was used to create 2-dimensional models of protein structures.

2.2.18 Growth curves of *L. monocytogenes*

Colonies from a plate of *L. monocytogenes* was inoculated into 10mls of TSB, and incubated overnight, shaking at 200rpm at the required temperature. Following overnight incubation cultures were diluted to a starting $OD500_{nm}$ of 0.1. 2ul of this diluted overnight culture was inoculated into 198ul of TSB in a well of a flat bottomed microtitre plate. Each strain was performed in triplicate. Microtitre plates were incubacter with shaking in the Multiskan Go plater reader (Thermo Fisher) at the appropriate temperature until stationary phase was reached.

2.3 Identification of mutants deficient in attachment

2.3.1 Arbitrary PCR of transposon mutants

Arbitrary PCR was performed based on the method by Knobloch (2003). Amplification of the flanking regions of the transposon was achieved by a two step PCR reaction of chromosomal DNA. Round one used the primers STAPHARB1 or STAPHARB2 to bind to the chromosomal DNA paired with a primer 5.3, 5.2, 3.3 or 3.2, which binds within the Tn917 transposon as demonstrated in Figure 2.3

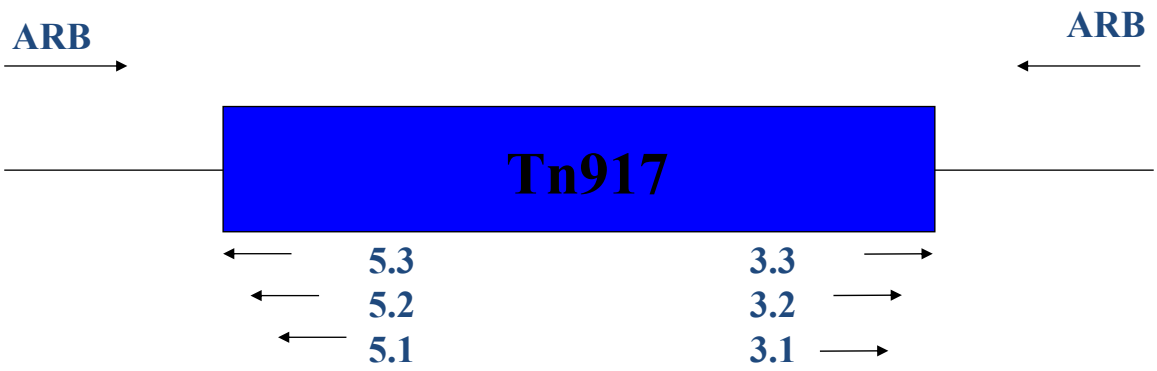

Figure 2.3- Diagram of the Arbitrary PCR reaction indicating where the primers bind within the chromosome and the transposon.

The inital reaction was set up as follows:

Template	1µg
Primer 1 (STAPHARB1 or 2)	200pmol
Primer 2 (5.3, 5.2, 3.3 or 3,2)	10pmol
dNTPs	5µl

10x Reaction buffer	5µl
$Mg2^{+}$	12µl
H_2O	6.75µl
Taq	0.25µl

PCR was performed under the following conditions: 95°C for 5 min; six cycles of 94°C for 30 s, 30°C for 30 s, and 72°C for 1 min; 30 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 1 min; and finally 72°C for 5 min. PCR products were cleaned using a Qiagen PCR purification kit according to the manufacturer's instructions and 5µl was analysed by running on a 1.5% (w/v) agarose gel electrophoresis in TAE buffer as described in 2.2.8. Any product giving a clear defined band was taken to be a positive. Reactions giving a positive result were used for the template of round two of the PCR. Round 2 used 5µl of this reaction in a final volume of 100µl, substituting STAPHarb3 paired with the respective Tn917 primer, and PCR was performed with the following programme: 30 cycles of 94ºC for 30 seconds, 45ºC for 30 seconds, 72ºC for one minute; followed by 72ºC for five minutes. Resulting PCR products were run on 1.5% (w/v) agarose gel electrophoresis as above.

2.3.2 Identification of Tn917 flanking regions

PCR products from the arbitrary PCR were purified using a Qiagen PCR purification kit (Qiagen, UK) and sent for sequencing (PNACL, University of Leicester). Sequencing results were entered into the NCBI BLAST website to allow identification of the flanking genes.

2.3.3 RTPCR

Reverse transcriptase was performed using the ProSTAR^{TM} HF Single-Tube RT-PCR system (High Fidelity) (Stratagene, UK). RNA that had been extracted from mid-exponential phase bacteria was DNase treated (Invitrogen, UK) prior to RTPCR. To do this, 1µg of RNA was added to 1x buffer, 1µl of DNase and dH_2O to a final volume of 10µl. This was incubated at room temperature for 15 minutes, and then the enzyme was inactivated by the addition of 1µl of 25mM EDTA (pH 8.0) and heated at 65°C for 10 minutes. This RNA was used to set up the RTPCR. For each RTPCR reaction, the following components were added to the RNA: 1x buffer, 100ng of both the forward and reverse primers, 1µl of dNTPs and RNase free water to a total volume of 48.5µl. To this 1µl of diluted strataScript was added, followed by 0.5µl of TaqPlus® Precision DNA polymerase mixture. Tubes were vortexed gently before undergoing the PCR program described in Table 2.4, on a thermal cycler (Biometra):

Cycles	Temperature	Duration
1	42°C	15 Minutes
1	95°C	1 Minute
40	95°C	30 Seconds
	60°C	30 Seconds
	68°C	2 Minutes
1	68°C	10 Minutes

Table 2.4 - Program used for the RTPCR

2.4 Characterisation of mutants

2.4.1 Growth in Sodium Chloride

Strains were grown on BHI Agar plates at 30°C, and then individual colonies were inoculated into a modified version of Trivett and Meyer broth (TM) (1971) as described in Appendix 1. Broths were incubated at 30°C overnight in a shaking incubator (New Brunswick) at 200 rpm. Cultures were diluted to an OD_{600nm} of 0.1 in fresh TM and then diluted 1 in 10 in TM containing the required concentration of NaCl in a 96-well flat bottomed microtitre plate. Growth in the microtitre plates were measured with a Multiskan plate reader (Thermo Fisher) at an optical density of 600nm, with measurements being taken every 20 minutes. Microtitre plates were incubated at 30°C during the whole process, with shaking at 200rpm. Strains were grown until stationary phase was reached.

2.4.2 Assay of osmotolerance

The ability of the mutants to mount an osmotolerant response was determined by the method of Faleiro *et al*. (2003).Strains were grown in TM medium to an OD_{655nm} of 0.15, and then centrifuged at 3000 x g for 15 minutes in a microcentrifuge. The resulting pellets were resuspended in TM medium containing 3.5% (w/v) NaCl for adapted cells or no added NaCl for non-adapted cells. Resuspended cells were incubated at 30°C for 2 hours shaking at 220rpm. Following incubation, adapted and non-adapted cells were centrifuged as before and resuspended into a flask containing 50ml TM medium with 20% (w/v) NaCl.

Flasks were incubated at 30°C shaking at 220rpm and samples were taken every 24hours. Samples were serially diluted and plated using Miles and Misra technique (see Section 2.2.7) onto tryptone soya agar in duplicate. Plates were incubated overnight and colonies counted to give colony forming units per ml.

2.4.3 Determination of minimum inhibitory concentrations of antimicrobial agents

The minimal inhibitory concentration was determined using a modified version of the method set out by Wiggins *et al*. (1978). The antimicrobial agents tested were Gentamicin, Ampicillin, Erythromicin, Spectinomycin, Streptomycin, Chloramphenicol and Tetracycline. The bile salt, sodium chlorate hydrate was used to test bile resistance. Strains were grown overnight in TSB at 37°C shaking at 220rpm until they reached stationary phase. Doubling dilutions of antimicrobial agents were made in a 96-well U-bottomed microtitre plate. Dilutions were made in TSB in a final volume of 198µl. 2µl of the overnight culture was added to each well, and plates were incubated, without shaking, at 37°C overnight. Following incubation the minimal inhibitory concentration was determined by identifying the lowest concentration of antibiotic which prevented growth in the well of the plate. Each dilution was performed in triplicate.

2.4.4 Adhesion and invasion to Caco-2 cells

2.4.4.1 Growth and maintenance of cell line

Adhesion and invasion assays were based on the methods of Van der Veen and Abee (2011), with some modifications. Caco-2 cells were obtained from Dr Richard Haigh (Department of Genetics, University of Leicester) but were originally derived from the National Collection of Type Cultures (NCTC, HPA, UK). Cells were routinely grown and maintained in DMEM containing 4.5g/L glucose, 2mM L-glutamine, 25mM HEPES and 10% (v/v) heat inactivated foetal bovine serum (all components were from Invitrogen, UK). All components were obtained sterile and complete medium was made using aseptic technique. All cell culture work was performed in a class II microbiological safety cabinet. Cells were routinely grown in 75cm^2 tissue culture flasks within a 37°C incubator under 5% CO_2. Cells were fed with fresh tissue culture medium every 2-3 days and grown to 70-80% confluence (as determined by light microscopy) before being subcultured (normally 4-5 days). To subculture cells, medium was aspirated and monolayers washed twice with 10ml of PBS. After washing 2ml of Trypsin:EDTA (0.25% (v/v):0.53mM) was added to the flasks, which were then incubated at 37°C for two minutes. 10ml of prewarmed tissue culture medium was added to deactivate the trypsin and cells were subcultured into new flasks containing fresh prewarmed medium at a dilution of 1:10. Frozen stocks of Caco-2 cells were also made from confluent flasks, with detached cells being transferred to 15ml centrifuge tubes which were spun in a bench top centrifuge for 5 minutes at 1500xg. The supernatant was removed and cells resuspended in prewarmed tissue culture medium containing 10% (v/v)

DMSO. Cells were transferred into cryotubes (Starstedt, UK) and placed into a Nalgene cryo freezing container, which contained 250ml isopropanol. This container was stored at -80°C for two hours and then Caco-2 stocks were transferred to liquid nitrogen for long-term storage.

2.4.4.2 Adhesion and invasion assays

Cells were prepared as described previously in section 2.4.4.1. After resuspension of detached cells in 10ml tissue culture medium, cells were made up to 100ml volume with tissue culture medium and used to seed 24-well (6.5mm) tissue culture plates (Costar). Cells were used at passage number 52-56 for all experiments. Wells were seeded with approximately $5x10^4$ cells in 1ml. Cells were incubated until confluent (approximately 4 days) and then left to differentiate for 7-8 days before being used for experiments. Frozen stocks of *L. monocytogenes* strains were defrosted at room temperature and diluted to a concentration of $1x10^8$ cfu/ml in prewarmed tissue culture medium. Cells were added to wells at a multiplicity of infection (MOI) of 100:1. Cells were incubated for 2 hours at 37°C under 5% CO_2. For adhesion assays, cell monolayers were washed five times with sterile pre-warmed PBS, enumeration of bacterial cells was performed on the 4th and 5th washing stages, by serial dilution and plating using the Miles and Misra technique (see Section 2.2.7). Washed monolayers were then lysed with 1(v/v)% Triton X-100 diluted in PBS, enumeration of bacterial cells in lysates was carried out. Cells were counted after overnight incubation at 37°C. For invasion assays,

after the two hours incubation period the bacterial suspension was removed and replaced with fresh sterile tissue culture medium containing 300µg/ml gentamicin, cells were incubated for three hours at 37°C under 5% CO_2 to allow the gentamicin to kill any cells still adhered to the cell surface. The monolayers are then washed three times with pre-warmed PBS and lysed with 1% (v/v) Triton X-100 diluted in PBS. Bacteria were enumerated on cell lysates.

2.4.5 Survival *in vivo*

All *in vivo* experiments were carried out using 8-9 week old MF1 outbred mice obtained from Charles River, UK. Mice were housed for a week in the Division of Biomedical Services, University of Leicester to acclimatise before starting any experiments. All experiments were carried out in accordance with the Home Office project licence and were approved by the University of Leicester ethical committee.

2.4.5.1 Preparation of bacteria for infection

L. monocytogenes strains were grown on BHI agar plates at 37°C overnight from frozen stocks. Individual colonies were picked and inoculated into 10ml BHI broth and incubated overnight at 37°C, shaking at 220rpm, until stationary phase. Cultures were centrifuged at 3000xg for 15min in a benchtop centrifuge (Beckman allegra) followed by resuspending in PBS. The cultures were then diluted to a concentration of $5x10^9$ in 0.5ml of PBS containing 0.5mg of calcium chloride.

2.4.5.2 Infection of mice

Mice were infected intragastrically with a volume of 0.5ml of bacteria using a 21g soft feeding tube (Instech Solomon, UK) attached to a 1ml syringe (BD plastipak). Each mouse was held by the scruff of the neck and held in a vertical position before the feeding tube was inserted into the mouse down to the stomach. Mice were returned to the cage and observed for any immediate signs of distress. Mice were then monitored for 10 days and disease signs recorded. Mice were assigned a score for the severity of disease as follows:

Normal	0
1+ Hunched	1
2+ Hunched	2
1+ Starey	3
2+ Starey	4
1+ Lethargic	5
2+ Lethargic	6
Culled	7

Mice reaching a severe (2+) lethargic state were culled by cervical dislocation, in accordance with the project licence. Any mice that survived for 10 days were considered to have survived the experiment.

2.5.1 Growth of mutants in different sugars

Strains were streaked onto TSA plates from frozen stock and incubated at the desired temperature. Colonies were inoculated into the TM medium (Appendix 1)

and incubated overnight shaking at 220rpm to stationary phase. TM medium with no sugar (TMNS) was made as shown in Appendix 1, to allow the medium to be supplemented with 10mM of B-glucosides. The B-glucosides tested were glucose, fructose, mannose, sucrose, galactitol, glucitol and mannitol. A no sugar control was also used in all experiments. 96 well-microtitre plates were set up with 198µl TM medium with different B-glucosides and 2µl of culture was added to each well. Each test was performed in triplicate. Plates were incubated overnight at the desired temperatures and growth was recorded after 24 hours of incubation at 37 and 30°C, and 48 hours of incubation at 20°C. Plates incubated at 10°C were agitated gently each day with a pipette and the OD_{595nm} was recorded using a Biorad 680 microtitre plate reader. Growth was recorded as a positive or when stationary phase appeared to have been reached.

2.5.2 API® *Listeria* test

Strains were tested for their ability to ferment sugar and perform enzymatic tests using the API® Listeria test strip (Biomerieux Inc.). All strains were tested at 10, 20, 30 and 37°C. Bacteria from frozen stocks were inoculated onto tryptone soya agar and incubated at the desired temperature overnight. Following incubation, colonies were suspended in the provided API suspension medium to an OD_{600nm} of approximately 0.25 (approximately equivalent to 1 McFarland). The suspension was used immediately to inoculate the test cupules. Test strips were prepared and inoculated in accordance with manufacturer's instructions, filling the cupule of all tests and the open part and the cupule of the DIM test. Strips were incubated at the

desired temperature overnight. Following incubation a drop of ZymB reagent was added to the DIM test and tests were read in accordance to manufacturer's instructions.

2.5.3 Quantitative RTPCR

2.5.3.1 cDNA production

2µg of RNA was treated with DNase1 (Qiagen) to remove any traces of genomic DNA in accordance with manufacturer's instructions. Treated RNA was cleaned using the RNeasy kit (Qiagen) in accordance with kit instructions and RNA was eluted in a volume of 50µl nanopure H_2O and the concentration was measured using a nanodrop spectrophotometer (ThermoFisher Scientific). The reverse transcriptase reaction was carried out using Superscript II reverse transcriptase (Invitrogen) and set up in a reaction volume of 40µl as following:

RNA	200ng
Random Primers (Invitrogen)	100nM
DNTPs (NEB)	10mM

Reactions were incubated at 65°C for 5 minutes in a thermal cycler (Biometra) before being submerged on ice and centrifuged at 13000 rpm briefly. 8µl of first strand buffer and 4µl of dTT was added to each tube and then incubated for 2 minutes at 25°C. Following incubation, 1µl Reverse transcriptase was added to

each reaction. At this point no reverse transcriptase negative controls were set up, using 1µl of nanopure H_2O instead of the enzyme.

2.5.3.2 Quantitative RTPCR

RNA was extracted from strains grown at 37°C, 30°C, 20°C and 10°C using the method described in Section 2.2.15. cDNA was produced on all samples as described in Section 2.5.3.1. Primers were designed to amplify a small region of the genes of interest, and the 16S gene to use as a house keeping reference gene. Table 2.5 shows the sequences of primers used. 20ng of cDNA was used in each reaction, with 200µM of each of the forward and reverse primer, 1x SYBR green Master mix (Bioline) and water to a volume of 25µl. Gene expression was quantitated on a Rotor gene 6000 instrument (Qiagen), with an initial denaturation period at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds. The quantitation program was followed by a melt curve analysis to confirm the presence of only one product in each reaction. The melt curve analysis was run at temperatures from 72-95°C.

Table 2.5 – List of primers used for qRTPCR

Primer	Sequence 5'-3'
16S Fw	TTAGCTAGTTGGTAGGGT
16S Rv	AATCCGGACAACGCTTGC
lmo0397 Fw	CGAATGGGACCAAGATGAAG
lmo0397 Rv	CGAAGAGCGTTTTGAACTCC
lmo0398 Fw	TGAAATCGGTATCACGACGA
lmo0398 Rv	ATCGGTTCAACGAGACGAAT
lmo0399 Fw	GTGCGGCGGATGTAGTTATT
lmo0399 Rv	CTTTTGTTTGCGTGCTTCAA
lmo0400 Fw	CGGCTGCTAGTTCTTTACCG
lmo0400 Rv	CAGGTCCACCAAGGTCAAAT
lmo0401 Fw	CTTCCGGTGAATCAATCGTT
lmo0401 Rv	TTTGGTAAACTTGCGGGTTC
lmo0402 Fw	AAGTCGGCAGTGAGCAATCT
lmo0402 Rv	GGCCGTGAAGAAATTCGATA
lmo0501 fw	TTCCGGCGAAATTTTAACTG
lmo0501 Rv	ACGCTTCGAATTCTTGGTTG
lmo0502 Fw	ATGCGATTTCACGTTTGCCGGA
lmo0502 Rv	TCGCAGCGACGCCAGAAGTT
lmo0503 Fw	CCGGGGTAGCGATTCCGCAT
lmo0503 Rv	AGGTCGTCATCACCTCCTCGTCT
lmo0504 Fw	ACGCCAAGTGGACACTCGCATA
lmo0504 Rv	TCGGCTTGGCACGAGAATGTACC
lmo0505 fw	ACGGAGTTCGCGCAGGAGTT
lmo0505 Rv	CCGCACCTGCAAGTGTGCATT
lmo0506 Fw	TTGGCGATAGGGCTGCCGGA
lmo0506 Rv	ACCACTGCGGAAGGCTCGAC
lmo0507 Fw	GTCAATGCACTGTTCAAGAGGTTGC
lmo0507 Rv	CTGGGACATCACCAACATCTCCTGT
lmo0508 Fw	TACCGGGTGGGCGCTTGTTG
lmo0508 Rv	GCAATGGCGGCAAGTCCACC

2.5.4 Use of a Enzyme linked lectinsorbent assay (ELLA)

An enzyme linked lectinsorbent assay was used to measure carbohydrate content of *Listeria* biofilms based on the method by Leriche *et al*. (2000). Peroxidase-labelled lectins, ConA from *Canavalia ensiformis* (jack bean) and WGA from *Triticum vulgaris* (both from Sigma Aldrich, UK) were used. ConA and WGA were suspended at a concentration of 1mg/ml in sterile nanopure H_2O and PBS respectively. Solutions were divided into smaller volumes and stored at -20°C until use. Attachment assays of the *L. monocytogenes* were set up in microtitre plates, as described in Section 2.2.4. Biofilms were left to develop for periods of 2 hours, 24 hours and 4 days at the desired temperature. After incubation of the assays, planktonic cells were removed and wells washed three times with PBS before being used for the assay.

Standard curves were set up by initially diluting the lectins to a working concentration of 10µg/ml (ConA) and 1.25µg/ml (WGA) in PBS containing 0.05% (v/v) Tween 20 (diluting buffer). Serial dilutions of lectin were made across the plate of attached cells by adding 200µl of the diluted lectin to the first row of 8 wells, 100µl of this was removed and added to the next well which contained 100µl of diluting buffer. This procedure was continued across the plate. Microtitre plates were left at room temperature for 1 hour to allow the lectins to bind to any carbohydrates present. Plates were then inverted and tapped gently to remove the lectin solution, and washed 3 times with diluting buffer. To allow visualisation of the peroxidase labelled lectins, 100µl of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate solutions, (Pierce) was added. The colour was left to

develop for 15 minutes in darkness and then absorbance was read at 405nm using a microtitre plate reader (BioRad, UK). The standard curve was drawn from the results and a concentration used for subsequent assays.

Chapter 3 - Identification of genes involved in attachment to abiotic surfaces

The first aim of this project was to modify an existing biofilm assay to allow screening of a previously constructed library of *L. monocytogenes* transposon mutants for their ability to attach to polystyrene. Transposon mutants showing a reduced level of attachment would be subject to further studies, allowing identification of the genes into which the transposon had inserted.

3.1 Modification of an existing attachment assay

A previous biofilm assay (Taylor *et al*., 2002) was utilised initially to test attachment of the *L. monocytogenes* wild type strain C52 to abiotic surfaces. This assay is a microtitre plate based assay which is used to measure surface attached growth utilising crystal violet to stain adhered bacteria. The assay by Taylor *et al*. (2002) was used initially to measure the adherence of this one strain of *L. monocytogenes* with an aim to establish a reliable assay. Figure 3.2 shows the stages of the originally method as described by Taylor *et al* (2002), the method was set up using the steps in box A, followed by the steps outlined in box C. To assess the validity of this assay to quantitatively measure surface attached *L. monocytogenes* the assay was set up using four independent overnight cultures each of which were used to inoculate the wells of an entire column; a column was also set up as a negative control containing TSB only. Results of assays set up using this method showed a high amount of inconsistency in terms of absorbance of adhered bacteria across the plate with inconsistency being observed between columns of wells or within individual wells, some wells exhibiting a much higher level of attached bacteria than others. A high level of variance within each

individual column was also observed, despite each column being set up from the same overnight culture. Table 3.1 demonstrates the results of a typical experiment set up using this method with four columns of biological replicates of the strain C52, and a column which contained TSB only. This result demonstrates the high variation observed using this assay within the optical density of the wells, either when comparing either down the columns, for example the column C52-1 which shows a range of absorbances from 0.4 - 1.39, or across the rows, for example row 5 which shows a range of absorbance's from 0.29 - 0.83. The negative control column containing TSB only did not show much variance with the exception of one well which had an OD of 0.635 (column E, row 6), however this was due to an error during the staining with crystal violet stage. Figure 3.1 shows this data in graph format, the high standard deviation is reflected by the large error bars.

	Column A	Column B	Column C	Column D	Column E
Row	C52-1	C52-2	C52-3	C52-4	TSB only
1	0.40	0.40	0.60	0.45	0.15
2	0.47	0.32	0.52	0.31	0.11
3	0.61	0.33	0.77	0.46	0.14
4	0.61	0.64	0.50	0.31	0.10
5	0.63	0.49	0.83	0.29	0.13
6	1.39	0.44	0.70	0.53	0.64
7	0.51	0.34	0.60	0.45	0.10
8	0.58	0.58	0.50	0.41	0.14

Table 3.1 - Results of a typical attachment assay with *L. monocytogenes* C52 measured using the method of Taylor *et al*. (2002). The numbers represent the OD_{595nm}. The columns A-D show attachment of four biologically independent samples of the strain C52, the final column contained TSB only.

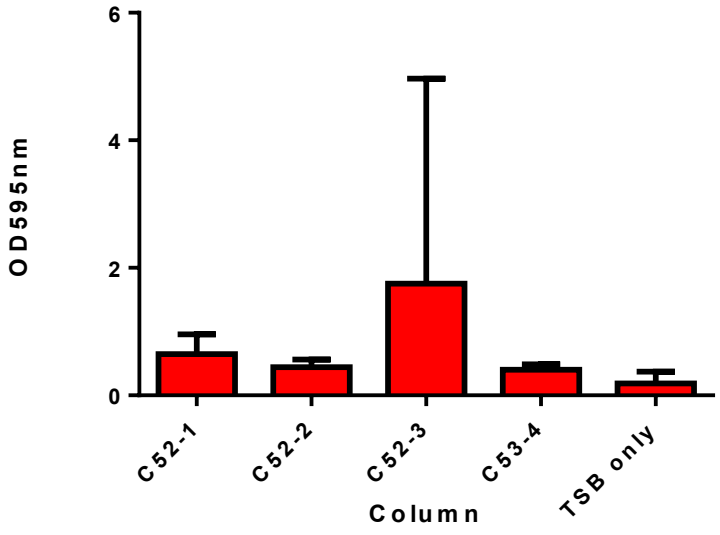

Figure 3.1 - Graph showing the data presented in Table 3.1. Typical attachment assay of *L. monocytogenes* C52. The columns represent each column of 8 wells on the plate. Error bars show standard deviation of the 8 technical replicates.

It was hypothesised that the observed variances in levels of attachment exhibited by this one strain of *L. monocytogenes* could be attributed to two things. Firstly, this method did not account for the fact that the overnight cultures of the bacteria could have grown at different rates resulting in a different OD of the initial culture. This could result in individual wells being inoculated with a different concentration of bacteria, as the initial method set out by Taylor *et al*. (2002) did not measure the optical density of the starting cultures. The second observation that could have led to variances within the assay is that the method did not take into account the growth rate of the bacteria within individual wells during the 6 hour incubation period: Some strains may grow faster than others during this period making

comparison between strains or between mutant strains and their isogenic parent problematic. The transposon mutants that were tested with this assay were visually observed to grow slower in liquid culture and the final OD of a stationary phase culture of a transposon mutant was lower than their parental strain, with a typical OD_{500nm} of an overnight culture being approximately 1.2, compared to an OD_{500nm} of approximately 1.6 for the parent strain. Growth curves of the transposon mutants at 37°C and 30°C were performed to confirm this; the results from this assay can be seen in Appendix 2. From this it was concluded that the OD of the initial culture used to set up the assay would have to be standardised to ensure all wells were inoculated with the same concentration of bacteria. Also the incubation time during the assay would have to be reduced to take into account differences in bacterial growth rates between mutants and their isogenic parents.

Based on these initial data, modifications were made to the assay of Taylor *et al*. (2002), as shown in Figure 3.2. Box A in Figure 3.2 show the initial steps in setting up the attachment assay for the published assay, whereas Box B highlights the modifications made to these steps for the set up of the modified attachment assay. The modifications made to the assay included the dilution of the overnight culture to an OD_{500nm} to 0.7 and 200µl of this diluted culture was added to each well of the microtitre plate. The incubation time of the assay was also reduced from 6 hours to 2 hours for the new assay. Both assays were then performed using the steps shown in Box C.

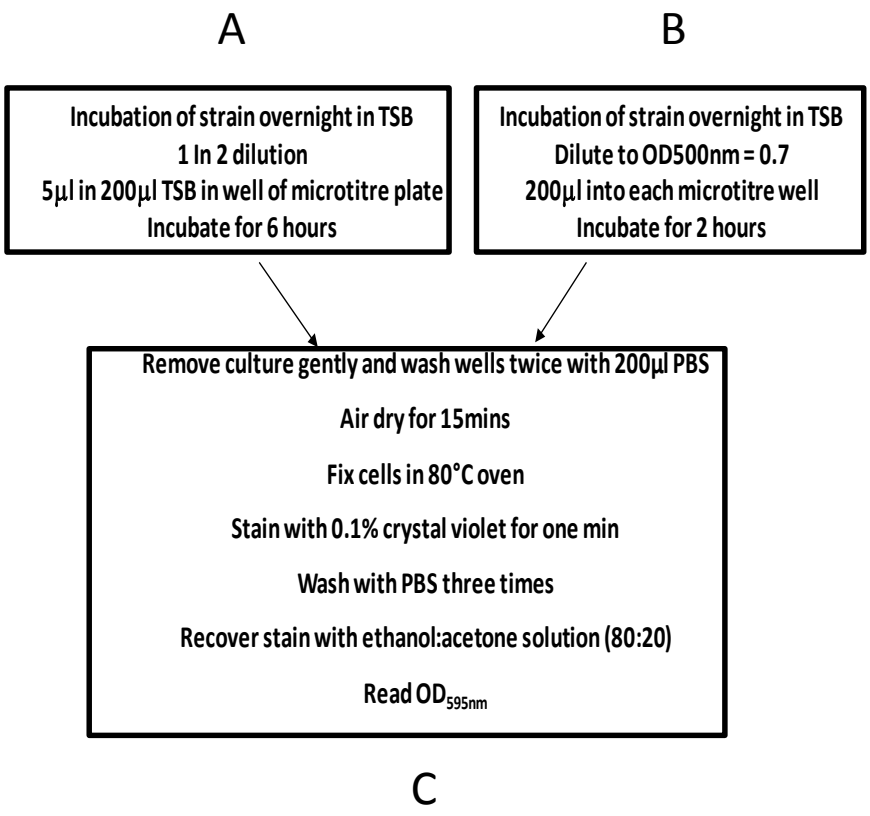

Figure 3.2 - Flow diagram showing how the assay of Taylor *et al*. (2002) was modified to the final assay. The published assay contained the steps in box A followed by box C, whereas the modified assay used the steps laid out in box B, and then continued to box C.

The first modification highlighted in box B introduced the step of diluting the overnight culture to a fixed OD_{500nm} it ensured that each well was inoculated with the same number of bacteria, this should ensure that any discrepancies that arose from different ODs of initial culture were removed. The cultures were subsequently diluted to an OD_{500nm} of 0.7, which was chosen to ensure bacteria were at mid exponential phase.

The second modification as shown in Box B reduced the incubation time of the assay from 6 hours to 2 hours to allow for measurement of initial attachment of bacteria to the surface of the microtitre plate, rather than surface attached growth. The work by Taylor *et al*. (2002) utilised this assay to measure surface attached growth over a period of 24 hours. The results of this work indicated that the bacteria remain within lag phase for over two hours, before entering exponential phase and starting to grow, hence 2 hours incubation time was chosen.

To test the modified assay five separate overnight cultures of the *L. monocytogenes* strain C52 were grown at 37°C and used for the assay. The result of this assay is shown in Figure 3.3. As can be seen from the figure, consistent results were seen over the entire plate, with no significant differences between the means of each of the columns ($P>0.05$). The data were also compared over each of the individual rows to check for consistency between different overnight cultures, the results from this analysis can be seen in Figure 3.4. Statistical analysis of the data across each of the rows also showed no significant difference ($P>0.05$).

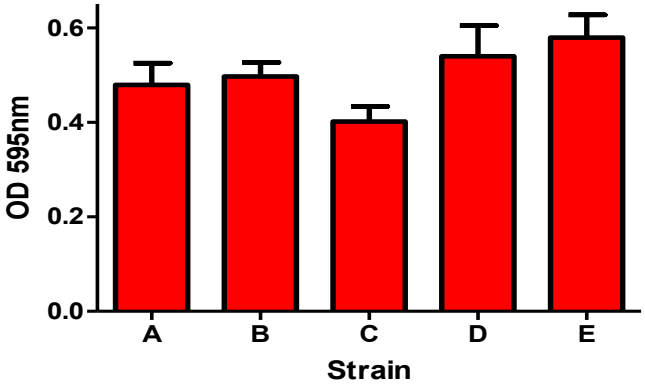

Figure 3.3 Adherence of *L. monocytogenes* C52 to wells of a microtitre plate measured using the modified assay. The letters A-E each represent a column of the microtitre plate containing a biologically independent sample of *L. monocytogenes* C52. Results are the mean of each of the 8 wells contained in the column, and indicate standard error of the mean (SEM).

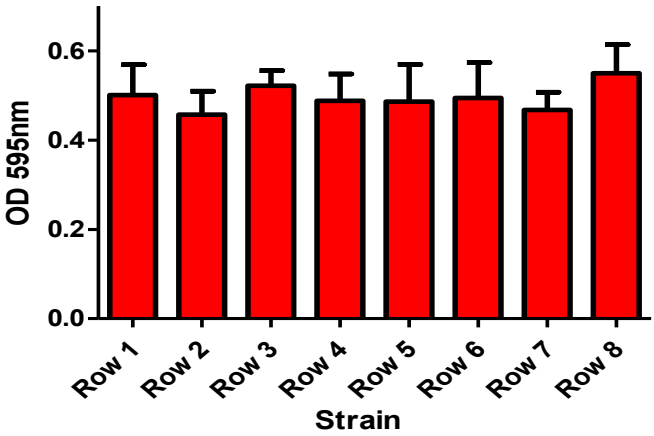

Figure 3.4 Adherence of *L. monocytogenes* C52 to wells of a microtitre plate measured using the modified assay. Each bar of the graph represents a row across the microtitre plate. Results are the mean of each of the 5 wells contained in the row, and indicate standard error of the mean (SEM).

3. 2 Screening of transposon mutants for strains deficient in attachment

L. monocytogenes transposon mutants from a previously constructed Tn917 transposon library created in the background strain 10403s had been screened for a reduction in attachment to stainless steel coupons and glass cover slips in comparison with their parent strain 10403s (Beresford, 2002). Based on the data obtained from adherence to glass and plastic, three mutant strains, all of which had shown reduced attachment to these two materials, were selected for further analysis: M113, M237 and B380. Lower numbers of cells of these transposon mutants adhered 2-3.5 log cfu lower than the parent strain 10403s to stainless steel coupons.

The strains B380, M237 and M113 were screened using the modified assay as described inSection 2.2.4. The assay was performed at the incubation temperatures 37, 30, 20 and 10°C, with all stages of the assay being done at the selected temperature (growth on TSA plate, overnight incubation in TSB and during the attachment assay). The assay was set up with each of the strains being inoculated into one column of 8 wells, from one overnight culture; assays were carried out as three biologically independent replicates. Figures 3.5 a, b, c & d show the results at 37, 30, 20 and 10°C respectively, all graphs show an average of the 3 independent experiments, with error bars showing standard error of the mean. A negative control containing TSB only was used in all experiments.

Figure 3.5a presents the data from the experiment at 37°C. The parent strain 10403s gave an average OD_{595nm} of approximately 0.65. The transposon mutants

M237 and B380 unexpectedly had a higher OD with values of 0.79 and 0.71 respectively, however one way analysis of variance (ANOVA) indicated this result was not significantly different ($P>0.05$). The only mutant to show a significantly lower level of attachment at 37°C in comparison to the parent strain was the transposon mutant M113. This mutant showed an average OD_{595nm} of 0.4 ($p<0.05$). The OD with all the strains was significantly higher ($P<0.05$) than the medium only control.

The same attachment assay was also performed at temperatures of 30°C, 20°C and 10°C (Figures 3.5b, 3.5c and 3.5d respectively). As can be seen from the Figures, at each of these temperatures a significant difference was observed between the attachment of all mutant strains and the isogenic parent strain 10403s ($p<0.05$). No significant difference was seen when the mutant strains were compared with each other ($P>0.05$). The biggest difference in attachment between the parent strain and the mutants was observed at 30°C, with the wild type giving an average OD_{595nm} of 1.64 in comparison to an OD_{595nm} of less than 0.75 with each mutant. Interestingly it can be noted that there is a big increase in the level of attachment of *L. monocytogenes* 10403s at 30°C in comparison to the other temperatures. The OD_{595nm} at the temperatures 37, 20 and 10°C did not exceed 0.63, whereas at 30°C the OD_{595nm} was on average 1.64.

These results from the attachment assays at different temperatures lead to the conclusion that the transposon mutant M113 contains a mutation which affects its

attachment to plastic surfaces at all temperatures, as the attachment of this mutant to microtitre plates was impaired at all temperatures. The strains M237, and B380 also show a reduction in attachment, but the phenotype caused by these mutations appears to be temperature-dependant, as the effect is only seen at temperatures of 30°C and less, indicating that the genes involved are expressed at lower temperatures. Because all three strains show reduced attachment to plastic all were selected for further investigation.

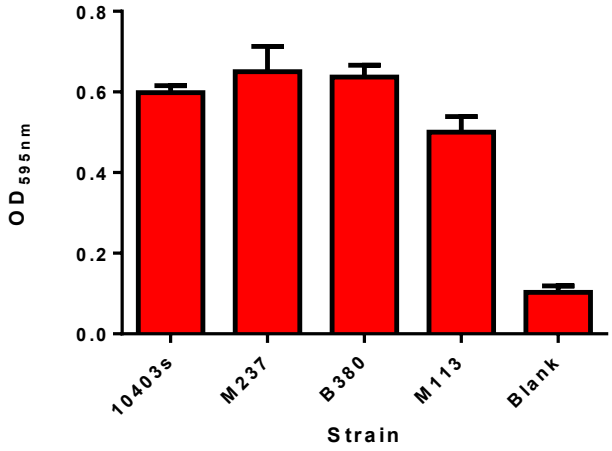

Figure 3.5a - Attachment of *L. monocytogenes* wild type and Tn917 mutants to wells of a microtitre plate. All incubations were performed at 37°C. Values are the mean of 3 biological replicates, each of which contained 8 technical replicates, error bars show standard error of the mean

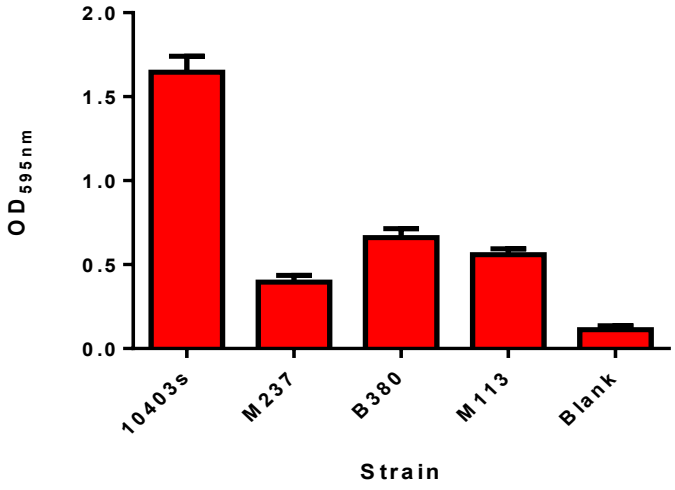

Figure 3.5b - Attachment of *L. monocytogenes* wild type and Tn917 mutants to wells of a microtitre plate. All incubations were performed at 30°C. Values are the mean of 3 biological replicates, each of which contained 8 technical replicates, error bars show standard error of the mean.

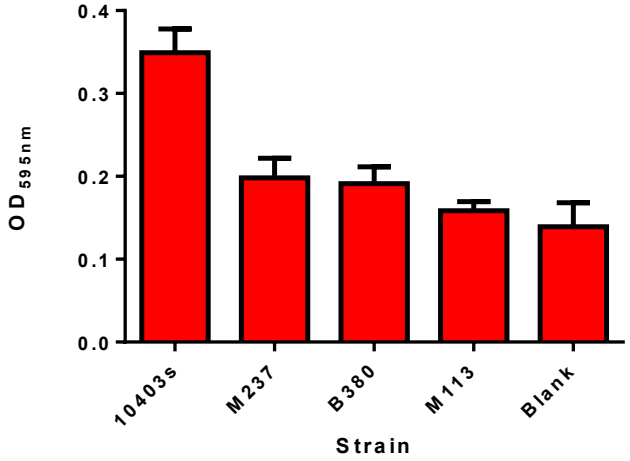

Figure 3.5c - Attachment of *L. monocytogenes* wild type and Tn917 mutants to wells of a microtitre plate. All incubations were performed at 20°C. Values are the mean of 3 biological replicates, each of which contained 8 technical replicates, error bars show standard error of the mean

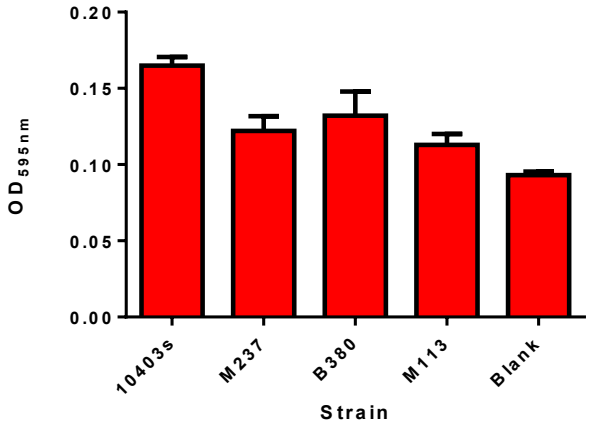

Figure 3.5d - Attachment of *L. monocytogenes* wild type and Tn917 mutants to wells of a microtitre plate. All incubations were performed at 10°C. Values are the mean of 3 biological replicates, each of which contained 8 technical replicates, error bars show standard error of the mean

3.3 Identification of Tn917 flanking regions by arbitrary PCR

Transposon mutants that showed a reduction in attachment to plastic microtitre plates were selected for arbitrary PCR, to allow identification of the region of the *L. monocytogenes* chromosome into which the transposon had located. Arbitrary PCR, using the method by Knobloch *et al*. (2003), was performed on the mutants M237 and M113, as described previously in Section 2.3.1. The identity of the gene into which the transposon had inserted in the transposon mutant B380 had previously been identified by cloning of the regions flanking the transposon (Beresford, 2002).

M237

Arbitrary PCR had previously been performed on mutant M237 (Jordan, S.J. Personal communication). After arbitrary PCR of this mutant using the primers 5.3 and STAPHarb2 in the first round of the PCR, bands were visible when the PCR products were analysed by agarose gel electrophoresis (Jordan, S.J. Personal communication). To continue with the arbitrary PCR that had already been performed, the PCR product from the first round was purified before being used as a template for the second round of PCR, using the primers STAPHarb3 and 5.2. The second round of PCR resulted in amplification that gave visible bands when analysed by agarose gel electrophoresis. The band was extracted from the gel as described in Section 2.2.8 and sequenced. The sequence was analysed using BLAST, and the resulting sequence matched to the *L. monocytogenes* EGD-e genome (Figure 3.6). The sequences corresponded to the base pairs 536434 - 536569 in the genome. This segment of DNA lies within the identified ORF

lmo0501, a 2.06kb ORF which shows significant homology to a transcriptional antiterminator of the BglG family. Further investigation of *L. monocytogenes* EGD-e genome identified 13 other BglG transcriptional antiterminators of this type within the EGD genome, *lmo2773*, *bvrA* (*lmo2788*), *gntR* (*lmo0020)*, *manR* (*lmo0785* and *lmo0873*), *licR* (*lmo0297*, *lmo2784*, *lmo0402*, *lmo0425*, *lmo2099* and *lmo0630*), *lacR* (*lmo1721*), *manR/mtlR* (*lmo2652*).

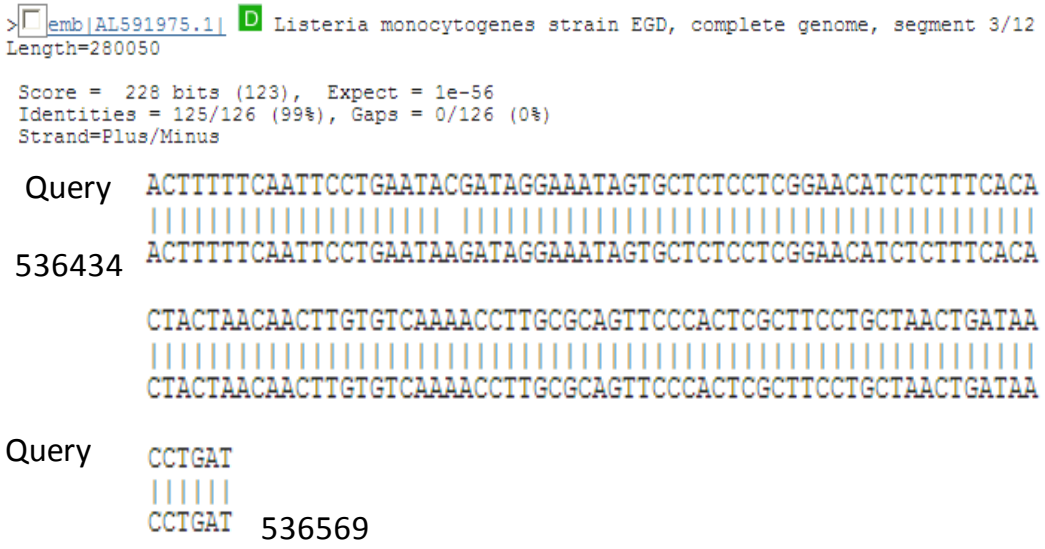

Figure 3.5 - Blast analysis of the sequence of the fragment after arbitrary PCR of the mutant M237. The sequence matches with the sequence between base pairs 536434 - 536569 in the *L. monocytogenes* genome. This sequence lies within the gene *lmo0501*

B380

Investigation of the surface attachment deficient *L. monocytogenes* mutant B380 had previously been done by Beresford (2002). The location was identified by cloning of the regions either side of the transposon. The recombinant plasmid was sequenced and subjected to analysis using BLAST to identify the location of the transposon into the chromosome. BLAST analysis revealed the location of the insertion of the transposon in this mutant as being the ORF *lmo0401*, coding a putative α-mannosidase, which had similarity to the *E. coli* YbgG protein. However it was interesting to note that the location of this ORF in *L. monocytogenes* EGD-e lay directly upstream to a second BglG transcriptional antiterminator (*lmo0402*) (Figure 3.7). Also within the vicinity of the α-mannosidase ORF, are ORFs' coding proteins involved in the phosphoenolpyruvate phosphotransferase system (PEP-PTS), *lmo0398-lmo0400*, Section 5.1 will discuss this in more detail.

Figure 3.7 - Location of the gene α-mannosidase *lmo0401*, the downstream bglG transcriptional antiterminator (*lmo0402*), and the genes involved in the phosphoenolpyruvate phosphotransferase system (PEP-PTS) (*lmo0398-lmo0400*)

Based on this result, it was decided to make deletion mutations in both of the transcriptional antiterminators, *lmo0501* and *lmo0402*, as well as the α-mannosidase *lmo0401*. This would allow clarification of the involvement in

attachment to abiotic surfaces by *L. monocytogenes* of the α-mannosidase gene and the two transcriptional antiterminators.

M113

Arbitrary PCR with the mutant M113 was performed using the same technique as for M237. Round one of the PCR gave a positive result using the primers STAPHarb1 and 3.3 (Jordan, S.J. Personal communication). To continue with this previous work the PCR product was purified and used as a template for round 2 using the primers STAPHarb3 and 3.2. Agarose gel electrophoresis was performed on the PCR products, which can be seen in Figure 3.8. As can be seen, the PCR gave two products, one of approximately 650 base pairs and one of approximately 400 base pairs. The PCR reaction was purified and sequenced as described in Section 2.3.2. Figure 3.9 shows the results of the BLAST analysis of the resulting sequence, BLAST analysis identified a matching sequence between base pairs 1249977 - 1250183 of the published genome sequence for *L. monocytogenes* EGD-e. This segment lies within the 3021bp ORF *lmo1226*. The protein coded by this gene is predicted to have 1066 amino acids and is similar to the transporter YdgH of *Bacillus subtilis*. This protein is identified by NCBI as a drug exporter of the resistance, nodulation and division (RND) superfamily.

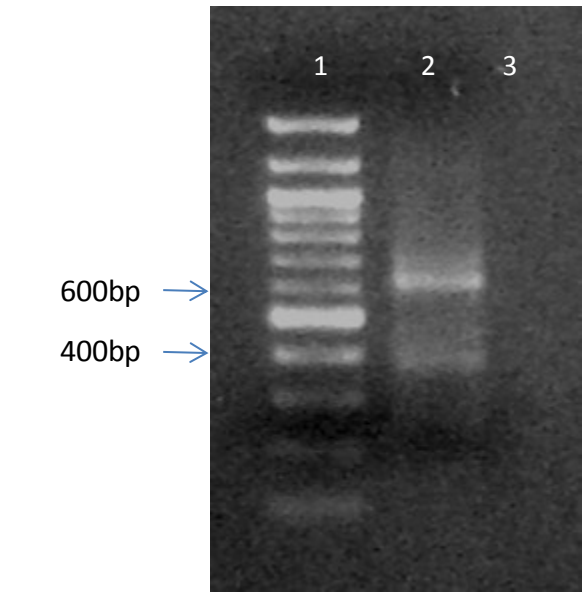

Figure 3.8 - Agarose gel electrophoresis of the PCR product of round 2 of arbitrary PCR of M113. Lane 1 shows a 100bp ladder, lane 2 shows the arbitrary PCR of M113, and lane 3 shows a negative control of the PCR containing water as a template

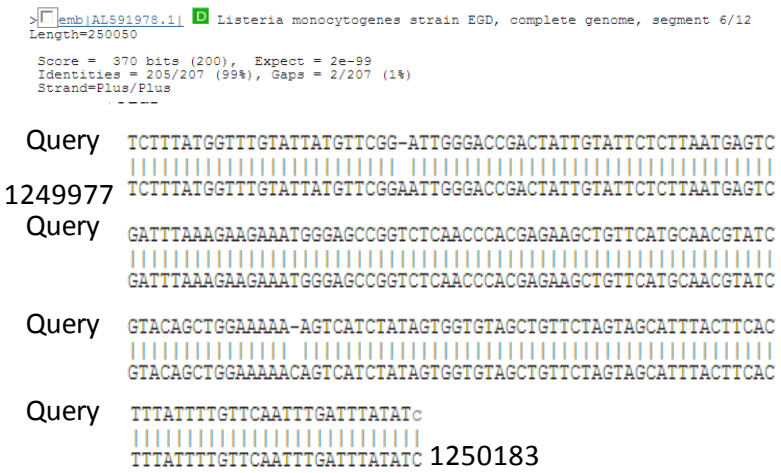

Figure 3.9 - Blast analysis of the sequence of the fragment after arbitrary PCR of the mutant M113. The sequence matches with the sequence between base pairs 1249977 - 1250183 in the *L. monocytogenes* genome. This sequence lies within the *lmo1226*.

3.4 Reverse transcriptase PCR of lmo0402

Due to the location of the transposon insertion in mutant B380 being identified as within the gene *lmo0401*, which was upstream from the proposed transcriptional antiterminator *lmo0402*, it was hypothesised that the expression of the gene *lmo0402* was being affected in the mutant B380 by the presence of the transposon. To test this hypothesis the transcription of *lmo0402* was analysed using RTPCR, performed by the method described in Section 2.3.3.

RNA was extracted from bacteria in mid exponential phase (OD_{600nm} of 0.7) for both the wild type 10403s and the mutant strain B380. The RNA concentrations after extraction and purification were 528µg/ml and 52µg/ml for 10403s and B380 respectively, from a starting volume of 200ml of bacterial culture. RTPCR was performed using the primers SJ27 and SJ28 to screen for expression of the *lmo0402* gene and the primers P60 Fw and P60 Rv were used to amplify the *iap* gene as a positive control for the reaction. Resulting PCR products were analysed by agarose gel electrophoresis, as shown in Figure 3.10. Both strains showed the expected size band (600 base pairs) for the positive control using the P60 primers (Figure 3.9, lanes 3 and 4). This confirmed that the reverse transcriptase reaction and subsequent PCR had worked successfully. The strain 10403s gave a band of 341 base pairs (Figure 3.10, Lane 7) which was the predicted band size for the transcript of the *lmo0402* gene. This band was absent from the reaction using the B380 RNA (Figure 3.10, Lane 2), although a band can be seen of less than 100bp in this and most of the other lanes, however this is just caused by mispriming of the primers to each other giving a 'primer dimer'. Lanes 5 and 8 in Figure 3.10 show

negative control reactions with water as a template with the primers for the P60 gene and the *lmo0402* primers respectively. Lane 6 shows a positive control reaction using the RNA supplied in the kit, which gave the expected band size of 500bp.

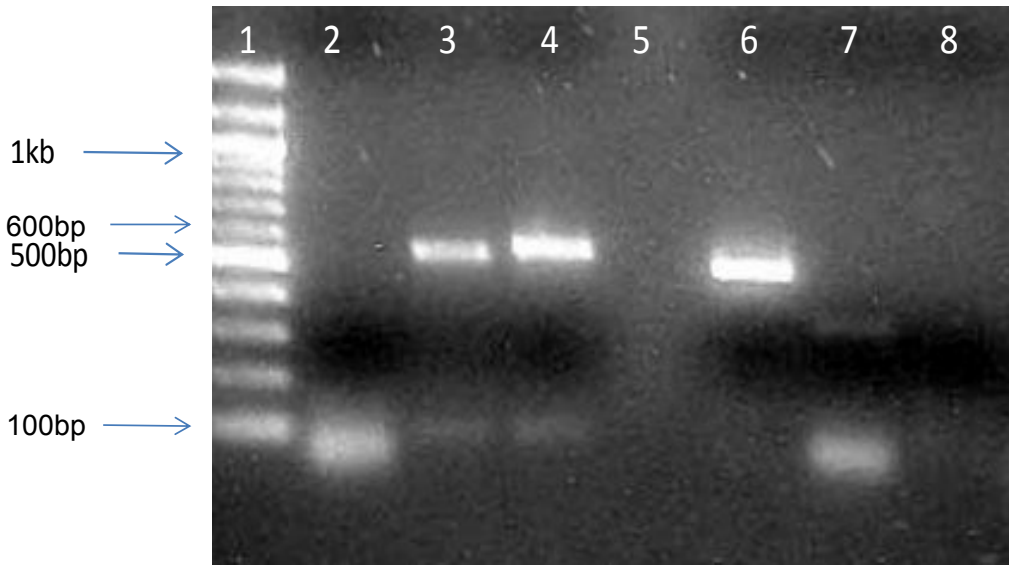

Figure 3.10 - RTPCR of the gene *lmo0402* in *L. monocytogenes*, Lane 1 - 100bp ladder, Lane 2 - Mutant B380 RNA with *lmo0402* primers, Lane 3 - Mutant B380 RNA with P60 primers, Lane 4 - 10403s RNA with P60 primers, Lane 5 - Negative control using water as a template with P60 primers, Lane 6 - Control reaction using kit RNA, Lane 7 - 10403s RNA with *lmo0402* primers, Lane 8 - Negative control using water as a template with *lmo0402* primers

3.5 Conclusion and discussion

This section of the work initially set out to identify transposon mutants that were deficient in attachment to polystyrene microtitre plates, to do this first a reliable consistent assay to quantitatively measure the extent of microbial adherence had to be designed. This chapter modified an existing assay by Taylor *et al*. (2002) to give a more reliable assay. This section then identified transposon mutants which were deficient in attachment in genes *lmo0402*, *lmo0501* and *lmo1226*. This discussion will look at the development of this new assay, and then discuss the use of this assay to test transposon mutants. Finally this section will discuss the location of the transposon into each of the mutants.

3.5.1 Development of a new assay to measure adherence

At the beginning of the project several assays existed to measure attachment or biofilm formation of bacteria and these are discussed in more detail in Section 1.2.4. For this study to measure attachment of *L. monocytogenes,* a microtitre plate-based method was chosen because it would allow quantitative analysis and multiple technical replicates to be carried out in each assay. Taylor *et al*. (2002) had previously described a microtitre plate method to measure surface attached growth of *L. monocytogenes* therefore this method was utilised first in this study. While using the method by Taylor *et* al. (2002) inconsistent results were observed, with wide variation in the optical density, between the wells of the microtitre plate. This variance was hypothesised to be due to two reasons: firstly the wells were

inoculated with 5µl of overnight culture and this led to some variance due to differences in the optical density of the overnight culture because variety in OD of the strains was observed frequently. Secondly, the transposon mutants that were selected for assays of attachment were observed to grow slower than the wild type (Appendix 2). This reduced growth rate meant that any reduction in surface attached growth seen using the method of Taylor *et* al. (2002) could not be definitely attributed to a reduction in the organism's ability to form a biofilm, as it could have been due to a slower growth rate of the strain being tested.

The inconsistency in Taylor *et* al. (2002) method led to the development of the modified assay to measure initial attachment to surfaces. Figure 3.2 shows the modifications that were made. Firstly, the OD_{500nm} of the inoculating cultures was measured cultures were adjusted to an OD_{500nm} to 0.7. This ensured that all wells were inoculated with the same concentration of bacteria and that the differences in attachment could not be attributed to differences in initial inoculum size. To confirm the concentration was the same at this OD, viable counts on the overnight culture were performed (data not shown). This eliminated the possibility that the same OD related to different bacteria concentrations due to differences in the size of the cell, or if the culture contained a higher proportion of dead cells. This modification, however, still did not account for the possibility that the strains were growing at different rates during the incubation period of the assay. To minimise any impact of this possibility this the incubation time was reduced from six hours to two hours. The incubation period was reduced to this amount due to the pattern of surface attached growth shown by Taylor *et al*. (2002). They investigated the surface

attached growth of *L. monocytogenes* wild type in comparison to two surface attachment deficient (SAD) insertion mutants, in the genes *relA* and *hpt*. To measure the surface attached growth, bacteria were left to adhere to plastic before removing TSB and washing the wells to remove any non-adhered bacteria before measuring the OD. They observed that the wild type showed the same level of growth as the SAD mutants for the first 3 hours staying within a lag phase of growth. After three hours the wild type began to grow faster than the mutants and enter the exponential phase of growth. Consequently the incubation time of 2 hours was chosen as it was concluded that the wild type would not have entered exponential growth phase at this point and therefore any apparent attachment measured would be attachment and not surface attached growth. To validate these modifications the new assay was performed using one strain of *L. monocytogenes* across all columns and rows of a microtitre plate. The results from showed that the new assay would give consistent results when used to test a strain of *L. monocytogenes* independent of the location of the strain on the plate.

A variety of assays currently exist for measuring attachment of bacteria to plastic surfaces. A full summary of these methods is given in Section 1.2.4. Many of these assays investigate surface attached growth rather than initial attachment to surfaces. For this study we wanted to investigate factors involved in the initial adherence of *L. monocytogenes* rather than surface attached growth. The microtitre plate method was picked specifically to enable a large amount of samples to be screened at once; also the method is quick and enabled the assay to be carried out under different environmental conditions, in this case different

incubation temperatures. It is also an inexpensive method and allowed measurement of the initial attachment of *L. monocytogenes*. The microtitre plate assay however has its disadvantages, the method does not allow for visualisation of the attached cells, or of the architecture of the biofilm. It would have been interesting to look at attachment of *L. monocytogenes* to plastics using scanning electron microscopy (SEM) because this would have allowed direct visualisation of the organisms adhering to the surfaces. SEM however would not allow for quantitative analysis, therefore could not be used to compare differences in attachment between strains. Studies by Beresford (2002) utilised this method to visualise any artefacts of the biofilm such as presence of exopolysaccharides and whether full formation of 3D biofilm occurred.

Using the modified assay led to the identification of three transposon mutants that appeared to be deficient in attachment to plastic surfaces, M113, M237 and B380. These data supported the previous work by Beresford (2002), which shown these mutants were deficient in attachment to stainless steel and glass, therefore these transposon mutants appear to demonstrate reduced attachment against more than one abiotic surfaces.

All attachment assays were performed at a variety of temperatures to attempt to establish if there is a difference in attachment. Data from these experiments identified that the mutant M113 was deficient at all temperatures tested, whereas the mutants M237 and B380 did not show deficiency in attachment when incubated at 37°C. However these two mutants did show a reduction in attachment at 30°C and lower. The phenotype exhibited by these two mutants indicates that the

transposon has inserted into the *L. monocytogenes* is in a gene that appeared to be temperature regulated (Section 3.3). The biggest difference in surface attachment was seen at 30°C with the parent strain shower a lot higher level of attachment than any of the transposon mutants. Interestingly from these data it can be seen that when attachment was measured at 30°C there was a large increase in numbers of surface attached bacteria in the parent strain 10403s, whereas the mutants show roughly the same level of attachment, as demonstrated at all of the other temperatures (Figures 3.5a, b, c, & d). From this it can be hypothesised that a particular gene is upregulated in the wild type bacteria at this temperature which is important in attachment at 30°C and lower, and that this gene is affected by the insertion of the transposon in the mutants M237 and B380. Alternatively the genes into which the transposon inserted could code for proteins which are only active at different temperatures.

3.5.2 Identification of genes involved in attachment

The arbitrary PCR technique used in this study was first used to identify the location of insertion of the transposon Tn917 in *Staphylococcus epidermidis* (Knobloch *et al*., 2003).The method uses primers designed to bind specifically inside the transposon paired with an arbitrary primer that binds to the chromosomal DNA flanking the transposon. The technique was used to identify the location of the transposon in the chromosome in each of the transposon mutants tested. Using this method, three genes were identified as being involved in adhesion of *L. monocytogenes* to polystyrene. Two of these genes were in a Bgl operon, which encodes for proteins involved in phosphoenolpyruvate-dependent carbohydrate

transport (transposon mutants M237 and B380), and a gene encoding a putative drug efflux protein belonging to the resistance, nodulation and division superfamily (mutant M113). The location of the transposon in each of the mutants will be discussed, along with the construction of deletion mutants in these genes and characterisation of these mutants.

3.5.2.1 Location of Tn917 insertion into mutant M113

Using arbitrary PCR performed on the transposon mutant M113 the location of insertion of the transposon Tn917 could be identified. Blast analysis of the sequence flanking the transposon identifed the gene into which it had inserted to be *lmo1226*, a putative drug exporter of the Resistance, Nodulation and Divsion (RND) superfamily. There is currently no literature available on the role of this gene within *L. monocytogenes*. It was decided to make deletion mutants of this gene to further characterise the role of *lmo1226* in attachment in *L. monocytogenes*. The creation of deletion mutants and the role of this gene in attachment and other characteristics will be discussed further in Chapter 4.

3.5.2.2 Location of Tn917 insertion into mutants M237 and B380

Following the results from the arbitrary PCR of the transposon mutants (Section 3.3), the location of insertion of the transposon into the mutant M237, was mapped to a gene coding for a BglG transcriptional antiterminator, *lmo0501*. This transcriptional antiterminator is thought to regulate expression of the β-glucoside

utilisation (*bgl*) operon (Stoll & Goebel, 2010). Arbitrary PCR of the location of the insertion of the transposon in the mutant B380 was identified to be within the gene *lmo0401*, a putative α-mannosidase. Upon further analysis of the sequence information available for *L. monocytogenes* EGD-e, this gene was identified to lie upstream from a second BglG transcriptional antiterminator (*lmo0402*). Due to this, it was decided to carry out a RTPCR assay to identify if the insertion of the transposon into *lmo0401* had a polar effect on the downstream gene, *lmo0402*. The RTPCR on *lmo0402* carried out in the mutant B380 indicated that *lmo0402* was affected by the transposon insertion as no PCR product was detected. As a positive control the reaction was also carried out on the strain 10403s, which gave a PCR product of correct size. Based on this result, and the result of the arbitrary PCR of the mutant M237, it was decided to create deletion mutants in the genes *lmo0401*, *lmo0402* and *lmo0501*.

Deletion mutants were created in each of the genes *lmo0401*, *lmo0402* and *lmo0501*. Full details of these deletions can be seen in Section 5.2. Each of these deletion mutants gave rise to a truncated form to the protein which they coded. Due to a problem in the construction of the mutants Δlmo0401 and Δlmo0501 these mutations gave rise to a mutant which was not in-frame, and coded for a stop protein within the DNA. Due to this any results observed could not be confirmed to be due to a deletion in this mutation, complementation of these mutants with the deleted gene would be required to confirm the phenotype observed was not due to a polar effect. Despite this, these deletion mutants were used for further characterisation experiments, which will be discussed further in the

following sections.

Chapter 4 - Construction and characterisation of mutants in *lmo1226*

The gene *lmo1226* was highlighted as being important in attachment of *L. monocytogenes* to abiotic surfaces at all temperatures tested (see Section 3.2). As already discussed (Section 3.3) this gene is a putative drug exporter of the RND superfamily, based on similarity with other proteins belonging to this superfamily. This section describes the construction of two deletion mutants in this gene and the characterisation of these mutants.

4.1 *In silico* analysis of the *lmo1226* ORF

In silico analysis, using the *L. monocytogenes* genome available on the NCBI website, of *lmo1226* identified a 3021 base pair open reading frame encoding a putative1066 amino acid protein (NCBI). Analysis of the protein using the web based program, SPLIT4 (Juretic *et al*., 2002), was performed to predict the structure of the protein and the web based program, pSORTB, (Yu *et* al., 2010) and identified the location of the protein to be within the cell membrane. The structure of the protein, as indicated by SPLIT4, is predicted to contain 12 transmembrane helices in the amino acid locations shown in Table 4.1

	Amino acid location	
Helix No.	Start	Finish
1	6	30
2	180	207
3	213	228
4	233	252
5	279	299
6	302	333
7	359	380
8	884	904
9	909	935
10	940	964
11	988	1012
12	1016	1040

Table 4.1 - Amino acid positions of the 12 transmembrane helices in the ORF Imo1226, as predicted by SPLIT4.

Using the web based program TOPO2 (Johns), it is possible to predict the 2D structure of the protein Lmo1226 (Figure 4.1). As well as the 12 transmembrane helices, two extracellular loops can be seen. Between helices 1 & 2 and again between 7 & 8, two regions of amino acids that protrude extracellularly out of the cell membrane and extend into the cell wall can be identified, as demonstrated in Figure 4.1 (termed Loop 1 and Loop 2, respectively, for the remainder of this thesis). Loop 1 is 150 amino acids long and Loop 2 is 504 amino acids long. The location of the transposon in *L. monocytogenes* in the M113 mutant discussed in Section 3.3, is located at the start of the 4th transmembrane domain as indicated by the red arrow in Figure 4.1.

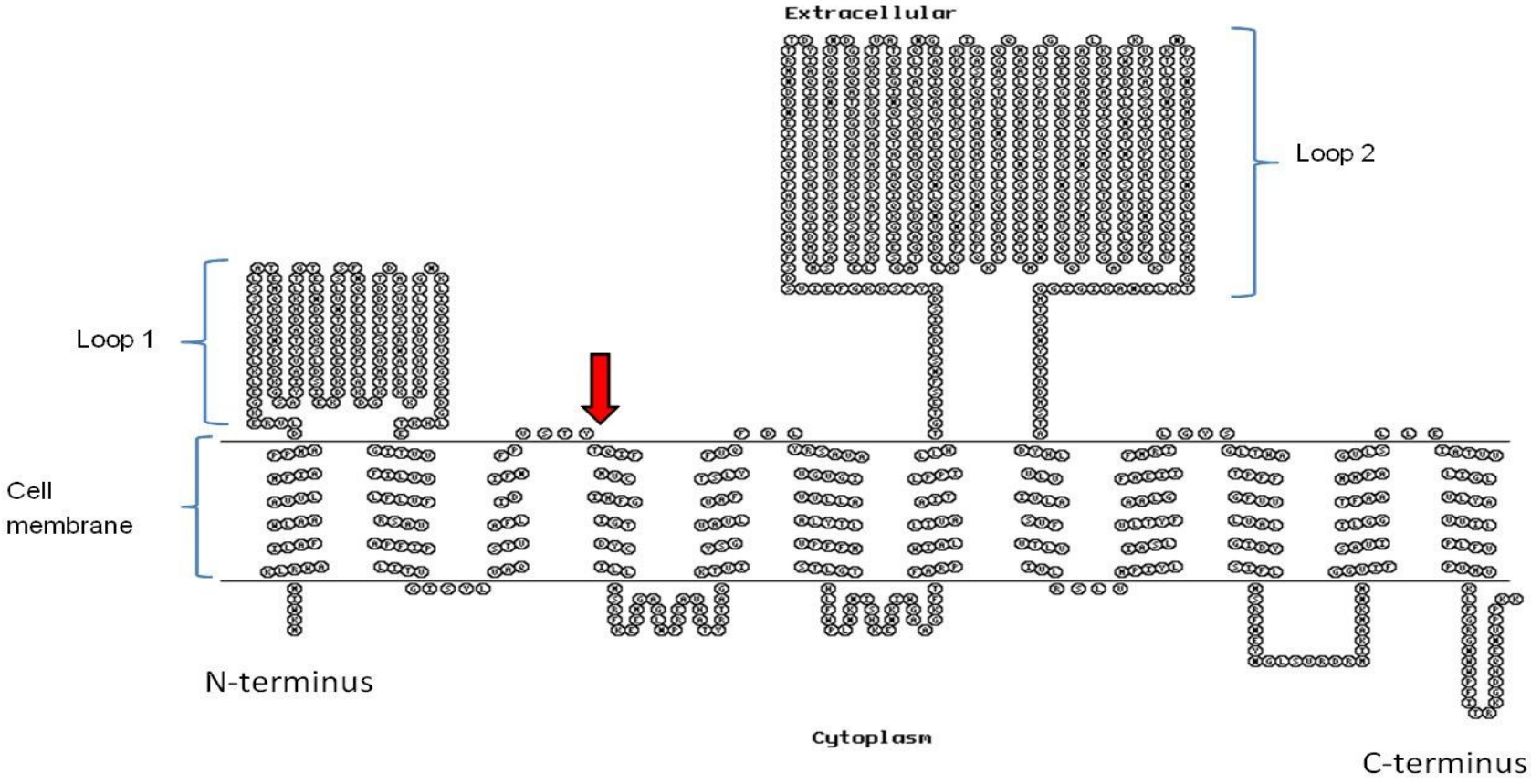

Figure 4.1 - Predicted topology of the protein LMO1226 made using TOPO2 software (Johns) . The protein shows 12 transmembrane helices spanning the cell membrane, and two extracellular loops, termed Loop 1 and Loop 2, which are 150 and 504 amino acids long, respectively. The red arrow indicates the start of the sequence downstream where the transposon is thought to be located in *lmo1226* in the mutant M113.

4.2 Construction of mutants in *lmo1226*

Based on *in silico* analysis, and the location of the insertion of the transposon Tn917 in M113, it was decided to construct three separate in-frame deletion mutants in *lmo1226*; two that produced a truncated form of the protein Lmo1226, with each having the region of DNA that encoded either one of the extracellular loops deleted, and one mutant with the entire gene deleted. This was done to help establish if the entire gene or a specific part of the gene is essential in attachment of *L. monocytogenes* to abiotic surfaces. It was decided to construct the mutants in the background strain 10403s, to allow direct comparison with the corresponding transposon mutants.

4.2.1 Construction of plasmids for mutagenesis

The initial step in the mutagenesis was the construction of the plasmids containing regions of DNA amplified by PCR. The resulting plasmids would be based on the temperature dependent plasmid pAUL-A. This plasmid was chosen as it contains a multiple cloning site and has temperature dependent replication, which allows selection of transformants in which the plasmid has recombined into the host chromosome. This plasmid has been used previously in constructing deletion mutations in *L. monocytogenes* (Chakraborty *et al*., 1992). The first step in constructing the plasmids was the linearization of the vector pAUL-A by restriction digest with the enzyme *Bam*H1. This enzyme cut the plasmid within the multiple cloning site (MCS). Digestion of the plasmid was confirmed by agarose gel

electrophoresis as shown in Figure 4.2. As can be seen, the digested plasmid gave a single band of approximately 9.2kb.

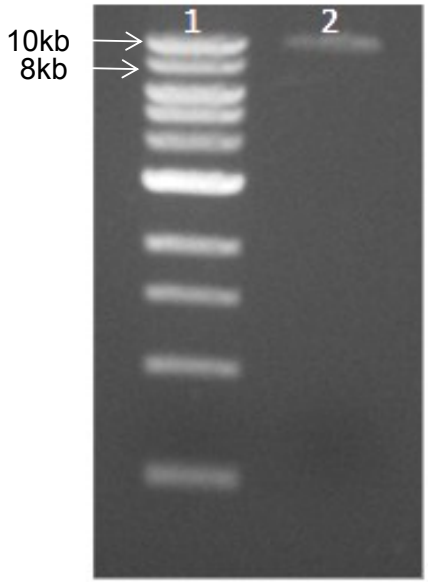

Figure 4.2 - A 1% agarose gel electrophoresis confirming digestion of the plasmid pAUL-A with the restriction enzyme *Bam*H1, giving a single band of approximately 9.2kb. Lane 1 - 1kb ladder, Lane 2 - Digested pAUL-A. Gel was ran in TAE buffer at 100 volts.

Plasmids were constructed by cloning regions of chromosomal DNA into the pre-digested pAUL-A plasmid. The regions of the gene were amplified using colony PCR as described in Section 2.2.7 amplifying two fragments of DNA for each construct that each had overlapping ends of DNA. The first fragment was designed to have a 5' overlap with the *Bam*H1 restriction site of the plasmid pAUL-A, and a 3' overlap with the 5' end of fragment 2. The second fragment was designed to have a 5' overlap with the 3' end of fragment 1 and a 3' overlap with the *Bam*H1

restriction site of the plasmid pAUL-A, as demonstrated in Figure 2.1. This allowed the inserts to be ligated into the plasmid with the use of the Infusion HD cloning kit.

The plasmid pINF1226-1 contained DNA encoding a truncated form of the protein Lmo1226, which would give a protein containing the 1st extracellular loop and the first six transmembrane helices of the protein Lmo1226. The plasmid was constructed as shown in Figure 4.3A. Firstly regions of DNA were amplified using the primer pair 1226_INF1 Fw1 & 1226_INF1 Rv1, to give fragment one. These primers amplified a 755bp region of DNA within the lmo1226 gene starting 43 base pairs downstream from the start codon. The fragments shown in Figure 4.3 A have green and red regions either side of the cloned portion of the *L. monocytogenes* chromosome. The red portion represents the region of the fragment with overlapping ends which would overlap and subsequently be ligated with the *Bam*H1 restriction site of the pre-digested plasmid pAUL-A whereas the green portion represents the region of the fragment with overlapping ends which would ligate to the overlapping ends of the other fragment. The primers 1226_INF1 Fw2 & 1226_INF1 Rv2 were used to amplify a 932bp region of DNA at the 3' end of the gene. PCR products were analysed by agarose gel electrophoresis (Figure 4.4). The figure shows the presence of a 755bp band for fragment one (Lane 4) and a 932bp band for fragment 2 (Lane 2). The two PCR products were ligated with the plasmid, using the Clontech HD infusion cloning kit as described in Section 2.2.10, giving an overall insert size of 1687bp. Ligation of these two fragments introduced a stop codon after the 6th transmembrane domain (380th amino acid). This plasmid was termed pINF1226-1 and was used for transformation into *E. coli*.

To allow the deletion of the entire gene *lmo1226*, two PCR products amplifying regions upstream and downstream from *lmo1226* were used to create the plasmid pINF1226-2 (Figure 4.3B), using the sets of primers 1226_INF2 Fw1 & 1226_INF2 Rv1 and 1226_INF2 Fw2 & 1226_INF2 Rv2 respectively. The region upstream of amplified DNA started 782 bp upstream from the gene start codon, until base pair 63 of the gene, giving a PCR product of 845bp (Figure 4.3B). The second product amplified the region of DNA downstream from the gene started at 54 bp prior to the stop codon located at the end of the gene, until 350 bp downstream from the stop codon, giving a PCR product of 404 bp (Figure 4.3B). Agarose gel electrophoresis was used to confirm the presence and size of the bands from the PCR reaction (Figure 4.5). Lane 2 shows the presence of a 845bp band of fragment 1 and lane 4 shows the 404bp band of fragment 2. The two fragments resulting from this PCR reaction were ligated together into the pre-digested plasmid pAUL-A using the Clontech HD infusion cloning kit as described in Section 2.2.10. This gave an overall insert size of 1249bp. The ligated plasmid was termed pINF1226-2 and was used for transformation into *E. coli*.

The final plasmid, pINF1226-3, was constructed in the same manner as the two previous plasmids, however it excluded the DNA encoding the 1st extracellular loop of the protein. The 1st fragment used for the creation of plasmid pINF1226-2 was used as the first fragment in this reaction as it already amplified the region of DNA upstream from the gene *lmo1226*, and the primers 1226_INF3 Fw1 and 1226_INF2 Rv2 were used to amplify the 2479bp region of DNA that encodes from

the second extracellular loop to the end of the protein (Figure 4.3C). Fragments were analysed using agarose gel electrophoresis (Figure 4.5) to confirm size and presence of insert. Lane 2 shows the already amplified fragment 1, and Lane 6 shows the 2479bp band indicating the presence of the 2nd fragment. Both these products were ligated together, as for the other two fragments, and ligated into the pre-digested plasmid pAUL-A, giving a combined product size of 3242 bp. The resulting plasmid was termed pINF1226-3 and was used for transformation into *E. coli*.

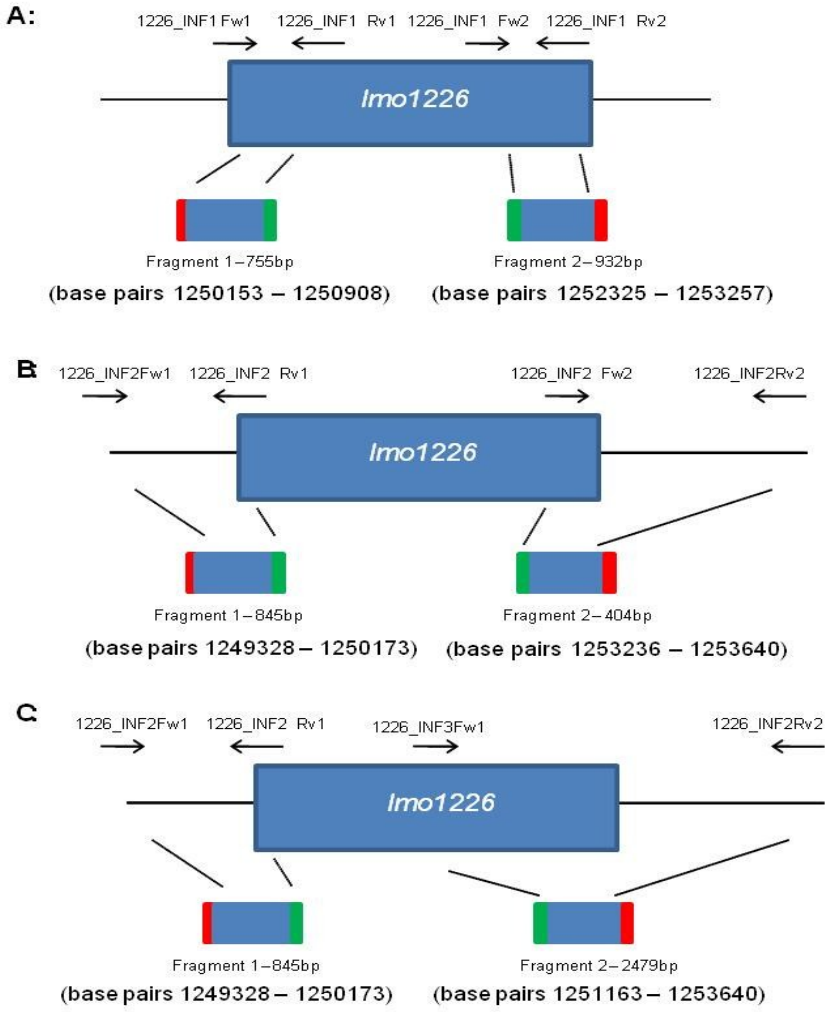

Figure 4.3 - Diagram showing the cloning of regions of the gene lmo1226 in *L. monocytogenes* and the up- and downstream regions of DNA to create two fragments to be ligated into the plasmid pAUL-A to create the plasmids pINF1226-1 (A), pINF1226-2 (B), pINF1226-3 (C). The regions of the fragment coloured red indicate the portion of the fragment which overlaps with the *Bam*H1 restriction site in the digested pAUL-A. The regions coloured green indicated the portion of the two fragments that overlap with complimentary sequences. Numbers in brackets indicate the location in base pairs on the *L. monocytogenes* chromosome which were amplified.

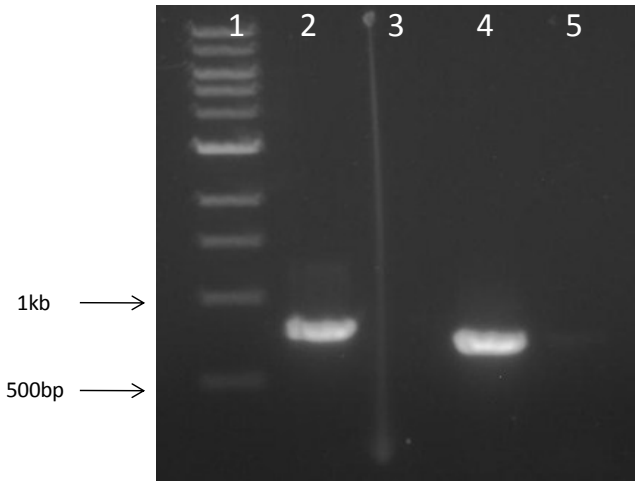

Figure 4.4 - A 1.5% agarose gel electrophoresis of the PCR to amplify the two fragments of DNA required for the construction of the plasmid pINF1226-1. Lane 1 contains a 1kb ladder, Lane 2 contains the 932bp fragment 2, Lane 4 contains the 755bp of fragment 1, Lanes 3 and 5 are negative controls with water instead of DNA as a template. Gel was run in TAE buffer at 100 volts.

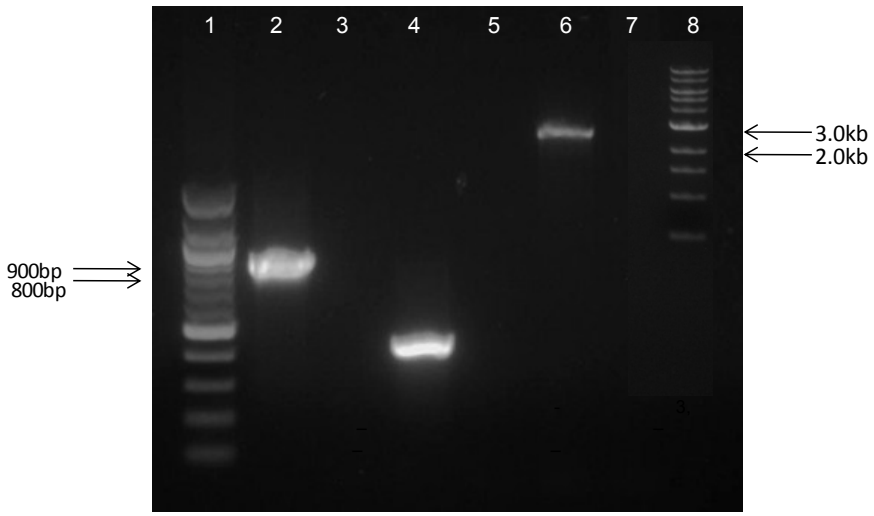

Figure 4.5 - A 1.5% agarose gel electrophoresis of the PCR reactions to amplify the two fragments of DNA required for the construction of the plasmids pINF1226-1 and pINF1226-2. Lane 1 contains a 100bp ladder, Lane 2 contains the 845bp fragment 1 which is used for the construction of both plasmids, Lane 4 contains the 404bp of fragment 2 used for construction of pINF1226-2, and lane 6 contains the 2479bp fragment 2 used for construction of pINF1226-3. Lanes 3, 5 and 7 are negative controls with water instead of DNA as a template and Lane 8 contains a 1kb ladder. The gel was run in TAE buffer at 100 volts.

4.2.2 Transformation of *E. coli* and *L. monocytogenes*

The plasmids created as described in Section 4.2.1 were transformed into XL-10 Gold competent *E. coli* cells using the method described in Section 2.2.4. Following transformation, cells were plated onto Luria agar containing IPTG/Xgal/Erythromycin and incubated overnight. Following incubation of cells white colonies were selected to be screened to confirm the insert.

Plasmid extractions were performed on the selected transformants as described in Section 2.2.11. To confirm the extracted plasmid contained the correct insert plasmids underwent PCR using the primers M13 universal forward and M13 universal reverse following the standard PCR protocol, using plasmid DNA as the template for the PCR reaction as described in Section 2.2.7. Resulting PCR products were purified and sequenced by PNACL, and sequences analysed using BLAST to confirm the presence and fidelity of the insert DNA.

Following transformation of *E. coli* with plasmids pINF1226-1 and pINF1226-2, a large number of white colonies were present after overnight incubation. PCR and subsequent sequencing of these colonies confirmed the correct insert had been successfully cloned within the plasmid. However, following transformation of *E. coli* with the plasmid pINF1226-3 no white colonies were present following incubation. To attempt to elucidate why this occurred, PCR was performed directly on the ligation reaction using the universal primers M13 forward and reverse. The PCR product was sequenced and the resulting sequence subjected to BLAST

analysis.The results of the PCR confirmed that the ligation reaction had worked and the ligated plasmid contained the correct insert yet despite this reaction being successful no transformants were obtained after transformation. It was hypothesized that the gene product of the insert was not compatible with the cells and was possibly toxic to the *E. coli*. Alternatively, this could have been due to the loss of a potential signal sequence in the protein. To investigate this, the potential signal sequence would have to be identified, and a mutant created with this sequence still present. Due to this problem the construction of a Δlmo1226-3 mutant could not continue. An alternative method to make this mutant could have involved transforming the ligation reaction directly into electrocompetent *L. monocytogenes* cells, but unfortunately due to time restrictions this was not possible within this study.

When the plasmids pINF1226-1 and PINF1226-2 had been confirmed as containing the correct insert, they were transformed into *L. monocytogenes* 10403s by electroporation as described in Section 2.2.13. After electroporation 1ml of SOC medium was added. Electroporated cells were allowed to recover in SOC medium at 30°C for three hours; following incubation transformed cells were plated onto BHI agar containing erythromycin and incubated for 48 hours at 30°C. Cells that grew by 48 hours were assumed to contain the plasmid and were selected for the recombination stage.

4.2.3 Recombination of plasmids into the *L. monocytogenes* chromosome

The first stage of the recombination was the integration of the plasmids pINF1226-1 and pINF1226-2 into the chromosome of *L. monocytogenes*. Cells from step 4.2.2 were subcultured on BHI agar containing erythromycin and incubated at 42°C. Following overnight incubation, this process was repeated for a second overnight culture to increase the number of recombinants with the plasmid inserted into the chromosome. Incubation at this temperature selected for insertion because free plasmids are not able to replicate at 42°C.

Colonies that grew after being subcultured twice on BHI agar at 42°C were screened for integration of the plasmids pINF1226-1 and pINF1226-2 into the *L. monocytogenes* chromosome, by colony PCR with the primers M113C and M13 universal reverse for pINF1226-1. For the plasmid pINF1226-2, the primer M113C was substituted by M113E, which annealed to the chromosomal DNA further upstream than the primer M113C. The primers M113C and M113E bind to the Listerial chromosome upstream of the region of insertion, so that any colonies that had an integrated plasmid would give a positive reaction with a band size of 1815bp and 1864bp for pINF1226-1 and pINF1226-2 respectively. Following PCR, products were analysed by agarose gel electrophoresis to confirm that integration had taken place. Colonies which gave the correct sized band on the gel were picked to continue the recombination. Figure 4.6 shows the gel electrophoresis of the colonies screened for integration of pINF1226-1 into the chromosome, with each lane of the gel representing a PCR reaction with an individual colony as the template. Positive colonies can be seen in lanes 2, 3, 6, 10, 11 and 12, as all

exhibit a band size of approximately1815bp. Some colonies did not show a correct result for the first stage of recombination, Lanes 2, 9 and 10 in Figure 4.6 show two bands, indicating possible incorrect insertion of the insert into the chromosome, or possible mispriming during the PCR reaction. Lanes 4, 5, 7 & 8 gave a negative result with no bands being present when the PCR product was run on agarose gel electrophoresis. The absence of bands in these reactions indicates insertion of the plasmid into the chromosome has not occurred in these colonies. Following the analysis by gel electrophoresis the colony in lane 11 was picked for continuation of the recombination process as it contained a correct size band.

Figure 4.7 shows the gel electrophoresis to screen for integration of the plasmid pINF1226-2 into the *L. monocytogenes* chromosome, with each lane representing an individual colony providing the template DNA for the PCR reaction. In this instance only the colony in lane 5 showed a positive band of approximately 1864bp, indicating that integration has taken place. All other reactions were negative for bands following gel electrophoresis, indicating integration had not occurred. Consequently colony 5 was chosen for continuation of the recombination process.

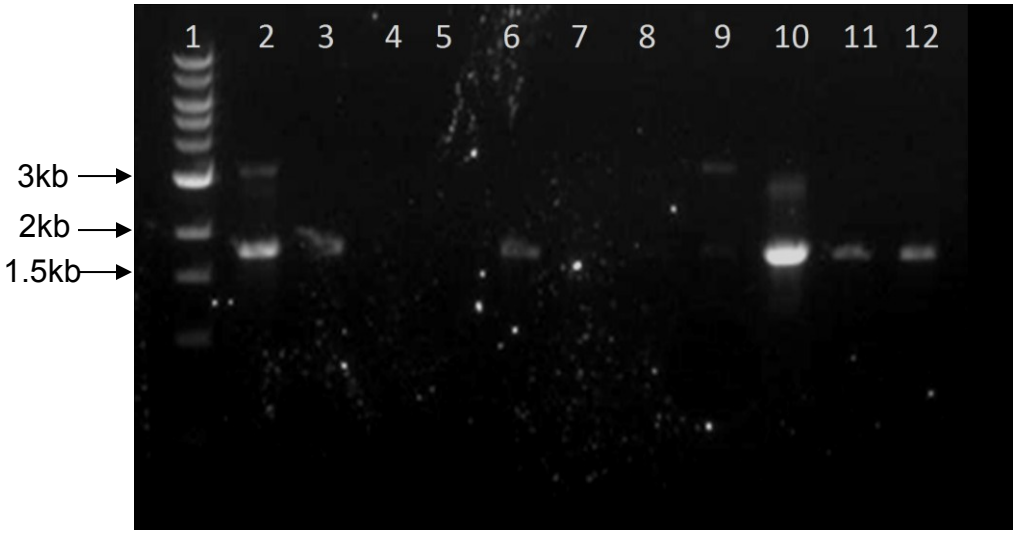

Figure 4.6 - A 1% agarose gel electrophoresis of PCR screening for integration of pINF1226-1 into the chromosome of *L. monocytogenes* 10403s. Lane 1 - 1 kb ladder, Lanes 2 to 12 - Individual colonies screened for integration, some showing the correct band size of 1815bp. Gel was ran in TAE buffer at 100 volts.

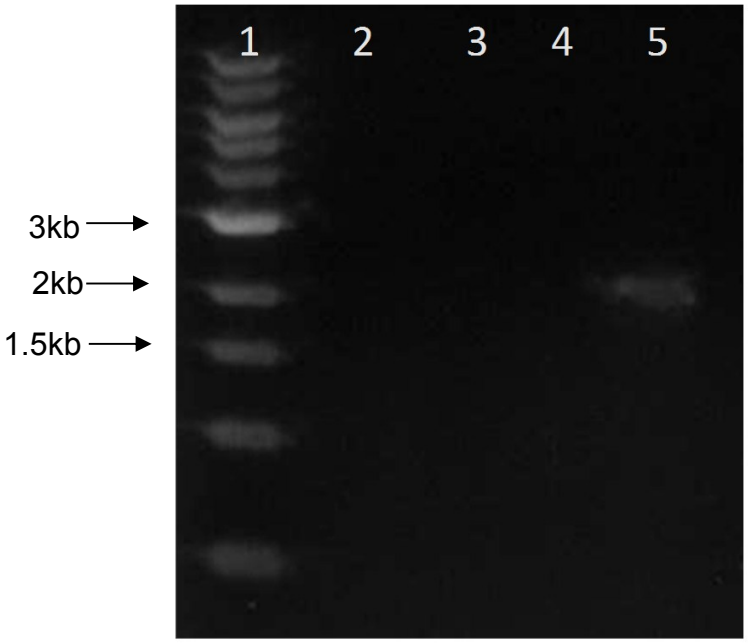

Figure 4.7 - A 1% agarose gel electrophoresis of PCR screening for integration of pINF1226-2 into the chromosome of *L. monocytogenes* 10403s. Lane 1 - 1 kb ladder, Lanes 2 to 5 - Individual colonies screened for integration, colony 5 showing the correct size band of 1864bp. Gel was ran at 100volts in TAE buffer.

The colonies selected following successful integration of the plasmids pINF1226-1 and pINF1226-2 into the *L. monocytogenes* chromosome were used for the continuation of the process of recombination, as discussed in Section 2.2.14. Following the process of recombination, cells were replica plated onto BHI agar, with and without erythromycin to allow identification of the loss of plasmid from the cell and incubated at 30°C. Any erythromycin sensitive cells were selected for screening for the occurrence of the desired mutations. Sensitive colonies were screened using colony PCR with the primers M113C and M113D, which bind to the chromosome up and downstream from *lmo1226* respectively. PCR products were

run on a 1% agarose gel with 1kb and 100bp ladders. The expected band size was 2417bp and 771bp for the mutants Δlmo1226-1 and Δlmo1226-2 respectively, the band size for the wild type was 3834bp. Figure 4.8 shows the agarose gel electrophoresis for the screening for the Δlmo1226-1 PCR products. Only one colony out of approximately 100 that were replica-plated was observed to be erythromycin sensitive (Figure 4.8, lane 2), which when screened gave a band size of approximately 2417bp, as expected for the mutant. Lane 3 of Figure 4.8 shows the PCR of the parent strain 10403s and Lane 4 is the negative control which used water for a template in the reaction. Figure 4.9 shows the agarose gel electrophoresis of the PCR screening of the colonies screened for the mutant Δlmo1226-2. In this instance two colonies were erythromycin sensitive and were screened (Lanes 2 and 3). Both of the colonies were positive for the mutation, showing a band size of 771bp. Lane 4 of this gel shows the negative control reaction.

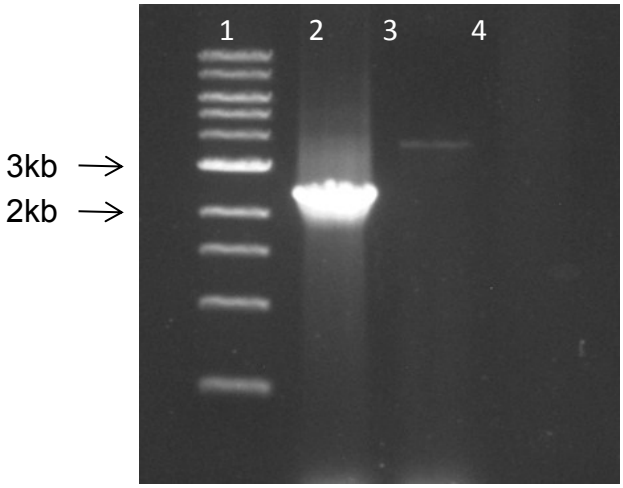

Figure 4.8 - A 1.5% agarose gel electrophoresis of PCR products of Δlmo1226-1 mutant. Lane 1 - 1kb ladder, Lane 2 - Δlmo1226-1 mutant showing the correct size band of 2417bp, Lane 3 - 10403s, Lane 4 - Negative control using water as a template. Gel was run at 10 volts in TAE buffer.

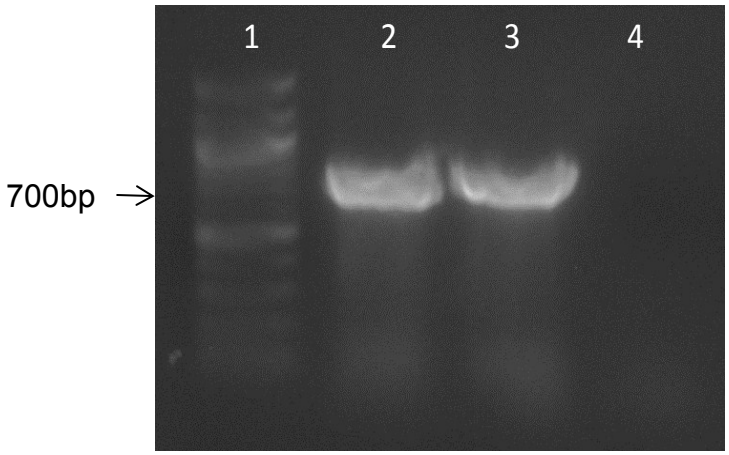

Figure 4.9 - Agarose gel electrophoresis of PCR products of Δlmo1226-2 mutant. Lane 1 - 1kb ladder, Lane 2 and 3 - Δlmo1226-2 mutant showing the correct size band of 771bp, Lane 4 - Negative control using water as a template. Gel was run at 100 volts in TAE buffer.

4.1.2 Attachment of *L. monocytogenes* ΔImo1226-1 and ΔImo1226-2 mutants to plastic

To confirm the involvement of *Imo1226* in attachment of *L. monocytogenes* to plastic surfaces, both of the deletion mutants ΔImo1226-1 and ΔImo1226-2 were tested for their ability to adhere to plastic, using the attachment assay described in Section 2.2.4. As shown in Section 3.2 the transposon mutant M113 had already shown a big reduction in initial attachment to plastic at all of the temperatures tested, therefore it was hypothesised that the same would occur in these deletion mutants, hence the attachment assay was carried out only at 37 and 30°C because it was assumed that the assay carried out at lower temperatures would give the same result as at the two higher temperatures. The attachment assays were performed as three biologically independent experiments and the data were collated to produce Figures 4.10 A and B. The data from these experiments are shown as an average of all experiments, and the error bars show standard error of the mean.

From these data it was observed that the mutant ΔImo1226-1 did not show reduction in adherence at either of the temperatures tested ($P>0.05$), which was in contrast to the effect seen with the transposon mutant M113. The data for the mutant ΔImo1226-2 correlated with those observed for the mutant M113 in that the result showed significantly less attachment to the microtitre plate at both 37 and 30° ($P<0.05$). Again as in the case of the transposon mutant M113, the effect did not appear to be temperature dependent. The results of these experiments confirm

the results observed previously with the transposon mutant M113 (Section 3.2). The deletion of the entire *lmo1226,* as in the mutant ∆lmo1226-2, led to a reduction in attachment at all temperatures, as seen with the transposon mutant, supporting the hypothesis that gene *lmo1226* is involved with attachment of *L. monocytogenes*. The attachment assay data for the mutant ∆lmo1226-1 also agree with the attachment assay data of the transposon mutant M113, as the region into which the transposon inserted into the chromosome (demonstrated by a red arrow, Figure 4.1) is still present in the mutant Δlmo1226-1. It can be hypothesised that these data indicate that the extracellular loop 2 or the transmembrane helices 7-12, both of which are deleted in the mutant ∆lmo1226-1 are not likely to be involved in the attachment of this organism to the microtitre plate. The fact that the deletion of the entire gene in mutant ∆1226-2 showed significantly lower attachment indicates that the region of DNA coding for extracellular loop 1 or the first 4 transmembrane helices is important in attachment of *L. monocytogenes* to abiotic surfaces.

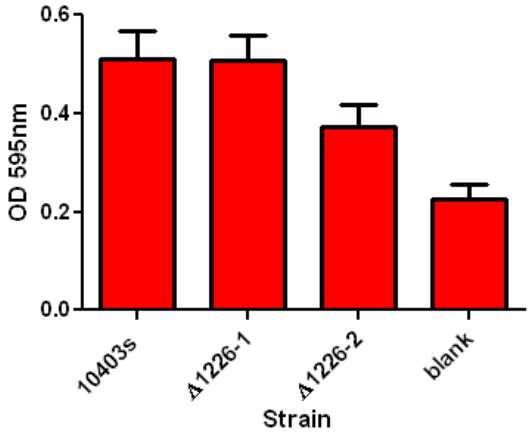

Figure 4.10a. - Attachment assay of *L. monocytogenes* mutants Δlmo1226-1 and Δlmo1226-2 in comparison to their wild type parent when the assay was carried out at 37°C. Data represent averages of 8 wells from three biologically independent experiments; error bars represent standard error of the mean.

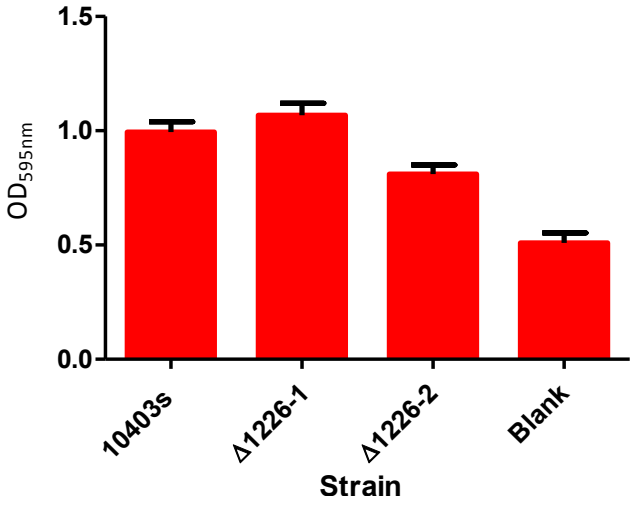

Figure 4.10b - Attachment assay of *L. monocytogenes* mutants Δlmo1226-1 and Δlmo1226-2 in comparison to their wild type parent when the assay was carried out at 30°C. Data represent averages of 8 wells from three biologically independent experiments; error bars represent standard error of the mean.

4.4.1 Growth of *L. monocytogenes* mutants' ∆lmo1226-1 and ∆lmo1226-2 in sodium chloride

Other drug exporters of the RND superfamily have been implicated in involvement of transport of bile salts and osmolytes (Hagman *et al*., 1997). To test if *lmo1226* was involved in salt transport in *L. monocytogenes*, the deletion mutants ∆*lmo1226*-1 and ∆*lmo1226*-2 were tested for their ability to grow in varying concentrations of sodium chloride. Bacterial growth curves were performed in TM medium supplemented with varying percentages of NaCl, as described in the method in Section 2.4.1. Growth curves were started in a microtitre plate by inoculating 2µl of an overnight culture into 198µl of TM with the required concentration of NaCl. Optical density over time was recorded using a Multiskan Go instrument (Thermo Fisher), with the OD_{600nm} being recorded every 20 minutes. The data from the growth curves are presented in Figures 4.11a to 4.11h, and Table 4.2 giving a summary of the length of lag phase, change in OD over time and the maximum yield obtained in the form of OD. All data are presented as the average of three biologically independent replicates.

From the Table 4.2 and Figure 4.11a it can be observed that there was no difference observed between the growth in TM with no added sodium chloride between the wild type and the mutants in terms of growth rate (change in OD/time) or length of lag phase ($P>0.05$). Both the mutants showed a significantly higher ($P<0.05$) yield than the wild type (OD_{600nm} of 0.303 and 0.278 for the mutants in comparison to 0.235 for the wild type strain) indicating higher amounts of bacteria

present. However these data are not necessarily indicative of a higher concentration present as the optical density of the culture would not exclude dead cells and the number does not represent the live cell concentration. Also there could be a morphological change in the bacterial cells, such as a change in size, which would also result in changes in optical density.

From the Figures 4.11a to 4.11h it can be seen that the length of the lag phase of the mutant strains slowly increased as the concentration of salt in the medium increases. At a concentration of 0.5% (w/v) NaCl there was still only a small difference between the lag phase of the mutants and the parent strain, however for concentrations greater than 0.5% (w/v) the length of lag phase was significantly longer for both of the mutants compared with the strain 10403s ($P<0.05$). This effect is highlighted most in 3% NaCl. At this concentration of sodium chloride the parent strain 10403s had a lag phase of 1580 minutes, whereas the lag phase for the mutant strains was 2560 and 2180 minutes for the mutant strains Δ*lmo1226*-1 and Δ*lmo1226*-2, respectively.

As observed in Table 4.2 the maximum yield of the mutants was significantly higher than that of the parent strain for concentrations of salt up to 2.5% (w/v). At the 3% (w/v) concentration of NaCl the mutants had not completed the growth curve at the end of the experiment so it is unclear whether they would still grow to a higher yield than the 10403s strain. To ascertain this, the growth curves needed

to be extended for a longer time period, but due to the constraints of the microtitre plate reader this was not possible.

The rate of bacterial growth was also calculated from these results and the data presented in Table 4.2 as OD/min. Overall the rate of growth showed a decrease as the percentage of NaCl in the medium increased, however there was no significant difference in the rate of growth between the wild type and the two mutant strains at most of the concentrations of NaCl. However at a concentration of 1% NaCl the mutant Δlmo1226-2 grew at a significantly lower rate than the wild type ($P<0.05$).

In 3.5% (w/v) NaCl the mutants do not appear to grow at all and the wild type only just entered exponential phase. Unfortunately due to the limitations of the equipment used to measure these data, it not possible to extend the time of this experiment to determine the full growth curve of the wild type in 3.5% (w/v) NaCl. A longer growth curve would have also shown whether the mutant strains would have recovered to grow eventually or whether this concentration was lethal to the mutants and they would not have grown.

The overall conclusion that can be drawn from these data is that the mutant strains take longer to adapt to higher concentrations of NaCl, as reflected by the increase in time spent in lag phase. This indicates that *lmo1226* is important in the adaptation of *L. monocytogenes* to NaCl. Once the strains have adapted to the

higher concentrations of NaCl however, they grew to a higher yield than their parent strain.

	10403s			Δlmo1226-1			Δlmo1226-2		
% NaCl	Length of Lag phase (min)	OD/min $x10^{-4}$	Maximum yield (OD 600nm)	Length of Lag phase (min)	OD/min $x10^{-4}$	Maximum yield (OD 600nm)	Length of Lag phase (min)	OD/min $x10^{-4}$	Maximum yield (OD 600nm)
0.0	560	3.9	0.235	460	3.1	0.3035	560	2.9	0.278
0.5	580	2.8	0.2338	620	2.3	0.2682	600	2.1	0.2609
1.0	600	2.6	0.2299	820	2.2	0.2518	880	1.5	0.2372
1.5	680	1.6	0.2125	1140	1.5	0.2365	1060	1.7	0.2468
2.0	860	1.5	0.2113	1220	1.3	0.2205	1400	1.5	0.2318
2.5	1060	1.3	0.1888	1460	1.7	0.2284	1540	1.4	0.2248
3.0	1580	1.0	0.1850[a]	2560	NG[b]	0.1163[a]	2180	1	0.1593[a]
3.5	2240	NG	0.1142[a]	NG	NG	NG	NG	NG	NG

Table 4.2 - Summary of data of growth curves of *L. monocytogenes* 10403s and the mutant strains Δlmo1226-1 and Δlmo1226-2 in different concentrations of NaCl. The length of lag phase, change in OD with time (presented as OD/min) and the maximum yield was calculated for each growth curve as an average from three independent experiments, [a] denotes a result from a growth curve in which the bacteria have not reached stationary phase, [b] NG indicates growth has not occurred in this experiment.

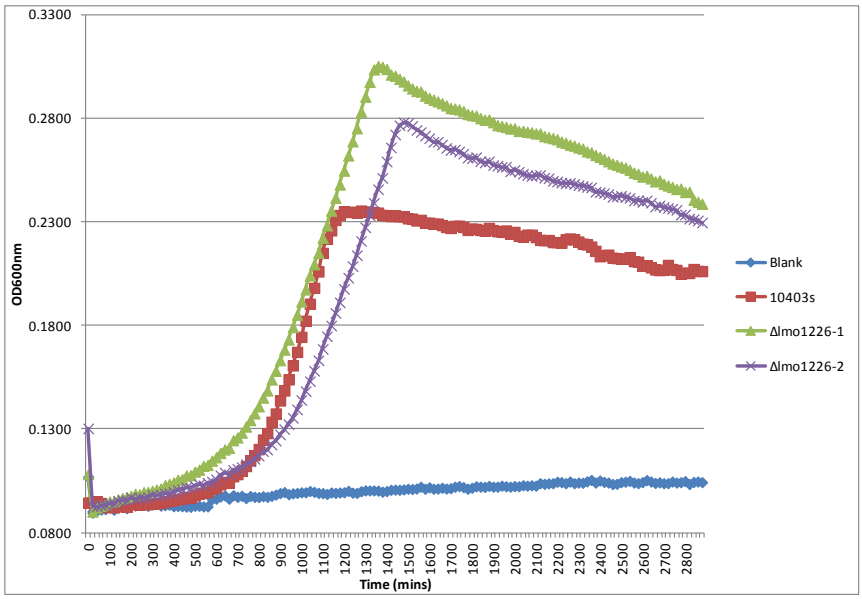

Figure 4.11a - Growth curve of *L. monocytogenes* 10403s and the deletion mutants in TM medium with 0% NaCl added. Results shown are an average of three biologically independent replicates

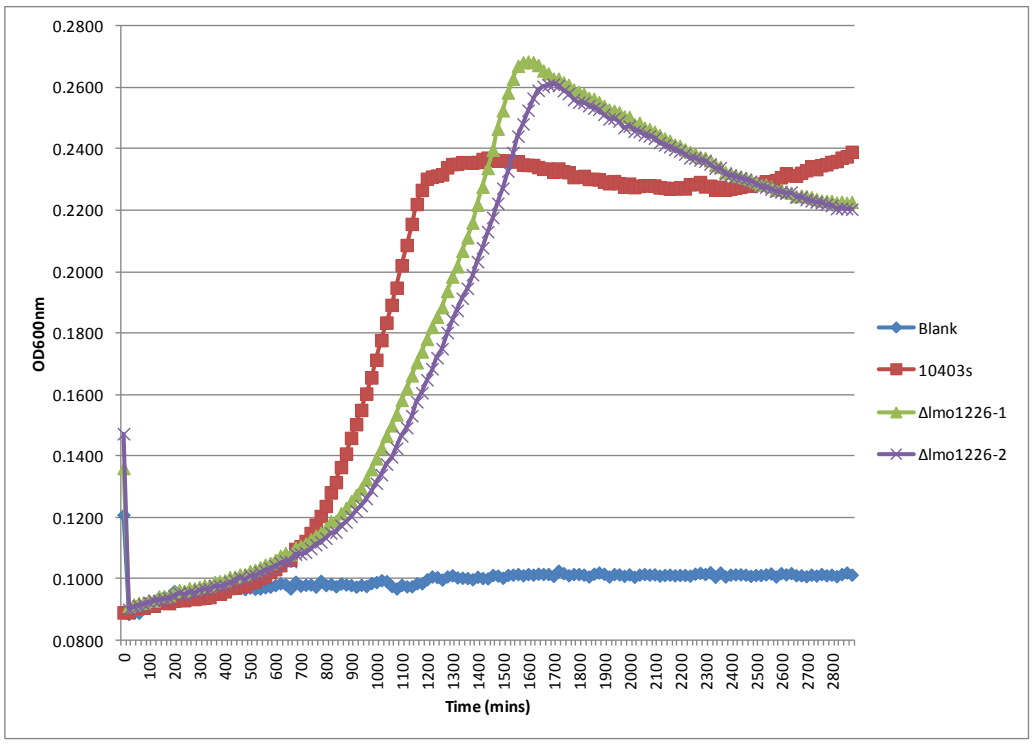

Figure 4.11b - Growth curve of *L. monocytogenes* 10403s and the deletion mutants in TM medium with 0.5% (w/v) NaCl

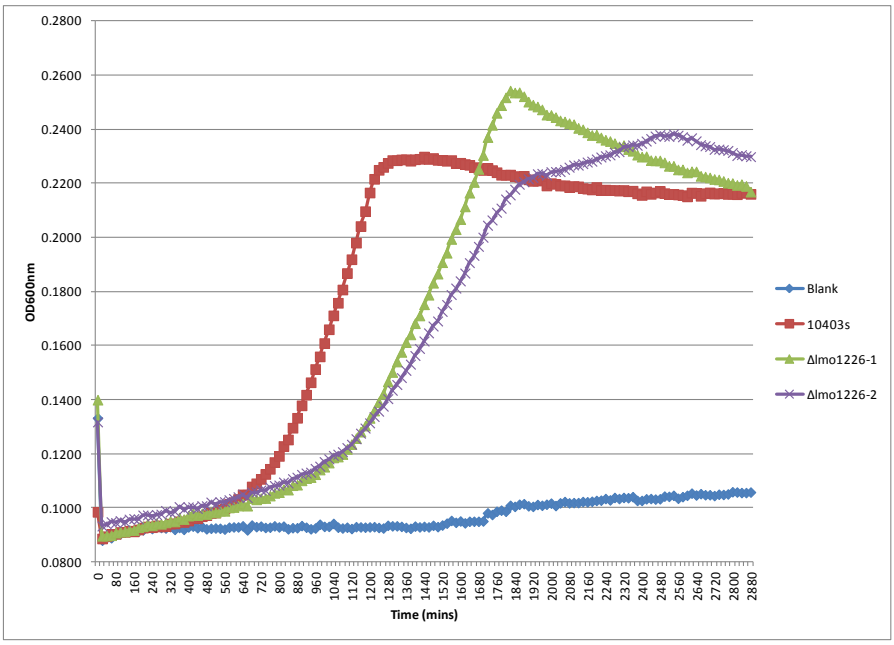

Figure 4.11c - Growth curve of *L. monocytogenes* 10403s and the deletion mutants in TM medium with 1.0% (w/v) NaCl

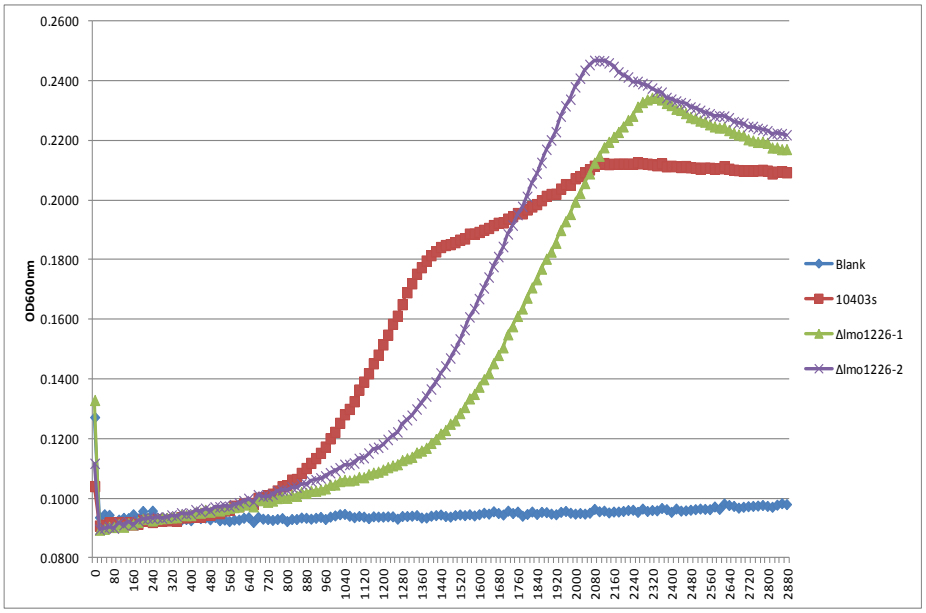

Figure 4.11d - Growth curve of *L. monocytogenes* 10403s and the deletion mutants in TM medium with 1.5% (w/v) NaCl

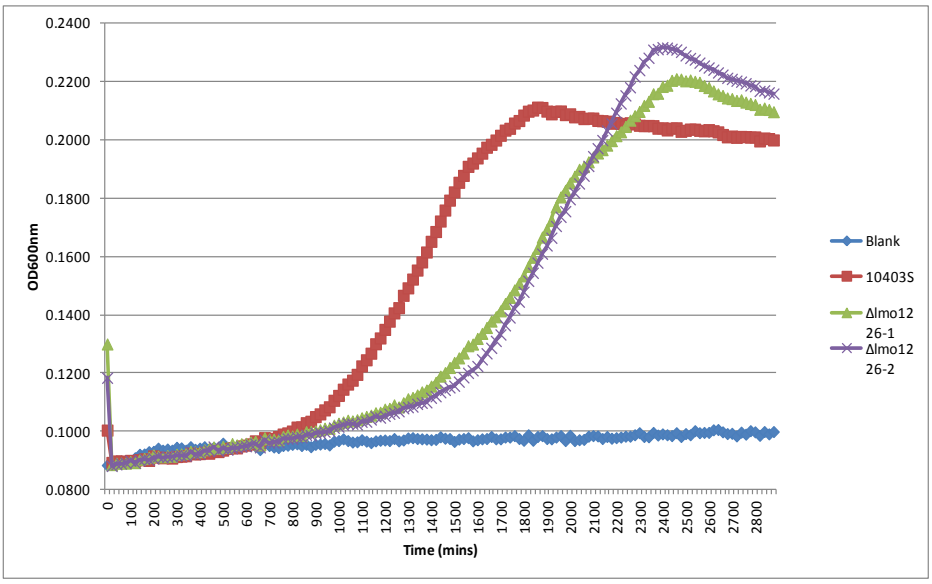

Figure 4.11e - Growth curve of *L. monocytogenes* 10403s and the deletion mutants in TM medium with 2% (w/v) NaCl

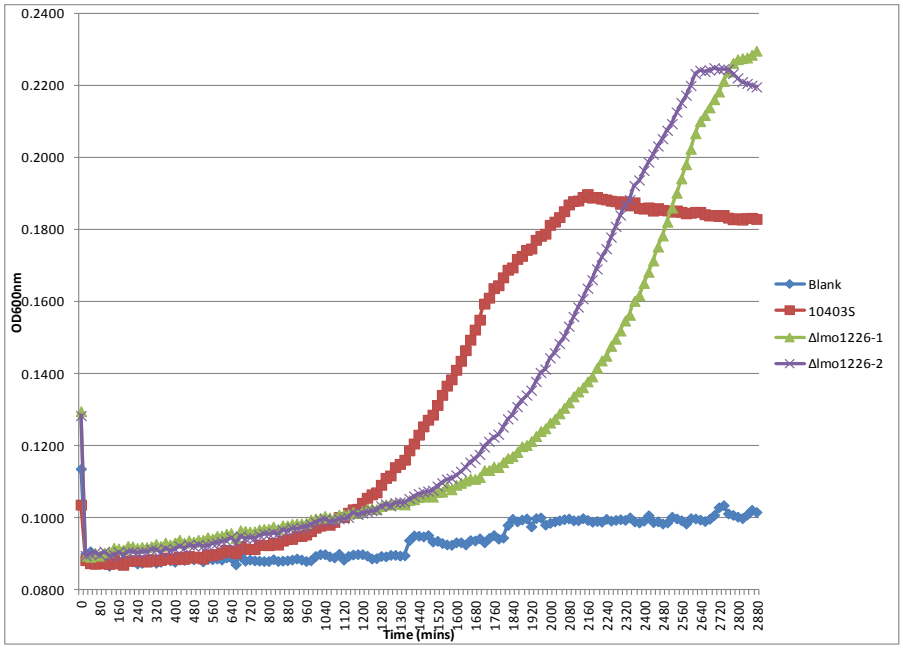

Figure 4.11f - Growth curve of *L. monocytogenes* 10403s and the deletion mutants in TM medium with 2.5% (w/v) NaCl

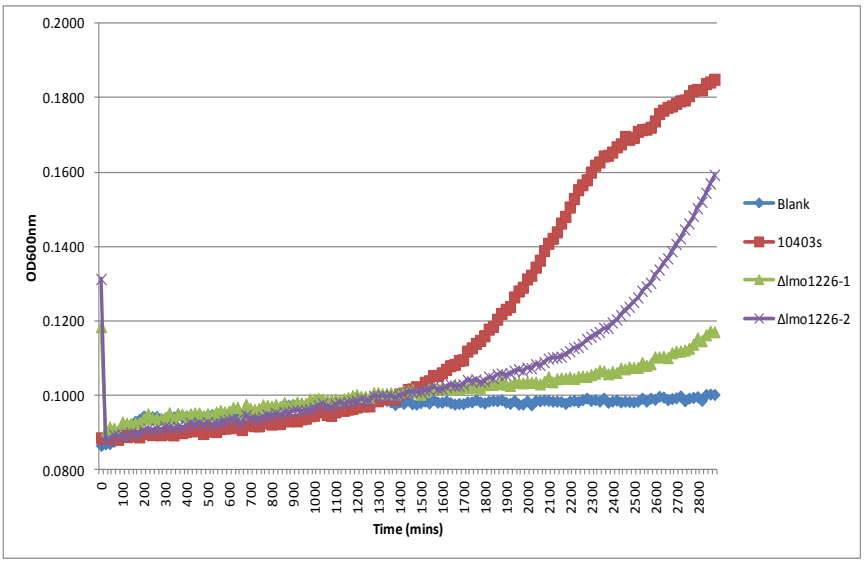

Figure 4.11g - Growth curve of *L. monocytogenes* 10403s and the deletion mutants in TM medium with 3% (w/v) NaCl

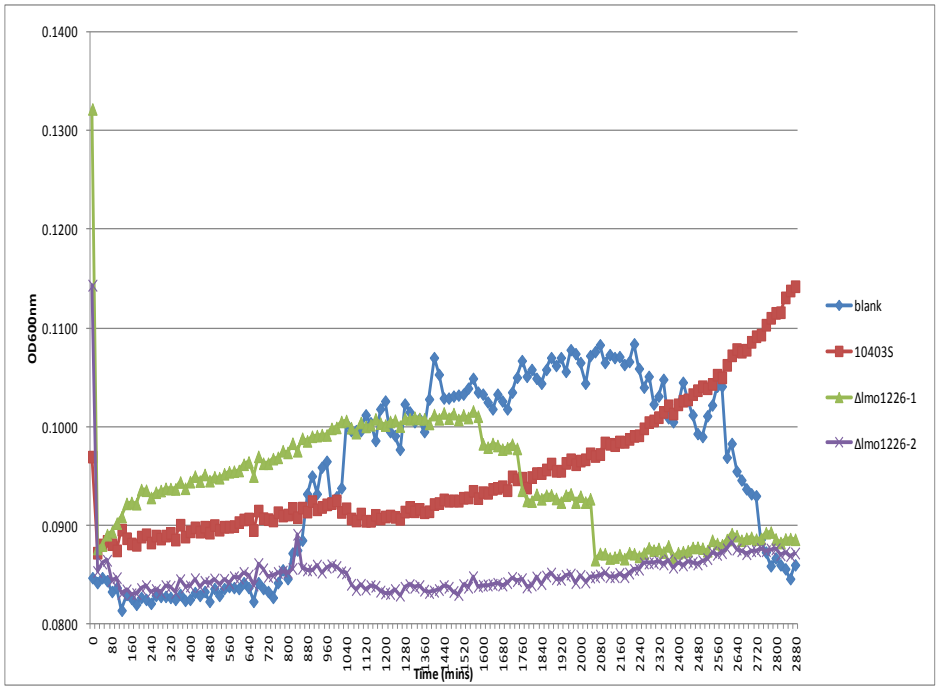

Figure 4.11h - Growth curve of *L. monocytogenes* 10403s and the deletion mutants in TM medium with 3.5% (w/v) NaCl

4.4.2 Osmotolerance of ΔImo1226-1 and ΔImo1226-2

Provisional data provided by Faleiro (Faleiro, personal communication) indicated that the transposon mutant M113 was involved in the osmotolerant response of *L. monocytogenes*. To test the hypothesis that *lmo1226* was important in the osmotolerant response, an osmotolerance assay was undertaken, using the method of Faleiro *et al.* (2003). The osmotolerant response is the response mounted by bacteria in response to a moderate salt concentration, which enables them to survive higher concentrations of sodium chloride. Bacterial cells were grown in TM medium and then resuspended in either 0% (w/v) NaCl (termed non-adapted cells) or 3.5% (w/v) NaCl (termed adapted cells). After adaptation to salt for three hours, cultures were resuspended in 50ml TM medium containing 20% (w/v) NaCl. Samples were taken daily and viable counts were determined for up to ten days. Results from this experiment are shown in Figure 4.12. Data were plotted as an average of two independent biological replicates, and are shown as a percentage survival compared to the number of cells present at time zero.

From Figure 4.12 it can be observed that the non-adapted parental strain 10403s showed a drop in viable counts in the non-adapted cells from 100% to 2.0% in a period of 24 hours. The percentage survival for the adapted cells exhibited a lower drop, from 100% survival to 3.5% survival at 24 hours. This shows that an osmotolerance response has taken place as the pre-adapted cells have a significantly higher percentage survival than the non-adapted cells ($P<0.05$).

The two mutant strains exhibited a much larger drop at the 24 hour point than that of the parent strain in the non-adapted cells, dropping from 100% to a survival percentage of 0.39% and 0.04% for the mutants *Δlmo1226*-1 and *Δlmo1226*-2 respectively ($P<0.05$). The adapted cells also saw a drop in numbers, but the drop was significantly lower than the drop seen in non-adapted cells ($P<0.05$), from 100% to 6.32% and 1.62% for Δlmo1226-1 and Δlmo1226-2 respectively. The percentage survival of the adapted cells showed no significant difference between any of the strains.

The large difference in percentage survival between the adapted and non-adapted cells observed in each of the mutant strains showed a statistically significant difference ($P<0.05$). This indicates this gene is probably not involved in the osmotolerant response of *L. monocytogenes* as the mutant strains survived better in the high salt concentration after pre-exposure to 3.5% sodium chloride, indicating an adaptive process has occured in those pre-exposed to 3.5% NaCl. The much larger drop in numbers in the non-adapted mutants than the wild type however indicates this gene is involved in survival in salt.

The general trend of these results shows that the non-adapted cells show a lower percentage of survival over the course of the study, although this is not true about all time points. The time point of 144 hours also demonstrates a clear difference in the percentage survival between the adapted and non-adapted cells, across all the strains with a significant difference ($P<0.05$) observed between the adapted and non-adapted cells. At this point however the percentage of survival is similar in the

strain 10403s and the mutants in non-adapted cells as opposed to the higher percentage observed in this strain at the 24 hour time point. However as the non-adapted cells are not lower in survival at all of the time points conclusions cannot be drawn over the role of *lmo1226* in osmotolerance at this point.

Looking at these data and the data obtained from the growth curves in different concentrations of NaCl it can be concluded that a concentration of 3% (w/v) of NaCl would have been a better concentration to use for the 3 hours that the strains are exposed to the lower concentration of salt. This lower percentage would probably have been better for adaptation due to the fact that the mutant strains do not seem to grow at all at the higher percentage of salt.

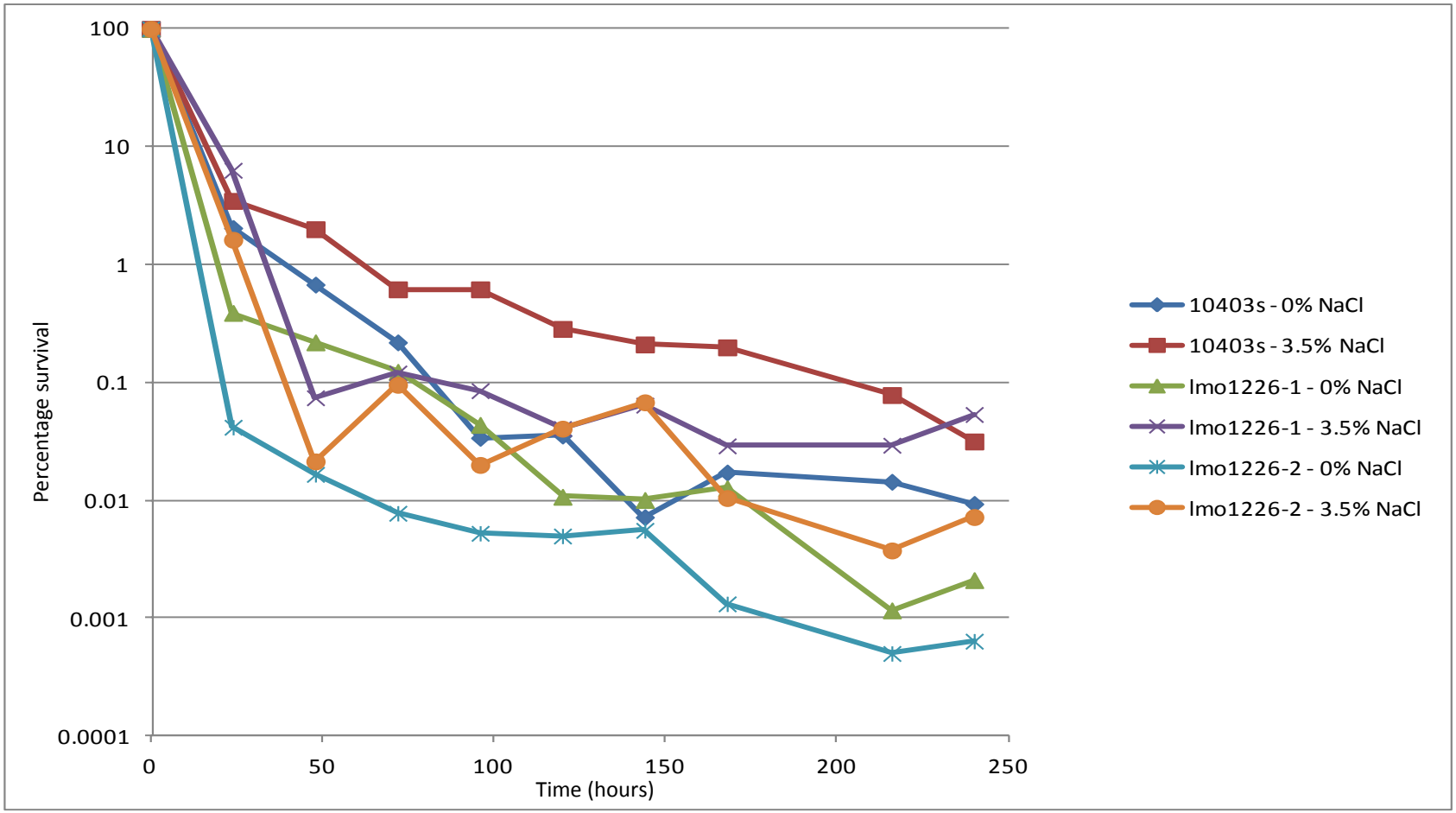

Figure 4.12 - Osmotolerance of the *L. monocytogenes* strain 10403s and the mutant strains Δlmo1226-1 and Δlmo1226-2.

Results are shown as percentage survival of the colony forming units at time zero

4.5 Antibiotic resistance of Δ1226-1 and Δ1226-2

As already discussed *lmo1226* is thought to encode for a putative drug exporter based on homology to other proteins within the RND superfamily. Drug exporters in other species of bacteria such as *E. coli* and *N. gonorrhoeae* have been identified to be involved in antibiotic resistance, sometimes in multidrug resistance (Putman *et al*., 2000). To test the hypothesis that *lmo1226* was involved in antibiotic resistance of *L. monocytogenes*, the mutant strains Δ1226-1 and Δ1226-2 were compared to the strain 10403s in terms of the minimum inhibitory concentration (MIC) of a variety of antibiotics. A wide range of antibiotics were chosen with different modes of action. Sodium chlorate hydrate, a bile salt, was also selected, as in *N. gonorrhoeae* a drug exporter of the RND superfamily, the MtrD protein, has been identified to be involved in survival in bile salts (Hagman *et al*., 1997) so it was hypothesised that gene *lmo1226* may also play a crucial role in bile sensitivity. The MIC for each antibiotic was determined as described in Section 2.4.3. Each antibiotic was tested in triplicate. Table 4.3 shows the MICs of the agents tested, all values are an average of three independent experiments.

As can be seen in Table 4.3, it was observed that the mutants exhibited the same MIC for the bile salt sodium chloride hydrate. From this it can be concluded that this gene is not important in the survival of *L. monocytogenes* in bile salts. The results also indicate *lmo1226* is not important in resistance of *L. monocytogenes* to gentamicin, ampicillin, erythromycin, spectinomycin, chloramphenicol, or tetracycline. There is however a highly significant difference ($P<0.001$) in the MIC

of streptomycin. The parent strain 10403s showed resistance at all concentrations of this antibiotic tested, (MIC>500µg/ml) this was in contrast to both of the mutants which had a MIC of 7.8µg/ml.

Antibiotic	10403s	Δ1226 -1	Δ1226 -2
Gentamicin	**3.1µg/ml**	**3.1µg/ml**	**3.1µg/ml**
Ampicillin	**1.3µg/ml**	**1.3µg/ml**	**1.3µg/ml**
Erythromycin	**0.3µg/ml**	**0.3µg/ml**	**0.3µg/ml**
Spectinomycin	**125µg/ml**	**125µg/ml**	**125µg/ml**
Streptomycin	**>500µg/ml**	**7.8µg/ml**	**7.8µg/ml**
Chloramphenicol	**10µg/ml**	**10µg/ml**	**10µg/ml**
Tetracycline	**0.3µg/ml**	**0.3µg/ml**	**0.3µg/ml**
Sodium chlorate hydrate	**4mg/ml**	**4mg/ml**	**4mg/ml**

Table 4.3 - The minimum inhibitory concentration (MIC) of different antibiotics for *L. monocytogenes* mutants and their isogenic parent strain 10403s. Each result is an average of three biologically independant replicates.

4.8 Screening for the presence of *lmo1226* in dairy isolates of *L. monocytogenes*

Colony PCR was used on strains isolated from artisanal dairies across Portugal (Chambel *et al*., 2007) to test for the presence of *lmo1226*, using the primers M113C & M113D (as described in Section 2.2.7). This screening was to determine whether this gene could be important for environmental persistence of *L. monocytogenes* in the environment. The absence of the gene in environmental isolates would indicate that this gene was not essential. PCR products were analysed by agarose gel electrophoresis (Figure 4.13), Lane 2 contains the positive control for PCR reaction done using strain 10403s as the template. Lanes 3-12 contain the PCR reactions with each lane containing the reaction from a different dairy isolate, and Lane 13 was the negative control reaction. As can be seen from the Figure, the gene was found in all of the dairy isolates that were screened with band size (3834 bp) being consistent in all the strains.

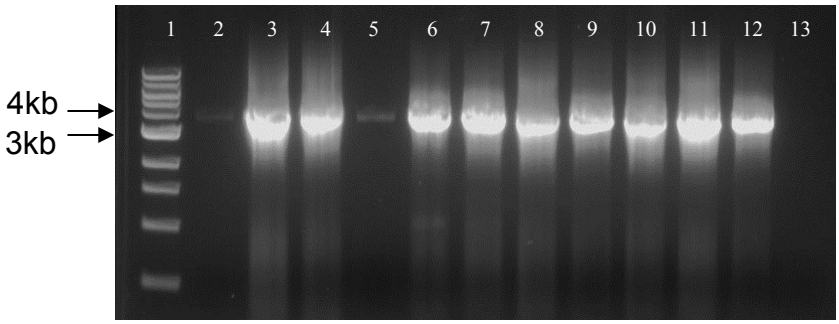

Figure 4.13 - A 1% agarose gel electrophoresis of the PCR screening dairy isolates for the presence of *lmo1226* using the primers M113C and M113 D. Lane 1 – 1kb ladder, Lane 2 – 10403s, Lane 3 – T8, Lane 4 – T46A, Lane 5 –SA11, Lane 6 – G8, Lane 7 – SA264, Lane 8 – A9, Lane 10 – G222, Lane 11 – Sa641, Lane 12 – C882, Lane 13 - Negative. All lanes containing DNA gave the correct size band of 3834bp.

4.9 Minimum inhibitory concentration for streptomycin in dairy isolates of *L. monocytogenes*

As screening of the dairy isolates identified that *lmo1226* was present in environmental strains, it was decided that the strains should also be analysed for the minimum inhibitory concentration of streptomycin given that this gene was identified as being involved in resistance of the strain 10403s to streptomycin. The assay was performed as described in Section 2.4.3. The results are shown in Table 4.4. As previously observed (Section 4.5.1), strain 10403s showed a MIC of >500µg/ml. This was much higher than the MIC observed for each of the dairy strains which showed a reduced MIC of either 31.3µg/ml or 62.5µg/ml.

Strain	MIC Streptomycin
10403s	>500µg/ml
G222	31.3µg/ml
SA264	31.3µg/ml
C882	62.5µg/ml
SA11	31.3µg/ml
T58-2	62.5µg/ml
SA641	31.3µg/ml
T46A	31.3µg/ml
T8	31.3µg/ml
G8	62.5µg/ml
A9	62.5µg/ml

Table 4.4 - The minimum inhibitory concentration of streptomycin in *L. monocytogenes* 10403s and *L. monocytogenes* isolates from cheese dairies.

4.6 ΔImo1226-1 and ΔImo1226-2 adhesion and invasion of Caco-2 cells

To establish if *Imo1226* was also important for the attachment of this bacterium to biotic surfaces, an adhesion and invasion assay was carried out using Caco-2 cells. Cells were grown as a monolayer and allowed to differentiate in 24 well tissue culture plates to enable a close representation of the human intestine before the assay was performed. Caco-2 cells were co-incubated with each of the mutants and the wild type at a multiplicity of infection of 100 bacteria per cell as described in Section 2.4.4.2.Colony forming units were determined in the supernatant following lysis of the cells by Triton X-100. This number represented the total number of viable bacteria that adhered to and invaded the Caco-2 cells. Figure

4.14 shows the results of the first experiment. This experiment measured the total cell-associated bacteria i.e those attached to the surface of the cells as well as those that had been internalised, but not killed. The parent strain 10403s had an average of 2.72 $x10^7$ cell-associated bacteria per ml of cell lysate (approximately 57 bacteria per cell), which was comparable with the mutant strain ∆lmo1226-1 which has an average of 5.11 $x10^7$ associated bacteria per ml of cell lysate (approximately 106 bacteria per cell). On average the mutant ∆lmo1226-2 showed more than 1 log reduction in the number of cell associated bacteria in comparison to its isogenic parent with an average number of bacteria of $1.34x10^6$ per ml of cell lysate (approximately 3 bacteria per cell), however statistical analysis of these data using one way ANOVA (Graph pad Prism) shows that this difference between the strain 10403s and either of the mutants is not significantly different ($P>0.05$).

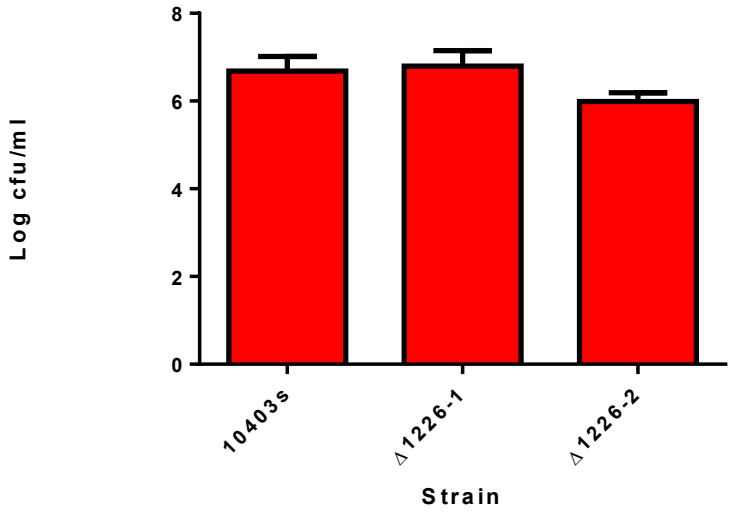

Figure 4.14 - Assay of cell-associated *L. monocytogenes* strain 10403s and mutant strains ΔImo1226-1 and ΔImo1226-2 following two hour incubation with a monolayer of Caco-2 cells. Counts are presented as log colony forming units per ml of lysate following lysis of Caco-2 cells.

To assess further the involvement of *Imo1226* in the virulence of *L. monocytogenes* the mutant strains were also analysed for their ability to invade Caco-2 cells using the method described in Section 2.4.4.2. To ensure any bacterial cells that had adhered to the cell surface but have not yet been internalised were removed, the Caco-2 cells, following incubation with the bacteria, were treated with 300ug/ml gentamicin. Cells were then lysed with Triton X-100 and bacterial cells enumerated. The strain 10403s showed an average cell count of $2.5x10^5$ colony forming units per ml of cell lysate, as shown in Figure 4.15 (on average approximately 1 bacterium per two cells), which is comparable with the average cell count of the mutant ΔImo1226-1, which had a cfu/ml of $2.33x10^5$ (on average

approximately 1 bacterium per 2 cells). The mutant ∆lmo1226-2 exhibited almost a log lower in numbers of bacteria internalised with an average of $4.34x10^4$ (on average approximately 1 bacterium per 100 cells). Statistical analysis of these data using ANOVA showed both mutants were significantly lower than that of parental strain 10403s ($P<0.05$ for ∆1226-1, $p<0.01$ for ∆1226-2).

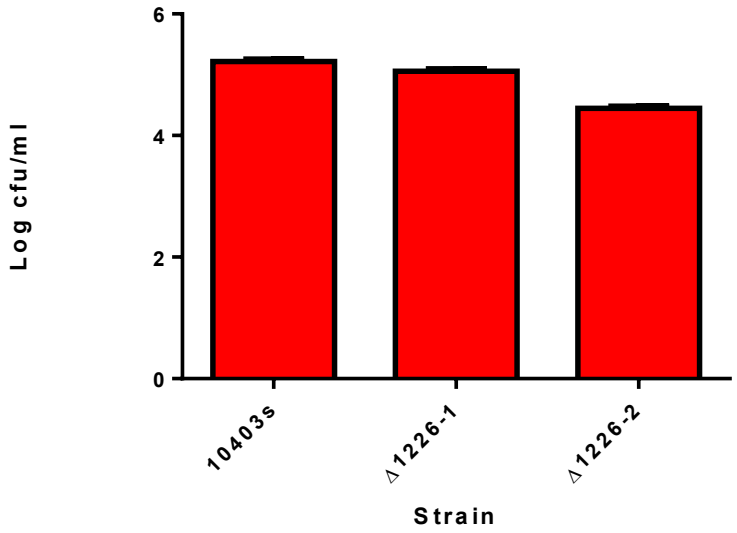

Figure 4.15 - Assay to measure invasion of *L. monocytogenes* parent strain in comparison to the two mutants Δlmo1226-1 and Δlmo1226-2. Counts are shown as log cfu/ml of cell lysate.

4.7.1 Construction of *L. monocytogenes* ΔImo1226-1 and ΔImo1226-2 mutants in a *L. monocytogenes* InlAm background

To allow intragastric infection of mice, the mutations made for the ΔImo1226-1 and ΔImo1226-2 mutants were created in a different background strain, namely the mutant strain InlAm. The InlAm mutant was created by Wollert *et al*. (2007) in an EGD-e background. This mutant contains two amino acids substitutions (S192N-Y369S) in the InlA protein, both of which are needed for adherence of *L. monocytogenes* to the E-cadherin receptor found on mouse intestinal epithelial cells. The wild type InlA protein of *L. monocytogenes* does not adhere to the mouse E-cadherin receptor but these amino acid substitutions modifies the protein so that it will adhere to the E-cadherin receptor.

The mutants were made in the *L. monocytogenes* strain InlAm as described in the method Section 2.2.14 using the plasmids pINF1226-1 and pINF1226-2 previously created in Section 4.2. Integration of the plasmids pINF1226-1 or pINF1226-2 into the chromosome was confirmed by colony PCR with the primers M113C or M113E respectively, paired with M13UR. Figure 4.16 shows the agarose gel electrophoresis to confirm integration of the plasmid pINF1226-1 into the *L. monocytogenes* InlAm chromosome. In lanes 2 and 3 of this figure a PCR amplicon of 1864bp can be seen, which confirms integration of the plasmid has taken place. Lanes 4 and 5 however show colonies where no integration had taken place, and therefore no band is present in the gel. A negative control reaction was

used during the screening (Lane 6) which used a colony from the non-transformed strain InlAm as a template. Figure 4.17 shows the gel electrophoresis of the PCR products from screening colonies for integration of the plasmid pINF1226-2 into the InlAm chromosome. Lanes 3, 4 and 5 on Figure 4.17 show a PCR fragment of 2312bp, confirming integration of the plasmid pINF1226-2 into the chromosome, Lane 2 on Figure 4.17 shows the negative control reaction again using an InlAm colony that has not been transformed.

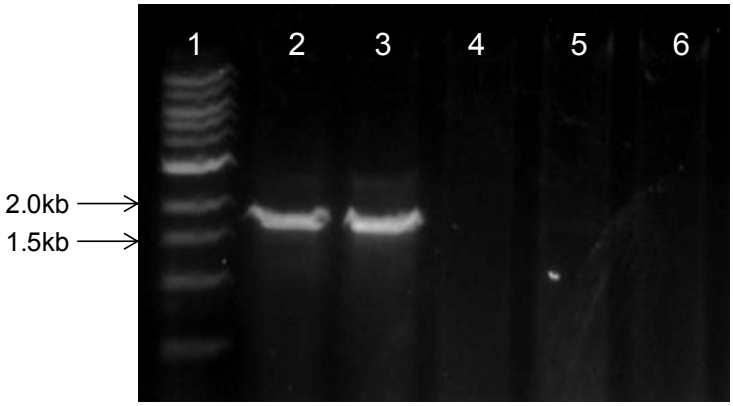

Figure 4.16 - Agarose gel electrophoresis of PCR for screening of integration of the plasmid pINF1226-1 into the InlAm chromosome. Lane 1 - 1kb Ladder, Lane 2 and 3 - Screened colonies giving a positive band of 1864bp confirming integration of the plasmid, Lanes 4 and 5 - Screened colonies that have not integrated the plasmid, Lane 6 - Negative control, using a InlAm colony which has not undergone recombination with the plasmid.

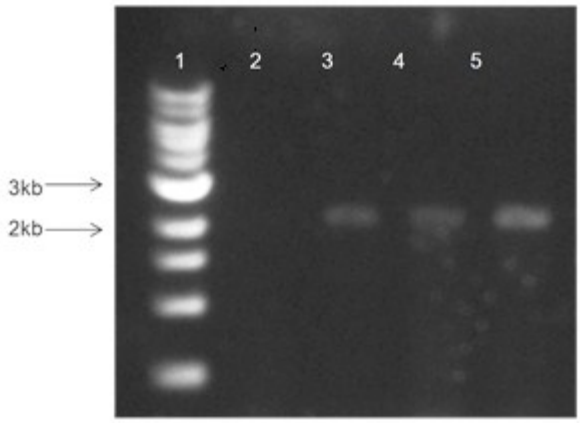

Figure 4.17 - Agarose gel electrophoresis of PCR screening of colonies for integration of the plasmid pINF1226-2 into the *L. monocytogenes* InlAm chromosome. Lane 1 - 1kb ladder, Lane 2 - negative control using colony of *L. monocytogenes* InlAm as a template, Lanes 3, 4 & 5 - Screened colonies confirming integration of the plasmid giving a band size of 2312 base pairs.

Colonies that showed a positive reaction for the integration of the plasmids into the chromosome underwent recombination as described in Section 2.2.14. Following recombination, erythromycin sensitive colonies were selected and screened by colony PCR using the primers M113C and M113D for the mutation. Deletion mutants gave a fragment size of 2417bp and 771bp for ∆lmo1226-1 and ∆lmo1226-2 respectively. Figure 4.18 shows the agarose gel electrophoresis of the PCR products with selected recombinants. The PCR product from the mutant Δlmo1226-2 can be seen in lane 3 of Figure 4.18, Lane 4 contains the PCR product for the mutant Δlmo1226-1, Lane 5 contains the positive control product using a InlAm colony that has not undergone the transformation and recombination reaction and Lane 2 shows the PCR product for the negative control.

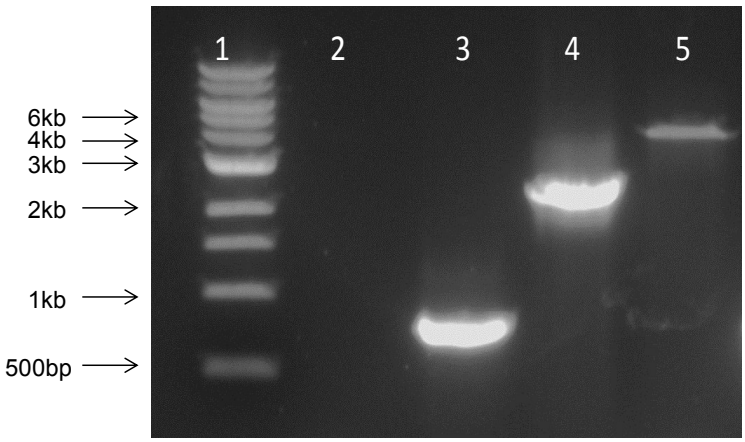

Figure 4.18 - Agarose gel electrophoresis showing the PCR products to confirm mutants Δlmo1226-1 and Δlmo1226-2 in *L. monocytogenes* InlAm. PCR was carried with the primers M113C and M113D. Lane 1 - 1kb ladder, Lane 2 - Negative control reaction, Lane 3 - Δlmo1226-2 mutant, giving a band size of 771bp, Lane 4 - Δlmo1226-1 mutant giving a band size of 2417 base pairs, Lane 5 - Positive control reaction using a InlAm colony

4.7.2 Survival of mice infected with *L. monocytogenes* InlAm∆1226-1 and InlAm∆1226-2 by oral gavage

In order to establish if *lmo226* is involved in virulence of *L. monocytogenes* in a mouse model of oral infection, mutants created in the InlAm background were used to infect mice by oral gavage, as described in methods Section 4.7.1. For this experiment ten MF1 mice were infected intragastrically with $5x10^9$ colony forming units in 0.5ml PBS containing 50mg calcium carbonate. Mice were observed at least twice a day for 10 days for signs of illness, and disease scores were recorded. Any mice reaching a 2+ lethargic state or surviving to the end of the experiment (240 hours) were culled.

Figure 4.19 shows the survival of mice orally infected with the three different strains of *L. monocytogenes*. The data are presented as the percentage survival of mice over time. Data presented for the mutants are for 10 mice, however the data for the mice infected with the background InlAm strain were analysed from nine mice, due to complications with one mouse during the dosing procedure (red line on Figure 4.19). From these data, it can be seen that only 44% of mice survived infection with the strain InlAm as represented by the red line. This survival rate was comparable with the group of mice infected with the mutant ∆1226-1, as represented by the blue line. Mice infected with this mutant showed a percentage survival of 40%. Comparison of the survival curve analysis using a Log-rank (Mantel-Cox) test showed no significant difference between the strains InlAm and ∆1226-1($P > 0.05$). Mice infected with the mutant ∆1226-2 (represented by the

green line) however had a much higher survival rate with 90% of mice alive at the end of the experiment (240 hours). Statistical analysis performed on this mutant in comparison with the background strain InlAm using a Log-rank (Mantel-Cox) test shows the mutant was significantly different from the parental strain InlAm ($P<0.05$).

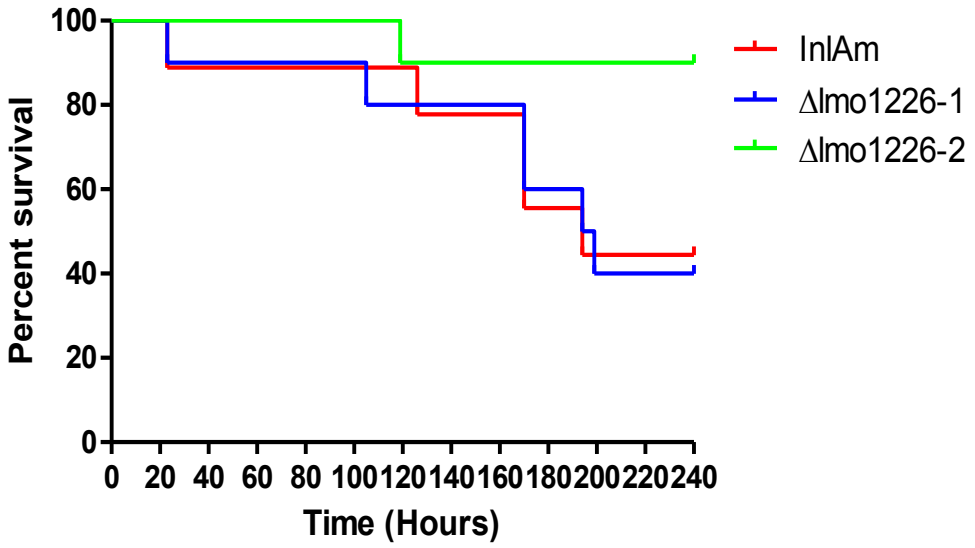

Figure 4.19 - Percentage survival of MF1 mice over the course of the 240 hour experiment following oral infection with $5x10^9$ cfu of either InlAm or the mutant strains InlAmΔlmo1226-1 and InlAmΔ1226-2.

To give an alternative representation of the course of infection, analysis of the disease signs of each mouse over the time course of the experiment was also performed. Mice were scored on three criteria, hunched, starey/piloerect or lethargic and scored as 1+ or 2+ depending on the severity of the disease signs.

Scores were assigned in accordance with the method in Section 2.4.5.2. Average scores were calculated for each group of mice, with scores being given for the most severe disease sign demonstrated. Average score for each group of mice was plotted against time, as shown in Figure 4.20. All groups of mice showed a very similar progression of disease signs for the first 150 hours ($P>0.05$), at which point the severity of the signs of disease of the groups of mice infected with InlAm and InlAmΔlmo1226-1 (represented by the red and the blue lines respectively) continued to increase whereas the signs of the group of mice infected with the mutant InlAmΔlmo1226-2 (represented by the green line) stabilised. Statistical analysis performed showed that there was a significant difference in sign scores between the wild type and the mutant strain InlAmΔlmo1226-2 from the time point 176 hours ($P<0.05$) to the time point 240 hours ($P<0.05$). There was no significant difference between sign scores of the wild type and the InlAmΔlmo1226-1 mutant.

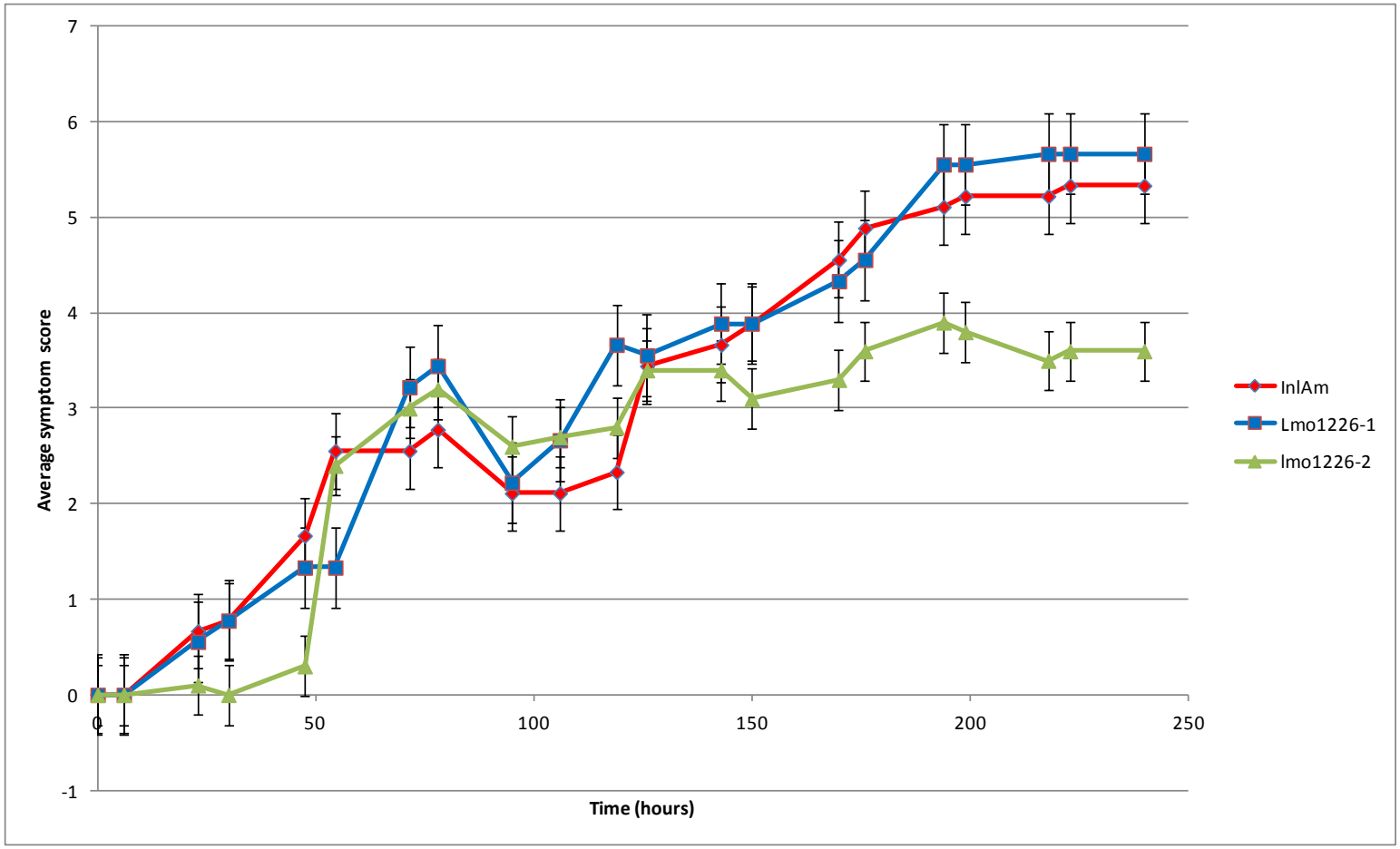

Figure 4.20 - Graph showing the progression of the average disease scores over the course of infection of MF1 mice infected intragastrically with $5x10^9$cfu/0.5ml *L. monocytogenes* strains InlAm, InlAmΔlmo12226-1 or InlAmΔlmo12226-2

4.8 Conclusions and Discussion

Chapter 3 identified the gene lmo1226 plays a putative role in attachment of L. *monocytogenes*. This chapter further characterised the role of the gene *lmo1226* in *L. monocytogenes* by first creating different deletion mutants to investigate which parts of the protein Lmo1226 was essential for attachment of *L. monocytogenes* to surfaces. The results from this chapter show that deletion of the entire gene *lmo1226* resulted in significant loss of attachment, whereas deletion of part of the gene did not result in a change in phenotype with regards to attachment. These mutants were also indetifiedto play a role in antibiotic tolerance, osmotolerance, adhesion and invasion of Caco-2 cells and in an in vivo murine model of infection. This discussion will start by discussing Lmo1226 in comparison to other drug efflux proteins of the RND superfamily, and then discuss each of the results of these assays.

4.8.1 Lmo1226 a putative drug efflux protein of the RND superfamily

As discussed in section 3 using arbitrary PCR, the location of the transposon in the mutant M113 was identified as having inserted into the gene *lmo1226* in *L. monocytogenes* based on sequence similarity of the sequence surrounding the transposon to the *L. monocytogenes* EGD-e sequence available on NCBI website. Annotation of this gene on the NCBI website identifies the sequence as encoding a putative drug efflux protein of the resistance, nodulation and division (RND) superfamily (Glasser 2000). In recent years understanding of drug efflux pumps and their role in antibiotic resistance has increased and efflux pumps of the RND superfamily have been shown to play an important role in antibiotic resistance and

solute transport in bacteria. Efflux pumps in this superfamily are found in a wide range of prokaryotic cells (Blair *et al*., 2009). These efflux pumps normally work as an antiporter, meaning that as a noxious substance, such as an antibiotic, is exported from the cell; protons are imported into the cell. Currently drug efflux pumps of this superfamily are not well characterised in Gram-positive bacteria, the YdfJ protein of *Bacillus subtilis* is the only identified RND drug export protein to date in Gram positive organisms (Serizawa *et al*. 2005). Until recently this superfamily was thought to be exclusive to Gram-negative bacteria. The *ydfJ* protein in B. subtilis has only been identified to be present in this organism, no work has yet been carried out to characterise this gene.

As drug efflux proteins of the RND superfamily have not yet been identified in Gram positive bacteria, comparisons have to be drawn to the information available about these proteins in Gram-negative bacteria. Several efflux pumps of this superfamily have already been well characterised in Gram-negative bacteria, these include the AcrB pump of *Escherichia coli* (Pos *et al*., 2009), the MexD pump of *Pseudomonas aeruginosa* (Mao *et al*., 2002) and the MtrD protein of *Neisseria gonorrhoeae* (Hagman *et al*., 1997). In Gram-negative bacteria these efflux pumps work as a tripartate system (see Figure 4.21), containing the efflux transporter protein located on the inner membrane, which expels molecules from inside the cell, an outer membrane channel to export any components exported to the periplasm to the exterior of the cell and a lipoprotein, which is thought to span the periplasm and co-ordinate efflux of the substrates (Elkins *et al*, 2003). The

formation of this tripartate system allows drugs to be exported directly outside the cell rather that accumulate in the periplasm. The MexD pump of *P. aeruginosa* is a typical example of these tripartate systems, being made up of three separate components, the efflux pump MexD which becomes associated with the outer membrane channel OmpR and a periplasmic lipoprotein, MexA. Another example is the AcrB pump system of *E. coli*, Figure 4.21 shows how the components of this system combine together to export antibiotics into the periplasm and outside the cell. As can be seen from the Figure the AcrB system is made up of the export pump, AcrB, the outer membrane channel, TolC, and the lipoprotein, AcrA. The protein Lmo1226 shows sequence similarity to the drug export pumps found on the inner membrane of *P. aeruginosa* and *E. coli*, the proteins MexB and AcrB respectively. As *L. monocytogenes* is a Gram-positive bacterium it could be assumed that there would be no need for the outer membrane channel, or the periplasmic pump in this bacterium as Gram-positive bacterium do not have an outer membrane. However from analysis of the *L. monocytogenes* genome available on the NCBI website it can be seen that genes encoding homologs to the *E. coli* outer membrane protein TolC, and the *E.coli* lipoprotein AcrA can be found in the *L. monocytogenes* strain10403s. Interestingly they are not present in any of the other sequence strains available on the NCBI website. The presence of these genes would suggest that they have other unknown functions within *L. monocytogenes*, at this time it is not possible to say if these proteins are working in conjunction with the efflux pump Lmo1226 in *L. monocytogenes*. Future work could involve making deletion mutants in these genes to try and assign a function.

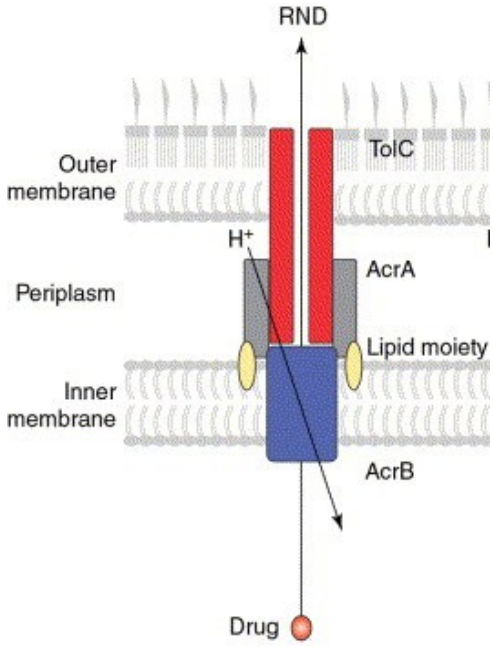

Figure 4.21 - The tripartate system of a drug efflux pump of the RND superfamily in *E. coli*. The TolC outer membrane protein exports substances from the periplasm to outside the cell. This protein is anchored by the lipoprotein AcrA to the drug efflux transporter AcrB on the inner membrane of the organism, which pumps drug outside the cell into the periplasm while importing protons. Picture adapted from McKeegan *et al*. (2002).

Proteins that are members of the RND superfamily seem to be similar in structure across all prokaryotic organisms. Proteins are approximately 1000 amino acids long and show the same structure of 12 transmembrane alpha helices, and two large extracytoplasmic domains between transmembrane helices 1 and 2 and helices 7 and 8. The structure of the *L. monocytogenes* protein Lmo1226 as predicted by the SPLIT 4 program (Figure 4.1) is consistent with the structures of other RND drug efflux proteins such as the MtrD protein of *Neisseria gonorrhoeae*,

as the protein Lmo1226 exhibits 12 transmembrane domains and the two extracellular loops as observed in other proteins.

One major difference which can be observed between Lmo1226 and proteins found in Gram-negative organisms, such as the AcrB protein of *E. coli*, is that AcrB has two periplasmic loops of approximately the same size (300 amino acids), whereas the two loop structures in Lmo1226 are of different sizes; with the first loop being 150 amino acids and the second loop having 540 amino acids. It has previously been hypothesised (McKeegan *et al*. 2003) that due to the similarity observed in the two halves of DNA sequence encoding these RND efflux proteins that the structure of these proteins has arisen from duplication of a single gene, which originally encoded a protein that contained 6 transmembrane helices, with a loop after the first transmembrane domain. 3D structural analysis of the AcrB protein has supported this hypothesis, as the two separate sets of 6 helices appear to arrange themselves as two distinct bundles. This hypothesis does not seem to be true for the protein Lmo1226 as one of the loops is considerably larger than the other. However it could be hypothesised that the protein may have originally been encoded by a duplicated gene as McKeegan suggested, with any difference observed now being due to naturally occurring mutations such as the insertion of transposable elements of DNA.

4.8.2 Creation of the deletion mutants Δlmo1226-1 and Δlmo1226-2

To further understand the role of *lmo1226* in the adhesion of *L. monocytogenes* to surfaces and to investigate any other phenotypes conferred by these gene

deletions, mutants were created in the *L. monocytogenes* strain 10403s. Section 4.2 describes the construction of two separate deletion mutants in this gene; one which deleted the entire gene from the *L. monocytogenes* chromosome and one which produced a truncated form of the protein, which encoded the first six transmembrane helices and the first extracellular loop. This study also attempt to create a deletion mutation in the other half of the gene *lmo1226*, which would mean the DNA would encode a protein containing the second 6 transmembrane helices and extracellular loop. Unfortunately the creation of this third mutant was not achieved. The ligation reaction of the insert DNA into the pAUL-A plasmid was confirmed using DNA sequencing, but the transformation of this plasmid into *E. coli* failed. It is possible that this was due to the region of DNA being cloned encoding for a gene product that was toxic for *E. coli*, effectively killing any cells that contained the plasmid which would explain the lack of colonies on the agar plate following the transformation. As discussed in the results section, this also could be due to the possibility that the protein contains a signal sequence which may have been removed during the mutation.To create this mutant in the future a different approach would have to be taken, one possible way to create this mutant is to transform the ligation reaction directly into *L. monocytogenes*. As this gene is cloned from the *L. monocytogenes* chromosome the same problems as observed when trying to transform the reaction into *E. coli* may not occur, however this will not solve the problem that the signal sequence may have been removed. Unfortunately time restrictions within this study prevented this method from being tested.

The creation of the two deletion mutants allowed the hypothesis that the gene *lmo1226* was involved in the attachment of *L. monocytogenes* to surfaces to be tested, and to identify whether one particular part of the gene was responsible. The following sections will discuss the phenotypic characterisation of these two mutants.

4.8.3 Characterisation of the deletion mutants Δlmo1226-1 and Δlmo1226-2

4.8.3.1 The role of Lmo1226 in adhesion to abiotic surfaces

Using the modified attachment assay described in Section 3.1 the transposon mutant M113 was identified to be deficient in attachment to plastic surfaces. The two deletion mutants created in *L. monocytogenes* 10403s were studied to test the hypothesis that the gene *lmo1226* was involved in attachment, and that the data seen with the transposon mutant were not a result of the transposon mutant having an effect on a gene downstream from *lmo1226*. Testing the two deletion mutants would also identify if the whole of the protein Lmo1226 was responsible for adhesion to surfaces or if one of these large loops of amino acids that protrude externally from the cell membrane was responsible. As hypothesised the mutant with the entire gene deletion (Δ1226-2) showed significantly lower attachment to polystyrene comparable to that with the transposon mutant (Section 4.3), which confirmed the hypothesis that the gene *lmo1226* is involved in attachment. This reduction in attachment was observed at both 37°C and 30°C. The mutant Δlmo1226-1 did not show significantly lower attachment than the wild type at either

of the temperatures tested. The mutant Δlmo1226-1 encodes a truncated form of the protein, containing the first 6 transmembrane helices and the first extracellular loop. Arbitrary PCR of the transposon mutant M113 identified the location of insertion of the transposon to be upstream of the 3' region of DNA which coded the amino acid just after the fourth transmembrane helix of the protein (indicated by the red arrow on Figure 4.1). From this sequencing result it was hypothesised that the insertion of the transposon would either alter the structure of the entire protein, or that the transposon may form a loop structure itself, possible plcking the upstream 150 amino acid extracellular loop, suggesting this region was responsible for attachment of *L. monocytogenes* 10403s to plastics. To confirm that the extracellular loop is the specific region responsible a mutant would need to be created that deleted the region of DNA which specifically encoded the first loop.

This study has identified a clear link between the protein Lmo1226, belonging to the RND superfamily, and attachment to surfaces, as the mutant Δlmo1226-2 showed significantly lower attachment to polystyrene than its isogenic parent. To date there is no explanation as to why drug efflux pumps of this superfamily are linked to attachment of bacteria to surfaces. Relatively few studies so far have been carried out to investigate this link; studies that have been carried out so far show an increase in expression of drug efflux pumps in bacteria during the formation of biofilm (Ito *et al*., 2009; May *et al*., 2009). Kvist *et al*. (2008) showed that the addition of efflux pump inhibitors such as thioridazine, 1-(1-naphthylmethyl)-piperazine or Phe-Arg β-naphthylamide (PA_N), reduced the

amount of biofilm formation in *E. coli* and *Klebsiella pneumoniae* when tested in a microtitre plate assay, they also demonstrated that a combination of efflux pump inhibitors could abolish bacterial biofilm formation completely. This study also demonstrated that the addition of efflux pump inhibitors increased antimicrobial effects of antibiotics against organisms within the biofilm. Matsumara *et al*. (2011) investigated the role of multidrug efflux proteins in biofilm formation in *E. coli*. To do this they looked at 22 mutants, each containing a mutation in a different gene, in *E. coli* that encoded proteins known to be involved in drug efflux, 10 of which express proteins which are members of the RND superfamily such as the genes *acrD* and *mdtE*. All mutants were tested for biofilm formation in a microtitre plate assay and all exhibited significantly less attachment than there isogenic parent strain, with the mutants in *acrD* and *mdtE* exhibiting two of the largest reductions in attachment. Matsumara *et al*. (2011) also looked at the differences in biofilm formation on both hydrophillic and hydrophobic plates in the wild type strain. They found that biofilm formation by *E. coli* was significantly higher on hydrophillic plates than hydrophobic plates. The work by Kvist *et al*. (2008) and Matsumara *et al.* (2011) support the hypothesis that drug efflux proteins are involved in attachment of bacteria to surfaces as observed in this study with *L. monocytogenes*.

As initial attachment of bacteria to surfaces is thought to be related to surface charge it can be hypothesised that the surface charge of the plate may be essential in determining how *L. monocytogenes* adheres to polystyrene. Surface charge has been previously shown to be important in attachment of bacteria to human tissue

(Heckels *et al*., 1976). This study already demonstrated that the region of Lmo1226 that is essential for attachment to surfaces is the region before the 4th transmembrane helix, as the deletion mutant Δlmo1226-2 showed a reduction in attachment. As the amino acids contained within the transmembrane helices are not exposed on the cell surface it can be hypothesised that the 150 amino acid extracellular loop located between the 1st and 2nd transmembrane helix could be responsible for attachment (Figure 4.1). This region of protein could either contain a specific amino acid sequence which allows it to bind to a surface or could carry a specific charge which enables it to bind to a surface which is oppositely charged.

To investigate the hypothesis that the extracellular loop could carry a specific charge, the resides of each of the two extracellular loops were examined for the net charge of the amino acids. Table 4.5 shows the result from this analysis; all charges were based on a pH of 7.4. As can be seen from the Table, loop 1 contains a higher number of charged and uncharged resides than loop 2, which contains a higher number of hydrophobic residues. The biggest difference that can be seen between the two loops is the higher percentage of negatively charged resides in loop 1 than loop 2. Polystyrene plates have a slight positive charge, and Gram positive bacteria have a slight negative charge due to the presence of the teichoic acids on the cell surface, so its possible that a slight disruption to that charge could alter the level of attachment. Adherence to stainless steel and glass could also be looked at as these carry a different surface charge, this will elucidate if deleting either loop alters the surface charge of the bacteria can affect its adherence to the plate.The two loops of Lmo1226 seem to have a higher level of

negatively charged amino acids than positive so deletion of these loops could disrupt the overall negative charge of the cell.

	Extracellular loop 1		Extracellular loop 2	
	Number	Percentage	Number	Percentage
Positively charged	20	13	46	9
Negatively charged	30	20	64	13
Uncharged	33	21	90	18
Hydrophobic	51	33	190	38

Table 4.5 - The amino acid composition of the two extracellular loops of the *L. monocytogenes* protein Lmo1226.

4.8.3.2 The role of *lmo1226* in adhesion to abiotic surfaces and in virulence

To establish if *lmo1226* was only involved in attachment to abiotic surfaces both of the mutants created were also tested for their ability to adhere to biotic surfaces, utilising an assay of adhesion and invasion to the human intestinal cell line, Caco-2. The mutant strains were also tested for virulence in an *in vivo* mouse model of infection.

As *L. monocytogenes* is not a natural pathogen to mice, to enable testing of the mutants in a murine model of listeriosis the mutants were created in the *L. monocytogenes* strain EGD-e InlAm (Wollert *et al*., 2007).The EGD-e InlAm strain was modified to make the strain infective to mice by the oral route by alteration of an amino acid in InlA of *L. monocytogenes*. This modification allows the internalin InlA to bind to the E-cadherin receptor found on mice epithelial cells, whereas the

wild type InlA would not normally bind to mouse E-cadherin. Mutants and the parent strain EGD-e InlAm were given orally as a dose of $5x10^9$ in 0.5ml PBS containing calcium carbonate, which was added to the dose to neutralise the acidity of the mouse stomach.

The results from the virulence testing of these mutants mirror the results of the attachment assay, with the mice infected with the mutant Δlmo1226-1 showing no difference in percentage survival to the mice infected with the parent strain. The mutant Δlmo1226-2 was significantly attenuated, with only one mouse dying through the course of infection. Analysis of the disease scores of the mice that survived the infection, showed that the mice that survived were still showing early signs of the infection, which persisted the entire time during the course of the study (240 hours). These data show that this gene is important in the virulence of *L. monocytogenes*, and that the region of this gene that is essential for virulence is the same region that is essential for adherence to polystyrene. As the same mutant is attenuated in the mice that showed significant reduced attachment to surfaces it is possible that this gene is also playing an important role in attachment of *L. monocytogenes* in the mice. The results of the disease score analysis support this as again the mutant shown to be deficient in attachment to surfaces showed less disease signs than the isogenic parent. This could also indicate that the bacteria are deficient in attachment to a particular tissue type such as the intestinal epithelial cells.

The hypothesis that Lmo1226 is required for attachment to tissue in mice is supported by work carried out in other organisms. An association has already been

made between drug efflux pumps and the virulence of certain species of bacteria. The *VexH* gene is one of six RND drug exporters found in *Vibrio cholera* (Taylor *et al*., 2012). Strains created to contain a deletion mutation in the *VexH* gene showed up to a 70% reduction in production of the cholera toxin in comparison to the parent strain. Mice infected with the deletion mutant also exhibited a much lower number of bacteria colonising the small intestine. This study supports the data presented in this thesis, as this study shows that RND drug exporter systems are involved in attachment and in virulence. The gene *lmo1226* could be involved in attachment of *L. monocytogenes* to intestinal epithelia.

To properly elucidate the role of this organism in the *in vivo* model further studies are required. Studies need to be carried out to look at bacterial load inside different tissue types at different type points of infection; this will give an indication as to the course of infection and the difference between mutant strains and their isogenic parent. As this gene is involved in attachment, it would be expected that the mutant strains exhibited a lower amount of bacteria associated with different types of tissue.

The infection data are supported by the data observed with the Caco-2 cell line. Each of the strains was tested for their ability to adhere to and invade the human intestinal epithelial cell line Caco-2. In this assay the whole gene deletion showing reduced attachment to the Caco-2 cells; with numbers of attached bacteria being almost a log lower than the parental strain. Despite the much lower numbers due to large variation between wells the difference between the two strains was not significant. The amount of attachment observed in the strain Δ1226-1 was again

comparable to that of the parent strain. A high degree of variance was seen in this study, consequently higher numbers of biological replicates are required in this assay to try and reduce the standard deviation between wells. Due to problems with growing the cells however this was not possible within the time constraints of this study. These data are in contradiction to the work by Taylor *et al*. (2012) in *V. cholerae*. The data from this experiment indicate that the gene *lmo1226* is not required for adhesion of *L. monocytogenes* to intestinal epithelial cells as there is no reduction in adherence in either mutant in this assay. Alternatively the gene *lmo1226* could be involved in adherence to another cell type such as brain cells, liver cells or macrophages. *L. monocytogenes* encounters a number of different cell types during the course of infection, as the disease scores from the *in vivo* study show that the signs of disease were comparable between all the strains for the first 150 hours this supports the hypothesis that the gene is not involved in adherence to intestinal epithelia. The involvement of *lmo1226* at a later stage of infection indicates this gene is more likely to be involved in adherence to organs infected during a systemic infection, such as the brain or liver, rather than the intestines which are colonised at the start of the infection.

The assay to measure invasion of the bacteria into the Caco-2 cells followed the same pattern, with the mutant Δlmo1226-1 showing no reduction in attachment and the mutant Δlmo1226-2 showing more than a log difference in amount of bacteria that had invaded the Caco-2 cells. Here, however, the difference between the whole gene deletion mutant and the parent strain was significant. From this assay it is not possible to identify if the reduction in numbers is due to less invasion by the

bacteria or due to the mutant bacteria not surviving within the Caco-2 cells. To clarify this, further experiments could include viable counting on the amount of *L. monocytogenes* inside the Caco-2 cells at different time points to give an indication of survival of the bacterium inside intestinal epithelial cells.

4.8.3.3 The involvement of *lmo1226* in antibiotic resistance

The understanding of the role of drug efflux proteins such as *lmo1226* is of increasing importance for development of future treatment of listeriosis. Currently the incidence of infection caused by drug resistant *L. monocytogenes* is on the increase (Lungu *et al*., 2011). An understanding of drug efflux proteins conferring resistance could lead to a massive increase in targets for potential new antimicrobial agents.

As the efflux pumps of the RND superfamily in Gram-negative bacteria have previously been shown to be involved in antibiotic resistance (Hagman *et al*., 2007; Blair & Piddock, 2009), both *lmo1226* deletion mutants created in the gene were tested for the MIC using a wide range of antibiotics. This experiment showed that the gene *lmo1226* is responsible for the streptomycin resistance in the clinical isolate 10403s, as indicated by a much higher MIC than the mutant strains. The strain 10403s is a streptomycin resistant derivative of the strain 10403. As both of the mutants exhibited the same reduction in MIC, it is clear that either the whole gene is required for streptomycin resistance in this strain, or that the 1st half of the gene is not essential.

Proteins belonging to the RND superfamily have long been recognised to be involved in drug resistance and in some instance multidrug resistance. Many of these proteins show broad substrate specificity, transporting many types of antibiotics, fatty acids, bile salts, detergent molecules and a range of noxious compounds such as ethidium bromide and crystal violet (Hagman *et al*., 1997; Putman, 2000). In contrast the gene *lmo1226* in *L. monocytogenes* has a smaller substrate specificity as it was only involved in resistance to one antibiotic, streptomycin, and not resistant to bile salts. Resistance to fatty acids, detergents, ethidium bromide or crystal violet were not tested in this study.

The data from this study are in direct contradiction to the studies into antibiotic resistance of the MtrD system of *Neisseria gonorrhoeae* (Hagman *et al*., 1997). This system confers resistance to a wide range of hydrophobic agents including two fatty acids (capric acid and palmitic acid), and a bile salt (cholic acid), but not hydrophillic agents such as streptomycin. Lmo1226 confers resistance to the hydrophillic antibiotic streptomycin in the *L. monocytogenes* strain 10403s, but not any hydrophobic agents tested, including the bile salt sodium chlorate hydrate, which is the opposite to that seen in *N. gonorrhoeae*. This difference may be explained by differences in helix structures of the protein. The alpha helices in the efflux pump will act together to form a pore through which drugs or other compounds would be transported in or out of the cell. The amino acids that make up these helices are normally hydrophobic; however they may contain charged

residues as well. As Lmo1226 seems to be involved in transport of hydrophilic molecules, this implies that the alpha helices form a predominately hydrophilic channel through which compounds can pass. This would be opposite to the hydrophobic channels you would expect to be formed in the MtrD protein of N. gonorrhoeae.

The efflux system MtrD is thought to be a key factor in the virulence of *N. gonorrhoeae* as this bacterium colonises mucosal sites which are covered in toxic fatty acids or bile salts, therefore MtrD allows organisms to colonise this environment. *L. monocytogenes* was also tested for resistance against the bile salt, sodium chlorate hydrate, but no decrease in resistance was found in the mutant strains. From this the conclusion can be drawn that *lmo1226* is not essential in bile resistance of *L. monocytogenes*. Comparative analysis of the amino acid sequence of the MtrD gene in N. gonorrhoeae and Lmo1226 in *L. monocytogenes* could be performed to try and explain the differences in substrate specificity of these two proteins.

Comparing the sequence Lmo1226 in the streptomycin resistant *L. monocytogenes* strain 10403s to that of the sequence of the streptomycin sensitive *L. monocytogenes* strain EGD-e, using BLASTP analysis, allows identification of changes in amino acid sequence that may account for the acquired antibiotic resistance of the strain 10403s. Direct comparison of the two sequences shows 99% homology, with only one amino acid difference. This amino acid is located at position 918 where there is an isoleucine residue in the strain 10403s, and a valine

residue found in *L. monocytogenes* streptomycin EGD-e. The substitution in this amino acid could account for the increased substrate specificity of the strain 10403s that leads to the strains resistance.

If the streptomycin resistance is indeed due to the change in the amino acid located at residue 918, this difference occurs in the 9th transmembrane helix. If this amino acid substitution is the cause of streptomycin resistance in *L. monocytogenes* 10403s, this is unusual in the case of drug exporters of this type, as substrate recognition appears to be determined by the two large loops found in these proteins. Elkins and Nikardo (2002) confirmed the importance of the loops in the protein by creating chimeras in the AcrB and AcrD pumps in *E. coli.* They did this by cloning the region of DNA encoding the periplasmic loops one and two of the pump AcrB into the DNA of the pump AcrD. In doing so they were able to identify substrate specificity of each of the periplasmic loops. Although in Gram-positive bacteria these loops do not span the periplasm and form the tripartate system with the lipoprotein and outer membrane protein as in Gram-negative organisms, these loops are still present.

The role of the large loops of amino acids in the RND drug efflux proteins has also been proven to be important for substrate recognition in *Pseudomonas aeruginosa*. The MexD system in *P. aeruginosa* has been studied to identify the region of DNA responsible for substrate specificity in the RND drug exporter protein (Mao *et al*., 2002). Mao *et al*. isolated spontaneous mutations that had occurred in *P. aeruginosa* which resulted in alterations in substrate specificity of the RND drug efflux proteins. The locations of these spontaneous mutants were located within

the large periplasmic loop, 4 within the first loop, and 2 in the second loop. All of these mutants led to an alteration in substrate specificity, in this case increasing the resistance to the β-Lactam antibiotic carbenicillin.

The studies by Mao *et al*. (2002) and Elkins and Nikaido (2003), which identified the large loops of amino acids present in this family of proteins as the region of the protein which confers substrate specificity, is contradictory to the hypothesis that the amino acid residue at location 918 is responsible, as this residue is in one of the transmembrane helices. To confirm the hypothesis that the change in amino acid residue 918 resulted in the acquisition of streptomycin resistance in the strain 10403s future experiments could use site directed mutagenesis to alter the 918th amino acid in the *L. monocytogenes* strain EGD-e to isoleucine from valine. If indeed this residue is responsible for the substrate specificity required for streptomycin resistance the mutagenesis of this amino acid would lead to a massive increase in streptomycin resistance in the *L. monocytogenes* strain EGD-e.

Based on the results of this experiment there is a possibility that streptomycin could be part of a possible future treatment regime in combination with some of the compounds that are known to be efflux pump inhibitors. The use of efflux pump inhibitors such as chlorpromazine and verapamil is of increasing interest these days as a potential treatment (Machado *et al*., 2012).

4.8.3.4 The presence of lmo1226 in dairy isolates

Strains isolated from artisanal cheese dairies across Portugal (Chambel *et al*., 2007) were screened by colony PCR for this gene to identify if *lmo1226* was present in these isolates, and therefore may play a role in these strains' survival within the food processing environment. The results from the PCR confirmed the presence of this gene in all strains tested and they all showed the same size PCR product. The dairy strains showed significantly lower MICs for streptomycin than 10403s, this result was expected as *L. monocytogenes* is not normally streptomycin resistant. However it is interesting to note than the MIC of the dairy isolates was higher than that of the deletion mutants created in this study. There are a number of possible explanations for this observation; (1) one that *lmo1226* is present in the dairy isolates but under-expressed or not expressed, which would account for the much lower MIC in the dairy strains compared to 10403s or (2) that this gene is present in environmental isolates as it has a second function or (3) single amino acid substitutions may exist that lower the resistance of the dairy isolates to strptomycin. As the MIC was higher in dairy isolates than the mutant strains it appears that this gene is expressed and playing a small role in streptomycin resistance in the dairy isolates. It is likely, however, that as this gene has been shown to be involved in attachment to abiotic surfaces in this study, that *lmo1226* is important in biofilm formation of these dairy isolates.

In future experiments to elucidate the role of this gene in antibiotic resistance in the dairy isolates the expression of mRNA in dairy isolates could be measured utilising

quantitative RTPCR in the dairy isolate strains. This will show if they exhibit a down regulation in expression of the gene lmo1226 in comparison to the strain 10403s. Also these genes could be amplified and sequenced to identify single nucleotide polymorphisms, which may have resulted in an amino acid substituion. However as this gene could also have secondary functions such as involvement in adhesion to surfaces or in involvement in sodium chloride transport, the hypothesis would be that gene expression is the same across all strains.

4.8.3.5 The role of Lmo1226 in adaptation to sodium chloride

From the data obtained in this study it is clear that the gene *lmo1226* plays an important role in survival and growth of bacteria in sodium chloride. Osmotolerance and growth in different concentrations of sodium chloride was assayed in both of the mutants created and their isogenic parent strain. The data from the growth experiments indicate that the gene is involved with growth of *L. monocytogenes* in medium containing 1.5% (w/v) and higher NaCl, as shown by the increased lag phase observed in both of the mutants in higher concentrations of NaCl. However once the bacteria adjust to the high percentage of NaCl the mutants grow as fast as the wild type, and sometimes to higher yields. These results indicate that *lmo1226* is essential for the adaptation of *L. monocytogenes* to NaCl. At this point it is not possible to explain why this gene has an involvement in adaptation to NaCl.

Based on these data and preliminary data on the osmotolerance of the transposon mutant M113 provided by Leanor Faleiro (Falerio, personnel communication) it was hypothesised that the gene *lmo1226* was involved in the osmotolerance of *L. monocytogenes*. An osmotolerance assay was carried out with both of the mutant strains but the data from this experiment were inconclusive. The experiment indicated that the gene was not important in osmotolerance at the 24 and 144 hour time point, as observed by the significantly lower percentage survival seen in the mutants which had not been pre-adapted to 3.5% (w/v) in comparison to the adapted mutants, however this pattern was not observed at all time points.

To further look at the involvement of this gene in osmotolerance the culture medium also needs to be taken into consideration. Growth experiments and osmotolerance assays carried out in this study were set up using bacteria that had been routinely cultured in TSB medium. Different culture medium contains different salt concentrations so to examine the osmotolerant response of *L. monocytogenes* further it would also be essential to try growing the bacterium in different culture media containing different salt concentrations prior to setting up the growth and osmotolerance assay.

Currently no conclusions can be drawn about the involvement of *lmo1226* in osmotolerance, or why the gene *lmo1226* seems to be important in growth in NaCl. However RND efflux transporters have been identified to be important with the import of Na+ ions into the cell, and it has been hypothesised that these transporters can work as an antiporter coupled with sodium ions rather than the

usual protons (Putman *et al*., 2000). Two studies have so far shown an association between NaCl and drug efflux transporters. A recent study on copper resistant halophiles isolated from a lake in Egypt (Osman *et al*., 2010) led to the discovery of a strain of *Halomonas spp.* that showed an increase in antibiotic resistance, copper resistance and an increased ability to grow in sodium chloride. Analysis of this organism identified a strain harbouring two plasmids, one of which encoded a drug exporter of the RND superfamily. Curing of these plasmids from the organism meant that the organism resulted in the loss of ability to grow in sodium chloride. The organism *Vibrio cholerae* has been shown to export ethidium bromide through a RND drug efflux system, VexEF-TolC(Vc) (Rahman *et* al., 2007).The ability of *V. cholerae* to export Ethidium bromide from the cell has been shown to require Na^+ to drive the antiporter motion of this pump, this shows the importance of RND drug exporters in import of sodium ions.

Chapter 5 - Construction and characterisation of mutants in the Bgl operon

To recapitulate, transposon mutations in *lmo0501* and *lmo0401* (mutants M237 and B380 respectively) were identified as having a lower level of attachment to abiotic surfaces at temperatures of 30°C and lower. These genes have been putatively identified as coding a BglG transcriptional antiterminator (*lmo0501*) and an α-mannosidase (*lmo0401*). Analysis of the *L. monocytogenes* genome has shown the α-mannosidase gene is upstream from a second BglG transcriptional antiterminator (*lmo0402*). It was hypothesised that the insertion of the transposon into the gene *lmo0401* had a polar effect on the downstream gene *lmo0402*. This chapter describes the creation of mutants in *lmo0401*, *lmo0402* and *lmo0501* and the characterisation of phenotypes displayed by each of these mutants.

5.1 *In silico* analysis of the *bglG* operon

Analysis of the *L. monocytogenes* chromosome using the NCBI website identified 15 putative BglG transcriptional antiterminators, which all appear to be located either up or downstream from the genes transcribing the components for the phosphoenolpyruvate dependent phosphotransferase system (PEP-PTS). As demonstrated in Figure 5.1a, *lmo0401*, encoding an α-mannosidase, and *lmo0402*, encoding a BglG transcriptional antiterminator, lie downstream from the three genes encoding the components of a PEP-PTS (*lmo0398*-*lmo0400*). This system is predicted to be involved in transport of mannose (Stoll & Goebel, 2010). Along with the α-mannosidase gene contained within this operon are genes for a phosphoenolpyruvate phosphotransferase (PEP-PTS) IIA component (*lmo0398*), a

PEP-PTS IIB component (*lmo0399*), and a PEP-PTS IIC component (*lmo0400*). Upstream from this is also a hypothetical protein (*lmo0397*), which may be in the same operon. In contrast the transcriptional antiterminator *lmo0501* is placed upstream of its putative associated PEP-PTS genes (Figure 5.1b), which is the opposite orientation of that observed for the operon containing *lmo0402*. This PEP-PTS system is thought to be involved in galactitol transport. Downstream from this operon are the genes encoding a putative sugar isomerase (*lmo0502*), a PEP-PTS EIIA component (*lmo0503*), an unknown protein (*lmo0504*), a ribulose-5-phosphate 3-epimerase (*lmo0505*), a polyol (sorbitol) dehydrogenase (*lmo0506*), a PEP-PTS EIIB component (*lmo0507*) and a PEP-PTS EIIC component (*lmo0508*).

BglG transcriptional antiterminators work at the hairpin loop that forms to stop transcription by binding to the hairpin and preventing termination of transcription (Raveh *et al*., 2009). The BglG operons were both analysed for the location of transcriptional terminators on which the BglG protein could be acting. The database TranstermHP was used to locate terminators (Kingsford *et al*, 2007) within the *L. monocytogenes* EGD-e chromosome. Terminators were identified that gave a hairpin-like structure with a confidence score of ≥30. Figure 5.1a also shows the hairpin structures that were identified using TranstermHP within the *lmo0402* operon. Terminator sequences were identified following *lmo0397*, *lmo0400, lmo0401*, and *lmo0402*. No transcriptional terminator appears to be between the genes *lmo0398 – lmo0400*. Using the same program, terminator sequences were also identified within the *lmo0501*-associated operon, Figure 5.1b

shows the location of these terminator sequences. Within the sequence of the *lmo0501* operon terminators were located after the genes *lmo0501*, *lmo0505*, and *lmo0508*.

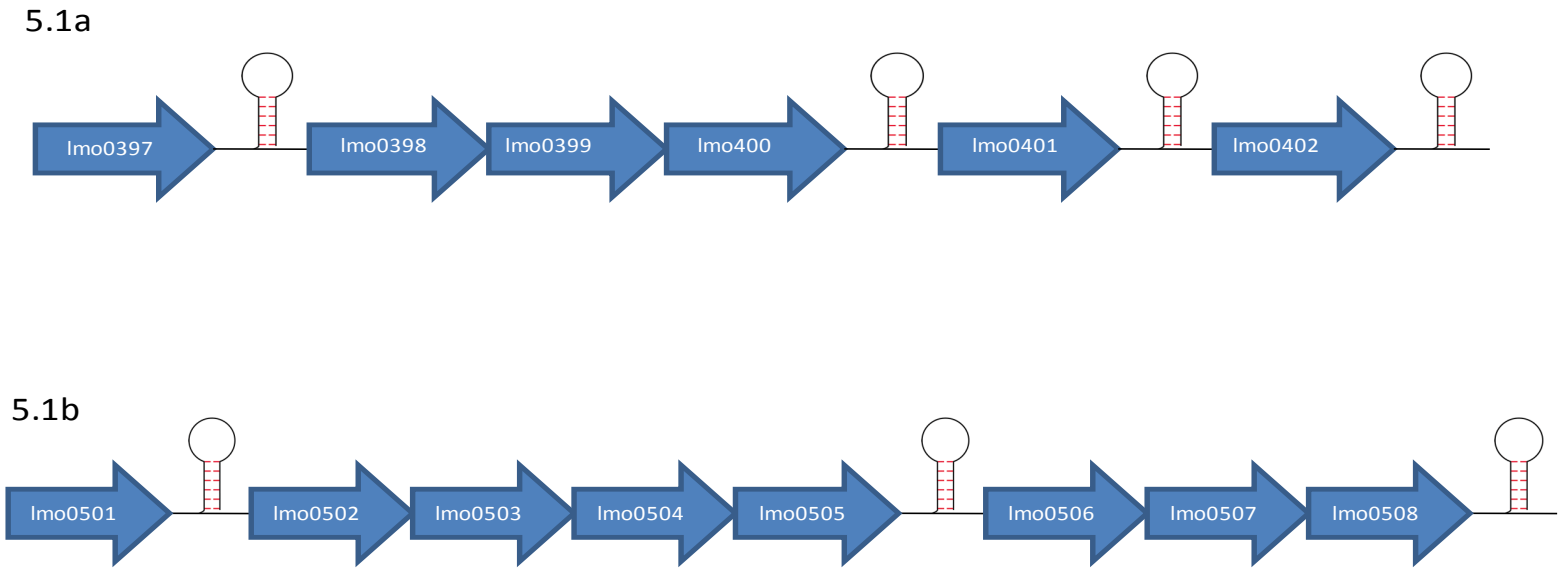

Figure 5.1 - Diagram identifying the location of the two BglG transcriptional antiterminators within the *L. monocytogenes* EGD-e genome and the genes coding the associated PEP-PTS. Figure a shows the location of the antiterminator *lmo0402* situated downstream from the PEP-PTS (genes *lmo0397-lmo0400*) and the location of the hairpin like transcriptional terminators located after *lmo0397*, *lmo0400*, *lmo0401* and *lmo0402*. Figure b shows the location of the antiterminator *lmo0501* situated upstream of the associated PEP-PTS (genes *lmo0503*, *lmo0507* and *lmo0508*) and the location of the hairpin like transcriptional terminators located after *lmo0501*, *lmo0505* and *lmo0508*.

5.2 Construction of the mutants ΔImo0401, ΔImo0402 and ΔImo0501

Mutants were constructed in a similar way as the mutants made in *Imo1226* (Section 4.2). Regions of DNA were amplified from the 3' and 5' end of the gene using primers with complimentary ends to each other, and the *Bam*H1 restriction site of the pAUL-A plasmid, which when cloned into the plasmid would give a truncated form of the protein (see Figure 5.2). The pAUL-A plasmid was used as the vector for the construction of these mutants and was digested with *Bam*H1 as previously described in section Section 2.2.9.

The plasmid pINF401 was constructed by amplification of two individual fragments with overlapping ends as described in Section 2.2.10. These were ligated into the *BamH1* digested pAUL-A plasmid. Colony PCR was carried out on *L. monocytogenes* 10403s as described in Section 2.2.7 to amplify the regions of interest. The first fragment was amplified using the primers 401_INF Fw1 & 401_INF Rv1 (Figure 5.2A), which amplified a 557bp fragment starting 184bp downstream from the 5’ end of the gene. The second fragment was amplified using the primers 401_INF Fw2 & 401_INF Rv2 (Figure 5.2A). These primers amplified a 671 bp region at the 3’ end of *Imo0401*, starting 374bp from the end of the gene. Agarose gel electrophoresis was performed on both of these PCR products to confirm the presence of fragments of correct sizes. Figure 5.3 shows the results of this agarose gel electrophoresis. As can be seen from this figure, the two fragments have amplified correctly giving the 557bp band for Fragment 1 (Figure 5.3, lane 2), and the 671bp band of fragment 2 (Figure 5.3, Lane 4). Following confirmation of the two fragments, PCR products were purified and cloned into the

pre-digested pAUL-A plasmid using an infusion HD cloning kit as described in Section 2.2.10. The combined fragments when inserted into the plasmid were 1228bp. This plasmid was used for transformation into *E. coli,* and termed pINF401.

The plasmid pINF402 was constructed in a similar manner (Figure 5.2B). Both fragments of *L. monocytogenes* were amplified using colony PCR with *L. monocytogenes* 10403s, as described in Section 2.2.8. As demonstrated in Figure 5.2B fragment 1 was amplified using the primer pair 402_INF Fw1 and 402_INF Rv1 which gave a fragment size of 371bp, which started 29bp downstream from the 5' end of *lmo0402*. The second region of DNA amplified for the creation of pINF402 was amplified using the primer pair 402_INF Fw2 and 402_INF Rv2. These primers amplified a 727bp region at the 3' end of the gene, 165bp from the stop codon. Agarose gel electrophoresis was performed on these PCR products to confirm size presence of the DNA fragments. Figure 5.4 shows the result of the agarose gel electrophoresis. Lane 2 shows the 371bp bands size for fragment 1 and lane 5 of the gel electrophoresis shows the 727bp PCR product of fragment 2. Following gel electrophoresis, the PCR products were purified using the Promega wizard S/V gel extraction kit. Purified fragments were ligated into the *Bam*H1 digested pAUL-A plasmid, giving a product of 1098bp long. The ligated plasmid was termed pINF402.

Finally the plasmid pINF501 was constructed by the same method. The first fragment was amplified using primers 501_INF Fw1 and 501_INF Rv1. This primer pair amplified a 457bp region of DNA which started 8bp from the 5' end of the gene. The primer pair 501_INF Fw2 and 501_INF Rv2 was then used to amplify a 625bp region of DNA starting 195 base pairs from the 3' end of the gene (Figure 5.2C). Agarose gel electrophoresis was performed on both of the PCR fragments to confirm the correct size of fragments were present. Figure 5.5 shows the gel. Lane 2 of the Figure contains the amplified fragment 1 and lane 4 of the figure shows the amplified fragment 2. PCR products were purified and ligated into pAUL-A as for the plasmids pINF401 and pINF402. The total size of the insert was 1082bp, and the resulting plasmid was termed pINF501.

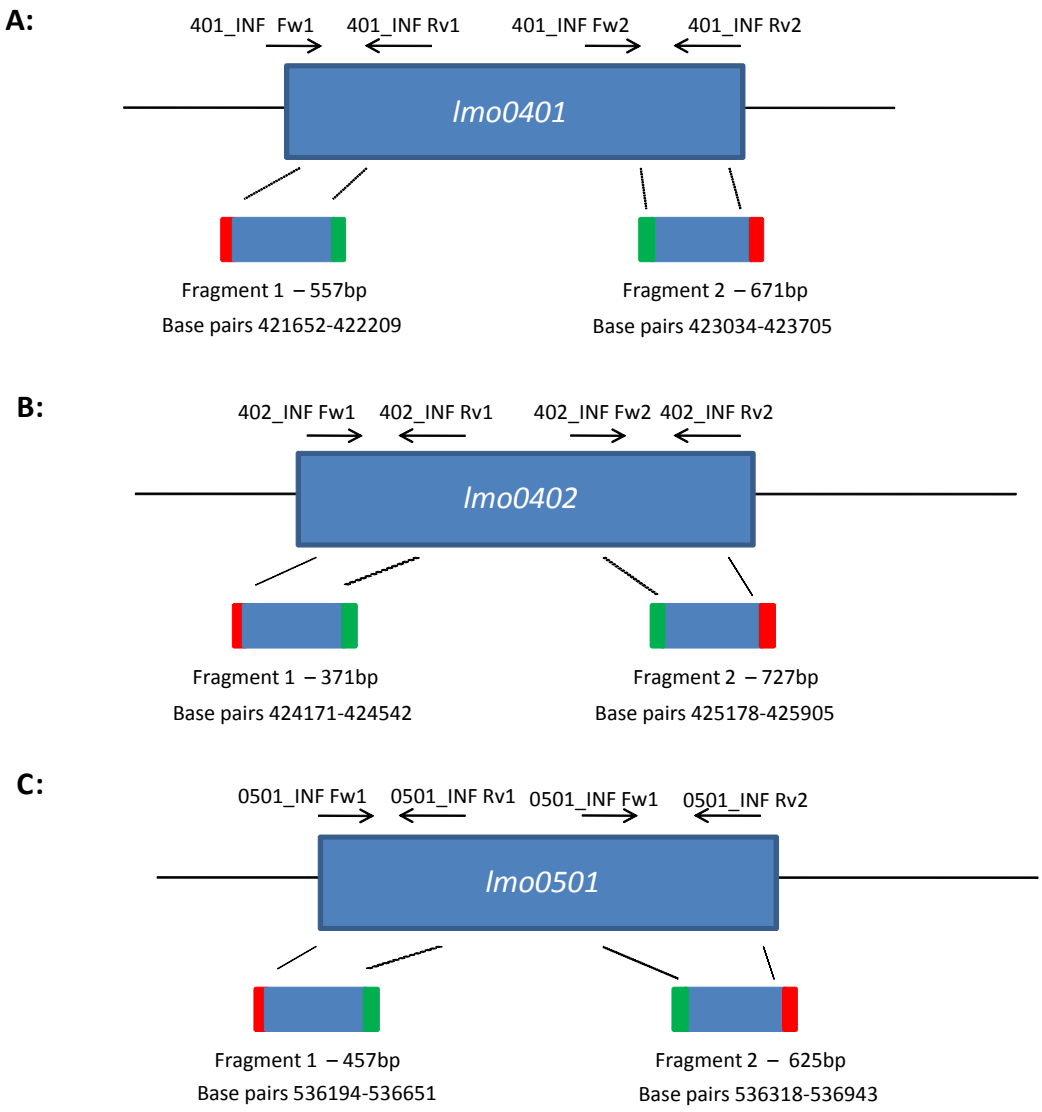

Figure 5.2 - Diagram showing the regions amplified from *lmo0401* (A), *lmo0402* (B) and lmo0501 (C) of *L. monocytogenes*. In each case two fragments were amplified which could be ligated together into the plasmid pAUL-A, creating the plasmids pINF401 pINF402 and pINF501, respectively. The regions of the fragment coloured red indicate the portion of the fragment which overlaps with the *Bam*H1 restriction site in the digested pAUL-A, regions coloured green indicated the portion of the two fragments with complementary sequences.

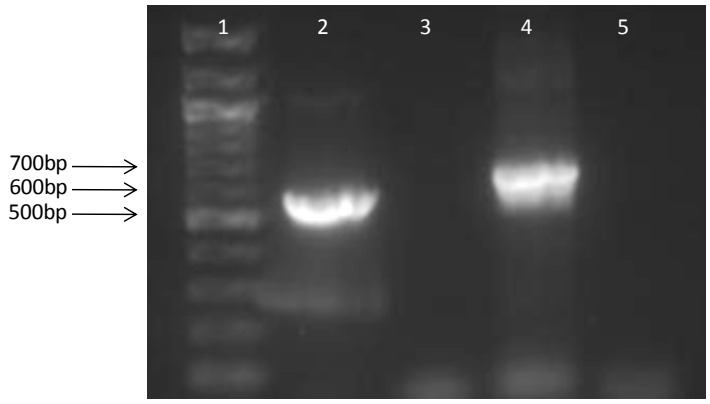

Figure 5.3 - A 1.5% agarose gel electrophoresis of PCR products of the two regions of *L. monocytogenes* DNA for construction of the plasmid pINF401. Lane 1 – 500bp ladder, Lane 2 – 557 bp Fragment 1, Lane 4 – 671 bp Fragment 2, Lanes 3 & 5 – Negative controls for reactions using water for template. Gel was ran in TAE buffer at 100 volts.

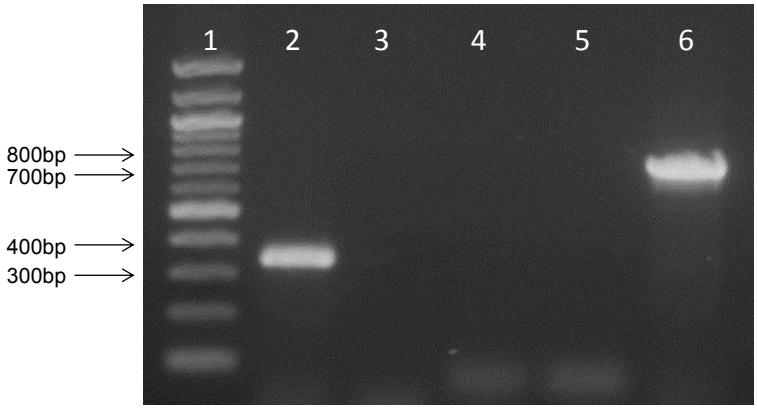

Figure 5.4 - A 1.5 % agarose gel electrophoresis of PCR products of the two regions of *L. monocytogenes* DNA for the construction of the plasmid pINF402. Lane 1 - 500bp ladder, Lane 2 - 371bp Fragment 1, Lane 6 - 727bp Fragment 2, Lanes 3, 4 & 5 - Negative control reactions. Gel was rran in TAE buffer at 100 volts.

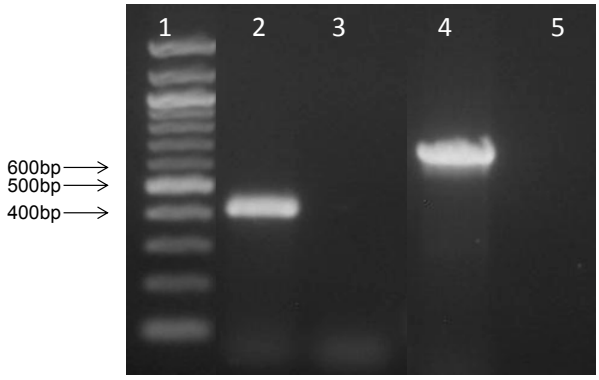

Figure 5.5 - A 1.5% agarose gel electrophoresis of PCR products of the two regions of *L. monocytogenes* DNA for the construction of the plasmid pINF501. Lane 1 - 500bp ladder, Lane 2 - 457bp Fragment 1, Lane 4 - 625bp Fragment 2, Lanes 3, & 5 - Negative control reactions. Gel was ran in TAE buffer at 100 volts.

5.2.2 Transformation of *E. coli* and *L. monocytogenes* with plasmids pINF401, pINF402 and pINF501

The plasmids pINF401, pINF402, and pINF501 created in Section 5.2.1 were transformed into XL-10 Gold competent *E. coli* cells using the method described in Section 2.2.11. Following transformation cells were plated onto Luria agar containing IPTG/Xgal/Erythromycin and incubated overnight. Following incubation of cells white colonies were selected to be screened to confirm the insert was correct. Transformation reactions with each of the plasmids gave a high number of white colonies following incubation of plates. White colonies were picked and

inoculated into Luria broth containing 300µg/ml erythromycin and incubated overnight for use for plasmid extraction.

Plasmid extractions were performed on the overnight incubations of the selected transformants as described in Section 2.2.9. To confirm the extracted plasmid contained the correct insert, plasmids underwent PCR using the primers M13 universal forward and M13 universal reverse following the standard PCR protocol, using plasmid DNA as the template for the PCR reaction as described in Section 2.2.7. Resulting PCR products were purified and sequenced by PNACL, and sequences analysed using BLAST to confirm the presence and fidelity of the insert DNA. The sequence for the plasmids pINF501 and pINF401 contained a mistake however, and as a result the transformed DNA sequence was no longer in-frame and introduced a stop codon earlier in the gene. Despite this, it was decided to continue with the creation of the deletion mutants.

Following confirmation of the insert, plasmids were transformed into *L. monocytogenes* 10403s by electroporation as described in Section 2.2.13. Electroporated cells were allowed to recover in SOC medium at 30°C for three hours; following incubation transformed cells were plated onto BHI agar containing erythromycin and incubated for 48 hours at 30°C. Cells that grew by 48 hours were assumed to contain the plasmid and were selected for the recombination stage. For all three plasmids that were electroporated, a large number of colonies were observed on the plates following incubation of the recovered cells.

5.2.3 - Recombination of the plasmids pINF401, pINF402, and pINF501 into the *L. monocytogenes* 10403s chromosome

Recombination was carried out as for the creation on the deletion mutants in lmo1226 following the method in Section 2.2.14. The first stage of the recombination was the integration of the plasmids pINF401, pINF402 and pINF501 into the *L. monocytogenes* chromosome. Colonies from step 5.2.2 were subcultured onto BHI agar containing erythromycin and incubated at 42°C. Following overnight incubation this process was repeated for a second overnight culture to increase the number of recombinants with the plasmid inserted into the chromosome.

Colonies that grew after being subcultured twice on BHI agar at 42°C were screened for integration of the plasmids pINF401, pINF402 and pINF501 into the *L. monocytogenes* chromosome by colony PCR. The PCR used an upstream primer which bound to the *L. monocytogenes* chromosome, and the M13 universal reverse primer, which bound to just outside the multiple cloning site in the vector pAUL-A. The primer pairs used for the screening of integration of each plasmid into the chromosome and the appropriate band size can be seen in Table 5.1. If integration had successfully taken place the relevant size band would be present, whereas a lack of band in the PCR reaction indicated that integration had not occurred, and a band of a different size would indicate that the plasmid had integrated into the wrong site in the chromosome. The size of the predicted band was calculated by adding the size of the insert to the size of the region of DNA from where the forward primer annealed to the start of the first fragment of the

insert plus 60 bases for the distance from the *Bam*H1 restriction site in the pAUL-A plasmid to the M13 universal primer.

Plasmid	Forward primer	Reverse primer	Expected band size
pINF401	401A	M13UR	1868bp
pINF402	402A	M13UR	1288bp
pINF501	501A	M13UR	1218bp

Table 5.1 - Details of primers used for the screening of the integration of the plasmids into the *L. monocytogenes* chromosome, and the expected band size.

Following PCR, products were analysed by agarose gel electrophoresis to confirm that integration had taken place. Colonies which gave the correct sized band on the gel after colony PCR were picked to continue the recombination. Figure 5.6 shows the gel electrophoresis of the colonies screened for integration of pINF401 into the chromosome, with each lane of the gel representing a PCR reaction containing an individual colony as the template. Only one colony gave a positive reaction which can be seen in lane 2 of the gel, with a band size of 1868bp. The remaining bands on the gel gave a negative result, with no bands being present when the PCR product was analysed by agarose gel electrophoresis. The absence of bands in these reactions indicates insertion of the plasmid into the chromosome has not occurred in these colonies. The colony in lane 2 of the gel was selected to continue with the recombination process.

Figure 5.7 shows the gel electrophoresis for integration of the plasmid pINF402 into the *L. monocytogenes* chromosome with each lane representing an individual colony providing the template DNA for each reaction. From this Figure it can be seen that the colonies in lanes 3, 4, 7 & 8 showed a positive band of approximately 1288bp, indicating that integration has taken place. These PCR products also showed a smaller (less than 500bp) band probably due to primer dimer formation in the primers. The other colonies gave bands on the electrophoresis, but these were not the correct size. The colony in lane 4 was selected for further recombination.

Figure 5.8 shows the gel electrophoresis for the screening for the integration of the plasmid pINF501 into the *L. monocytogenes* chromosome. Four of the colonies screened for integration of the plasmid into the chromosome gave a band size of 1218 bp. The colony used for the PCR reaction in lane 2 was used to continue the recombination.

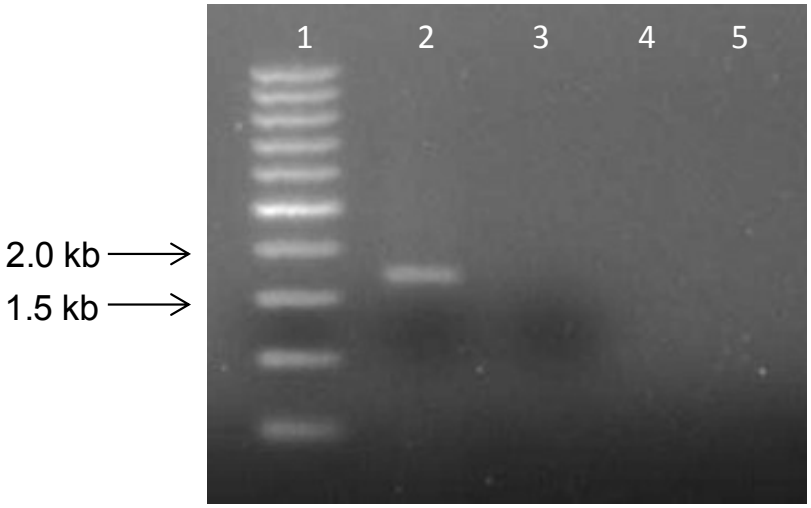

Figure 5.6 - A 1% agarose gel electrophoresis of the PCR products from screening of the integration of the plasmid pINF401 into the *L. monocytogenes* 10403s chromosome. Lane 1 indicates the 1kb ladder, and lanes 2-5 each represent an individual colony. The expected band size was 1868bp. The gel was run in TAE buffer at 100 volts.

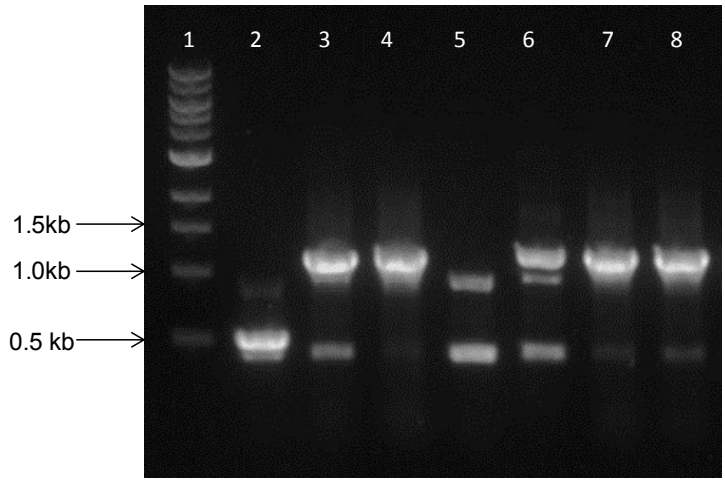

Figure 5.7 - A 1% agarose gel electrophoresis of the PCR products from screening of the integration of the plasmid pINF402 into the *L. monocytogenes* 10403s chromosome. Lane 1 indicates the 1kb ladder, and lanes 2-8 each represent an individual colony. Lanes 3, 4 7 & 8 all show the correct band size for integration of the plasmid into the chromosome (1288bp). The gel was run in TAE buffer at 100 volts.

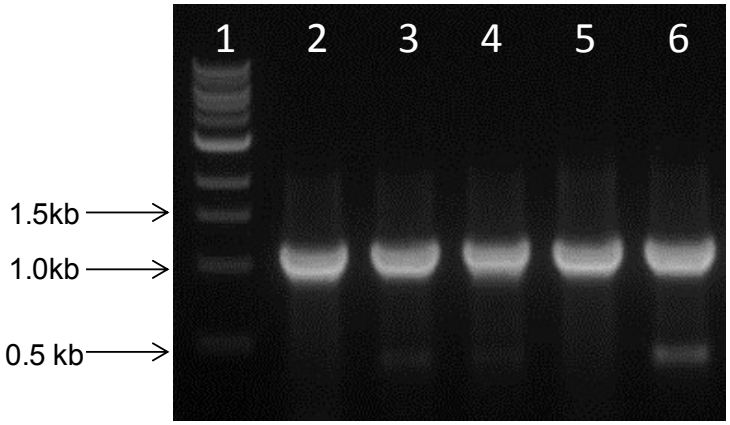

Figure 5.8 - A 1% agarose gel electrophoresis of the PCR products from screening of the integration of the plasmid pINF501 into the *L. monocytogenes* 10403s chromosome. Lane 1 indicates the 1kb ladder, and lanes 2-6 each represent an individual colony. All colonies show the correct band size for integration of the plasmid into the chromosome (1218bp). The gel was run at 100 volts in TAE buffer.

The colonies selected following successful integration of the plasmids pINF401, pINF402 and pINF501 into the *L. monocytogenes* chromosome were used for the continuation of the process of recombination as discussed in Section 2.2.14. Following the process of recombination cells were replica-plated onto BHI agar, with and without erythromycin to allow selection for the loss of plasmid from the cell. Any erythromycin sensitive cells were selected for screening for the occurrence of the desired mutations. Sensitive colonies were screened using colony PCR with the primers 401A and 401B, 402A and 402B or 501A and 502B for the corresponding plasmids pINF401, pINF402 and pINF501. PCR products were analysed by agarose gel electrophoresis. Figure 5.9 shows the gel with the PCR products for the screening for the deletion mutant Δlmo0401. Four colonies,

out of approximately 100 that were replica-plated, were observed to be erythromycin sensitive. Out of these colonies two showed the expected band size for the mutant at 2708bp. Lane 6 of Figure 5.9 shows the PCR products from the parent strain 10403s, with a band size of 3513bp. Lanes 4 and 5 also have bands of the same size as the parent, showing that the bacteria have reverted to the wild type.

Figure 5.10 shows the agarose gel electrophoresis of the PCR screening of the colonies screened for the mutant Δlmo0402. In this instance eight colonies were screened (Lanes 2-7, 9&10), but only one was positive for the mutation (lane 2), showing the truncated size of 1596bp. The other colonies had reverted to the size of the parent strain (2224bp).

Figure 5.11 shows the gel electrophoresis for the PCR screening for the mutant Δlmo0501. In this instance eight colonies were screened (lanes 2-9), only one showed the correct size band for the mutant of 1550bp (lane 9). The remaining colonies had reverted to wild type with a band size of 2296 base pairs.

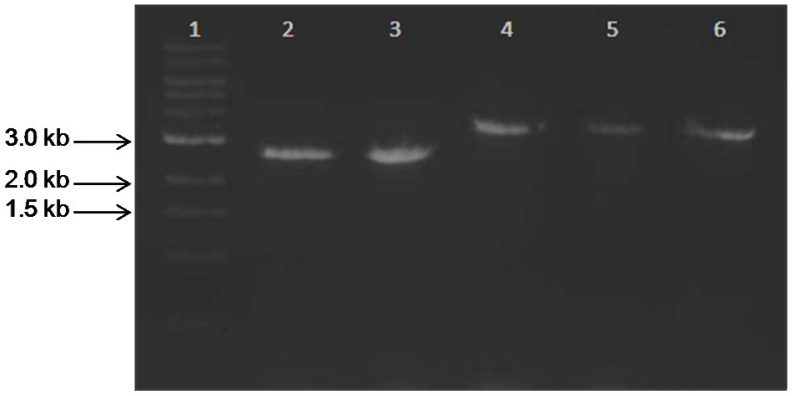

Figure 5.9 - A 1% agarose gel electrophoresis for screening of the PCR products for the Δlmo0401 mutant. Lane 1 - 1kb ladder, lanes 2-5 each contain PCR product using DNA from an individual colony as a template. Lane 6 - 10403s. The expected band size was 2708bp for mutants and 3513bp for the wild type strain. THe gel was ran in TAE buffer at 100 volts.

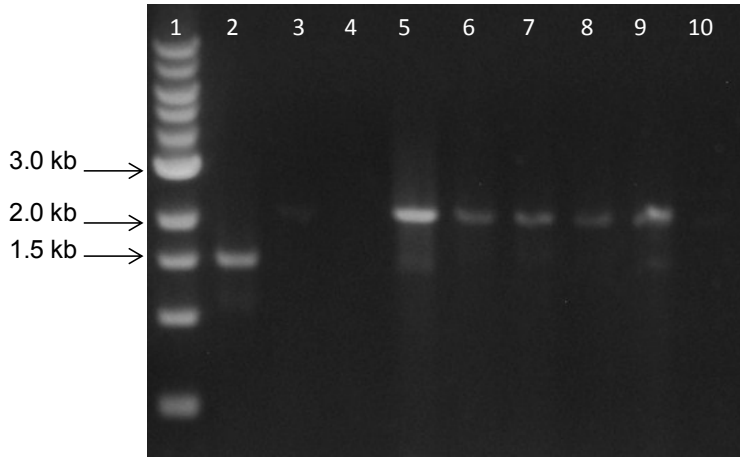

Figure 5.10 - A 1% agarose gel electrophoresis for screening of the PCR products for the Δlmo0402 mutant. Lane 1 - 1kb ladder, lanes 2-7, and 9-10 each contain PCR product using DNA from an individual colony as a template. Lane 8 - 10403s. The mutant strains show a band size of 1596bp and the wild type strain show a band size of 2224bp. The gel was ran at 10 volts in TAE buffer.

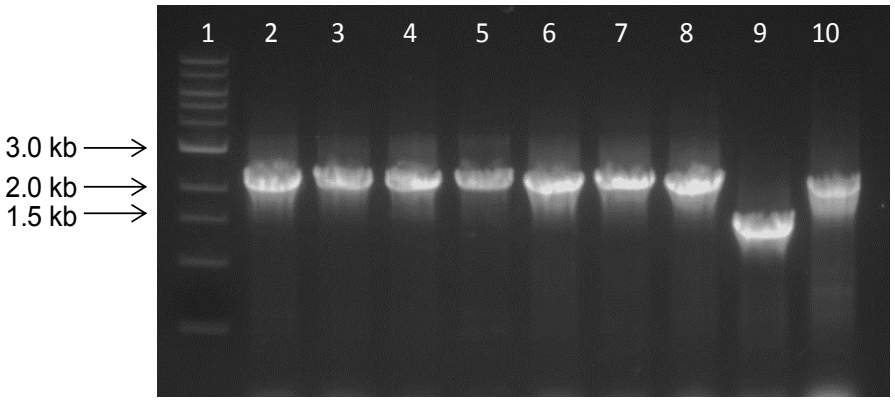

Figure 5.11 - A 1% agarose gel electrophoresis for screening of the PCR products for the Δlmo0501 mutant. Lane 1 - 1kb ladder, lanes 2-9 each contain PCR product using DNA from an individual colony as a template. Lane 10 - 10403s. The expected band sizes for the mutant was 1550bp, and 2296bp for the wild type. The gel was run in TAE buffer at 100 volts.

5.3 - Attachment of the strains Δlmo0401, Δlmo0402 and Δlmo0501 to plastics

The deletion mutants, Δlmo0401, Δlmo0402 and Δlmo0501, were tested for their ability to attach to plastic surfaces, using the method described in Section 2.2.4. It was hypothesised that the mutants Δlmo0401 and Δlmo0501 would show temperature-dependant behaviour, with reduction in attachment only being observed at 30°C and less. This was due to the observed results with the respective transposon mutants B380 and M237 (Section 3.3).

Figure 5.12a shows the results of the attachment assay performed at 37°C. As hypothesised, there was no significant difference between the mutant strains and their isogenic parent in the levels of attachment when tested at this temperature, as shown by similar levels of optical density. This result is in agreement with those data observed by the corresponding transposon mutants, as shown in Section 3.3.

Figure 5.12b shows the result of the same attachment assay carried out at 30°C. As expected the Δlmo0501 mutant showed a significant reduction in attachment in comparison to the parent 10403s ($P<0.05$), which is consistent with the data for the corresponding transposon mutant, M237. It was hypothesised that a polar effect on the gene lmo0402 in the transposon mutant B380 was causing the reduction in attachment of this mutant. The deletion mutant Δlmo0401 shows as much reduction at lower temperatures as the other mutants, however due to the deletion mutation not being an in-frame mutation; it can still not be proved that the reduced attachment is due to the mutation in *lmo0401*. Complementation of this mutant would be needed to clarify if this gene was important in attachment. The bglG

antiterminator (lmo0402) also showed significantly less attachment than the wild type, indicating that these transcriptional antiterminators are essential for attachment of *L. monocytogenes* to plastic.

Figures 5.12c and 5.12d show the results of this experiment at both 20 and 10°C. As with the result at 30°C the mutants exhibited significantly less attachment to the surface than the wildtype ($P<0.05$).

In conclusion these data indicate the genes *lmo0401*, *lmo0402* and *lmo0501* are required for attachment to abiotic surfaces and their involvement is temperature dependent.

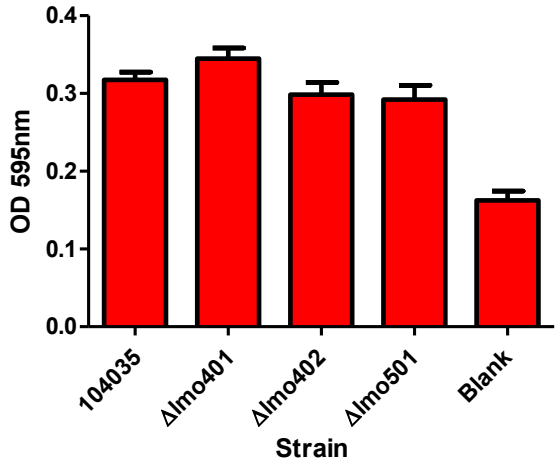

Figure 5.12a - Attachment of *L. monocytogenes* 10403s and deletion mutants Δlmo0401, Δlmo0402, and Δlmo0501 to a microtitre plate at 37°C. All data are an average of three biologically independent replicates; error bars represent standard error of the mean.

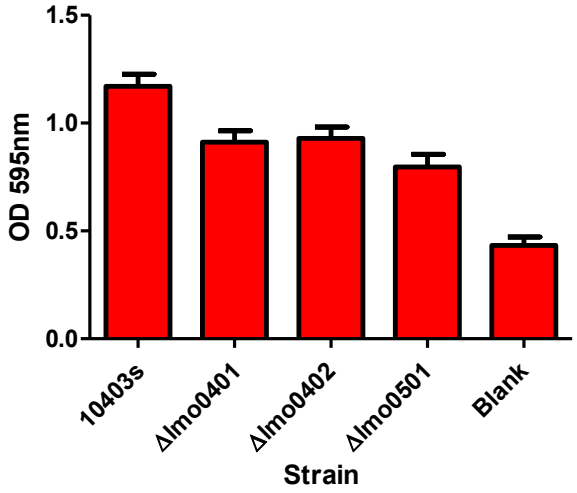

Figure 5.12b - Attachment of *L. monocytogenes* 10403s and deletion mutants Δlmo0401, Δlmo0402, and Δlmo0501 to a microtitre plate at 30°C. All data are an average of three biologically independent replicates; error bars represent standard error of the mean.

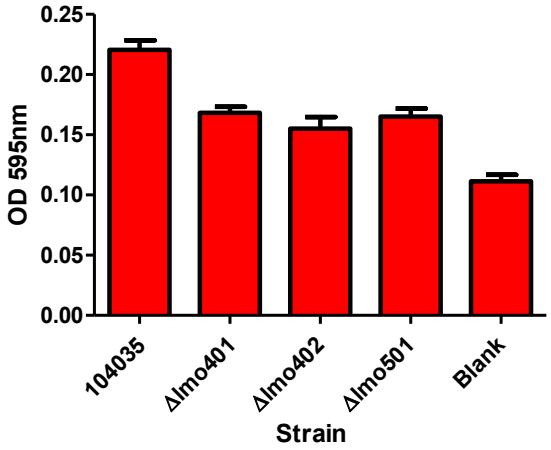

Figure 5.12c - Attachment of *L. monocytogenes* 10403s and deletion mutants Δlmo0401, Δlmo0402, and Δlmo0501 to a microtitre plate at 20°C. All data are an average of three biologically independent replicates; error bars represent standard error of the mean.

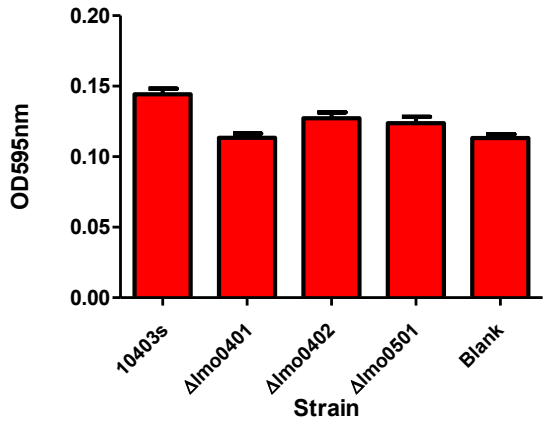

Figure 5.12d - Attachment of *L. monocytogenes* 10403s and deletion mutants Δlmo0401, Δlmo0402, and Δlmo0501 to a microtitre plate at 10°C. All data are an average of three biologically independent replicates; error bars represent standard error of the mean.

5.4.1 - Relative expression of the bglG transcriptional antiterminators

RNA was extracted from *L. monocytogenes* 10403s incubated at temperatures of 37, 30, 20 and 10°C as described in Section 2.2.15. RNA concentration was determined by use of a nanodrop spectrophotometer and used for quantitative RNA. RNA was DNase treated and used for production of cDNA using the method in Section 2.6.3.1, which, was used for the qRTPCR.

Quantitative RTPCR for the genes *lmo0501* and *lmo0402* was performed on the cDNA produced from RNA extracted at each of the temperatures. Each experiment was performed as two biologically independent replicates, each with two technical replicates. qRTPCR was performed using the relative expression ($\Delta\Delta C_T$) method (Livak, 2001). This method uses a housekeeping gene to which the expression of the gene of interest is normalised. The gene for 16S rRNA was used as the house keeping gene for *L. monocytogenes* because literature reports that this gene is stably expressed across many conditions, including different temperatures (Tasara & Stephan, 2007). $\Delta\Delta C_T$ was calculated with respect to the level of expression of each of the genes at 37°C, to establish if at lower temperatures these genes are up- or down-regulated. qRTPCR Primers were designed based on the *L. monocytogenes* EGD-e sequence, using the Primer design tool available on the NCBI website. These were used to amplify regions of the gene of between 150-300bps. The primers used for amplification of the genes *lmo0402* and *lmo0501* were the primers lmo0402fw and lmo0402Rv, and lmo0501Fw and lmo0501Rv

respectively (see Table 2.15 for sequences). Primer binding was also checked during the qRTPCR reaction by dissociation curve analysis, to ensure only one amplicon was present and no nonspecific binding had occurred. The qRTPCR was set up using the conditions described in Section 2.5.3.

Figure 5.13 demonstrates the relative expression of the gene *lmo0402* in *L. monocytogenes* at different temperatures, and Table 5.1 shows the Ct values for the individual runs, and the fold change in expression. As can be seen from these data *lmo0402* is upregulated at temperatures of 30 and 20°C, with a fold difference in expression of 1.48 fold and 2.06 fold respectively. Figure 5.13 shows the average of two biological independent samples, error bars show standard deviation. A students T-test was used to statistically analyse the data, the level of expression at 20°C was significantly higher than at 37°C ($P<0.05$), all other temperatures were not significantly different ($P>0.05$).

The expression of *lmo0501* in the *L. monocytogenes* strain 10403s was also tested at the same temperatures. Figure 5.14 shows the relative expression of this gene at each of the temperatures tested with respect to 37°C, the histogram represents the average $\Delta\Delta C_T$ value from two biologically independent experiments. Table 5.2 shows the numerical data. Expression at all the temperatures appeared to be upregulated with respect to 37°C. But statistical analysis showed this was not statistically different. This was probably due to the large variation in $\Delta\Delta C_T$ values

between the two values. Further experiments need to be done to reduce the standard deviation of these values.

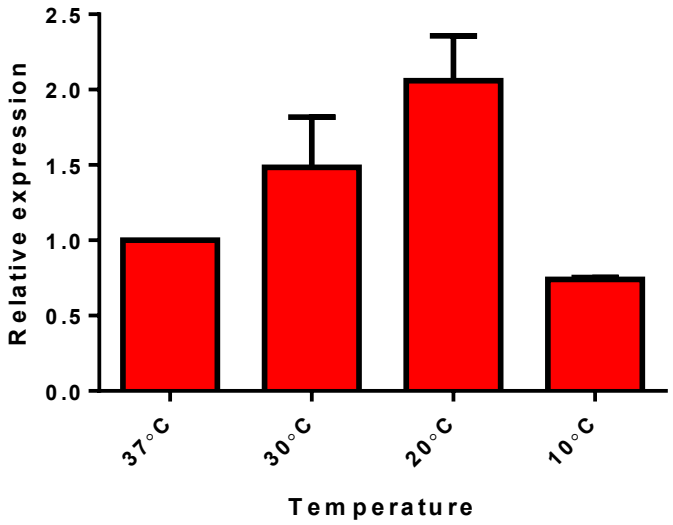

Figure 5.13 - Relative expression of *lmo0402* in *L. monocytogenes* 10403s at temperatures of 37, 30, 20 and 10°C. All data are shown relative to the level of expression at 37°C, and are the average of two biologically independent replicates

	$\Delta\Delta C_T$ Run 1	$\Delta\Delta C_T$ Run 2	Average $\Delta\Delta C_T$	SD	Fold difference
37°C	1.00	1.00	1.00	0.00	0.00
30°C	1.72	1.25	1.49	0.34	1.48
20°C	2.27	1.85	2.06	0.30	2.06
10°C	0.75	0.73	0.74	0.01	0.74

Table 5.1 - $\Delta\ \Delta C_T$ results from two independent qRTPCR runs showing expression of *lmo0402* in *L. monocytogenes* from RNA extracted at different temperatures. All values are relative to expression at 37°C.

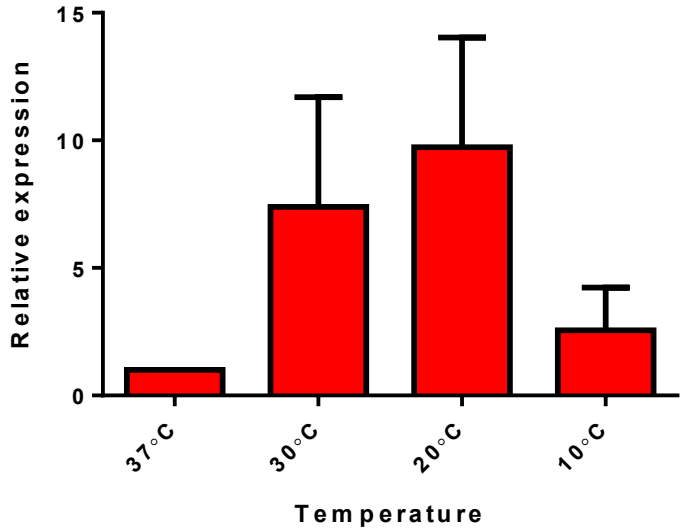

Figure 5.14 - Relative expression of *lmo0501* in *L. monocytogenes* 10403s at temperatures of 37, 30, 20 and 10°C. All data are shown relative to the level of expression at 37°C, and are the average of two biologically independent replicates

	$\Delta\Delta C_T$ Run 1	$\Delta\Delta C_T$ Run 2	Average $\Delta\Delta C_T$	SD	Fold difference
37°C	1.00	1.00	1.00	0.00	0.00
30°C	4.36	10.44	7.40	4.30	7.40
20°C	6.70	12.77	9.74	4.29	9.74
10°C	1.37	3.74	2.56	1.68	2.56

Table 5.2 - $\Delta\ \Delta C_T$ results from two independent qRTPCR runs showing expression of *lmo0501* in *L. monocytogenes* from RNA extracted at different temperatures. All values are relative to expression at 37°C.

5.4.2- Relative expression of the genes in the bglG operons

5.4.2.1 - Lmo0402 operon

The Bgl operon regulated by the transcriptional antiterminator, *lmo0402*, is thought to contain the genes *lmo0398*-*lmo0400*. To confirm this hypothesis and identify where in the operon the BglG transcriptional antiterminator acted, the relative expression of the genes *lmo0397* to *lmo0401* were quantified by qRTPCR on RNA extracted at 37°C and 10°C. Extracted RNA was first DNase treated to remove any traces of genomic DNA, before being subjected to the reverse transcriptase reaction to produce cDNA (Section 2.5.3.1), no reverse transcriptase controls were also made at this point by omitting the reverse transcriptase enzyme, and this step was to confirm that traces of genomic DNA contamination were removed. Primers were designed for qRTPCR using the BLAST primer design tool available on NCBI website (Table 2.5 shows full primer sequences). Primers were designed to amplify regions of less than 300bps. As for the expression analysis of the transcriptional antiterminators at different temperatures, colony PCR was used as described in Section 2.2.7 to check validity of the primers before being used for qRTPCR. Reactions for qRTPCR were set up as described in Section 2.5.3.2. Each gene to be tested was performed in duplicate alongside a no reverse transcriptase control, and a negative control using water instead of RNA.

The relative expression of these data is presented in Figures 5.15 and 5.16, a relative expression of 1 would indicate the expression is the same in the mutant and the parent strain, expression of less than 1 indicates down regulation in the mutant, and an expression of more than 1 indicated up regulation. The data from

these experiments can be seen in Tables 5.3 and 5.4. As can be seen from both the figures and the tables, the genes *lmo0398-lmo0400* were down regulated in the mutant ΔImo0402 in respect to the parent strain 10403s ($P<0.05$). This result was seen at both of the temperatures tested indicating that the BglG transcriptional terminator is expressed and active at both 37 and 10°C. The data for the qRTPCR appear to have a larger fold difference for the genes *lmo0398-lmo0400*, with respect to the parent strain, at 37°C than at 10°C. However these experiments were run at different times, on different cDNA preparations therefore cannot be directly compared.

The data indicate that expression of the genes *lmo0397* and *lmo0401* is not effected by *lmo0402*. These genes encode a hypothetical protein (*lmo0397*) and an alpha-mannosidase (*lmo0401*). This confirms that the BglG transcriptional antiterminator controls expression of the Bgl operon.

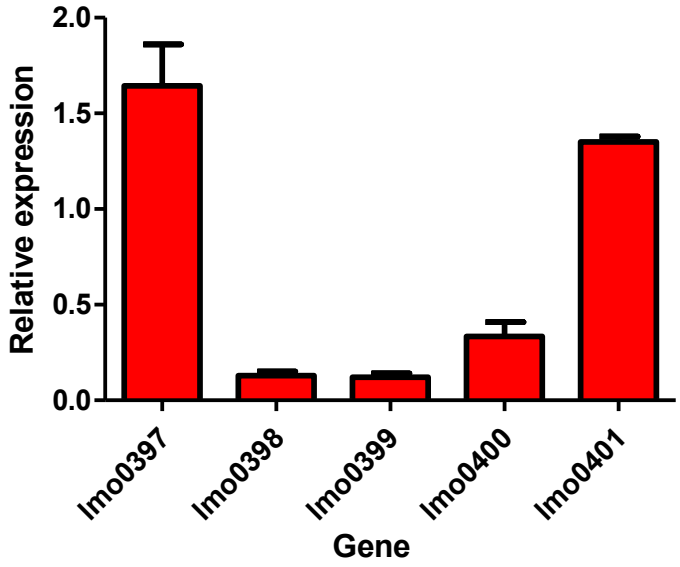

Figure 5.15 - Relative expression of the genes within and flanking the Bgl operon, thought to be regulated by the antiterminator, *lmo0402*, in *L. monocytogenes* 10403s at 10°C. All data shown give the relative expression of each gene in the mutant Δlmo0402, with respect to the wild type, and are the average of two biologically independent replicates

	$\Delta\Delta C_T$ Run1	$\Delta\Delta C_T$ Run 2	Average $\Delta\Delta C_T$	SD	Fold difference
lmo0397	1.43	1.86	1.65	0.30	1.65
lmo0398	0.15	0.11	0.13	0.03	-7.69
lmo0399	0.1	0.14	0.12	0.03	-8.33
lmo0400	0.26	0.41	0.34	0.11	-3.03
lmo0401	1.38	1.32	1.35	0.04	1.35

Table 5.3 - Δ ΔC_T results from two independent qRTPCR runs showing expression of the genes *lmo0397-lmo0401* in *L. monocytogenes* from RNA extracted at 10°C. All values show the relative expression and the fold difference of the mutant Δlmo0402 in comparison to the parent strain 10403s

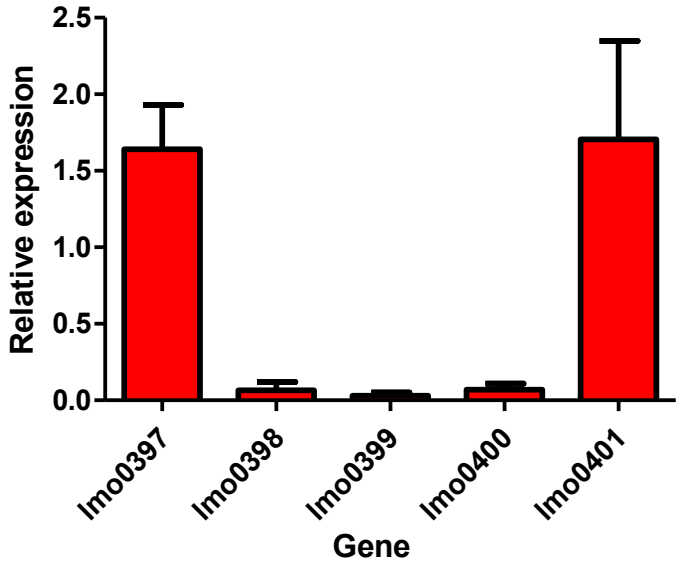

Figure 5.16 - Relative expression of the genes within and flanking the Bgl operon, thought to be regulated by the antiterminator, *lmo0402*, in *L. monocytogenes* 10403s at 37°C. All data shown give the relative expression of each gene in the mutant Δlmo0402, with respect to the wild type, and are the average of two biologically independent replicates

	$\Delta\Delta C_T$ Run1	$\Delta\Delta C_T$ Run 2	Average $\Delta\Delta C_T$	SD	Fold difference
lmo0397	1.93	1.35	1.64	0.41	0.61
lmo0398	0.01	0.12	0.07	0.08	-15.38
lmo0399	0.01	0.05	0.03	0.03	-33.33
lmo0400	0.03	0.11	0.07	0.06	-14.29
lmo0401	1.06	2.35	1.71	0.91	0.59

Table 5.4 - Δ ΔC_T results from two independent qRTPCR runs showing expression of the genes *lmo0397-lmo0401* in *L. monocytogenes* from RNA extracted at 37°C. All values show the relative expression and the fold difference of the mutant Δlmo0402 in comparison to the parent strain 10403s

5.4.2.2 - Lmo0501 operon

The Bgl operon regulated by the transcriptional antiterminator, *lmo0501*, is thought to be the genes *lmo0503, lmo0507* and *lmo0508*. The organisation of this operon is different to that of the *lmo0402* Bgl operon, as other genes lie in between the genes making up the Bgl operon, including the gene coding sugar phosphate isomerase and a poly(sorbitol) dehydrogenase. To identify where on this region of DNA the BglG transcriptional antiterminator acted, the relative expression of the genes *lmo0502* to *lmo0508* were quantified by qRTPCR on RNA extracted at 37°C and 10°C. Extracted RNA was first DNase treated to remove any traces of genomic DNA, before being subjected to the reverse transcriptase reaction to produce cDNA (Section 2.5.3.1), controls were used as Section 5.4.2.1. Primers were designed for qRTPCR using the BLAST primer design tool available on NCBI website (Table 2.5 shows full primer sequences). Reactions for qRTPCR were set up as described in Section 2.5.3.2. Each gene tested was performed in duplicate alongside a no reverse transcriptase control, and a negative control using water instead of RNA.

The relative expression of these data is presented in Figures 5.17 and 5.18, relative expression of 1 would indicate the expression is the same in the mutant Δlmo0501 and the parent strain 10403s. Expression of less than 1 indicates down regulation in the mutant, and an expression of more than 1 indicated up regulation. The data from these experiments can be seen in Tables 5.5 and 5.6. As can be seen from both the figures and the tables, all of the genes tested (*lmo0502*-

lmo0508) were down regulated in the mutant Δlmo0501 in respect to the parent strain 10403s ($P<0.05$). As for the *lmo0402* operon, this result was seen at both 37 and 10°C, indicating that the BglG transcriptional terminator is expressed and active at both temperatures. However these data are not conclusive as the mutant Δlmo0501 contains an out-of-frame deletion mutant, therefore the down regulation of the expression of these genes could be due to a polar effect from the deletion.

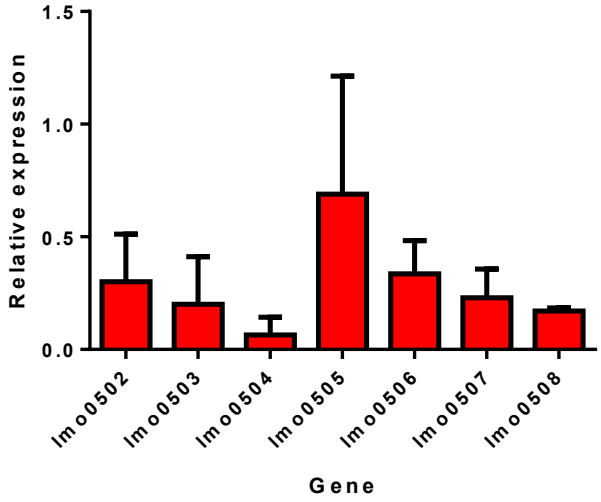

Figure 5.17 - Relative expression of the genes within and flanking the Bgl operon, thought to be regulated by the antiterminator, *lmo0501*, in *L. monocytogenes* 10403s at 10°C. All data shown give the relative expression of each gene in the mutant Δlmo0501, with respect to the wild type, and are the average of two biologically independent replicates

	$\Delta\Delta C_T$ Run1	$\Delta\Delta C_T$ Run 2	Average $\Delta\Delta C_T$	SD	Fold difference
lmo0502	0.45	0.15	0.30	0.21	-4.55
lmo0503	0.05	0.35	0.20	0.21	-4.00
lmo0504	0.01	0.12	0.07	0.08	-6.25
lmo0505	0.32	1.06	0.69	0.52	-4.35
lmo0506	0.44	0.23	0.34	0.15	-3.45
lmo0507	0.14	0.32	0.23	0.13	-5.00
lmo0508	0.18	0.16	0.17	0.01	-5.00

Table 5.5 - Δ ΔC_T results from two independent qRTPCR runs showing expression of the genes *lmo0502-lmo0508* in *L. monocytogenes* from RNA extracted at 10°C. All values show the relative expression and the fold difference of the mutant Δlmo0501 in comparison to the parent strain 10403s.

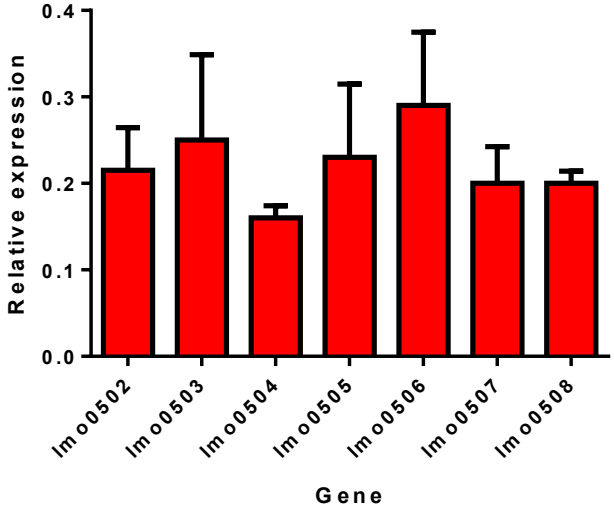

Figure 5.18 - Relative expression of the genes within and flanking the Bgl operon, thought to be regulated by the antiterminator, *lmo0501*, in *L. monocytogenes* 10403s at 37°C. All data shown give the relative expression of each gene in the mutant Δlmo0501, with respect to the wild type, and are the average of two biologically independent replicates

	$\Delta\Delta C_T$ Run1	$\Delta\Delta C_T$ Run 2	Average $\Delta\Delta C_T$	SD	Fold difference
lmo0502	0.25	0.18	0.22	0.05	4.65
lmo0503	0.18	0.32	0.25	0.10	-4.00
lmo0504	0.17	0.15	0.16	0.01	-6.25
lmo0505	0.29	0.17	0.23	0.08	-4.35
lmo0506	0.23	0.35	0.29	0.08	-3.45
lmo0507	0.23	0.17	0.2	0.04	-5.00
lmo0508	0.21	0.19	0.2	0.01	-5.00

Table 5.6 - Δ ΔC_T results from two independent qRTPCR runs showing expression of the genes *lmo0502-lmo0508* in *L. monocytogenes* from RNA extracted at 10°C. All values show the relative expression and the fold difference of the mutant Δlmo0501 in comparison to the parent strain 10403s.

5.4.3 - Growth of mutant strains Δlmo0401, Δlmo0402 and Δlmo0501 in different sugars

To establish the role of these genes in sugar transport, strains were grown in modified TM media containing different sugars as a carbon source. All mutants were tested in each sugar at 10, 20, 30 and 37°C. Growth was recorded by eye as a positive or negative following the incubation period. The sugars tested were glucose, mannose, sucrose, fructose, galactose, mannitol, glucitol, and d-galactitol. As can be seen from Table 5.7 no difference was observed in the growth of these strains. All strains exhibited growth in glucose, mannose, fructose, sucrose and mannitol. No growth was seen in galactose, sorbitol or D-galactitol.

	37				30				20				10			
	10403s	lm0401	lm0402	lm0501	10403s	lm0401	lm0402	llm0501	10403s	lm0401	lm0402	lm0501	10403s	lm0401	lm0402	lm0501
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Galactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-galactitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 5.7 - Growth of the *L. monocytogenes* strains 10403s, Δlmo0401, Δlmo0402 and Δlmo0501 in TM medium containing different sugars. Each strain was tested at temperatures of 37, 30, 20 and 10°C. Growth was assessed by eye and denoted by a + indicating growth, no growth is denoted by a -.

5.4.4 - Use of enzyme linked lectinsorbent assay to measure EPS of *L. monocytogenes*

To determine whether *L. monocytogenes* produced carbohydrate containing exopolysaccharide an Enzyme Linked Lectinsorbent Assay (ELLA), designed by Leriche *et al*. (2000), was used to quantitate the binding of lectins to extracellular polysaccharides. This assay was also used to try and compare the composition of EPS production between the parent strain and the mutants to see if the mutations resulted in differences in EPS quantity or composition. The assay was performed as described in Section 2.5.4. The lectins Concanavalin A (ConA) and the Wheat germ agglutinin (WGA) were used for the assay because these bind to the carbohydrates D-Glucose or D-Mannose and *N*-acetyl-D-glucosamine (GlcNAc) or *N*-acetylneuraminic acid (Neu5Ac), respectively. From the previous attachment assays in this study it was clear that the highest number of adhered cells was observed at the temperature of 30°C, therefore it was decided to carry out this assay at 30°C.

Before using the assay to compare the amount of extracellular polysaccharides produced by the mutant strains with the parent strain 10403s, a standard curve was set up with the wild type strain 10403s and a negative control of TSB only. The amount of binding of lectins was then detected using ABTS substrate, and measured by reading optical density at 405nm. Figure 5.19 shows the result of the standard curve using the lectin WGA, and Figure 5.20 shows the standard curve

with the lectin ConA. This standard curve showed a higher OD at all concentrations of WGA in the strain 10403s than the medium only control.

The standard curve using the lectin ConA (Figure 5.20), however exhibited a higher OD_{405nm} for the medium only controls for all concentrations above 3µg/ml. At concentrations lower that this as can be seen from the figure, the OD_{405nm} was roughly the same in the wells containing the bacteria and the medium only control. Based on this result it was decided not to continue with the assay using the lectin ConA.

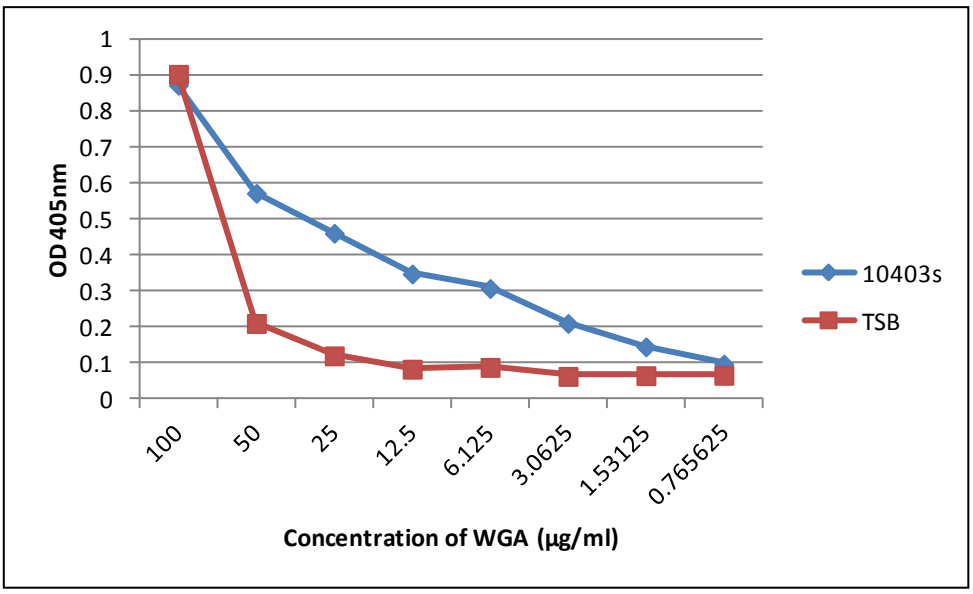

Figure 5.19 - Standard curve of concentration of WGA bound to *L. monocytogenes* 10403s. The curve with the negative control containing TSB only is also shown.

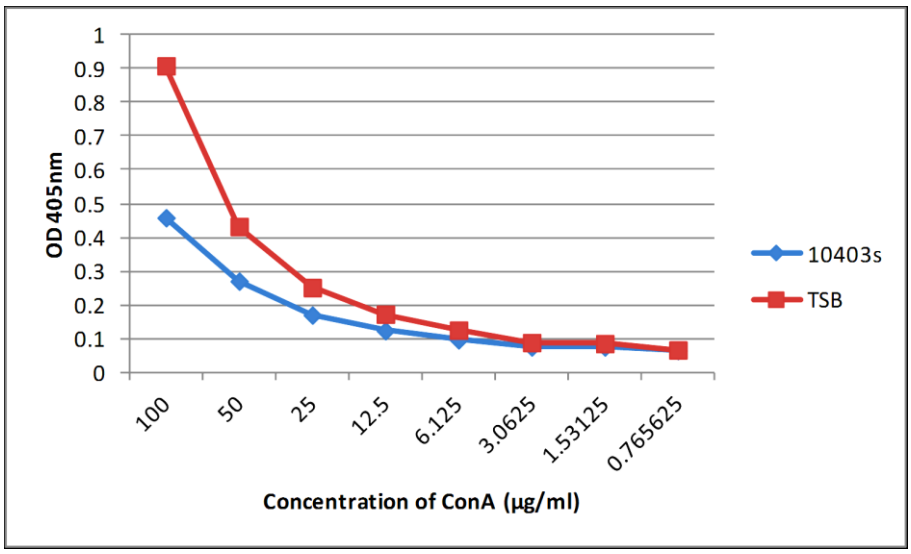

Figure 5.20 - Standard curve of concentration of ConA bound to *L. monocytogenes* 10403s. The curve with the negative control containing TSB only is also shown.

The lectin WGA was used at a concentration of 1.5µg/ml, as this was the lowest concentration that gave binding to *L. monocytogenes* higher than to the medium only control, to quantitate the binding of lectin to adhered cells that have been incubated for 2, 24 and 48 hours (Figures 5.21a, b and c respectively). As can be seen from these Figures the strain 10403s exhibited higher amount of binding of WGA (as exhibited by a higher absorbance) than the mutant strains Δlmo0401 and Δlmo0402 at all time points ($P<0.05$). There was no significant difference between the mutant strains ($P>0.05$). The strain Δlmo0501 showed significantly less ($P<0.05$) binding than the wild type at the 2 and 48 hour time points but interestingly not at the 24 hour time point ($P>0.05$).

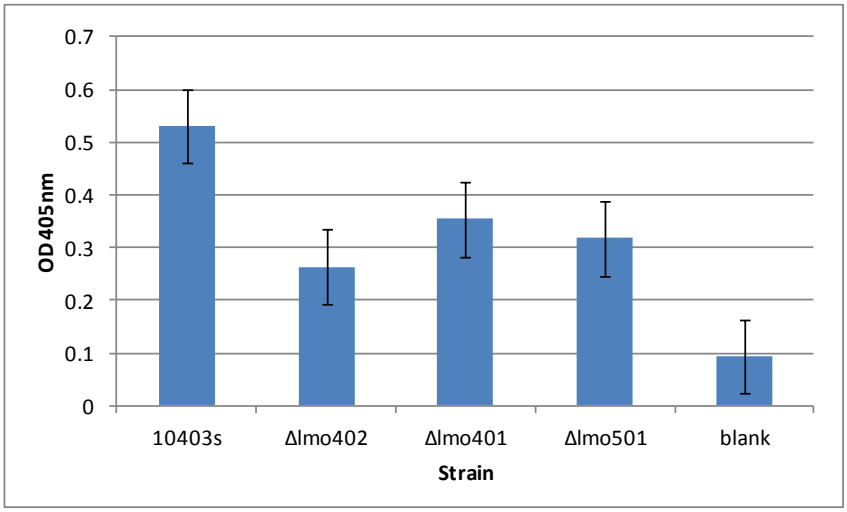

Figure 5.21a - Assay to measure the amount of lectin WGA that bound to adhered cells after the cells had been allowed to adhere to polystyrene for 2 hours. Results shown are from 3 biological replicates each containing 8 technical replicates.

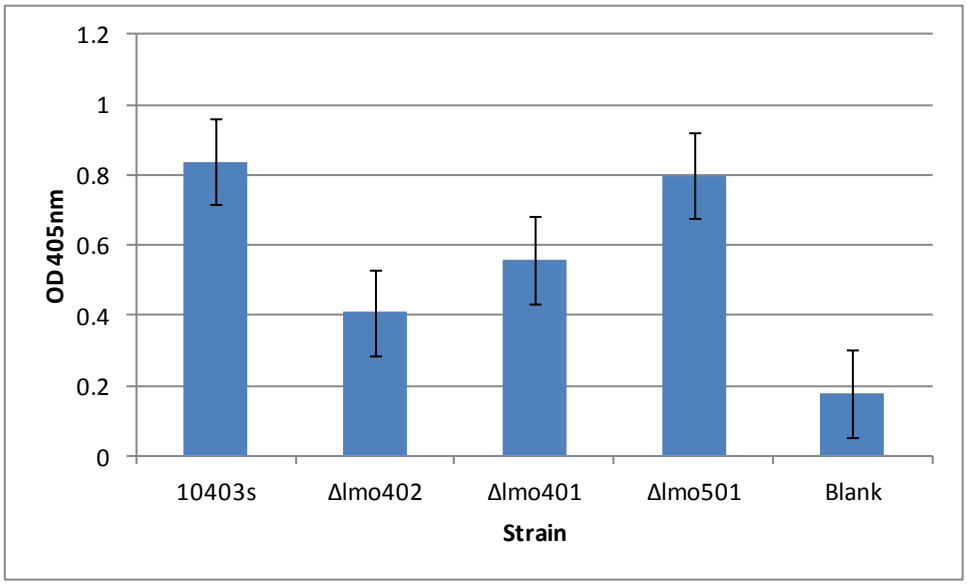

Figure 5.21b - Assay to measure the amount of lectin WGA that bound to adhered cells after the cells had been allowed to adhere to polystyrene for 24 hours. Results shown are from 3 biological replicates each containing 8 technical replicates.

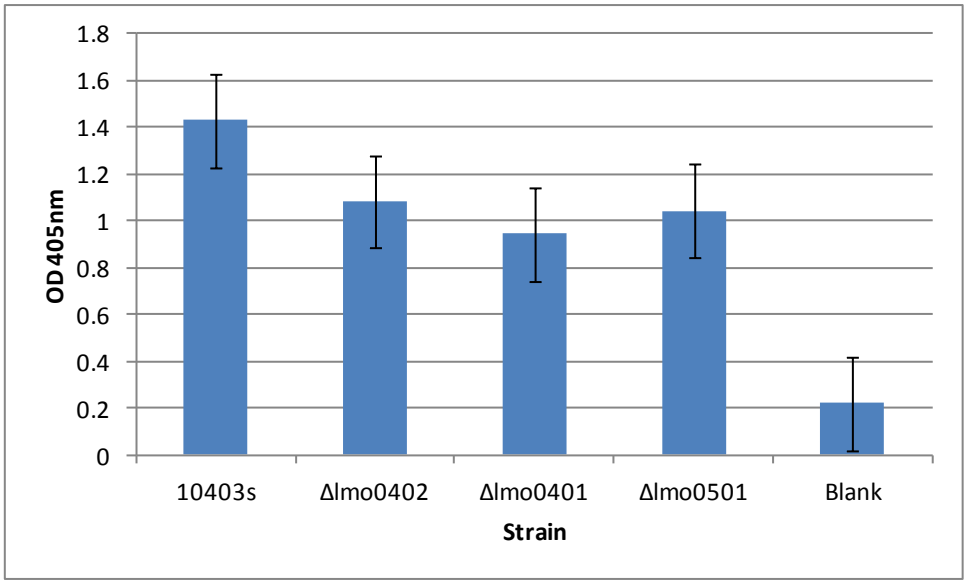

Figure 5.21c - Assay to measure the amount of lectin WGA that bound to adhered cells after the cells had been allowed to adhere to polystyrene for 48 hours. Results shown are from 3 biological replicates each containing 8 technical replicates.

5.4.5 - Assay of metabolic profile of *L. monocytogenes* using the API test system

The API *Listeria* test is a commercially available test normally used for the identification of *Listeria* spp. It contains 10 microtubes containing substrates for enzymatic reactions or fermentation. The strips were inoculated with each of the bglG operon mutants (Δlmo0401, Δlmo0402 and Δlmo0501) or the parent strain10403s.Tests were carried out at 10, 20, 30 and 37°C. Results were recorded after incubation using the key in Table 5.8.

TESTS	REACTION	POSITIVE RESULT	NEGATIVE RESULT
DIM	Differentiation	Pale orange/beige	Orange
ESC	Hydrolysis	pale yellow	black
a -MAN	a -mannosidase	yellow	colourless
DARL	acidification (D-arabitol)	red/orange-red	yellow/orange yellow
WYL	acidification (Xylose)	red/orange-red	yellow/orange yellow
RHA	acidification (Rhamnose)	red/orange-red	yellow/orange yellow
MDG	acidification (Methyl- a D-glucopyranoside)	red/orange-red	yellow/orange yellow
RIB	acidification (Ribose)	red/orange-red	yellow/orange yellow
G1P	acidification (Glucose 1-phosphate)	red/orange-red	yellow/orange yellow
TAG	acidification (Tagatose)	red/orange-red	yellow/orange yellow

Table 5.8 - Key to interpret the results of the API test used to check the metabolic profile of *L. monocytogenes*

At the temperatures 20, 30, and 37°C no difference was observed between the wild type and the mutants in any of the tests, all of the results showed the typical metabolic profile seen for *L. monocytogenes* (as seen for *L. monocytogenes* 10403s at 10°C, Figure 5.22). At 10°C a difference was observed between the wild type and the mutants ∆lmo0401 and ∆lmo0402 and the parental strain in the test for α-mannosidase activity (Figure 5.22, 3rd tube from the left). Both the mutants ∆lmo0401and ∆lmo0402 showed a negative result in this test as indicated by the colourless result. These shows a lack of α-mannosidase activity at 10°C (see Figure 5.22).

Figure 5.22 - API test of *L. monocytogenes* strains 10403s (top strip), Δlmo0401 (2nd strip), Δlmo0402 (3rd strip) and Δlmo0501 (4th strip) carried out at 10°C.

5.5 Conclusion and Discussion

As discussed in Chapter 3 the transposon mutants B380 and were shown to have the transposon inserted into the genes *lmo0401*, an α-mannosidase, and *lmo0501*, a bglG transcriptional antiterminator. These genes are thought to work within an operon involved in sugar transport into the cell. This chapter explained how deletion mutants were made in this genes and a second transriptional antiterminator (*lmo0402*) located downsteam from the α-mannosidase. This chapter also discussed futher characterisation of these mutants to invesitgate their role in attachment, expression of other genes thought to be in the operon, their role in growth in sugars and in production of EPS. This section will discuss the role of the Bgl operon, and discuss the results presented in this chapter.

5.5.1 The Bgl operon and α-mannosidase genes

Bgl operons contain genes encoding proteins involved in phosphoenolpyruvate phosphate transferase systems. This section will discuss the role of PEP-PTS', and discuss the characterisation of deletion mutants made in those genes regulating their transcription and in the gene coding an α-mannosidase.

5.5.1.1 Function of PEP-PTS systems and BglG transcriptional antiterminators

PEP-PTS systems were first described in *E. coli* as early as 1949 (Barabote & Saier, 2005). Since then many attempts have been made to understand these systems and their role in carbohydrate transport, in a wide range of organisms. PEP-PTS systems are involved in transport and phosphorylation of a large number of carbohydrates, and are thought to be involved in chemotaxis towards carbohydrate sources (Deutscher *et al*., 1989; Lengeler & Jahreis, 2009). They have been recently implicated as important in biofilm formation (Abranches *et al*., 2006; Lazazzera, 2010; Koerdt *et al*., 2012). The main function of the PEP-PTS systems is to transport sugars into the cell in conditions of high sugar, phosphorylating the sugar during the process. The systems consist of two major parts, termed EI and EII. EI consists of two minor parts, the enzyme EI and the histidine phosphocarrier protein HPr, and EII consists of three, or sometimes four, membrane bound protein sugar specific permeases. The EII component is split into two hydrophillic proteins, EIIA and EIIB, which are involved in the phosphorylation of the sugar and one (or sometimes two) membrane proteins that allow the sugar

to be transported into the cell (termed EIIC and EIID) (Barabote & Saier, 2005). All PEP-PTS systems consist of an EIIC protein, but very few also have an EIID protein. The EII complexes show sugar specificity, whereas the EI complex does not.

PEP-PTS systems are thought work in the presence of high levels of β-glucosides. Sugars are phosphorylated as they enter the cell by way of transfers of phosphoryl groups from one component of the system to another. Initially phophorylation of EI occurs using the phopsphate from phosphoenol pyruvate. Phosphoylation then occurs as a relay with EI phosphorylating HPr, which in turn phosphorylates EIIA, and then EIIB. Finally as the sugar is transferred into the cell, via the membrane transporter EII, the sugar is phosphorylated.

The β-glucoside utilisation operon contains the genes required to encode for the EII component of the PEP-PTS (sometimes collectively referred to a BglF). Regulation of this operon is co-ordinated by a transcriptional antiterminator of the BglG family which is usually located at the start of the operon (Raveh *et al*., 2009). The summary of the action of this system is shown in Figure 5.23. Transcriptional regulation by the BglG antiterminator occurs by binding of the Bgl operon mRNA, at sites overlapping the hairpin termination structures that form on the mRNA. Binding to these terminators is known to occur at sites flanking the BglG operon (Schnetz & Rak, 1998). The binding of BglG prevents the formation of hairpin structures and stops premature termination (Raveh *et al*., 2009; Rothe *et al*., 2012) but for binding of mRNA to occur, BglG must first form a dimer.

To form a dimer, BglG is modulated by EI and HPr. In the presence of high amounts of β-glucosides, HPr and EI also phosphorylate the BglG transcriptional antiterminator. Phosphorylation occurs at histidine residue 208 (Rothe *et al*., 2012) and this allows dimer formation and for the antiterminator to bind to mRNA and prevent the formation of the hairpin structures which result in termination of transcription.

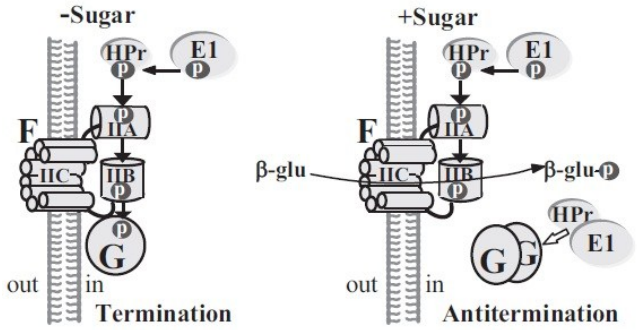

Figure 5.23 - Demonstrating the processes of the PEP PTS system, taken from (Raveh *et al*., 2009)

L. monocytogenes are known to be able to utilise a wide range of carbohydrates using these PEP-PTS systems. These sugars include glucose, mannose, fructose and cellobiose (Stoll & Goebel, 2010). *In silico* analysis of the *L. monocytogenes* genome identifies 84 PTS-associated genes (Glasser *et al*, 2001), which is in fact the highest known number of PTS associated genes found within a single organism. The systems are grouped according to the sugar they are thought to transfer, e.g. glucose-glucoside, fructose-mannitol (see Table 6.2 for details). Some of these systems appear to be complete, whereas some only contain

components of the system, for example the gene *lmo0027* does not seem to be contained within a Bgl operon (Stoll & Goebel, 2010). The systems are grouped into seven groups according to the scheme of Barabote and Saier (2005), these groups are PTS^{glc}, PTS^{Man}, PTS^{lac}, PTS^{fru}, PTS^{asc}, PTS^{gat} and PTS^{guc}. Table 6.2 shows the genes in *L. monocytogenes* that encode for components of these systems, and their respective transcriptional activators or antiterminators. *In silico* analysis revealed only four of these systems contained a fourth component, EIID, all of which appear to be mannose specific (Stoll & Goebel, 2010).

PTS	Encoded by	Known antiterminator/activator
PTS glucose–glucoside (Glc) family		
PTS^{glc}-1	*lmo0027*	
PTS^{glc}-2	*lmo0738*	
PTS^{glc}-3	*lmo1035*	
PTS^{glc}-4	*lmo2772*	*lmo2773*
PTS^{glc}-5	*lmo2787*	*lmo2788 (bvrA)*
PTS^{glc}(EIIA)	*lmo1017*	
PTS^{glc}(EIIBC)	*lmo1255*	
PTS mannose–fructose–sorbose (Man) family		
PTS^{Man}-1	*lmo0021–0024*	GntR (*lmo0020*)
PTS^{Man}-2 (mpt)	*lmo0096–0098* (*mptACD*)	ManR (*lmo0785*)
PTS^{Man}-3 (mpo)	*lmo0781–0784** (*mpoABCD*)	ManR (*lmo0785*)
PTS^{Man}-4	*lmo1997*/*lmo2000–2002*	
PTS lactose–N,N'-diacetylchitobiose–β-glucoside (Lac) family		
PTS^{Lac}-1	*lmo0298/0299/0301*	LicR (*lmo0297*)
PTS^{Lac}-2	*lmo0874–0876*	ManR (*lmo0873*)
PTS^{Lac}-3	*lmo0914–0916*	
PTS^{Lac}-4	*lmo2683–2685*	LacR (*lmo1721*)
PTS^{Lac}-5	*lmo2762*/*2763*/*2765*	
PTS^{Lac}-6	*lmo2780*/*2782*/*2783*	LicR (*lmo2784*)
PTS^{Lac}(EIIC)	*lmo0034*	
PTS^{Lac}(EIIBC)	*lmo0373*/*0374*	
PTS^{Lac}(EIIC)	*lmo0901*	
PTS^{Lac}(EIIB)	*lmo1095*	
PTS^{Lac}(EIIAB)	*lmo1719*/*1720* (lpo)	LacR (*lmo1721*)
PTS^{Lac}(EIIA)	*lmo2259*	
PTS^{Lac}(EIIB)	*lmo2373*	
PTS^{Lac}(EIIC)	*lmo2708*	
PTS fructose–mannitol (Fru) family		
PTS^{Fru}-1	*lmo0357*/*0358*	
PTS^{Fru}-2	*lmo0398–0400*	LicR (*lmo0402*)
PTS^{Fru}-3	*lmo0426–0428*	LicR (*lmo0425*)
PTS^{Fru}-4	*lmo0631–0633*	LicR (*lmo0630*)
PTS^{Fru}-5	*lmo2135–2137*	MtlR (*lmo2138*)
PTS^{Fru}-6	*lmo2335*	
PTS^{Fru}-7	*lmo2733*	
PTS^{Fru}-8	*lmo2797*/*2799*	
PTS galactitol (Gat) family		
PTS^{Gat}-1	*lmo0503*/*0507*/*0508*	MtlR (*lmo0501*)
PTS^{Gat}-2	*lmo2096–2098*	LicR (*lmo2099*)
PTS^{Gat}-3	*lmo2665–2667*	MtlR (*lmo2668*)
PTS L-ascorbate (L-Asc) family		
PTS^{Asc}-1	*lmo1971–1973*	
PTS^{Asc}-2	*lmo2649–2651*	ManR/MtlR (*lmo2652*)
PTS glucitol (Gut) family		
PTS^{Gut}-1	*lmo0542–0544*	

Table 5.9 - Name of the 84 PTS associated genes within the *L. monocytogenes* EGD-e genome. Taken from (Stoll & Goebel, 2010).

5.5.1.2 The role of the BglG transcriptional antiterminators and a α-mannosidase in adhesion to abiotic surfaces

To confirm the observation that the *L. monocytogenes* transposon mutants had a reduction in attachment to polystyrene, each of the deletion mutants were tested for attachment. The results of this experiment confirmed that the gene *lmo0402* is essential for attachment of *L. monocytogenes* to polystyrene and that this effect is temperature dependent. The mutants in the genes *lmo0401* and *lmo0501* also showed a temperature dependent reduction in attachment, however due to the fact that these deletion mutations were not in frame deletions, this effect could still be due to a polar effect on the genes downstream. To confirm the role of these genes in attachment complementation of the deleted genes back into these mutants is required. If these genes are essential for attachment, complementation of the genes should restore the phenotype giving levels of attachment comparable to those of the strain 10403s.

The BglG transcriptional antiterminators are known to regulate the genes belonging to the *bgl* operon, which encodes the components for PEP-PTS systems. These β-glucoside transport systems have become increasing implicated to have a role in bacterial attachment although the method of involvement is not yet clear. Gorski *et al*. (2003) have already demonstrated a clear link between BglG transcriptional antiterminators and attachment to radishes in *L. monocytogenes* at lower temperatures, supporting the work carried out in this study. This study did not come to any definite conclusions about the role of the transcriptional

antiterminators or the α-mannosidase in attachment of *L. monocytogenes* to abiotic surfaces, just that they were essential in attachment at lower temperatures. Currently theories behind the involvement of these genes in attachment include the involvement in these genes in carbon catabolite response (CCR), and the contribution of the PEP-PTS in exopolysaccharide production, each of these theories will be discussed briefly below, along with another hypothesis for why these genes are involved in attachment.

One hypothesis for the involvement of PEP-PTS is that PEP-PTS are involved with the production of exopolysaccharide in many organisms. Currently it is thought that these systems are used for transport of the carbohydrates that go on to be utilised in the production of EPS, which is reported to be composed of between 3-7% sugar in some organisms (Myszka *et al*., 2009). Previous studies indicated that the PEP-PTS systems were required to transport precursors that were then involved in the alginate pathway which is thought to be an important component of EPS in *Pseudomonas* biofilms (Ghafoor *et al*., 2011).

Alongside the link between the *bgl* genes with EPS production, an α-mannosidase has also been associated with EPS production. The role of the α-mannosidase in biofilm formation has been investigated in *Sulfolobus solfataricus* (Koerdt *et al*., 2012). By complementation of a mutant strain of this bacterium, which is deficient in 50 different genes, with the gene encoding an α-mannosidase the attachment and biofilm forming ability of the mutant strain was restored. This supports the work in our study which also showed that the α-mannosidase was essential for

attachment. The work by Koerdt *et al*. (2012) however observed this effect at much higher temperatures than was observed in this study in *L. monocytogenes* as all growth stages of *S. solfataricus* were carried out at 76°C. Koerdt *et al*. (2012) visualised the bacteria in biofilms with the use of the fluorescently labelled lectin ConA. In this organism they observed that the restoration of the α-mannosidase gene led to either a production of more GLc/Man residues in the EPS of the bacterium, and/or a significant change had occurred on the glycosylation of the cell surface associated or extracellular proteins.

To test the hypothesis that the α-mannosidase gene, *lmo0401*, or the BglG transcriptional antiterminators, *lmo0402* and *lmo0501*, were essential for putative EPS production in *L. monocytogenes* an enzyme linked lectinsorbent assay (ELLA) was utilised using the method by Leriche *et al*. (2000). This assay used the lectins ConA and WGA which bind to mannose/glucose and N-acetyl glucosamine residues respectively. Firstly the parent strain 10403s was used to set up a standard curve of lectin binding to be able to determine which concentration to use for the assay. The standard curve for the lectin WGA allowed the best concentration of lectin to be determined. However when trying to establish a standard curve with the lectin ConA, using the strain 10403s, no lectin binding can be seen. A higher amount of lectin binding was observed in the negative control cells, which contained only TSB, than the wells that contained adhered *L. monocytogenes*. This result implies that in contrast to the work seen by Koerdt *et al*. (2012), no mannose residues are present in EPS in this organism; therefore either the α-mannosidase is not important in the role of EPS production in *L.*

monocytogenes or there is no EPs produced. Alternatively mannose could be present, but at levels too low for detection by this assay. Strathmann *et al*. (2002) also utilised the binding of these lectins to characterise the biochemical composition of *Ps. aeruginosa* biofilms. They also observed a high amount of ConA binding indicating a large amount of mannose/glucose residues within the EPS of *Ps. aeruginosa* biofilms.

The ELLA was utilised for two reasons within this study; firstly to attempt to see if EPS was present during attachment of *L. monocytogenes* and secondly to determine if there was a change in composition, primarily mannose or glucose residues as indicated by a change in an amount of ConA binding. The lectin WGA was also used to check presence of N-acetyl glucosamine residues. Past studies have hypothesised that these are present in bacterial EPS during biofilm formation (Renier *et al*., 2011). This study showed a high amount of WGA binding to *L. monocytogenes* 10403s, which was consistently lower in mutant strains. However it was concluded that this was not indicative of presence of EPS in *L. monocytogenes* or that the mutants exhibited EPS with a different composition due to the fact that this lectin has been shown previously to bind to N-acetyl glucosamine residues found on the cell surface (Sizemore *et al*., 1990). Therefore the reduction in WGA binding that was observed in the mutant strains is probably a reflection of the lower numbers of cells adhered to the surfaces. From the data acquired in this study it seems unlikely that the BglG transcriptional antiterminators or the α-mannosidase play a role in production of EPS in *L. monocytogenes*, or even that EPS is present in this model of attachment.

The second hypothesis surrounding the role of PEP-PTS systems in attachment and biofilm formation is the involvement in this system in the processes of carbon catabolite repression (CCR). CCR is a regulatory mechanism which is utilised by the bacteria to control their use of carbon sources in response to an environmental signal (Loo *et al*., 2003). Several studies have now shown a link between PEP-PTS, attachment and CCR (Loo *et al*., 2003; Abranches *et al*., 2006; Houot & Watnik, 2008). Abranches *et al.* investigated the roles of $EIIAB^{Man}$ and EII^{glc} in biofilm development in *Streptococcus mutans*. They identified that the $EIIAB^{Man}$ is essential for biofilm formation. Upon microarray analysis of mutant strains with the genes for $EIIAB^{Man}$ deleted they observed many changes in expression of genes involved in energy metabolism including genes involved in other PEP-PTS systems, suggesting these genes play a regulatory role on other PEP-PTS systems. Similar studies in V. cholerae have also hypothesised about the role of PEP-PTS' in biofilm formation (Houot & Watnik 2008; Lazazzera *et al*., 2010). The PEP-PTS systems in V. cholerae appear to be very important in the role of biofilm formation in this organism, with three systems being shown to be of vital importance to date (Houot & Watnik, 2008), one of which is responsible for nitrogen transport, rather than the traditional transport of carbohydrates. V. cholerae biofilm formation is known to be largely controlled by transcription of the *vps* operon, which encodes for the exopolysaccharide. The three PEP-PTS systems have been identified to control expression of this operon (Lazazzera *et al*., 2010). Till now there has been no similar studies undertaken on *L. monocytogenes*, and no link is yet known between carbon catabolite repression

and biofilm formation in this bacterium. This study did not investigate whether CCR was involved in attachment of *L. monocytogenes* or if the genes looked at in this study are involved in CCR. To test the hypothesis that these genes are involved in expression of the CCR operon, as seen in *V. cholerae*, qRTPCR could be performed on the genes within the CCR operon.

From the observations that have been made in this study, a further hypotheses can be made about the role of PEP-PTS' and the α-mannosidase in attachment in *L. monocytogenes* which can be made from the observation that attachment of the mutant strains is only impaired at 30°C and lower. Flagella are only present on *L. monocytogenes* at 30°C and lower, which could indicate that the PEP-PTS systems play a role in expression of *L. monocytogenes* flagella. This hypothesis is supported by the fact that flagella have already been identified to be important in *Listeria* attachment at 22°C during the early stages of attachment (Vatanyoopaisarn *et al*., 2000). This study compared surface attachment of *L. monocytogenes* to stainless steel between a strain lacking flagella and its parent strain. The flagella negative mutant showed a significantly lower level of attachment for the first four hours of this assay, proving the importance of flagella in initial attachment at lower temperatures. The study by Vatanyoopaisarn supports the data of this study.

It is possible that the transcriptional antiterminators control expression of flagella at lower temperatures. To test whether BglG transcriptional antiterminators effect the transcription of flagella genes or other genes important in attachment, microarray

analysis could be carried out. By comparing expression of the genes in the parent strain to that of the mutants it would be possible to see which genes were over or under expressed.

The role of the α-mannosidase in attachment could also be involved in flagella mediated attachment. It is possible that the α-mannosidase is required for the glycosylation of flagella. Flagella are known to undergo O-linked glycosylation with β-O--N-linked N-acetylglucosamine (Shen *et al*., 2006; Logan *et al*., 2006). Figure 5.24 shows the pathway for N-glycan precursor biosynthesis in *L. monocytogenes*, and the modifications to the glycan to form the core structure (KEGG pathway, Kaneisha laboratories). The modifications to the core structure are catalysed by a series of enzymatic reactions including the modification of 2Manα1, catalysed by α-mannosidase. The pathway for O-glycan biosynthesis is not yet known in *L. monocytogenes*, but O-glycan is only thought to differ in structure to N-glycan by the replacement of the side chain Asn-X-Ser or Asn-X-Th (where X denotes any amino acid except proline) with a side chain composed of a serine or threonine linked to another amino acid, which is likely to be a proline. The PEP-PTS systems, regulated by the BglG transcriptional antiterminators in this study, could also be involved in this process. It is possible that these systems are required for the transport of the carbohydrates required for the structure of the glycan molecules.

The involvement of an α-mannosidase, and the need for carbohydrate molecules for the biosynthesis of glycan, indicates that this hypothesis could explain why the bgl genes and the α-mannosidase are required for attachment, as mutant strain

may expresses flagella that are glycosylated with glycans of a different structure, or that are not glycosylated at all. Flagella glycosylation has previously been shown to be important in biofilm formation of *Campylobacter jejuni* to glass (Howard *et al*., 2009) as mutants in flagella glycosylation showed significantly less biofilm formation that the wild type parents. However this difference was observed in 7 day old biofilms rather than looking at differences in initial attachment.

A recent study in *L. monocytogenes* also supports the idea that these genes may be involved in flagella glycosylation (Shen *et al*., 2006). The study by Shen and colleagues looked at gene expression in a deletion mutant in the gene encoding a O-GlcNAc transferase, required for the glycosylation of flagella in *L. monocytogenes*. Microarray analysis identified two genes (*lmo0398* and *lmo0400*) that were downregulated in the mutant compared to the wildtype strain that belong to the PEP-PTS system regulated by the transcriptional antiterminator *lmo0402*. This study indicates that these genes are potentially involved in glycosylation of flagella. To clarify the role of PEP-PTS and the α-mannosidase in flagella glycosylation, flagellin molecules could be isolated and the composition of the glycans could be determined.

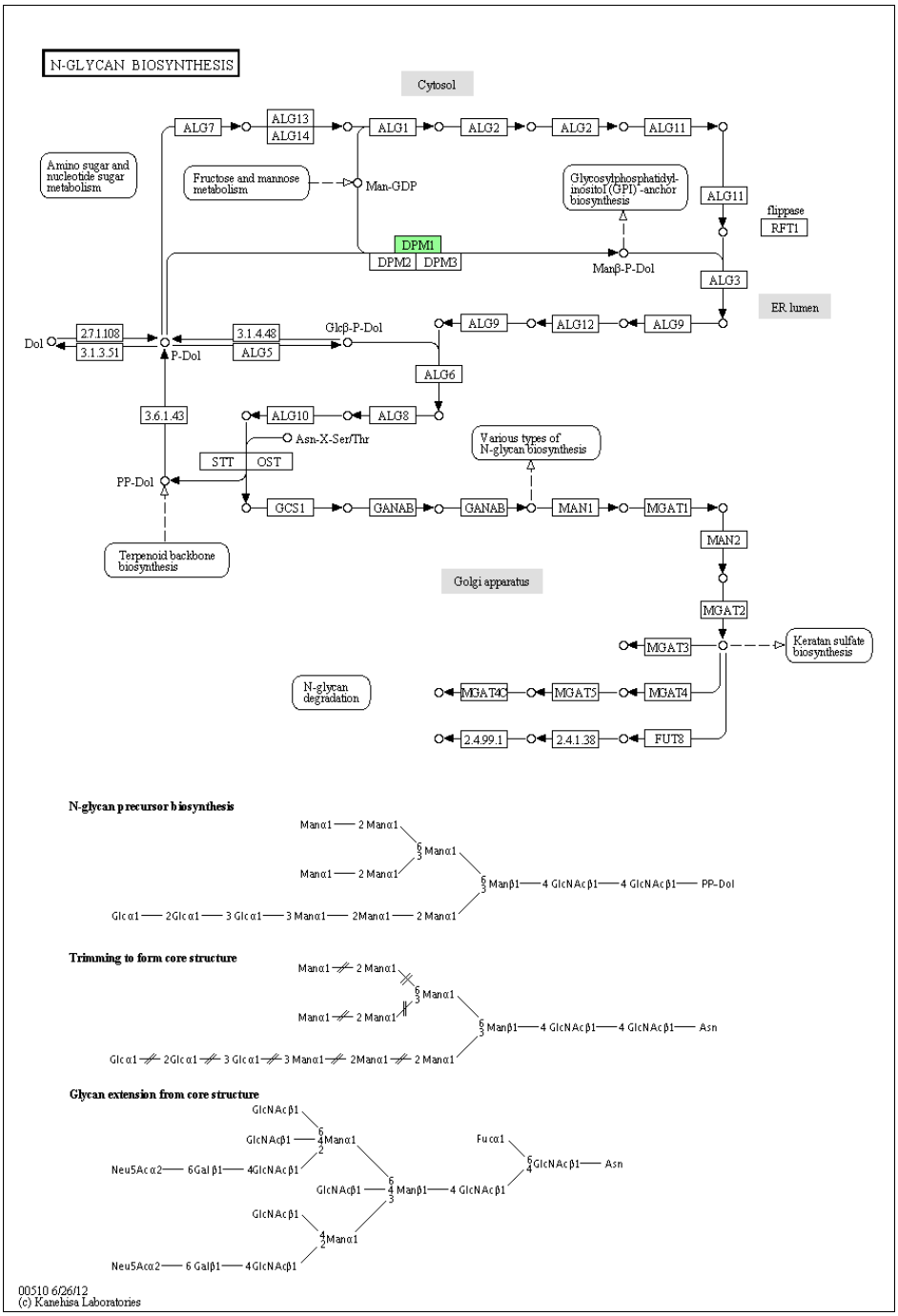

Figure 5.24 - The biosynthesis of N-glycan precursor pathway, and the post translation modifications to form the core structure of N-glycan in *L. monocytogenes* (KEGG Pathways, Kaneisha laboratories).

5.5.2 Expression of ΔImo0402 and ΔImo0501 at different temperatures

The work in this study highlighted three genes involved in attachment of *L. monocytogenes* that appeared to show a temperature dependent response. Attachment of the mutantsΔImo0401, ΔImo0402 and ΔImo0501 was shown to exhibit significantly lower attachment than there isogenic parent at temperatures of 30°C and lower, but not at 37°C.

The data from these attachment assays also showed the highest level of attachment of *L. monocytogenes* 10403s to polystyrene occurred at 30°C. The assay shows a large increase of attachment observed at 30°C in the parent strain that is not observed in any of the mutant strains. This was hypothesised to be due to an up regulation of the BglG transcriptional antiterminators and the α-mannosidase at 30°C. Genes encoding BglG transcriptional antiterminators have already been shown to be involved with attachment of *L. monocytogenes* to surfaces at lower temperatures (Gorski *et al*., 2003). This study which investigated the levels of adhesion of *L. monocytogenes* to radishes also identified mutants from a transposon library created in the 10403 strain background that showed a reduction in attachment to surfaces at temperatures of 30°C and lower. Gorski *et al*. identified three transposon mutations which showed a temperature dependent reduction in attachment, as observed in this study. The location of transposon insertion in the mutants in the study by Gorski was mapped to three different genes, two genes of which had unknown function but were in a region known to contain genes essential for flagella assembly, and the third gene was identified to be that of a BglG transcriptional antiterminator. The work by Gorski *et al*. supports

the work of this study as they also identified a role of a BglG transcriptional antiterminator in *L. monocytogenes* attachment at lower temperatures. The study by Gorski *et al*. also agreed with the observation made in this study that the highest level of attachment of *L. monocytogenes* to radishes occurred at a temperature of 30°C using the wild type strain 10403; this is consistent with the result seen with the streptomycin resistant derivative of this strain 10403s which also exhibits the highest level of attachment to plastics at a temperature of 30°C.

As reduced attachment was only seen in the mutants at temperatures of 30°C and lower, this led to the hypothesis that the gene involved in attachment was temperature regulated. To test this hypothesis quantitative RTPCR was performed on the two transcriptional antiterminators, *lmo0402* and *lmo0501*. Analysis of the qRTPCR results showed an increase in expression at temperatures of less than 37°C. In both of these genes the level of expression was highest at 20°C. However statistical analysis of these data indicated that the gene *lmo0402* is only significantly higher at a temperature of 20°C, and the data for the gene *lmo0501* are not statistically higher at any of the temperatures analysed. The data from the qRTPCR does not support the data from the attachment assay, as the attachment assay saw a difference in attachment between parent and mutant strains at temperatures less than 30°C, therefore implying expression of these genes should be upregulated at all of these temperatures. Also the data from the attachment assay shows the difference is greatest between the parent strain 10403s and the mutants at 30°C indicating that the expression would be highest at this temperature. One explanation for the differences observed in the results of the

attachment assay and the qRTPCR may be due to the fact that the RNA was extracted from planktonic cells, rather than from adhered cells. RNA was isolated for these experiments at mid exponential phase, which ensured the bacteria were at the same stage of growth as they were for the attachment assay. However RNA extraction from adhered cells can be very problematic due to the large numbers of bacteria required for RNA extraction. Alternatively the differences could be due to problems with reproducibility within the qRTPCR. The two biological independent runs gave very different results giving rise to a large standard deviation, a larger number of samples need to be tested to try and reduce the standard deviation.

Expression of the gene *lmo0501* has already been shown to be up regulated at lower temperatures (Liu *et al*., 2002). This study used SCOTS (selective capture of transcribed sequences) analysis, which already identified that the gene *lmo0501* is upregulated at 10°C with respect to 37°C. This result was confirmed by Northern blot analysis. The work of Liu *et al*. is in direct contradiction to the work in this study, as this study showed no significant difference in expression of the gene *lmo0501* at any of the temperatures tested. Again, however, the high variance in the results between the two biologically independent samples led to a high amount of variance in the data. Northern blot analysis could be used as in the study of Liu *et al*. to confirm this result.

5.5.3 Transcriptional regulation of the Bgl operon

The *bglG* transcriptional antiterminators have been shown to regulate transcription in other organisms such as *E. coli* and in other Bgl operons within *L. monocytogenes (*Barabote & Saier, 2005; Raveh *et al*, 2009; Rothe *et al*., 2012). This study investigated the direct effect of the BglG transcriptional antiterminators, *lmo0402* and *lmo0501*, on the genes contained within the putative Bgl operon. Levels of expression were looked at for the genes lmo0397-lmo0401 in the Δlmo0402 mutant and lmo0502-lmo0508 in the Δlmo0501 mutant. Levels of expression were looked at both temperatures of 37°C and 10°C, to see if there is a difference in mode of action of BglG transcriptional antiterminators at different temperatures.

In silico analysis revealed that the BglG transcriptional antiterminator lmo0501 lies at the start of the putative Bgl operon (Figure 5.1). Studies into the Bgl operons have already indicated that the BglG transcriptional antiterminator usually lies at the start of the Bgl operon, and that the antiterminator works by biding to the mRNA either side of the mRNA coding for the antiterminator (Barabote & Saier, 2005). However Bgl operons are thought to contain just the genes coding the EIIA, B and C components of the PEP-PTS (*lmo0503*, *lmo0507* and *lmo0508*, respectively), which normally lie next to each other in the genome. The putative *lmo0501* operon contains a number of other genes too, which separate the genes encoding the EII components of the PEP-PTS. These genes code for a sugar phosphate isomerase (*lmo0502*), an unknown protein (*lmo0504*), a ribulose phosphate 5-epimerase (*lmo0505*) and a poly(sorbitol) dehydrogenase (*lmo0506*).

At this point no conclusions can be drawn as to the role of these other genes in this operon.

In silico analysis was used to identify where transcriptional terminators may lie within this operon, to attempt to hypothesis where the BglG antiterminator may bind. Hairpin like termination structures were identified to lie following *lmo0501*, *lmo0505* and *lmo0508*. Based on the *in silico* analysis it was hypothesised that the transcriptional antiterminator would act on the terminator structures found following the genes *lmo0501*, and *lmo0505*, this confirms that the BglG transcriptional antiterminator is controlling expression of the Bgl operon. The qRTPCR confirmed this result as all of the genes exhibited significantly less expression in the mutant Δlmo0501 than the isogenic parent, supporting the theory that the BglG transcriptional antiterminator is essential for expression of the *bgl* operon. However due to the mutation in *lmo0501* creating a deletion mutation that is not in frame, and thereby introducing a stop codon, it is not possible to conclude in this experiment that the reduction in expression of the genes in the Bgl operon is due to the deletion of *lmo0501*. As this gene lies upstream from the Bgl operon, it is possible that the reduction in expression of these genes is due to a polar effect of this mutation.

The *lmo0402* operon has the opposite lay out to the *lmo0501* operon, with the transcriptional antiterminator lying at the end of the operon, which disagrees with what has been seen in past studies. *In silico* analysis indentified the location of the terminator structures in this operon to lie after the gene *lmo0397*, which lies

upstream of the Bgl genes *lmo0398-lmo0400*, after *lmo0400*, and after the alpha-mannosidase gene, *lmo0401.* From this *in silico* analysis it was hypothesised that the transcriptional antiterminator would work on the terminator structure following the gene *lmo0397*. This hypothesis was confirmed by qRTPCR of these genes in the mutant Δlmo0402 and its isogenic parent. The reduction in expression observed in the mutant in respect to the parent strain of the genes *lmo0398-lmo0400* shows that the transcriptional antiterminator *lmo0402* is essential for expression of these genes.

To analyse whether this effect was temperature dependent the expression of the genes in both operons was analysed at temperatures of 37°C and 10°C. No difference was seen between these results, indicating that the action of the Bgl transcriptional antiterminators in these operons is not temperature dependent. This indicates that although the genes lmo0402 and lmo0502 are essential for attachment of *L. monocytogenes* to polystyrene, the temperature dependent effect observed is not due to temperature dependent expression of the Bgl operon.

5.5.4 α-mannosidase activity

To test for α-mannosidase activity in these mutants, along with utilisation of a range of other sugars, the API *Listeria* test was utilised. This test was carried out on all strains (Δlmo0401, Δlmo0402 and Δlmo0501 and 10403s). The results of this experiment show no difference in the metabolic profile between the parent strain and the strain Δlmo0501 at any of the temperatures tested (37, 30, 20 and 10°C). The mutant strains Δlmo0401 and Δlmo0402 however both had a lack of α-

mannosidase activity at 10°C. This result was expected in the α-mannosidase mutant, however it was expected to be important at all temperatures tested. Previous work by Beresford (2002) already showed a reduction in α-mannosidase activity in the corresponding transposon mutant at 30°C, which contradicts the result seen here, where the loss of α-mannosidase activity was only observed at 10°C. As the transposon mutant is know to grow slower than the parent strain it is possible the previous observed result of lack of α-mannosidase activity by Beresford (2002) was caused by slower growth of the mutant, the deletion mutant does not have this growth defect. This study cannot currently explain the loss of α-mannosidase activity in the Δlmo0402 mutant.

5.5.5 Growth in sugars

The role of PEP-PTS systems in sugar transport has been investigated in many different studies (Stoll & Goebel, 2010). This study tested the ability of each of the mutants, Δlmo0401, Δlmo0402 and Δlmo0501 to grow in TM medium with different sugars. The genes *lmo0402* and *lmo0401* were expected, based on similarity to genes in other organisms, to be involved in transport of fructose and mannitol (see Table 5.9) and the gene *lmo0501*, was expected to be involved in transport of galactitol (see Table 5.9). However the results from this data indicate that these genes are not required for growth in any of the eight sugars tested, at any of the temperatures tested, as no difference was seen between any of the mutant strains and their isogenic parent.

Previous studies in *L. monocytogenes* have already shown a requirement for these systems in transport of carbohydrates. (Michel *et al*., 2011) investigated the role of one of the genes looked at in this study, *lmo0501*, in carbohydrate transport. They found that this gene was required for growth of *L. monocytogenes* in defined medium containing either glucose or fructose as the sole carbon source, which is in contrast to the result of this study which observed no difference at all between the mutant and wild type with these sugars. However the study by Michel *et al*. found the difference in growth with a concentration of 1% glucose or fructose, but not at concentrations of 10%, this study used a defined medium with a final concentration of sugar of 2%. This higher concentration of sugar could account for the lack of differences observed between the wild type and all of the mutant strains in different sugars. Further experiments with a concentration of 1% glucose or fructose would be required to confirm the role of *lmo0501*.

Other genes involved in PEP-PTS systems in *L. monocytogenes* have also been investigated for their role in growth in sugar, with systems so far being identified as involved with glucose, mannose, lactose or cellobiose transport (Dalet *et* al., 2003; Arous *et al*, 2004; Stoll & Goebel, 2010). From this work it would appear that neither of the *bgl* operons looked at are required for growth in sugar in *L. monocytogenes*. This could be due to the fact that as there are a large number of PEP-PTS systems in *L. monocytogenes* it is possible that some of these systems are compensating for those missing in the deletion mutants. Alternatively it is possible that the genes within these operons transport sugars required for alternate reasons rather than bacterial growth.

Chapter 6 - Conclusions and future work

6.1 Conclusion

Listeria monocytogenes is still a major problem in the food processing industry (Wong *et al.*, 1998; Chambel *et al*., 2007). With cases of listeriosis on the increase, over the last decade it is an increasingly important pathogen (Gillespie *et al*., 2010; Mook *et al*., 2011). The first bacterial biofilms were observed in 1938 by Henrici, since then the processes of attachment and biofilm formation have been investigated (Monds and O'Toole, 2009). A number of listerial genes have been identified to be involved in the process (Renier *et al*., 2011), but the process is still not well characterised and not much is known about how *L. monocytogenes* forms biofilms, or, whether this organism produces a characteristic exopolysaccharide, as other organisms, such as *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* (Hall-Stoodley *et al*., 2009). The aim of this study was to elucidate genes involved in attachment of *L. monocytogenes* to abiotic surfaces and identify genes involved in the initial attachment stage of biofilm formation. The identification of important genes and the process that *L. monocytogenes* undergo to form attachment is important, information into this process could help identify new techniques for preventing biofilm formation within food processing factories. Identification of genes may also help identify new potential targets for treatment of Listeriosis.

This work first established a new reliable method for looking at surface attachment of *L. monocytogenes* to polystyrene microtitre plates. This was an improvement on an existing method with examine surface attached growth (Taylor *et al*., 2002).

This was important as it allowed testing of mutant strains of *L. monocytogenes* that were impaired in *in vitro* growth. The development of this assay led to more consistency in results than previous assay, although was not without technical limitations. These will be discussed briefly in Section 6.2.

This assay was then utilised to examine three transposon mutants that were previously identified to be impaired in surface attachment to stainless steel and glass. This assay verified that these mutants were also deficient in attachment to polystyrene. Following on from this arbitrary PCR and previous work by Beresford (2002) was used to show that the transposon had inserted into three different genes inside *L. monocytogenes*; *lmo0401*, *lmo0501* and *lmo1226*. Deletion mutants were then created in these genes as well as *lmo0402,* as it was hypothesised that the insertion of the transposon into the gene lmo0401 in mutant M237 may affect this downstream gene.

The gene lmo1226 was hypothesised to be a drug exporter of the RND superfamily. The deletion mutants created in this gene confirmed that deletion of the whole gene resulted in a loss of attachment. Further characterisation of this gene proved the hypothesis that this was a drug export protein as this gene is responsible for streptomycin resistance in the *L. monocytogenes* strain 10403s. Other characterisation experiments also showed that this gene was essential in an *in vivo* model of infection, invasion into Caco-2 cells and in osmotolerance. This is the first study to the authors' knowledge into this gene in *L. monocytgoenes* and the first link of a drug export protein in *L. monocytgoenes* withbiofilm formation,

although links have been made in other organisms (Kvist *et* al., 2008; Matsumara *et al*, 2011).

The genes *lmo0401*, *lmo0402* and *lmo0501* are all known to be part of the Bgl operon, encoding genes in the PEP-PTS sugar transport system. This system is responsible for sugar phosphorylation and transport into the cell. The genes *lmo0402* and *lmo0501* both encode a BglG transcriptional antiterminator, and *lmo0402* encodes an α-mannosidase. Deletion mutants in these genes showed they are important for attachment of *L. monocytogenes* at lower temperatures which is consistent with past studies (Gorski *et al*., 2003). Although studies have shown a link between this operon and attachment, the involvement of the PEP-PTS system which is encoded by the BglG operon is still not clear. Unfortnately from these studies it's still not possible to say why this operon is important for attachment of *L. monocytogenes*.

To look further at the transcriptional antiterminators, expression levels of genes *lmo0402* and *lmo0501* were measured at different temperatures. This showed that these genes were upregulated at lower temperatures than 37°C. The expression of other genes thought to be within the operon was also measured in the Δlmo0402 and Δlmo0501 mutants to clarify the role the antiterminators play in transcription of the operon. Deletion of these antiterminators led to reduced expression of other genes in the operon, confirming these genes are essential in transcriptional antitermination.

As sugars such as mannose are known to be key components of EPS (Koerdt *et al*., 2012) an enzyme linked lectinsorbent assay was performed to try and identify if EPS was present in *L. monocytogenes*, and if mutations in these genes led to a reduction in amount of EPS. However this assay failed to detect any EPS containing mannose in this study. The question of whether *L. monocytogenes* produces EPS is highly disputed, from the results presented in this thesis it is possible to conclude that *L. monocytogenes* does not produce any mannose containing EPS.

This thesis met its original aims in the development of an assay to better assess attachment of *L. monocytogenes* and identification and characterisation of some genes involved in the attachment process. Further studies on this work are discussed in Section 6.3.

6.2 Technical Limitations of the study

Tehcnical limitations of the experiments used are discussed individually in each of the discussion sections; however the microtitre plate assay designed in Chapter 3 was used throughout so the limitations of this assay will be discussed briefly here. This study initially set out to develop a reliable consistent method to measure adherence of *L. monocytogenes* to polystyrene microtitre plates. The method previously used by Taylor *et al*. (2002) was developed to produce a more reliable assay which looked at inital attachment of the bacteria to the plate as opposed to looking at surface attached growth which is what most of the current methods utilised to invesitgate biofilms measure. Despite the consitency of this method

within a microtitre plate, there was still some variation in the method when comparing assays to each other. From one assay to another there is a high amount of variance in the OD_{595nm} of the blank wells. One explanation for this inconsitency could be due to variations within the medium. The medium contains amino acids which would also stain with crystal violet. The consistency of the medium changing from one batch to anoother could result in variation between each assay. Alternatively sometimes during autoclaving the pH of the medium can sometimes be altered which could have also led to inconsistencies in the assay. To try and maintain consistency for each set of replicates of experiments, batches of broth were made up at the same time to try and limit variation from the medium.

Another limitation to this method could have arisen from differences from different batches of plates as the composition of the polystyrene may have vaired slightly. However this was accounted for in this study and a large number of plates were purchased at the beginning of the assay which was used throughout.

To validate the data produced in the microtitre plate assay it would have been better to have utilised a second method to confirm the results seen. This could have been electron microscopy which would have allowed visualisation of the biofilm, and alothough it would not have gave a quantitative result, the differences in attachment should have been visable in the images produced. This would also give more insight into biofilm architecture and may have allowed visualisation of any EPS if it was produced.

6.3 Future work

The work done in this thesis established four potential genes involved in attachment of *L. monocytogenes* to abiotic surfaces, but further work needs to be carried out to continue to try and explain why these genes are so important for attachment in *L. monocytogenes* and to further characterise other phenotypes observed in these mutants.

To further establish the involvement of *lmo1226* in attachment *in vivo*, experiments could be carried out including *ex vivo* attachment and invasion assays involving a variety of cells such as the J774 macrophage line and brain epithelial cells to try and identify whether *lmo1226* is involved in attachment to a particular cell type. Also further *in vivo* assays can be carried out to quantitate the amount of bacterial load in different organs at various time points; this will help establish the role of this gene in the pathogenesis of *L. monocytogenes*. Experiments into efflux drug inhibitors could also be carried out, with a view to using these as future treatment.

Further work also needs to be carried out to identify how this gene is involved in streptomycin resistance. Site directed mutagenesis could be performed on the amino acid at location 918 in Lmo1226 in the strain 10403s, to see if this leads to a reduction in streptomycin resistance in this strain.

Further work needs to be done to characterise fully the role of the Bgl operon on attachment to surfaces. Complementation of the genes deleted in Δlmo0401 and Δlmo0501 is needed to confirm the direct role of these genes in attachment.

To investigate further why these genes are involved in attachment, microarrays could be performed to see if mutations in these genes lead to a down regulation in other genes, such as flagella expression, or genes involved in carbon catabolite repression. Assays could also be carried out on *L. monocytogenes* flagella extracted from each of the mutants Δlmo0401, Δlmo0402 and Δlmo0501, to confirm the hypothesis that the operon is involved in O-linked glycosylation of flagella. The other genes in the bglG operon could also be deleted to characterise the role of these genes too.

Appendix 1 - Trivett & Meyer medium no sugar (TMNS)

For 1L Salt solution:
Mix solution B with solution C, then add solution A, and finally solution D

Solution A

K_2HPO_4	8.5g
$NaH_2PO_4.H_2O$	1.5g
NH_4Cl	0.5g
H_2O	440ml

Solution B

NaOH	0.048g
Nitrilotriacetic acid	0.48g
H_2O	200ml

Solution C

$FeCL_3.6H_2O$	0.048g
H_2O	200ml

Solution D

$MgSO_4.7H_2O$	0.41g
H_2O	100ml

Amino acid solution

L-Cysteine HCl	200mg
L-Leucine	200mg
CL-Isoleucine	400mg
DL-Valine	400mg
DL-Methionine	400mg
L-Arginine HCl	400mg
L-Histidine HCl	400mg
H_2O	100ml

Vitamin solution

Riboflavine	10mg
Thiamine	10mg

Dissolve in 99ml preheated H_2O

Biotin	10mg
DL thiotic acid	1mg

Dissolve in 10ml H_2O, add 1ml to the 99ml

For TMNS medium add 50ml of amino acid solution and 10ml of the vitamin solution to the salt solution

For TM medium, add Glucose (or other sugar) to the amino acid solution, to give a final concentration of 2%.

Appendix 2

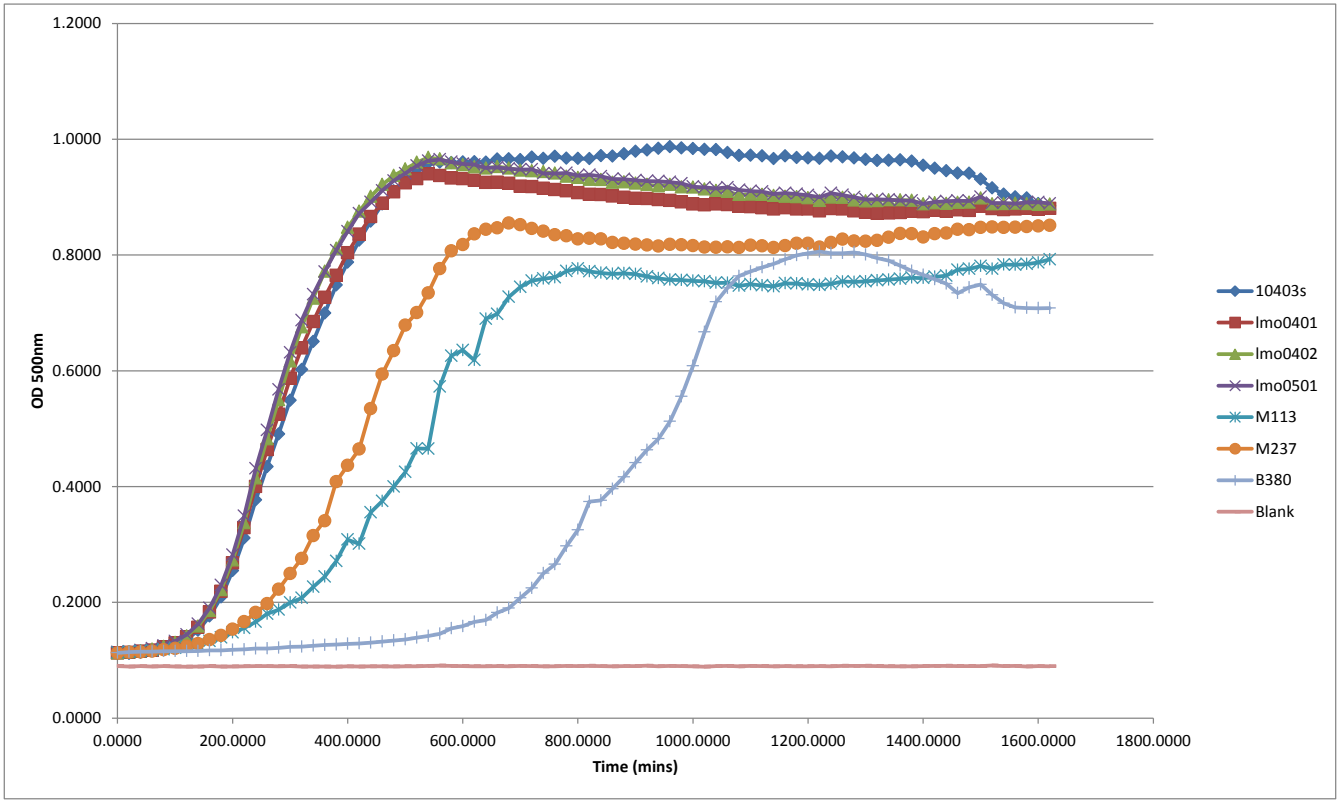

Figure 7.1 – Growth curve of transposon mutants M133, M237 and B380 with their isogenic parent strain 10403s at 37°C. Also included are growth curves of the deletion mutants Δlmo0401, Δlmo0402 and Δlmo0501 for comparison. All data is an average of three replicates.

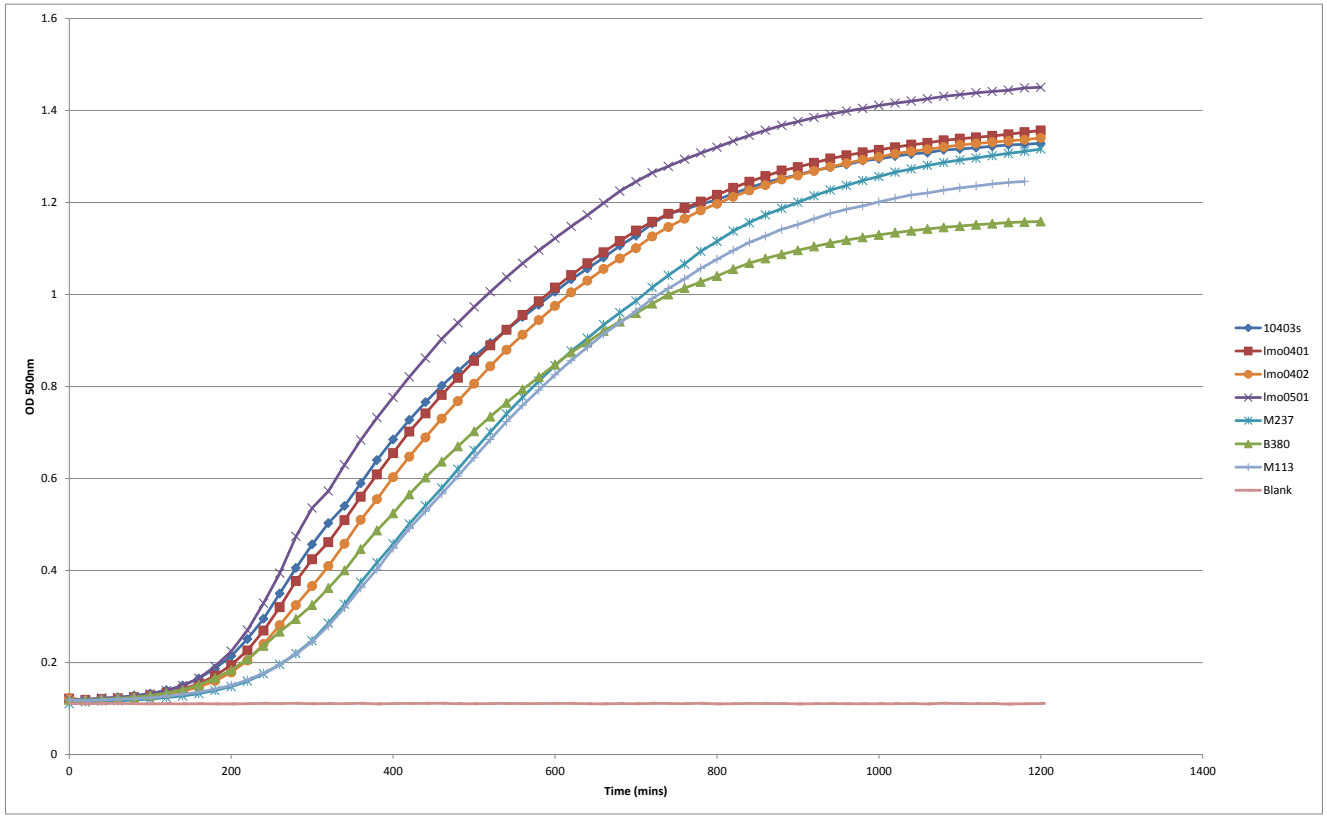

Figure 7.2 – Growth curve of transposon mutants M133, M237 and B380 with their isogenic parent strain 10403s at 30°C. Also included are growth curves of the deletion mutants Δlmo0401, Δlmo0402 and Δlmo0501 for comparison. All data is an average of three replicates.

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