Mechanisms of Biofilm formation by

Listeria monocytogenes

Thesis Submitted for the Degree of

Doctor of Philosophy

at the University of Leicester

by

Salwa Abdalla

Department of Infection, Immunity and Inflammation

University of Leicester

March 2013

### **Statement of Originality**

This accompanying thesis submitted for the degree of PhD entitled "Mechanisms of Biofilm formation by *Listeria monocytogenes*" is based on work conducted by author in the Department of Infection, Immunity and Inflammation of the University of Leicester during the period between October 2008 and Marsh 2013

All the work recorded in this thesis is original unless otherwise acknowledged text or by references.

None of this work has been submitted for another degree in this or any other University

Signed \_\_\_\_\_

Date \_\_\_\_\_

### Abstract

#### Title: Mechanisms of biofilm formation by Listeria monocytogenes

#### By: Salwa Abdalla

*Listeria monocytogenes* is a food-borne bacterial pathogen which has the ability to attach to and form biofilm on food-processing surfaces. It is thought that *L. monocytogenes* biofilm formation in food processing environments is a major source of contamination because, once established, the biofilm appears to have greater resistance to disinfectants and other cleaning agents.

In the first stage of this thesis, *L. monocytogenes* wild type 10403s and *Tn*917transposon mutant strains were surveyed for their ability to attach to polystyrene surfaces at different temperatures (37°C, 30°C and 18°C). The results indicated that two mutants, B265 and I366, showed a significant reduction in their attachment at 18°C and 30°C but not at 37°C. These data have revealed that there is a temperature-dependent involvement of some genes in surface attachment. Subsequently Arbitrary PCR was used to analyse sequences flanking the transposon insertions in both attachment-deficient transposon mutants. Two open reading frames for a putative NADH oxidase in B265 mutant and for a putative penicillin-binding protein in I366 mutant were identified. In the second stage of investigation, deletion mutants were subsequently made successful in these ORFs. Deletion mutation in each of these ORFs also resulted in a significant decrease in attachment of *L. monocytogenes* to polystyrene at 18°C and 30°C with no effect at 37°C.

In the third stage, *L. monocytogenes* strains were screened for their ability to attach to polystyrene at different NaCl concentrations and pH values. Both the wild type and deletion mutants showed a decrease in attachment at high NaCl concentrations and a loss of attachment at high pHs. But neither of the mutants differed from wild type which showed a similar pattern of response.

The molecular basis of biofilm mechanisms of *L. monocytogenes* is considered and this has been ascribed to the presence or absence of specific genes. Recently, there has been a growing interest in understanding the molecular basis of these processes. This study has demonstrated that NADH oxidase and penicillin-binding protein may play a role in *L. monocytogenes* attachment.

#### Acknowledgments

First I would like to thank almighty God and all praises and gratitude from Him.

There are many people who helped, guided and supported me through my PhD experience. Firstly I would like to express my deepest gratitude to my research PhD supervisor Prof. Peter Andrew for offering excellent guidance and enormous support throughout my studies.

I am very thankful to my sponsors, the Libyan government, for providing me with the funds to enable me to complete my PhD successfully.

I take this opportunity to thank my postgraduate tutor Dr. Caroline Beardsmore for her encouragement and support. I am very also grateful to my progress review panel members Aras Kadoglu and Ed Galyov for their advice, support and positive feedback; it was much appreciated.

I would like to thank all my 227 lab mates and all my friends in the Infection, Immunity and Inflammation department: A special thanks to Sarah Glenn for her assistance in the laboratory over the past three years. I would also like to thank Dr Hasan Yesilkaya who has taken the time to listen, discuss and argue over almost every problem I faced.

I dedicate this body of work to my father who is no longer with us. Thank you, my father. To my mother and all members of my family who inspired me to pursue this research even though they are away. To all my friends: Souad, Fatima, Aisha, Najat, Souher, and Noria. To my sister Amal, my brother in law Abdallah and their kids Maram and Marwa.

Finally, I would like to thank all who have helped me - I would not have been able to complete this journey without their support and encouragement.

# List of Figures

Figure 1-1 Distribution of <i>L. monocytogenes</i> in the different sections of Portuguese
cheese dairies during the production cycle11
Figure 1-2 Schematic representation of the virulence gene cluster located in $L$ .
monocytogenes EGD-e genome16
Figure 1-3 Scheme of Intracellular invasion by <i>L. monocytogenes</i> 20
Figure 1-4 Listeriosis incidence in six EU countries 1999–2007
Figure 1-5 The stages of bacterial biofilm development
Figure 2-1 Schematic drawing demonstrate cloning procedure using In-Fusion HD kit
Figure 3-1 Assay of attachment with L. monocytogenes wild type 10403s to
polystyrene at 37°C75
Figure 3-2 The result for assay of attachment of L. monocytogenes strains to
polystyrene at 37°C76
Figure 3-3 Assay of attachment <i>L. monocytogenes</i> strains to polystyrene at 30°C77
Figure 3-4 Assay of attachment <i>L. monocytogenes</i> strains to polystyrene at 18°C79
Figure 3-5 The result for assay of attachment of L. monocytogenes wild type to
polystyrene at 37°C, 30°C and 18°C during 2 h incubation80
Figure 3-6 The optical density of L. monocytogenes strains in TSB after 2 h incubation
at 18°C81
Figure 3-7 The optical density at 595nm of L. monocytogenes strains in TSB after 2h
incubation at 30°C
Figure 3-8 SEM of attachment of L. monocytogenes strains; wild type 10403s, B265
and I366 to stainless steel coupons at 30°C after 2h incubation time

Figure 3-9 SEM of attachment of L. monocytogenes strains; wild type 10403s, B265
and I366 strains to polystrayne coupons at 37°C 30°C, and 18°C for 2h incubation
time
Figure 3-10 SEM of attachment of L. monocytogenes strains; wild type 10403s, I366
and B265 strains to polystryrene coupons at 37°C, 30°C, and 18°C for 24h incubation
time
Figure 3-11 Schematic drawing to explain the Arbitrary PCR primers
Figure 3-12 Agarose gel electrophoresis of the PCR products generated from the I366
mutant in the first round
Figure 3-13 Agarose gel electrophoresis of the PCR products generated from the B265
mutant in the first round
Figure 3-14 Agarose gel electrophoresis of the PCR products generated from the B265
mutant in the second round91
Figure 3-15 Agarose gel electrophoresis of the PCR products generated from the I366
mutant in the second round91
Figure4-1 Agarose gel electrophoresis showing the amplification of 321bp fragment of
<i>lmo0103</i> by NO1F and NO1R primers in <i>L. monocytogenes</i> 10403s102
Figure 4-2 Agarose gel electrophoresis of the <i>EcoRI</i> restriction digest104
Figure 4-3 Restriction digestion of pAUL-A plasmid with enzyme <i>EcoRI</i> :105
Figure 4-4 Agarose gel electrophoresis of plasmid pAUL-03 transformed into in L.
monocytogenes 10403s
Figure 4-5 Agarose gel electrophoresis confirming PCR amplification of <i>lmo2235</i> and
<i>lmo2471</i> in <i>L. monocytogenes</i> 10403s107
Figure 4-6 Design PCR primers for gene of interest with 15bp extensions (5') that are
complementary to the ends of linearized pAUL-A

Figure 4-7 Diagram showing the strategy for mutagenesis of L. monocytogenes using
In-Fusion HD kit
Figure 4-8 Restriction digestion of the plasmid pAUL- A with <i>BamHI</i> 109
Figure 4-9 Agarose gel electrophoresis showing ligation of inset into plasmid pAUL-
A110
Figure 4-10 Agarose gel electrophoresis to test integration of plasmid pl2235 into the L.
monocytogenes 10403s genome112
Figure 4-11 Agarose gel electrophoresis to test integration of plasmid pl2471 into the <i>L</i> .
monocytogenes 10403s strain112
Figure 4-12 Agarose gel electrophoresis of the recombination of plasmid pl2235 into <i>L</i> .
monocytogenes 10403s chromosome114
Figure 4-13 Agarose gel electrophoresis of the recombination of plasmid pl2471 into <i>L</i> .
monocytogenes 10403s chromosome114
Figure 4-14 Agarose gel electrophoresis confirming PCR amplification of <i>lmo0540</i>
gene in L. monocytogenes 10403s115
Figure 4-15 Agarose gel electrophoresis showing ligation of inset into plasmid pAUL-
A117
Figure 4-16 Agarose gel electrophoresis to test integration of plasmid pl0540 into the L.
monocytogenes 10403s genome118
Figure 4-17 Agarose gel electrophoresis of the recombination of plasmid pl0540 into <i>L</i> .
monocytogenes 10403s strain119
Figure 4-18 Agarose gel electrophoresis confirming PCR amplification of <i>lmo2236</i>
gene in <i>L. monocytogenes</i> 10403s121
Figure 4-19 Agarose gel electrophoresis showing ligation of inset into pAUL-A122

Figure 4-20 Agarose gel electrophoresis of integration of plasmid pl2236 into the $L$ .
monocytogenes 10403s strain
Figure 4-21 Agarose gel electrophoresis of the recombination of plasmid pl2236 into <i>L</i> .
monocytogenes 10403s strain
Figure 5-1 Growth curves of <i>L. monocytogenes</i> strains performed in TSB at 37°C130
Figure 5-2 Growth curves of <i>L. monocytogenes</i> strains cultuted in TSB at 37°C and in
2% (w/v) NaCl
Figure 5-3 Growth curves of L. monocytogenes strains cultured in TSB at 37°C and in
4% (w/v) NaCl
Figure 5-4 Growth curves of <i>L. monocytogenes</i> strains cultured in TSB at 37°C and pH
6.5
Figure 5-5 Growth curves of <i>L. monocytogenes</i> strains cultured in TSB TSB at 37°C
and pH 5.5
Figure 5-6 Assay of attachment assay of L. monocytogenes strains to polystyrene at
37°C
Figure 5-7 Assay of attachment <i>L. monocytogenes</i> strains to polystyrene at 30°C136
Figure 5-8 Assay of attachment <i>L. monocytogenes</i> strains to polystyrene at 18°C138
Figure 5-9 Assay of attachment L. monocytogenes wild type, B265, $\Delta lmo0103$ ,
$\Delta lmo2235$ , $lmo\Delta2471$ , I366 and $\Delta lmo0540$ at 37°C in TSB with different NaCl
concentrations
Figure 5-10 Assay of attachment L. monocytogenes wild type, B265, $\Delta lmo0103$ ,
$\Delta lmo2235$ , $lmo\Delta2471$ , I366 and $\Delta lmo0540$ at 30°C in TSB with different NaCl
concentrations
Figure 5-11 Assay of attachment L. monocytogenes wild type, B265, $\Delta lmo0103$ ,
$\Delta lmo2235$ , $lmo\Delta2471$ , I366 and $\Delta lmo0540$ at 37°C in TSB at different pHs144

Figure 5-12 Assay of attachment L. monocytogenes wild type, B265, $\Delta lmo0103$ ,
$\Delta lmo2235$ , $lmo\Delta 2471$ , I366 and $\Delta lmo0540$ at 30°C in TSB at different pHs145
Figure 5-13 Assay of attachment L. monocytogenes wild type, $\Delta lmo2236$ and
$\Delta lmo2235$ to polystyrene at 37°C and 30°C146
Figure 5-14 Agarose gel electrophoresis showing isolated listerial RNA148
Figure 5-15 Gene expression of <i>lmo2235</i> gene and <i>lmo0540</i> gene in <i>L. monocytogenes</i>
wild type at 18°C and 30°C relative to expression at 37°C
Figure 5-16 The expression pattern of <i>lmo2235</i> and <i>lmo0540</i> at 18°C and 30°C relative
to 37°C in planktonic and detached cells after attachment
Figure 5-17 Schematic drawing to show the location of neighboring gene lmo2236 to
lmo2235 and lmo0539 and lmo0541 to lmo0540 in L. monocytogenes EGD-e153
Figure 5-18 Eexpression of <i>lmo2236</i> in B265 and <i>lmo0530</i> and <i>lmo0541</i> in the I366
compared to wild type153
Figure 5-19 Photograph of the E-test demonstrating the point at which the zone of
inhibition of bacterial growth intersects the antimicrobial strip
Figure 5-20 Photograph of the E-test demonstrating the point at which the zone of
inhibition of bacterial growth intersects the antimicrobial strip
Figure 5-21 NADH oxidase activity in <i>L. monocytogenes</i> strains157
Figure 6-1Terminator map of <i>lmo2235</i> in <i>L. monocytogenes</i> EGD-e indicates
terminator at end of gene170
Figure 6-2 Terminator map of Imo0540 L. monocytogenes EGD-e indicates terminator
at end of gene

## List of Tables

Table 1-1 Serovars of L. monocytogenes with the detected O and H antigens
Table 1-2 Episodes of <i>L. monocytogenes</i> contamination of milk and cheese in USA and
European countries10
Table 1-3 Strains of L. monocytogenes with completely sequenced genomes.       14
Table 1-4 List of genes regulated directly by PrfA and their function
Table 1-5 The mortality rate among food-borne pathogens in the United States20
Table 1-6 Summary of data on major outbreaks of listeriosis, 1980-1999
Table 1-7 Summary of the features of microscopy used to study biofilm formation39
Table 2-1 Bacteria strains
Table 2-2 Plasmids44
Table 2-3 Oligonucleotides used as primers    55
Table 2-4 PCR primer pairs during the first and second round of arbitrary PCR55
Table 2-5 Oligonucleotides used as primers to prepare L. monocytogenes mutants60
Table 2-6 Primers used for analysis of gene expression    68
Table 3-1Viable counts of planktonic cells of L. monocytogenes wild type10403s in the
attachment assay after 2 hours incubation at 37°C75
Table 3-2 Viable counts of planktonic cells in the attachment assay after 2 hours
incubation at 37°C76
Table 3-3 Viable counts of planktonic cells in the attachment assay after 2 hours
incubation at 30°C78
Table 3-4 Viable counts of planktonic cells in the attachment assay after 2 hours
incubation at 18°C

Table 3-5 The number of L. monocytogenes strains attaching to stainless steel 304
coupons after 2 hrs incubation at 30°C82
Table 3-6 The primer pairs used for the second round amplified DNA in each mutant.
Table 5-1 The growth rate constant $\mu$ was calculated for the <i>L. monocytogenes</i> and six
mutants in TSB at 37°C and at different NaCl and pH concentration 130
Table 5-2 Viable counts of planktonic cells in the attachment assay after 2 hours
incubation at 37°C136
Table 5-3 Viable counts of planktonic cells in the attachment assay after 2 hours
incubation at 30°C137
Table 5-4 Viable counts of planktonic cells in the attachment assay after 2 hours
incubation at 18°C

## Abbreviations

arbPCR	arbitrary PCR
BHI Agar	brain heart infusion agar
BHI	brain heart infusion broth
BSA	bovine serum albumin
c.f.u	colony forming units
DNA	deoxy ribonucleic acid
dNTP	deoxynucleotide acid
EDTA	ethylenediaminetetraacetic acid
Erm	erythromycin
HKG	housekeeping gene
LA	luria- bertani agar
LB	luria-bertani
O.D	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PNACL	protein nucleic acid chemistry laboratory
qRT-PCR	quantitative real-time PCR
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
TE buffer	tris-EDTA buffer
TSA	tryptone soya agar
TSB	tryptone soya broth
UV	ultraviolet

# Symbols

 $\Delta$  deletion mutant

### List of contents

Chapte	r One: Introduction	1
1.1 <i>Lis</i>	teria monocytogenes	1
1.1.1	The history and classification of <i>Listeria monocytogenes</i>	1
1.1.2	Characteristics of the Listeria genus	2
1.1.3	Morphology and Growth Requirements of L. monocytogenes	3
1.1.4	Serotyping of L. monocytogenes	6
1.1.5	Habitats of <i>L. monocytogenes</i>	6
1.1.6	L. monocytogenes in soil and vegetation	7
1.1.7	L. monocytogenes in dairy products	9
1.1.8	L. monocytogenes in other foods and food processing	11
1.1.9	Virulence factors of <i>L. monocytogenes</i>	14
1.1.10	Pathogenesis and intracellular infectious cycle	17
1.1.11	Listeriosis	20
1.1.12	Outbreaks of food-borne listeriosis	22
1.2 Bic	ofilms and attachment	24
1.2.1	Microbial biofilms	24
1.2.2	Biofilm Matrix	25
1.2.3	Biofilm formation stages	26
1.2.4	Factors affecting attachment	28

1.	2.5	Biofilm in environments and medicine	31
1.	2.6	Bacteria biofilms in the food processing industry	32
1.	2.7	Listerial biofilms	33
1.	2.8	Methods for studying biofilm formation in laboratory	35
	1.2.8	3.1 Microtitre plate	36
	1.2.8	3.2 Flow cells devices	37
	1.2.8	3.3 Coupon systems	37
	1.2.8	.4 Microscopy	38
	1.2.8	.5 Total viable counts	40
1.3	Tra	nsposons	40
1.4	Ain	n and Objectives	42
C	hapte	r Two: Materials and Methods	43
2.1	Che	emical and Biological Material	43
2.2 ]	Bacter	ial strains and plasmids	43
2.1	2.1 Tn	917 Transposon mutants	45
2.3	Cul	ture media and growth conditions	45
2.4	Gro	owth curves of <i>L. monocytogenes</i>	46
2.5	Ass	ay of attachment to polystyrene (adapted from Taylor et al., 2002)	48
2.	5.1	Viable Counts	48
2.6	Ass	say of attachment to stainless steel (based on quantitative adhesion assay l	эу
Bere	esford	<i>et al.</i> , 2001)	50

2.6	5.1	Preparation of coupons	0
2.6	5.2	Preparation of bacteria	0
2.6	5.3	Attachment assays	0
2.7	Pro	cedures for DNA extraction5	1
2.7	7.1	Extraction of listerial chromosomal DNA	1
2.7	7.2	Extraction of plasmid DNA using a HiSpeed Maxi kit5	1
2.7	7.3	Extraction of plasmid DNA using a miniprep kit	2
2.8	Pol	ymerase Chain Reaction (based on (Sambrook et al., 1989)53	3
2.8	3.1	Arbitrary primer PCR	3
2.9	DN	A extraction from agarose gels using a Promega S/V system	5
2.10	DN	A purification extraction using a QIAquick purification kit	5
2.11	Aga	arose gel electrophoresis57	7
2.12	Nuo	cleotide sequence analysis57	7
2.13	Cor	nstruction of mutants in <i>L. monocytogenes</i> 10403s58	8
2.1	3.1	Preparation of L. monocytogenes cells for electroporation with plasmid	
Dì	ΝA		3
2.1	3.2	Electroporation of <i>L. monocytogenes</i> with plasmid DNA (based on Park	
&	Stewa	art, 1990)	8
2.1	3.3	Recombination of plasmid into <i>L.monocytogenes</i>	9
2.14	Clo	ning reactions6	1
2.1	4.1	Ligation of DNA fragment using pGEM-T Easy vector	1
2.1	4.2	Ligation of DNA fragment using pAUL-A vector	1

2.1	4.3	Ligation of DNA fragment using In-fusion HD cloning kit	.61
2.1	4.4	Transformation protocol	.63
2.15	DN	A restriction digestion	.63
2.16	Sca	nning Electron Microscopy	64
2.1	6.1	Preparation of coupons	64
2.1	6.2	Preparation of bacteria	64
2.17	Imp	pact of temperature on <i>L. monocytogenes</i> gene expression	65
2.1	7.1	Analysis of gene expression by Real Time Reverse Transcriptase	
Pol	yme	rase Chain Reaction (RT-PCR)	.65
	2.1	17.1.1 RNA extraction	.66
	2.1	17.1.2 Extraction RNA from attached and planktonic cells	.67
	2.1	17.1.3 First-strand cDNA synthesis using superscript II RT	67
	2.1	17.1.4 Real-Time reverse Transcriptase PCR	.68
2.18	Me	asurement of NADH oxidase activity	69
2.1	8.1	Preparation of cell lysate (adapted from (Lopez de Felipe et al., 2006).	.69
2.1	8.2	Enzyme activity measurement (Lopez de Felipe et al., 2006)	.69
2.1	8.3	Quantification of protein using Bradford Protein Assay	69
2.19	Mi	nimum Inhibitory concentration MIC determinations	70
2.20	An	tibiotic susceptibility test (Etest)	.70
2.21	Sta	tistical analysis	70
Ch	apte	r Three: <i>Tn</i> 917 mutants characterisation and identification	72

3.1 Assay of attachment to polystyrene72
3.1.1 Assay of attachment of wild type 10403s at 37°C74
3.1.2 Assay of attachment of wild type and transposon mutants to polystyrene
at 37°C
3.1.3 Assay of attachment of wild type and transposon mutants to polystyrene
at 30°C
3.1.4 Assay attachment of wild type and transposon mutants to polystyrene at
18°C
3.2 Assay of attachment to stainless steel (quantitative adhesion assay)
3.3 Scanning Electron Microscopy83
3.4 Arbitrary PCR to identify the genes disturbed by <i>Tn</i> 917 in <i>L. monocytogenes</i>
transposon mutants
3.5 Discussion
Chapter Four: Deletion mutant creation101
4.1 Construction of mutants in genes encoding three putative NADH oxidase in <i>L</i> .
monocytogenes 10403s101
4.1.1 Amplification of gene <i>lmo0103</i> by PCR101
4.1.2 Cloning <i>lmo0103</i> into pGEM-T-Easy vector and small scale plasmid
preparation102
4.1.3 Restriction digestion of plasmid pl0103103
4.1.4 Digestion of plasmid pAUL-A with <i>EcoRI</i> 104

4.1.5	Subcloning of <i>lmo0103</i> insert into pAUL-A and transformation of	
pAUL-(	03 into <i>L. monocytogenes</i> 10403s105	
4.1.6	Amplification of the <i>lmo2235</i> and <i>lmo2471</i> ORF by PCR106	
4.1.7	Preparation and digestion of plasmid pAUL-A with <i>BamHI</i> 108	
4.1.8	Cloning and analysis by PCR of clones of <i>lmo2235 and lmo2471</i> in	
pAUL-	A109110	
4.1.9	Recombination of pl2235 and pl2471 into <i>L</i> .monocytogenes111	
4.2 Co	nstruction of a mutant in lmo0504, an ORF which encode a putative	
penicillin	binding protein, in <i>L. monocytogenes</i> 10403s115	
4.2.1	Amplification of the genes <i>lmo0540</i> by PCR115	
4.2.2	Cloning and analysis by PCR of clone <i>lmo0540</i> in pAUL-A116	
4.2.3	Recombination of pl0540 into L.monocytogenes 10403s117	
4.3 Co	nstruction of deletion mutant in <i>lmo2236</i> , a putative oxidoreductase in <i>L</i> .	
monocyto	genes 10403s120	
4.3.1	Amplification of the genes <i>lmo2236</i> PCR120	
4.3.2	Cloning and analysis by PCR of clone of <i>lmo2236</i> in pAUL-A121	
4.3.3	Recombination of pl2236 into <i>L</i> .monocytogenes122	
4.4 Discu	126 Ission	
Chapter Five: Characterisation of <i>L monocytogenes</i> mutants attachment		
Error! Bookmark not defined.		
5.1 Gr	owth of <i>L. monocytogenes</i> wild type and mutants129	

	5	0	51	
5.2	Attachment studies			

5.2.	.1	Assay for attachment of <i>L. monocytogenes</i> wild type and mutants at $37^{\circ}C$
5.2.	.2	Assay attachment of <i>L. monocytogenes</i> wild type with mutants at $30^{\circ}$ C
•••••		
5.2.	.3	Assay of attachment assay of <i>L. monocytogenes</i> wild type and mutants at
18°	С	
5.3	Effe	ect of NaCl concentration and pH on the attachment of L. monocytogenes
strains	s at d	ifferent temperatures
5.3.	.1	Effect of NaCl on <i>L. monocytogenes</i> attachment at 37°C139
5.3.	.2	Effect of NaCl on <i>L. monocytogenes</i> attachment at 30 °C141
5.3.	.3	Effect of acidic pH on <i>L. monocytogenes</i> attachment at 37°C143
5.3.	.4	Effect of acidic pH on <i>L. monocytogenes</i> attachment at 30°C143
5.4	Ass	ay of the attachment of wild type and $\Delta lmo2236$
5.5	Qua	intitative real-time PCR (qRT-PCR) analysis of <i>lmo2235</i> and <i>lmo0540</i>
gene e	expre	ssion in <i>L. monocytogenes</i> 10403s147
5.5.	.1	Listerial RNA extraction and purification147
5.5.	.2	Impact of temperature on <i>L. monocytogenes</i> 10403s gene expression148
5.5.	.3	The impact of temperature on planktonic cells of L. monocytogenes
104	03s a	and cells detached after attachment
5.6	Stuc	dy of genes <i>lmo2236</i> , <i>lmo0539</i> and <i>lmo0541</i> in B265 and I366 transposon
mutan	nts	
5.7	Ant	ibiotic susceptibility test (Etest)154
5.8	Min	imum inhibitory concentration MIC determinations154

5.9	NADH oxidase activity	156
5.10	Discussion	159
Cha	apter Six: General Discussion	166
Арре	endix	176
Refe	erence	

#### **1** Introduction

#### 1.1 Listeria monocytogenes

#### 1.1.1 The history and classification of *Listeria monocytogenes*

The official discovery of *Listeria* dates back to 1926 when Murray, Webb and Swann investigated the cause of sudden death of rabbits and guinea pigs at the University of Cambridge. They isolated a *Bacterium monocytogenes* (Murray *et al.*, 1926). The name of the organism was derived from the characteristic monocytosis in infected laboratory animals (Farber & Peterkin, 1991). A year later, in 1927, Pirie discovered a similar organism from the liver of an infected gerbil. He named the organism *Listerella hepatoltica*, dedicating it in honour of the famous surgeon Lord Lister (Wagner& McLauchlin, 2008). Because of the identity between the Murray and Pirie discoveries, the National Type Collection at the Lister Institute in London decided to rename this bacterium *Listerella monocytogenes*. Thirteen years later in 1939, the generic name *Listerella* was rejected by the Judicial Commission of the International Committee on Systematic Bacteriology, because it had been previously assigned to name other microorganisms, such as a *mycetozoan* in 1906 and for a species of foraminifera in 1933, in honour of Joseph Jackson. Although the name *Listeria* was proposed by Pirie in 1949, numerous names were used to designate *L. monocytogenes* after that.

Several earlier reports may have described *Listeria* isolation before the report of Murray. The first was by Hulphers (1911) who named the organism *Bacillus hepatis* after isolation from a rabbit (Rocourt & Buchrieser, 2007). This was followed by isolation by Atkinson in 1917 and Dick and Dumont in 1921 (Rocourt & Buchrieser, 2007).

*Listeria* was classified for a time in the family of *Corynebacteriaceae* in the sixth and seventh editions of Bergey's Manual of Determinative Bacteriology, published in 1948 and 1957 respectively (Stuart & Pease, 1972). Finally *Listeria* was reclassified with *Lactobacillus, Erysipelothrix, Borchothrix, Renibacterium, Kurthia* and *Caryophanon* as a nonsporing Gram positive rod in the eighth edition of Bergey's Manual of Systematic Bacteriology (Farber & Peterkin, 1991). Later, reverse transcriptase sequencing of 16S rRNA showed that *Listeria* is phylogenetically remote from *Lactobacillus* and should merit a separate family, the *Listeriaceae*, due to the distance between *Lactobacillus* and *Listeria* 23SrRNA with other Gram positive bacteria that have low G+C contents, *Listeria* exhibited the highest match with *Bacillus* and *Staphylococcus* (Sallen *et al.*, 1996).

*Listeria monocytogenes* was the only recognised species in the genus *Listeria* until 1961 (Roccourt *et al.*, 1982). DNA, DNA hybridization and 16S rRNA sequencing data indicated that the genus *Listeria* consisted of six species (Rocourt *et al.*, 1992).

#### **1.1.2** Characteristics of the *Listeria* genus

The *Listeria* genus is characterised as Gram positive cocco-bacilli and motile nonspore-forming, non-capsulated bacteria. They are facultative anaerobes, which prefer microaerophillic conditions (Wagner& McLauchlin, 2008). The genus consists of seven low G+C content species; *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. grayi*, *L. seeligeri*, *L. welshimeri* and *L. marthii* (Rocourt *et al.*, 1992; Graves *et al.*, 2010). *L. ivanovii* was divided into two subspecies, *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *Londoniensis* (Boerlin *et al.*, 1992). Two of these species, *L. monocytogenes* and *L. ivanovii*, are known to cause infections in humans and animals (Seeliger *et al.*, 1984); (Vázquez-Boland *et al.*, 2001). A third species, *L. seeligeri*, was considered non-pathogenic (Rocourt & Grimont, 1983; McLauchlin, 1996). Recently it has been implicated in at least one case of human listeriosis (Rocourt *et al.*, 1986).

The cell wall composition in *L. monocytogenes* and other *Listeria* spp. has been studied (Wagner & McLauchlin, 2008). The listerial cell well showed it to be typical of a Gram-positive bacterium, i.e. it is composed mainly of a multilayer of peptidoglycan (35%) consisting of cross-linked meso-diaminopimelic acid (meso-DAP). The remaining carbohydrate cell wall content consists of teichoic acids, in addition to N-acetylmuramic acid and N-acetylglucosamine, Ribitol and lipoteichoic acid Wagner& McLauchlin, 2008)

#### **1.1.3** Morphology and Growth Requirements of *L. monocytogenes*

*L. monocytogenes* is a regular, short rod, 0.4 to 0.5  $\mu$ m in diameter and 1-2  $\mu$ m in length with parallel side and blunt ends and usually occurs either singly or in short chains or filaments, 6  $\mu$ m in length, also may develop (Wagner & McLauchlin, 2008) *L. monocytogenes* possesses peritrichous flagella, which give it a characteristic tumbling motility (Low & Donachie, 1997). *L. monocytogenes* is catalase positive and oxidase negative and expresses a  $\beta$ -haemolysin (Farber & Peterkin, 1991). The haemolytic activity of *L. monocytogenes* is due to the action of listeriolysin O (LLO), which is a major listerial virulence factor (Cossart & Mengaud, 1989).

*L. monocytogenes* is psychrotrophic in nature. It can grow at temperatures between 0 and 45°C (Junttila *et al.*, 1988; Bajard *et al.*, 1996) with optimal growth between 30°C

and 37°C (Low & Donachie, 1997). It is highly flagellated and motile at temperatures  $30^{0}$ C and below and typically is not motile at temperature of 37°C or above (Peel *et al.*, 1988). *L. monocytogenes* strains was found to be non-motile and produced little or no flagellin at 37°C compared to *L. innocua* which latter produces substantial amounts of flagellin and is motile at this temperature (Kathariou *et al.*, 1995). Flagella synthesis is temperature regulated and is associated with virulence and adherence to abiotic materials (Gründling *et al.*, 2004).

*L. monocytogenes* growth can occur at a permissive pH range from 4.3 - 9.5 (Te Giffel & Zwietering, 1999; Giotis *et al.*, 2008). Further to this, *L. monocytogenes* is capable of surviving pH well beyond 4.3 - 9.5 and culture in a pH or less than 5.2 may result in loss of growth but which does not eliminate *L. monocytogenes* from foods (Wagner & McLauchlin, 2008).

*L. monocytogenes* is halotolerant (Tienungoon *et al.*, 2000), and has been reported to grow in 10% (w/v) NaCl and survive in a high concentration of salt. *L. monocytogenes* is one of the few foodborne pathogens that can grow at an  $a_w$  below 0.93 (Petran & Zottola, 2006) although the optimal  $a_w$  for its growth has been determined to be between 0.98 and 0.99 (Ross *et al.*, 2000).

In the laboratory, *L. monocytogenes* requires biotin, riboflavin, thiamine, thioctic acid and some amino acids for growth (Premaratne *et al.*, 1991). *L. monocytogenes* can grow well on a number of non-selective microbiological media, such as tryptone soy broth and agar, tryptose blood base agar, and brain-heart infusion (BHI) broth. These support excellent growth, presenting colonies with a characteristic blue-green sheen appearance under obliquely transmitted light on nutrient agar between 24-48 hours at 37°C (Pearson & Marth, 1990). Other media have been developed for isolation and selection of *Listeria*, including modification of Stuart's Transport medium and Lovett's medium for enrichment of *Listeria* in milk (Wagner & McLauchlin, 2008).

#### 1.1.4 Serotyping of L. monocytogenes

Serotyping is a classic phenotypic tool for epidemiological studies. Serotyping of *L. monocytogenes* is based on ribitol and lipoteichoic acid (O-factor) and flagella antigens (H-factor). To date at least thirteen serotypes have been described in *L. monocytogenes*: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 (Allerberger, 2006; Wagner & McLauchlin, 2008) as shown in Table 1-1.

Based on molecular subtyping studies, *L. monocytogenes* is divided into three main lineages: I, II and III. The lineage I cluster includes serotypes 1/2b, 3b, 4d, 4e and most strains of serotype 4b. Lineage II includes serotypes 1/2a, 1/2c, 3a and 3C, while lineage III includes serotypes 4a, 4c and some serotype 4b strains (Zhang *et al.*, 2003; Nightingale *et al.*, 2005). The lineage III is the less common lineage and has recently been subdivided into three subgroups based on genotypic and phenotypic characterisation; these subgroups are termed lineages IIIA, IIIB and IIIC (Roberts *et al.*, 2006).

All serotypes have the capability to cause disease (Lukinmaa *et al.*, 2003); however, epidemiological data suggests that serotypes 4b, 1/2a and 1/2b are involved in the majority of human *L. monocytogenes* infections which are implicated in 90% of outbreaks of listeriosis (Lukinmaa *et al.*, 2003; Gasanov *et al.*, 2005). However the most serotyping *L. monocytogenes* strains involved in human infection was found to differ from strains found in contaminated food (Gasanov *et al.*, 2005). Serotype 4b was found responsible for 45-70% of sporadic cases of listeriosis (Rocourt, 1996).

Serotypes also appear to be related to the reported difference in the ability of some serotypes to adhere to stainless steel (Kalmokoff *et al.*, 2001). Serotype 1/2c demonstrated the highest degree of adsorption to stainless steel of any serotype isolated from a food-processing environment (Lunden *et al.*, 2000). A recent study also found that the *L. monocytogenes* 1/2c serotype adheres better to stainless steel than serotypes 1/2a and 4b (Norwood & Gilmour, 1999). Kalmokoff and his workers (2001) found that most *L. monocytogenes* strains from biofilm are isolated from listeriosis outbreaks covering serotypes 1/2a, 1/2b, 1/2c, 4b, and 4c. Djordjevic *et al.*, (2002) reported that lineage I *L. monocytogenes* strain produced a biofilm greater than those observed for lineage II and III strains.

Serovar designation	O-antigen	H-antigen
1/2a	I II (III)	AB
1/2b	I II (III)	ABC
1/2c	I II (III)	BD
3a	II (III) IV	AB
3b	II (III) IV (XII) (XIII)	ABC
3c	II (III) IV (XII) (XIII)	BD
4a	(III) (V) VII IX	ABC
4ab	(III) V VI VII IX X	ABC
4b	(III) V VI	ABC
4c	(III) V VII	ABC
4d	(III) (V) VI VII	ABC
4e	(III) V VI (VIII) (IX)	ABC
7	(III) XII XIII	ABC

**Table 1-1** Serovars of L. monocytogenes with the detected O and H antigens (adapted from Allerberger, 2006)

#### 1.1.5 Habitats of L. monocytogenes

*L. monocytogenes* is a ubiquitous organism, which is present in many animals (including ruminants, birds, insects, fish, crustaceans and humans (Schoder *et al.*, 2003; Nightingale *et al.*, 2004; Wagner *et al.*, 2005), soil and waste water, fruits (Heaton & Jones, 2008), dust, and surface water (Weis & Seeliger, 1975; Farber & Peterkin,

1991). *L. monocytogenes* has also been found in silage, sewage, slaughterhouse waste, milk, and human and animal faeces (Cox *et al.*, 1989). It also has been found in poultry, dairy products (Cox *et al.*, 1989; Sauders & Wiedmann, 2007; Chambel *et al.*, 2007) and on the surfaces of equipment in food-processing factories. The main source of *L. monocytogenes* is soil and rotting vegetation, where they survive as saprophytic organisms (Sauders & Wiedmann, 2007). *L. monocytogenes* can also be a commensal organism living in the intestinal tract, as it has been isolated from the faeces of humans and animals not exhibiting signs or symptoms of disease (Wagner & McLauchlin, 2008). *L. monocytogenes* has also been detected in various types of seafood, including shrimps, lobster, crab, mussels and fish (Brackett & Beuchat, 1990; Elliot & Kvenberg, 2000).

#### 1.1.6 L. monocytogenes in soil and vegetation

L. monocytogenes is a saprophytic organism which can live widely in the natural environment, associated with soil and vegetation (Sauders & Wiedmann, 2007). L. monocytogenes is able to survive and may even multiply in many soil types and decaying vegetation (Sauders & Wiedmann, 2007. In early studies, L. monocytogenes was isolated from plant samples collected from cornfields, grain fields, cultivated fields, wildlife feeding areas and forests (Weis & Seeliger, 1975). Although L. monocytogenes has rarely been isolated from growing grass and vegetables, it has been detected in cut grass samples (leaves and stems) from some crops that had wilted before the ensiling process (Fenlon *et al.*, 1996). Although the prevalence of L. monocytogenes on plants is low, some human listerial infection has been linked with

consumption of lettuce and vegetables contaminated by *L. monocytogenes* (García-Gimeno *et al.*, 1996).

It is believed that animal faeces, waste and sewage sludge are the source of plant and soil contamination (Fenlon *et al.*, 1996). For example, an outbreak of 42 cases of human listeriosis was linked to consumption of coleslaw produced from cabbage obtained from a farm with a history of ovine listeriosis (Sauders & Wiedmann, 2007).

#### 1.1.7 L. monocytogenes in dairy products

Milk and milk products, including ice cream, butter, frozen desserts and frozen yogurt, have been shown to support growth of *L. monocytogenes* (Rosenow & Marth, 1987). Milk can be contaminated directly from cows with mastitis or from environmental sources (Surak & Barefoot, 1987). Although it was suggested that Pasteurized milk was the probable cause for an outbreak in Massachusetts (Fleming *et al.*, 1985), the World Health Organisation claimed that *L. monocytogenes* cannot survive even at minimum Pasteurisation levels. Several studies have been done to determine the incidence of *L. monocytogenes* in milk and cheese. These are summarised in Table 1.2.

As *L. monocytogenes* can survive and grow at refrigeration temperature, it is a serious threat to consumers and to the food industry. The risk from *L. monocytogenes* contamination in cheese is high compared with other milk products and this is due to the ability of the organism to survive for long durations of time at a pH of 5.0 (Ryser & Marth, 1989). The storage temperature also may permit growth during prolonged storage and transport time (Farber, 1989). In contrast, the incidence of *L. monocytogenes* is very low in other milk products, such as ice cream, since the storage temperature does not permit growth during storage ( $-18^{\circ}$ C) (Chen *et al.*, 2003). *L.* 

*monocytogenes* was also found to reach potentially hazardous levels in fluid dairy products held at 4°C (Rosenow & Marth, 1987). More recent data suggest that slow growth of *L. monocytogenes* can even occur in milk held at 0°C (Lado & Yousef, 2007).

*L. monocytogenes* serotype 12/b was isolated from a washed cheese in an environmental sample in Japan during routine listerial monitoring of 123 domestic cheeses (Makino *et al.*, 2005). A survey of dairy processing plants in California in 1987 showed that 46 plants (29.5%), of 156 sampled, were positive for *Listeria* (Brocklehurst *et al.*, 1987). Other studies have pointed to the isolation of *L. monocytogenes* from dairy processing plants, on processing and packing equipment, especially in wet, difficult-to-clean environments such as conveyor belts, floor drains, condensate, storage tanks, hand trucks, and wooden shelves in the cheese ripening room (Charlton *et al.*, 1990; Cox *et al.*, 1989; Nelson, 1990).

In a study focusing on the environment rather than the cheese itself, eight artisanal cheese-making dairies in Portugal were sampled for the presence of *Listeria* spp. at three different times in the production cycle over the course of a single year (2004). The investigation concerned the internal environment of each dairy, such as walls, drains, shelves and most of the various types of equipment used in the dairies to make cheese. *L. monocytogenes* and *L. innocua* were the two most prevalent species. *L. monocytogenes* was isolated from three production sections as shown in Figure 1.1. It was found in the working room where it was isolated from multiple sites, whereas it was isolated from only two sites in the ripening room. In the washing room there were very few instances of *L. monocytogenes* isolation (Chambel *et al.*, 2007).

Dairy cows and other animals can carry the organism in their intestinal tract without becoming sick. In rare cases, contact with infected cows has caused listerial skin infection in veterinarians (Surak & Barefoot, 1987).

Year	Reference	Source	Deaths	Overall incidence %
1983 (Fleming et al., 1985)	New England	Pasteurized milk	14	
1985 (James et al., 1985)	California	Mexican-style fresh cheese	29	-
1985 (Malinverni et al., 1985)	Switzerland	Vacherin Mont d'Or cheese	31	-
1987 (Lovett et al., 1987)	Massachusetts and California	Raw milk from farm bulk tanks	-	4.2
1988 (Liewen & Plautz, 1988)	Nebraska	Raw milk	-	4
1987 (Beckers et al., 1987)	Netherlands	Soft cheese made from raw milk	-	10
1987 (Boer & Kuik, 1987)	Netherlands	Blue-veined cheese	-	7
1986 (Garayzábal <i>et al.</i> , 1986)	Spain	Pasteurized milk	-	21.4
1995(McLauchlin et al., 2004)	France	Soft cheese	4	-
1998-1999 (McLauchlin <i>et al.</i> , 2004)	Finland	Butter	6	-
2005 (Jemmi & Stephen, 2006)	Switzerland	Soft cheese	1	-

**Table 1-2** Episodes of *L. monocytogenes* contamination of milk and cheese in USA and European countries.

- = Data not available



**Figure 1-1** Distribution of *L. monocytogenes* in the different sections of Portuguese cheese dairies during the production cycle (Chambel *et al.*, 2007, with permission). Represents *L. monocytogenes* isolated from various sections of the site of production: 1. whey drain; 2. whey stand; 3. vat; 4. bench; 5. shelves. Arrows refer to the direction of the cycle of cheese production.

#### 1.1.8 L. monocytogenes in other foods and food processing

*L. monocytogenes* has been isolated from many food products, including fruit and vegetables, meat (particularly ready-to-eat meats), and seafood (particularly smoked fish) (Petran *et al.*, 1988; Glass & Doyle, 1989; Gitter, 1976; Weagant *et al.*, 1988; Chasseignaux *et al.*, 2002; Lianou & Sofos, 2007). Gitter (1976) isolated *L. monocytogenes* serotype 4 and 1/2 from 14.7% of oven-ready poultry from supermarkets (Gitter, 1976). Petran *et al.* (1988) did not isolate *L. monocytogenes* from fresh lettuce, potato peel, corn husks, broccoli stems, cabbage outer leaves, carrot peels, cauliflower stems, mushroom stems, spinach, and beet peels from a local supermarket (Petran *et al.*, 1988). Additionally, frozen beans, pea pods, and spinach were examined and were found to be *Listeria*-free (Petran *et al.*, 1988). However, Steinbruegge *et al.* 

(1988) isolated *L. monocytogenes* from lettuce and also showed that the population of *L. monocytogenes* increased during storage (Steinbruegge *et al.*, 1988). More recently, *L. monocytogenes* has been isolated from a variety of plant foods including, salad vegetables (Sagoo *et al.*, 2003), green table olives (Caggia *et al.*, 2004), spinach (Jablasone *et al.*, 2005), mushrooms (Samadpour *et al.*, 2006) and melons (Varma *et al.*, 2007).

*L. monocytogenes* was reported to be present in over a quarter of all frozen seafood product sampled, such as shrimp, crabmeat, lobster tail, langoustines, scallops and surimi based imitation seafood (Weagant *et al.*, 1988; Brackett & Beuchat, 1990; Elliot & Kvenberg, 2000).

Studies have detected *L. monocytogenes* in raw chicken with a recovery rate of 11.4% (Wang *et al.*, 1992) and 59% of total samples (Lawrence & Gilmour, 1994). In Spain *L. monocytogenes* was found on 15% of the chicken carcasses tested (Capita *et al.*, 2001).

Floors, floor drains, racks and rollers, conveyor belts, slicer blades, packing equipment, doors, and benches in processing plants are believed to present the primary source of food industries contamination (Pearson & Marth, 1990; Lunden *et al.*, 2002; Lunden *et al.*, 2002). Therefore contamination of food by *L. monocytogenes* may occur directly from farms or other environments, or via contact with those surfaces used to prepare processed foods, or indirectly, by contact with a person or other piece of equipment that has come into contact with a contaminated surface (Lunden *et al.*, 2002).

Contamination of food by *L. monocytogenes* can occur at several stages of the food production and processing cycle (Lianou & Sofos, 2007). Food survey studies demonstrated that the initial point of listerial contamination often begins in the food processing environment during processing or post-processing food rather than being

due to survival during the processing itself (Van den Elzen & Snijders, 1993; Ojeniyi *et al.*, 2000; Norton *et al.*, 2001). Thus, the best way to avoid a public health problem with food products is to prevent *L. monocytogenes* from entering these products before, during, and after manufacture.

In 2002, the largest food recall incident in the United States was linked to ready-to-eat (RTE) deli turkey contaminated with *L. monocytogenes*. The outbreak affected multiple states in the north-eastern region of the United States, with a total of 46 confirmed cases of infection. There were seven deaths and three stillbirths reported during the outbreak. The mortality rate from this outbreak reached 22% (CDC, 2002). Subsequently, 27.4 million pounds of product was recalled and the processing plant involved suspended operation (CDC, 2002). This outbreak prompted the United States Food and Drug Administration (FDA), the Food Safety and Inspection Service (FSIS), and the United States Department of Agriculture (USDA) to mandate a 'zero tolerance' policy (no detectable level) on RTE food products (USDA-FSIS, 2003).

*L. monocytogenes* is one of the most difficult organisms to eliminate from foodprocessing plants (Rocourt & Buchrieser, 2007), due to its ability to grow at refrigeration temperatures (Lado & Yousef, 2007), and in conditions of high salinity and acidic pH (George *et al.*, 1988; Parish & Higgins, 1989; Sorrells *et al.*, 1989). *L. monocytogenes* can also survive and multiply under various stress conditions including those likely to be encountered on food processing plant surfaces (Garner *et al.*, 2006). It can be found not only in food products but also attached to and forming biofilm on many materials that are used in food processing plants (Beresford *et al.*, 2001). Biofilm formed by *L. monocytogenes* in food-processing environments can be a potential source of contamination and has been implicated in several food-borne outbreaks. It is a matter of concern that environmental conditions may actually contribute to this bacterial adherence in specific food-processing operations and to increased resistance to disinfection. Two factors that may enhance *L. monocytogenes* biofilm formation are low pH values and salt addition (Adrião *et al.*, 2008). Recently, Adrião *et al.* (2008) examined the effect of low pH and high salt concentration on *L. monocytogenes* in food processing (particularly in cheese-manufacturing processes) and their data revealed that a sub-lethal pH and salt concentration may protect cells of some strains of *L. monocytogenes* attached to stainless steel and polystyrene surfaces and enhance the survival of attached cells (Adrião *et al.*, 2008).

#### 1.1.9 Virulence factors of L. monocytogenes

To date a number of *L.monocytogenes* genome sequences have been completely sequenced. The genome is approximately 3.0 Mb in length (Michel & Cossart, 1992). Six *L. monocytogenes* genome sequences are available, and these are shown in Table 1-3. Although the genome sequences of *L. monocytogenes* have been published, a large number of proteins are still hypothetical or are of unknown function.

Strain	Size of the chromosome (bp)	Protein codes
<i>L. monocytogenes</i> EGDe (1/2a) <sub>a</sub>	2944528	2846
L. monocytogenes HCC23 4a <sub>b,c</sub>	2976212	2974
L. monocytogenes F2365 (4b) <sub>a</sub>	2905187	2848
L. monocytogenes Clip80459 4b b,c	2912690	2766
L. monocytogenes 08-5578b 1/2a b	3109342	2766
L. monocytogenes 08-5923b 1/2a b	2999054	2966

 Table 1-3 Strains of L. monocytogenes with completely sequenced genomes.

a. (Buchrieser, 2007). b, KEGG Genome. c, National Centre for Bioinformation

Virulence and virulence-like genes are factors which help L. monocytogenes to adapt and survive in diverse environments, including hosts, during infection (Bierne & Cossart, 2007). L. monocytogenes contains a virulence genes cluster (8.2 kb) which is located between prs and orfB genes in the Listeria genome and it is the only pathogenicity island identified in *Listeria* (Chakraborty et al., 2000). The genes in this pathogenicity island are regulated by the regulatory factor A prfA (Chakraborty et al., 1992). The cluster encodes ten genes located into two distinct loci on the chromosome. Figure 1-2 shows seven genes (prfA, plcA, hly, mpl, actA, plcB, and orfX) clustered together in LIPI-1. The other three PrfA dependent transcriptional units inIAB, which encodes *inlA*, *inlB*, *inlC* and *hpt*, are located at different points of the *L*. *monocytogenes* genome. These genes and their function are listed in Table 1-4. The function of the three small open reading frames (orf X) X, Y, and Z are still unknown. The other virulence genes result in the protein products of hly, plcA, plcB and actA encode listeriolysin O (LLO), phosphatidylinositol-specific phospholipase C (PI-PLC), phosphatidylcholine-specific phospholipase C (PC-PLC), and an actin polymerisation protein (ActA) respectively. LLO is a secreted protein of 58 kDa belonging to the cholesterol-dependent family of toxins, which can promote the intracellular growth by promoting listerial escape from the phagosomes of phagocytes (Berche et al., 1987). Loss of listeriolysin O production is associated with loss of virulence (Gaillard et al., 1986). The other three PrfA dependent transcriptional units genes are still related to the gene cluster (Kuhn et al., 1988) and are responsible for producing surface proteins necessary for internalization of the Listeria to the host cell; these include the inlAB (encodes internalin A and B) and *inlC* genes which encodes for internalin C (Lingnau et al., 1995).

Gene	Function
prfA	Encoding positive regulatory factor
hly	Encoding listeriolysin O (LLO), escaping from the phagocytic vacuole
plcA	Encoding phosphatidylinositol-specific phospholipasee C (PI-PLC), escaping from the phagocytic vacuole
plcB	Encoding phosphatidylcholine-specific phospholipase C (PC-PLC) escaping from vacuoles
mpl	Encoding metalloprotease, maturation of the <i>plcB</i>
ActA	Encoding an actin polymerization protein A, necessary for intracellular actin- based motility and cell-to-cell spread
InlA	Encoding internalin A mediate, invasion of non-phagocytic cells
InlB	Encoding internalin B, invasion of non-phagocytic cells

**Table 1-4** List of genes regulated directly by PrfA and their function (Adapted from Roberts &Wiedmann, 2003)



**Figure 1-2** Schematic representation of the virulence gene cluster located in *L. monocytogenes* EGD-e genome. The listerial pathogenicity island-1 (LIPI-1) genes and the three other prfA regulated loci, *inlA*, *inl, inlC* and *hpt*. The PrfA boxes are indicated by black boxes (Adapted from (Scortti *et al.*, 2007).
# 1.1.10 Pathogenesis and intracellular infectious cycle

L. monocytogenes has three distinct life styles, (i) an extracellular free-living as a saprophyte, (ii) an extracellular bacterium colonised in a biofilm and (iii) an intracellular facultative parasite capable of causing serious foodborne illness (Gray & Killinger, 1966; Vazquez-Boland et al., 1992; Vázquez-Boland et al., 2001a). The transcriptional regulator PrfA has previously been reported as a factor that switches L. monocytogenes from extracellular bacterium to intracellular pathogen (Freitag 2009). The intestinal tract is the major portal of entry for L. monocytogenes following ingestion of contaminated food (Lundén et al., 2004; Mead et al., 2006). The liver is thought to be the first target after intestinal translocation (Vázquez-Boland et al., 2001). In the liver the bacteria multiply until the cell-mediated immune response eliminates the infection during the first 6 hours after infection. However, any surviving bacteria replicate and re-enter the blood stream and spread to various organs, including the central nervous system (CNS) and placenta of pregnant women (Vázquez-Boland et al., 2001; Drevets & Bronze, 2008). L. monocytogenes needs to be present in high numbers, probably in the order of  $10^8$ , for the infection to establish. Each step of the intracellular parasitism by L. monocytogenes is dependent upon the production of virulence factors. (Portnoy et al., 1992).

The initial attachment of *L. monocytogenes* to host cell surface is due to the recognition of listerial ligands by host receptors (Figure 1-3a). These ligands are characterised as surface proteins. It has been proposed that a cellular surface protein of *L. monocytogenes* interacts with receptors in the intestinal lining and induces phagocytosis of the bacterium (Brooks *et al.*, 1998). The surface proteins InIA and InIB (Braun & Cossart, 2000) were found to be responsible for promoting the binding and

internalisation by either E-cadherin or the Met receptor tyrosine kinase and PI3-kinase activation respectively (Cossart, 2001). ActA has also been reported to be one of the listerial ligands (Lecuit *et al.*, 2001). The infection cycle starts with adhesion of bacteria cells to host cell surfaces and is then followed by penetration into the host cell by phagocytosis (Vázquez-Boland *et al.*, 2001a).

After entry, Listeria disrupts the host phagosomes and starts proliferation (Goebel & Kreft, 1997) (Figure 1-3b). The bacteria escape the phagosomes by the action of Listeriolysin-O (LLO) (Kuhn et al., 1988; Portnoy et al., 1988; Gedde et al., 2000). A phosphatidylinositol-specific phospholipase C (PI-PLC) also promotes escape from the phagocytic vacuole (Goldfine et al., 2000; Wadsworth & Goldfine, 2002). Subsequent to its escape from the phagosomes, the bacterium multiplies in the cytosol and acquires actin-based motility, which enables it to form filopods at one end of the bacterium, thus facilitating movement and spread from cell to cell (Tilney & Portnoy, 1989; Kocks et al., 1993) (figure 1-3c,d). This actin-based motility depends on actin polymerization of protein (ActA) by actA (Domann et al., 1992; Kocks et al., 1992). Once the bacteria come into contact with the plasma membrane of neighbour cells they produce protrusions (Figure 1-3e) and are then engulfed in a new vacuole forming a double membrane vacuole. A phosphatidylcholine-specific phospholipase C (PC-PLC) of the bacteria then degrades the double membrane vacuole (Vazquez-Boland et al., 1992) and starts the process again with new host cells. The other cells move to the host cytoplasm and start proliferating (Figure 1-3f, g).



**Figure 1-3** Scheme of Intracellular invasion by *L. monocytogenes* (Adopted from (Tilney & Portnoy, 1989). a, Attachment and internalization (InIA and InIB). b, escape from phagosomes (*hly* and *plcA*). c, replication within cytoplasm. d, Actin filament assembly (*actA*). e, tail generation motility to invade neighbour cells (*actA*). f, formation of double membrane phagosomes. g, escaping from phagosomes and reinitiating of the cycle (*plcB* and *hly*).

# 1.1.11 Listeriosis

*L. monocytogenes* is recognized as the causative agent of listeriosis. Although listeriosis is a serious disease, it still remains an uncommon, representing a very small fraction of all illness due to food-borne pathogens (Mead *et al.*, 1999). Annually, there are between two and eight sporadic cases of human listeriosis per million inhabitants in the United States and Europe (Tappero *et al.*, 1995). The average mortality rate of human listeriosis is high at about 30%, resulting from food industry contamination and food recalls (Schlech III & Acheson, 2000; Hof, 2001; Hamon *et al.*, 2006). This is much higher than that for other common food-borne pathogens such as *Salmonella*,

*Campylobacter* and *E. coli*, as shown in Table 1-5 (Mead *et al.*, 1999). Globally, it is estimated that *L. monocytogenes* causes approximately 1600 cases of listeriosis annually, resulting in 400 to 500 deaths (Ramaswamy *et al.*, 2007). It has also been estimated that 2500 cases of *L. monocytogenes* infection occur in the United States each year (Mead *et al.*, 1999). *L. monocytogenes* was also found to be responsible for the highest hospitalisation rate (91%) amongst known food-borne pathogens (Mead *et al.*, 1999)

Pathogens	Total Cases	Mortality Rate (%)	Hospitalisation Rate (%)
Campylobacter spp	~1.9 million	<1	17.3
<i>E. coli</i> O157:H7	~62,500	<1	3.0
Salmonella spp	~1.3 million	<1	25.6
L. monocytogenes	2500	20-30	3.8

 Table 1-5 The mortality rate among food-borne pathogens in the United States (Mead *et al.*, 1999).

Listeriosis is clinically defined as the presence of *L. monocytogenes* in a normally sterile site of the body (Ramaswamy *et al.*, 2007). Serovars 1/2a, 1/2b and 4b of *L. monocytogenes* are responsible for the majority of cases of human listeriosis, constituting more than 95% of human clinical isolates (Tran & Kathariou, 2002). The minimum infective dose of *L. monocytogenes* is unknown, but is believed to vary in accordance with the immunity of the infected individual and the strain type (Bailey *et al.*, 1989). However infectious doses of *L. monocytogenes* associated with human infection range from ~10<sup>6</sup> cells/g to  $10^3$  cells/g (Farber & Peterkin, 1991).

Listeriosis is normally caused by ingestion of contaminated food. Briefly, *L. monocytogenes* invades the intestinal epithelium following passage through the stomach (Vázquez-Boland et al., 2001). Once past the intestinal barrier, *L.*  *monocytogenes* spreads to the lymph nodes, the spleen, or the liver via the lymph / blood system (Vázquez-Boland et al., 2001). *L. monocytogenes* is rapidly eliminated from the blood stream by macrophages in the liver and spleen (Vázquez-Boland et al., 2001)

Infection by L. monocytogenes can result in invasive or non-invasive listeriosis (Franciosa et al., 2001). Non-invasive gastrointestinal listeriosis is mild, non-specific and has influenza-like symptoms including chills, fatigue, headache, muscular pains, diarrhoea, vomiting and joint pain, as well as gastroenteritis. This non-invasive manifestation normally occurs in healthy adults and requires ingestion of a large dose  $(\sim 10^6 \text{ cells/g})$  of *Listeria* to result in an infection (Chen *et al.*, 2003). The invasive form of the disease results in serious systemic illness, including septicaemia, meningitis, encephalitis, spontaneous abortions in pregnant females, and in some cases death (Gellin et al., 1991). The highest incidence of invasive listeriosis has been in the elderly and in pregnant women, in whom it can lead to miscarriage or stillbirth or to meningitis in the newborn (McLauchlin, 1996; Rocourt et al., 2003). The risk of listeriosis is greatest among certain well-defined high-risk groups, including immunocompromised patients such as those with AIDS, diabetes mellitus, alcoholism, cardiovascular and renal collagen disease, and haemodialysis failure (Farber & Peterkin, 1991; Jurado et al., 1993). In North America, AIDS patients have an estimated 100-500-fold increased risk over the normal population (Schlech, 1996). L. monocytogenes is considered to be the fifth most common form of bacterial meningitis (Ramaswamy et al., 2007).

#### 1.1.12 Outbreaks of food-borne listeriosis

Food-borne outbreaks caused of listeriosis have been recognised in the USA and European countries over the early eighties, and sources in contaminated food have been determined in each continent (see Table 1-6). In Japan the first verified outbreak of food-borne listeriosis was in a neonate and was to be due to the mother eating washed-type cheese (Makino *et al.*, 2005). Since then *L. monocytogenes* has been involved in several listeriosis outbreaks in many food products. For example, Pasteurized milk, cheese made from non-pasteurized milk and other dairy products (Dalton *et al.*, 1997; Schuchat *et al.*, 1997; Schlech III & Acheson, 2000; Goulet *et al.*, 2001). However, a few listeriosis outbreaks have been associated with the consumption of contaminated vegetables. Recently, in the USA listeriosis outbreaks were linked to the consumption of melon or hummus prepared in commercial establishments (Varma *et al.*, 2007).

The food and dairy industry started to pay attention to *L. monocytogenes* as a potential food-borne pathogen due to two major cheese-related outbreaks of listeriosis. In 1985, consumption of Mexican-style cheese in California caused 142 cases of listeriosis, leading to 48 deaths (James *et al.*, 1985; Linnan *et al.*, 1988). Another outbreak of listeriosis was due to the consumption of contaminated Vacherin Mont d'Or soft-ripened cheese, which resulted in 31 deaths (Malinverni *et al.*, 1985). In the UK, it has been highlighted that pâté was the cause of an increase in the incidence of listeriosis between 1987 and 1989 (McLauchlin *et al.*, 1991). Subsequent these outbreaks, guidance was issued to those in at-risk groups such as pregnant women and those with impaired immune systems, advising avoidance of pâté and soft cheese (HPA, 2006).

The incidence of listeriosis in pregnancy is estimated to be approximately 1 in 30,000 live and stillbirths. Moreover *L. monocytogenes* is the third most common pathogen

causing bacterial meningitis among neonates, after group B *streptococcus* and *E. coli*. (Dawson *et al.*, 1999). Because of the risk of listeriosis, it is recommended that pregnant women should avoid eating certain ripened soft cheeses and meat-based pâtés because these may also contain high levels of *L. monocytogenes*.

A recent report indicates a dramatic increase in listeriosis incidence in European countries in those over 60 years of age, who are not otherwise linked by geography, gender, ethnicity, socioeconomic factors or infectious serotypes (Goulet *et al.*, 2008; Kasper *et al.*, 2009; Cairns & Payne, 2009). A recent study by Allerberger and Wagner (2009) reported that the listeriosis incidence rate in six EU countries between 1999 and 2007 was 0.3 per 100,000 populations, as shown in Figure 1.4.

Years(s)	Location (reference)	No. of cases	Mortality rate, %	Source(s)
1980-1981 (Schlech III <i>et al.</i> , 1983)	Maritime Provinces /Canada	41	34	Coleslaw
1986-1987 (Schwartz <i>et al.</i> , 1989)	Pennsylvania	36	44	Unknown
1989 (Riedo <i>et al.</i> , 1994)	Connecticut	10	10	Shrimp
1987-1989 (McLauchlin <i>et al.</i> , 1991)	United Kingdom	355	26	Pâté
1993 (Salamina <i>et al</i> ., 1996)	Italy	39	-	Rice salad
1992 (Goulet <i>et al.</i> , 1998)	France	38	32	Rillettes (pork)
1994 (Dalton <i>et al.</i> , 1997)	Illinois	45	0	Chocolate milk
1997 (McLauchlin <i>et al.</i> , 2004)	Italy	1566	0	Corn salad
1989-1999 (Schlech III & Acheson, 2000)	United States	101	21	Hot dogs/ deli meat
1999-2000 (McLauchlin <i>et al.</i> , 2004)	France	32	31	Pork tongue

**Table 1-6**Summary of data on major outbreaks of listeriosis, 1980-1999 (Schlech III &Acheson, 2000)

- = information not provided



**Figure 1-4** Listeriosis incidence in six EU countries 1999–2007 (Allerberger & Wagner, 2009, with permission)

# **1.2 Biofilms and attachment**

# **1.2.1** Microbial biofilms

Biofilms are defined as a community of microbial sessile cells that are irreversibly (not removed by gentle rinsing) attached to a substratum, interface or each other, with production of extracellular polymeric matrix and with respect to growth rate and gene transcription (Costerton *et al.*, 1995; Donlan, 2000). Biofilm in the environment is found in a multicellular sessile form and cannot exist in a unicellular planktonic form (Costerton *et al.*, 1995; Davey & O'Toole, 2000)

The first discovery of a microbial biofilm dates back to the 17th century and Van Leeuwenhoek, who observed the microbial biofilms on tooth surfaces using his simple microscope (Donlan, 2002). Later Zobell also found that the population of bacteria on solid surfaces can be higher than in surrounding medium (Zobell, 1943). After presenting the biofilm formation theory with an explanation of its mechanisms by

Costerton and his co-workers (1978), more studies in industrial and environmental biofilms were published in respect to public health (Hood & Zottola, 1995; Sinde & Carballo, 2000; Donlan & Costerton, 2002, Hall-Stoodley *et al.*, 2004).

Biofilms consist primarily of viable and nonviable microorganisms embedded in an extracellular polymeric substance (EPS) anchored to a surface (Carpentier & Cerf, 1993), which provides the bacteria within biofilm with protection from a broad range of antimicrobial and sterilising agents (Mittelman, 1998). It is known that biofilm formation is a complex process, and a number of cell surface structures have been implicated in the initial stage of biofilm formation. Flagella, fimbrae (pili), adhesion protein and surface charge have been implicated in the initial stage of biofilm formation by various bacteria (Frank, 2001).

# **1.2.2 Biofilm Matrix**

Biofilms are composed primarily of microbial cells and exopolysaccharides (EPS). Extracellular polysaccharides (EPS) were later renamed as 'extracellular polymeric substances' because of the presence of other biopolymers (Flemming et al., 2007). In most biofilms, the matrix consist of less than 10% cells, 90% extracellular polysaccharide and other polymeric substances hydrated to 85 to 95% water (Allison, 2003; Flemming & Wingender, 2010). Water provides a hydrophobic property and thus protects the biofilms from water fluctuations in the environment (Sutherland, 2001). The EPS is composed of polysaccharides, proteins, phospholipids, teichoic and extracellular DNA (Flemming et al., 2007). Recently many biofilm matrix polysaccharide components have been identified, for example polysaccharide intercellular adhesion/poly-N-acetylglucosamine (PIA/PNAG) polymer in Staphylococcus aureus and Staphylococcus epidermidis (Mack et al., 1999; Otto, 2009). EPS components identified as produced by Gram-negative organisms, such as colanic acid in *E. coli* alginate, glucose extracellular polysaccharide in *Pseudomonas aeruginosa* and B-1,6-GlcNac polymer in *Salmonella* and *E. coli* have been reported to play important roles in biofilm formation (Solano *et al.*, 2002; Friedman & Kolter, 2003; Branda *et al.*, 2005).

EPS is a key element that shapes and provides structural support for bacterial biofilms. EPS is hydrophilic and hydrophobic; a hydrophilic structure of EPS is highly hydrated and varies in its solubility and this property prevents desiccation in some natural biofilms. The rigidity of EPS is due to 1.3- or 4- $\beta$ -linked hexose residues, which give the biofilm the structure and then the conformation (Sutherland, 2001).

EPS production is increased by time and bacteria produce a varying amount of EPS during biofilm development (Leriche *et al.*, 2000). The antimicrobial resistance properties of biofilms may be due to EPS which impedes the transfer of antibiotics through the biofilm, probably by binding directly to these agents (Donlan, 2000). *L. monocytogenes* cannot produce EPS within biofilm formation and it has been suggested that *L. monocytogenes* synthesize little EPS (Chavant *et al.*, 2003).

### **1.2.3** Biofilm formation stages

Biofilm formation was originally thought to require three main sequential stages: attachment, growth and detachment. First, primary attachment of cells to a solid substrate occurs followed by cell-cell adhesion, thus creating multiple layers of cells (Stoodley *et al.*, 2002).

Recently, five stages of biofilm development have been proposed based on proteomic studies in *Pseudomonas aeruginosa* (Sauer *et al.*, 2002). These five stages are shown in

Figure 1-5: reversible attachment where individual cells attach to the solid surface and some may become planktonic (Stage1); irreversible attachment comes over time along with production of extracellular polysaccharide (Stage2); maturation and development of biofilm; attached cells multiply by binary division leading to cell clusters (Stage3 and 4) and dispersal of some cells which detach from the mature biofilm because of internal factors or external factors like shear stress (Lemon *et al.*, 2008). These detached cells become planktonic and may start entering another biofilm cycle in appropriate conditions (Stage5). Each stage of the biofilm formation process depends on the microbial genera, species, attachment surface characteristics, environmental conditions and physiological status of the microorganism.

A reversible stage of attachment of cells to surfaces occurs within 5-10 seconds; cells may return to the planktonic state and are experimentally removed easily by gentle washing (Mittelman, 1998). Irreversible attachment takes from 20 minutes to a maximum of 4 hours (Mittelman, 1998). After initial attachment, the bacterial cells start dividing and spreading on the surface as a monolayer to form microcolonies and EPS is produced as developmental changes during this stage.

The initial attachment of the microorganism is also called adsorption and is influenced by electrical charges of the bacteria, by Van der Waals forces, electrostatic factors, hydrophobic interactions, and Brownian motion (Mittelman, 1998; Donlan & Costerton, 2002). The initial colonisation is controlled by environmental and genetic factors (Hall-Stoodley *et al.*, 2004). The temporal adherence can result in irreversible attachment or cells becoming free-floating planktonic (Palmer & White, 1997).

Biofilms produced by *L. monocytogenes* are architecturally simple in comparison to those of many other microorganisms, but their contribution to resistance and

environmental persistence is widely reported (Chae & Schraft, 2000; Kalmokoff *et al.*, 2001; Rieu *et al.*, 2008).



**Figure 1-5** The stages of bacterial biofilm development (adopted from Monroe, 2007).Stage1, reversible attachment, individual cells attach to the solid surface and some may become planktonic; Stage2, irreversible attachment comes over time with extracellular polysaccharide production; stage3and 4, maturation and development of biofilm; stage5, dispersion of cells from biofilm.

# **1.2.4** Factors affecting attachment

Many factors influence the attachment of microorganism. Attachment may be affected by the structural and physiological characteristics of the cell, as well as the nature and temperature of the suspension. Surface hydrophobicity and charge of the bacterial cell, the ionic strength of the medium, and smoothness or roughness of the material surface have been identified as factors that influence the bacterial attachment (Hood & Zottola, 1997; Ong *et al.*, 1999; Seo & Frank, 1999; Arnold & Bailey, 2000; Sinde & Carballo, 2000).

The type of surface can be divided into two categories: hydrophilic, which has high surface energy materials carrying a negative charge, such as glass, minerals, and metals; and hydrophobic, which has low surface energy carrying positive or negative charge, for example plastics and rubber surfaces (Farber *et al.*, 1996; Sinde & Carballo, 2000; Chae *et al.*, 2006). Smoot and Pierson (1998) demonstrated that a lower number of *L. monocytogenes* cells adhered to Buna-N rubber and stainless steel in alkaline conditions over a short contact time. In addition they found increasing numbers of adhered cells on both surfaces with increasing temperature (from 10 to  $45^{\circ}$ C). They concluded that exposing cells of *L. monocytogenes* to suboptimal levels of environmental stress, such as pH, temperature, and the hydrophobicity of the surface determine the microorganisms' ability to attach to common food contact surfaces. (Mafu *et al.*, 1991). Other work by Rodriguez *et al.* (2008) suggests that *Listeria* biofilms adhere more strongly to hydrophobic surfaces than hydrophilic surfaces at the cellular level. In other investigations *L. monocytogenes* produced biofilm at comparable levels on glass and stainless steel and higher than on polystyrene (Di Bonaventura *et al.*, 2008).

Other factors affecting attachment have been studied; Kim *et al.* (1995) investigated the effect of nutrients on the development of biofilm using modified Welshimer's broth (MWB). They claimed that replacement of amino acids in MWB with tryptone was important in the initial phase of biofilm formation. However, the effect was not significant in biofilm formation after 12 days at 21°C, in comparison with shorter times of 1, 4 and 7 days the concentration of amino acids affected the area covered by the microorganism. In the same study, different carbohydrates were examined and it was found that the concentration of glucose in the range 1 to 20 g/L did not affect biofilm growth, while other carbohydrates, for example mannose and trehalose, improved the development of the biofilm. Stoodley and his colleagues (1997) found that decreasing the pH of media increased the thickness of the structure of biofilm by about 69% of its

original thickness. Similarly, the effect of temperature and growth medium was investigated by Mai and his colleague (2007); they found the number of cells attached to stainless steel in nutrient-rich medium and minimal medium increased with increasing temperature, with the exception at  $42^{\circ}$ C where the number of attached cells was lower than at 37°C and 30°C. In the same study *L. monocytogenes* showed greater attachment when cultivated in rich medium versus minimal medium. Recently the parameters of contact time and pressure, surface composition, and relative humidity of biofilm were evaluated and a significant effect of these parameters on biofilm adhesiveness was found at cellular level. This study also suggested that *L. monocytogenes* adheres more strongly to hydrophobic surfaces than to hydrophilic surfaces (Rodriguez *et al.*, 2008).

Data from Vatanyoopaisarn *et al.* (2000) confirmed the role of flagella in the adhesion to stainless steel under static conditions, in the early stage of attachment, as the number of cells of a non-flagellated mutant was significantly lower than the flagellated *L. monocytogenes* wild type, after only a short period of contact with the surface at 22°C but not after 24 hours or at 37°C. Importantly *L. monocytogenes* produce flagella only at temperatures below 35°C (Peel *et al.*, 1988), but biofilm may also be established at temperatures at which flagella are not expressed (Vatanyoopaisarn *et al.*, 2000). In addition Todhanakasem and his colleague (2008) demonstrated that the loss of flagellar motility alters the *L. monocytogenes* biofilm development and decreased initial bacterial surface attachment but subsequently resulted in formation of hyperbiofilms. Frank and Koffi (1990) showed that *L. monocytogenes* adhering to glass survived more than 10 times longer than free-living cells (planktonic cells) when exposed to benzalkonium chloride, anionic acid sanitizer or heat. Also, Lee and Frank (1991) found that *L. monocytogenes* adhering for 8 days were 100 times more resistant to hypochlorite than those adhering for only 4 days.

The genetic and molecular processes involved in transition to surface attachment remain obscure. Although several genes have been identified, they are only involved indirectly in attachment and biofilm formation of *L. monocytogenes*. A recent study investigated the role of Biofilm Associated Protein *BapL* in the surface attached of *L. monocytogenes*; it was found that *BapL* may be involved in surface attachment for some strains but it is not required for other strains (Jordan *et al.*, 2008).

# **1.2.5** Biofilm in environments and medicine

Bacterial biofilms can be found on a wide variety of biotic and abiotic surfaces. Biofilms have been found in Antarctica, thermal springs and deep sea environments (Costerton *et al.*, 1987; Costerton *et al.*, 1995; Davey & O'Toole, 2000). They can also be formed in soil and wet environments, on plants and on and within animals (Costerton *et al.*, 1987; Costerton *et al.*, 1995). In plants, for example, biofilms occur on the external tissues on the roots in symbiotic relationships (Davey & O'Toole, 2000). In animals biofilms can be either external, such as on teeth, or internal, throughout the mucosa of the digestive tract (Dale & Fredericks, 2005).

Biofilms become a problem and influence industrial productivity, and occur in almost in every water-based industrial process (Costerton *et al.*, 1987; Davey & O'Toole, 2000). Biofilms have been found to be involved in up to 80% of human infections based on a National Institute of Health statement in 2002 (Davies, 2003) this high incidence of biofilm related infection can be attributed to organisms within a biofilms becoming less susceptible to antimicrobial agents (Donlan, 2001). Biofilms also have been shown to be related with some human chronic and persistent infections such as cystic fibrosis, otitis media and native valve endocarditis, and they also colonise on some medical devices, such as prosthetic devices or implants (Donlan & Costerton, 2002; Hall-Stoodley *et al.*, 2004).

Biofilms can be beneficial in many cases, for example commensal organisms found in the intestine and female genito-urinary tract (Habash & Reid, 1999). Biofilms are also beneficial, as in bioremediation of hazardous waste sites or treatment of municipal or industrial waste water that require complex microbial community interactions (Costerton *et al.*, 1987; Davey & O'Toole, 2000).

# 1.2.6 Bacteria biofilms in the food processing industry

Microbial contamination of food is becoming a problem in food processing factories and it presents great health risks since as few as ten colony forming units (CFUs) of certain pathogens can cause life-threatening infections such as *Salmonella* and *E. coli* O157:H7 (Ekperigin & Nagaraja, 1998; Kaper *et al.*, 2004). Biofilms in the food industry have mainly been studied in conjunction with *L. monocytogenes* and *Salmonella* (Hood & Zottola, 1995; Sinde & Carballo, 2000). Contamination of food occurs when food comes in contact with contaminated surfaces (Eginton *et al.*, 1995; Barnes *et al.*, 1996). EPS plays an important role in initial bacterial attachment and facilitates the formation of biofilms by providing a firm adherence to solid surfaces (Marshall, 1992; Costerton *et al.*, 1995). Once formed, biofilms become highly resistant to disinfectants and other cleaning agents (Mittelman, 1998). Several studies have indicated that cells in biofilm are more resistant to sterilants and antibiotics than planktonic cells (Costerton *et al.*, 1987; Norwood & Gilmour, 1999; Parsek & Singh, 2003). Attachment to surfaces may take about 15 minutes and then microorganisms can trap nutrients and develop biofilms or detach and become planktonic again.(Mafu *et al.*, 1990). Several studies in the food processing industry demonstrated that bacterial biofilm was found on surfaces and crevices of a lot of equipment, such as stainless steel pipes, vessels, valves, tables, drains and conveyors (Austin & Bergeron, 1995).

Much research has been done to examine biofilm formation on the different types of surfaces that are used in the food processing industries, for example on stainless steel (Ronner & Wong, 1993; Norwood & Gilmour, 1999), rubber (Ronner & Wong, 1993; Smoot & Pierson, 1998), nylon and polyester (Blackman & Frank, 1996), glass, polypropylene (Mafu *et al.*, 1990). Bacterial biofilms showed their effectiveness against a range of detergents and sterilisers and then persisted in contamination of these devices (Austin & Bergeron, 1995). Once biofilms are established bacteria can survive for long periods of time in the presence of nutrients and environmental conditions such as temperatures, relative humidity, attachment surfaces, pressure and contact time (Lee Wong, 1998; Rodriguez *et al.*, 2008). The presence of attached microorganisms in drinking water has been widely reported (Ridgway & Olson, 1981). It is also known that attached bacteria can be found in milk cooling and storage tanks, heat exchangers, stirrers and packing products (Criado *et al.*, 1994).

# **1.2.7** Listerial biofilms

Many foodborne bacteria have been found to form typical biofilms; however *L. monocytogenes* has been reported to form only attached monolayers on surfaces (Mafu *et al.*, 1990; Sashara & Zottola, 1993). The highest incidence of *L. monocytogenes* was reported in moist environments (Lee Wong, 1998). Recently listerial attachment in the food industries has been widely studied (Krysinski *et al.*, 1992; Blackman & Frank, 1996).

Although it is widely accepted that *L. monocytogenes* has the ability to attach to and to form biofilm on different materials (Herald & Zottola, 1988; Jeong & Frank, 1994; Norwood & Gilmour, 2001), there has been little direct microscopical evidence to support this. Hood and his colleague (1997) reported that *L. monocytogenes* V7 strains were able to attach to stainless steel surfaces, but did not form biofilm. Chae and his co-worker (2000) found that *L. monocytogenes* strains can attach to glass coupons under static conditions and all strains were found to form biofilms within 24 hours. Using confocal scanning laser microscopy the three-dimensional structure of *in situ* biofilm was determined (Chae & Schraft, 2000). Kalmokoff *et al.*, (2001) also claimed that *L. monocytogenes* under static conditions on stainless steel and form biofilm but merely adsorbed to surfaces at much lower levels than some other Gram positive and Gram negative isolates. Biofilm formation by *L. monocytogenes* under static conditions on stainless steel has been confirmed (Djordjevic *et al.*, 2002; Marsh *et al.*, 2003). Quantitative studies of attached bacteria in the materials of the food industry have become more common.

*L. monocytogenes* can attach and grow on abiotic surfaces including all types of foods processing surface such as glass, stainless steel, polypropylene, and rubber after contact times as short as 20 minutes (Mafu *et al.*, 1990). More recent work by Beresford (2001) has reported that *L. monocytogenes* is able to attach to a wide range of material commonly used in food processing such as stainless steel, different polymers and aluminium. He also reported that the number of cells adhered to coupons increased during the first two hours of contact, regardless of nature of the material.

Krysinski (1992) evaluated listerial attachment on cleaned and sanitized stainless steel, polyester and polyesters/polyurethane; he found that *Listeria* was resistant to detergent and also that these surfaces showed different resistance to sanitizing agents. Arizcun *et* 

*al.*, (1998) investigated several decontamination procedures for removal of *L. monocytogenes* from glass surfaces. They found that the most efficient decontamination procedure for *L. monocytogenes* was conducted at  $55^{\circ}$ C, with high osmolarity (10.5% sodium chloride) together with acetic acid (pH 5.4). Once bacteria were detached, cells would be more susceptible to sanitizing conditions. The results of this work may suggest that different sanitation protocols should be used when using different materials (Rodriguez *et al.*, 2008).

# 1.2.8 Methods for studying biofilm formation in laboratory

A variety of methods have been developed to study bacterial attachment and biofilm formation in the laboratory. These methods ideally should reflect the natural environment of the attachment and biofilm formation under study. Although, biofilm formation is a dynamic process, most studies have been carried out under static conditions with fluctuation of nutrient availability, growth rate, temperature, pH, and shear rate (Marsh *et al.*, 2003). However, Perni *et al.* (2006) developed a novel system to study listerial biofilm under turbulent flow conditions (Perni *et al.*, 2006).

### **1.2.8.1** Microtitre plate

Biofilms mainly have been studied in static batch culture using microtitre plates. This procedure is based on estimating the level or the number of cells attach to a 96-well plate surface, which can be quantified by staining with the appropriate stain or total viable counting. Optical density is recorded with a standard ELISA plate reader, typically at 595 nm (Riss, 1992). This assay has been modified by various researchers

who have used different staining solutions and different brands of microtitre plates (Djordjevic *et al.*, 2002; Taylor *et al.*, 2002; Marsh *et al.*, 2003).

# **1.2.8.2** Flow cells devices

Flow cells devices are used to study biofilm on different surfaces under controllable parameters such as temperature, pressure and nutrient concentration. They are designed to be compatible with high-resolution imaging techniques such as confocal microscopy. Flow cell systems are open systems which allow biofilms to be grown in observation chambers with the continual supplement of fresh nutrients. There are many types of flow cells used to study biofilm including the modified Robbins<sup>'</sup> device (MRD) (McCoy *et al.*, 1981), radial flow device (Dickinson *et al.*, 1995), and parallel plate flow (Bos *et al.*, 1999). Recently, there is the flow system designed by Perni to eliminate problems of turbulent flow (Perni *et al.*, 2007).

### **1.2.8.3** Coupon systems

Coupons are systems which are used to study the early stage of attachment, when a microbial suspension is kept statically with respect to an exposed substratum. This system allows the running of a large number of comparative experiments over a short time. Coupons are usually small squares, 1 mm in thickness, which can be made from different materials, such as stainless steel, copper, glass, or plastic (Beresford *et al.*, 2001). These coupons are submerged in a cell suspension and then washed before the attached cells are removed for investigation either by direct microscopic visualisation or by detaching and plating them (total viable count) (Beresford *et al.*, 2001). Mechanical removal of attached cells includes swabbing the surface, vortexing,

sonication, and shaking with beads (Bos *et al.*, 1999; Beresford, 2002) The detached cells are then transferred into a bottle of sterile water or buffer and analysed by estimating the number of attached cells by culture on agar (Beresford, 2002). Lindsay *et al.* (1997) found no significant difference between the bacterial counts resulting from three different removal methods (vortexing, sonication, or shaking with beads).

# 1.2.8.4 Microscopy

Microscopy and computer image analysis is the most common direct method for enumeration and morphological observation of microorganism attachment on surfaces. This includes light microscopy, scanning electron microscopy (SEM), epifluorescence microscopy (EPM), confocal laser-scanning microscopy (CLSM), transmission electron microscopy (TEM), environmental scanning electron microscopy (ESEM), Hoffman modulation contrast microscopy (HMC), atomic force microscopy (AFM).

SEM is used in the visualisation of the surfaces of the biofilm at high magnification (Stewart *et al.*, 1995). EPM techniques are used to study the structure of thin multispecies biofilms by applying staining with 4, 6-diamidino-2-phenylindole (DAPI) or acridine orange (AO) to differentiate the species in the biofilm (Wirtanen *et al.*, 1996). CLSM has become a powerful tool in biofilm research, allowing *in situ* analysis of biofilms and providing three-dimensional information without sample processing or using chemical fixation (Surman *et al.*, 1996). EPM is used to generate three-dimensional images of biofilms. A wide range of specific and non-specific fluorescent compounds such as DAPI, Syto9, and acridine orange are available for use in EPM for staining biofilms (Donlan & Costerton, 2002). TEM is used to visualise the internal cross-sectional of the individual microorganisms and their relationship to each other

within the overall biofilm (Surman *et al.*, 1996). ESEM is a form of SEM adapted to visualise hydrated specimens (Sutton *et al.*, 1994). HMC allows direct visualisation of hydrated specimens without staining or fixation (Surman *et al.*, 1996). AFM can be used to study the topography of the biofilm structure or the interaction between surfaces and cells (Bowen *et al.*, 2001). SCLM allows a three-dimensional visualisation with little inclusion of artefacts (Surman *et al.*, 1996). Surman *et al.* (1996) reviewed the use of different types of microscopy for biofilm study. Each of the techniques described in his publication has its own advantages and disadvantages, which are listed in Table 1-7. They also used episcopic differential interference contrast microscopy (DIC) to examine biofilm on opaque surfaces and they succeeded in showing a three-dimensional visualisation of the biofilm population.

The disadvantage of using microscopic methods is that they underestimate biofilm levels, since the thickness of biofilm is not measured. In SEM, EPS often appears as fibres rather than as a thick gelatinous matrix surrounding the cells (Wimpenny *et al.*, 2000). Staining the EPS can also be utilised using stains, such as ruthenium red. However, this can result in overestimating the areas covered by cells (Blackman & Frank, 1996). In spite of these disadvantages, direct microscopy is a reliable method and is still used when studying the attachment of bacteria to different surfaces. For instance, using SEM it was shown that shaking with beads is the most suitable method to remove attached cells from the stainless steel and polyurethane test surfaces (Lindsay & Von Holy, 1997).

Microscopy	Advantages	Disadvantages
SEM	A good analysis of surface shape of the biofilm to be examined at a high magnification <sup>(a)</sup>	The preparation required and electron beam may damage the biofilm and result in the inclusion of artefacts <sup>(b)</sup>
TEM	Useful spatial information relationships of microorganisms within the biofilm matrix <sup>(c)</sup>	The preparation required may damage the biofilm and result in the inclusion of artefacts <sup>(b)</sup>
SCLM	Investigates the presence and the viability of the biofilm and also interactions between cells and surfaces <sup>(c)</sup>	<i>In situ</i> analysis under low magnification. Requires flat samples <sup>(c)</sup>
Light microscopy	Allows examination of intact biofilms <sup>(d)</sup>	Unable to reproduce high magnification compared with the other microscopy technique <sup>(d)</sup>
НМС	Allows <i>in situ</i> examination and a clear image is produced without artefacts <sup>(c)</sup> Can be used successfully to examine dense areas of the biofilm <sup>(c)</sup>	Quantification possibilities are poor when examining denser areas of biofilm <sup>(c)</sup>
ESEM	Direct visualisation of intact hydrated specimens <sup>(d)</sup> ; does not require prior fixation and staining of the biofilm <sup>(c)</sup>	Electron beam may damage the biofilm and result in the inclusion of artefacts <sup>(b)</sup> The magnification achieved by ESEM is much lower than for conventional SEM <sup>(e)</sup>
AFM	Direct visualisation of intact hydrated specimens at high magnification. Allows a 3-D image and provides accurate measurement of individual microorganisms <sup>(f);</sup> does not require prior fixation and staining of the biofilm <sup>(c)</sup>	May result in poor image quality with sample immersed in a liquid <sup>(c)</sup>

Table 1-7 Summary of the features of microscopy used to study biofilm formation.

(a), (Stewart *et al.*, 1995). (b), (Slayter, 1992). (c), (Surman *et al.*, 1996). (d), (Bakke & Olsson, 1986). (e), (Sutton *et al.*, 1994). (f), (Bowen *et al.*, 2001)

# **1.2.8.5** Total viable counts

Viable counting is a classical microbiological identification method that is based on cultivation of the organisms on a suitable medium. Viable counts are determined after removing the biofilm from a surface, other washing steps are carried out to eliminate any planktonic cells. The microorganisms forming the biofilm are detached by different mechanical forces, for example using a sonic bath (Beresford *et al.*, 2001) or by swabbing the surface (Chae & Schraft, 2000).

#### 1.3 Transposons

Transposons are important tools for the genetic analysis of both bacteria and eukaryotes. A large number of transposon derivatives based on Mu, *Tn5* or *Tn10* have been used (Trun & Trempy, 2004). Transposon mutagenesis was the original strategy used to identify virulence genes in *L. monocytogenes* (Portnoy *et al.*, 1992). The first use of transposons in *L. monocytogenes* dates back to 1986 by Gaillard and his colleagues, who used transposons to study the role of haemolysin in the virulence of *L. monocytogenes*.

The first isolation of *Tn*917 was by Tomich *et al.* (1989) and was found in a plasmid pAD2, encoding resistance to streptomycin, kanamycin and erythromycin. Cossart and his co-worker (1989) introduced the *Tn*3-like transposon *Tn*917 into *L. monocytogenes*, carried on vector pTV1 and demonstrated its utility for insertional mutagenesis. Later Camilli *et al.* (1990) subsequently developed a mutant library of a *L. monocytogenes* using the temperature sensitive vector pLTV3 carrying a *Tn*917-lac fusion. This transposon exhibits a high degree of insertional mutations (Youngman *et al.*, 1983), including species in the genera *Lactococcus* (Israelsen *et al.*, 1995), *Clostridium* (Babb *et al.*, 1993), *Staphylococcus* (Thomas & Archer, 1989), and *Enterococcus* (Clewell *et al.*, 1982).

The transposon Tn917 is related to the Tn3 family of transposons. Recent results have demonstrated significant homology between regions of the Tn917 and Tn3 transposon, and its transposase has 80% similarity to the Tn3 transposase (Shaw & Clewell, 1985; Kingsman *et al.*, 1988). It contains the homologous short terminal inverted repeats and generates a 5bp duplication of the target DNA during transposition as was the case with

40

Tn3 (Perkins & Youngman, 1984). Tn917 transposes both to chromosomal and plasmid sites with preference transposes to plasmid (Kingsman *et al.*, 1988). The nucleotide sequences of Tn917 was determined and found to be 5.257 base pairs in length with inverted repeats at its termini; this small size allows a simple cloning and mapping of the DNA flanking its insertion (Nelson *et al.*, 1997).

Although transposon insertional mutagenesis was recently demonstrated in *L. monocytogenes* by introduction of conjugative transposons *Tn*1545 and *Tn*916, *Tn*917 showed low frequencies of transposition  $(10^{-5})$  compared to *Tn*916 and *Tn*1545 (Camilli *et al.*, 1990), which do not significantly harm the bacterial chromosome (Kingsman *et al.*, 1988). Furthermore, *Tn*917 exhibits a preference for A+T rich regions, therefore it is a convenient transposon for insertional mutagenesis in *L. monocytogenes* which possess markedly A+T rich genomes (33% G+C) (Kingsman *et al.*, 1988); because of this *Tn*917 does not show high site specificity in bacteria compared with other members of the *Tn*3 family, therefore this transposon can transpose non-specifically and randomly in nature higher than other transposons.

# **1.4 Aim and Objectives**

*L. monocytogenes* has the ability to attach to and form biofilm on different abiotic surfaces and thus is of great concern for food industries. The overall aim of this project is to investigate the genes that are involved in biofilm formation by *L. monocytogenes*. The first stage was to compare the ability of *L. monocytogenes* wild type and transposon insertion mutants of *L. monocytogenes* to attach to abiotic surfaces (polystyrene and stainless steel) at different temperatures. Subsequently transposon mutants that have significantly reduced levels of attachment were examined to determine the site of transposon insertion; Arbitrary PCR was used for this purpose. Deletion mutants will be subsequently made in these genes to investigate their role in listerial attachment. Finally the effect of NaCl and pH on listerial attachment will be examined.

# 2 Materials and Methods

# 2.1 Chemical and Biological Material

Unless stated in the text, all chemicals were from Sigma (Sigma-Aldrich, UK Ltd) or Fisher (Fisher Scientific, UK Ltd), all bacteriological media were from Oxoid (Thermo Fisher Scientific, UK), and all the PCR reagents were from Bioline (Bioline, UK).

# 2.2 Bacterial strains and plasmids

The bacteria and plasmids used in this project are shown in Table 2-1 and Table 2-2.

Bacteria strain	Genotype and description	Reference
<i>L. monocytogenes</i> strain 10403s wild type		Dr D. Portnoy, Department of Microbiology, University of Pennsylvania, Philadelphia, USA.
<i>L. monocytogenes Tn</i> 917 transposon mutants of 10403s I366	L. monocytogenes 10403s harbouring Tn917 element	Collection of Lab 227, Department of infection, Immunity and Inflammation, University of Leicester. UK. Preliminary data from Beresford (2002)
<i>L. monocytogenes Tn</i> 917 transposon mutants of 10403s M237	L. monocytogenes 10403s harbouring Tn917 element	Collection of Lab 227, Department of infection, Immunity and Inflammation, University of Leicester. UK. Preliminary data from Beresford (Beresford, 2002)
<i>L. monocytogenes Tn</i> 917 transposon mutants of 10403s B265	<i>L. monocytogenes</i> 10403s harbouring <i>Tn</i> 917 element	Collection of Lab 227, Department of infection, Immunity and Inflammation, University of Leicester. UK. Preliminary data from Beresford (Beresford, 2002)
L. monocytogenes $\Delta lmo0103$		This study
L. monocytogenes $\Delta lmo2235$		This study
L. monocytogenes $\Delta lmo2236$		This study
L. monocytogenes $\Delta$ lmo2471		This study
L. monocytogenes $\Delta lmo0540$		This study

Table 2-1 Bacteria strains

Bacteria strain	Genotype and description	Reference
<i>Escherichia coli</i> XL-10- Gold Ultracompetent Cells	Tet <sup>r</sup> $\Delta$ (mcrA) 183 $\Delta$ (mcrCB-hsdSMR- mrr)173 endA1supE44 thi-1 recAI gyrA96 relA1 lac Hte [F proAB lac1 <sup>q</sup> $Z\Delta MI5$ Tn10(Tet <sup>r</sup> ) Amy Cam <sup>r</sup> ]	Agilent Technologies, UK
<i>Escherichia coli</i> Stellar <sup>TM</sup> competent cells	F-, endA1, supE44, thi- 1, recA1, relA1, gyrA96, phoA, Φ80d lacZΔ M15, Δ (lacZYA - argF) U169, Δ (mrr - hsdRMS - mcrBC), ΔmcrA, λ-	Takara BioEurope/Clontech, France

 Table 2-1 Bacteria strains

## Table 2-2 Plasmids

Plasmid	Characteristics	Reference
pGEM-T-Easy vector	<i>E.coli</i> T-A cloning vector, ampicillin resistance. Blue white selection	Promega UK, England
pAUL-A vector	<i>E.coli, Listeria</i> <i>monocytogenes</i> shuttle vector, Erythromycin resistance marker, blue- white selection	Chakraborty <i>et al</i> . 1992

# 2.2.1 Tn917 Transposon mutants

*Tn*917 transposon mutants used in this project were obtained from a library of mutants previously prepared in University of Leicester (Beresford, 2002). Transposition of Tn917-LTV3 in *L. monocytogenes* 10403s was carried out according to the methods of Camilli *et al.* (1990), using the temperature sensitive plasmid pLTV3. A derivative of *Tn*917 (Tn917-LTV3) carried on plasmid pLTV3 (Camilli *et al.*, 1990)was used for transposon mutagenesis of *L. monocytogenes* 10403s.

pLTV3 is temperature sensitive plasmid, it does not replicate at temperature above 37°C, because it a highly temperature-sensitive derivative of vector pE194Ts (Villafane

*et al.*, 1987). The plasmid contains a copy of *Tn*917 conferring inducible  $\text{Em}^r$  (erythromycin resistance), a promoterless copy of the *E. coli lacZ* gene positioned so that insertions into chromosomal genes can generate transcriptional *lacZ* funsions, the kanamycin resistance gene (*neo*) selectable in *E. coli*, the chloramphenicol resistance gene (*cat*), the lincomycin (*ble*) and tetracycline resistance gene (*tet*) selectable in *Listeria*, ColE1 replication functions, an M13 origin of replication, and cluster of polylinker cloning sites which facilities the recovery in *E. coli* of chromosomal DNA adjacent to sites of insertion (Camiili *et al.*, 1990). Any colonies surviving incubation at 41°C (the non-permissive threshold for plasmid replication), which had the phenotypes  $\text{Em}^r$  (erythromycin resistance),  $\text{Lm}^r$  (lincomycin resistance) and Tc<sup>s</sup> (tetracycline sensitive) were assumed to have acquired a chromosomal insertion of Tn917-LTV3 and had lost the plasmid portion of Tn917-LTV3.

Southern Blots were performed to chromosomal DNA of transposon mutants to confirm that Tn917-LTV3 had integrated randomly into the chromosome of *L*. *monocytogenes* (Beresford, 2002)

### 2.3 Culture media and growth conditions

*L. monocytogenes* was grown on Tryptone Soya Agar (TSA) or Brain Heart Infusion agar (1.5% (w/v) bacteriological agar added), Tryptone Soya Broth (TSB) or Brain Heart Infusion broth (BHI) with shaking at 220 rpm at appropriate temperatures (37°C, 30°C and 18°C). Transposon mutants I366, M237 and B265 were grown in the presence of lincomycin and erythromycin at a concentration of 25µg/ml and 1µg/ml respectively. Insertion mutants, of *L monocytogenes*  $\Delta lmo0103$  were grown in the presence of erythromycin at concentration of 5µg/ml. *Escherichia coli* strains XL 10 Gold ultracompetent cells and Stellar competent Cells were grown on Luria-Bertani (LB) medium (Davis *et al.*, 1980) 10g/l of Tryptone, 5g/l yeast Extract, 5g/l sodium chloride at 37°C with shaking or on Luria- Bertani Agar (LA) with added 1.5% (w/v) bacteriological agar. Antibiotics were used, when appropriate, at the following concentrations: erythromycin 100ug/ml and 300ug/ml respectively.

Super optimal broth with catabolite Repression (SOC) recovery medium was used to recovery transformants of *E. coli*. It was prepared as follows, 2g Tryptone, 0.5g yeast extract, 10mM sodium chloride, 2.5mM potassium chloride, 20mM magnesium stock (MgCl<sub>2</sub> and MgSO<sub>4</sub>), 20mM Glucose per 100ml (Sambrook *et al.*, 1989).

All media were prepared using distilled water and were sterilised by autoclaving at 121°C at 15psi (pound per square inch) for 15 minutes.

For long-term storage, bacterial frozen stocks (Davis & Botstein, 1980) were prepared as follows; 10 ml TSB was inoculated with a sweep of bacteria cells from an overnight culture on TSA and incubated at 37°C for 24 hours, with shaking at 220 rpm. Sterile glycerol was added to give 10% (v/v). Bacteria were then dispensed into 500  $\mu$ l aliquots and stored at -80°C until required.

### 2.4 Growth curves of *L. monocytogenes*

The tests were done with wild type and all mutants at different concentration of salt and different pHs. Each strain was grown on TSA at 37°C overnight (with the appropriate antibiotic for the mutant). A colony was subcultured into 10 ml of TSB (with the appropriate antibiotic for the mutants) and incubated with shaking (220 rpm) overnight at 37°C. 100 ml of TSB in an Erlenmeyer flask (500ml) was then inoculated with 1% of the overnight culture. These cultures were incubated at 37°C with shaking (220

rpm); 2 ml samples were taken every 20 minutes for measuring the optical density and for duplicate viable counts as described in section 2.5.1. All tests were done in duplicate from two independent stocks. Optical density measurements were made using a UV spectrophotometer (Jenway), in 1 ml plastic cuvettes, at a wavelength of 500nm. Sterile TSB was used as a blank. All cultures were vortexed prior to measurement and 1 ml was pipette into a cuvette, then the sample readings were taken. The viable counts were used to plot growth curves and to calculate growth rate constant ( $\mu$ ) for all strains, using the formula

$$\mu = \left( \left( \log_{10} N - \log_{10} N_0 \right) 2.303 \right) / (t - t_0)$$

Bacterial growth was also assessed using Varioscan in Nunc 96 wells plate with the same conditions mentioned above. This was done in triplicate starting from three different stocks.

### 2.5 Assay of attachment to polystyrene (adapted from Taylor *et al.*, 2002)

Overnight cultures were diluted to OD 0.7 with TSB; 200 µl of the diluted culture was added into eight separate wells of a 96-well microtitre plate (Greiner polystyrene, Frickenhausen, Germany). Only alternate wells of the microtitre tray were inoculated. Eight wells of TSB without *L. monocytogenes* were included as control wells. The tray was incubated at 37°C, 30°C or 18°C (as appropriate to the experiment) for 2 hours.

After 2 hours incubation the supernatant was aspirated from each of the wells. The loosely associated bacterial cells were removed by gently washing two times with 200  $\mu$ l sterile phosphate-buffered saline (PBS). The plate was air dried and the attached cells were fixed by incubation at 80°C for 30 minutes. Attached cells were stained by adding 220  $\mu$ l 0.1% (v/v) crystal violet in distilled water to each well for 1 minute. Unbound dye was removed by rinsing three times with 220  $\mu$ l PBS. The crystal violet was recovered by adding 220  $\mu$ l decolourisation solution (ethanol: acetone [80:20]) for 15 minutes at room temperature. Crystal violet was quantified by measurement of A<sub>595nm</sub> in a microtitre plate reader (BIO-RAD).

A duplicate tray prepared as described above was used to estimate the total number of viable cells in each well as described in 2.5.1.

### 2.5.1 Viable Counts

Viable counts were determined using an adaptation of the Miles and Misra plating technique (Miles and Misra, 1938). Serial ten-fold dilution was made in sterile PBS in a 96 well microtitre plate up to  $10^6$  and 20 µl (you said you plated 50ul in section above) each were plated in duplicate. The plate was allowed to dry and incubated overnight at  $37^{\circ}$ C. Next day only dilutions which resulted in counts of between 20-150 colonies

were counted. The number of colony forming units per ml is calculated using the following formula:

Number of colonies counted in the sector

X Sector dilution X 1000

Volume plated (20 µl)

# 2.6 Assay of attachment to stainless steel (based on quantitative adhesion assay by Beresford *et al.*, 2001)

### **2.6.1 Preparation of coupons**

Coupons of stainless steel grade 304 (2B finish) ( $1.6 \text{ cm}^2$  and thickness 0.78 mm thick) were used. Each coupon was placed in a sterile universal tube containing 10 ml 1% (v/v) Teepol solution to remove any grease. Tubes were sonicated in a sonication bath for 15 minutes to remove any debris. After sonication the coupons were thoroughly washed in 20ml sterile distilled water. The coupons were finally transferred to a clean beaker and autoclaved at 121°C for 15 minutes.

# 2.6.2 Preparation of bacteria

*L. monocytogenes* was recovered from frozen stocks and was grown on TSA at 30°C. A sweep then was inoculated into 20 ml 20% (w/v) TSB and incubated overnight statically at  $30^{\circ}$ C. The overnight culture then was harvested at 1400 g at 4°C for 15 minutes. The cell pellet was resuspended in 20% (w/v) fresh TSB and cell suspensions were diluted to an OD<sub>600nm</sub> between 0.15 and 0.2 for use.

# 2.6.3 Attachment assays

A single coupon was placed into 10 ml 20% (w/v) Listerial cell suspensions (OD 0.15-0.2) in a universal tube. The coupons were incubated statically for 2 hours at 30°C, then was removed and transferred to a Petri dish containing 10 ml sterile phosphate buffered saline (PBS). The dish was swirled twice gently and the coupon transferred to 10 ml sterile 20% (w/v) TSB in a plastic universal tube. The coupon was then sonicated in a sonic bath for 1 minute, and detached bacteria enumerated as in section 2.5.1.

### 2.7 Procedures for DNA extraction

### 2.7.1 Extraction of listerial chromosomal DNA

Chromosomal DNA was extracted from *L. monocytogenes* using a method perversely published (Dillard & Yother, 1994); 10 ml of *L. monocytogenes* culture was grown in TSB at 37°C overnight to stationary phase. The bacteria were centrifuged at 4000 x g for 10 minutes and the pellet resuspended in 2.5ml of Tris-EDTA buffer (TE buffer) (10mM Tris-HCl, 1mM Ethylenediaminetetraacetic acid (EDTA), pH 8.0). Sodium dodecyl sulphate (SDS) was added to 1% (w/v), and the cells were lysed at 65°C for 15 min, followed by incubation at 4°C for 60 minutes. Cell debris was removed by centrifugation at 11000 x g for 10 minutes. The supernatant was added to 2vols of ethanol and kept at -20°C overnight. The DNA was then spun down at 11000 x g at 4°C for 45 minutes and the supernatant carefully removed. The pellet was air-dried for 30 minutes and then resuspended in 20  $\mu$ l sterile nanopure water. The DNAs were cleaned using QIAquick PCR Purification Kit (Qiagen) as described in section 2.10.

### 2.7.2 Extraction of plasmid DNA using a HiSpeed Maxi kit

A HiSpeed plasmid Maxi kit (Qiagen) was used and the methods were performed according to the manufacturer's instruction; 150 ml bacterial cell culture was grown to stationary phase and then harvested by centrifugation at 6000g for 15 min at 4°C. The bacterial pellet was resuspended in 10 ml Buffer P1; 10 ml Buffer P2 was then added and mixed thoroughly. Chilled Buffer P3, 10 ml was added to the lysate and mixed immediately and thoroughly. The lysate was incubated in a QIAfilter Cartridge at room temperature for 10 min.

A HiSpeed Maxi Tip was equilibrated by adding 10 ml Buffer QBT, before transferring the cell lysate from the QIAfilter. Then, 60 ml Buffer QC was used to wash the Hispeed Maxi Tip. Plasmid DNA was eluted from the Hispeed Maxi Tip by adding 15 ml Buffer QF, and then 10.5ml volume of Isopropanol was added to precipitate plasmid DNA. Plasmid DNA was washed with 2 ml 70% (v/v) ethanol. Finally, the plasmid DNA was eluted from the QIA precipitator in 1 ml of Buffer TE. Plasmid DNA was stored at -20°C for further use.

### 2.7.3 Extraction of plasmid DNA using a miniprep kit

A single bacterial colony was picked from a fresh overnight culture plate and inoculated into 10 ml of LB broth supplement with appropriate selective antibiotic. The culture was incubated at 37°C with vigorous shaking over 16 hours. 5ml of culture was transferred into a new universal tube, and the bacterial cells were harvested by centrifugation at 4000g for 10 min at room temperature. Plasmid extraction was performed according to the manufacturer's instruction (miniprep kit, Qiagen). Following these steps, the pellet was then resuspended in 250 µl Buffer P1, and transferred to a micro-centrifuge tube (1.5 ml). Then, 250 µl of Buffer P2 was added and the preparation mixed by inverting the tube gently, 4 to 6 times to give a homogeneously coloured suspension; 350 µl of Buffer N3 was added to precipitate DNA, followed by immediately mixing the tube by inversion 4 to 6 times. The preparation became cloudy. The suspension was then centrifuged for 10 min at 13000 rpm in a micro centaur bench top centrifuge, and the supernatant transferred to the QIAprep column, by decanting and pipetting the traces. The tube was once again centrifuged for 1 min at 13000 rpm in a micro centaur bench top centrifuge and the flow-through discarded. To remove any trace of nucleases, 0.5 ml of Buffer PB\* was added to the column, followed by centrifugation at 13000 rpm in a micro centaur bench top centrifuge for 1 min, and discarding the flow-through after. Then the column was washed by adding 0.75 ml of Buffer PE and centrifuging twice, discarding the flow-
through between and after each centrifugation. The column was placed into a new microcentrifuge tube and to elute DNA 50  $\mu$ l of Buffer EB was added and left to stand for 1 min at room temperature and then centrifugation in a micro centaur bench top for 1 min at 13000 rpm. Plasmid DNA concentration was measured using Nanodrop before stored in the freezer (-20°C) for further use.

#### 2.8 Polymerase Chain Reaction (based on (Sambrook et al., 1989)

PCR was typically performed in 50  $\mu$ l volumes, using about 200ng of *L. monocytogenes* DNA. Primers (20 pmol) were added to a PCR mixture containing; PCR buffer (1x), 200  $\mu$ M each deoxynucleotide, 1.25 U of Taq polymerase, 1.5 mM MgCl<sub>2</sub> and 0.1-0.5  $\mu$ g template chromosomal DNA used in a final volume of 50 $\mu$ l. PCR was performed under the following conditions; 35 cycles of 94°C for 30s, 52°C for 30s and 72°C for 1 min, followed by 72°C for 5 min. The product was separated on a 1.5% (w/v) agarose gel containing 0.2 $\mu$ g/ml ethidium bromide solution, as described in section 2.11. For sequencing 10ng/8 $\mu$ l PCR product was sent for analysis as described in 2.12.

# 2.8.1 Arbitrary primer PCR

The DNA sequence flanking the transposon insertion sites was determined by Arbitrary PCR in a method adapted from (Knobloch *et al.*, 2003). PCR amplification was performed with a primer specific to the end of the transposon in combination with a primer of random sequence, which could anneal to chromosomal sequences flanking the transposon (Figure 3-11)

PCR was performed in two rounds. In the first round of PCR an arbitrary primer (STAPHarb1 or STAPHarb2; 20 pmol/reaction) and a primer unique to one end of the transposon [917-5.2, 917-5.3, 917-3.2 and 917-3.3]; 10 pmol/reaction) were used. The

PCR mixture contained PCR buffer (1x), each deoxynucleotide at 200  $\mu$ M, 1.25 U of Taq polymerase, 1.5 mM MgCl2 and 0.5  $\mu$ g template chromosomal DNA in a total volume of 50  $\mu$ l. PCR was performed under the following conditions: 95°C for 5 min; six cycles of 94°C for 30s, 30°C for 30s 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. samples were kept at 4°C until required.

The second round of PCR was performed in a final volume of 100 µl using 5 µl of the first round product used as the template DNA. A transposon specific primer ([917-5.2, 917-5.1, 917-3.2 or 917-3.1]; 20 pmol/reaction) and the arbitrary primer arb3 (20 pmol/reaction) (Table 2-3 and Table 2-4) were used in the second round. The reactions for the second round were performed under the PCR described above in the first round of PCR, except that both primers were used at a concentration of 20 pmol/ reaction and the annealing temperature was 45°C. PCR was performed under the following conditions; 30 cycles of 94°C for 30s, 45°C for 30s and 72°C for 1 min, followed by 72°C for 5 min. The products from the second round of PCR were separated on a 1.5% (w/v) agarose gel containing 2 µl (0.2mg/ml ethidium bromide solution as described in section 2.11.

Primer	Localization (bp) <sup>a</sup>	Primer sequence
917-5.1	124-103	5'-ATC GAT ACA AAT TCC TCG TAG G-3'
917-5.2	274-255	5'-AAC CGA TAC CTG TTT GTG CC-3'
917-5.3	353-333	5'-CCA-ATC ACT CTC GGA CAA TAC-3'
917-3.1	5298-5318	5'-TTT AGT GGG AAT TTG TAC CCC-3'
917-3.2	5194-5215	5'-GGG AGC ATA TCA CTT TTC TTG G-3'
917-3.3	5102-5122	5'-GAA CGC GGT CTA CTT ACA AGC-3'
STAPHarb1	Arbitrary	5'-GGC CAC GCG TCG ACT AGT CAN NNN NNN NNN GAT AT-3'
STAPHarb2	Arbitrary	5'-GGC CAC GCG TCG ACT AGT CAN NNN NNN NNN GAT CT-3'
Arb3	Arbitrary	5'-GGC CAC GCG TCG ACT AGT CA-3'

Table 2-3 Oligonucleotides used as primers (Knobloch et al., 2003)

<sup>a</sup> Localization in the published nucleotide sequence of Tn917 (accession number M11180).

Table 2-4 PCR primer pairs during the first and second round of arbitrary PCR

round	1	round 2		
STAPHarb1	917-5.3	917-5.2	Arb3	
STAPHarb1	917-5.2	917-5.1	Arb3	
STAPHarb1	917-3.3	917-3.2	Arb3	
STAPHarb1	917-3.2	917-3.1	Arb3	
STAPHarb2	917-5.3	917-5.2	Arb3	
STAPHarb2	917-5.2	917-5.1	Arb3	
STAPHarb2	971-3.3	917-3.2	Arb3	

#### 2.9 DNA extraction from agarose gels using a Promega S/V system

A Promega s/v kit was used to extract DNA fragments from agarose gels. The desired DNA fragments were produced by digestion with the appropriate enzyme and were separated by agarose gel electrophoresis as described in section 2.11. The gel containing the fragment of interest was determined under UV light from a long wave UV transilluminator and then excised using a clean scalpel and transferred to a 1.5 ml microcentrifuge tube. The agarose gel slice containing the fragment of interest was dissolved at 65°C for 10 min until the gel slice is completely dissolved using membrane binding (10µl of solution per10mg of agarose gel slice) solution. The dissolved gel was transferred to a minicolumn provided with the kit and the other steps were applied according to the supplier's instruction manual (Promega). All centrifugation steps were done at 13,000g.

#### 2.10 DNA purification extraction using a QIAquick purification kit

DNA was cleaned according to the manufacturer's instructions (Qiagen); 1 ml of Buffer PB was added to 20  $\mu$ l of DNA solution. The mixture was then centrifuged at 13,000*g* for 1 minute in a microcentrifuge (Beckman Coulter). The eluate was discarded and the QIAguick column washed with 0.75 ml Buffer PE, following centrifugation at 13,000 for 1 minute. The eluate was discarded and the centrifugation repeated for an additional 1 minute. Finally, 30  $\mu$ l Buffer EB was added to eluate DNA from the column. The column was left to strand for 1 minute and then centrifuged for 1 minute at 13,000 rpm. The DNA was stored at -20°C ready for use.

#### 2.11 Agarose gel electrophoresis

The agarose gel was prepared according to the size of DNA fragments (Sambrook *et al.*, 1989). Fragment sizes bigger than 2Kb was analysed using 1% (w/v) agarose gel electrophoresis (Lab M) and 1.5% (w/v) agarose concentration for separating fragments smaller than 2Kb. Agarose was prepared in TE buffer (10 mM Tris-HCl + 1 mM EDTA, pH 8.0) and electrophoresis was performed in TAE buffer with  $0.2\mu$ g/ml ethidium bromide. A 1Kb DNA ladder and 100bp DNA ladder were used as the DNA size marker. DNA and PCR products were mixed with 1/5th (v/v) of 6 x gel-loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 15% (w/v) Ficoll Type 400, Pharmacia) in water). The electrophoresis was carried out at 75-85 volts for one hour. DNA fragments were visualised under UV light from a long-wave UV transilluminator.

#### 2.12 Nucleotide sequence analysis

DNA was sequenced by PNACL (Protein Nucleic Acid Chemistry Department-University of Leicester), after cleaning of DNA with a Promega Wizard SV kit. Nucleotide sequence analysis was performed PCR products with 1 pmol/µl primers. Resulting sequence was identified using a basic local alignment search tool (BLAST) and the NCBI database of the finished genome for *L. monocytogenes* EGD-e.

#### 2.13 Construction of mutants in L. monocytogenes 10403s

# 2.13.1 Preparation of *L. monocytogenes* cells for electroporation with plasmid DNA

The method followed for the electroporation of *L. monocytogenes* was adapted from the penicillin treatment protocol described by Park and Stewart (1990). 1ml of *L. monocytogenes* in BHI broth supplemented with sterile 0.25M sucrose was added to 500ml of fresh BHI and incubated at 37°C with shaking at 150 rpm until an  $OD_{600nm}$  of 0.2 was attained. Then 10 µg/ml of Penicillin G was added, and the incubation was continued to an  $OD_{600nm}$  of 0.4. The cells were then harvested by centrifugation at 3000xg for 10 minutes at 4°C and then washed three times in 250, 100 and 30 ml respectively in volume of 1mM sterile HEPES (pH 7.0) plus 0.25M sucrose. The cell pellet was drained carefully and resuspended in 500 µl volume of 1mM HEPES, 0.25M sucrose and 15% (v/v) sterile glycerol. Finally, the cells were dispended into 50 µl aliquots and kept at -70°C until use, or were kept on ice for immediate use.

# 2.13.2 Electroporation of *L. monocytogenes* with plasmid DNA (based on Park & Stewart, 1990)

For electroporation, 100ng of plasmid was added to 40  $\mu$ l of competent *L. monocytogenes* strain10403s cells (thawed on ice) and mixed gently and then incubated on ice for 1 minute. The mixture was transferred into a 0.2 cm prechilled electroporation cuvettes (Bio Rad) and placed into the electrodes of a gene-Pulser (Bio-Rad). The settings used for electroporation with *L. monocytogenes* were 25  $\mu$ F, 2.5kv/cm<sup>2</sup>, and 200 $\Omega$ . The cells were electroporated using a pulse with a time constant of about 4.2ms. Immediately, 1 ml prewarmed SOC medium was added and mixed by pipetting twice and then transferred to 15ml Falcon tubes and the culture incubated at 30°C for 3 hours with gentle shaking at 150rpm. The cells were then plated onto BHI agar with 1.5% (w/v) agar, containing  $5\mu$ g/ml erythromycin. Plates were incubated at 30°C for 2 days. Next, small white colonies were picked and PCR was done to confirm integration of plasmid into the bacterial chromosome, as described in section 2.8

# 2.13.3 Recombination of plasmid into L.monocytogenes

Colonies from electroporation were streaked on BHI agar containing  $5\mu$ g/ml erythromycin and incubated twice at 42°C. Integration of the plasmids into bacterial chromosome was confirmed by PCR using specific gene primer and universal primer M13 reverse. Colonies which showed a positive result of fragment size were picked and inoculated overnight into 10 ml BHI broth with shaking. This was incubated at  $30^{0}$ C with shaking at 220 rpm. Following subculture twice in BHI a day for three days 100 µl of -6 and -7 dilutions was plated on BHI agar and incubated at 42°C. Next day viable colonies were plated on BHI with and without erythromycin (5µg/ml) and then incubated at 30°C. Erythromycin sensitive colonies were screened for double crossover (Table 2-5).

Primer	Sequence	Gene target	Product size	
Fw 540 INF FW1	5' CGGTACCCGGGGATCCGTGGCGAGCTTTGATTGCT 3'	lmo0540		
Fw 540 INF RV1	5'GACACGTCCCATCCGGTCACAAACACGATAATCATGTTCTTG CGTC 3'	lmo0540	540bp	
Fw 540 INF FW2	5'GACGCAAGAACATGATTATCGTGTTTGTGACCGGATGGGAC GTGTC 3'	lmo0540	471hp	
Fw 540 INF RV2	5' CGACTCTAGAGGATCTACTCCGGCGCGGGTTTCAC 3'	lmo0540	4710p	
CpbpFW	5' AAGAAGTTAAAAGTCCGCTACC 3'	lmo0540	1152hn	
CbpbRV	5' CCAAAGAAGCAAAAGCAACAC 3'	lmo0540	11520p	
NO1F	5'ACGGTGAAAATTAGCCAAGAG3'	lmo0103	210hm	
NO1R	5'GCGTGGAATGGAAGCAAAG3'	lmo0103	5180p	
NOWF	5'TGCTGCCGATTTTGTTGC3'	lmo0103	512bn	
NOWR	5'AATATCAGCTATTTCCTTGCCC3'	lmo0103	5120p	
M13F	5'TGTAAAACGACGGCCAGT3'	-		
M13R	5'CAGGAAACAGCTATGACC3'	-		
2236FW1	5 'CGGTACCCGGGGATCCAAATGCGATTAGCTGCCTATCCT3'	lmo2236		
2236RV1	5'CAGTCTTACAACCAGCAGCTTCAGCGCCAAGAAGCCTAGTCG TTCCTGA3'	lmo2236	773bp	
2236FW2	5'TCAGGAACGACTAGGCTTCTTGGCGCTGAAGCTGCTGGTTGT AAGACTG3'	<i>lmo2236</i> 517bp		
2236RV2	5' CGACTCTAGAGGATCGAGTTTGCTGCTCCTCCGACAAAAG3'	lmo2236	51700	
C236F	5' GTGAATCCGAAGCTTACTTACC3'	lmo2236		
C236R	5' GCCGTCAATAACGATTAAAGCC3'	lmo2236	2336bp	
INF 471 FW1	5' CGGTACCCGGGGATCTGTTAGTCAGCCCAGAAGTTACG 3'	lmo02471		
INF 471 RV1	5'AAAGACGGGATTGCAACAGACTTTCGATGAAAGCAGATGGC GTGGAAC3'	lmo02471	566bp	
INF 471 FW2	5'GTTCCACGCCATCTGCTTTCATCGAAAGTCTGTTGCAATCCC GTCTTT3'	lmo02471	656bp	
INF 471 RV2	5' CGACTCTAGAGGATCATGAGGAGCACAACGAGGCAATTTG3'	lmo02471		
C471F	5' GAGGAAGGCAACACCATAAC3'	lmo02471	1264hm	
C471R	5' AGGCGAAATGCTGACAAC 3'	lmo02471	13646p	
INF235 FW1	5'CGGTACCCGGGGATCAGGAAGACTCCCTGTGAAAGGAATG 3'	lmo2235		
INF235 RV1	5'GCATGAATCAATCCTGCGTCCTCGTTTGTTTGTCCGTGCCGAT AAGA 3'	lmo2235	577bp	
INF235 FW2	TCTTATCGGCACGGACAAACAAACGAGGACGCAGGATTGAT Imo2235		582bp	
INF235 RV2	5' CGACTCTAGAGGATCCCAATTAAGCCAAGAAGCCTAGTCG3' <i>lmo2235</i>		r	
C235F	5' TCTCCACGCTAAACACCTAC 3' Imo2235		20521-	
C235R	5' CGAATGAATCCTACTCGCTAC 3'		203200	

Table 2-	5 Oli	gonucleotides	used as	primers	to pre	pare L.	monocyto	genes	mutants
		G							

#### 2.14 Cloning reactions

# 2.14.1 Ligation of DNA fragment using pGEM-T Easy vector

DNA ligation was performed according to the manufacturer's recommendations. pGEM-T-Easy vector (Promega, UK) a ratio of 1:3 vector to insert was used. Ligations were performed at room temperature for an hour, and then used for transformation using XL 10 Gold ultracompetent cells (Agilent Technologies UK Limited) as described in section 2.14.4. Transformation mixtures were plated on LB containing the appropriate antibiotic. Successful transformation was confirmed by PCR (Section 2.8).

#### 2.14.2 Ligation of DNA fragment using pAUL-A vector

DNA ligation was performed as described above in section-2.14.1. A ratio of 1:3 and 1:6 vector to insert was used. Ligations were performed at room temperature for an hour, and then used for transformation using either XL-10-Gold ultracompetent cells (Agilent Technologies UK Limited) or Stellar<sup>™</sup> competent cells (Clontech, USA) as described in section 2.14.4. Transformation mixtures were plated on LB containing erythromycin antibiotic. Successful transformation was confirmed by PCR (Section 2.8).

#### 2.14.3 Ligation of DNA fragment using In-fusion HD cloning kit

An In-fusion HD cloning kit (Clontech, USA) is designed to join multiple DNA fragments into any vector in a single reaction. PCR primers for interest of genes (Table 2-5) were designed with 15 bp extensions (5<sup>'</sup>) that homology to the ends of the linearsed vector. Molar ration of insert to vector was calculated at a 6:1 ratio of insert to linearised pAUL-A vector, following the protocol instruction. Ligation reaction was incubated at 50°C for 15 min, and then used for transformation using Stellar<sup>TM</sup> competent cells (Clontech, USA) protocol. Transformation was plated on LB

containing the appropriate antibiotic. Successful transformation was confirmed by PCR (Section 2.8).



Figure 2-1 Schematic drawing demonstrate cloning procedure using In-Fusion HD kit

#### 2.14.4 Transformation protocol

Transformation was performed as instructed by the manufacturer (Agilent Technologies, UK). Competent cells were thawed on ice and mixed gently and 50  $\mu$ l was transferred into Falcon polypropylene round-bottom tubes; 2  $\mu$ l of plasmid DNA was added directly into the competent cells and gently swirled. The tubes then were incubated on ice for 30 min. followed by heat shock for 30 sec at 42°C and then incubated again on ice for exactly 2 min. Then, 950  $\mu$ l of pre-warmed SOC medium was added to each tube and incubated at 37°C with 220 rpm shaking for 1 h. Finally the transformation reaction was plated onto LA supplement with the appropriate selective antibiotic, X-Gal and IPTG and then incubated at 37°C overnight.

#### 2.15 DNA restriction digestion

The restriction endonucleases used for routine DNA manipulation were purchased from Biolabs (New England Biolabs, UK). DNA was digested in 50 µl reaction mixture with 10 units of enzyme and 500ng of DNA at 37°C in water bath for 2 hours, with the tubes sealed with nescofilm. The successful digestion was confirmed by run on a gel. The restriction endonucleases used were *BamHI* and *EcoRI* (New England Biolabs, UK). Finally, digests were purified using Promega kit and the concentration was measured using a Nanodrop spectrophotometer. Finally DNAs were stored at -20°C for further use.

## 2.16 Scanning Electron Microscopy

# **2.16.1 Preparation of coupons**

Coupons of polystyrene and stainless steel grade 304 (2B finish) (1.6 cm<sup>2</sup> and thickness 0.78 mm thick) were used for scanning electron microscopy. Each coupon was cleaned and sterilised as described in section 2.6.1, with the addition of sterilisation with 70% (v/v) ethanol for 20 min.

# 2.16.2 Preparation of bacteria

A 3 ml *Listeria* overnight cultures diluted to OD 0.7 with TSB were used to inculcate the well of a 12-well microtitre plate containing a single coupon. The plates were then incubated at 18°C, 30°C and 37°C for 2 hours and 24 hours.

After 2 and 24 hours incubation, each coupon was removed from the wells using sterile forceps (the coupon was held by two edges) and placed into a new 12 well microtitre plate containing 1ml 2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer pH 7.4. The sample was allowed to fix at room temperature and imaged by scanning electron microscopy (SEM) using a Hitachi S3000H scanning electron microscope. The SEM was done by the Electron Microscope Laboratory, University of Leicester.

### 2.17 Impact of temperature on *L. monocytogenes* gene expression

# 2.17.1 Analysis of gene expression by Real Time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

# 2.17.1.1 RNA extraction

The extraction of RNA was done as described previously by Stewart et al. (2002) using the GTC/TRIZOL method, with the exception on that an extra chloroform extraction was introduced. Briefly, 10 ml of a mid-log phase L. monocytogenes culture was mixed rapidly by swirling with 4 volume of GTC lysis solution (5M guanidine isothiocyanate, 0.5% sodium N-lauryl sarcosine, 25 mM tri-Sodium citrate pH 7.0, 0.1 M 2mercaptoethanol, 0.5% Tween 80). These were subsequently centrifuged at 3000g (Allegra<sup>™</sup> X-22 centrifuge, Beckman coulter) for 20 min to pellet the cells. The supernatant was discarded and the pellet was resuspended in 1 ml GTC and then the suspension transferred to a microcentrifuge tube and centrifuged at 13000g for 20 seconds. The supernatant discarded and the pellet was resuspended in 1.2 ml Trizol (Invitrogen) and immediately transferred into a Ribolyser blue matrix tube (Fisher Scientific, UK) and processed in a Ribolyser (Thermo) at 6.5 power setting for 45 seconds. The bacterial protein was removed by adding 200µl chloroform, vortexing and incubating at room temperature for 2-3 min. Then the sample was centrifuged at 12000g (Mikro 22R, Hettich Zentrifugen) for 15min to remove bacterial debris. The aqueous phase was transferred to a fresh tube and re-extracted with addition of 0.5ml chloroform, and centrifugation at 12000g for 15 min at 4°C. RNA was precipitated and washed with 500µl isopropanol followed by centrifugation at 12000g for 10 min at 4°C and the RNA pellet was resuspended in 87.5µl RN/DNase free water. RNA was treated with RNase-free DNase (Qiagen), by incubating for 10 min at room temperature.

The RNA was cleaned to eliminate DNA from the samples using a Qiagen RNeasy mini kit as described by the manufacturer (Qiagen). Finally, the RNA was eluted in 30  $\mu$ l of nuclease-free water. The RNA concentration was measured using a NanoDrop 1000 spectrophotometer (Termo Scientific). In addition, the integrity of RNA was assessed by electrophoresis using a 1.5% (w/v) agarose gel (Section 1.8). Samples were stored at -80°C until needed.

#### 2.17.1.2 Extraction RNA from attached and planktonic cells

A 5 ml diluted culture of  $OD_{500}$  0.7 of *L. monocytogenes* wild type and mutants were grown in flat-bottom polystyrene 6-well plate (Greiner polystyrene, Frickenhausen, Germany) as described in section 2.5. Plates were incubated at 18, 30 and 37°C. After incubation for 2 hours, 5ml of culture in each well was collected into a centrifuge tube and wells were washed twice with 5ml PBS. Then, 5ml TSB was added and sonicated for 1 min to detach attached cells. Finally the suspension of detached cells was removed for RNA extraction as described in section 2.17.1.1.

#### 2.17.1.3 First-strand cDNA synthesis using superscript II RT

The reverse transcriptase reaction was performed using SuperScript II reverse transcriptase (Invitrogen, UK). Approximately 1  $\mu$ g of RNA template for each desired temperature was converted into first strand cDNA in 20  $\mu$ l volumes by following the manufacturer's instructions. First strand cDNA synthesis used random primers (Invitrogen, UK) using superScript II Reverse Transcriptase at 42°C for 55 min, and then the reaction was inactivated at 70°C for 15 min.

As a negative control similar amount of total RNA from each desired temperature was subjected to cDNA synthesis reaction without the reverse transcriptase enzyme. This provides a way to assess the potential DNA contamination of each sample during the real time PCR assay. Although DNase treatment removed a large portion of contamination, to eliminate interference of residual DNA to results, cDNA samples were diluted to the extent that in cycling conditions employed, no amplification in negative control could be detected.

# 2.17.1.4 Real-Time reverse Transcriptase PCR

cDNA was amplified in a 20µl reaction volume which contained 1X SYBR Green PCR master mix and 20pmol of each primer (Table 2-6). The RT-PCR reactions were performed in a real time PCR machine (Corbett Research). The transcription level of specific gene primers was normalized to *gyrB* expression. The primers were designed by standard procedures and amplified approximately 150bp length product. The annealing efficiency of primers was determined by using different dilutions of template. The reactions were performed in triplicate using following cycle. 1 cycle of 10 min at 95°C for followed by 40 cycles of amplification at 95°C for 20s; 55°C for 30s, 72°C for 20s. The expression of target genes in different conditions was normalised against the expression of *gyrB*. The results were interpreted using the comparative cycle threshold (C<sub>T</sub>) (Schmittgen & Livak, 2008). Change fold of gene expression was calculated using formula below:-

 $2^{-\Delta\Delta C}_{T} = [(C_{T} \text{ gene of interest} - C_{T} \text{ internal control}) \text{ sample } 1$ - (C<sub>T</sub> gene of interest - C<sub>T</sub> internal control)] sample 2

	=	=		_
Gene name	Forward prime	Reverse primer	Product size	reference
NADH oxidase lmo2235	gtcatttctgagcaccgtaag	tcaaaaccatcgattcgctg	149bp	This study
penicillin binding protein lmo0540 cccatcggaaaatgcagcaac		cgaactacaagtgccgaacc	150bp	This study
gyrB (House keeping gene)	caagcaacatcatttcattcgc	tcaccagaaagattgtcatcac	151bp	This study
Similar to oxidoreductase <i>lmo2236</i>	tccgaatgaacatgaaacacc	aaagcattcctaacccgcc	152bp	This study
similar to tagatose-1,6- diphosphate aldolase <i>lmo0541</i>	actgcatctccaccattttc	ctccagcaatcaaagctcg	148bp	This study
similar to ABC transporter (binding protein) <i>lmo0539</i>	cattaccaacactttctgtccc	agaagttcctacaacgccg	146bp	This study

 Table 2-6 Primers used for analysis of gene expression

#### 2.18 Measurement of NADH oxidase activity

#### 2.18.1 Preparation of cell lysate (adapted from (Lopez de Felipe et al., 2006)

*L. monocytogenes* strains were grown at 30°C and harvested at the end of stationary growth phase ( $OD_{500} = 1.6$ ) by centrifugation at 3000g for 15 min. The pellet was washed once in 50mM potassium phosphate buffer pH 7.0. The suspension was centrifuged at 3000g for 15min and then the pellet was resuspended in 1 ml of 50mM potassium buffer. The cell suspension was sonicated on ice for 5 times at amplitude 7 for 15s. The mixture was kept on ice for 45s between each disruption. The cell lysate was kept at -80°C until required.

#### 2.18.2 Enzyme activity measurement (Lopez de Felipe *et al.*, 2006)

NADH oxidase activity in cell lysates was measured in Nunc 96 wells plate at 25°C, in a total volume of 200µl containing 50mM potassium phosphate buffer (pH 7), 0.29mM NADH, 10uM FAD and 0.3mM EDTA. The reaction was initiated by adding 20 µl cell lysate and monitored the decrease in  $A_{340}$  with a Varioscan spectrophotometer. Enzyme activity of each strain was calculated using formula below:-

# Specific Activity= $\Delta A_{340}$ /min/mg protein

#### 2.18.3 Quantification of protein using Bradford Protein Assay (Bradford, 1976)

A Bradford protein assay was performed according to manufacturer's instructions (BioRad). The dye reagent was prepared by dilution of 1 part dye reagent with 4 parts of PBP pH 0.7. A standard curve was prepared with 4-20  $\mu$ g/ml of Bovine Serum Albumin (BSA) protein to Nunc 96 wells plate, 10  $\mu$ l of each standard and samples was added in triplicate. Then, 190  $\mu$ l of the Bradford reagent concentrate was added to each well and mixed by pipetting. The plate was incubated at room temperature for 5 min and the absorbance was measured at 595nm a Bio-Rad microplate reader. The

standards were plotted and the concentration of proteins was determined by using the equation from the trendline adjusted to the points obtained by plotting the standards.

# 2.19 Minimum Inhibitory concentration MIC determinations (Korsak *et al.*, 2010)

The MIC value of Penicillin G against *L. monocytogenes* 10403s and mutant  $\Delta lmo0540$  were determined in liquid culture using serial two-fold dilution of Penicillin G antibiotic (30µg/ml to 0.05µg/ml) in Nunc 69 well plate in triplicate. The inoculums  $10^4$  cells per ml were used and the plate was finally incubated statically for 24h at 37°C before recording the minimum concentration of penicillin that inhibited growth. The susceptibility to Penicillin G antibiotic was analysed by population of bacteria growth each wells.

# 2.20 Antibiotic susceptibility test (Etest)

Etest strip containing pencilling G was done according the manufacturer's instructions (bioMérieux, France). Overnight culture was diluted to  $OD_{600} \sim 0.132$  (1.5 X10<sup>8</sup> CFU/ml) using fresh BHI broth and 100 µl aliquots was carefully streaked over the entire agar surface evenly using a sterile spreader over a 90 mm- diameter Brain Heart Infusion (BHI) agar plate. Using sterile forceps an Etest strip was applied to the agar surface with the MIC scale facing upwards (towards the opening of the plate). The plate was incubated in an inverted position for 24h at 37°C. The MIC value was taking where the edge of the inhibition ellipse intersects the strip.

#### 2.21 Statistical analysis

One-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test and two-way analysis of variance (ANOVA) with Bonferroni multiple comparison post tests were used. The software Graph-Pad prism 5 for windows (Graphpad, San Diego California, USA) was used. Tow Statistical significance was taken as P<0.05.

# 3 *Tn*917 mutants characterisation and identification

Previous work by Beresford (2002) showed that a Tn917 transposon mutant library of *L. monocytogenes* contained a number of mutants which appeared to be defective in surface attachment. Therefore, because so little is currently understood about the genes involved in *L. monocytogenes* biofilm formation, an investigation was carried out to confirm that the original Tn917 attachment mutants were defective and to identify the genes which were disrupted, so that defined, non-polar mutants could be created.

Transposon mutants B265 and I366 were selected at random as candidates for this study after showing a reduced attachment level to stainless steel in comparison to wild type 10403s in the quantitative adhesion assay. Transposon mutant M235 was used as a control for assay of attachment to polystyrene because it had been shown previously to have a low level of attachment to polystyrene at  $30^{\circ}$ C compared to  $37^{\circ}$ C.

The first stage of this project was to compare the ability of transposon mutants B265 and I366 and *L. monocytogenes* wild type 10403s to attach to polystyrene at different temperatures using the microtitre plate method. Three different incubation temperatures were investigated, namely 18°C, 30°C and 37°C. The first of these, 18°C, was chosen as an intermediary temperature whilst 37°C and 30°C were used because both these temperatures provide the optimal conditions for bacterial growth, attachment and biofilm formation.

The results show that the transposon mutants B265 and I366 are a deficient in the ability to attach to polystyrene at 30°C and 18°C but not at 37°C, compared to wild type (P<0.05). Results from the quantitative adhesion assay to stainless also showed that both transposon mutants, B265 and I366 attached, to stainless steel in lower numbers than the wild type (P<0.05).

Subsequently, Arbitrary PCR was performed in order to determine the site of transposon insertion by the Tn917 in the transposon mutants B265 and I366. The Tn917 insertion site of both mutants was successfully identified. Analysis of the nucleotide sequences of PCR products from the B265 mutant identified that this matched a sequence that is annotated as an open reading frame (ORF) for a hypothetical protein similar to NADH oxidase (lmo2235). Similarly, the nucleotide sequence flanking Tn917 in I366 matched a sequence that is annotated as an ORF for encoding a penicillin-binding protein.

#### 3.1 Assay of attachment to polystyrene

An early objective of this study was to devise an assay for analysis of the attachment of *L. monocytogenes* strains, which would allow detection of a quantitative difference in the level of attachment to polystyrene. To achieve this, *L. monocytogenes* wild type 10403s and a selection of transposon mutants were used.

Variables such as growth medium, incubation time, performance of washing steps, and fixation and staining procedures were carefully controlled during the assay. Also, several factors had to be considered during performance of the attachment assay. One of those factors was to dilute the overnight culture of *L. monocytogenes* to an  $OD_{500}$  =0.7, because all the strains did not reach the stationary phase at the same time. Therefore to ensure that OD differences did not affect the behaviour in attachment, cultures were diluted to the same OD.

Following investigation of the attachment of *L. monocytogenes* wild type to polystyrene at 37°C, the attachment assay was utilised to explore the attachment of transposon insertion mutants I366, M237, and B265 compared to wild type, at different temperatures (37°C, 30°C and 18°C).

# 3.1.1 Assay of attachment of wild type 10403s at 37°C

The attachment assay was first applied only to *L. monocytogenes* wild type 10403s at  $37^{\circ}$ C to investigate the assay reproducibility. A preliminary assay was first conducted by adding 200 µl diluted wild type 10403s culture (OD<sub>500</sub> =0.7) to four sets of eight separate wells of a 96-well microtitre plates (columns A-D) and incubating statically for 2 hours at 37°C, following by removed of non-adherent cells and medium from the microtitre wells. These were gently washed, dried and attached cells fixed. Attached cells were stained with crystal violet, and then the absorbance measured. The numbers

of planktonic cells were determined after 2 hours, using the Miles and Misra technique. There was no significant difference in the level of attachment in each set of eight wells as shown in Figure 3-1 (P>0.05). Table 3-1shows the viable counts of planktonic cells of wild type from wells (A-D) after 2 hours incubation were preformed, and were found to be the same for each set of eight well (P>0.05)



**Figure 3-1** Assay of attachment with *L. monocytogenes* wild type 10403s to polystyrene at 37°C. WTA-WTD represents the mean wild type (WT) ( $\pm$  S.D.) of each column (A-D) of eight wells. Control, represents TSB. There was no significant difference between the columns (mean of eight wells) in the attachment (P>0.05).

**Table 3-1**Viable counts of planktonic cells of *L. monocytogenes* wild type10403s in the attachment assay after 2 hours incubation at  $37^{\circ}$ C.

Strains	Log <sub>10</sub> of the mean viable counts
wild type A	9.1 ±0.05
wild type B	9.1 ±0.01
wild type C	9.1 ±0.07
wild type D	9.2 ±0.03

Data are the mean  $\pm$  standard deviation of the result of eight experiments

# 3.1.2 Assay of attachment of wild type and transposon mutants to polystyrene at 37°C

The next series of experiments was to compare the ability of *L. monocytogenes* strains to attach to polystyrene. Different incubation temperatures were investigated; there were 18°C, 30°C and 37°C. Figure 3-2 shows the results for the attachment assay of wild type and mutants; M237, B265, I366 at 37°C to polystyrene. Wild type and transposon mutant strains showed the same level of attachment at 37°C. There was found no significant difference between strains at 37°C (P>0.05) and no difference in the number of planktonic cell counts (P>0.05) after 2 hours incubation (Table 3-2)



**Figure 3-2** The result for assay of attachment of *L. monocytogenes* strains to polystyrene at  $37^{\circ}$ C. Data are the mean (± S.D.) of eight experiments. Control, represents TSB. There was no significant difference between any of the stains (P>0.05).

**Table 3-2** Viable counts of planktonic cells in the attachment assay after 2 hours incubation at 37°C.

Strains	Log <sub>10</sub> of the mean viable counts
wild type 10403s	9.0 ±0.05
I366	9.0 ±0.04
M237	9.0 ±0.02
B265	9.0 ±0.04

Data are the mean  $\pm$  standard deviation of the result of eight experiments.

# 3.1.3 Assay of attachment of wild type and transposon mutants to polystyrene at 30°C

The attachment assay was also conducted at  $30^{\circ}$ C to compare the ability to *L. monocytogenes* strains to attach to polystyrene. Figure 3-3, shows the results for the attachment assay of wild type and mutants; M237, B265, I366 to polystyrene at  $30^{\circ}$ C. As can be seen, the wild type strain and all mutants showed a better adherence at  $30^{\circ}$ C compared to  $37^{\circ}$ C. However the mutants showed about 50% reduction in attachment compared to wild type (P<0.05) at  $30^{\circ}$ C. On the other hand, there was no significant difference between the mutants in their attachment at  $30^{\circ}$ C (P>0.05). The deficiency of attachment of mutants shows that there is an effect of temperature on the manifestation of the transposon mutation. The total numbers of planktonic cell counts were the same for all four cultures (P>0.05) after 2 hours incubation (n= 8) (Table 3-3)



**Figure 3-3** Assay of attachment *L. monocytogenes* strains to polystyrene at 30°C. Control, represents TSB. Data are the mean ( $\pm$  S.D.) of eight experiments. All the mutants showed a 50% reduction in attachment compared to the wild type (P<0.05)

Strains	log <sub>10</sub> of the mean viable counts
wild type 10403s	9.1 ±0.09
I366	9.2 ±0.1
M237	9.2 ±0.08
B265	9.2 ±0.1

**Table 3-3** Viable counts of planktonic cells in the attachment assay after 2 hours incubation at 30°C.

Data are the mean  $\pm$  standard deviation of the result of eight experiments.

# 3.1.4 Assay attachment of wild type and transposon mutants to polystyrene at 18°C

The ability of *L. monocytogenes* strains to attach to polystyrene was compared at  $18^{\circ}$ C during 2 hours incubation. Figure 3-4 shows the results for the attachment assay of wild type and mutants M237, B265 and I366 at  $18^{\circ}$ C to polystyrene. All the mutants showed a significant reduction in the level of attachment in comparison to wild type at  $18^{\circ}$ C (P<0.05), whereas there was no difference in the level of attachment between the mutants (P>0.05). The numbers of planktonic cell numbers were found to be equivalent in all *L. monocytogenes* strains (P>0.05) after 2 hours incubation at  $18^{\circ}$ C (Table 3-4)



**Figure 3-4** Assay of attachment *L. monocytogenes* strains to polystyrene at  $18^{\circ}$ C. Data are the mean of eight experiments. Control, represents TSB. Wild type showed a significantly greater level of attachment than that of mutant strains (P<0.05)

**Table 3-4** Viable counts of planktonic cells in the attachment assay after 2 hours incubation at 18°C.

Strains	log <sub>10</sub> of the mean viable counts
wild type 10403s	9.1 ±0.1
I366	9.0 ±0.09
M237	9.1 ±0.08
B265	9.0 ±0.1

Data are the mean  $\pm$  standard deviation of the result of eight experiments.

Comparison of the data in Figure 3-2, 3-3 and 3-4 demonstrates that the *L*. *monocytogenes* wild type showed significantly greater attachment at 30°C, comparing to the other temperatures of 18°C and 37°C (P<0.05), the data also showed no difference in its level of attachment at 18°C and 37°C (P>0.05) (Figure 3-5).



**Figure 3-5** The result for assay of attachment of *L. monocytogenes* wild type to polystyrene at 37°C, 30°C and 18°C during 2 h incubation. Data are the mean of eight experiments. Control, represents TSB. Wild type showed the highest level of attachment at 30°C (P<0.05) comparing with 37°C and 18°C where There was no significant difference (P>0.05)

The data show in Table 3-3 and 3-4 were confirmed by measuring the optical density of the planktonic cells of the wild type and mutants. As can be seen in Figure 3-6 and Figure 3-7, there was no difference in the  $OD_{595}$  after 2 hours incubation at 18°C and 30°C.



**Figure 3-6** The optical density of *L. monocytogenes* strains in TSB after 2 h incubation at 18°C. Control, represents TSB.



**Figure 3-7** The optical density at 595nm of *L. monocytogenes* strains in TSB after 2 h incubation at 30°C. Control, represents TSB.

#### **3.2** Assay of attachment to stainless steel (quantitative adhesion assay)

To investigate the numbers of *L. monocytogenes* strains attached to stainless steel and to confirm the previous data from Beresford (Beresford, 2002), which indicated that these mutants were deficient in attachment to stainless steel at  $30^{\circ}$ C, an attachment assay was conducted by immersing sterile stainless steel 304 coupons in a diluted culture of *L. monocytogenes* wild type 10403s and transposon mutants B265 and I366. The coupons were removed from the culture using sterile forceps and washed. The number of attached cells was estimated after two hours at  $30^{\circ}$ C

The total number of *L. monocytogenes* strains attached to stainless steel after two hours is shown in Table 3-5. There was a significant difference in the number of cells of *L. monocytogenes* wild type attached to stainless steel after 2 hours incubation compared to the numbers of transposon mutant B265 and I366 (P<0.05) whereas the mutants showed the same number of attached cells (P>0.05) The results of this experiment show that transposon mutants B265 and I366 have a deficiency to attach to stainless steel as well as to polystyrene.

**Table 3-5** The number of *L. monocytogenes* strains attaching to stainless steel 304 coupons after 2 hrs incubation at  $30^{\circ}$ C (n=4).

Strains	log <sub>10</sub> attached cells/coupon		
wild type10403s	$6.2 \pm 0.10$		
B265	5.50 ±0.29		
I366	5.51 ±0.37		

Data are the mean  $\pm$  standard deviation of the result of three replicates

# 3.3 Scanning Electron Microscopy

Scanning electron microscopy was performed to visualise the density of *L. monocytogenes* strains to attach to different surfaces at different incubation time, different temperatures, and also see how attachment differed between strains of *L. monocytogenes* wild type 10403s, I366 and B265 were allowed 2 hours and 24 hours contact time on stainless steel 304 and polystyrene coupons. After attachment occurred, the samples were fixed and prepared for scanning electron microscopy, as described in section 2.16.

Figure 3-8 shows the attachment of *L. monocytogenes* strains to stainless steel at  $30^{\circ}$ C after 2 hours incubation and after 24 hours incubation. Overall there was no difference in the extent of attachment between the *L. monocytogenes* strains at different incubation times. As can be seen from the images, a cluster form was formed with wild type after 2 hours but not after 24 hours. Increasing cell length was observed with attached cells of B265 and I366 strains after 24 hour incubation.



Wild type 10403s



**B265** mutant



### I366 mutant

**Figure 3-8** SEM of attachment of *L. monocytogenes* strains; wild type 10403s, B265 and I366 to stainless steel coupons at  $30^{\circ}$ C after 2 hours incubation time (left) and after 24 hours incubation (right). Red arrows refer to culsters formed by wild type cells after two hours incubation time. Yellow arrows refer to increasing cell length was observed with attached cells of B265 and I366 strains after 24 hour incubation. No evidence of EPS production was observed. The scale bars for each image are shown at the bottom of the figure.

Figure 3-9 shows the attachment of *L. monocytogenes* strains to polystyrene at different temperatures (37°C, 30°C and 18°C) after 2 hours incubation. Apparently the extent of attachment of strains at 30°C is characterized as a compact compared to 37°C. The attached cells at 37°C tended to form short chains with small numbers of cells with all strains. However filament formation was observed with the three strains at 18°C.

Figure 3-10 show the attachment of *L. monocytogenes* 10403s wild type, I366 and B265 to polystyrene surface at different temperatures at 37°C 30°C and 18°C after 24 hours incubation. Images indicate an irregular curved cells and increased cell length. Also the amount of burst dead bacteria was observed with all the examined strains. Filament formation occurred at three different temperatures after 24 hours. No difference in surface attachment was observed between *L. monocytogenes* strains.

In general *L. monocytogenes* strains did not appear to form complex biofilm structures on either stainless steel or polystyrene surface and at different incubation time. Only a monolayer of cells was formed and no visual evidence of the production of sticky exopolysaccharides was observed



#### I366 mutant

**Figure 3-9** SEM of attachment of *L. monocytogenes* strains; wild type 10403s, B265 and I366 strains to polystrayne coupons at 37°C 30°C, and 18°C for 2 hours incubation time. Number of attached cells were more at 30°C copmared to 37°C. yellow arrows refer to filment formatiom was observed with three strains at 18°C. No EPS production was seen. The scales for each image are shown at the bottom of the figure (5µm).



#### I366 mutant

**Figure 3-10** SEM of attachment of *L. monocytogenes* strains; wild type 10403s, I366 and B265 strains to polystryrene coupons at  $37^{\circ}$ C,  $30^{\circ}$ C, and  $18^{\circ}$ C for 24 hours incubation time. An irregular curve shapes and filaments was formed with all strains at different temperatures. All strains produced the same attachment pattern at different temperture. No EPS was observed. The scales for each image are shown at the bottom of the figure (5µm).

# 3.4 Arbitrary PCR to identify the genes disturbed by *Tn*917 in *L*.

#### monocytogenes transposon mutants

Arbitrary PCR was performed in order to identify the genes mutated by the *Tn*917 transposon in the attachment deficient mutants I366 and B265. DNA of wild type 10403s and mutants was used as the template DNA in the reaction (Section 2.8.1). Six primers, specific for the transposon *Tn*917, were used. Three were homologous to the 5' end of the transposon, and the other three were homologous to the 3' end. These were used alongside arbitrary PCR primers for the bacterial chromosome as described in Figure 3-11. Arbitrary PCR was performed in two rounds. In the first round, primers STAPHarb1 and STAPHarb2 for the bacteria chromosome were paired with the specific primers 917-3.3, 917-3.2, 917-5.3 and 917-5.2, as described in the material and methods (Table 2-4). A low annealing temperature was used for the first round of arbitrary PCR (30°C) to allow non-specific binding of the primers to the chromosomal DNA.



Figure 3-11 Schematic drawing to explain the Arbitrary PCR primers.
In figure 3-12 and 3-12 smears of DNA can be seen in the lane (3-10) containing PCR products from the I366 and B265 mutants using all of the pairs of primers



**Figure 3-12** Agarose gel electrophoresis of the PCR products generated from the I366 mutant in the first round using arbitrary primers (STAPHarb1 and STAPHarb2) paired with transposon specific primers (917-5.3, 917-5.2, 917-3.3 and 917-3.2). Lane1- 100bp DNA marker, Lane 2 and 7 negative controls (wild type DNA). Lane 3- STAPHarb1 and 917-5.3. Lane 4-STAPHarb1 and 917-5.2. Lane 5 STAPHarb1 and 917-3.3. Lane 6 STAPHarb1 and 917-3.2. Lane 8- STAPHarb2 and 917-5.3. lane 9- STAPHarb2 and 917-5.2. Lane 10- STAPHarb2 and 917-3.3. Lane 11- STAPHarb2 and 917-3.2.



**Figure 3-13** Agarose gel electrophoresis of the PCR products generated from the B265 mutant in the first round using arbitrary primers (STAPHarb1 and STAPHarb2) paired with transposon specific primers (917-5.3, 917-5.2, 917-3.3 and 917-3.2). Lane 1- 100bp DNA marker, Lane 2-negative controls (wild type DNA). Lane 3- STAPHarb1 and 917-5.3. Lane 4- STAPHarb1 and 917-5.2. Lane 5- STAPHarb1 and 917-3.3. Lane 6- STAPHarb1 and 917-3.2. Lane 7- STAPHarb2 and 917-5.3. Lane 8- STAPHarb2 and 917-5.2. Lane 9- STAPHarb2 and 917-3.3. Lane 10- STAPHarb2 and 917-3.2.

In the second round of PCR, STAPHarb1 and SATPHarb2 primers were replaced with a more specific primer (Arb3), with the positive PCR of round 1 as a template. Also to avoid nonspecific binding of the Tn917 primers, the annealing temperature was increased to 45°C. Despite the positive reaction with all the pairs of primers that were used in the first round of PCR, only some primer pairs in the second round of arbPCR gave positive results. Figure 3-14 shows that DNA from the B265 mutant was detected in lanes 3, 4, 8 and 10 in B265 mutant. Similarly in figure 3-15 a DNA fragment can be seen in lanes 3, 4, 5, 7, 9 and 10 in I366 mutant. Table 3-6 summarizes the primer pairs used for the second round amplified DNA in each mutant.

Table 3-6 The primer pairs used for the second round amplified DNA in each mutant.

Mutants	STAPHarb1 and first/second <i>Tn</i> 917 primers				STAPHarb2 and first/second <i>Tn</i> 917 primers			
Primers	9175.3 917-5.2	9175.2 917-5.1	9173.3 917-3.2	9173.2 917-3.1	9175.3 917-5.2	9175.2 917-5.1	9173.3 917-3.2	9173.2 917-3.1
I366	+	+	+	-	+	+	+	-
B265	+	+	-	-	+	-	+	-

(+) Positive result (Amplification) (-) Negative result (No amplification)



**Figure 3-14** Agarose gel electrophoresis of the PCR products generated from the B265 mutant in the second round using arbitrary primers (Arb3) paired with transposon specific primers (917-5.2, 917-5.1, 917-3.2 and 917-3.1). Lane 1- 100bp DNA marker, Lane 2 and 7 negative controls (wild type DNA). Lane 3- Arb3 and 917-5.2. Lane 4- Arb3 and 917-5.1. Lane 5- Arb3 and 917-3.2. Lane 6- Arb3 and 917-3.1. Lane 8- Arb3 and 917-5.2. Lane 9- Arb3 and 917-5.1. Lane 10- Arb3 and 917-3.2. Lane 11- Arb3 and 917-3.1.



**Figure 3-15** Agarose gel electrophoresis of the PCR products generated from the I366 mutant in the second round using arbitrary primers (Arb3) paired with transposon specific primers (917-5.2, 917-5.1, 917-3.2 and 917-3.1). Lane 1- 100bp DNA marker, Lane 2 and 7 negative controls (wild type DNA). Lane 3- Arb3 and 917-5.2. Lane 4- Arb3 and 917-5.1. Lane 5- Arb3 and 917-3.2. Lane 6- Arb3 and 917-3.1. Lane 8- Arb3 and 917-5.2. Lane 9- Arb3 and 917-5.1. Lane 10- Arb3 and 917-3.2. Lane11- Arb3 and 917-3.1

The detected DNAs from the second PCR round presented in lanes (3, 4, 8 and 10) and lanes (3, 4, 5, 7, 9 and 10) from the B265 and I366 mutants respectively were sent to the protein nucleic acid chemistry laboratory PNACL (University of Leicester) for sequencing with Tn917 primers used in second PCR round. The DNA Sequences received from the PNACL are shown in Appendix (Figure 1 and 2). To identify the Tn917 insertion sites, the nucleotide sequence of the DNA fragments generated by the Arbitrary PCR were compared to the *L. monocytogenes* EGD-e genome sequence using nucleotide BLAST algorithm. The Tn917 insertion site of both mutants was successfully identified. In the mutant I366 the transposon was identified to be in *lmo0540*, an open reading frame (ORF) which encodes a penicillin binding protein whereas the location of the transposon in the B265 mutant was identified to be within *lmo2235*, an ORF which encodes a hypothetical protein, similar to NADH oxidase. The result alignment of the nucleotide sequences from B265 and I366 in Basic Local Alignment Search Tool (BLAST) are shown in Appendix (Figure 3 and 4).

### 3.5 Discussion

The ability of *L. monocytogenes* to attach to different surfaces has been reported (Mafu *et al.*, 1990; Beresford *et al.*, 2001). The quantitative study by Beresford *et al.* (2001) exhibited the ability of *L. monocytogenes* strain 10403s to adhere to seventeen tested surfaces, including stainless steel, aluminum, polycarbonate and nitryl rubber after 2 hours contact time without exception, with only polypropylene showing lower number of adherent cells; meanwhile stainless steel 405 was recorded to have the highest number of adherent cells.

Many techniques have been developed to study bacterial attachment, some of which are based on counting the number of detached cells from the surface, such as standard plate counts and sonication (Green & Pirrie, 1993; Doolittle *et al.*, 1995; Poulsen, 1999). In this study reported in this thesis sonication was used to study the attachment of *L. monocytogenes* transposon mutant strains to stainless steel and to compare them to their parent strain.

*L. monocytogenes* wild type 10403s was found to attach more to stainless steel compared to the transposon mutants B265 and I366 (P<0.05) (Table 3-5). Despite the fact that researchers have found that using a dislodging method, for example scraping and vortexing, may remove up to 97% and 99% of the cells from a surface respectively (Anwar *et al.*, 1992; Jeong & Frank, 1994), Lindsay and Van Holy (1997) reported that there was no significant difference between the numbers of detached cells removed by different dislodging methods such as vortexing, shaking with beds and sonication.

The other methods utilised for looking at biofilm involve direct observation of attached cells to the surface, including microtitre plate methods and microscopy techniques. These methods were also used in this project. Attachment assays using microtitre plates are widely utilised to investigate the ability of bacteria to attach to abiotic surfaces. This assay has been modified by various researchers to study the ability of *L. monocytogenes* to attach to surfaces and form biofilms (Marsh *et al.*, 2003; Taylor *et al.*, 2002; Di Bonaventura *et al.*, 2008). The microtitre plate technique has been also modified to enable evaluation of the effect of various growth conditions and environment signals on bacterial biofilm. Djordjevic *et al.* (2008) conducted a study using a rapid microtitre plate assay in which they indicated that the polyvinyl chloride (PVC) microtitre plate assay can be used as a simple and fast method to compare the ability of *L. monocytogenes* strains to form biofilm.

In the study in this thesis the attachment assay was modified to compare the ability of transposon mutants of *L. monocytogenes* and wild type strains to attach to polystyrene at a range of temperatures. Here, overnight cultures were diluted to the same  $OD_{500}$  to ensure that concentration differences between strains did not affect the behaviour in attachment. Also, two hours incubation time was chosen. Transposon mutants were found to grow slower in vitro than wild type over two hours; this will result deficient in transposon mutant attachment

The attachment assay was first used with *L. monocytogenes* 10403s wild type to check assay reproducibility (Figure 3-1) and then the assay was subsequently conducted comparing the transposon mutants to the wild type at different temperatures (Figures 3-2, 3-3 and 3-4). Some variables should be considered and controlled carefully during attachment assay, for example performance of pipetting, washing steps, and fixation and staining procedures.

The results of this thesis reported an effect of different temperatures on the ability of *L*. *monocytogenes* wild type to attach to polystyrene the results showed that a higher level

of attachment to polystyrene by the wild type was observed at 30°C (P<0.05) compared with 37°C and 18°C (Figure 3-3). The results suggest that the mechanism of attachment at 30°C is different from that at 37°C and 18°C. The finding of this thesis is in accordance with the results of Chavant et al. (2002) who investigated the ability of L. monocytogenes LO28 to attach to and form biofilms on abiotic surfaces at different temperatures and growth phases. Their findings demonstrated that L. monocytogenes significantly attach to and form biofilms on stainless steel LO28 and polytetrafluoroethylene (PTFE) surfaces at 37°C after 5 days incubation, but at 8°C there was only a bacterial monolayer formed on stainless steel. Another study demonstrated that the number of L. monocytogenes ATCC 19111 attached to stainless steel in nutrient-rich medium and minimal medium increased with increasing temperature, with an exception at 42°C where the number of attached cells was lower than at  $37^{\circ}$ C and  $30^{\circ}$ C (Mai & Conner, 2007).

A recent study by Di Bonaventura *el al.*, (2008) investigated the influence of temperature on biofilm formation by *L. monocytogenes* on different food surfaces. The biofilm levels were significantly higher at 37°C than at other selected temperatures (8°C and 20°C). It can also be concluded from the data in this thesis that the mechanism involved in surface attachment is temperature dependent. In contrast to the previously published works on studying the effect of temperatures on attachment of *L. monocytogenes* strains, there is no published research on the insertion transposon mutants used in this study (M237, I366 and B265) and whether a temperature dependent effect is observed.

*L. monocytogenes* wild type demonstrated elevated attachment to polystyrene at 30°C, whereas transposon mutants attach to polystyrene in lowest frequency at 30°C

95

compared to wild type but at higher levels of attachment compared to other temperatures of 18°C and 37°C (Figure 3-3). At 37°C when flagella are not expressed, attachment of *L. monocytogenes* strains was identical (Figure 3-2). In this study, at a lower temperature (18°C) the wild type attached significantly less than at 30°C but was not significantly different from at 37°C (Figure 3-4). The results suggest that flagella expression at 30°C may have an effect in initiating attachment of *L. monocytogenes* strains. Expression of listerial flagella occurs at 30°C and below (Peel *et al.*, 1988). The wild type develops peritrichous flagella and become flagellated and motile between 20°C and 30°C, this can give an explanation for not being attached well at 18°C as well. In contrast to this result observed in *L. monocytogenes, a* non-flagellated mutant strain of *S. typhimurium* has been reported to attach in numbers similar to those of cells that possess flagella at 30°C (Lillard, 1986), indicating in this organism flagella are not important in initial attachment.

Scanning electron microscopy (SEM) can be used to verify the data obtained with the attachment assay, as lower numbers of cells would be observed under microscopy in those strains, showing a significant reduction in attachment using the microtitre plate assay. SEM can also be used to note any changes in morphology of the cells or the biofilm. Lindsay and Holy (1997) reported that, using scanning electron microscopy, vortexing and sonication only remove the surface layers of biofilm but do not remove the EPS residue. In this study it was decided to use scanning electron microscopy to better compare the attachment assay results, to show that attachment of *Listeria* is temperature dependent.

In this study SEM showed that the density of attached cells on polystyrene was slightly affected by different temperatures i.e. the monolayer of cells formed on polystyrene

was denser at 30°C, compared to 37°C and 18°C, which correlates with the increase in attachment observed at 30°C using the microtitre plate assay. Although there is always a possibility of damage and dislodging of attached cells during preparation for electron microscopy, the results (Figures 3.8, 3.9 and 3.10) revealed that all strains attached and formed similar monolayers of cells on polystyrene and stainless steel surfaces over contact times of 2 hours to as long as 24 hours. This result is in agreement with the work of Beresford (2002), who demonstrated the number of L. monocytogenes cells attached on different surfaces was significantly increased between short contact time and two hours contact time but not after a week. These findings do not support the hypothesis that bacterial attachment is time dependent. The lack of a significant increase in the numbers of attached cells after the two hour contact time suggests that the Listeria may have reached a maximum level of attachment to the material and will not continue attaching to form the advanced microcolonies or the biofilm structures described by Costerton et.al (1978). Using an atomic force microscope to study adherence of L. monocytogenes Scott A to stainless steel, one study pointed out that attached cells did not colonize and form true biofilm at the surface but grew as single cells attached to the surfaces (Rodriguez et al., 2008). Extracellular polysaccharide has been hypothesised to be produced during attachment of L. monocytogenes. Extracellular polysaccharide of attached L. monocytogenes was found to be produced after only short time of bacteria contact with the surfaces (Mafu et al., 1990). Ronner and his colleague (1993) demonstrated that L. monocytogenes attached to stainless steel and buna-nitryl rubber appeared to produce an extracellular polysaccharide matrix. They reported that extracellular polysaccharide production was observed more on stainless steel surfaces compared to buna-nitryl surfaces (Ronner & Wong, 1993). The scanning electron microscopy photomicrograph shown in the study by Ronner and

Wong (1993) does not resemble those in scanning electron photomicrographs of this thesis. No complex biofilm structures were observed on either stainless steel or polystyrene surfaces, even after different incubation times, and no visual evidence of the production of exopolysaccharides could be seen. The data in this thesis agreed with the observations made by Borucki *et al.* (2003) who investigated the actual production of EPS in *L. monocytogenes* strains using a carbohydrate-binding dye and found that *Listeria* strains produced some type of extracellular carbohydrate with biofilm matrix; this was hypothesised to be due to the binding of the dye to the carbohydrate on the cell surface but not correlated with EPS formation.

Arbitrary PCR has been used widely to analyse sequences flanking transposon insertions (Dale & Fredericks, 2005). In the technique a primer of arbitrary sequence is paired with a primer that binds to a region of known sequence in the transposon. The technique is performed in two rounds; in the first round, arbitrary primers, which differ in their 3' pentameric sequences, are bound with transposon-specific primers in a PCR reaction resulting in multiple amplicons. This due to the arbitrary primers binding at many regions in the genome, including adjacent to the known transposon sequences. The resulting products of the first round are used as a DNA template for a second round of reaction. In the second round, a primer composed of the common 5' region of the arbitrary primers is used to bind with a transposon-specific primer (see Table 2-3 and 2-4). To identify the insertion site of *Tn*917, the resulting sequences of arbitrary PCR fragments were analysed with the BLAST tool using the *L. monocytogenes* EGD-e genome at NCBI (www.ncbi.nlm.nih.gov/).

The analysis of nucleotide sequence of PCR products from B265 was matched to a sequence that is annotated as a gene for a hypothetical protein similar to NADH oxidase (*lmo2235*) of *L. monocytogenes. lmo2235* encodes a protein comprising of 210

amino acids in length and is positioned on the chromosome downstream of a hypothetical protein encoded by gene *lmo2234* and upstream of gene *lmo2236*, which encodes a hypothetical protein similar to an oxidoreductase. The terminator program of the WebGeSTer DB (<u>http://pallab.serc.iisc.ernet.in/gester</u>) was used to investigate intrinsic transcription terminators at the end of *lmo2235* gene. There was a putative terminator detected at the end of the *lmo2235* which is shown in Figure 6-1. The presence of the terminator means the end of transcription of *lmo2235*. Using this information, it was concluded that the *lmo2235* is an independent gene and the transcription of gene ceases at the putative terminator, suggesting that *lmo2235* is not part of an operon.

In a similar way, nucleotide sequences of I366 matched a sequence in the published genome for *L. monocytogenes* strain EGD-e that is annotated as a gene for a penicillinbinding protein (*lmo0540*). This protein consists of 397 amino acids in length and encodes PBPC1. The *lmo0540* gene is located downstream of the gene *lmo0539* which is thought to encode a protein similar to tagatose-1,6 diphosphate aldolase gene and is upstream from a putative ABC transporter (*lmo0541*). The *lmo0540* is an independent genes and are not part of an operon as shown in Figure 6-2.

As the results of the attachment assay for the transposon mutants I366 and B265 indicate, *lmo0540* and *lmo2235* respectively play a role in attachment; deletion mutation in *lmo2235* and *lmo0540* was constructed to investigate whether these genes were required for *L. monocytogenes* attachment or not. The direct role of both genes in attachment has not yet been demonstrated in *L. monocytogenes*.

### **4** Creation of Deletion mutants

The deletion mutant were created to test the hypothesis that the phenotype change in transposon mutants B265 and I366 is caused by the insertion of a transposon into NADH oxidase and penicillin-binding protein ORFs and is not due to polar effects on other neighbouring genes. The deletion mutations were generated using two different strategies, as described in the Material and Methods (Section 2.13) and detailed in sections 4.1. Cloning vectors pGEM-T-Easy and pAUL-A were used in cloning and genomic DNA from wild type *L. monocytogenes* 10403s was used in the gel as a negative control. The length of desired fragments in each mutant is calculated in advance and depends on the position of genomic DNA of the restriction enzyme used. Deletion mutants in NADH oxidase genes (*lmo2235*, *lmo2471*, and *lmo0103*) and the penicillin-binding protein gene (*lmo0540*) were successfully obtained and the mutants were selected and named, and were used during the following stage of the work.

## 4.1 Construction of mutants in genes encoding three putative NADH oxidase in *L. monocytogenes* 10403s

In order to confirm that NADH oxidase was the cause of the adhesion defect in B265 and to check for polar effects, a deletion mutation in *L. monocytogenes* 10403s was made. As *L. monocytogenes* has three NADH oxidase ORF; *lmo0103*, *lmo2237* and *lmo2471*, it is decided to introduce deletion mutations into each ORF.

### 4.1.1 Amplification of gene *lmo0103* by PCR

In order to introduce a deletion into the NADH oxidase ORF, *lmo0103*, a 321bp fragment located at the 5<sup>°</sup> end at the ORF was amplified by PCR using primers NO1F and NO1R (Table 2-5). That the correct fragments had been amplified was confirmed by agarose gel electrophoresis (Lane 3 and 4) Figure 4-1.



**4-1** Agarose gel electrophoresis showing the amplification of 321bp fragment of *lmo0103* by NO1F and NO1R primers in *L. monocytogenes* 10403s. Lane 1- 100bp DNA marker. Lane 2- negative control (no DNA). Lane 3 and 4 amplicon from *lmo0103*.

## 4.1.2 Cloning *lmo0103* into pGEM-T-Easy vector and small scale plasmid preparation.

In order to facilitate 321bp fragments amplified by PCR it was necessary to clone the fragment into pGEM-T Easy cloning vector before subcloning into pAUL-A vector. PCR product of *lmo0103* (321bp) prepared in the previous section was clean-up, the fragment was then A-tailed at 72°C for 35 minutes in the presence of *Taq* DNA polymerase. The purified A-tailed PCR fragment was then cloned into the pGEM-T-Easy cloning vector following the protocol described in the Material and Methods section (2.14.1).The molar ratio of insert to vector in ligation reaction was determined and a 1:3 ratio insert DNA to the vector was used.

pGEM-T-Easy vector derives from *Eco*RV cut pGEM-<sub>®</sub>-5Zf(+) and has a 3; terminal thymidine on each end to improve efficiency of ligation of PCR products produced from thermostable enzyme leaving A tails.

pAUL-A vector was chosen for subcloning due to the fact that it is capable of autonomous replication in both *E. coli* and *Listeria* spp (Schaferkordt & Chakraborty,

1995). However, in *Listeria spp* pAUL-A is incapable of replication at temperatures higher than 30°C because of the temperature-sensitive origin of replication derived from the *Staphylococcus aureus* plasmid pE194ts (Schaferkordt & Chakraborty, 1995). Also it carries an erythromycin resistance marker that expresses in both hosts. pAUL-A also contains the *lacZ* gene of *E. coli* with multiple cloning site of plasmid pUC19. Insertion of the DNA fragment into any of their sites disrupts the *lacZ* gene, and results in *Lac*-negative phenotype in the appropriate *E. coli* host strain.

In order to determine if the cloning had been successful, small scale preparation of the plasmid DNA was done according the protocol described in the Material and Methods (Section 2.7.3) and the plasmid DNA from a number of resulting transformants was extracted prior to the sequencing with primers that were homologous to the sequences of the *lmo0103*. The sequences were analyzed with nucleotide BLAST for homology with gene sequences in database. The sequencing analysis proved to be positive and sequences displayed a 90% match to that of strain EGDs (from the NCBI database). Results of comparisons are summarized in the Appendix Figure 5. One of the selected plasmids DNA was named pl0103 and the work was continued with this plasmid.

### 4.1.3 Restriction digestion of plasmid pl0103

Pl0103 plasmid was single digested with *EcoRI* as there is an *Eco*RI site flanking the multiple cloning site of the pGEM-T-Easy vector thus avoiding the need for double digests into a shuttle vector. The digested plasmid then electrophoresed through a 1% (w/v) low-melting-point agarose gel. A gel slice containing the 321bp fragment was excised and DNA purified using the Promega S/V system. Figure 3-19 shows the agarose gel containing digested plasmid DNA. Two bands were produced, one corresponding to the plasmid itself (3015bp) and another corresponding to the inset (321bp).



**Figure 4-2** Agarose gel electrophoresis of the *EcoRI* restriction digest. The pl0103 is presented in lane 2 with two band, insert size of 321bp and vector size of 3015bp: Lane1-1Kb DNA marker; Lane 3- 100bp DNA marker.

### 4.1.4 Digestion of plasmid pAUL-A with *EcoRI*

pAUL-A vector was cut with *EcoRI* following the protocol described in the Material and Methods, (Section 2.15). The digestion of plasmid was checked by electrophoreses. As can be seen in Figure 4-3 one fragment (9200bp) was appeared in lane 2 which suggests that restriction endonucleases cut at one sites.



**Figure 4-3** Restriction digestion of pAUL-A plasmid with enzyme *EcoRI*: Lane1- 1Kb DNA marker; Lane 2- Digest of pAUL-A with *EcoRI* resulting in one band in the gel.

## 4.1.5 Subcloning of *lmo0103* insert into pAUL-A and transformation of pAUL-03 into *L. monocytogenes*10403s

The subcloning was done as described in the Material and Methods (Section2.14.1). The excised 321bp insert from pl0103 was ligated into pre-digested pAUL-A. The molar ratio of insert to vector in ligation reaction was determined and a 1:3 ratio insert DNA to the vector was used. The resulting plasmid was extracted from a number of transformants before starting transformation into *L. monocytogenes*. The plasmid was then named pAUL-03

pAUL-03 plasmid was transformed into electrocompetent *L. monocytogenes* 10403 cells following the protocol in Material and Methods (Section 2.14.4). To facilitate the entry of the plasmid into *L. monocytogenes*, cells were penicillin treated prior to transformation to increase the permeability of the cells (Park & Stewart, 1990). The transformation of plasmid pAUL-A-03 into the *L. monocytogenes* was confirmed by screening a number of transformants by PCR using primers binding to the *lmo0103* ORF (NOWF primer) and the vector (M13 primer). Confirmation of successful

transformation is shown in Figure 4-4 (Lane 4) where a product of 521bp of the expected size can be seen. The results suggest that the insertion of pAUL-03 into the chromosome had successfully occurred, and so one mutant was picked and named  $\Delta lmo0103$ . Comparison of the nucleotide sequences showed a significant match (>90%) to the *lmo0103* ORF (see Appendix, Figure 61).

Attempts to integrate of pAUL-03 into the *L. monocytogenes* genome was tried several times unsuccessfully, for this reason it was decided to change the methods of mutagenesis.



**Figure 4-4** Agarose gel electrophoresis of plasmid pAUL-03 transformed into in *L. monocytogenes* 10403s. Lane 1- 100bp DNA marker; Lane 2- negative controls (no DNA). Lane 3- negative control (10403s DNA); Lane 4- positive result of *L. monocytogenes lmo*0103 mutant amplified using NOWF and M13R. The expected size was 521bp.

Further mutagenesis was done using previous methods, pGEM-T-Easy cloning vector and pAUL-a shuttle vector to construct other mutants in NADH oxidase (*lmo2235* and *lmo2471*) and penicillin binding protein ORFs (*lmo0540*) Unexpectedly, sequencing analysis of resulting pGEM-T Easy derived clones was found to be negative and there was no matching in the database when comparisons of these sequences obtained with the gene of interest, meaning that the pGEM-T Easy vector was not harboring the correct insert in the multiple cloning site although white transformants were recovered on LA agar with X-Gal. This result could have been due to the problem with efficiency of pGEM-T-Easy vector itself. Thus, it was decided to change the methods and clone pAUL- A plasmid into *L. monocytogenes* using In-Fusion<sup>®</sup> HD cloning kit. With this method short, long and multiple DNA fragment can be cloned into any vector in a single reaction.

### 4.1.6 Amplification of the *lmo2235* and *lmo2471* ORF by PCR

To introduce a deletion mutation into other NADH oxidase *lmo2235* and *lmo2471* ORF, In-Fusion HD kit used for direct transformation of ligation mixture into *L. monocytogenes*. pAUL-A containing 5' and 3' regions of *lmo2235* and *lmo2471* were inserted into the chromosomal copy of each ORF by homologous recombination, as described in the Figure 4-7. Mutagenesis was started by amplifying *lmo2235* and *lmo2471* in of *L .monocytogenes* 10403s by PCR. Primers were designed with 15 bp (5') extensions that were complementary to the both ends of the linearized pAUL-A (Figure 4-6).

Primers INF235 FW1, INF235 RV, INF235 FW2 and INF235 RV2 (Table 2-5) were designed to amplify a region of 577bp at the 5' end of *lmo2235* and another region of 582bp at 3' the end of the gene. In the same way 566bp of DNA from the 5<sup>'</sup> end of *lmo2471* and another region of 656bp at 3' were amplified by INF 471 FW1, INF 471

RV1, INF 471 FW2 and INF 471 RV2 (Table 2-5). The results are shown in Figure 4-5 and the amplified DNA of both genes was confirmed to have the expected sizes of 566bp and 656bp (Lane 3 and, *lmo2471* gene) and 577bp and 582bp fragment (Lane7 and 9, *lmo2235* gene) in *L. monocytogenes* 10403s.



**Figure 4-5** Agarose gel electrophoresis confirming PCR amplification of *lmo2235* and *lmo2471* in *L. monocytogenes* 10403s. Lane 1 and 10-100bp DNA markers. Lane 2, 4, 6 and 8 negative controls (no DNA). Lane 3 and 5 shows the amplified 566bp and 656bp fragments of the *lmo2471* gene. Lane7 and 9 shows the amplified 577bp and 582bp fragment of the *lmo22235* gene.



**Figure 4-6** Design PCR primers for gene of interest with 15bp extensions (5') that are complementary to the ends of linearized pAUL-A (blue and red boxes).



Figure 4-7 Diagram showing the strategy for mutagenesis of *L. monocytogenes* using In-Fusion HD kit

### 4.1.7 Preparation and digestion of plasmid pAUL-A with BamHI

The large scale prep of the plasmid pAUL-A was performed according the protocol described in the Material and Methods (Section 2.7.2). Plasmid pAUL-A was digested with *BamHI* following the protocol described in the Material and Methods (Section 2.15). The restriction digestion results are shown in Figure 4-8. Lane 2 shows the uncut pAUL-A plasmid and land 3 shows one band from digested pAUL-A with the expected size of 9.2kb.



**Figure 4-8** Restriction digestion of the plasmid pAUL- A with *BamHI*. Lane 1- 1Kb DNA ladder; Lane 2 – uncut plasmid PAUL-A; Lane 3 – plasmid PAUL-A cut with *BamHI*.

# **4.1.8** Cloning and analysis by PCR of clones of *lmo2235 and lmo2471* in pAUL-A *lmo2235* and *lmo2471* fragments were cloned into digested pAUL-A vector cut with *BamHI* using the in-fusion DH cloning kit. The cloning procedure was carried out following the protocol described in the Material and Methods (Section 2.14.3).

Ligation of the 1159bp and 1222bp fragments of *lmo2235* and *lmo2471* respectively into pAUL-A was confirmed by screening a number of pl2235 and pl2471 plasmids recovered from transformants by PCR using primers M13F and M13R. Also the ligation of insert into pAUL-A was confirmed by sequencing. The nucleotide sequences of plasmid DNA from a number of transformants was determined using primers M13F and M13R. Comparison of the nucleotide sequences showed a significant match (>90%) to the *lmo2235* and *lmo2471* ORFs (see Appendix, Figure 7 and 8).

The resulting plasmids carrying a deletion mutation in lmo2235 and lmo2471were termed pl2235 and pl2471 respectively and were used to introduce the mutation into the chromosome of *L*.*monocytogenes* as described earlier. Following growth at 42°C overnight, erythromycin sensitive recombinants were selected. PCR amplification of these recombinants with primers M13F and M13R gave size to products of 1255bp and 1318bp in lmo2235 and lmo2471 respectively.

The results confirm the ligation of the insert to pAUL-A and successful transformation was achieved. The work was carried with plasmids pl2235 and pl2471.



**Figure 4-9** Agarose gel electrophoresis showing ligation of inset into plasmid pAUL-A. Lane 1 and 9- 1kb DNA markers. Lane 2- negative control (no DNA). A. ligation of insert of *lmo2235* into pAUL-A; expected size is 1255bp. Lane 3, 4 and 5- screening of different transformants using M13F and M13R. B. ligation insert of *lmo2471* gene into pAUL-A; expected size is 1318bp. Lane 6, 7 and 8- screening of different transformants using M13F and M13R.

### 4.1.9 Recombination of pl2235 and pl2471 into L.monocytogenes.

The resulting plasmid pl2235 and pl2471 were transformed into *L. monocytogenes* 10403s as described in section 2.13.2. Transformants were grown at 30°C overnight and colonies were then streaked onto plates and incubated at 42°C overnight, a temperature restrictive for the replication of pAUL-A. Following these incubation erythromycin-resistant colonies should contain the plasmid integrated into the genome Integration of both pl2235 and pl2471 into the bacteria chromosome was confirmed by screening a number of colonies by PCR, using primers C235F for *lmo2235* and C471F primer for *lmo2471* in conjunction with vector M13 primer. Products of 1358bp and 1476bp consistent with integration of pAUL-A235 and pAUL-A471 into the respectively genome were successfully amplified (as indicated in Figure 4-10 and 4-11).



**Figure 4-10** Agarose gel electrophoresis to test integration of plasmid pl2235 into the *L. monocytogenes* 10403s genome. Expected size is 1358bp. Lane 1 and 8- 1KB DNA marker. Lane 2- negative controls (no DNA). Lane 3- negative control (10403s DNA). Lane 4 to 7-screening of different transformants using C235F and M13R. PCR products were visualised on a 1.5% (w/v) agarose gel.



**Figure 4-11** Agarose gel electrophoresis to test integration of plasmid pl2471 into the *L. monocytogenes* 10403s strain. Expected size is 1476bp. Lane1 and 8- 1KB DNA marker. Lane 2- negative control (no DNA). Lane 3- negative control (10403s DNA). Lane 4 to 7 screening of different transformants using C471Fand M13R. PCR products were visualised on a 1.5% (w/v) agarose gel.

One mutant was selected to continue recombination as following, colonies from which a 1358bp and 1476bp PCR products had been amplified from the first crossover were selected and grown in BHI broth and subsequently plated onto BHI agar with and without erythromycin at 42°C, following the protocol described in the Material and Methods (Section 2.13.3). Erythromycin sensitive colonies were screened using primers C235F, C235R and C471F, C471R which were designed to anneal to the chromosome flanking the amplified region of *lmo2235* and *lmo2471* respectivily.

A product of 1156bp was successfuly amplified in the deletion mutant in the *lmo2235* gene compared with product of 2052bp from the *L. monocytogenes* 10403s wild type strain. Figure 4-12 shows two distinguished bands, lane 4, 5, 6, and 7 are products of 1156bp size was amplified from the deletion mutant in *lmo2235* gene, band was with size product of 2052bp refers to *L. monocytogenes* wild type 10403s.

Similarly, A product of 777bp was successfuly amplified in the deletion mutant in the *lmo2471* gene compared with product of 1364bp from the *L. monocytogenes* 10403s wild type strain, as shown in figure 4-13 lanes 3 and 4 shows a product of 777bp was successfuly amplified from the deletion mutant in *lmo2471* gene compared with a product of 1364bp (Lane 5 and 6) from the *L. monocytogenes* 10403s wild type strain.

This inducated that deletion of the *lmo2235* and *lmo2471* ORF had been successful and one mutant from each was selected and designated *L. monocytogens*  $\Delta lmo2235$  and  $\Delta lmo2471$  respectively.



**Figure 4-12** Agarose gel electrophoresis of the recombination of plasmid pl2235 into *L. monocytogenes* 10403s chromosome. Lane 1 and 8- 1kb DNA markers. Lane 2- negative control (no DNA). Lane 3- positive control expected size in *L. monocytogenes* 10403s was 2052bp. Lane- 4 to 7. *L. monocytogenes*  $\Delta lmo2235$  expected size 1156bp using C235F and C235R primers. PCR products were visualised on a 1.5% (w/v) agarose gel.



**Figure 4-13** Agarose gel electrophoresis of the recombination of plasmid pl2471 into *L. monocytogenes* 10403s chromosome. Lane 1 and 7- 1kb DNA markers. Lane 2- negative control (no DNA). Lane 3 and 4- positive control expected size in *L. monocytogenes* 10403s was 1364bp. Lane 5 and 6- *L. monocytogenes*  $\Delta lmo2471$  expected size 777bp using C471F and C471 primers. PCR products were visualised on a 1.5% (w/v) agarose gel.

# 4.2 Construction of a mutant *in lmo0504*, an ORF which encode a putative penicillin binding protein, in *L. monocytogenes* 10403s

According to the result obtained from Arbitrary PCR and sequencing analysis a deletion mutation in *L. monocytogenes* 10403s was constructed in order to check the polar effects. Therefore to confirm that *lmo0540* was the cause of the adhesion defect in I366 mutant.

### 4.2.1 Amplification of the genes *lmo0540* by PCR

Primers were designed to amplify region of *lmo0540* gen which contain 15bp extension complementary to the both end of the linearized pAUL-A vector. Fw540 INF FW1 and Fw540 INF RV1 primers were designed to amplify a region of 540bp at the 5' end of *lmo*0540. Another region of 471bp, at 3' the end of the gene was amplified using Fw540 INF FW2 and Fw540 INF RV2 primes. Figure 4-14 shows products of 540bp and 471bp of the expected size in lane 3 and 4.



**Figure 4-14** Agarose gel electrophoresis confirming PCR amplification of *lmo0540* gene in *L. monocytogenes* 10403s using Fw540 INF FW2 and Fw540 INF RV2 primes. Lane 1- 100bp DNA marker. Lane 2 and 4- negative controls (no DNA). Lane 3 and 5- from *L. monocytogenes* shows the amplified 540bp and 471bp fragments of the *lmo0540* gene.

### 4.2.2 Cloning and analysis by PCR of clone *lmo0540* in pAUL-A

Fragments of the 5' and 3' region of *lmo0540* of sizes 540bp and 471bp respectively, were cloned into digested pAUL-A using the in-fusion HD cloning kit to give a total fragment size of 1011bp. Ligation of the 1011bp fragment into pAUL-A was confirmed by screening a number of pl0540 plasmids recovered from transformants by PCR using primers M13F and M13R. Also, the ligation of insert into pAUL-A was confirmed by sequencing. The nucleotide sequences of plasmid DNA from a number of transformants was determined using primers M13F and M13R. Comparison of the nucleotide sequences showed a significant match (>90%) to the *lmo0540* ORF (see Appendix, Figure 11).

The resulting plasmid carrying a deletion mutation in lmo0540 was termed pl0540 and was used to introduce the mutation into the chromosome of *L*.*monocytogenes* as described earlier. Following growth at 42°C overnight, erythromycin sensitive recombinants were selected. PCR amplification of these recombinants with primers M13F and M13R gave size to products of 1100bp.

The results obtained are shown in Figure 4-15. As can be seen in lanes 3, 4 and 5 *lmo0540* fragments ligated into the pAUL-A vector with expected product size of 1100bp. The result confirm the ligation of the insert to pAUL-A and successful transformation was achieved. The work was carried with plasmid pl0540.



**Figure 4-15** Agarose gel electrophoresis showing ligation of inset into plasmid pAUL-A. Lane 1-100bp DNA marker; Lane 2- negative control (no DNA). Lane 3, 4 and 5- ligation of insert of *lmo0540* into pAUL-A; expected size is 1100bp using M13F and M13R by screening a number of transformants. Lane 6- 1kb DNA marker. PCR products were visualised on a 1 % (w/v) agarose gel.

### 4.2.3 Recombination of pl0540 into *L*.monocytogenes 10403s.

The resultant plasmid was transformed into *L. monocytogenes* 10403s using electroporation. And recombinants selected by growth on erythromycin and incubated overnight at 24°C to allow integration of the plasmid onto the chromosome to occur. To confirm that this had occurred PCR was carried out using primers CpbpFW and M13 primer. Figure 4-16 shows pl0540 integrated into the *L. monocytogenes*10403s genome with size products of 1095bp from different transformants. One mutant was selected to continue the recombination.



**Figure 4-16** Agarose gel electrophoresis to test integration of plasmid pl0540 into the *L. monocytogenes* 10403s genome. Expected size is 1095bp. Lane 1- 1kb DNA marker. Lane 2-negative control (no DNA). Lane 3 to 7- screening of different transformants using CpbpFW and M13R. PCR products were visualised on a1.5% (w/v) agarose gel.

For recombination, colonies giving a positive band 1095bp for the first crossover were selected and grown in BHI broth and subsequently plated onto BHI with and without erythromycin at 42°C as described before (Section 2.13.3). to force a second crossover event, erythromycin sensitive colonies were screened using primers which was designed to amplify outside the region of interest. As can be seen in Figure 4-17 a product with 1152bp was successfuly amplified from the deletion mutant in the *lmo0540* (Lane 4) compared with a product of 2154bp from the *L. monocytogenes* wild type 10403s (Lane 3) and one mutant was selected and designated *L. monocytogenes*  $\Delta lmo0540$ .



**Figure 4-17** Agarose gel electrophoresis of the recombination of plasmid pl0540 into *L. monocytogenes* 10403s strain. Lane 1- 1kb DNA marker. Lane 2- negative control (no DNA). Lane 3- positive control expected size in *L. monocytogenes* 104003s was 2154bp. Lane4- *L. monocytogenes*  $\Delta lmo0540$  expected size1152bp using CpbpFW and CpbpRV primers. PCR products were visualised on a 1.5% (w/v) agarose gel.

### 4.3 Construction of deletion mutant in *lmo2236*, a putative oxidoreductase in *L. monocytogenes* 10403s

According to the result obtained from qRT-PCR analysis (Section 5-6) indicating that the expressing level of lmo2236 in transposon B265 was a significantly lower compared to the expression in the wild type .and this result suggested that the Tn917transposon may alter expression of lmo2236 which is located downstream to lmo2235. Accordingly it is possible that this reduced expression is responsible for the adhesion defect and not disruption of lmo2235. A deletion mutation in Lmo2236 in L. monocytogenes 10403s was therefore constructed.

### 4.3.1 Amplification of the genes *lmo2236* PCR

Primers were designed as described above in section (4.1.6). Primers were designed to amplify a region of 773bp at the 5' end and 517bp at 3' the end of *lmo2236* ORF. The results shown in Figure 4-18 show that the PCR products amplified from *L*. *monocytogenes* 10403s had the expected sizes of 773bp (Lane 3 and 4) and 517bp (Lane 6 and 7) in *L. monocytogenes* wild type 10403s.



**Figure 4-18** Agarose gel electrophoresis confirming PCR amplification of *lmo2236* gene in *L. monocytogenes*. Lane 1-100bp DNA marker. Lane 2 and 5- negative controls (no DNA). Lane 3and 4- from *L. monocytogenes* 10403s show the amplified 773bp fragment of the *lmo2236* gene by 2236FW1 and 2236RV1. Lane 6 and 7- show the amplified 517bp fragment of the *lmo2236* gene by 2236FW2 and 2236RV2.

### 4.3.2 Cloning and analysis by PCR of clone of *lmo2236* in pAUL-A

Fragments of the 5' and 3' region of *lmo2236* of sizes 773bp and 517bp respectively, were cloned into digested pAUL-A using the in-fusion HD cloning kit to give a total fragment size of 1290bp. Ligation of the 1290bp fragment into pAUL-A was confirmed by screening a number of pl2236 plasmids recovered from transformants by PCR using primers M13F and M13R. Also the ligation of an insert into pAUL-A was confirmed by sequencing. The nucleotide sequences of plasmid DNA from a number of transformants was determined using primers M13F and M13R. Comparison of the nucleotide sequences showed a significant match (>90%) to the *lmo2236* ORF (see Appendix, Figure 13).

The resulting plasmid carrying a deletion mutation in lmo2236 was termed pl2236 and was used to introduce the mutation into the chromosome of *L*.*monocytogenes* as

described earlier. Following growth at 42°C overnight, erythromycin sensitive recombinants were selected. PCR amplification of these recombinants with primers M13F and M13R gave size to products of 1387bp

The results obtained are shown in Figure 4-19. As it can be seen in lanes 3 to 7, the *lmo2236* fragment ligated into pAUL-A vector with expected product size of 1387bp. The result confirm the ligation of the insert to pAUL-A and successful transformation was achieved. The work was carried with plasmid pl2236.



**Figure 4-19** Agarose gel electrophoresis showing ligation of inset into pAUL-A. Lane 1-1kbDNA marker; Lane 2- negative control (no DNA). Lane 3 to 7- ligation of insert of *lmo2236* into pAUL-A; expected size 1387bp recovered from transformant using M13F and M13R primer. PCR product were visualised on a 1% (w/v) agarose gel.

### 4.3.3 Recombination of pl2236 into *L*.monocytogenes.

Plasmid pl2236 was transformed into *L. monocytogenes* 10403s by electroporation and transformants harbouring the plasmid pl2236 from electroporation were streaked on BHI agar with erythromycin (5µg/ml) and incubated at 42°C overnight. Plasmid

integration into the bacteria chromosome was confirmed by screening a number of transformants by PCR using the C236F primers which binding to the chromosome outside the *lmo2236* gene with M13R primer. PCR products of 1546bp were successfully amplified from number of transformants as indicated in Figure 4-20 (Lanes 4-13). One mutant was selected to continue recombination.



**Figure 4-20** Agarose gel electrophoresis of integration of plasmid pl2236 into the *L. monocytogenes* 10403s strain. Expected size is 1546bp. Lane 1 and 14- 1kb DNA marker. Lane 2- negative control (no DNA). Lane 3- negative control (10403s DNA). Lane 4 to 13- screening of different colonies using C2236FW and M13R confirming the integration of plasmid into gene at expected size of 1546bp. PCR products were visualised on a 1.5% (w/v) agarose gel.

Colonies giving a positive band 1546bp for first crossover were selected for recombination as described above (Section 2.13.3). Following a second round of recombination, erythromycin sensitive colonies were screened using primers C236F and C236R which was designed to anneal to the chromosome flanking the amplified region. A product with 1669bp was successfully amplified from the deletion mutant in *lmo2236* gene compared with a product of 2336bp from the *L. monocytogenes* 10403s

strain. In Figure 4-21 lane 4 shows a product of 1669bp was successfully amplified from the deletion mutant in *lmo2236* ORF. Lane 3 shows a product size of 2336bp from th *L. monocytogens* 10403s strain. The mutant was selected and designated *L. monocytogenes*  $\Delta lmo2336$ .



**Figure 4-21** Agarose gel electrophoresis of the recombination of plasmid pl2236 into *L. monocytogenes* 10403s strain. Lane 1 and 5- 1kb DNA markers. Lane 2- negative control (no DNA). Lane3- positive control expected size in *L. monocytogenes* 104003s was 2336bp. Lane-4 *L. monocytogenes*  $\Delta lmo2336$  expected size1669bp. Using C236F and C236R primer. PCR products were visualised on a 1.5% (w/v) agarose gel.
#### 4.4 Discussion

Amplification of genes was performed by PCR (Section 2.8) and products were cloned into pGEM T-easy. Following transformation of plasmids into E. coli, resulting transformants for each of the inserted genes were sequenced to confirm that the correct gene had inserted into the plasmid. Only the transformants containing the lmo0103 gave a positive match when submitted to BLAST analysis. The positive transformant containing the correct sequence for *lmo0103* was then ligated into pAUL-A and electroporated into L. monocytogenes 10403s to enable the deletion mutant to be made using recombination. The deletion mutant of  $\Delta lmo0103$  in L. monocytogenes was successfully achieved. However, none of the other resulting plasmids harbouring other DNA fragments showed any matching against genes in L. monocytogenes EGD-e. This meant the pGEM-T Easy vector was not harbouring the correct insert in the multiple cloning sites although white transformants were recovered on LA agar. The process was repeated a couple of times with other DNA fragments of genes and the same problem was presented. Consequently, it was decided to change this method using In-Fusion<sup>®</sup> HD cloning kit to clone DNA fragment into the pAUL-A plasmid vector and electroporate into L. monocytogenes 10403s without using cloning pGEM-T Easy cloning vector; with this method short, long and multiple DNA fragment can be cloned into any vector in a single reaction. Further mutagenesis was done using this method to construct other mutants in NADH oxidase (*lmo2235* and *lmo2471*) and penicillin binding protein genes (*lmo0540*)

The plasmid pAUL-A is capable of autonomous replication in both *E. coli* and *Listeria* spp (Schaferkordt & Chakraborty, 1995). It also carries an erythromycin resistance marker that expresses in both hosts at  $300\mu$ g/ml in *E. coli* and  $5\mu$ g/ml in *L. monocytogenes*. This characteristic is important because direct transformation of

ligation mixtures into *L. monocytogenes* is very inefficient (Schaferkordt & Chakraborty, 1995). pAUL-A also contains the *lacZ* gene of *E. coli* which includes the respective operator and promoter and encloses the multiple cloning site of plasmid pUC19. Cloning into any of these sites disrupts the *lacZ* gene, causing a loss of  $\alpha$ -complementation and resulting in lac<sup>-</sup> phenotype in the appropriate *E. coli* host strain. The recombinant plasmid can then be readily isolated and transformed into *L. monocytogenes*.

pAUL-A with correct DNA fragments were transformed into L. monocytogenes using the electroporation of penicillin treated cells (Park & Stewart, 1990). This method takes advantage of the fact that cell wall damage, caused either by degradative enzymes or by incorporation of cell wall active agents in the growth media, typically improves transformation efficiencies in Gram positive bacteria (Park & Stewart, 1990). Therefore a pre-treatment with penicillin caused a dramatic increase in the transformation efficiency of L .monocytogenes. The optimal conditions for electroporation of penicillin-G treated L. monocytogenes (maximum number of transformation  $4 \times 10^6 / \mu g$ DNA) was achieved in the presence of 10  $\mu$ g penicillin-G/ml and electroporated at a field strength of 10kV/cm (pulse duration, 5ms) (Park & Stewart, 1990). The voltage used in this study to electroporate L. monocytogenes was 2.5kV/cm (pulse duration about 5ms) (Section 2.13.2). Although the voltage value was, successful transformants were recovered and this suggests that the field strength used was enough to produce permeability of *Listeria* cell membrane, thus allowing the entry of the plasmid DNA. Electroporation therefore has proved to be effective for transferring DNA into eukaryotic and prokaryotic organisms (Luchansky et al., 1988). Electroporation is a quick technique and less tedious and inexpensive than other transformation methods.

To recover deletion mutants, bacteria were at first cultured at 30°C with erythromycin, to remove the plasmid. The bacteria were then cultured at the non-permissive temperature of 42°C with erythromycin. The high temperature of incubation does not allow the pAUL-A to replicate since it contains pE194<sub>ts</sub>, an origin of replication which is sensitive to temperatures higher than 30°C (Schaferkordt & Chakraborty, 1995). Surviving bacteria should have lost plasmid encoding erythromycin resistance. PCR was used to confirm that the genes of interest have been mutated. Mutants also were sent for sequencing with appropriate primers. Resulting sequences was aligned against the sequenced *L. monocytogenes* EDG-e genome held at NCBI and the result was positive with all mutants (see appendix, Figures 6, 9, 10, 11and 14).

## 5 Characterisation of attachment of *L. monocytogenes* mutants

Deletion mutants of *L. monocytogenes* ( $\Delta lmo2235$ ,  $\Delta lmo2471$ ,  $\Delta lmo0103$ ,  $\Delta lmo2236$ , and  $\Delta lmo0540$ ) were successfully generated as described in the previous chapter. These mutants, with their parent *L. monocytogenes* 10403s and transposon mutants (B265 and I366), were used for further investigation. In this chapter the work done was intended to determine the growth rate of *L. monocytogenes* strains, at different NaCl concentrations and pHs.

-To examine the effect of NaCl and acidic pH on the attachment of *L. monocytogenes* strains to polystyrene.

-To measure the expression of *lmo2235*, *lmo0540* in the planktonic and attached cells after listerial attachment at 18°C and 30°C.

NaCl and acidic pH were chosen in this study as both are used in food processing industries as major barriers to prevent bacterial growth in food. Thus, this may provides valuable pathway for prevention of biofilm formation.

The growth of *L. monocytogenes* strains was tested at different NaCl concentrations and pHs and there was no significant difference in the mean growth curve between all strains (P>0.05).

Microtitre plates were used in the investigation of the effect of NaCl and pH on listerial attachment. All strains including the wild type showed a decrease in their ability to adhere with increasing NaCl concentration. On the other hand, *L. monocytogenes* strains lost their attachment at pH below 7.3. Therefore these results suggest that the mutated genes have no impact on the behavior of *L. monocytogenes* in these tested conditions.

These results indicate that NaCl and acidic pH had an effect on the attachment of *L*. *monocytogenes* strains, and they are extremely sensitive to lactic pH.

Microtitre plate was also used to investigate the expression of *lmo2235* and *lmo0540* genes in the planktonic and detached cells after attachment at 18°C and 30°C. The pattern of *lmo2235* expression in detached and planktonic cells after attachment was very similar at 18°C and 30°C. However the expression of *lmo0540* was higher in the planktonic cells compared to detached cells at 18°C and 30°C.

#### 5.1 Growth of *L. monocytogenes* wild type and mutants

Growth of *L. monocytogenes* strains was determined at different concentrations of NaCl and acidic pHs in order to investigate whether these conditions have an impact on the growth rate of *L. monocytogenes* strains, before deciding to examine their attachment in these conditions. To determine the growth rate, *L. monocytogenes* strains were cultured in TSB at different concentrations of NaCl (2% (w/v) and 4% (w/v)) and pH (6.5 and 5.5). Each strain was prepared as described in the Materials and Method (Section 2.4). Optical density and viable counts were carried out every 20 minutes. The viable counts were used to calculate the growth rate constant ( $\mu$ ) for all strains. The wild type and the seven mutants listed in Table 2.1 were examined and all the strains were grown with shaking until they reached the stationary phase.

In Figure 5-5 5-1 the growth of the *L. monocytogenes* wild type 10403s and mutants in TSB at 37°C is shown. As can be seen, the pattern of growth displayed by the strains was similar; all strains grew well and demonstrated an exponential growth phase of two hours from the beginning of growth. One-way ANOVA was used to compare the growth rates constants ( $\mu$ ) and they listed in (Table 5-1) the growth rate constants for growth curves. There was no effect on the growth rate of all strains compared to the

wild type and compared to each other (P>0.05). The mean growth rate for all strains was between 1.6 and 2.1 /hour.



**Figure 5-1** Growth curves of *L. monocytogenes* strains performed in TSB at 37°C. Each point is the mean of two replicates. The pattern of growth of strains was similar, demonstrating an exponential growth phase of two hours from the beginning of growth.

**Table 5-1** The growth rate constant  $\mu$  was calculated for the *L. monocytogenes* and six mutants in TSB at 37°C and at different NaCl and pH concentration. There was no significant difference (P>0.05) in the growth rate of all the stains at the different conditions (n=2).

Strains	Growth rate (hour <sup>-1</sup> )							
Strams	37°C	2%(w/v) Nacl	4% (w/v) Nacl	pH6.5	pH5.5			
wild type 10103s	1.700	1.255	1.573	1.674	1.433			
$\Delta lmo0540$	1.913	1.525	1.984	1.549	1.415			
B265	1.636	1.198	1.806	1.302	1.030			
I366	1.869	1.063	1.260	1.209	1.205			
$\Delta lmo0103$	1.856	1.869	1.254	1.191	1.656			
Δ <i>lmo2235</i>	2.296	1.869	1.384	1.821	1.493			
$\Delta lmo2471$	2.109	1.156	1.378	1.791	1.247			

Growth curves also were used to assess whether NaCl had an effect on growth of *L.monocytogenes* strains. The following concentrations of NaCl were tested; 2, 4, and 6% (w/v) in TSB. These concentrations are in addition to the 0.5% (w/v) NaCl TSB already contains. Wild type and deletion mutants grew on the TSA plates with 6% (w/v) NaCl concentration, but transposon mutants B265 and I366 did not show growth under these conditions, so it was decided to not examine the strains with 6% (w/v) NaCl.

Figure 5-2 shows the growth of the *L. monocytogenes* wild type 10403s and mutants in TSB with 2% (w/v) NaCl. As can be seen, the pattern of growth displayed by the strains was similar with no difference between them. The mean growth rate of all strains was between 1.0 and 1.8/hour and statistically no difference in the growth rate were detected between the wild type and mutants at this concentration (P>0.05), as indicated in Table 5-1.

Similarly the results in Figure 5-3 show the growth of wild type and mutants in TSB containing 4% (w/v) NaCl. Again the wild type and the mutant exhibited similar pattern of growth. However, the stationary phase was reached after 3 hours. The growth rate of 1.9 and 1.2/hour was found when comparing wild type with mutants as shown in Table 5-1. The statistical analysis to the growth rate values indicated that there is no significant difference (P>0.05) between strains to grow in TSB with 4 % (w/v) NaCl.



**Figure 5-2** Growth curves of *L. monocytogenes* strains cultured in TSB at  $37^{\circ}$ C in 2% (w/v) NaCl. Each point is the mean of two replicates. The pattern of growth of strains was similar, with no difference between them. The stationary phase was reached after 2 hours.



**Figure 5-3** Growth curves of *L. monocytogenes* strains cultured in TSB at  $37^{\circ}$ C and in 4% (w/v) NaCl. Each point is the mean of two replicates. Wild type and the mutant exhibited similar pattern of growth and the stationary phase was reached after 3 hours from the beginning of growth.

The effects of pH on the growth of *L. monocytogenes* strains were examined. The pHs of 6.5, 5.5 and 4.5 were used. It was reported previously that growth of *L*.

*monocytogenes* occurred between pH 5.2 and 9 and that they are not able to grow below pH 5.2 (Wagner & McLauchlin, 2008). However, *L. monocytogenes* strains did not show growth in TSB with pH 4.5 (data not shown). Therefore it was decided to test *Listeria* strains growth at pH 6.5 and 5.5.

Figure 5-4 shows the growth of wild type and mutants in TSB at pH 6.5. *L. monocytogenes* mutant strains also showed no difference in their growth pattern and the stationary phase was reached after 2 hours. The growth rates of strains were between 1.9 and 1.2/hour (Table 5-1). ANOVA of these data revealed that there were no significant differences in the mean of growth between strains (P>0.05).

Also the growth of *L. monocytogenes* strains was examined at pH 5.5. The results are shown in Figure 5-5 *L. monocytogenes* strains exhibited similar pattern of growth. Strains began to grow exponentially after 3hours. Data revealed that the difference in the mean of growth was not significant (P>0.05) and the growth rates of strains were between 1.0 and 1.6/hour as demonstrated in Table 5-1.

None of the mutants presented a significant difference in growth rate when compared to the wild type or each other (P>0.05). The analysis of these results suggested that,  $\Delta lmo2235$ ,  $\Delta lmo2471$ ,  $\Delta lmo0103$  and  $\Delta lmo0540$  genes deleting have no impact on the growth of *L. monocytogenes* even under different conditions, and subsequently it was decided to check their adherence to polystyrene

In order to confirm previous data, growth curves were determined in TSB in microtitre plates. The  $OD_{550nm}$  was measured at regular intervals with a Varioscan (see Appendix, Figure 19 to 22). There was no differences (P>0.05) in the growth pattern detected between the strains in TSB at different concentrations of NaCl and pH.



**Figure 5-4** Growth curves of *L. monocytogenes* strains cultured in TSB at 37°C and pH 6.5. Each point is the mean of two replicates. *L. monocytogenes* strains exhibited similar pattern of growth. Strains began to grow exponentially after 2hours.



**Figure 5-5** Growth curves of *L. monocytogenes* strains cultured in TSB at 37°C and pH 5.5. Each point is the mean of two replicates. *L. monocytogenes* wild type and mutants in. No difference in their growth pattern and the stationary phase was reached after 3hours.

#### 5.2 Attachment studies

Attachment assays were done using the deletion mutants, in order to confirm whether the NADH oxidase and penicillin binding protein genes were involved in attachment of *L. monocytogenes* to polystyrene. The attachment assay was performed following the protocol described in the Material and Methods (Section 2.5)

## 5.2.1 Assay for attachment of *L. monocytogenes* wild type and mutants at 37<sup>°</sup>C

The results for the attachment assay of *L. monocytogenes* wild type and mutants at  $37^{\circ}$ C are shown in Figure 5-6. There was no significant difference in the ability of the mutant strains to adhere to polystyrene at  $37^{\circ}$ C (P>0.05). Also no difference in the total number of planktonic cells counts (P>0.05) was found after 2 hours incubation as, shown in Table 5-2.



**Figure 5-6** Assay of attachment assay of *L. monocytogenes* strains to polystyrene at 37°C. Data are the mean ( $\pm$  S.D.) of three experiments. There was no significant difference between all the stains (P>0.05).

Strains	log <sub>10</sub> of the mean viable counts					
wild type 10403s	9.0 ±0.05					
B265	9.3 ±0.03					
$\Delta lmo0103$	9.2 ±0.02					
$\Delta lmo 2235$	9.0 ±0.04					
$\Delta lmo2471$	9.1 ±0.01					
I366	9.2 ±0.1					
$\Delta lmo0540$	9.1 ±0.08					

**Table 5-2** Viable counts of planktonic cells in the attachment assay after 2 hours incubation at 37°C.

Data are the mean  $\pm$  standard deviation of the result of four experiments.

## 5.2.2 Assay attachment of L. monocytogenes wild type with mutants at $30^{\circ}$ C

The attachment assay of wild type and mutants was conducted at  $30^{\circ}$ C. As can be seen in Figure 5-7 all mutants showed a clear reduction in the ability of attachment compared to wild type (P<0.05) at  $30^{\circ}$ C. On the other hand, there was no significant difference between the mutants in their attachment at  $30^{\circ}$ C (P>0.05). The deficiency of mutants suggests a role for these genes in ability to adhere to polystyrene, without difference in the total number of planktonic cells in the end of assay as, can be seen in Table 5-3.



**Figure 5-7** Assay of attachment *L. monocytogenes* strains to polystyrene at 30°C. Data are the mean ( $\pm$  S.D.) of three experiments. All the mutants showed a reduction in attachment compared to the wild type (P<0.05).

Strains	log <sub>10</sub> of the mean viable counts				
wild type 10403s	9.1 ±0.1				
B265	9.0 ±0.2				
$\Delta lmo0103$	9.1 ±0.08				
$\Delta lmo2235$	9.0 ±0.09				
$\Delta lmo2471$	9.1 ±0.05				
I366	9.0 ±0.1				
$\Delta lmo0540$	9.2 ±0.02				

**Table 5-3** Viable counts of planktonic cells in the attachment assay after 2 hours incubation at 30°C.

Data are the mean  $\pm$  standard deviation of the result of four experiments.

# 5.2.3 Assay of attachment assay of *L. monocytogenes* wild type and mutants at 18°C

The attachment assay of wild type and mutants was done at  $18^{\circ}$ C, shown in Figure 5-8. All the mutants showed a significant reduction in the level of attachment when compared to the wild type at  $18^{\circ}$ C (P<0.05). On the other hand there was no difference in the level of attachment between the mutants (P>0.05). The numbers of planktonic cell numbers were found to be similar in all *L. monocytogenes* strains (P>0.05) after 2 hours incubation at  $18^{\circ}$ C, as shown in Table 3-10. These results suggested that the influence of NADH oxidase and penicillin binding protein on surface attachment is temperature dependent.



**Figure 5-8** Assay of attachment *L. monocytogenes* strains to polystyrene at 18°C. Data are the mean of three experiments. Wild type showed a higher level of attachment than that of mutant strains (P<0.05).

Table 5-4	Viable	counts of	of plankto	nic cell	s in th	e attachment	assay	after 2	2 hours	incubation	at
18°C.											

Strains	log <sub>10</sub> of the mean viable counts				
wild type 10403s	9.2 ±0.1				
B265	9.1 ±0.09				
$\Delta lmo0103$	9.0 ±0.05				
$\Delta lmo2235$	9.0 ±0.1				
$\Delta lmo2471$	9.1 ±0.01				
I366	9.1 ±0.4				
$\Delta lmo0540$	9.1 ±0.1				

Data are the mean  $\pm$  standard deviation of the result of four experiments.

# 5.3 Effect of NaCl concentration and pH on the attachment of *L. monocytogenes* strains at different temperatures

The aim of this study was to examine the effect of NaCl and pH on the attachment of *L. monocytogenes* strains to polystyrene at two different temperatures,  $37^{\circ}C$  and  $30^{\circ}C$ . The listerial cells listed in (Table 2-1) were cultured overnight  $37^{\circ}C$  and  $30^{\circ}C$  in medium containing different NaCl concentrations (0.5% (w/v), 2% (w/v) and 4% (w/v)) and different pHs (7.3, 6.5 and 5.5). The medium was adjusted using NaCl and HCl before autoclaving. The assay was done following the protocol in the Material and Methods (Section 2.5). Diluted cultures (OD<sub>500</sub> =0.7) were added to a 96-well microtitre plate and incubated for 2 hours. The total number of planktonic cells was estimated in duplicate at the end of the assay. Also *L. monocytogenes* strains did not grow in TSA at pH 4.5 (data not provided).

#### 5.3.1 Effect of NaCl on *L. monocytogenes* attachment at 37°C

Figure 5-9 panels A and B show that all *L. monocytogenes* strains could attach to the polystyrene at the different concentrations of NaCl. However the efficiency of attachment declined as the concentration of NaCl increased. A significant difference in their ability of the strains to attach to the surface was observed at NaCl 2% (w/v) and 4% (w/v) NaCl compared to 0.5% (w/v) NaCl (P<0.05). However, there was no significant difference in their ability to attach at NaCl 2% (w/v) and 4% (w/v) when compared to each other (P>0.05). It is obvious the level of attachment significantly decreased gradually at the higher concentrations of NaCl (2% (w/v) and 4% (w/v).

The mutants showed no significant difference in their attachment compared to the wild type at all NaCl concentrations (P>0.05). Similarly, there was also no significant difference between the mutants when compared to their attachment to polystyrene at

different concentrations of NaCl (P>0.05). The numbers of planktonic cells were equivalent at the end of the experimental period (P>0.05).



**Figure 5-9** A) Assay of attachment *L. monocytogenes* wild type, B265,  $\Delta lmo0103$ ,  $\Delta lmo2235$  and  $lmo\Delta2471$  at 37°C in TSB with different NaCl concentrations. B) Assay of attachment *L. monocytogenes* wild type, I366 and  $\Delta lmo0540$  at 37°C in TSB with different NaCl concentrations. Data are the mean (± S.D.) of three experiments. All the strains in A and B showed an ability to attach to polystyrene at different NaCl concentrations compared to control (P>0.05). The mutants showed the same level of attachment compared to the wild type (P<0.05). All strains showed a low level of attachment at high concentration of NaCl (2% and 4% (w/v)).

#### 5.3.2 Effect of NaCl on L. monocytogenes attachment at 30 °C

*L. monocytogenes* strains were also examined for their ability to attach to polystyrene in TSB containing different NaCl concentrations. As indicated in Figure 5-10 panels, A and B all strains were able to adhere to polystyrene at all NaCl concentrations tested. However, there was a significant difference in the extent of attachment at the higher concentrations of NaCl (0.5% (w/v), 2% (w/v) and 4% (w/v)) (P<0.05). The attachment significantly decreased at high NaCl concentration (P<0.05). Although *L. monocytogenes* wild type had the highest level of attachment in 0.5% (w/v) NaCl compared to the mutants (P<0.05), it also showed a decreased in ability to adhere with increasing NaCl concentration. Mutants showed significantly lower levels of attachment at 0.5% NaCl (w/v), when compared to the wild type (P<0.05) but there was no significant difference between mutants and wild type at NaCl levels of 2%(w/v) and 4% (w/v) (P>0.05). On the other hand, the mutants showed no significant difference in their attachment when compared to each other at different NaCl concentrations (P>0.05). The numbers of planktonic cells of the wild type and mutants were the same (P>0.05).





**Figure 5-10** A) Assay of attachment *L. monocytogenes* wild type, B265,  $\Delta lmo0103$ ,  $\Delta lmo2235$  and  $lmo\Delta2471$  at 30°C in TSB with different NaCl concentrations. B) Assay of attachment *L. monocytogenes* wild type, I366 and  $\Delta lmo0540$  at 30°C in TSB with different NaCl concentrations Data are the mean (± S.D.) of three experiments.

#### 5.3.3 Effect of acidic pH on L. monocytogenes attachment at 37°C

Attachment assays were conducted with *L. monocytogenes* wild type and mutants at different pHs (7.3, 6.5, and 5.5) at 37°C. The obtained results shown in Figure 5-11 A and B highlighted that all strains were able to attach well to polystyrene at pH 7.3, while no attachment was observed at pHs of 6.5 and 5.5 compared to control (P>0.05). However, there was no significant difference in the ability to attach of all strains at pH 7.3 (P>0.05). The numbers of planktonic cells of the wild type and mutants were the same at the end of assay (P>0.05).

#### 5.3.4 Effect of acidic pH on *L. monocytogenes* attachment at 30°C

Figure 5-12 A and B shows the attachment assay of *L. monocytogenes* strains at different pHs at 30°C. It can be observed that *L.monocytogenes* strains attached well at pH 7.3 and the level of attached cells of the wild type and mutants was higher than observed at  $37^{\circ}$ C (P<0.05). The wild type showed a high level of attachment at pH 7.3 compared to the mutants (P<0.05). The ability of strains to attach was significantly diminished at the pH 6.5 and 5.5 (P<0.05) i.e. they lost their attachment to polystyrene with lower pHs. There was no difference between all the mutants in their attachment at the different pHs (P>0.05). The numbers of planktonic cell of the wild type and mutants were the same for all strains (P>0.05). These results indicate that pH had an effect on the attachment of *L. monocytogenes* strains, and they and wild type are extremely sensitive to acidic pH.





**Figure 5-11** A) Assay of attachment *L. monocytogenes* wild type, B265,  $\Delta lmo0103$ ,  $\Delta lmo2235$  and  $lmo\Delta2471$  at 37°C in TSB at different pHs. B) Assay of attachment *L. monocytogenes* wild type, I366 and  $\Delta lmo0540$  at 37°C in TSB at different pHs. Data are the mean (± S.D.) of three experiments. All the strains were able to attach at pH 7.3 compared to the control (P<0.05) while no attachment was observed at pHs of 6.5 and 5.5 (P>0.05)





**Figure 5-12** A) Assay of attachment *L. monocytogenes* wild type, B265,  $\Delta lmo0103$ ,  $\Delta lmo2235$  and  $lmo\Delta2471$  at 30°C in TSB at different pHs. B) Assay of attachment *L. monocytogenes* wild type, I366 and  $\Delta lmo0540$  at 30°C in TSB at different pHs. Data are the mean (± S.D.) of three experiments. *Listeria strains* attached well at pH 7.3 (P<0.05). Wild type showed a high level of attachment at pH 7.3 compared to other mutants (P<0.05), mutants ability to attach were significantly decreased at the pH 6.5 and 5.5 (P>0.05)

#### 5.4 Assay of the attachment of wild type and $\Delta lmo2236$

It was hypothesised that, *lmo2236* gene may regulate adhesion and attachment in *L. monocytogenes*. In order to test this hypothesis  $\Delta lmo2236$  was examined for its ability to attach to polystyrene compared to wild type and  $\Delta lmo2235$  at 37°C and 30°C. The attachment assay was conducted as described in the Material and Methods (Section 2.5). The result obtained is shown in Figure 5-13.  $\Delta lmo2236$  showed the same level of attachment compared to the wild type and  $\Delta lmo2235$  at 37°C (P>0.05). There was also no significant difference (P>0.05) between  $\Delta lmo2236$  and wild type in their attachment at 30°C. However,  $\Delta lmo2235$  showed a reduction in attachment compared to wild type and  $\Delta lmo2236$  (P<0.05). The results suggest that *lmo2236* does not play a major role in listerial adherence to polystyrene.



**Figure 5-13** Assay of attachment *L. monocytogenes* wild type,  $\Delta lmo2236$  and  $\Delta lmo2235$  to polystyrene at 37°C and 30°C. Data are the means (± S.D.) of three experiments. There was no significant difference between all the stains (P>0.05) at 37°C.  $\Delta lmo2235$  mutants showed a reduction in attachment compared to the wild type and  $\Delta lmo2236$  (P<0.05) at 30°C. Wild type and  $\Delta lmo2236$  showed the same level of attachment (P<0.05) at 30°C.

# 5.5 Quantitative real-time PCR (qRT-PCR) analysis of *lmo2235* and *lmo0540* gene expression in *L. monocytogenes* 10403s

As reported earlier in the attachment study of *L. monocytogenes* wild type 10403s, it was demonstrated that *L. monocytogenes* has a significantly greater attachment at  $30^{\circ}$ C compared to 18°C and 37°C. In the same study wild type showed a high level of attachment compared to transposon mutants B256 and I366 at 18°C and 30°C (Section 3.1.3 and 3-1-4). *Imo02235* and *Imo0450* genes were chosen to test their expression in *L. monocytogenes* wild type, as these genes found to be involved in the deficiency of attachment to polystyrene at 18°C and 30°C in transposon mutants B265 and I366 respectively, thus it was decided to test the expression of these genes at different temperatures. qRT-PCR was used to determinate expression of these genes in *L. monocytogenes* wild type.

#### 5.5.1 Listerial RNA extraction and purification

RNA was extracted by the GTC/TRIZOL method and treated with DNase following the protocol in the Material and Methods section (2.17.1.1). The RNA preparations were analysed on agarose gel electrophoresis as shown in Figure 5-14.



**Figure 5-14** Agarose gel electrophoresis showing isolated listerial RNA. Lane 1- 100bp DNA Ladder. Lane 2- RNA from *L. monocytogenes* wild type grown at 18°C. Lane 3- RNA from *L. monocytogenes* wild type grown at 30°C, Lane 4- RNA from *L. monocytogenes* wild type grown at 37°C.

### 5.5.2 Impact of temperature on *L. monocytogenes* 10403s gene expression

Initially, the expression of the *lmo2235* gene and *lmo0540* gene was determined at three different temperatures;  $18^{\circ}$ C,  $30^{\circ}$ C and  $37^{\circ}$ C, in bacteria grown to mid log phase. The qRT-PCR assay was optimised by determining primer annealing efficiency for each primer pairs. This was done to ensure that the differential expression of genes in the assay condition reflects the biological events taking place rather than artefact of differences in primer annealing efficiency. To determine the primer annealing efficiency, reactions were set up using different concentrations of *L. monocytogenes* genomic DNA samples and the Ct values were plotted against the amount of initial template. A line of best fit was imposed on the different data points and the annealing efficiency. The melting curve analysis for all the selected primer pairs showed single peaks confirming that there was no DNA contamination. The PCR efficiency for the primers ranged between 95 to 96% (Appendix Figure 15 and 17). Hence, it was

concluded that the annealing efficiency of the primers used in this study was similar, and any difference in expression will be due to the experimental condition employed.

Figure 5-15 shows the result of gene expression analysis for the *lmo2235* gene and *lmo0540* gene in *L. monocytogenes* wild type at 30°C and 18°C relative to at 37°C. The result showed that the expression of *lmo2235* increased by  $1.82 \pm 0.12$  fold (n=3) and  $1.92 \pm 0.04$  fold (n=3) at 18°C and 30°C, respectively relative to the expression at 37°C. In addition it was observed that the expression of *lmo0540* at 18°C and 30°C was similar  $1.13 \pm 0.01$  fold (n=3) and  $1.07 \pm 0.09$  (n=3) respectively to the expression at 37°C.

It was expected that the expression of lmo2235 and lmo0540 in wild type at 30°C higher according to previous data of assay of attachment (Section 3.1.3), the expression of lmo0540 at 30°C was similar relative to at 37°C. In contrast the expression of lmo2235 increased at 30°C relative to at 37°C.



**Figure 5-15** Gene expression of *lmo2235* gene and *lmo0540* gene in *L. monocytogenes* wild type at 18°C and 30°C relative to expression at 37°C. Data are the mean ( $\pm$  *S.D.*) of three experiments

# 5.5.3 The impact of temperature on planktonic cells of *L. monocytogenes* 10403s and cells detached after attachment

In order to measure the expression of *lmo2235*, *lmo0540* level in the planktonic and attached cells during listerial attachment, qRT-PCR was used to define the expression of these genes in the planktonic and detached cell after attachment. The experimental models were designed to assess the expression of *lmo2235* and *lmo0540* genes under temperature changes after attachment are described in the Material and Methods (Section 2.17). An attachment of *L. monocytogenes* wild type to polystyrene was performed in flat-bottom polystyrene 6-well plate for 2 at 18°C, 30°C and 37°C (2.17.1.2).

RNA was extracted from planktonic cells (by aspirating the culture from the wells) and detached cells (by sonication) after 2 hours incubation. The gene expression of planktonic and detached cells at  $18^{\circ}$ C and  $30^{\circ}$ C was compared to expression at  $37^{\circ}$ C after normalising the expression of each gene with the housekeeping gene *gyrB*. C<sub>T</sub> values of each planktonic and detached cell were determined. Fold changes in gene expression were calculated.

Figure 5-16 shows the result of expression of *lmo2235* and *lmo0540* at 18°C and 30°C relative to 37°C during attachment. It was observed that expression of *lmo2235* at 18°C was not significantly different in both detached and planktonic cells  $0.18 \pm 0.18$  fold (n3) and  $0.17 \pm 0.2$  fold (n=3), respectively, relative to 37°C. The expression of *lmo2235* at 30°C was  $1.34 \pm 0.03$  fold (n=3) and  $0.73 \pm 0.21$  fold (n=3) in detached and planktonic cells, respectively, relative to expression at 37°C. It was expected that the *lmo2235* expression in detached cells was no significantly different to that in planktonic cells under 18°C and 30°C.

The expression of *lmo0540* was reduced in detached cells at 18°C compared to the expression in the planktonic cells; it was  $0.47 \pm 0.11$  fold and  $1.1 \pm 0.25$  fold, respectively, relative to 37°C. Similarly, the expression of *lmo0540* at 30°C in planktonic cells was higher than the expression of detached cells; the expression was  $0.42 \pm 0.13$  fold and  $0.76 \pm 0.08$  fold in the detached and planktonic cells respectively relative to the expression at 37°C.

In summary, the pattern of *lmo2235* expression in detached and planktonic cells after attachment was not significantly different at 18°C and 30°C. However the expression of *lmo0540* was higher in the planktonic cells compared to detached cells at 18°C and 30°C.



**Figure 5-16** The expression pattern of *lmo2235* and *lmo0540* at 18°C and 30°C relative to 37°C in planktonic and detached cells after attachment. Data are the mean ( $\pm$  *S.D.*) of three experiments.

# 5.6 Study of genes *lmo2236*, *lmo0539* and *lmo0541* in B265 and I366 transposon mutants

In order to confirm that the Tn917 element was not influencing the expression of flanking genes in the transposon mutant of B265 and I366 (Figure 5-17), qRT-PCR was used to evaluate the expression of *lmo2236*, the downstream gene to *lmo2235* and *lmo0539*, and *lmo0541*, the neighbouring genes to *lmo0540*, in the B235 and I366 mutant, respectively. The level of expression of these genes in transposon mutants B265 and I366 was compared to their expression in the wild type strain. The house keeping gene *gyrB* was used for normalisation.

Figure 5-18 shows the expression of lmo2236 in the B265 and lmo0530 and lmo0541 in the I366 compared to wild type. lmo0539 and lmo0541 genes were upregulated in I366. The mRNA level for these genes increased by  $41 \pm 1.5$  fold (n=3) and  $4.41 \pm 1.6$  fold (n=3) for lmo0539 and lmo0541 respectively, whereas lmo2236 exhibited  $0.51 \pm 0.42$  fold (n=3) downregulation in B265. The decrease in expression of lmo2236 gene in the B265 mutant suggests that, the gene might be influenced by the transposon location on the genome. Thus it was decided to mutate this gene and examine its action in the attachment of *L. monocytogenes*.



Figure 5-17 Schematic drawing to show the location of neighboring gene *lmo2236* to *lmo2235* and *lmo0539* and *lmo0541* to *lmo0540* in *L. monocytogenes* EGD-e.



**Figure 5-18** Expression of *lmo2236* in B265 and *lmo0530* and *lmo0541* in mutant I366 compared to wild type. Data are the mean ( $\pm$  *S.D.*) of three experiments

#### 5.7 Antibiotic susceptibility test (Etest)

To determine whether disruption of the penicillin binding protein encoding genes had an impact on  $\beta$ -lactam resistance of *L. monocytogenes*, Etest strips containing benzylpenicillin at 0.002 to 32µg/ml were used. *L. monocytogenes* wild type and  $\Delta lmo0540$  suspensions used for E-test were matched, to a 0.5 McFarland standard with a cell density of ~10<sup>8</sup> CFU/ml. The results were read after 24 h of incubation at 37°C.

The result was read in accordance with the manufacturer's directions. The MIC was taken where the edge of the inhibition ellipse of growth intersects the strip. Figure 5-19 and 5-20 show the MIC values of benzylpenicillin for *L. monocytogenes* wild type and  $\Delta lmo0540$  mutant was similar 0.125µg/ml.

#### 5.8 Minimum inhibitory concentration MIC determinations

The MIC methodology was also used to determine whether disruption of the penicillin binding protein encoding genes had an impact on  $\beta$ -lactam resistance of *L. monocytogenes* and to obtain identical results that achieved by Etest. The results demonstrated that the lack of function  $\Delta lmo0540$  genes had little effect on the sensitivity of the mutant strain to the panel of  $\beta$ -lactam antibiotics tested

MIC values of Penicillin G in the  $\Delta lmo0540$  mutant were determined against L. monocytogenes wild type in TSB culture was  $0.11\mu g/ml$  and similar results were obtained for  $\Delta lmo0540$  MIC values was  $0.11\mu g/ml$ .



**Figure 5-19** Photograph of the E-test demonstrating the point at which the zone of inhibition of bacterial growth intersects the antimicrobial strip. The MIC scale is printed on the upper surface of the strip and the antimicrobial agent is on the underside of the strip. MIC values of in *L. monocytogenes* wild type were  $0.125\mu$ g/ml.



**Figure 5-20** Photograph of the E-test demonstrating the point at which the zone of inhibition of bacterial growth intersects the antimicrobial strip. The MIC scale is printed on the upper surface of the strip and the antimicrobial agent is on the underside of the strip. MIC values of  $\Delta lmo0540$  mutant was 0.125µg/ml.

#### 5.9 NADH oxidase activity

This experiment was performed to characterise the NADH oxidase enzyme in the transposon mutant and deletion mutants against the wild type strains. The cell extracts were prepared in TSB as described in the Material and Methods (Section 2.18.1). Total protein concentration of *L. monocytogenes* strains was determines by the Bradford assay as described in the Material and Methods (Section 2.18.3) and 1mg/ml was used in the reactions. A straight line standard curve was obtained, which used as a reference to determine the concentration of protein. The enzyme activity was tested in wild type and four mutants,  $\Delta lmo0103$ ,  $\Delta lmo2235$ ,  $\Delta lmo2471$  and B265 compared to control. The absorbance was measured at 340nm in a Varioscan for 10 minutes

Figure 5-21 shows the activity obtained from *L. monocytogenes* strains against control (negative control). The mutants  $\Delta lmo0103$  showed a decreased in the activity (1.25 mU/mg of protein) compared to the other strains (P<0.05), whereas  $\Delta lmo2235$  and  $\Delta lmo2471$  had the same enzyme activity (2.5 mU/mg of protein). Wild type and B265 showed a significantly greater activity (5 mU/mg of protein) compared to the other strains (P<0.05). No detectable activity was seen in the negative control.

This data suggest that, the NADH oxidase enzyme is either not being expressed or expressed at a lower level in  $\Delta lmo0103$ . Furthermore, *L. monocytogenes* have three copies of the NADH oxidase genes and only one gene has been knocked out in each mutant.



**Figure 5-21** NADH oxidase activity in *L. monocytogenes* strains. The curves represent mean from three individually measurement of each strain. Dotted lines represent initial maximum activity of each strain.  $\Delta lmo0103$  showed a decreased in activity (p<0.05).  $\Delta lmo2235$  and  $\Delta lmo2471$  showed a similar (P<0.05) activity but decreased in activity compared to wild type and B265. Wild type and B2653 showed had more activity compared to the other mutants (P<0.05). Data are the mean ( $\pm$  *S.D.*) of three experiments.

#### 5.10 Discussion

In the work performed in this thesis, a reduced ability to adhere to polystyrene *in vitro* was proved in mutants *lmo2235*, *lmo2471*, *lmo0103* and *lmo0540* compared to wild type at 18°C and 30°C (P<0.05) (Figures 5-6, 5-7, and 5-8). There also was no significant difference between these mutants and transposon mutants B265 and I366 (P>0.05). They showed the same attachment deficiency at 18°C and 30°C. Mutant  $\Delta lmo2236$  was also screened for the ability to attach to polystyrene compared to wild type and  $\Delta lmo2235$  at 30°C and 37°C (Figure 5-13). There was no significant difference (P>0.05) between  $\Delta lmo2236$  and wild type in the attachment at 30°C and 37°C. These results confirmed that the downstream *lmo2236* gene has no effect on *Listeria* attachment.

*L. monocytogenes* is able to grow in a wide range of food protection conditions, such as refrigeration, acidic pH and high salinity (Junttila *et al.*, 1988; Bajard *et al.*, 1996; Te Giffel & Zwietering, 1999; Tienungoon *et al.*, 2000). The importance of this work is to extend the knowledge about the effect of these different conditions for better understanding of biofilm in *Listeria*, which may provide valuable pathways for prevention of biofilm formation. NaCl and pH were chosen as in the food processing industry acids and salts are routinely used as major barriers to prevent bacterial growth in food (Shabala *et al.*, 2008).

The growth of *L. monocytogenes* wild type and six mutants was tested on TSB at low pH (6.5 and 5.5) and high NaCl (2% and 4% (w/v)) concentrations at  $37^{0}$ C (Figures 5-2, 5-3, 5-4 and 5-5). The data represented shows that all mutants showed markedly similar behaviour in growth compared to wild type and compared to each other. All strains were able to grow at pH 5.5 and above, at  $37^{\circ}$ C. Statistical analysis of the growth rates of mutants and wild type growth curve incubating at  $37^{\circ}$ C was applied

(Table 5-1) revealed no significant difference in means of wild type and mutants growth rates (P>0.05), therefore these results suggest that the mutated genes have no impact on the growth of these mutants in these conditions. These results from wild type are in agreement with the results obtained by El-Shenawy and Marth (1989). They indicated that pH 5.0 to pH 5.5 was minimum for *L. monocytogenes* growth. In a similar way, Falerio *et al.* (2003) reported that, Portuguese cheese isolates were able to grow at pH 5.5 and above at 30°C and 8°C. In contrast, a recent study by Shabala *et al.* (2008) found that the majority (95%) of strains habituated at pH 5.0 grew subsequently at pH 4.2, while 25% were able to grow at pH 4.1. However, the results of this work were found to have growth limits different to those described within Shabala's *et al.* (2008) finding.

*L. monocytogenes* strains were able to grow in the presence of 4% (w/v) NaCl and below at 37°C (Figure 5-2 and 5-3). In this study the transposon mutant B265 and I366 did not grow at 6% (w/v) NaCl. The defective growth of transposon mutants B265 and I366 at 6% (w/v) NaCl may be due the interaction between erythromycin and lincomycin and NaCl in the medium, which may affect growth of transposon mutants. The result from wild type is this study is also in accordance with data from Falerio *et al.* (2003). They demonstrated that none of the cheese, meat and fish related isolates grew at 8% (w/v) NaCl and below the concentration of 10% (w/v).

*L. monocytogenes* has been reported to be NaCl tolerant in complex medium (Tienungoon *et al.*, 2000). Autolysis is common in *Listeria* strains and this like other bacteriolytic systems was associated with cell wall degradation (Tyrrell, 1973). Recently the growth limit in NaCl has been evaluated in 127 *L. monocytogenes* strains at 25°C. They demonstrated that the majority of strains grew in the presence of 11.3%

to 11.6% (w/v) NaCl, although a few strains were able to grow in 13.9% (w/v) NaCl (Shabala *et al.*, 2008). Results from this work were found to be opposite to those found by Shabala *et al.* (2008). Growth limits of NaCl in study of this thesis were determined as 4% (w/v) NaCl in TSB at  $37^{\circ}$ C.

The influence of salt on biofilm formation has been poorly investigated, although the effect of NaCl during biofilm formation has been explored. The effect of different concentrations of NaCl (0.5%, 2% and 4% (w/v) and pH (7.3, 6.5 and 5.5) on bacterial attachment in the wild type strain and six mutants using microtitre plate at 37°C and 30°C was investigated. Independent of temperature, all strains showed a decrease in their ability to adhere with increasing NaCl concentration (2% and 4% (w/v) compared to 0.5% (w/v) NaCl (Figure 5-9 and 5-10). This result is in agreement with data from Xu et al. (2010); they found that adherent cells of L. monocytogenes (KACC 12671) decreased with increasing NaCl (>4% (w/v)) concentration, with a decline in the numbers of adherent cells observed at concentration levels of NaCl (<2%). These observations were also in line with the data reported by Chorianopoulos et al. (2011), who found that increasing NaCl up to 5.5% (w/v) did not enhance attachment in nonadapted and acid-adapted L. monocytogenes cells. In contrast, Jensen et al. (2007) observed that up 5% (w/v) NaCl concentration increased the adherence of L. monocytogenes strains to plastic microtitre plate. Cataldo et al. (2007) found that attachment increased with NaCl up to 5% (w/v) at 37°C because of cell aggregation. Caly et al. (2009) also demonstrated effect of NaCl on L. monocytogenes attachment. They found adhesion between 0% and 6% (w/v) NaCl was similar, whereas adhesion at 11% (w/v) NaCl was significantly lower. Similar observations were made in this thesis, the increase of NaCl resulting in a significant decrease in Listeria attachment at 4% (w/v) NaCl when compared to data observed at 0.5% (w/v) NaCl.
The reduction in attachment is probably due to the hydrophobic difference of strains among the NaCl concentration which may affect the ability to adhere to the surface in the early stage of incubation (Xu *et al.*, 2010). Previous determination of physicalchemical properties of polystyrene indicates that it was uncharged and highly hydrophobic compared to stainless steel surfaces (Tresse *et al.*, 2006). The work of Beresford (2001) also demonstrated the weak effect of the surface on the *L. monocytogenes* 10403s adhesion.

*L. monocytogenes* strains were analysed for their ability to attach to polystyrene at different pHs. Regardless of temperatures, all *L. monocytogenes* strains lost their attachment at pH below 7.3 (Figures 5-11 and 5-12). *L. monocytogenes* attached well at neutral pH (pH 7.3). The effect of pH on listerial attachment has been studied: Tresse *et al.* (2006) indicated that *L. monocytogenes* Scott A showed a decrease of adhesion to polystyrene microplates at pH 5, though Briandet *et al.*, (1999) found that the ability of *L. monocytogenes* Scott A increased in a lactic acid-supplemented medium at pH 6.

The effect of pH on the bacterial attachment may be due to the influence on the degree of D-alanylation in cell wall and hence on the surface charge properties (Nostro *et al.*, 2012). The deficiency of attachment at low pHs may be explained by the role of pH in regulation of flagellum expression (Tresse *et al.*, 2006). As known, flagella were suspected to play a role on the initial attachment of *L. monocytogenes* which expressed at 30°C. It was therefore not surprising to find these results.

QRT-PCR is now being widely applied as a rapid and sensitive technique for mRNA quantification. Several factors may result in inaccurate quantitative analysis; for example, variation associated with RNA isolation, enzymatic efficiencies and normalization of quantification results to an internal reference gene (Bustin *et al.*,

2005). Since there is no universal internal control reference gene in *L. monocytogenes* (Vandecasteele *et al.*, 2001). The housekeeping gene gyrB was used as a reference gene for normalisation. The gyrB is found in a single copy in *L. monocytogenes*. The expression of this gene is known not to change under different environmental condition hence it has been used for normalisation of gene expression commonly both for *L. monocytogenes* (Bhattacharya *et al.*, 2003) and for other bacteria (Martens *et al.*, 2008).

Previously, *lmo2235* and *lmo0540* genes were found to be involved in the attachment defect in transposon mutants B265 and I366 respectively at 18°C and 30°C but not at 37°C. To investigate the impact of temperature on the expression of *lmo2235* and *lmo0540* genes in *L. monocytogenes* wild type in the exponential phase of growth in TSB, qRT-PCR was used. The expression of genes lmo2235 and lm0540 was quantified at 18°C and 30°C relative to 37°C (Figure 5-15). An increase in gene expression (twofold) was seen in *lmo2235* at both tested temperature; meanwhile, the expression of *lmo0450* gene was unchanged at 18°C and 30°C relative to 37°C. The increase in *lmo2235* expression suggests that this gene may have an important role in attachment in *L. monocytogenes*, which correlates with the previous data obtained from the attachment assay as lmo2235 was found to be involved in the attachment 30°C of L. monocytogenes. Unexpectedly, lmo0540 had no increase in expression at 18°C and 30°C. Data from *lmo0540* does not agree with the attachment result where the *lmo0540* proved to be responsible for the attachment redaction in transposon I336 mutant; in this case the redaction in the attachment may be due to the indirect effect of *lmo0540* on the *Listeria* attachment, for example affecting the growth of cells during initial attachment. qRT-PCR was also used to investigate the impact of temperature on the expression of lmo2235 and lmo0540 in detached and planktonic cells after attachment of L.

monocytogenes wild type at 18°C and 30°C (Figure 5-16). Results from the present study are shown in Figure 5-16. The expressions of *lmo2235* and *lmo0540* genes in detached cells were compared to the expression of these genes in planktonic cells following 2 hours contact time of bacteria to polystyrene. The expression of *lmo2235* in detached cells and planktonic cells at 30°C was identical. Likewise at 18°C the expression in both detached and planktonic cells were similar. Again *lmo0540* showed a decrease in expression at 18°C and 30°C and the expression was not identical in detached and planktonic cells. It was expected that both genes should be expressed at higher levels at 30°C in wild type in the detached cells in comparison to planktonic, indicating that these genes are upregulated in attached cells, and therefore they have a role in attachment. The results of this thesis found a poor match with the data of Oggioni et al., (2006). They found that the expression of nox of Streptococcus pneumoniae in biofilm (detached state) was less (fold change between 0.02 and 0.07). Also in the same study they found the gene expression patterns of 29 genes in S. pneumoniae were not identical in the detached cells and planktonic cells (Oggioni et al., 2006).

The expression of *lmo2235* and *lmo0540* genes in the exponential phase of growth was not fit to record data from the attachment assay (detached and planktonic cells). This could be explained by the behaviour of *Listeria* cells during attachment; the transition from a planktonic to an attached position may occur during the initial attachment and this may respond to the change in environment factors in the medium. Another factor that can affect the qRT-PCR result is the chosen appropriate internal control, because the efficiency of the housekeeping gene has been found to be affected and vary with experimental conditions (Vandecasteele *et al.*, 2001). Therefore the validity of reference genes should be the first choice in specific experimental conditions. Recently

Tasara and Stephan (2007) evaluated the suitability of five housekeeping genes as reference genes in 16 *L. monocytogenes* strains under three stress adaption models including cold, acid and high NaCl concentration. They found that the expression of these references genes varied under the different stress adaption models, except in 16s which was consistent at all conditions.

In order to confirm that Tn917 transposon was not influencing the expression of flanking genes in the transposon mutants B265 and I366, qRT-PCR was used to evaluate the expression of lmo2236 in I366 and lmo0539 and lmo0541 in B235 mutant (Figure 5-18). The regulation of the genes in the transposon mutants was compared to the expression in the wild type at 37°C. The genes lmo0539 and lmo0541 were upregulated in I366, showing an increase of 41 fold and 4.4 fold respectively (P>0.05) whereas lmo2236 exhibited 0.51 fold downregulation in B265 (P<0.05). The decrease in expression of lmo2236 in the B265 mutant suggests that the gene might be influenced by the transposon location on the genome and lmo2236 may be involved in the attachment defect in B265. Therefore it was decided to also create a mutation in this gene and examine its action in the attachment of *L. monocytogenes*.

NADH oxidase activity assay was done to investigate the hypothesis that NADH oxidase activity was downregulated in mutated *lmo2235*, *lmo2471* and *lm0103*. The result showed that all tested *L. monocytogenes* were able to produce NADH oxidase but at different concentrations (Figure 5-21). The results also showed a significant reduction of NADH activity in the mutant *lmo0103* compared to the other *Listeria* strains (P<0.05). This result suggests that the enzyme is being synthesized in each mutant but may be expressed at a lower level, e.g the organism have three copies of the NADH genes and only one copy has been knocked out in each mutant, thus the other two genes can be expressed in mutants.

Recently *lmo0540* was identified as  $\beta$ -lactamase class C domain which plays a control role in the  $\beta$ -lactam resistance of *L. monocytogenes* (Korsak *et al.*, 2010). An antibiotic susceptibility test (Etest) and minimum inhibitory concentration MIC determination was used to determine whether disruption of the *lmo0540* encoding gene had influenced the  $\beta$ -lactam ring in the *L. monocytogenes*  $\Delta lmo0540$  mutant. Results from both tests were identical and *lmo0540* mutant and wild type showed similar results (Figures 3-57 and 3-58). These results confirm that the interrupting of *lmo0540* did not change the sensitivity *to*  $\beta$ -lactam.

# 6 General Discussion

*Listeria monocytogenes* causes problems in food-processing industries because of its ability to survive and grow in a wide range of environmental conditions. Furthermore *L. monocytogenes* can attach to and produce biofilm on a wide variety of different surfaces in the food-processing environment, allowing the bacterium to be resistant to antimicrobial and sanitizing agents.

The aim of this study was to identify and investigate genes that are involved in attachment of L. monocytogenes to abiotic surfaces. This was done by screening a number of Tn917 transposon insertion mutants of L. monocytogenes, using assays of attachment to polystyrene and to stainless steel. Transposons are powerful tools in genetic manipulation for identifying genes related to a characteristic phenotype (Knobloch et al., 2003; Gaillard et al., 1986). The use of transposon insertion mutants then provides methods by which one can identify those genes responsible for attachment of bacteria. For example, in a study by Knobloch et al. (2003) a number of well characterised biofilm-negative Tn917 mutants of Staphylococcus epidermidis was screened to identify *Tn*917 insertion sites using an arbitrary PCR technique. The *Tn*917 transposon is small in size (5.2kb) and therefore easier to work with; also, this transposon has not been shown to have a strong preference for intergenic regions compared to Tn916 (16kb) (Nelson et al., 1997), making insertion of the transposon into open reading frames more likely. Several published reports have shown that Tn917 insertion is sufficiently random to find mutations throughout the genome (Clewell et al., 1982; Coulter et al., 1998), but there have also been contradicting reports indicating that *Tn*917 does not insert randomly (Garsin *et al.*, 2004).

Some genes have been indirectly determined as factors required for attachment in *L. monocytogenes*, such as superoxide dismutase gene (*sod*) (Tremoulet *et al.*, 2002) and the *relA* gene that encodes (p)ppGpp synthetase (Taylor *et al.*, 2002). Mutated *relA* gene in *L. monocytogenes* was found to indirectly impact on attachment by decreasing the growth after attachment. They found that the *relA* mutant was unable to accumulate (p)ppGpp in response to amino acid starvation compared to wild type that accumulated (p)ppGpp within 30 min under the same condition. A recent study by Lemon *et al.* (2010) demonstrated a new role for virulence regulator PrfA in promoting biofilms in *L. monocytogenes* outside host cells. A mutant lacking PrfA resulted in a biofilm defect in several different strains and serotypes.

This thesis covers the investigation of the ability of a wild type of *L. monocytogenes* strain and *Tn*917 transposon mutants to attach to abiotic surfaces at different temperatures. The results showed that certain transposon mutants display a considerably reduced level of attachment and that this reduction was temperature-dependent. The results showed that a higher level of attachment to polystyrene by the wild type was observed at 30°C (P<0.05) compared with 37°C and 18°C (Figure 3-3). The results suggest that the mechanism of attachment at 30°C is different from that at 37°C and 18°C. The influence of temperature on attachment of *L. monocytogenes* strains has been investigated by several studies (Di Bonaventura *et al.*, 2008; Chavant *et al.*, 2002). Also B265 and I366 transposon mutants showed a significant reduction in the level of attachment in comparison to wild type at 18°C (P<0.05), whereas there was no difference in the level of attachment between the mutants (P>0.05). The defect of mutants in their attachment is obviously temperature dependent and the result from the attachment assay at 30°C and 18°C suggested that the influence of genes involved in

surface attachment may be temperature dependent. The results also suggest that the mechanism of attachment at 37°C is different from that at 30°C and 18°C.

Methods involving direct observation of biofilm, such as microscopy were used to allow observation of bacteria attached directly on the surfaces. The microphotographs of the scanning electron microscopy experiment (Figure 3-8, 3-9 and 3-10) showed that *L. monocytogenes* strains can attach to stainless steel 304 and polystyrene surface at different temperatures. Attachment occurs following exposure times of both 2 and 24 hours. A similar study by Beresford (2002) demonstrated that *L. monocytogenes* 10403s can attach to different surfaces but no EPS matrix production was observed. It has been hypothesised that *L. monocytogenes* does not form classic densely populated biofilm but more a sparse monolayer of cells (Mafu *et al.*, 1990; Sashara & Zottola, 1993). The microphotographs from scanning electron microscopy in this study are in accordance with the hypothesis of *L. monocytogenes* does not form true biofilm: single cells are shown attaching to each of the stainless steel and polystyrene surfaces. At no point was a dense biofilm of cells observed in this study. Electron microscopy experiments by Sasahara and Zottola (1993) also reported that *L. monocytogenes* only attach to glass in sparse numbers.

Subsequently two mutants with significantly reduced levels of attachment, B265 and I366, were chosen for this study and were examined to determine the site of transposon insertion, using Arbitrary PCR. Arbitrary PCR has been used widely to analyse sequences flanking transposon insertions (Dale & Fredericks, 2005). As a result of Arbitrary PCR and following nucleotide sequence analysis, two ORFs were identified into which the *Tn*917 transposon had been inserted. *lmo2235* ORF was found to be similar to a gene for NADH oxidase in B265 strain and *lmo0540* ORF was found to be

mutants in *lmo2235* and *lmo0540* were constructed to investigate whether these genes were required for *L. monocytogenes* attachment.

Putative terminators were detected at the end of *lmo2235* and *lmo0540* genes and are shown in Figure 6-1 and 6-2. The presence of the terminator at the end of *lmo2235* and *lmo0540* means the end of transcription of *lmo2235* and *lmo0504*, it was concluded that the *lmo2235* and *lmo0540* an independent genes and are not part of an operon.



**Figure 6-1**Terminator map of *lmo2235* in *L. monocytogenes* EGD-e indicates terminator at end of gene. Terminator is represented as lollipops at end of gene.



Figure 6-2 Terminator map of *lmo0540 L. monocytogenes* EGD-e indicates terminator at end of gene. Terminator is represented as lollipops at end of gene.

The effect of salt and pH on biofilm formation has been investigated, Regardless of temperatures all strains showed a decrease in their ability to adhere with increasing NaCl concentration (Figure 5-9 and 5-10) and a loss of attachment at high pHs (Figures 5-11 and 5-12). But neither of the mutants differed from the wild type. These results suggest that the mutated genes have no impact on the attachment of these mutants in these conditions.

Reduction in adherence by deletion mutants ( $\Delta lmo2235$ ,  $\Delta lmo2471$ ,  $\Delta lmo0103$  and  $\Delta lmo0540$ ) compared to wild type at 18°C and 30°C (P<0.05) (Figures 5-6, 5-7, and 5-8) was proved but the question that can be raised is how NADH oxidase (*lmo2235*) and penicillin binding protein (*lmo0540*) genes could have such an impact on the ability of attachment in *L. monocytogenes*. It is not yet understood why these genes are involved in attachment, but the following section will present some hypotheses as to why these genes may be involved.

*L. monocytogenes* possesses NADH oxidase activity associated with the cell wall and membrane (Patchett *et al.*, 1991). Therefore it was hypothesised that the mutagenesis to knock out one of the NADH oxidases in *L. monocytogenes* may influence the cell wall structure, which would indirectly affect the attachment mechanism in *Listeria*. NADH oxidase is hypothesised to be involved in defense against oxidative stress, by detoxifying molecular  $O_2$  by catalysing its reduction by NADH into either  $H_2O$  or  $H_2O_2$ and by regulation of competence which that allows the capture of DNA as a source of nucleotides and DNA fragments for repair of  $O_2$ -induced damage to the chromosome (Auzat *et al.*, 1999; Echenique & Trombe, 2001). This possible involvement of NADH oxidase suggests that its activity is important for attachment of *L. monocytogenes* possibly by sensing and adapting to  $O_2$  concentration in the environment. Although the three NADH oxidase mutants have shown a reduction in attachment, further work would be required to investigate the effect of NADH oxidases by knocking out all the three genes in *L. monocytogenes*. NADH oxidase activity has been shown to be upregulated during growth in *L. monocytogenes* at a temperature of  $30^{\circ}$ C as the wild type strain demonstrated a two fold increase in gene expression. However, NADH Oxidase expression has been shown to be similar during attachment in the *L. monocytogenes* wild type strain. The role of NADH oxidase in adherence was investigated recently, since a recombinant *nox* in *Streptococcus pneumoniae* showed significant reduction in its adhesion to A549 cells *in vitro* (europneumo 2011, abstract by Nurith). This data can be used to determine the role of NADH in vitro in *L. monocytogenes*.

The gene *lmo*0540 was found to be similar to a penicillin binding protein in I366 strain. The first identification of penicillin binding protein in *L. monocytogenes* dates back to 1990, by Vicente *et al.* So far here are ten penicillin binding proteins predicted in *L. monocytogenes*; *lmo0441*(PBPB3), *lmo1438*(PBPB1), *lmo1892*(PBPA1), *lmo2039*(PBPB2), *lmo2229*(PBPA2), *lmo1916*(PBPC2), *lmo1855*(PBPD3), *lmo2754*(PBPD1), *lmo2818*(PBPD2) and *lmo0540*(PBPC1) (Korsak *et al.*, 2010; Korsak *et al.*, 2002; Guinane *et al.*, 2006). Five of these proteins -PBPB3, PBPB1, PBPA1, PBPB2 and PBPA2 belong to high molecular class of PBPs, whereas PBPD1 and PBPC1 - belong to low molecular weight (Korsak *et al.*, 2005).

As in other bacteria, penicillin-binding proteins are a group of enzymes involved in the final stage of the biosynthesis of peptidoglycan (transpeptidation and transglycosylation), which is the major component in the bacteria cell wall (Ghuysen, 1991). Inactivation of one of these genes could have a detrimental effect on cell wall construction and subsequently cell division and growth rate. Therefore a decrease in the

attachment in *L. monocytogenes*  $\Delta lmo0540$  may be due to the effect on cell wall formation or changes in the structure of the cell wall.

The work presented in this thesis has fulfilled the main objectives of the study which were to investigate the gene involved in listerial attachment. The results gained from the mutants indicated that both *lmo2235* and *lmo0540* ORF were involved in *L. monocytogenes* attachment to polystyrene. The role played by *lmo2335* and *lmo0540* genes in reducing attachment of *L. monocytogenes* is not yet known. Further experiments will be required in order to better understand the molecule mechanism of the NADH oxidase and penicillin-binding protein in *L. monocytogenes* attachment.

# **Further work**

Further study is required to understand the role of both NADH oxidase and penicillinbinding protein ORF in listerial attachment. The involvement of both ORF in listerial attachment is now clear and in this context, further work with these mutants could focus on establishing the reasons why  $\Delta lmo2235$  and  $\Delta lmo0540$  are involved in the attachment process.

Research is also needed to survey the presence of the identified ORF in a large collection of field *L. monocytogenes* isolates from different sources, such as dairy, food, and clinical samples, and therefore to screen their ability to attach.

In the more immediate future, a genetic complementation experiment of deletion mutants should be carried out to determine if the wild type phenotype is restored and confirm the roles of NADH oxidase and penicillin-binding protein ORFs in biofilm formation in *L. monocytogenes*.

Deletion mutations could be made to knock out different penicillin-binding protein genes in *L. monocytogenes* to investigate their role on biofilm formation. Extra work is also required to investigate the effect of NADH oxidase on *Listeria* biofilm by knocking out the three genes ( $\Delta lmo2235$ ,  $\Delta lmo2471$  and  $\Delta lmo0103$ ) in *L. monocytogenes* 10403s.

Further work can be done to investigate if antibodies against the *L. monocytogenes* NADH oxidase can block *Listeria* from binding to polystyrene and stainless steel surfaces.

Analysis of the effect of different surfaces on listerial biofilm formation on a range of industry devices could be investigated using mutants with wild type for example, stainless steel, glass, aluminium and rubber.

# Appendix

## 917-3.2 primer

## 917-5.2 primer

nnnnnnnnnnnnnnnnnnnggttganactgtaccactaataactcacaatagagagatgtcaccgtcaagttaaatgtacaa aataacagcgaaattttaaatctatttcttatcgatacaaattcctcgtaggcgctcgggaccccnaatagtttcttctagtggattataagctc ttgatccgggtcaanttgactagtcgacgcgtggccacnnttgnnnngatttgttatctgntatntaaaangttaccgnncatncaacgat ncttgttacttttgcattcanaacccttttaattttatattcttttaagtcacgcatcatgaatttaccgtgttcagaaataacgtctgctccgacttc gtcgcgaagttcgncaactgtgacatcatgaccnncttnnnnnnaaaatgnnnttgtttcnntacngancataccgccnccnactacna gnacnttnnncnnnnatttnnnntgactanncgacncgtgnnan

## 917-5.2 primer

#### 917-5.1 primer

Figure1- The DNA sequences resulting from arbitrary PCR with different primers of B265 strain

#### 917-3.2 primer

nnnnnnnttnntnattnnnnggttgnnactgtaccactaataactcacaatagagagatgtcaccgtcaagttaaatgtacaaataacagcgaaattttaaatctatttcttatcgatacaaattectcgtaggcgctcgggaccacnttgatacgttttttcccccatcaacatcttttgatagtatcctgtatgcttcattcctgcagggttaaatatattttcttgaagatatgtggctaaaggttgcccactaattttttctacaagataacccactacagtataattagcatccattaatcccatttcccaggttggatttaattcccgttgtggttaacaatttgatcccattccttg actagtcnnncgtgncaannnnntggacttgtngngctacattttttccgttgatcaannntanatgactagccgacncttgnnnnnncttatacnncnctgttggngngcnnaaaangaanangannnaaancnannannnaanntccgcgnncnaannatttngagtagn naacnnnngnnnnnnanntnnnntnnancn

#### 917-5.2 primer

#### 917-5.1 primer

#### 917-3.2 primer

#### 917-5.2 primer

Nnnnnnnnttnntnnattnnnnggttgnnactgtaccactaataactcacaatagagagatgtcaccgtcaagttaaatgtacaaaata acagcgaaattttaaatctatttettatcgatacaaatteetegtaggegetegggaccaenttgatacgtttttteeceetaacaattttttg atagtateetgtatgettcatteetgeagggttaaatattttettgaagatatgtggetaaaggttgeccaetaattttttettacaagataacce actacagtataattagcateetattaateeceattteetaggttggatttaatteetegttgatcaaggttgaacaattttttettgaagatatgtggetaaaggttgaccaetttgatgeteetaattgeteetaatteettgaggegttggatttaatteetegtaggegetegggaccaenttgatacgtttaacaatttgateeceatteetttg actaggttgattaattageateetattaateeceattteetaggttggatttaatteetegttgateaannntaagettgateetettgannnnnnntggacttgtngngetacatttttteegttgateaannntanatgaetageegaenettgnnnnn nettataennenetgttggngngennaaaangaanangannnaaanenannannnaannteegegnnenaannatttngagtagn naaennnngnnnnnnanntntnanen

#### 9175.1 primer

Figure 2- The DNA sequences resulting from arbitrary PCR with different primers of I366 strain

## Gene *lmo0103* nucleotide sequence

# Gene *lmo2471* nucleotide sequence

## Gene *lmo2236* nucleotide sequence

#### Gene *lmo0540* nucleotide sequence

## Gene *lmo2235* nucleotide sequence

ttgaaatttaattcgatgttttcacctattgatatcgggccgatgaaagtgcctaacagatttgtcgtatcaccaatgtgcaacaactatgcaaatacagatggaactttatcagacacttcccttgcttactataaagaacgagcacttggagggttcgggcttattacttttgaagcaacggttgtagetggagcaaaaatttetgtteagttacagcacgetggaccagaaggaaattecaaagtatetggttateetttaagagcageeteagegat tgceteggetgcagggcgaaatactccagaagccatttegcgagaagaactttatgagttgattgaactttacggagaagctgcettacga agatgaattcggcggctgttttgaaaatagaatgcgattacctcgacttatcattgaaagtattcgtaaacgtgttggtcattctattgcaattctt tgccgaattaatagtactgacggtgtcgacggtggattaagtgttcaagacagcgcgacggttgcagcttatttggaagattgtggtttggatggtttacatgtctctcgcagtgttcatattcgcgatgaatacatgtgggcgccaaccgtcttgcatgctggatttagctctgatcttgtaacgca aattaaacgcgctgtttctatcccagtaattactgtgggtcgtttcacagagccgcattacgcggaattaatggttcgtgaaggccgtgctga cttggttgetttegggegteagtetttageagateeagagacaeeaaaeaaagetgeageagaaaaetagaegagttaetteettgtattg cttgtcttcaaggttgtgtggctaacatgtacgccggaaaaccaattacttgtctagtaaatccattactcggacgtgaatccgaagcttacttgtgacagtttatgaagcatcagatatgattggtggtcaaatgcgattagctgcctatcctcctggtaaaggcgacttaacaaatatggttcgta ccgttgtagagcttcgcgatgaagttggggcagatgtcatttctgagcaccgtaagtttcttatgcatgatttcgatgaatataaaatcaaaag tgttactaatgccaaagtaacgagtttctttgaagacggagtaacctattccctagctgacaacaagaacagcgaatcgatggttttgactc tgtagttctagcaatgggatcaagagcctataatccactagaagaagctattaaggcaattgtaccggaaacctatgttatcggtgatgctgt acgcgcacgccgcgcactcgacgcaacgaaagaagcacttgacgctgtattacaattataa

## Gene *lmo0541* nucleotide sequence

ttatttaccagtaatttgttttacagcatcttccatttgataaagttgagcagcggcactgtagtcgctccatttagtattatccactttatacacttg gtttttettcacggetggaatatetttecaaacacettttagcattteatccacagaatccgtgttgccatcagetggcattaagatgaagagac gatetgetccagetgcatattcaggaattgettcactagaaatagettttgtttetttgtttgetttagcagegtetgttggtggaaaccegege eggagtataageegtcaaagaettttgeategtgcacatagatttetttaccataaaattgaattaetgeagetttttegttegtagaaaccegege tetttgagtgttgettttgettetttggagettgeegegtaatetttgtceeattttettettettettetttattaaaagttaggaagtagaaagt tgtttgteeggagtatcaccaaaatgegtgatataaacaggageeaetttttegttegtaaaagtttgttagaaagt tgtttgteeggagtatcaccaaaatgegtgatataaacaggageeaetttttgteettttgttgeategggatagtagtagtagtaataa agtetggttttaaattagegatttttteaatactaggtttgteattaccaacactttetgteeettttgttgeaggaacttetaetttge ggteegttgtaccagggttaaggaccgtttttgttgeggatteettttetttgettgaggaactgeagaactgecaaagggttaataatta aggteegttgtateagtgacegtttttgttgeggatteettttetttgettgagteettaetgagaactgecaaagggttaataatetgeag ggteegttgtateagtgacegtttttgttgggatteettttetttgettgagteettaetgagaactgecaaaggggttaatactaetgetaa aagaacagteatcaaaaatageageeceatttagettttggtacat

#### Gene *lmo0539* nucleotide sequence

A

Query	219	CGCTGTATTACAATTATAATTTACAT-T-CTACTCGCTGTAATGTGCAGCGAGTAGGATT	276
Subject	227384	CGCTGTATTACAATTATAATTTA-TTGTCCTACTCGCTCTAATATGTAGCGAGTAGGATT	227442
Query 27	7	CATTCGGAGGAAAACAAAATGGAAAATANAATTTCNGGAACGACTAGGCTTCTTGGCTTA	336
Subject 2	27443	CATTCGGAGGAAAACAAAATGGAAAATAGAATTTCAGGAACGACTAGGCTTCTTGGCTTA	227502
Query 33	17	ATTGGGACACCCATAGACCATTCAAAATCTCCAATCATGTATAATTACAGTTTTCAAAAA	396
Subject	227503	ATTGGGACACCCATAGACCATTCAAAATCTCCAATCATGTATAATTACAGTTTTCAAAAA	227562
Query 39	7	GCGGGTTTGGATTATGCTTACCTAGCTTTTGACACACCTA-CTGAAAAAGTTACTGATGC	455
Subject	227563	GCGGGTTTGGATTATGCTTACCTAGCCTTTGACACCCTATCC-AAAAAGTTGCAGATAC	227621
Query 45	6	TATAAAGGCAATCAAGACATTTAATCTGCGTGGTGCAAACGTGACCATGCCTTGTAAAAG	515
Subject	227622	TATTAAGGCAATCAAGACATTTAATCTTCGTGGTGCAAATGTAACCATGCCTTGTAAAAG	227681
Query 51	. 6	TGAAGTTTTAAAATACATGGATGACCTTTCCCCTGC 551	
Subject	227682	TGAAGTTTTAAAACACATGGATGACCTTTCCCCTGC 227717	

# B

Query 153	TAATAG-TTCTTCTAGTGGATTATAAGCTCTTGATCCCATTGCTAGAACCACAGAGTCAA	211
Subject 227301	TAATAGCTTCTTCTAGTGGATTATAGGCTCTTGATCCCATTGCTAGAACTACAGAGTCAA	227242
Query 212	ATCCGTCAATTCGGTGTTCTTTGTTATCCGCTAGGGAATAGGTTACCCCGTCTTCAAAGA	271
Subject 227241	AACCATCGATTCGCTGTTCTTTGTTGTCAGCTAGGGAATAGGTTACTCCGTCTTCAAAGA	227182
Query 272	AACTTGTTACTTTTGCATTCGTAACACTTTTGATTTTATATTCATCGAAATCACGCATCA	331
Subject 227181	AACTCGTTACTTTGGCATTAGTAACACTTTTGATTTTATATTCATCGAAATCATGCATAA	227122
Query 332	GGAATTTACGGTGTTCAGAAATAACGTCTGCTCCGACTTCATCGCGAAGTTCTACAACTG	391
Subject 227121	GAAACTTACGGTGCTCAGAAATGACATCTGCCCCAACTTCATCGCGAAGCTCTACAACGG	227062
Query 392	TGACATCATGACCAGCTTCACCTAAAAATGCAGCTGTTTCACTACCAACCA	451
Subject 3 22706:	1 TAACATCATGACCAGCCTCACCTAAAAATGCAGCTGTTTCACTACCAACCA	C 227002
Query 452 0	CAACTAGAATANCNNNNNGACCGCNNNNNTCTTTCCCNTNGANTannnnnnGNGNGN	511
Subject 227001	CTACAACAAGAACTTTTT-GACCACATTTCTCTTTTCCATCCAGTAAGTCAACTGCATGA	226943
Query 512	AN-AAGTCCAGCATCCTCAATACCTGGAATCGGCAAAACAAGTGNAGTGGCGCCCAGTTGC     I   IIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIIII	570
Subject 226942	ATCAA-TCCTGCGTCCTCGATACCTGGGATTGGTAAAACTAGTGGAGTGGCGCCAGTTGC	226884
Query 571	AATAATGACGGCATCTGGGGCAACTTCTTGAATAAGTTCTGGTGTTACTGGTGTGTTTGT	630
Subject 226883	AATAATGACAGCATCTGGTGCAACTTCTTGAATAAGTTCTAATGTTACGGGTGTGTTTGT	226824
Query 631	TTTAATTTCGACATCGAACTGTTC 654	
Subject 226823	TTTAATTTCGACATCAAACTGTTC 226800	

С

Query 30	ATAAGCTCTTGATCCCATTGCTAGAACCACAGAGTCAAATCCGTCAATTCGGTGTTCTTT	89
Subject 227279	ATAGGCTCTTGATCCCATTGCTAGAACTACAGAGTCAAAACCATCGATTCGCTGTTCTTT	227220
Query 90	GTTATCCGCTAGGGAATAGGTTACCCCGTCTTCAAAGAAACTTGTTACTTTTGCATTCGT	149
Subject 227219	GTTGTCAGCTAGGGAATAGGTTACTCCGTCTTCAAAGAAACTCGTTACTTTGGCATTAGT	227160
Query 150	AACACTTTTGATTTTATATTCATCGAAATCACGCATCAGGAATTTACGGTGTTCAGAAAT	209
Subject 227159	AACACTTTTGATTTTATATTCATCGAAATCATGCATAAGAAACTTACGGTGCTCAGAAAT	227100
Query 210	AACGTCTGCTCCGACTTCATCGCGAAGTTCTACAACTGTGACATCATGACCAGCTTCACC	269
Subject 227099	GACATCTGCCCCAACTTCATCGCGAAGCTCTACAACGGTAACATCATGACCAGCCTCACC	227040
Query 270	TAAAAATGCAGCTGTTTCACTACCAACCATATCGCCGCCAACTTGAATaannnnnnatg	329
Subject 227039	TAAAAATGCAGCTGTTTCACTACCAACCATACCGCCACCTACAACAAGAACTTTTTGACC	226980
Query 330	gcannnctctttcccatngantannnnnnngngncnannaGTCCAGCATCCTCAATACC	389
Subject 226979	ACATTTCTCTTTTCCATCCAGTAAGTCAACTGCATGAATCCAATCCTGCGTCCTCGATACC	226920
Query 390	TGGAATCGGCAAAACAAGNGNANNGGCGCCAGTTGCAATAATGACGGCATCTGGGGCAAC	449
Subject 226919	TGGGATTGGTAAAACTAGTGGAGTGGCGCCAGTTGCAATAATGACAGCATCTGGTGCAAC	226860
Query 450	TTCTTGAATAAGTTCTGGTGTTACTGGTGTGTGTTTGATTTCGACATCGAACTGTTC	509
Subject 226859	TTCTTGAATAAGTTCTAATGTTACGGGTGTGTTTGTTTTAATTTCGACATCAAACTGTTC	226800
Query 510		569
Subject 226799	GCATTTTTTAATGTAACTACGAACCATATTTGTTAAGTCGCCTTTACCAGGAGGATAGGC	226740
Query 570	AGCTAAGCGCATTTGACCGCCAATCATATC 599	
Subject 226739	AGCTAATCGCATTTGACCACCAATCATATC 226710	

**Figure 3-** Results showing the alignment of the DNA sequences from B265 in Basic Local Alignment Search Tool (BLAST) with the *L. monocytogenes* EGD-e genome available on NCBI.

# Key:

**Query**, refers to nucleotide sequences from DNA amplified and sequenced in *L. monocytogenes* 10403s. **Subject**, refers to nucleotide sequences from nucleotide BLAST database of *L. monocytogenes* EGDs: putative NADH oxidase *lmo*2235. A, **B**, and **C**, refer DNA amplified with 917-3.2, 917-5.2 and 917-5.1 primers respectively.

# A

Query 154	TTGATACGTTTTTTCCCCCATCAACATCTTTTTGATAGTATCCTGTATGCTTCATTCCTG	213
Subject 219702	TTGATACGTTTTTTCCCCCATCAACATCTTTTTGATAGTATCCTGTATGCTTCATTCCTG	219643
Query 214	CAGGGTTAAATATATTTTCTTGAAGATATGTGGCTAAAGGTTGCCCACTAATTTTTTCTA	273
Subject 219642	CAGGGTTAAATATATTTTCTTGAAGATATGTGGCTAAAGGTTGCCCACTAATTTTTTCTA	219583
Query 274	CAAGATAACCCACTACAGTATAATTAGCATCCATATAATCCCATTTCCCAGGTTGTGATT	333
Subject 219582	CAAGATAACCCACTACAGTATAATTAGCATCCATATAATCCCATTTCCCAGGTTGTGATT	219523
Query 334	TAATTCCCCGCTTCTCAATAGCTTTAACAATTTGATCC 371	
Subject 219522	TAATTCCCCGCTTCTCAATAGCTTTAACAATTTGATCC 219485	

# B

Query 153	TTGATACGTTTTTTCCCCCATCAACATCTTTTTGATAGTATCCTGTATGCTTCATTCCTG	212
Subject 219702	TTGATACGTTTTTTCCCCCATCAACATCTTTTTGATAGTATCCTGTATGCTTCATTCCTG	219643
Query 213	CAGGGTTAAATATATTTTCTTGAAGATATGTGGCTAAAGGTTGCCCACTAATTTTTTCTA	272
Subject 219642	CAGGGTTAAATATATTTTCTTGAAGATATGTGGCTAAAGGTTGCCCACTAATTTTTCTA	219583
Query 273	CAAGATAACCCACTACAGTATAATTAGCATCCATATAATCCCATTTCCCAGGTTGTGATT	332
Subject 219582	CAAGATAACCCACTACAGTATAATTAGCATCCATATAATCCCATTTCCCAGGTTGTGATT	219523
Query 333	TAATTCCCCGCTTCTCAATATCTTTAACAATTTGA-CCAGNCGTNNCnnnnnnnTAAG	391
Subject 219522	TAATTCCCCGCTTCTCAATAGCTTTAACAATTTGATCCGGT-GTCATATTACTTGTTAAG	219464
Query 392	TTTGGTCGTCCAACAATACCAGATGTATGATTCATAAAGTTCTTCACTAAAATCTTATTA	451
Subject 219463	TTTGGTCGTCCAACAATACCAGATGTATGATTCATAAAGTTCTTCACTAAAATCTTATTA	219404
Query 452	CCATTAGGAAAATCAGGTATATACTTCGAAATTGGATCATTCGTGTTTATTTTACCTTTT	511
Subject 219403	CCATTAGGAAAATCAGGTATATACTTCGAAATTGGATCATTCGTGTTTATTTTACCTTTT	219344
Query 512	TCTTCTAATTGTAAAAGTGCAGTTGCTATGATTGCTTTTTGAGAAGATCCAATATAAAAC	571
Subject 219343	TCTTCTAATTGTAAAAGTGCAGTTGCTATGATTGCTTTTTGAGAAGATCCAATATAAAAC	219284
Query 572	AAAGTATCTGGTGTATTCAATACTTTGTCATCACGATTTGCATACATA	631
Subject 219283	AAAGTATCTGGTGTATTCAATACTTTGTCATCACGATTTGCATACATA	219224
Query 632	ATGATAGTTTCTCCATCGCGAACTACAAGTGCCGAACCACTAAAACCAATTTTTTGTAAA	691
Subject 219223	ATGATAGTTTCTCCATCGCGAACTACAAGTGCCGAACCACTAAAACCAATTTTTTGTAAA	219164
Query 692	TAGTTATCAATTTCTTGATTTTTTACAATTGTTTCCTTTGGCTTTTCTTCTACTGGTTCN	751
Subject 219163	TAGTTATCAATTTCTTGATTTTTTACAATTGTTTCCTTTGGCTTTTCTTCTACTGGTTCA	219104
Query 752	NC-TCTTTCGTTTCTTGGGCTCATTAGTTGCTGCATTTTCCGATGGGTTTGTCGCAGTT	810
Subject 219103	GGATCTTTCGTTTCTTCGGCTCATTAGTTGCTGCATTTTCCGATGGGTTTGTCGCAGTT	219044
Query 811	GCTGCTTTCTTCTTAGTTGGTGCGGANACTGTTTGCTCTTGTTTACTTTTGAATTCGTAT	870
Subject 219043	GCTGCTTTCTTCTTAGTTGGTGCGGATACTGTTTGCTCTTGTTTACTTTTGAATTCGTAT	218984
Query 871	ATC 873	
Subject 218983	ATC 218981	

Query 17	TTTTTT-CCCCNTCAACATCTTTTTGATAGTATCCTGTATGCTTCATTCCTGCAGGGTTA	75
Subject 219694	TTTTTTCCCCCATCAACATCTTTTTGATAGTATCCTGTATGCTTCATTCCTGCAGGGTTA	219635
Query 76	AATATATTTTCTTGAAGATATGTGGCTAAAGGTTGCCCACTAATTTTTTCTACAAGATAA	135
Subject 219634	AATATATTTTCTTGAAGATATGTGGCTAAAGGTTGCCCACTAATTTTTTCTACAAGATAA	219575
Query 136	CCCACTACAGTATAATTAGCATCCATATAATCCCATTTCCCAGGTTGTGATTTAATTCCC	195
Subject 219574	CCCACTACAGTATAATTAGCATCCATATAATCCCATTTCCCAGGTTGTGATTTAATTCCC	219515
Query 196	CGCTTCTCAATATCTTTAACAATTTGATCCGGTGTCATATTnnnnnnaaGTTTGGTCGT	255
Subject 219514	CGCTTCTCAATAGCTTTAACAATTTGATCCGGTGTCATATTACTTGTTAAGTTTGGTCGT	219455
Query 256	CCAACAATACCAGATGTATGATTCATAAAGTTCTTCACTAAAATCTTATTACCATTAGGA	315
Subject 219454	CCAACAATACCAGATGTATGATTCATAAAGTTCTTCACTAAAATCTTATTACCATTAGGA	219395
Query 316	AAATCAGGTATATACTTCGAAATTGGATCATTCGTGTTTATTTTACCTTTTTCTTCTAAT	375
Subject 219394	AAATCAGGTATATACTTCGAAATTGGATCATTCGTGTTTATTTTACCTTTTTCTTCTAAT	219335
Query 376	TGTAAAAGTGCAGTTGCTATGATTGCTTTTTGAGAAGATCCAATATAAAACAAAGTATCT	435
Subject 219334	TGTAAAAGTGCAGTTGCTATGATTGCTTTTTGAGAAGATCCAATATAAAACAAAGTATCT	219275
Query 436	GGTGTATTCAATACTTTGTCATCACGATTTGCATACATATACCCTTTTTGTATGATAGTT	495
Subject 219274	GTGTATTCAATACTTTGTCATCACGATTTGCATACATATACCCTTTTTGTATGATAGTT	219215
Query 496	TCTCCATCGCGAACTACAAGTGCCGAACCACTAAAAACCAATTTTTTGTAAATAGTTATCA	555
Subject 219214	TCTCCATCGCGAACTACAAGTGCCGAACCACTAAAACCAATTTTTTGTAAATAGTTATCA	219155
Query 556	ATTTCTTGATTTTTTACAATTGTTTCCTTTGGCTTTTCTTCTACTGGTTCAGGCTCTTTC	615
Subject 219154	ATTTCTTGATTTTTTACAATTGTTTCCTTTGGCTTTTCTTCTACTGGTTCAGGATCTTTC	219095
Query 616	GTTTCTTCTGGCTCATTAGTTGCTGCATTTTCCGATGGGTTTGTCGCAGTTGCTGCTTTC	675
Subject 219094	GTTTCTTCTGGCTCATTAGTTGCTGCATTTTCCGATGGGTTTGTCGCAGTTGCTGCTTTC	219035
Query 676	TTCTTAGTTGGTGCGGATACTGTTTGCTCTTGTTTACTTTTGAATTCGTATATCa 730	
Subject 219034	TTCTTAGTTGGTGCGGATACTGTTTGCTCTTGTTTACTTTTGAATTCGTATATCA 2189	80

**Figure 4-** Results showing the alignment of the DNA sequences from I366 in Basic Local Alignment Search Tool (BLAST) with the *L. monocytogenes* EGD-e genome available on NCBI.

# Key:

**Query**, refers to nucleotide sequences from DNA amplified and sequenced in *L. monocytogenes* 10403s. **Subject**, refers to nucleotide sequences held at nucleotide BLAST database of *L. monocytogenes* EGDs: putative penicillin binding protein *lmo*0540. **A**, **B**, and **C**, refer to DNA amplified with 917-3.2, 917-5.2 and 917-5.1 primers respectively.

Query	28	GCTCCTTCTTGTAAACATGCAACCATGGCGTTTTGTTGTCGTAGAAAGTGACGCGGGG	87
Sbjct	108747	GCTCCTTCTTCTGTAAACATGCAACCATGGCGTTTTGTTGTCGTAGAAAGTGACGCGGGG	108806
Query	88	AAAGAAAAATTAAGCTCGTTAGTTCGCTTTAATACACGTCAAAATGAATCTTCTTCCGCG	147
Sbjct	108807	AAAGAAAAATTAAGCTCGTTAGTTCGCTTTAATACACGTCAAAATGAATCTTCTTCCGCG	108866
Query	148	ATGATTTTAGTTTTTGGAGACTTACAGAATTTTGAAAACGGTGAAAAAATTTACGGAGAA	207
Sbjct	108867	ATGATTTTAGTTTTTGGAGACTTACAGAATTTTGAAAACGGTGAAAAAATTTACGGAGAA	108926
Query	208	TCAGTTGAACGGGGCTTGATGCCGGCAGAAGTGAAAGAACAACAAATGGCTAAATTAAGC	267
Sbjct	108927	TCAGTTGAACGGGGCTTGATGCCGGCAGAAGTGAAAGAACAACAAATGGCTAAATTAAGC	108986
Query	268	CAGTACTTTGCTTCCATTCC 287	
Sbjct	108987	CAGTACTTTGCTTCCATTCC 109006	

**Figure 5-** Aalignment of the *lmo0103* insert in plasmid pGEM-T-Easy with the *L. monocytogenes* EGD-e genome available on NCBI.

Query	15	ACGCCNCTTGCGAGANAAGGAGGanaaaaTATGACTTATTTAAATAATGATTTTGAAGA	74
Sbjct	108593	ACGCCACTTGCGAGATAAGGAGGAGAAAAATATGACTTATTTAAATAATGATTTTGAAGA	108652
Query	75	CTTAATGAAAAACCGCCGCTCGATTCGAGAATACGATGAAAACGGTGAAAATTAGCCAAGA	134
Sbjct	108653	CTTAATGAAAAACCGCCGCCCGATTCGAGAATACGATGAAACGGTGAAAATTAGCCAAGA	108712
Query	135	GGAAATGAAAGCTATTTTAGAGGATGCGGTAACTGCTCCTTCTTCTGTAAACATGCAACC	194
Sbjct	108713	GGAAATGAAAGCTATTTTAGAGGATGCGGTAACTGCTCCTTCTTCTGTAAACATGCAACC	108772
Query	195	ATGGCGTTTTGTTGTCGTAGAAAGTGACGCGGGGAAAGAAA	254
Sbjct	108773	ATGGCGTTTTGTTGTCGTAGAAAGTGACGCGGGGAAAGAAA	108832
Query	255	CTTTAATACACGTCAAAATGAATCTTCTTCCGCGATGATTTTAGTTTTTGGAGACTTACA	314
Sbjct	108833	CTTTAATACACGTCAAAATGAATCTTCTTCCGCGATGATTTTAGTTTTTGGAGACTTACA	108892
Query	315	GAATTTTGAAAACGGTGAAAAAATTTACGGAGAATCAGTTGAACGGGGCTTGATGCCGGC	374
Sbjct	108893	GAATTTTGAAAACGGTGAAAAAATTTACGGAGAATCAGTTGAACGGGGCTTGATGCCGGC	108952
Query	375	AGAAGTGAAAGAACAACAAATGGCTAAATTAAGCCAGTACTTTGCTTCCATTCCACGC 4	32
Sbjct	108953	AGAAGTGAAAGAACAAACAAATGGCTAAATTAAGCCAGTACTTTGCTTCCATTCCACGC 1	09010

**Figure 6-** Alignment of the *lmo0103* gene sequences from  $\Delta lmo0103$  with the *L. monocytogenes* EGD-e genome available on NCBI. Confirming *lmo0103* has been mutated.

# Appendix

Query	37	TCCCAATTAAGCCAAGAAGCCTAGTCGTTCCTGAAATTCTATTTTCCATTTTGTTTTCCT	96
Sbjct	227509	TCCCAATTAAGCCAAGAAGCCTAGTCGTTCCTGAAATTCTATTTTCCATTTTGTTTTCCT	227450
Query	97	CCGAATGAATCCTACTCGCTACATATTAGAGCGAGTAGGACAATAAATTACAATTGTAAT	156
Sbjct	227449	CCGAATGAATCCTACTCGCTACATATTAGAGCGAGTAGGACAATAAATTATAATTGTAAT	227390
Query	157	ACAGCGTCAAGTGCTTCTTTCGTTGCGTCGAGTGCGCGGGGGGGG	216
Sbjct	227389	ACAGCGTCAAGTGCTTCTTTCGTTGCGTCGAGTGCGCGGCGTGCGCGTACAGCATCACCG	227330
Query	217	ATAACATAGGTTTCCGGTACAATTGCCTTAATAGCTTCTTCTAGTGGATTATAGGCTCTT	276
Sbjct	227329	ATAACATAGGTTTCCGGTACAATTGCCTTAATAGCTTCTTCTAGTGGATTATAGGCTCTT	227270
Query	277	GATCCCATTGCTAGAACTACAGAGTCAAAACCATCGATTCGCTGTTCTTTGTTGTCAGCT	336
Sbjct	227269	GATCCCATTGCTAGAACTACAGAGTCAAAACCATCGATTCGCTGTTCTTTGTTGTCAGCT	227210
Query	337	AGGGAATAGGTTACTCCGTCTTCAAAGAAACTCGTTACTTTGGCATTAGTAACACTTTTG	396
Sbjct	227209	AGGGAATAGGTTACTCCGTCTTCAAAGAAACTCGTTACTTTGGCATTAGTAACACTTTTG	227150
Query	397	ATTTTATATCATCGAAATCATGCATAAGAAACTTACGGTGCTCAGAAATGACATCTGCC	456
Sbjct	227149	ATTTTATATTCATCGAAATCATGCATAAGAAACTTACGGTGCTCAGAAATGACATCTGCC	227090
Query	457	CCAACTTCATTGCGAAGCTCTACAACGGTAACATCATGACCAGCCTCACCTAAAAATGCA	516
Sbjct	227089	CCAACTTCATCGCGAAGCTCTACAACGGTAACATCATGACCAGCCTCACCTAAAAATGCA	227030
Query	517	GCTGTTTCACTACCAACCATACCGCCACCTACAACAAGAACTTTTTGACCACATTTCTCT	576
Sbjct	227029	GCTGTTTCACTACCAACCATACCGCCACCTACAACAAGAACTTTTTGACCACATTTCTCT	226970
Query	577	TTTCCATCCAGTAAGTCAACTGCATGAATCAATCCTGCGTCCTCG 621	
Sbjct	226969	TTTCCATCCAGTAAGTCAACTGCATGAATCAATCCTGCGTCCTCG 226925	

# **Figure 7-** Alignment of the *lmo2235* insert in pAUL-A with the *L. monocytogenes* EGD-e genome available on NCBI. Using M13R

37	TGAGGAGCACAACGAGGCAATTTGCGCTTTGATGAACCAATTTGATCTTAACGCGCGAAC	96
152040	TGAGGAGCACAACGAGGCAATTTGCGCTTTGATGAACCAATTTGATCTTAACGCGCGAAC	151981
97	GCTTGAACGGAAAAATGGTTTTATTACCTATTTGAAAGAAGCGGAAAAAATCACGGAATT	156
151980	GCTTGAACGGAAAAATGGTTTTATTACCTATTTGAAAGAAGCGGAAAAAATCACGGAATT	151921
157	CTTGAGTATTATCGGAGCAACTAGCGCGCTTCTTCATTTTGAGGATGTTCGGATTATGCG	216
151920	CTTGAGTATTATCGGAGCAACTAGCGCGCTTCTTCATTTTGAGGATGTTCGGATTATGCG	151861
217	AGATATGCGAAATTCGGTGAACAGGCTAGTAAACTGCGAAACAGCGAATCTCAACAAGAC	276
151860	AGATATGCGAAATTCGGTGAACAGGCTAGTAAACTGCGAAACAGCGAATCTCAACAAGAC	151801
277	AATTAATGCGGCTGTTAGGCAAATCGATAATATCAAATATATCCAATCAACCGTTGGACT	336
151800	AATTAATGCGGCTGTTAGGCAAATCGATAATATCAAATATATCCAATCAACCGTTGGACT	151741
337	AGAAGCTTTACCAGAACGATTGCGTGAGATTGCAGCGCTTCGTATAGCTAATGAGGATGT	396
151740	AGAAGCTTTACCAGAACGATTGCGTGAGATTGCAGCGCTTCGTATAGCTAATGAGGATGT	151681
397	TACCTTAAAAGAGCTAGGCGAAATGCTGACAACGGGTCAAGTAAGT	456
151680	TACCTTAAAAGAGCTAGGCGAAATGCTGACAACGGGTCAAGTAAGT	151621
457	TCATCGTCTCCCGGAAACTAGATCGGATTGCAGAGCGCCTTAGAAGCGGAGAAACACCAGC	516
151620	TCATCGTCTCCGGAAACTAGATCAGATTGCAGAGCGCCTTAGAAGCGGAGAAACACCAGC	151561
	37 152040 97 151980 157 151920 217 151860 277 151800 337 151740 397 151680 457	37TGAGGAGCACAACGAGGCAATTTGCGCTTTGATGAACCAATTTGATCTTAACGCGCGCAAC 111111111111111111111111111111111111

Query	517	GCAAGTAGGGCTGAAAATCAGTAATAGTTAATTAAAAAGAAAAGGATGCGAATTAGAATG	576
Sbjct	151560	GCAAGTAGGGCTGAAAATCAGTAATAGTTAATTAAAAAGAAAAGGATGCGAATTAGAATG	151501
Query	577	TCAAAATTATTTTCAGAATATAAAATTAAAAGATGTAACGCTAAAAAAACAGAATTGTTATG	636
Sbjct	151500	TCAAAATTATTTTCAGAATATAAATTAAAAGATGTAACGCTAAAAAACAGAATTGTTATG	151441
Query	637	TCGCCAATGTGTATGTATTCAGTAGAAAATAAAGACGGGATTGCAACAGACTTTC 691	
Sbjct	151440	TCGCCAATGTGTATGTATTCAGTAGAAAATAAAGACGGGATTGCAACAGACTTTC 1513	86

**Figure 8-** Results show the alignment of the *lmo2471* insert in pAUL-A with the *L. monocytogenes* EGD-e genome available on NCBI. Using M13R primer.

Query	496	CGTTTGTTTGTCCGTGCCGATAAGAAACTACTTACTAAATAACCGTGTGCACAGTGAACT	555
Sbjct	226030	CGTTTGTTGTCCGTGCCGATAAGAAACTACTTACTAAATAACCGTGTGCACAGTGAACT	225971
Query	556	TCAACTGCATCAGCACCAGCtttttttGCTCGTAAGGCAGCTTCTCCGTAAAGTTCAATC	615
Sbjct	225970	TCAACTGCATCAGCACCAGCTTTTTTTGCTCGTAAGGCAGCTTCTCCGTAAAGTTCAATC	225911
Query	616	AACTCATAAAGTTCTTCTCGCGAAATGGCTTCTGGAGTATTTCGCCCTGCAGCCGAGGCA	675
Sbjct	225910	AACTCATAAAGTTCTTCTCGCGAAATGGCTTCTGGAGTATTTCGCCCTGCAGCCGAGGCA	225851
Query	676	ATCGCTGANGCTGCTCTTAAAGGATAACCAGATACTTTGGAATTTCCTTCTGGTCCAGCG	735
Sbjct	225850	ATCGCTGAGGCTGCTCTTAAAGGATAACCAGATACTTTGGAATTTCCTTCTGGTCCAGCG	225791
Query	736	TGCTGTAACTGAACAGAAATTTTTGCTCCAGCATCGTGACATACAT	795
Sbjct	225790	TGCTGTAACTGAACAGAAATTTTTGCTCCAGCATCGTGACATACAT	225731
Query	796	AAACTAGCAATTTGATGATCACTATATAGGCAAGCTTTATTTGCGCCACCTTTTGCGCGT	855
Sbjct	225730	AAACTAGCAATTTGATGATCACTATATAGGCAAGCTTTATTTGCGCCACCTTTTGCGCGT	225671
Query	856	ACATCTACAACCGTTGCTTCAAAAGTAATAAGCCCGAACCCTCCAAGTGCTCGTTCTTTA	915
Sbjct	225670	ACATCTACAACCGTTGCTTCAAAAGTAATAAGCCCGAACCCTCCAAGTGCTCGTTCTTTA	225611
Query	916	TAGTAAGCAAGGGAAGTGTCTGATAAAGTTCCATCTGNATTTGCATAGTTGNTGCACATT	975
Sbjct	225610	TAGTAAGCAAGGGAAGTGTCTGATAAAGTTCCATCTGTATTTGCATAGTTGTTGCACATT	225551
Query	976	GGNGATACGACNAATCNNNTAGGCACTTTCATC 1008	
Sbjct	225550	GGTGATACGACAAATCTGTTAGGCACTTTCATC 225518	

**Figure 9-** Alignment of the *lmo2235* gene sequences from  $\Delta lmo2235$  with the *L. monocytogenes* EGD-e genome available on NCBI. Confirming *lmo2235* has been mutated.

Query	467	GAAAGTCTGTTGCAATCCCGTCTTTATTTTCTACTGAATACATAC	526
Sbjct	151386	GAAAGTCTGTTGCAATCCCGTCTTTATTTTCTACTGAATACATAC	151445
Query	527	CAATTCTGTTTTTTAGCGTTACATCTTTTAATTTATATTCTGAAAATAATTTTGACATTC	586
Sbjct	151446	CAATTCTGTTTTTTAGCGTTACATCTTTTAATTTATATTCTGAAAATAATTTTGACATTC	151505
Query	587	TAATTCGCATCCTTTTCTTTTTTTTTTTTTTTTTTTTTT	646
Sbjct	151506	TAATTCGCATCCTTTTCTTTTTTTTTTTTTTTTTTTTTT	151565
Query	647	TGTTTCTCCGCTTCTAAGGCGCTCTGCAATCTGATCTAGTTTCCGGAGACGATGATTAAT	706
Sbjct	151566	TGTTTCTCCGCTTCTAAGGCGCTCTGCAATCTGATCTAGTTTCCGGAGACGATGATTAAT	151625
Query	707	ACCTGATTTACTTACTTGACCCGTTGTCA 735	
Sbjct	151626	ACCTGATTTACTTGACCCGTTGTCA 151654	

**Figure 10-** Alignment of the lmo2471 gene sequences from  $\Delta lmo2471$  with the *L.* monocytogenes EGD-e genome available on NCBI. Confirming lmo2471 has been mutated.

Query	1	GCAAACAAAGAAACAAAAGCTATTTCTAGTGAAGCAATTCCTGAATATGCAGCTGGAGCA	60
Sbjct	220376	GCAAACAAAGAAACAAAAGCTATTTCTAGTGAAGCAATTCCTGAATATGCAGCTGGAGCA	220317
Query	61	GATCGTCTTCATCTTAATGCCAGCTGATGGCAACACGGATTCTGTGGATGAAATGCTA	120
Sbjct	220316	GATCGTCTCTTCATCTTAATGCCAGCTGATGGCAACACGGATTCTGTGGATGAAATGCTA	220257
Query	121	AAAGGTGTTTGGAAAGATATTCCAGCCGTGAAGAAAAACCAAGTGTATAAAGTGGATAAT	180
Sbjct	220256	AAAGGTGTTTGGAAAGATATTCCAGCCGTGAAGAAAAACCAAGTGTATAAAGTGGATAAT	220197
Query	181	ACTAAATGGAGCGACTACAGTGCCGCTGCTCAACTTTATCAAATGGAAGATGCTGTAAAA	240
Sbjct	220196	ACTAAATGGAGCGACTACAGTGCCGCTGCTCAACTTTATCAAATGGAAGATGCTGTAAAA	220137
Query	241	CAAATTACTGGTAAATAATaaaaaaaCTCGATTCTACAAACAGAATCGAGTTTTTTATTA	300
Sbjct	220136	CAAATTACTGGTAAATAATAAAAAAAACTCGATTCTACAAACAGAATCGAGTTTTTTATTA	220077
Query	301	TTTATTTAAGAAACCATAAATATCATTATTCACTTTTCCGAATGAAGCAATGTAGTTTTG	360
Sbjct	220076	TTTATTTAAGAAACCATAAATATCATTATTCACTTTTCCGAATGAAGCAATGTAGTTTTG	220017
Query	361	GACGTTGGAGAATAATATAACGTAAGTTCTTCCGGTATGGCTAAAGCTATTAGACACGTC	420
Sbjct	220016	GACGTTGGAGAATAATATAAACGTAAGTTCTTCCGGTATGGCTAAAGCTATTAGACACGTC	219957
Query	421	CCATCCGGTCACAA 434	
Sbjct	219956	IIIIIIIIIII CCATCCGGTCACAA 219943	

**Figure 11-** Alignment of the *lmo0540* gene sequences from  $\Delta lmo0540$  with the *L. monocytogenes* EGD-e genome available on NCBI. Confirming *lmo0103* has been mutated.

Query	20	GTGGCGAGCTTTGATTGCTGGAGTACCATATTCTAAATCAAGCAAAATTGCAGAAGCATA	79
Sbjct	218403	GTGGCGAGCTTTGATTGCTGGAGTACCATATTCTAAATCAAGCAAAATTGCAGAAGCATA	218462
Query	80	AGGAGTTAGTTCTTCAGAAACAAGTTGTTTGAAGTCCTCTACATCCTTTTATTTTCTGT	139
Sbjct	218463	${\tt AGGAGTTAGTTCTTCAGAAACAAGTTGTTTGAAGTCCTCTACATCCTTTTATTTTCTGT}$	218522
Query	140	TCCTTTAGCTTGTTGGATCAttttttGAGTGAGCCACGTTGGTCAATGGCAAGCGCTGC	199
Sbjct	218523	${\tt TCCTTTAGCTTGTTGGATCATTTTTTTGAGTGAGCCACGTTGGTCAATGGCAAGCGCTGC$	218582
Query	200	AATGACACCTTTATCGTTGGAGAGTCTTTGTAAACCATCAAATTTACCTTTTGTTATTTG	259
Sbjct	218583	AATGACACCTTTATCGTTGGAGAGTCTTTGTAAACCATCAAATTTACCTTTTGTTATTTG	218642
Query	260	TACCATTTTTATCTAGTCTCCTTTATAGCAATTTGTTGTTGCTTTAATCAATTTATCCTT	319
Sbjct	218643	${\tt TACCATTTTTATCTAGTCTCCTTTATAGCAATTTGTTGTTGCTTTAATCAATTTATCCTT}$	218702
Query	320	CCTTATCATACCACTGAATAAtttttttAAAACATAGGTATATTTCCGCATTGATTTATC	379
Sbjct	218703	${\tt CCTTATTATACCACTGAATAATTTTTTTTTTTTTTTTTT$	218762
Query	380	TGAAGTAATAAATTACGATGTATGTGGGAGTGTTAGGTATTAATAGAAATTTGGGATAATT	439
Sbjct	218763	TGAAGTAATAAATTACGATGTATGTGGAGTGTTAGGTATTAATAGAAATTTGGGATAATT	218822
Query	440	TCTACTAATACATTCTCTTTTGGGAAAGTCATTTAACGATAAGACTAGACCAGGAGTGAT	499
Sbjct	218823	TCTACTAATACATTCTCTTTTGGGAAAGTCATTTAACGATAAGACTAGACCAGGAGTGAT	218882
Query	500	GAAATGAATCGACGCGAAAGACGTAAACACACAAAAGACGCAAGAACATGATTATCGTGTT	558
Sbjct	218883	GAAATGAATCGACGCGAAAGACGTAAACACAAAAGACGCAAGAACATGATTATCGTGTT	218941

**Figure 12-** Alignment of the *lmo0540* insert in pAUL-A with the *L. monocytogenes* EGD-e genome available on NCBI.

Query	21	ATGCGATTAGCTGCCTATCCTCCTGGTAAAGGCGACTTAACAAATATGGTTCGTAGTTAC	80
Sbjct	226728	ATGCGATTAGCTGCCTATCCTCCTGGTAAAGGCGACTTAACAAATATGGTTCGTAGTTAC	226787
Query	81	ATTAAAAAATGCGAACAGTTTGATGTCGAAATTAAAACAAAC	140
Sbjct	226788	ATTAAAAAATGCGAACAGTTTGATGTCGAAATTAAAACAAAC	226847
Query	141	CTTATTCAAGAAGTTGCACCAGATGCTGTCATTATTGCAACTGGCGCCACTCCACTAGTT	200
Sbjct	226848	CTTATTCAAGAAGTTGCACCAGATGCTGTCATTATTGCAACTGGCGCCACTCCACTAGTT	226907
Query	201	TTACCAATCCCAGGTATCGAGGACGCAGGATTGATTCATGCAGTTGACTTACTGGATGGA	260
Sbjct	226908	TTACCAATCCCAGGTATCGAGGACGCAGGATTGATTCATGCAGTTGACTTACTGGATGGA	226967
Query	261	AAAGAGAAATGTGGTCAAAAAGTTCTTGTTGTAGGTGGCGGTATGGTTGGT	320
Sbjct	226968	AAAGAGAAATGTGGTCAAAAAGTTCTTGTTGTAGGTGGCGGTATGGTTGGT	227027
Query	321	GCTGCATTTTTAGGTGAGGCTGGTCATGATGTTACCGTTGTAGAGCTTCGCGATGAAGTT	380
Sbjct	227028	GCTGCATTTTTAGGTGAGGCTGGTCATGATGTTACCGTTGTAGAGCTTCGCGATGAAGTT	227087
Query	381	GGGGCAGATGTCATTTCTGAGCACCGTAAGTTTCTTATGCATGATTTCGATGAATATAAA	440
Sbjct	227088	GGGGCAGATGTCATTTCTGAGCACCGTAAGTTTCTTATGCATGATTTCGATGAATATAAA	227147
Query	441	ATCAAAAGTGCTACTAATGCCAAAGTAACGAGTTTCTTTGAAGACGGAGTAACCTATTCC	500
Sbjct	227148	ATCAAAAGTGTTACTAATGCCAAAGTAACGAGTTTCTTTGAAGACGGAGTAACCTATTCC	227207
Query	501	CTAGCTGACAACAAGAACAGCGAATCGATGGTTTTGACTCTGTAGTTCTAGCAATGGGA	560
Sbjct	227208	CTAGCTGACAACAAGAACAGCGAATCGATGGTTTTGACTCTGTAGTTCTAGCAATGGGA	227267

Query	561	TCAAGAGCCTATAATCCACTAGAAGAAGCTATTAAGGCAATTGTACCGGAAACCTATGTT	620
Sbjct	227268	TCAAGAGCCTATAATCCACTAGAAGAAGCTATTAAGGCAATTGTACCGGAAACCTATGTT	227327
Query	621	ATCGGTGATGCTGTACGCGCCGCCGCCGCCACTCGACGCAACGAAAGAAGCACTTGACGCT	680
Sbjct	227328	ATCGGTGATGCTGTACGCGCACGCGCGCGCCACCGAACGAA	227387
Query	681	GTATTACAATTATAATTTATTGTCCTACTCGCTCTAATATGTAGCGAGTAGGATTCGTTC	740
Sbjct	227388	GTATTACAATTATAATTTATTGTCCTACTCGCTCTAATATGTAGCGAGTAGGATTCATTC	227447
Sbjct Query	227388 741	GTATTACAATTATAATTTATTGTCCTACTCGCTCTAATATGTAGCGAGTAGGATTCATTC	227447

**Figure 13-** Alignment of the *lmo2236* insert in pAUL-A with the *L. monocytogenes* EGD-e genome available on NCBI. Using M13R primer.

Query	557	GCCAAGAAGCCTAGTCGTTCCTGAAATTCTATTTTCCATTTTGTTTTCCTCCGAATGAAT	616
Sbjct	227499	GCCAAGAAGCCTAGTCGTTCCTGAAATTCTATTTTCCATTTTGTTTTCCTCCGAATGAAT	227440
Query	617	CCTACTCGCTACATATTAGAGCGAGTAGGACAATAAATTATAATTGTAATACAGCGTCNA	676
Sbjct	227439	CCTACTCGCTACATATTAGAGCGAGTAGGACAATAAATTATAATTGTAATACAGCGTCAA	227380
Query	677	GTGCTTCTTTCGTTGCGTCGAGTGCGCGGCGTGCGCGTACAGCATCACCGATAACATAGG	736
Sbjct	227379	GTGCTTCTTTCGTTGCGTCGAGTGCGCGGCGTGCGCGTACAGCATCACCGATAACATAGG	227320
Query	737	TTTCCGGTACAATTGCCTTAATAGCTTCTTCTAGTGGATTATAGGCTCTTGATCCCATTG	796
Sbjct	227319	TTTCCGGTACAATTGCCTTAATAGCTTCTTCTAGTGGATTATAGGCTCTTGATCCCATTG	227260
Query	797	CTAGAACTACAGAGTCAAAAACCATCGATTCGCTGTTCTTTGTTGTCAGCTAGGGAATAGG	856
Sbjct	227259	CTAGAACTACAGAGTCAAAAACCATCGATTCGCTGTTCTTTGTTGTCAGCTAGGGAATAGG	227200
Query	857	TTACTCCGTCTTCAAAGAAACTCGTTACTTTGGCATTAGTAACACTTTTGATTTTATATT	916
Sbjct	227199	TTACTCCGTCTTCAAAGAAACTCGTTACTTTGGCATTAGTAACACTTTTGATTTTATATT	227140
Query	917	CATCGAAATCATGCATNANANN-TTANNN-GCTCANAAATGACATCTGCCCCNACTTCAT	974
Sbjct	227139	CATCGAAATCATGCATAAGAAACTTACGGTGCTCAGAAATGACATCTGCCCCAACTTCAT	227080
Query	975	CGCGAAGCTCTACNACGGTAACATCATGACCAGCCTC 1011	
Sbjct	227079	CGCGAAGCTCTACAACGGTAACATCATGACCAGCCTC 227043	

**Figure 14-** Alignment of the lmo2236 gene sequences from  $\Delta lmo2236$  with the *L.* monocytogenes EGD-e genome available on NCBI. Confirming lmo2236 has been mutated.



**Figure 15-** The PCR efficiency for RT235 and RT540 primers used in qRT-PCR (primers efficiency was 0.95)



**Figure 16-** The melting curve analysis of RT235 and RT 540 primer pairs showing single peaks.



**Figure 17-** The PCR efficiency for RT236, RT 539 and RT 541 primers used in qRT-PCR (primers efficiency was 0.96)



Figure 18- The melting curve analysis of qRT-PCR primer of RT236, RT 539 and RT 541 showing single peaks.



**Figure 19-** Growth curves of *L. monocytogenes* strains performed in TSB at  $37^{\circ}$ C using Varioscan. This was done in triplicate starting from three different stocks.



**Figure 20-** Growth curves of *L. monocytogenes* strains performed in TSB  $37^{\circ}$ C and in 2% (w/v) NaCl using Varioscan. This was done in triplicate starting from three different stocks.



**Figure 21-** Growth curves of *L. monocytogenes* strains performed in TSB at  $37^{\circ}$ C and in 4% (w/v) NaCl using Varioscan. This was done in triplicate starting from three different stocks.



**Figure 22-** Growth curves of *L. monocytogenes* strains performed in TSB at 37°C and pH 6.5 using Varioscan. This was done in triplicate starting from three different stocks.



**Figure 23-** Growth curves of *L. monocytogenes* strains performed in TSB at 37°C and pH 5.5 using Varioscan. This was done in triplicate starting from three different stocks.

# References

Adrião, A., Vieira, M., Fernandes, I., Barbosa, M., Sol, M., Tenreiro, R., Chambel, L., Barata, B., Zilhao, I., Shama, G., 2008. Marked intra-strain variation in response of *Listeria monocytogenes* dairy isolates to acid or salt stress and the effect of acid or salt adaptation on adherence to abiotic surfaces. *International Journal of Food Microbiology*. **123**, 142-150.

Allerberger, F. & Wagner, M., 2009. Listeriosis: a resurgent foodborne infection. *Clinical Microbiology and Infection.* **16**, 16-23.

Allerberger, F., 2006. *Listeria*: growth, phenotypic differentiation and molecular microbiology. *FEMS Immunology & Medical Microbiology*. **35**, 183-189.

Allison, D.G., 2003. The biofilm matrix. Biofouling. 19, 139-150.

Anwar, H., Strap, J., Costerton, J., 1992. Eradication of biofilm cells of *Staphylococcus aureus* with tobramycin and cephalexin. *Canadian Journal of Microbiology*. **38**, 618-625.

Arizcun, C., Vasseur, C., Labadie, J.C., 1998. Effect of several decontamination procedures on *Listeria monocytogenes* growing in biofilms. *Journal of Food Protection*. **61**, 731-734.

Arnold, J. & Bailey, G., 2000. Surface finishes on stainless steel reduce bacterial attachment and early biofilm formation: scanning electron and atomic force microscopy study. *Poultry Science*. **79**, 1839-1845.

Austin, J.W. & Bergeron, G., 1995. Development of bacterial biofilms in dairy processing lines. *Journal of Dairy Research*. **62**, 509-519.

Auzat, I., Chapuy-Regaud, S., Le Bras, G., Dos Santos, D., Ogunniyi, A.D., Le Thomas, I., Garel, J., Paton, J.C., Trombe, M., 1999. The NADH oxidase of *Streptococcus pneumoniae*: its involvement in competence and virulence. *Molecular Microbiology*. **34**, 1018-1028.

Babb, B.L., Collett, H.J., Reid, S.J., Woods, D.R., 1993. Transposon mutagenesis of *Clostridium acetobutylicum* P262: isolation and characterization of solvent deficient and metronidazole resistant mutants. *FEMS Microbiology Letters*. **114**, 343-348.

Bailey, J., Fletcher, D., Cox, N., 1989. Recovery and serotype distribution of *Listeria monocytogenes* from broiler chickens in the southeastern United States. *The Lancet.* **1**, 148-150

Bajard, S., Rosso, L., Fardel, G., Flandrois, J., 1996. The particular behaviour of *Listeria monocytogenes* under sub-optimal conditions. *International Journal of Food Microbiology*. **29**, 201-211.

Bakke, R. & Olsson, P., 1986. Biofilm thickness measurements by light microscopy. *Journal of Microbiological Methods*. **5**, 93-98.

Barnes, B., Cassar, C., Halablab, M., Parkinson, N., Miles, R., 1996. An in situ method for determining bacterial survival on food preparation surfaces using a redox dye. *Letters in Applied Microbiology*. **23**, 325-328.
Beckers, H., Soentoro, P., Delgou-van Asch, E., 1987. The occurrence of *Listeria monocytogenes* in soft cheeses and raw milk and its resistance to heat. *International Journal of Food Microbiology*. 4, 249-256.

Berche, P., Gaillard, J.L., Geoffroy, C., Alouf, J.E., 1987. T cell recognition of listeriolysin O is induced during infection with *Listeria monocytogenes*. *The Journal of Immunology*. **139**, 3813-3821.

Beresford, M., 2002. Attachment of L. monocytogenes to materials commonly forn in a food-processing environment. Thesis. University of Leicester: UK.

Beresford, M., Andrew, P., Shama, G., 2001. *Listeria monocytogenes* adheres to many materials found in food-processing environments. *Journal of Applied Microbiology*. **90**, 1000-1005.

Bhattacharya, B., Karak, K., Ghosal, A.G., Roy, A., Das, S., Dandapat, P., Khetawat, D., Mondal, D.K., Bhattacharya, S., Chakrabarti, S., 2003. Development of a new sensitive and efficient multiplex polymerase chain reaction (PCR) for identification and differentiation of different mycobacterial species. *Tropical Medicine & International Health.* **8**, 150-157.

Bierne, H. & Cossart, P., 2007. *Listeria monocytogenes* surface proteins: from genome predictions to function. *Microbiology and Molecular Biology Reviews*. **71**, 377-397.

Blackman, I.C. & Frank, J.F., 1996. Growth of *Listeria monocytogenes* as a biofilm on various food-processing surfaces. *Journal of Food Protection* **®**. **59**, 827-831.

Boer, E.d. & Kuik, D., 1987. A survey of the microbiological quality of blue-veined cheeses. *Netherlands Milk and Dairy Journal.* **41**, 227-2.

Boerlin, P., Rocourt, J., Grimont, F., Grimont, P.A.D., Jacquet, C., Piffaretti, J.C., 1992. *Listeria ivanovii* subsp. *londoniensis* subsp. nov. *International Journal of Systematic Bacteriology*. **42**, 69-73.

Borucki, M.K., Peppin, J.D., White, D., Loge, F., Call, D.R., 2003. Variation in biofilm formation among strains of *Listeria monocytogenes*. *Applied and Environmental Microbiology*. **69**, 7336-7342.

Bos, R., Mei, H.C., Busscher, H.J., 1999. Physico-chemistry of initial microbial adhesive interactions-its mechanisms and methods for study. *FEMS Microbiology Reviews.* 23, 179-230.

Bowen, W.R., Lovitt, R.W., Wright, C.J., 2001. Atomic Force Microscopy Study of the Adhesion of *Saccharomyces cerevisiae*. *Journal of Colloid and Interface Science*. **237**, 54-61.

Brackett, R.E. & Beuchat, L.R., 1990. Pathogenicity of *Listeria monocytogenes* grown on crabmeat. *Applied and Environmental Microbiology*. **56**, 1216-1220.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* **72**, 248-254.

Branda, S.S., Vik, Å., Friedman, L., Kolter, R., 2005. Biofilms: the matrix revisited. *Trends in Microbiology.* **13**, 20-26.

Braun, L. & Cossart, P., 2000. Interactions between *Listeria monocytogenes* and host mammalian cells. *Microbes and Infection.* **2**, 803-811.

Briandet, R., Leriche, V., Carpentier, B., Bellon-Fontaine, M., 1999. Effects of the growth procedure on the surface hydrophobicity of *Listeria monocytogenes* cells and their adhesion to stainless steel. *Journal of Food Protection.* **62**, 994-998.

Brocklehurst, T., Zaman-Wong, C., Lund, B.M., 1987. A note on the microbiology of retail packs of prepared salad vegetables. *Journal of Applied Microbiology*. **63**, 409-415.

Brooks, G.F., Butel, J.S. and Morse S. A., 1998. *Jawetz, Melnick & Adlbergs Medical Microbiology*. 21st ed. Stamford, CT: Appleton & Lange.

Buchrieser, C., 2007. Biodiversity of the species *Listeria monocytogenes* and the genus *Listeria. Microbes and Infection.* **9**, 1147-1155.

Busscher, H.J. & Weerkamp, A.H., 1987. Specific and non-specific interactions in bacterial adhesion to solid substrata. *FEMS Microbiology Letters*. **46**, 165-173.

Bustin, S., Benes, V., Nolan, T., Pfaffl, M., 2005. Quantitative real-time RT-PCR-a perspective. *Journal of Molecular Endocrinology*. **34**, 597-601.

Cairns, B.J. & Payne, R.J., 2009. Sudden increases in listeriosis rates in England and Wales, 2001 and 2003. *Emerging Infectious Diseases.* **15**, 465-468.

Caggia, C., Randazzo, C.L., Di Salvo, M., Romeo, F., Giudici, P., 2004. Occurrence of *Listeria monocytogenes* in green table olives. *Journal of Food Protection.* **67**, 2189-2194.

Camilli, A., Portnoy, A., Youngman, P., 1990. Insertional mutagenesis of *Listeria monocytogenes* with a novel *Tn*917 derivative that allows direct cloning of DNA flanking transposon insertions. *Journal of Bacteriology*. **172**, 3738-3744.

Capita, R., Alonso-Calleja, C., Moreno, B., García-Fernández, M.C., 2001. Occurrence of *Listeria* species in retail poultry meat and comparison of a cultural/immunoassay for their detection. *International Journal of Food Microbiology*. **65**, 75-82.

Carpentier, B. & Cerf, O., 2008. Biofilms and their consequences, with particular reference to hygiene in the food industry. *Journal of Applied Microbiology*. **75**, 499-511.

Carpentier, B. & Cerf, O., 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. *Journal of Applied Microbiology*. **75**, 499-511.

Center for Disease Control and Prevention (CDC). 2002. Public Health Dispatch: Outbreak of Listeriosis. Northeastern United States, 2002. *Morbidity and Mortality Weekly Report.* 51(42): 950-951.

Chae, M.S. & Schraft, H., 2000. Comparative evaluation of adhesion and biofilm formation of different *Listeria monocytogenes* strains. *International Journal of Food Microbiology*. **62**, 103-111.

Chae, M.S., Schraft, H., Truelstrup Hansen, L., Mackereth, R., 2006. Effects of physicochemical surface characteristics of *Listeria monocytogenes* strains on attachment to glass. *Food Microbiology*. **23**, 250-259.

Chakraborty, T., Leimeister-Wächter, M., Domann, E., Hartl, M., Goebel, W., Nichterlein, T., Notermans, S., 1992. Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the prfA gene. *Journal of Bacteriology*. **174**, 568-574.

Chakraborty, T., Hain, T., Domann, E., 2000. Genome organization and the evolution of the virulence gene locus in *Listeria* species. *International Journal of Medical Microbiology*. **290**, 167-174.

Chambel, L., Sol, M., Fernandes, I., Barbosa, M., Zilhão, I., Barata, B., Jordan, S., Perni, S., Shama, G., Adrião, A., 2007. Occurrence and persistence of *Listeria* spp. in the environment of ewe and cow's milk cheese dairies in Portugal unveiled by an integrated analysis of identification, typing and spatial–temporal mapping along production cycle. *International Journal of Food Microbiology*. **116**, 52-63.

Charlton, B., Kinde, H., Jensen, L., 1990. Environmental survey for *Listeria* species in California milk processing plants. *Journal of Food Protection*. 53, 198-201.

Chasseignaux, E., Gérault, P., Toquin, M., Salvat, G., Colin, P., Ermel, G., 2002. Ecology of *Listeria monocytogenes* in the environment of raw poultry meat and raw pork meat processing plants. *FEMS Microbiology Letters*. 210, 271-275.

Chavant, P., Martinie, B., Meylheuc, T., Bellon-Fontaine, M.N., Hebraud, M., 2002. *Listeria monocytogenes* LO28: surface physicochemical properties and ability to form biofilms at different temperatures and growth phases. *Applied and Environmental Microbiology*. **68**, 728-737.

Chavant, P., Folio, P., Hebraud, M., 2003. Growth of *Listeria monocytogenes* in biofilm according to the surface and the temperature and effects of different treatments on its survival. *Sciences Des Aliments*. **23**, 116-119.

Chen, Y., Ross, W.H., Scott, V.N., Gombas, D.E., 2003. *Listeria monocytogenes*: low levels equal low risk. *Journal of Food Protection*. **66**, 570-577.

Chorianopoulos, N., Giaouris, E., Grigoraki, I., Skandamis, P., Nychas, G.J., 2011. Effect of acid tolerance response (ATR) on attachment of *Listeria monocytogenes* Scott A to stainless steel under extended exposure to acid or/and salt stress and resistance of sessile cells to subsequent strong acid challenge. *International Journal of Food Microbiology.* **145**, 400-406.

Clewell, D., Tomich, P., Gawron-Burke, M., Franke, A., Yagi, Y., An, F., 1982. Mapping of *Streptococcus faecalis* plasmids pAD1 and pAD2 and studies relating to transposition of *Tn*917. *Journal of Bacteriology*. **152**, 1220-1230.

Collins, M., Wallbanks, S., Lane, D., Shah, J., Nietupski, R., Smida, J., Dorsch, M., Stackebrandt, E., 1991. Phylogenetic analysis of the genus *Listeria* based on reverse transcriptase sequencing of 16S rRNA. *International Journal of Systematic Bacteriology*. **41**, 240-246.

Cossart, P., 2001. Met, the HGF–SF receptor: another receptor for *Listeria monocytogenes*. *Trends in Microbiology*. **9**, 105-107.

Cossart, P. & Mengaud, J., 1989. *Listeria monocytogenes*. A model system for the molecular study of intracellular parasitism. *Molecular Biology & Medicine*. **6**, 463-474.

Costerton, J.W., Cheng, K., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., Marrie, T.J., 1987. Bacterial biofilms in nature and disease. *Annual Reviews in Microbiology*. **41**, 435-464.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., Lappin-Scott, H.M., 1995. Microbial biofilms. *Annual Reviews in Microbiology*. **49**, 711-745.

Costerton, J.W., Geesey, G., Cheng, K.J., 1978. How bacteria stick. *Scientific American.* 238, 86-95.

Coulter, S.N., Schwan, W.R., Ng, E.Y., Langhorne, M.H., Ritchie, H.D., Westbrock-Wadman, S., Hufnagle, W.O., Folger, K.R., Bayer, A.S., Stover, C.K., 1998. *Staphylococcus aureus* genetic loci impacting growth and survival in multiple infection environments. *Molecular Microbiology*. **30**, 393-404.

Cox, L., Kleiss, T., Cordier, J., Cordellana, C., Konkel, P., Pedrazzini, C., Beumer, R., Siebenga, A., 1989. *Listeria* spp. in food processing, non-food and domestic environments. *Food Microbiology*. **6**, 49-61.

Criado, M., Suarez, B., Ferreiros, C., 1994. The importance of bacterial adhesion in the dairy industry. *Food Technology*. **48**, 123-126.

Dale, B.A. & Fredericks, L.P., 2005. Antimicrobial peptides in the oral environment: expression and function in health and disease. *Current Issues in Molecular Biology*. **7**, 119-133.

Dalton, C.B., Austin, C.C., Sobel, J., Hayes, P.S., Bibb, W.F., Graves, L.M., Swaminathan, B., Proctor, M.E., Griffin, P.M., 1997. An outbreak of gastroenteritis

and fever due to *Listeria monocytogenes* in milk. *New England Journal of Medicine*. **336**, 100-106.

Davey, M.E. & O'toole, G.A., 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews*. **64**, 847-867.

Davies, D., 2003. Understanding biofilm resistance to antibacterial agents. *Nature Reviews Drug Discovery.* **2**, 114-122.

Davis, R.W. & Botstein, D.j., R., 1980. Advanced Bacterial Genetics: A Manual for Genetic Engineering . Cold Spring Harbor, Cold Spring Harbor Laboratory Press.

Di Bonaventura, G., Piccolomini, R., Paludi, D., D'orio, V., Vergara, A., Conter, M., Ianieri, A., 2008. Influence of temperature on biofilm formation by *Listeria monocytogenes* on various food-contact surfaces: relationship with motility and cell surface hydrophobicity. *Journal of Applied Microbiology*. **104**, 1552-1561.

Dickinson, R.B., Nagel, J.A., McDevitt, D., Foster, T.J., Proctor, R.A., Cooper, S.L., 1995. Quantitative comparison of clumping factor-and coagulase-mediated *Staphylococcus aureus* adhesion to surface-bound fibrinogen under flow. *Infection and Immunity*. **63**, 3143-3150.

Dillard, J.P. & Yother, J., 1994. Genetic and molecular characterization of capsular polysaccharide biosynthesis in *Streptococcus pneumoniae* type 3. *Molecular Microbiology*. **12**, 959-972.

Djordjevic, D., Wiedmann, M., McLandsborough, L., 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Applied and Environmental Microbiology*. **68**, 2950-2958.

Domann, E., Wehland, J., Rohde, M., Pistor, S., Hartl, M., Goebel, W., Leimeister-Wächter, M., Wuenscher, M., Chakraborty, T., 1992. A novel bacterial virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the proline-rich region of vinculin. *The EMBO Journal.* **11**, 1981-1990.

Donlan, R.M., 2002. Biofilms: microbial life on surfaces. *Emerging Infectious Diseases.* **8**, 881-890.

Donlan, R.M. & Costerton, J.W., 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*. **15**, 167-193.

Donlan, R.M., 2001. Biofilms and device-associated infections. *Emerging Infectious Diseases.* **7**, 277-281.

Donlan, R.M., 2000. Role of biofilms in antimicrobial resistance. *Asaio Journal.* **46**, 47-52.

Donlan, R.M. & Costerton, J.W., 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*. **15**, 167-193.

Doolittle, M., Cooney, J., Caldwell, D., 1995. Lytic infection of *Escherichia coli* biofilms by bacteriophage T4. *Canadian Journal of Microbiology*. **41**, 12-18.

Drevets, D.A. & Bronze, M.S., 2008. *Listeria monocytogenes*: epidemiology, human disease, and mechanisms of brain invasion. *FEMS Immunology & Medical Microbiology*. **53**, 151-165.

Echenique, J.R. & Trombe, M.C., 2001. Competence Modulation by the NADH Oxidase of Streptococcus pneumoniae Involves Signal Transduction. Journal of Bacteriology. **183**, 768-772.

Eginton, P., Gibson, H., Holah, J., Handley, P., Gilbert, P., 1995. Quantification of the ease of removal of bacteria from surfaces. *Journal of Industrial Microbiology* & *Biotechnology*. **15**, 305-310.

Ekperigin, H.E. & Nagaraja, K.V., 1998. Microbial food borne pathogens. *Salmonella*. *The Veterinary Clinics of North America.Food Animal Practice*. **14**, 17-29.

Elliot, E.L. & Kvenberg, J.E., 2000. Risk assessment used to evaluate the US position on *Listeria monocytogenes* in seafood. *International Journal of Food Microbiology*. **62**, 253-260.

El-Shenawy, M.A. & Marth, E.H., 1989. Behavior of *Listeria monocytogenes* in the presence of sodium propionate. *International Journal of Food Microbiology*. **8**, 85-94.

Faleiro, M., Andrew, P., Power, D., 2003. Stress response of *Listeria monocytogenes* isolated from cheese and other foods. *International Journal of Food Microbiology*. **84**, 207-216.

Farber, J., Ross, W., Harwig, J., 1996. Health risk assessment of *Listeria* monocytogenes in Canada. International Journal of Food Microbiology. **30**, 145-156.

Farber, J. & Peterkin, P., 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiological Reviews*. **55**, 476.

Farber, J., 1989. Thermal resistance of *Listeria monocytogenes* in foods. *International Journal of Food Microbiology*. **8**, 285-291.

Fenlon, D., Wilson, J., Donachie, W., 1996. The incidence and level of *Listeria monocytogenes* contamination of food sources at primary production and initial processing. *Journal of Applied Microbiology*. **81**, 641-650.

Fleming, D.W., Cochi, S.L., MacDonald, K.L., Brondum, J., Hayes, P.S., Plikaytis, B.D., Holmes, M.B., Audurier, A., Broome, C.V., Reingold, A.L., 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. *New England Journal of Medicine*. **312**, 404-407.

Flemming, H., Neu, T.R., Wozniak, D.J., 2007. The EPS matrix: the "house of biofilm cells". *Journal of Bacteriology*. **189**, 7945-7947.

Flemming, H. & Wingender, J., 2010. The biofilm matrix. *Nature Reviews Microbiology*. **8**, 623-633.

Franciosa, G., Tartaro, S., Wedell-Neergaard, C., Aureli, P., 2001. Characterization of *Listeria monocytogenes* strains involved in invasive and noninvasive listeriosis outbreaks by PCR-based fingerprinting techniques. *Applied and Environmental Microbiology*. **67**, 1793-1799.

Frank, J.F. & Koffi, R.A., 1990. Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. *Journal of Food Protection.* **53**, 550-554.

Friedman, L. & Kolter, R., 2003. Genes involved in matrix formation in *Pseudomonas aeruginosa* PA14 biofilms. *Molecular Microbiology*. **51**, 675-690.

Gaillard, J., Berche, P., Sansonetti, P., 1986. Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. *Infection and Immunity*. **52**, 50-55.

García-gimeno, R.M., zurera-Cosano, G., Amaro-López, M., 1996.Incidence, survival and growth of *listeria monocytogenes* in ready-to-use mixed vegetable salads in Spain. *Journal of Food Safety.* **16**, 75-86.

Garner, M.R., James, K.E., Callahan, M.C., Wiedmann, M., Boor, K.J., 2006. Exposure to salt and organic acids increases the ability of *Listeria monocytogenes* to invade Caco-2 cells but decreases its ability to survive gastric stress. *Applied and Environmental Microbiology*. **72**, 5384-5395.

George, S.M., Lund, B.M., Brocklehurst, T., 1988. The effect of pH and temperature on initiation of growth of *Listeria monocytogenes*. *Letters in Applied Microbiology*. **6**, 153-156.

Gitter, M., 1976. *Listeria monocytogenes* in" oven-ready" poultry. *Veterinary Record*. **99**, 336-336.

Glass, K.A. & Doyle, M.P., 1989. Fate of *Listeria monocytogenes* in processed meat products during refrigerated storage. *Applied and Environmental Microbiology*. **55**, 1565-1569.

Goulet, V., de Valk, H., Pierre, O., Stainer, F., Rocourt, J., Vaillant, V., Jacquet, C., Desenclos, J., 2001. Effect of prevention measures on incidence of human listeriosis, France, 1987-1997. *Emerging Infectious Diseases.* **7**, 983-989.

Goulet, V., Rocourt, J., Rebiere, I., Jacquet, C., Moyse, C., Dehaumont, P., Salvat, G., Veit, P., 1998. Listeriosis outbreak associated with the consumption of rillettes in France in 1993. *Journal of Infectious Diseases*. **177**, 155-160.

Kaper, J.B., Nataro, J.P., Mobley, H.L., 2004. Pathogenic *Escherichia coli*. *Nature Reviews Microbiology*. **2**, 123-140.

Garsin, D.A., Urbach, J., Huguet-Tapia, J.C., Peters, J.E., Ausubel, F.M., 2004. Construction of an *Enterococcus faecalis Tn*917-mediated-gene-disruption library offers insight into *Tn*917 insertion patterns. *Journal of Bacteriology*. **186**, 7280-7289.

Gasanov, U., Hughes, D., Hansbro, P.M., 2005. Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: a review. *FEMS Microbiology Reviews*. **29**, 851-875.

Gedde, M.M., Higgins, D.E., Tilney, L.G., Portnoy, D.A., 2000. Role of Listeriolysin O in Cell-to-Cell Spread of *Listeria monocytogenes*. *Infection and Immunity*. **68**, 999-1003.

Gellin, B.G., Broome, C.V., Bibb, W.F., Weaver, R.E., Gaventa, S., Mascola, L., 1991. The epidemiology of listeriosis in the United States—1986. *American Journal of Epidemiology*. **133**, 392-401.

Ghuysen, J.M., 1991. Serine beta-lactamases and penicillin-binding proteins. *Annual Reviews in Microbiology*. **45**, 37-67.

Giotis, E.S., Muthaiyan, A., Blair, I.S., Wilkinson, B.J., McDowell, D.A., 2008. Genomic and proteomic analysis of the Alkali-Tolerance Response (AITR) in *Listeria monocytogenes* 10403S. *BMC Microbiology*. **8**, 102. Goebel, W. & Kreft, J., 1997. Cytolysins and the intracellular life of bacteria. *Trends in Microbiology.* **5**, 86-88.

Goldfine, H., Wadsworth, S.J., Johnston, N.C., 2000. Activation of host phospholipases C and D in macrophages after infection with *Listeria monocytogenes*. *Infection and Immunity*. **68**, 5735-5741.

Goulet, V., Hedberg, C., Le Monnier, A., De Valk, H., 2008. Increasing incidence of listeriosis in France and other European countries. *Emerging Infectious Diseases*. 14, 734-740.

Graves, L.M., Helsel, L.O., Steigerwalt, A.G., Morey, R.E., Daneshvar, M.I., Roof, S.E., Orsi, R.H., Fortes, E.D., Milillo, S.R., Den Bakker, H.C., 2010. *Listeria marthii* sp. nov., isolated from the natural environment, Finger Lakes National Forest. *International Journal of Systematic and Evolutionary Microbiology*. **60**, 1280-1288.

Gray, M.L. & Killinger, A., 1966. *Listeria monocytogenes* and listeric infections. *Bacteriological Reviews.* **30**, 309-382.

Green, P. & Pirrie, R., 1993. A laboratory apparatus for the generation and biocide efficacy testing of *Legionella* biofilms. *Journal of Applied Microbiology*. **74**, 388-393.

Gründling, A., Burrack, L.S., Bouwer, H., Higgins, D.E., 2004. *Listeria monocytogenes* regulates flagellar motility gene expression through MogR, a transcriptional repressor required for virulence. *Proceedings of the National Academy of Sciences of the United States of America.* **101**, 12318-12323.

Guinane, C.M., Cotter, P.D., Ross, R.P., Hill, C., 2006. Contribution of penicillinbinding protein homologs to antibiotic resistance, cell morphology, and virulence of Listeria monocytogenes EGDe. Antimicrobial Agents and Chemotherapy. 50, 2824-2828.

Habash, M. & Reid, G., 1999. Microbial biofilms: their development and significance for medical device-related infections. *The Journal of Clinical Pharmacology*. **39**, 887-898.

Hall-Stoodley, L., Costerton, J.W., Stoodley, P., 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology*. **2**, 95-108.

Hamon, M., Bierne, H., Cossart, P., 2006. *Listeria monocytogenes*: a multifaceted model. *Nature Reviews Microbiology*. **4**, 423-434.

Heaton, J. & Jones, K., 2008. Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: a review. *Journal of Applied Microbiology*. **104**, 613-626.

Herald, P., J & Zottola, E., A, 1988. Attachment of *Listeria monocytogenes* to stainless steel surfaces at various temperatures and pH values. *Journal of Food Science*. **53**, 1549-1562.

Hof, H., 2001. *Listeria monocytogenes*: a causative agent of gastroenteritis? *European Journal of Clinical Microbiology & Infectious Diseases*. **20**, 369-373.

Hood, S.K. & Zottola, E.A., 1997. Adherence to stainless steel by foodborne microorganisms during growth in model food systems. *International Journal of Food Microbiology*. **37**, 145-153.

Hood, S. & Zottola, E., 1995. Biofilms in food processing. Food Control. 6, 9-18.

HealthProtectionAgency(HPA).2006.Listeria.(http://www.hpa.org.uk/webc/HPAwebFile/HPAweb\_C/1194947407995AccessedOctober, 2013

Israelsen, H., Madsen, S.M., Vrang, A., Hansen, E.B., Johansen, E., 1995. Cloning and partial characterization of regulated promoters from *Lactococcus lactis Tn*917-lacZ integrants with the new promoter probe vector, pAK80. *Applied and Environmental Microbiology*. **61**, 2540-2547.

Jablasone, J., Warriner, K., Griffiths, M., 2005. Interactions of *Escherichia coli* O157:
H7, *Salmonella typhimurium* and *Listeria monocytogenes* plants cultivated in a gnotobiotic system. *International Journal of Food Microbiology*. 99, 7-18.

James, S., Fannin, S., Agee, B., Hall, B., Parker, E., Vogt, J., Run, G., Williams, J., Lieb, L., Salminen, C., 1985. Listeriosis outbreak associated with Mexican-style cheese-California. *Morbid.Mortal.Weekly Rep.* **34**, 357-359.

Jemmi, T. & Stephen, R., 2006. *Listeria monocytogenes*: food-borne pathogen and hygiene indicator. *Revue Scientifique Et Technique-Office International Des Épizooties*. **25**, 571-580.

Jensen, A., Larsen, M.H., Ingmer, H., Vogel, B.F., Gram, L., 2007. Sodium chloride enhances adherence and aggregation and strain variation influences invasiveness of *Listeria monocytogenes* strains. *Journal of Food Protection*. **70**, 592-599.

Jeong, D.K. & Frank, J.F., 1994. Growth of *Listeria monocytogenes* at 10C in biofilms with microorganisms isolated from meat and dairy processing environments. *Journal of Food Protection.* **57**, 576-586.

Jordan, S.J., Perni, S., Glenn, S., Fernandes, I., Barbosa, M., Sol, M., Tenreiro, R.P., Chambel, L., Barata, B., Zilhao, I., Aldsworth, T.G., Adriao, A., Faleiro, M.L., Shama, G., Andrew, P.W., 2008. *Listeria monocytogenes* biofilm-associated protein (BapL) may contribute to surface attachment of *L. monocytogenes* but is absent from many field isolates. *Applied & Environmental Microbiology*. **74**, 5451-5456.

Junttila, J.R., Niemelä, S., Hirn, J., 1988. Minimum growth temperatures of *Listeria monocytogenes* and non-haemolytic *listeria*. *Journal of Applied Microbiology*. **65**, 321-327.

Jurado, R.L., Farley, M.M., Pereira, E., Harvey, R.C., Schuchat, A., Wenger, J.D., Stephens, D.S., 1993. Increased risk of meningitis and bacteremia due to *Listeria monocytogenes* in patients with human immunodeficiency virus infection. *Clinical Infectious Diseases.* **17**, 224-227.

Kalmokoff, M., Austin, J., Wan, X., Sanders, G., Banerjee, S., Farber, J., 2001. Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. *Journal of Applied Microbiology*. **91**, 725-734.

Kasper, S., Huhulescu, S., Auer, B., Heller, I., Karner, F., Würzner, R., Wagner, M., Allerberger, F., 2009. Epidemiology of listeriosis in Austria. *Wiener Klinische Wochenschrift.* **121**, 113-119.

Kathariou, S., Mizumoto, C., Kanenaka, R., Allen, R., Fok, A., 1995. Repression of motility and flagellin production at 37 C is stronger in *Listeria monocytogenes* than in the nonpathogenic species *Listeria innocua*. *Canadian Journal of Microbiology*. **41**, 572-577.

Kim, K.Y. & Frank, J.F., 1995. Effect of nutrients on biofilm formation by *Listeria monocytogenes* on stainless steel. *Journal of Food Protection*. **58**, 24-28.

Kingsman, A.,J, Chater, K.,.F and Kingsman, S.,M, 1988. *Transposition*. UK: Cambridge University Press.

Knobloch, J.K.M., Nedelmann, M., Kiel, K., Bartscht, K., Horstkotte, M.A., Dobinsky,
S., Rohde, H., Mack, D., 2003. Establishment of an arbitrary PCR for rapid identification of *Tn*917 insertion sites in *Staphylococcus epidermidis*: characterization of biofilm-negative and nonmucoid mutants. *Applied and Environmental Microbiology*. 69, 5812-5818.

Kocks, C., Gouin, E., Tabouret, M., Berche, P., Ohayon, H., Cossart, P., 1992. *L. monocytogenes*-induced actin assembly requires the *actA* gene product, a surface protein. *Cell.* **68**, 521-531.

Kocks, C., Hellio, R., Gounon, P., Ohayon, H., Cossart, P., 1993. Polarized distribution of *Listeria monocytogenes* surface protein ActA at the site of directional actin assembly. *Journal of Cell Science*. **105**, 699-710.

Korsak, D., Markiewicz, Z., Gutkind, G.O., Ayala, J.A., 2010. Identification of the full set of *Listeria monocytogenes* penicillin-binding proteins and characterization of PBPD2 (Lmo2812). *BMC Microbiology*. **10**, 239.

Korsak, D., Popowska, M., Markiewicz, Z., 2005. Analysis of the murein of a Listeria monocytogenes EGD mutant lacking functional penicillin binding protein 5 (PBP5). *Polish Journal of Microbiology*. **54**, 339-342.

Korsak, D., Zawadzka, J.J., Siwińska, M.E., Markiewicz, Z., 2002. Penicillin-binding proteins of *Listeria monocytogenes* a re-evaluation. *Acta Microbiologica Polonica*.
51, 5-12.

Krysinski, E., Brown, L., Marchisello, T., 1992. Effect of cleaners and sanitizers on *Listeria monocytogenes* attached to product contact surfaces. *Journal of Food Protection.* 55, 246.

Kuhn, M., Kathariou, S., Goebel, W., 1988. Hemolysin supports survival but not entry of the intracellular bacterium *Listeria monocytogenes*. *Infection and Immunity*. **56**, 79-82.

Lawrence, L.M. & Gilmour, A., 1994. Incidence of *Listeria* spp. and *Listeria monocytogenes* in a poultry processing environment and in poultry products and their rapid confirmation by multiplex PCR. *Applied and Environmental Microbiology*. **60**, 4600-4604.

Lecuit, M., Vandormael-Pournin, S., Lefort, J., Huerre, M., Gounon, P., Dupuy, C., Babinet, C., Cossart, P., 2001. A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier. *Science*. **292**, 1722-1725.

Lee Wong, A.C., 1998. Biofilms in food processing environments. *Journal of Dairy Science*. **81**, 2765-2770.

Lee, S.H. & Frank, J., 1991. Inactivation of surface-adherent *Listeria monocytogenes* hypochlorite and heat. *Journal of Food Protection.* **54**, 4-6.

Lemon, K.P., Earl, A.M., Vlamakis, H.C., Aguilar, C. and Kolter, R., 2008. Biofilm development with an emphasis on *Bacillus subtilis In:* Romeo, T. *Bacterial biofilms* 

*current topics in microbiology and immunology*. Springer-Verlag Berlin Heidelberg: Cambridge University Press. 1-16.

Lemon, K.P., Freitag, N.E., Kolter, R., 2010. The virulence regulator PrfA promotes biofilm formation by *Listeria monocytogenes*. *Journal of Bacteriology*. **192**, 3969-3976.

Leriche, V., Sibille, P., Carpentier, B., 2000. Use of an enzyme-linked lectinsorbent assay to monitor the shift in polysaccharide composition in bacterial biofilms. *Applied and Environmental Microbiology*. **66**, 1851-1856.

Lianou, A. & Sofos, J.N., 2007. A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. *Journal of Food Protection*. **70**, 2172-2198.

Lillard, H., 1986. Role of fimbriae and flagella in the attachment of *Salmonella typhimurium* to poultry skin. *Journal of Food Science*. **51**, 54-56.

Lindsay, D. & Von Holy, A., 1997. Evaluation of dislodging methods for laboratorygrown bacterial biofilms. *Food Microbiology*. **14**, 383-390.

Lingnau, A., Domann, E., Hudel, M., Bock, M., Nichterlein, T., Wehland, J., Chakraborty, T., 1995. Expression of the *Listeria monocytogenes* EGD inIA and inIB genes, whose products mediate bacterial entry into tissue culture cell lines, by PrfAdependent and-independent mechanisms. *Infection and Immunity*. **63**, 3896-3903.

Linnan, M.J., Mascola, L., Lou, X.D., Goulet, V., May, S., Salminen, C., Hird, D.W., Yonekura, M.L., Hayes, P., Weaver, R., 1988. Epidemic listeriosis associated with Mexican-style cheese. *New England Journal of Medicine*. 319, 823-828. Lopez de Felipe, F., Starrenburg, M.J.C., Hugenholtz, J., 2006. The role of NADH-oxidation in acetoin and diacetyl production from glucose in *Lactococcus lactis* subsp. lactis MG1363. *FEMS Microbiology Letters*. **156**, 15-19.

Low, J. & Donachie, W., 1997. A review of *Listeria monocytogenes* and listeriosis. *The Veterinary Journal.* **153**, 9-29.

Luchansky, J., Muriana, P., Klaenhammer, T., 1988. Application of electroporation for transfer of plasmid DNA to *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Listeria*, *Pediococcus*, *Bacillus*, *Staphylococcus*, *Enterococcus* and *Propionibacterium*. *Molecular Microbiology*. **2**, 637-646.

Lukinmaa, S., Miettinen, M., Nakari, U.M., Korkeala, H., Siitonen, A., 2003. *Listeria monocytogenes* isolates from invasive infections: variation of sero-and genotypes during an 11-year period in Finland. *Journal of Clinical Microbiology*. **41**, 1694-1700.

Lundén, J., Tolvanen, R., Korkeala, H., 2004. Human listeriosis outbreaks linked to dairy products in Europe. *Journal of Dairy Science*. **87**, 6-12.

Lunden, J.M., Autio, T.J., Korkeala, H.J., 2002. Transfer of persistent *Listeria monocytogenes* contamination between food-processing plants associated with a dicing machine. *Journal of Food Protection*. 65, 1129-1133.

Lunden, J.M., Miettinen, M.K., Autio, T.J., Korkeala, H.J., 2000. Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. *Journal of Food Protection*. **63**, 1204-1207.

Mack, D., Riedewald, J., Rohde, H., Magnus, T., Feucht, H.H., Elsner, H., Laufs, R., Rupp, M.E., 1999. Essential functional role of the polysaccharide intercellular adhesin of *Staphylococcus epidermidis* in hemagglutination. *Infection and Immunity*. **67**, 1004-1008.

Mafu, A.A., Roy, D., Goulet, J., Magny, P., 1990. Attachment of *Listeria monocytogenes* to stainless steel, glass, polypropylene, and rubber surfaces after short contact times. *Journal of Food Protection*. **53**, 742-246.

Mafu, A.A., Roy, D., Goulet, J., Savoie, L., 1991. Characterization of physicochemical forces involved in adhesion of *Listeria monocytogenes* to surfaces. *Applied and Environmental Microbiology*. **57**, 1969-1973.

Mai, T.L. & Conner, D.E., 2007. Effect of temperature and growth media on the attachment of *Listeria monocytogenes* to stainless steel. *International Journal of Food Microbiology*. **120**, 282-286.

Makino, S., Kawamoto, K., Takeshi, K., Okada, Y., Yamasaki, M., Yamamoto, S., Igimi, S., 2005. An outbreak of food-borne listeriosis due to cheese in Japan, during 2001. *International Journal of Food Microbiology*. **104**, 189-196.

Malinverni, R., Bille, J., Perret, C., Regli, F., Tanner, F., Glauser, M., 1985. Epidemic listeriosis-Observation of 25 cases during 15 months at the Vaudois-University-Medical-Center. *Schweizerische Medizinische Wochenschrift.* **115**, 2-10.

Marsh, E.J., Luo, H., Wang, H., 2003. A three-tiered approach to differentiate *Listeria monocytogenes* biofilm-forming abilities. *FEMS Microbiology Letters*. **228**, 203-210.

Marshall, K.C., 1992. Biofilms: An overview of bacterial adhesion, activity, and control at surfaces. Control of biofilm formation awaits the development of a method to

prevent bacterial adhesion. ASM American Society for Microbiology News. 58, 202-207.

Martens, M., Dawyndt, P., Coopman, R., Gillis, M., De Vos, P., Willems, A., 2008. Advantages of multilocus sequence analysis for taxonomic studies: a case study using 10 housekeeping genes in the genus Ensifer (including former Sinorhizobium). *International Journal of Systematic and Evolutionary Microbiology*. **58**, 200-214.

McCoy, W., Bryers, J., Robbins, J., Costerton, J., 1981. Observations of fouling biofilm formation. *Canadian Journal of Microbiology*. **27**, 910-917.

McLauchlin, J., 1996. The relationship between *Listeria* and listeriosis. *Food Control*. **7**, 187-193.

McLauchlin, J., Hall, S., Velani, S., Gilbert, R., 1991. Human listeriosis and paté: a possible association. *BMJ: British Medical Journal.* **303**, 773.

McLauchlin, J., Mitchell, R., Smerdon, W., Jewell, K., 2004. *Listeria monocytogenes* and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods. *International Journal of Food Microbiology*. **92**, 15-33.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V., 1999. Food-related illness and death in the United States. *Emerging Infectious Diseases.* **5**, 607-625.

Mead, P., Dunne, E., Graves, L., Wiedmann, M., Patrick, M., Hunter, S., Salehi, E., Mostashari, F., Craig, A., Mshar, P., 2006. Nationwide outbreak of listeriosis due to contaminated meat. *Epidemiology and Infection.* **134**, 744-751.

Michel, E. & Cossart, P., 1992. Physical map of the *Listeria monocytogenes* chromosome. *Journal of Bacteriology*. **174**, 7098-7103.

Miles, A., Misra, S., Irwin, J., 1938. The estimation of the bactericidal power of the blood. *Journal of Hygiene*. **38**, 732-749.

Mittelman, M.W., 1998. Structure and functional characteristics of bacterial biofilms in fluid processing operations. *Journal of Dairy Science*. **81**, 2760-2764.

Monroe, D., 2007. Looking for chinks in the armor of bacterial biofilms. *PLoS Biology*. **5**, 2458-2461.

Murray, E.G.D., Webb, R.A., Swann, M.B.R., 1926. A disease of rabbits characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed *bacillus Bacterium monocytogenes* (n. sp.). *The Journal of Pathology and Bacteriology*. **29**, 407-439.

Nelson, J., 1990. Where are *Listeria* likely to be found in dairy plants?. *Dairy, Food* and Environmental Sanitation. **10**, 344-345.

Nelson, K., E, Richardson, D., L, Dougherty, B., A, 1997. *Tn*916 transposition in *Haemophilus influenzae* Rd: preferential insertion into noncoding DNA. *Microbial & Comparative Genomics.* **2**, 313-321.

Nightingale, K., Schukken, Y., Nightingale, C., Fortes, E., Ho, A., Her, Z., Grohn, Y., McDonough, P., Wiedmann, M., 2004. Ecology and transmission of *Listeria monocytogenes* infecting ruminants and in the farm environment. *Applied and Environmental Microbiology*. **70**, 4458-4467.

Nightingale, K., Windham, K., Wiedmann, M., 2005. Evolution and molecular phylogeny of *Listeria monocytogenes* isolated from human and animal listeriosis cases and foods. *Journal of Bacteriology*. **187**, 5537-5551.

Norton, D.M., McCamey, M.A., Gall, K.L., Scarlett, J.M., Boor, K.J., Wiedmann, M., 2001. Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. *Applied and Environmental Microbiology*. **67**, 198-205.

Norwood, D. & Gilmour, A., 2001. The differential adherence capabilities of two *Listeria monocytogenes* strains in monoculture and multispecies biofilms as a function of temperature. *Letters in Applied Microbiology.* **33**, 320-324.

Norwood, D. & Gilmour, A., 1999. Adherence of *Listeria monocytogenes* strains to stainless steel coupons. *Journal of Applied Microbiology*. **86**, 576-582.

Nostro, A., Cellini, L., Di Giulio, M., D'Arrigo, M., Marino, A., Blanco, A.R., Favaloro, A., Cutroneo, G., Bisignano, G., 2012. Effect of alkaline pH on staphylococcal biofilm formation. *Apmis.* **120**, 733-742.

Oggioni, M.R., Trappetti, C., Kadioglu, A., Cassone, M., Iannelli, F., Ricci, S., Andrew, P.W., Pozzi, G., 2006. Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. *Molecular Microbiology*. **61**, 1196-1210.

Ojeniyi, B., Christensen, J., Bisgaard, M., 2000. Comparative investigations of *Listeria monocytogenes* isolated from a turkey processing plant, turkey products, and from human cases of listeriosis in Denmark. *Epidemiology and Infection.* **125**, 303-308.

Ong, Y., Razatos, A., Georgiou, G., Sharma, M.M., 1999. Adhesion Forces between E. coli Bacteria and Biomaterial Surfaces. *Langmuir*. **15**, 2719-2725.

Otto, M., 2009. *Staphylococcus epidermidis*—the'accidental'pathogen. *Nature Reviews Microbiology*. **7**, 555-567.

Palmer, R.J. & White, D.C., 1997. Developmental biology of biofilms: implications for treatment and control. *Trends in Microbiology*. **5**, 435-440.

Patchett, R.A., Kelly, A.F., Kroll, R.G., 1991. Respiratory activity in *Listeria* monocytogenes. FEMS Microbiology Letters. **78**, 95-98.

Parish, M. & Higgins, D., 1989. Survival of *Listeria monocytogenes* in low pH model broth systems. *The Lancet.* **1**, 144-147.

Park, S.F. & Stewart, G.S., 1990. High-efficiency transformation of *Listeria monocytogenes* by electroporation of penicillin-treated cells. *Gene.* **94**, 129-132.

Parsek, M.R. & Singh, P.K., 2003. Bacterial biofilms: an emerging link to disease pathogenesis. *Annual Reviews in Microbiology*. **57**, 677-701.

Pearson, L.J. & Marth, E.H., 1990. *Listeria monocytogenes* –Threat to a Safe Food Supply: A Review. *Journal of Dairy Science*. **73**, 912-928.

Peel, M., Donachie, W., Shaw, A., 1988. Temperature-dependent Expression of Flagella of *Listeria manocytogenes* Studied by Electron Microscopy, SDS-PAGE and Western Blotting. *Journal of General Microbiology*. **134**, 2171-2178.

Perkins, J.B. & Youngman, P.J., 1984. A physical and functional analysis of *Tn*917, a *Streptococcus* transposon in the *Tn*3 family that functions in *Bacillus*. *Plasmid*. **12**, 119-138.

Perni, S., Aldsworth, T.G., Jordan, S.J., Fernandes, I., Barbosa, M., Sol, M., Tenreiro, R.P., Chambel, L., Zilhão, I., Barata, B., 2007. The resistance to detachment of dairy strains of *Listeria monocytogenes* from stainless steel by shear stress is related to the fluid dynamic characteristics of the location of isolation. *International Journal of Food Microbiology*. **116**, 384-390.

Perni, S., Jordan, S.J., Andrew, P.W., Shama, G., 2006. Biofilm development by *Listeria innocua* in turbu

Petran, R. & Zottola, E., 2006. A study of factors affecting growth and recovery of *Listeria monocytogenes* Scott A. *Journal of Food Science*. **54**, 458-460.

Petran, R., Zottou, E., Gravani, R., 1988. Incidence of *Listeria monocytogenes* in market samples of fresh and frozen vegetables. *Journal of Food Science*. **53**, 1238-1240.

Portnoy, D.A., Chakraborty, T., Goebel, W., Cossart, P., 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. *Infection and Immunity*. **60**, 1263-1267.

Portnoy, D.A., Jacks, P.S., Hinrichs, D., 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. *The Journal of Experimental Medicine*. **167**, 1459-1471.

Poulsen, L.V., 1999. Microbial biofilm in food processing. *LWT-Food Science and Technology*. **32**, 321-326.

Premaratne, R.J., Lin, W., Johnson, E.A., 1991. Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. *Applied and Environmental Microbiology*. **57**, 3046-3048.

Ramaswamy, V., Cresence, V.M., Rejitha, J.S., Lekshmi, M.U., Dharsana, K., Prasad, S.P., Vijila, H.M., 2007. *Listeria*-review of epidemiology and pathogenesis. *Journal of Microbiology Immunology and Infection.* **40**, 4-13.

Ridgway, H. & Olson, B., 1981. Scanning electron microscope evidence for bacterial colonization of a drinking-water distribution system. *Applied and Environmental Microbiology*. **41**, 274-287.

Riedo, F.X., Pinner, R.W., de Lourdes Tosca, M., Cartter, M.L., Graves, L.M., Reeves, M.W., Weaver, R.E., Plikaytis, B.D., Broome, C., 1994. A point-source foodborne listeriosis outbreak: documented incubation period and possible mild illness. *Journal of Infectious Diseases.* **170**, 693-696.

Rieu, A., Briandet, R., Habimana, O., Garmyn, D., Guzzo, J., Piveteau, P., 2008. *Listeria monocytogenes* EGD-e biofilms: no mushrooms but a network of knitted chains. *Applied and Environmental Microbiology*. **74**, 4491-4497.

Riss, T., 1992. Comparison of attachment factors using the CellTiter 96 TM Assay. *Promega Notes Mag.* **38**, 18.

Roberts, A. & Wiedmann, M., 2003. Pathogen, host and environmental factors contributing to the pathogenesis of listeriosis. *Cellular and Molecular Life Sciences CMLS*. **60**, 904-918.

Roberts, A., Nightingale, K., Jeffers, G., Fortes, E., Kongo, J.M., Wiedmann, M., 2006. Genetic and phenotypic characterization of *Listeria monocytogenes* lineage III. *Microbiology*. **152**, 685-693. Roccourt, J., Grimont, F., Grimont, P.A.D., Seeliger, H.P.R., 1982. DNA relatedness among serovars of *Listeria monocytogenes* sensu lato. *Current Microbiology*. **7**, 383-388.

Rocourt, J., 1996. Risk factors for listeriosis. Food Control. 7, 195-202.

Rocourt, J., BenEmbarek, P., Toyofuku, H., Schlundt, J., 2003. Quantitative risk assessment of *Listeria monocytogenes* in ready-to-eat foods: the FAO/WHO approach. *FEMS Immunology & Medical Microbiology*. **35**, 263-267.

Rocourt, J. & Grimont, P.A.D., 1983. Notes: *Listeria welshimeri* sp. nov. and *Listeria seeligeri* sp. nov. *International Journal of Systematic Bacteriology*. **33**, 866-869.

Rocourt, J., Hof, H., Schrettenbrunner, A., Malinverni, R., Bille, J., 1986. Acute purulent *Listeria seelingeri* meningitis in an immunocompetent adult. *Schweizerische Medizinische Wochenschrift.* **116**, 248-251.

Rocourt, J., Boerlin, P., Grimont, F., Jacquet, C., Piffaretti, J.C., 1992. Assignment of *Listeria grayi* and *Listeria murrayi* to a single species, *Listeria grayi*, with a revised description of *Listeria grayi*. *International Journal of Systematic Bacteriology*. **42**, 171-174.

Rodriguez, A., Autio, W.R., McLandsborough, L.A., 2008. Effects of contact time, pressure, percent relative humidity (% RH), and material type on *Listeria* biofilm adhesive strength at a cellular level using atomic force microscopy (AFM). *Food Biophysics.* **3**, 305-311.

Ronner, A.B. & Wong, A., 1993. Biofilm development and sanitizer inactivation of *Listeria monocytogenes* and *Salmonella typhimurium* on stainless steel and Buna-n rubber. *Journal of Food Protection.* **56**, 750-758.

Rosenow, E. & Marth, E., 1987. Growth of *Listeria monocytogenes* in skim, whole and chocolate milk, and in whipping cream during incubation at 4, 8, 13, 21 and 35 degrees C. *Journal of Food Protection.* **50**, 452-463.

Ross, T., Dalgaard, P., Tienungoon, S., 2000. Predictive modelling of the growth and survival of *Listeria* in fishery products. *International Journal of Food Microbiology*.62, 231-245.

Rocourt, J., & Buchrieser, C., 2007. The Genus *Listeria monocytogenes*: phylogenetic position, taxonomy and identification *In:* Ryser, E.T. & Marth, E.H., 3rd ed. *Listeria, listeriosis, and food safety*. Boca Raton: Taylar & Francis CRC Pres, pp. 1-20

Ryser, E.T. & Marth, E.H., 1989. Behaviour of *Listeria monocytogenes* during manufacture and ripening of brick cheese. *Journal of Dairy Science*. **72**, 838-853.

Sagoo, S., Little, C., Mitchell, R., 2003. Microbiological quality of open ready-to-eat salad vegetables: effectiveness of food hygiene training of management. *Journal of Food Protection* **®**. **66**, 1581-1586.

Salamina, G., Donne, E.D., Niccolini, A., Poda, G., Cesaroni, D., Bucci, M., Fini, R., Maldini, M., Schuchat, A., Swaminathan, B., 1996. A foodborne outbreak of gastroenteritis involving *Listeria monocytogenes*. *Epidemiology and Infection*. **117**, 429-436.

Sallen, B., Rajoharison, A., Desvarenne, S., Quinn, F., Mabilat, C., 1996. Comparative analysis of 16S and 23S rRNA sequences of *Listeria* species. *International Journal of Systematic Bacteriology*. **46**, 669-674.

Samadpour, M., Barbour, M., Nguyen, T., Cao, T., Buck, F., Depavia, G., Mazengia, E., Yang, P., Alfi, D., Lopes, M., 2006. Incidence of enterohemorrhagic *Escherichia coli*, *Escherichia coli* O157, *Salmonella*, and *Listeria monocytogenes* in retail fresh ground beef, sprouts, and mushrooms. *Journal of Food Protection*®. **69**, 441-443.

Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989. *Molecular cloning: a laboratory manual*. 3rd ed. Cold Spring harbour: Cold Spring Harbor Laboratory press.

Sashara, K. & Zottola, E.A., 1993. Biofilm formation by *Listeria monocytogenes* utilizes a primary colonizing microorganism in flowing systems. *Journal of Food Protection.* **56**, 1022-1028.

Sauer, K., Camper, A.K., Ehrlich, G.D., Costerton, J.W., Davies, D.G., 2002. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *Journal of Bacteriology*. **184**, 1140-1154.

Sauders, D. B., & Wiedmann, M., 2007. Ecology of *Listeria* species and *L. monocytogenes* in natural environment *In:* Ryser, E.T. & Marth, E.H., 3rd ed. *Listeria, listeriosis, and food safety.* Boca Raton: Taylar & Francis CRC Pres, pp. 21-45

Shaw, J. & Clewell, D., 1985. Complete nucleotide sequence of macrolidelincosamide-streptogramin B-resistance transposon *Tn*917 in *Streptococcus faecalis*. *Journal of Bacteriology*. **164**, 782-796. Schaferkordt, S. & Chakraborty, T., 1995. Vector plasmid for insertional mutagenesis and directional cloning in *Listeria* spp. *BioTechniques*. **19**, 720-2, 724-5.

Schlech III, W.F. & Acheson, D., 2000. Foodborne listeriosis. *Clinical Infectious Diseases.* **31**, 770-775.

Schlech, W.F., 1996. Overview of listeriosis. Food Control. 7, 183-186.

Schlech III, W.F., Lavigne, P.M., Bortolussi, R.A., Allen, A.C., Haldane, E.V., 1983. Epidemic listeriosis: evidence for transmission by food [*Listeria monocytogenes*]. *New England Journal of Medicine*. **308**, 203-206

Schmittgen, T.D. & Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. *Nature Protocols.* **3**, 1101-1108.

Schoder, D., Winter, P., Kareem, A., Baumgartner, W., Wagner, M., 2003. A case of sporadic ovine mastitis caused by *Listeria monocytogenes* and its effect on contamination of raw milk and raw-milk cheeses produced in the on-farm dairy. *Journal of Dairy Research.* **70**, 395-401.

Schuchat, A., Robinson, K., Wenger, J.D., Harrison, L.H., Farley, M., Reingold, A.L., Lefkowitz, L., Perkins, B.A., 1997. Bacterial meningitis in the United States in 1995. *New England Journal of Medicine*. **337**, 970-976.

Schwartz, B., Hexter, D., Broome, C.V., Hightower, A.W., Hirschhorn, R.B., Porter, J.D., Hayes, P.S., Bibb, W.F., Lorber, B., Faris, D.G., 1989. Investigation of an outbreak of listeriosis: new hypotheses for the etiology of epidemic *Listeria monocytogenes* infections. *Journal of Infectious Diseases*. **159**, 680-685.

Scortti, M., Monzó, H.J., Lacharme-Lora, L., Lewis, D.A., Vázquez-Boland, J.A., 2007. The PrfA virulence regulon. *Microbes and Infection*. **9**, 1196-1207.

Seeliger, H.P.R., Rocourt, J., Schrettenbrunner, A., Grimont, P.A.D., Jones, D., 1984. Notes: *Listeria ivanovii* sp. nov. *International Journal of Systematic Bacteriology*. **34**, 336-337.

Seo, K. & Frank, J., 1999. Attachment of *Escherichia coli* O157: H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. *Journal of Food Protection.* **62**, 3-9.

Shabala, L., Lee, S.H., Cannesson, P., Ross, T., 2008. Acid and NaCl limits to growth of *Listeria monocytogenes* and influence of sequence of inimical acid and NaCl levels on inactivation kinetics. *Journal of Food Protection*. **71**, 1169-1177.

Sinde, E. & Carballo, J., 2000. Attachment of Salmonella spp. and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluorethylene: the influence of free energy and the effect of commercial sanitizers. *Food Microbiology*. **17**, 439-447.

Slayter, E.M., 1992. Light and electron microscopy. Cambridge University Press.

Smoot, L.M. & Pierson, M.D., 1998. Effect of environmental stress on the ability of *Listeria monocytogenes* Scott A to attach to food contact surfaces. *Journal of Food Protection.* **61**, 1293-1298.

Solano, C., García, B., Valle, J., Berasain, C., Ghigo, J., Gamazo, C., Lasa, I., 2002.
Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose. *Molecular Microbiology*. 43, 793-808.

Sorrells, K., Enigl, D., Hatfield, J., 1989. Effect of pH, acidulant, time, and temperature on the growth and survival of *Listeria monocytogenes*. *Journal of Food Protection*. **52**, 571-573

Steinbruegge, E., Maxcy, R.B., Liewen, M., 1988. Fate of *Listeria monocytogenes* on ready to serve lettuce. *Journal of Food Protection*. **51**, 596-599

Stewart, P.S., Murga, R., Srinivasan, R., de Beer, D., 1995. Biofilm structural heterogeneity visualized by three microscopic methods. *Water Research.* **29**, 2006-2009.

Stoodley, P., deBeer, D., Lappin-Scott, H.M., 1997. Influence of electric fields and pH on biofilm structure as related to the bioelectric effect. *Antimicrobial Agents and Chemotherapy*. **41**, 1876-1879.

Stoodley, P., Sauer, K., Davies, D., Costerton, J., 2002. Biofilms as complex differentiated communities. *Annual Reviews in Microbiology*. **56**, 187-209.

Stuart, M. & PEASE, P.E., 1972. A numerical study on the relationships of *Listeria* and *Erysipelothrix. Journal of General Microbiology*. **73**, 551-565.

Surak, J.G. & Barefoot, S.F., 1987. Control of *Listeria* in the dairy plant. *Veterinary* and Human Toxicology. **29**, 247-249.

Surman, S., Walker, J., Goddard, D., Morton, L., Keevil, C., Weaver, W., Skinner, A., Hanson, K., Caldwell, D., Kurtz, J., 1996. Comparison of microscope techniques for the examination of biofilms. *Journal of Microbiological Methods*. **25**, 57-70.

Sutherland, I.W., 2001. Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology*. **147**, 3-9.

Sutton, N.A., Hughes, N., Handley, P.S., 1994. A comparison of conventional SEM techniques, low temperature SEM and the electroscan wet scanning electron microscope to study the structure of a biofilm of *Streptococcus crista* CR3. *Journal of Applied Microbiology*. **76**, 448-454.

Tappero, J.W., Schuchat, A., Deaver, K.A., Mascola, L., Wenger, J.D., Swaminathan,
B., Hayes, P.S., Graves, L.M., Reeves, M.W., Weaver, R.E., 1995. Reduction in the incidence of human listeriosis in the United States. *JAMA: The Journal of the American Medical Association.* 273, 1118-1122.

Tasara, T. & Stephan, R., 2007. Evaluation of housekeeping genes in *Listeria monocytogenes* as potential internal control references for normalizing mRNA expression levels in stress adaptation models using real-time PCR. *FEMS Microbiology Letters.* **269**, 265-272.

Taylor, C.M., Beresford, M., Epton, H.A.S., Sigee, D.C., Shama, G., Andrew, P.W., Roberts, I.S., 2002. *Listeria monocytogenes* relA and hpt mutants are impaired in surface-attached growth and virulence. *Journal of Bacteriology*. **184**, 621-628.

Te Giffel, M. & Zwietering, M., 1999. Validation of predictive models describing the growth of *Listeria monocytogenes*. *International Journal of Food Microbiology*. **46**, 135-149.

Thomas, W. & Archer, G., 1989. Identification and cloning of the conjugative transfer region of *Staphylococcus aureus* plasmid pGO1. *Journal of Bacteriology*. **171**, 684-691.

Tienungoon, S., Ratkowsky, D., McMeekin, T., Ross, T., 2000. Growth limits of *Listeria monocytogenes* as a function of temperature, pH, NaCl, and lactic acid. *Applied and Environmental Microbiology*. **66**, 4979-4987.

Tilney, L.G. & Portnoy, D.A., 1989. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. *The Journal of Cell Biology*. **109**, 1597-1608.

Todhanakasem, T. & Young, G.M., 2008. Loss of flagellum-based motility by *Listeria* monocytogenes results in formation of hyperbiofilms. *Journal of Bacteriology*. **190**, 6030-6034.

Tomich, P.K., An, F., Clewell, D., 1980. Properties of erythromycin-inducible transposon *Tn*917 in *Streptococcus faecalis*. *Journal of Bacteriology*. **141**, 1366-1374.

Tran, H.L. & Kathariou, S., 2002. Restriction fragment length polymorphisms detected with novel DNA probes differentiate among diverse lineages of serogroup 4 *Listeria monocytogenes* and identify four distinct lineages in serotype 4b. *Applied and Environmental Microbiology*. **68**, 59-64.

Tresse, O., Lebret, V., Benezech, T., Faille, C., 2006. Comparative evaluation of adhesion, surface properties, and surface protein composition of *Listeria monocytogenes* strains after cultivation at constant pH of 5 and 7. *Journal of Applied Microbiology*. **101**, 53-62.
Trun, N. & Trempy, J., 2004. *Fundamental bacterial Genetics*. UK: Blackwell Science Ltd.

Tyrrell, E., 1973. Autolysis of *Listeria monocytogenes*. *Journal of Bacteriology*. **113**, 1046-1048.

USDA-FSIS. (United States Department of Agriculture - Food Safety and Inspection Service) 2003. FSIS Rule Designed To Reduce *Listeria monocytogenes* In Ready-To-Eat Meat And Poultry Products. United States Department of Agriculture Food Safety and Inspection Service, Washington, DC.

http://www.fsis.usda.gov/Frame/FrameRedirect.asp?main=http://www.fsis.usda.gov/O A/background/Imfinal.htm. Accessed: October, 2013

Vandecasteele, S., Peetermans, W., Merckx, R., Van Eldere, J., 2001. Quantification of expression of *Staphylococcus epidermidis* housekeeping genes with Taqman quantitative PCR during in vitro growth and under different conditions. *Journal of Bacteriology*. **183**, 7094-7101.

Van den Elzen, A. & Snijders, J., 1993. Critical points in meat production lines regarding the introduction of *Listeria monocytogenes*. *Veterinary Quarterly*. **15**, 143-145.

Varma, J.K., Samuel, M.C., Marcus, R., Hoekstra, R.M., Medus, C., Segler, S., Anderson, B.J., Jones, T.F., Shiferaw, B., Haubert, N., 2007. *Listeria monocytogenes* infection from foods prepared in a commercial establishment: a case-control study of potential sources of sporadic illness in the United States. *Clinical Infectious Diseases*.
44, 521-528.

Vatanyoopaisarn, S., Nazli, A., Dodd, C.E.R., Rees, C.E.D., Waites, W.M., 2000. Effect of flagella on initial attachment of *Listeria monocytogenes* to stainless steel. *Applied and Environmental Microbiology*. **66**, 860-863.

Vázquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Domínguez-Bernal, G., Goebel, W., González-Zorn, B., Wehland, J., Kreft, J., 2001. *Listeria* pathogenesis and molecular virulence determinants. *Clinical Microbiology Reviews*. **14**, 584-640.

Vazquez-Boland, J., Kocks, C., Dramsi, S., Ohayon, H., Geoffroy, C., Mengaud, J., Cossart, P., 1992. Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. *Infection and Immunity*. **60**, 219-230.

Vázquez-Boland, J.A., Domínguez-Bernal, G., González-Zorn, B., Kreft, J., Goebel, W., 2001a. Pathogenicity islands and virulence evolution in *Listeria*. *Microbes and Infection*. **3**, 571-584.

Vicente, M.F., Berenguer, J., de Pedro, M.A., Perez-Diaz, J.C., Baquero, F., 1990. Penicillin binding proteins in *Listeria monocytogenes*. *Acta Microbiologica Hungarica*. **37**, 227-231.

Villafane, R., Bechhofer, D., Narayanan, C., Dubnau, D., 1987. Replication control genes of plasmid pE194. *Journal of Bacteriology*. **169**, 4822-4829.

Wadsworth, S.J. & Goldfine, H., 2002. Mobilization of protein kinase C in macrophages induced by *Listeria monocytogenes* affects its internalization and escape from the phagosome. *Infection and Immunity*. **70**, 4650-4660.

Wagner, M. & McLauchlin, J., 2008. biology and pathology In: Liu, D., eds.*Handbook of Listeria monocytogenes*. Boca Raton: Taylar & Francis CRC Press, pp. 3-25.

Wagner, M., Melzner, D., Bago, Z., Winter, P., Egerbacher, M., Schilcher, F., Zangana, A., Schoder, D., 2005. Outbreak of clinical listeriosis in sheep: evaluation from possible contamination routes from feed to raw produce and humans. *Journal of Veterinary Medicine, Series B.* **52**, 278-283.

Wang, G., Yan, K., Feng, X., Chen, S., Lui, A., Kokubo, Y., 1992. Isolation and identification of *Listeria monocytogenes* from retail meats in Beijing. *Journal of Food Protection.* **55**, 56-58.

Weagant, S.D., Sado, P.N., Colburn, K.G., Torkelson, J., Stanley, F., Krane, M.H., Shields, S., Thayer, C., 1988. The incidence of *Listeria* species in frozen seafood products. *Journal of Food Protection.* **51**, 655-657.

Weis, J. & Seeliger, H., 1975. Incidence of *Listeria monocytogenes* in nature. *Applied Microbiology*. **30**, 29-32.

Wimpenny, J., 2000. An overview of biofilms as functional communities. *In:* Allison, D.G., Gilbert, P., Lappin-Scott, H.M., & Wilson, M. 1st ed. *Community structure and co-operation in biofilms*, Cambridge: Cambridge University Press, pp. 1-24.

Wimpenny, J., Manz, W., Szewzyk, U., 2000. Heterogeneity in biofilms. *FEMS Microbiology Reviews*. 24, 661-671.

Wirtanen, G., Alanko, T., Mattila-Sandholm, T., 1996. Evaluation of epifluorescence image analysis of biofilm growth on stainless steel surfaces. *Colloids and Surfaces B: Biointerfaces.* **5**, 319-326.

Xu, H., Zou, Y., Lee, H.Y., Ahn, J., 2010. Effect of NaCl on the biofilm formation by foodborne pathogens. *Journal of Food Science*. 75, M580- M585.

Youngman, P.J., Perkins, J.B., Losick, R., 1983. Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with *Streptococcus faecalis* transposon *Tn*917. *Proceedings of the National Academy of Sciences*. **80**, 2305-2309.

Zhang, C., Zhang, M., Ju, J., Nietfeldt, J., Wise, J., Terry, P.M., Olson, M., Kachman, S.D., Wiedmann, M., Samadpour, M., 2003. Genome diversification in phylogenetic lineages I and II of *Listeria monocytogenes*: identification of segments unique to lineage II populations. *Journal of Bacteriology*. **185**, 5573-5584.

Zobell, C.E., 1943. The effect of solid surfaces upon bacterial activity. *Journal of Bacteriology*. **46**, 39-56.