

LONDON
SCHOOL of
HYGIENE
& TROPICAL
MEDICINE



LSHTM Research Online

Naz, N; (2014) Reinvestigation into the mechanisms of *Campylobacter jejuni* invasion of intestinal epithelial cells. PhD thesis, London School of Hygiene & Tropical Medicine. DOI: <https://doi.org/10.17037/PUBS.02115599>

Downloaded from: <https://researchonline.lshtm.ac.uk/id/eprint/2115599/>

DOI: <https://doi.org/10.17037/PUBS.02115599>

Usage Guidelines:

Please refer to usage guidelines at <https://researchonline.lshtm.ac.uk/policies.html> or alternatively contact researchonline@lshtm.ac.uk.

Available under license. To note, 3rd party material is not necessarily covered under this license: <http://creativecommons.org/licenses/by-nc-nd/3.0/>

<https://researchonline.lshtm.ac.uk>

**Reinvestigation into the mechanisms of
Campylobacter jejuni invasion of intestinal
epithelial cells**

Thesis submitted for the degree of Doctor of Philosophy

By

Navida Naz

Pathogen Molecular Biology Department
Faculty of Infectious and Tropical Diseases
London School of Hygiene and Tropical Medicine
University of London

2014

Funding Details – No funding was received.

Declaration

I Navida Naz confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. All experiments were performed at the London School of Hygiene and Tropical Medicine.

Abstract

Background: *Campylobacter jejuni* is an important foodborne pathogen and the leading cause of bacterial gastroenteritis. Despite the importance of *C. jejuni* infection and decades of research, the mechanisms for colonisation of the human intestinal tract by *C. jejuni* and how this causes diarrhoeal disease remain unclear, with a significant number of conflicting reports in the literature creating controversy in this area.

Methods: The effect of different inhibitors of host cell pathways on the ability of the *C. jejuni* 81-176 wild-type strain to interact with and invade intestinal epithelial cells (IECs) was investigated. Defined isogenic *C. jejuni* 81-176 & 11168H *ciaB*, *cadF* and *flpA* mutants were constructed and characterised for the ability to interact with and invade host cells.

Results: Disruption of microfilaments with Cytochalasin D increased *C. jejuni* invasion. Disruption of caveolae-mediated endocytosis with Methyl-beta-cyclodextrin, disruption of microtubules with Colchicine, disruption of clathrin-mediated endocytosis with Monodansylcadaverine, inhibition of phosphatidylinositol 3-kinase with Wortmannin all decreased *C. jejuni* invasion. Infection of the *Galleria mellonella* insect model with *ciaB*, *cadF* and *flpA* mutants resulted in a significantly reduced cytotoxic effect on the larvae. The ability of *ciaB*, *cadF* and *flpA* mutants to interact with and invade Caco-2 and T84 IECs was significantly reduced. The *ciaB*, *cadF* and *flpA* mutants exhibited a more significant decrease in the number of invasive bacteria when co-cultured with IECs in the Vertical Diffusion Chamber model. Pre-treatment of Caco-2 IECs with OMVs isolated from the *ciaB*, *cadF* and *flpA* mutants reduced interactions and invasion of these IECs by live *C. jejuni*.

Conclusion: *C. jejuni* invasion of IECs involves modulation of many host cell pathways. CiaB, CadF and FlpA all play an important role in *C. jejuni* interactions with IECs leading to bacterial invasion. Further studies are still required to elucidate the exact roles that these important *C. jejuni* virulence factors play during interactions with and invasion of host cells.

Table of Contents

Declaration	2
Abstract	3
Acknowledgements	12
List of Abbreviations:	13
Chapter 1: Introduction	19
1.1 Discovery of <i>Campylobacter jejuni</i>	19
1.1.1 Biology of <i>Campylobacter jejuni</i>	20
1.1.2 Transmission of <i>Campylobacter jejuni</i>	21
1.1.3. Epidemiology of <i>Campylobacter jejuni</i>	21
1.1.4 Pathogenesis and Symptoms of <i>Campylobacter jejuni</i>	24
1.1.5 The human gastrointestinal tract	25
1.1.6 Post-infectious sequelae of <i>Campylobacter jejuni</i>	26
1.2 Models Used to Study <i>Campylobacter jejuni</i> Pathogenesis	26
1.2.1 <i>in vivo</i> models used to study <i>C. jejuni</i>	27
1.2.2 <i>Galleria mellonella</i> model of <i>C. jejuni</i> infection	29
1.2.3 <i>in vitro</i> Models Used to Study <i>Campylobacter jejuni</i>	30
1.2.4 Cell Line Models	31
1.2.5 The Vertical Diffusion Chamber Model	33
1.3 Molecular basis of <i>Campylobacter jejuni</i> pathogenesis	35
1.3.1 <i>C. jejuni</i> Flagellum	37
1.3.2 <i>C. jejuni</i> Glycan structures	38
1.3.3 Capsule	38
1.3.4 Lipooligosaccharide	40
1.3.5 Glycosylation pathways	41
1.3.5.1 <i>O</i> -linked protein glycosylation	41
1.3.5.2 <i>N</i> -linked protein glycosylation	43
1.3.6 Cytotoxic distending toxin	44
1.4 Host response to <i>C. jejuni</i> infection	45

1.4.1 Humoral Immune Response to <i>C. jejuni</i>	48
1.5 Adhesion to and invasion of host epithelial cells	48
1.5.1 <i>C. jejuni</i> Invasion	49
1.5.2 The putative role of <i>C. jejuni</i> outer membrane vesicles in invasion of IEC ..	54
1.5.3 Inhibition of <i>C. jejuni</i> adhesion to and invasion of intestinal epithelial cells	56
1.5.4 Role of CiaB	59
1.5.5 Role of CadF	62
1.5.6 Role of FlpA	65
1.5.7 Intracellular survival of <i>C. jejuni</i>	67
1.5.8 Imaging of <i>C. jejuni</i> adhesion to and invasion of Caco-2 intestinal epithelial cells	69
Chapter 2: Materials and Methods	72
2.1 Bacterial strains and plasmids	72
2.1.1 Chemicals and reagents	72
2.1.1.1 Sterilisation	72
2.1.1.2 Reagent preparation	72
2.1.2 Microbiological procedures	76
2.1.2.1 Bacterial strains, media and growth conditions	76
2.1.2.2 Culturing conditions for <i>E. coli</i>	76
2.1.3 Examination of <i>C. jejuni</i> morphology	76
2.1.3.1 Gram staining	76
2.1.4 Preparation of specific OD ₆₀₀ suspension of <i>C. jejuni</i>	77
2.1.5 Glycerol stock preparation of <i>C. jeuni</i> and <i>E. coli</i> strains	77
2.1.6 Preparation of electrocompetent <i>C. jejuni</i> cells	77
2.1.7 Resuscitation of <i>C. jeuni</i> and <i>E. coli</i> frozen stocks	78
2.1.8 Motility assays	78
2.1.9 <i>C. jejuni in vitro</i> growth kinetics assay	78
2.1.10 Quantifying bacterial numbers by counting colony forming units	79

2.1.11 <i>C. jejuni</i> autoaggregation studies	79
2.1.12 <i>Galleria mellonella</i> model of <i>C. jejuni</i> infection	79
2.2 Mammalian cell culture.....	80
2.2.1 Tissue culture	80
2.2.2 Maintenance and passaging of tissue culture cell lines.....	80
2.2.3 Passaging of tissue culture cells.....	81
2.2.4 Enumerating the number of cells using a haemocytometer	81
2.2.5 Storage of tissue culture cells.....	82
2.2.6 Resuscitation of tissue culture cells from frozen stocks	82
2.2.7 Bacterial interaction assay.....	82
2.2.8 Bacterial invasion assays.....	83
2.2.9 Triton and gentamicin sensitivity assays.....	83
2.2.10 Immunofluorescence staining of Caco-2 IECs	83
2.2.11 Anti- <i>C. jejuni</i> antibody staining of <i>C. jejuni</i> for confocal microscopy.....	84
2.2.12 Quantification of mean fluorescence intensity (MFI).....	85
2.2.13 Pre-treatment of Caco-2 IECs with biochemical inhibitors	85
2.2.14 Cytotoxicity assays.....	86
2.2.15 Intracellular survival assays	86
2.2.16 Macrophage survival assays.....	86
2.2.17 Maintenance of IECs for Vertical Diffusion Chamber experiments.....	87
2.2.18 Measurement of transepithelial electrical resistance of IEC monolayer for Vertical Diffusion Chamber experiments	87
2.2.19 Assembly of the Vertical Diffusion Chamber model system for <i>C. jejuni</i> Caco-2 and T84 IECs invasion studies.	87
2.2.20 Disassembly of the VDC for enumeration of intracellular <i>C. jejuni</i>	88
2.2.21 Cytokine analysis of IEC supernatants following co-culture with <i>C. jejuni</i>	88
2.3 Molecular biology techniques	88
2.3.1. Isolation of <i>C. jejuni</i> genomic DNA	88

2.3.2 Bacterial gDNA extraction for PCR screening	88
2.3.3 Isolation of plasmid DNA from <i>E. coli</i>	89
2.3.4 Oligonucleotide design.....	89
2.3.5 Amplification of <i>C. jejuni</i> genes for cloning	91
2.3.6 Polymerase chain reaction PCR.....	91
2.3.7 Analysing PCR products by gel electrophoresis.....	93
2.3.8 Purification of PCR products	93
2.3.9 Restriction enzyme digestion	94
2.3.10 Dephosphorylation of plasmid DNA	94
2.3.11 Cloning of purified PCR products into a plasmid vector.....	94
2.3.12 Transformation of <i>E. coli</i>	95
2.3.13 PCR screening of transformants.....	95
2.3.14 Insertion of a Kanamycin cassette.....	96
2.3.15 DNA sequencing	96
2.3.16 Electroporation of <i>C. jejuni</i>	97
2.3.17 Inverse Polymerase Chain Reaction Mutagenesis	98
2.3.18 Construction of complements	100
2.4 Outer membrane vesicles assays.....	100
2.4.1 Pre-treatment of <i>C. jejuni</i> OMVs with Caco-2 IECs	100
2.4.2 <i>C. jejuni</i> fibronectin binding assays.....	101
2.4.3 Statistical Analysis	101
Chapter 3. Reinvestigation of <i>C. jejuni</i> adhesion to and invasion of intestinal epithelial cells	102
3.1 Introduction	102
3.2. Results.....	102
3.2.1 Motility.....	102
3.2.2 The <i>C. jejuni</i> 81-176 wild-type strain exhibits higher levels of interaction with and invasion of Caco-2 IECs.	104

3.2.3 Inhibitors of bacterial invasion of eukaryotic cells	106
3.2.4 Investigation of the cytotoxicity of individual inhibitors.....	107
3.2.5 Disruption of microfilaments by inhibition of actin polymerisation using Cytochalasin D enhances <i>C. jejuni</i> invasion	109
3.2.6 Disruption of lipid rafts by removal of cholesterol from the cell plasma membrane using methyl-beta-cyclodextrin reduces <i>C. jejuni</i> invasion	111
3.2.7 Disruption of microtubules using colchicine reduces <i>C. jejuni</i> invasion.....	113
3.2.8 Disruption of the Phosphatidylinositol 3-kinase pathway with wortmannin reduces <i>C. jejuni</i> invasion	115
3.2.9 Disruption of clathrin-mediated endocytosis with monodansylcadaverine reduces <i>C. jejuni</i> invasion.	117
3.2.10 Comparison of the inhibition of <i>C. jejuni</i> 81-176 wild-type strain interactions with and invasion of Caco-2 intestinal epithelial cells.....	119
3.3 Imaging of <i>C. jejuni</i> adhesion to and invasion of Caco-2 intestinal epithelial cells	120
3.3.1 Construction of Evoglow® expressing <i>C. jejuni</i> strains.....	121
3.3.2 Construction of eGFP construct expressing <i>C. jejuni</i> strains.....	123
3.3.3 Comparison of eGFP versus Evoglow® expressing <i>C. jejuni</i> strains.....	124
3.3.4 Comparison of the fluorescence intensity of <i>C. jejuni</i> expressing eGFP and Evoglow® using Volocity software.....	126
3.3.5 Infection of Caco-2 intestinal epithelial cells with Evoglow® expressing <i>C.</i> <i>jejuni</i>	128
3.3.6 Anti- <i>C. jejuni</i> antibody staining to distinguish between adhered and invaded bacteria using Caco-2 IECs	129
3.4 Discussion	132
3.4.1 Inhibition of invasion studies	132
3.4.2 Visualisation of <i>C. jejuni</i> within IECs	141
3.4.3 Limitations of the study	143

Chapter 4. Reinvestigation of the role of CiaB in <i>C. jejuni</i> invasion of intestinal epithelial cells.....	144
4.1 Introduction.....	144
Results 4.2.....	145
4.2.1 Chromosomal location of <i>ciaB</i>	145
4.2.2 Construction of 81-176 & 11168H <i>ciaB</i> mutants	146
4.2.3 Construction 81-176 & 11168H <i>ciaB</i> complements	147
4.2.4 Characterisation of 81-176 and 11168H <i>ciaB</i> mutants	149
4.2.4.1 Growth rate assays and cfu counts	149
4.2.4.2 Motility.....	152
4.2.4.3 Autoaggregation Assays.....	154
4.2.4.4 <i>Galleria mellonella</i> infection model	156
4.2.5 Interaction and invasion assays.....	157
4.2.5.1 The role of CiaB of <i>C. jejuni</i> interactions with and invasion of Caco-2 IECs.....	157
4.2.5.2 The role of CiaB in <i>C. jejuni</i> interactions with and invasion of T84 IECs	159
4.2.6 Intracellular survival assays	162
4.2.7 Macrophage survival assays.....	164
4.2.8. Sensitivity of <i>C. jejuni</i> to Triton X-100	165
4.2.9 Vertical Diffusion Chamber assays.....	166
4.2.10 ELISA assays	168
4.2.11 Role of outer membrane vesicles in <i>C. jejuni</i> interactions with IECs	172
4.3 Discussion	174
Chapter 5. Reinvestigation of the roles of CadF and FlpA in <i>C. jejuni</i> adhesion to and invasion of intestinal epithelial cells.....	180
5.1 Introduction.....	180
5.2 Results.....	181
5.2.1 Chromosomal location of <i>cadF</i> and <i>flpA</i>	181

5.2.2 Construction of 81-176 and 11168H <i>cadF</i> mutants.....	183
5.2.3 Construction of 81-176 and 11168H <i>flpA</i> mutants using Inverse PCR Mutagenesis.....	184
5.2.4 Construction of 81-176 and 11168H <i>cadF</i> and <i>flpA</i> complements.....	187
5.2.4.1 Growth rate assays and cfu counts.....	190
5.2.4.2 Motility assays.....	195
5.2.4.3 Autoaggregation.....	196
5.2.4.4 <i>Galleria mellonella</i> infection model.....	199
5.2.5 81-176 <i>cadF</i> and <i>flpA</i> mutants exhibit reduced binding to fibronectin	201
5.2.6 Interaction and invasion assays.....	202
5.2.6.1 The role of CadF in <i>C. jejuni</i> interactions with and invasion of Caco-2 IECs.....	202
5.2.6.2 The role of CadF in <i>C. jejuni</i> interactions with and invasion of T84 IECs.....	204
5.2.6.3 The role of FlpA in <i>C. jejuni</i> interactions with and invasion of Caco-2 IECs.....	206
5.2.6.4 The role of FlpA in <i>C. jejuni</i> interactions with and invasion of T84 IECs.....	207
5.2.7 Intracellular survival assays.....	210
5.2.8 Macrophage survival assays.....	212
5.2.9 Sensitivity of <i>C. jejuni</i> to Triton X-100 and gentamicin.....	213
5.2.10 Vertical Diffusion Chamber Assays.....	215
5.2.11 ELISA assays.....	217
5.2.12 Role of outer membrane vesicles in <i>C. jejuni</i> interactions with IECs	221
5.3 Discussion.....	223
Chapter 6: Final Discussion.....	231
6.1 Invasion.....	231
6.2 Visualisation of <i>C. jejuni</i>	231
6.3 <i>ciaB</i> mutant.....	232

6.4 <i>cadF</i> mutant	233
6.5 <i>flpA</i> mutant	233
6.6 Final comments	234
7 Appendices	238
7.1 Appendix 1	238
7.2 Appendix 2	242
8. References	246

Acknowledgements

Acknowledgement of Academic Support:

Completing my PhD has been one of the most challenging things in my life. It was a privilege and an honour to spend several years at LSHTM and be given the chance to work on such an interesting and sometimes difficult area of research. First and foremost I would like to express my sincere gratitude to my supervisor Dr Nick Dorrell for his support, expertise, advice on experimental procedures, his patience and encouragement over the past 5 years and for proof reading my thesis. I could not have asked for a better supervisor. I am grateful to Professor Brendan Wren for supporting my work and allowing to me to be part of a great lab group. I am also obliged to thank many of my colleagues, Dr Ozan Gundodgdu for his guidance and making those long hours in the lab entertaining. Dr Abdi Elmi for his assistance and advice, I owe a lot to my colleagues who supported me in the lab, Dr Dominic Mills, Dr Laura Yates and Dr Vanessa-Terra for all their help and advice. I would like to thank Emma Taylor and Emma Cobb for sacrificing their free time and assisting with lab work. I am grateful to Dr Elizabeth McCarthy, Dr Sam Willcocks, Rachel Gregory and Dr Priyanka Naranga for their invaluable knowledge and expertise in cell staining and confocal microscopy studies.

Acknowledgement of Other Support:

I am truly indebted and thankful to my father Said Iqbal Shah and my mother Zakia Shah for giving me the opportunity that I appreciate is rare for many young middle eastern women and be able to pursue my studies. Thank you for all your support and encouragement. I owe my deepest gratitude to James Heelan, you are a grandfather, a mentor, a dear friend and without your encouragement and help, this would not have been possible. I would like to express my gratitude to Professor Paul Fine, for his advice and support this past year. To Kerry and Joe, thank you for the endless moral support. Finally, I would like to show my greatest appreciation to Sarah McLean, the most selfless, thoughtful human being I know. Thank you for your love, kindness and support. I'm so blessed to have you in my life.

List of Abbreviations:

AAG	Autoaggregation
ABC	ATP binding cassette
ABI	Applied Biosystems
AgCl	Silver chloride
AIDS	Acquired immune deficiency syndrome
ATP	Adenosine triphosphate
BA	Blood agar
BamHI	Restriction enzyme (recognises GGATCC sites) <i>Bacillus amyloliquefaciens</i>
BglII	Restriction enzyme (recognises AGATCT sites) <i>Bacillus globigii</i> .
H β D2	Human β Defensin
bp	Base pairs
BSA	Bovine serum albumin
C57 BL	C57 standard Black mice
Caco-2	Intestinal epithelial cells (colon)
CadF	Campylobacter Adhesion to Fibronectin
Cag A	Cytotoxin associated gene A
CCV	Campylobacter containing vacuole
CDC	United States Centre for Disease Control and Prevention
Cdc42	Cell division control protein
CDT	Cytolethal distending toxin
CFU	Colony forming units
CgtA	GTP binding protein
Cia	Campylobacter invasion antigen
CiaB	Campylobacter invasion Antigen B
CiaI	Campylobacter invasion Antigen I
CO ₂	Carbon dioxide
CoA	Coenzyme A
Cos-7	Cell line derived from the kidney of the African Green Monkey, <i>Cercopithecus aethiops</i>
CPS	Capsular polysaccharide
CysK	Cysteine synthase A
CytD	Cytochalasin D

DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DMEM	Dulbeccos modified eagles medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPS	Deoxynucleotide triphosphates
DpnI	Restriction enzyme (recognises and cuts methylated DNA) <i>Diplococcus pneumoniae</i>
<i>E. coli</i>	<i>Escherichia coli</i>
EBF	Ice cold wash buffer
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
EHEC	Enterohaemorrhagic <i>Escherichia coli</i>
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic <i>Escherichia coli</i>
ERK	Extracellular signal regulated kinase
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FedA	Flagellar co-expressed colonisation determinant A
FedB	Flagellar co-expressed colonisation determinant B
FedC	Flagellar co-expressed colonisation determinant C
FedD	Flagellar co-expressed colonisation determinant D
FITC	Fluorescein isothiocyanate
FlaA	Flagella filament A
FlaB	Flagella filament B
FlgE	Flagella Hook associated protein E
FlgK	Flagella Hook associated protein K
FlgL	Flagella Hook associated protein L
FlgM	Anti-sigma factor
FlgRS	Two-component regulatory system
FlhA	Flagella biosynthesis protein A

FlhB	Flagella biosynthesis protein B
FlhF	Putative GTPase
FliA	Flagellar biosynthesis gene encoding alternative sigma factor (σ^{28})
FliR	Flagellar biosynthesis gene
FlpA	Fibronectin-like protein A
FMN	Flavin mononucleotide
Fops	Flagellar outer proteins
FP	Fluorescent protein
FSA	Food standards agency
<i>G. mellonella</i>	<i>Galleria mellonella</i> (The greater wax moth larvae)
G1/S	Cell division
G2/M	DNA damage check point
GBS	Guillian-Barré syndrome
gDNA	Genomic DNA
GFP	Green fluorescent protein
GI	Gastrointestinal
GIT	Gastrointestinal tract
GPA	Gentamicin protection assay
GTPases	Guanine-nucleotide binding proteins
HCA-7 cells	Human colon adenocarcinoma
HEP 2	Human epithelial type 2
Hsp90 α	Heat shock protein
IEC	Intestinal epithelial cells
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgG2a	Immunoglobulin g2a
IL-10	Interleukin-10
IL-8	Interleukin-8
IM	Inner membrane
INT 407	Epithelial-like cells derived from embryonic intestine
IpaB	Invasion plasmid antigen B
IPCRM	Inverse polymerase chain reaction mutagenesis
IPTG	Isopropyl-beta-D-thiogalactopyranoside
JlpA	<i>C. jejuni</i> lipoprotein A

Kan ^r	Kanamycin resistant cassette
KatA	Catalase A
Kb	Kilobase
kDa	Kilodalton
LB	Luria Bertani
LDH	Lactate dehydrogenase
LOS	Lipo oligosaccharide
LPS	Lipo polysaccharide
MAPKs	Mitogen activated protein kinases
MβCD	Methyl-beta-cyclodextrin
MDC	Monodansylcadaverine
MFI	Mean fluorescence intensity
MFs	Microfilaments
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MSCRAMMS	Microbial surface components recognising adhesive matrix molecules
MTs	Microtubules
MyD88	Myeloid differentiation factor 88
N ₂	Nitrogen
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NOD	Nucleotide binding oligomerisation domain
N-ramp1	Natural resistance associated macrophage protein
O ₂	Oxygen
OD	Optical density
OMV	Outer membrane vesicles
PAMPS	Pathogen associated pattern molecules
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFR	Platelet derived growth factor receptor
PEB1	Periplasmic binding protein
PFA	Paraformaldehyde
pg/ml	Picogram/ml

PglB	Oligosaccharyltransferase
PHE	Public Health England
PI3-Kinase	Phosphatidylinositol 3-kinases
PKC	Protein kinase C
PRRS	Pathogen recognition receptors
pVir	Putative virulence plasmid
Rac1	Signalling G protein member of the Rac subfamily of the Rho family of GTPase
Rho	Small GTP binding proteins
RNA	Ribonucleic acid
rpm	Revolutions per minute
rpoN	gene encoding Sigma factor ⁵⁴
rpoZ	DNA-directed RNA polymerase subunit omega
SD	Standard deviation
SipB	Salmonella invasion protein B
siRNA	Small interfering RNA
SodB	Superoxide dismutase B
Src	Non-receptor protein tyrosine kinase
T3SS	Type 3 secretion system
T4SS	Type 4 secretion system
T84	Colonic adenocarcinoma cell line
TAE buffer	(Tris-acetate-EDTA)
TEER	Trans epithelial electrical resistance
TH2	T helper cell
TLR	Toll like receptor
T _m	Melting temperature
TNF α	Tumour Necrosis Factor α
tRNA	Transfer RNA
v/v	Volume/volume
VAIN	Variable atmospheric incubator
Vav2	Guanine nucleotide exchange factor
VDC	Vertical diffusion chamber
w/v	Weight/volume
WlaN	Encodes a β -1,3-galactosyltransferase

XbaI	Restriction enzyme recognises AGATCT <i>Xanthomonas badrii</i>
YopB	Yersinia outer protein B
μm	Micrometre
σ^{54}	Sigma ⁵⁴ factor
σ^{28}	Sigma ²⁸ factor

Chapter 1: Introduction

1.1 Discovery of *Campylobacter jejuni*

In 1906, two British veterinary surgeons Sir John McFadyean and Sir Stewart Stockman identified an unknown microorganism with spiral shaped morphology in the uterine mucous tissue of the aborted fetuses of sheep (McFadyean, 1913, Skirrow, 2006). McFadyean and Stockman isolated and designated the unknown microorganisms to the genus *Vibrio*. However, there is evidence that similar bacteria had been isolated even earlier. Theodor Escherich published a series of articles in 1882 in the *Weekly Munich Medical Review* after studying stool samples of 72 children suffering from intestinal diseases, from which he isolated bacteria with a spiral morphology from 35 of the 72 samples (Butzler *et al.*, 1973). Marian Taylor and Theobald Smith also observed similar microorganisms from aborting cattle in the USA, which they designated as *Vibrio fetus* due to the spiral shape and the ability to grow on a medium of agar supplemented with gelatine and serum (Smith 1919). In 1938, the first case of human Campylobacteriosis due to contaminated milk was reported in the USA, with bacteria observed from faecal smears. However, these bacteria could not be cultured (Levy 1946). Further reports occurred in 1947, where Vinzent *et al.* isolated *Vibrio*-like microorganisms from three pregnant women admitted to hospital due to sepsis (Vinzent, 1947). Elizabeth King was one of the first people to analyse human strains and she found that many were a classic type whilst other strains formed a distinct group which she termed “related vibrios”. King was the first to distinguish between these two groups based on serological differences and growth at 42°C, as *Vibrio fetus* does not grow at this temperature, highlighting the importance of using appropriate culturing procedures (King, 1957). The isolation of such bacteria from patients suffering from gastrointestinal disease was mainly from blood (Bokkenheuser, 1970). Attempts to culture the organisms from the faeces of patients always resulted in contamination and over growth of coliforms (Sellu, 1986). In order to avoid contamination, two methods were introduced to isolate *Campylobacter* species. The first method for isolation was reported in 1969 by Dekeyser and colleagues in Brussels, using a 0.64 µm membrane to filter stool samples. This method removed other bacteria but allowed the smaller *Campylobacter* species to pass through (Dekeyser *et al.*, 1972). The second method was reported by Cooper and Slee in the early 1970’s, who isolated a *Campylobacter* species from the blood of a

patient suffering from diarrhoea, which was found to be resistant to cephalothin. Antibiotic discs were placed on the agar plates that were inoculated with the faecal samples of the patients. The plates were also incubated under a reduced oxygen tension level. *Campylobacter* colonies were found in the zone of the cephalothin disc (Cooper and Slee, 1971, Slee, 1972). The method of isolation of *Campylobacter* species was further improved by Skirrow in 1977, whilst working as a microbiologist at the Worcester Royal Infirmary, who introduced a new method of isolating *Campylobacter* species from faecal samples that did not require filtration techniques. Skirrow inoculated faecal samples directly onto media supplemented with the antibiotics vancomycin, trimethoprim and polymyxin B followed by incubation at 43°C under atmospheric conditions of 5% oxygen, 10% carbon dioxide and 85% nitrogen (Skirrow, 1977). *Campylobacter* species require microaerophilic conditions for growth, so the reduced oxygen tension allows optimum growth. Skirrow's examination of faecal samples from over 800 patients revealed that *Campylobacter* species were the most common microorganism cultured, more so than *Salmonella* species, *Shigella* species or *Escherichia coli* (Skirrow, 1977). The delay in understanding the specific growth requirements of *Campylobacter* species was partly responsible for the delay in acknowledging the importance of this organism as a human pathogen (Sellu, 1986). Following extensive improvements in isolation of the bacteria and surveillance systems, there has been a significant increase in the number of cases reported (Hermans *et al.*, 2012). *C. jejuni* is now the predominant cause of bacterial diarrhoeal disease in England and Wales (PHE 2011) and one of the leading causes of foodborne bacterial gastroenteritis worldwide (Man, 2011).

1.1.1 Biology of *Campylobacter jejuni*

The *Campylobacter* taxonomy has changed considerably since the microorganism's classification in 1963 (Sebald and Veron, 1963). Initially the *Campylobacter* genus consisted of two species; *Campylobacter fetus* and *Campylobacter bubulus*, the latter now renamed *Campylobacter sputorum* (Sebald and Veron, 1963). *Campylobacter jejuni* and *Campylobacter coli*, which were misclassified previously as *Vibrio* species, were added to the new *Campylobacter* genus based on DNA similarities and serology tests (Hore *et al.*, 1973). The genus of *Campylobacter* currently contains 25 species, 2 provisional species (not yet denoted a scientific name) and 8 sub-species (Man, 2011).

C. jejuni is a member of the epsilon class of proteobacteria and belongs to the order of *Campylobacteriales*. *C. jejuni* are small (0.2–0.9 µm wide and 0.2–5.0 µm long), spiral shaped, Gram-negative rods with a single flagellum at one or both poles, which gives the bacteria a rapid, darting mobility (Alm *et al.*, 1991, Butzler, 2004). *C. jejuni* is oxidase positive and hydrolyses both hippurate and indoxyl acetate (Kaur *et al.*, 2011). *C. jejuni* grows optimally at 42°C and is a microaerophilic and capnophilic organism, requiring 85% N₂, 10% CO₂ and 5% O₂ for optimal growth. Despite such specific growth requirements, *C. jejuni* is ubiquitous in the environment and can withstand many different environmental stresses (Fields and Thompson 2008).

1.1.2 Transmission of *Campylobacter jejuni*

C. jejuni is the major cause of foodborne bacterial diarrhoeal disease in humans (Friedman C. R., 2000). Most cases of Campylobacteriosis in humans are caused by *C. jejuni*, the remaining cases predominantly by *C. coli* (Janssen *et al.*, 2008). *C. jejuni* is a commensal in avian species and colonises the intestines of chickens at a few days old (less frequently those of turkeys and ducks) in which overt disease is not observed (Welkos 1984). In humans, the consumption of contaminated chicken products or cross-contamination with other food products seems to be responsible for more than 90% of all sporadic human cases. Transmission can occur from the consumption of infected poultry, and contaminated water or milk (Hopkins *et al.*, 1984, Schmid *et al.*, 1987, Schildt *et al.*, 2006). In addition, the handling or cross contamination of raw poultry products (Newell *et al.*, 2011), bird droppings, contact with pets, consumption of undercooked beef and pig meat have been reported as consistent sources of *C. jejuni* human infection (Dasti *et al.*, 2010). Other sources of human infection include contact with wild birds (Kapperud *et al.*, 1992, Wilson *et al.*, 2008, Dasti *et al.*, 2010).

1.1.3. Epidemiology of *Campylobacter jejuni*

C. jejuni is found ubiquitously in the environment despite the microorganism's strict microaerobic growth requirements. As such, *C. jejuni* poses a potential threat to public health (Dasti *et al.*, 2010).

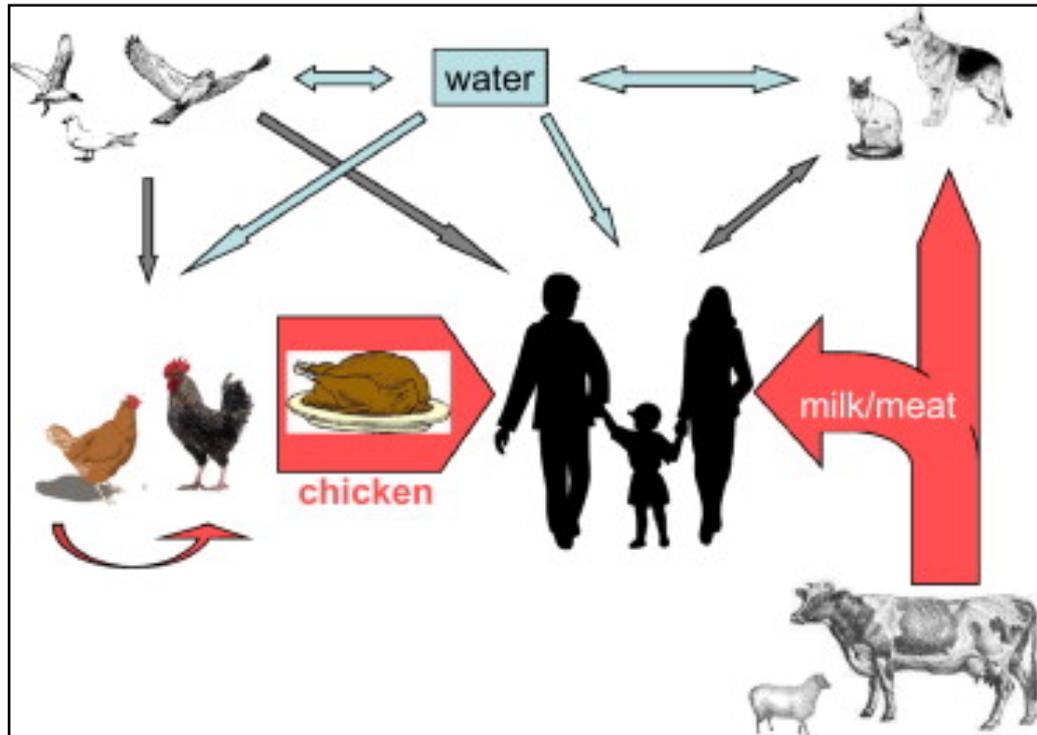


Figure 1.1 Important routes for human infection by *Campylobacter jejuni* (Dasti *et al.*, 2010)

C. jejuni is the main cause of diarrhoeal disease in England and Wales causing a significant economic impact and is one of the leading causes of bacterial gastroenteritis in the world. In the United States, the Centre for Disease Control (CDC) report that *C. jejuni* is estimated to cause 1.3 million infections every year with the risk being highest in those that travel to Africa and South America. The CDC also reports that the microorganism is isolated more frequently in males than in females, predominantly in infants and young adults (CDC 2012).

The Food Standards Agency (FSA) report 460,000 cases, 22,000 hospitalisations and 110 deaths each year due to *Campylobacter*. Many *C. jejuni* infections go unreported and so the actual number of cases is presumed to be considerably higher (Butzler, 2004). The FSA reported 72,571 laboratory confirmed cases of *Campylobacter* in 2012 compared with 72,249 reported in 2011, costing the UK economy an estimated £580 million (FSA). The incidence rate shows an increasing trend over the last 10 years (see Figure 1.2).

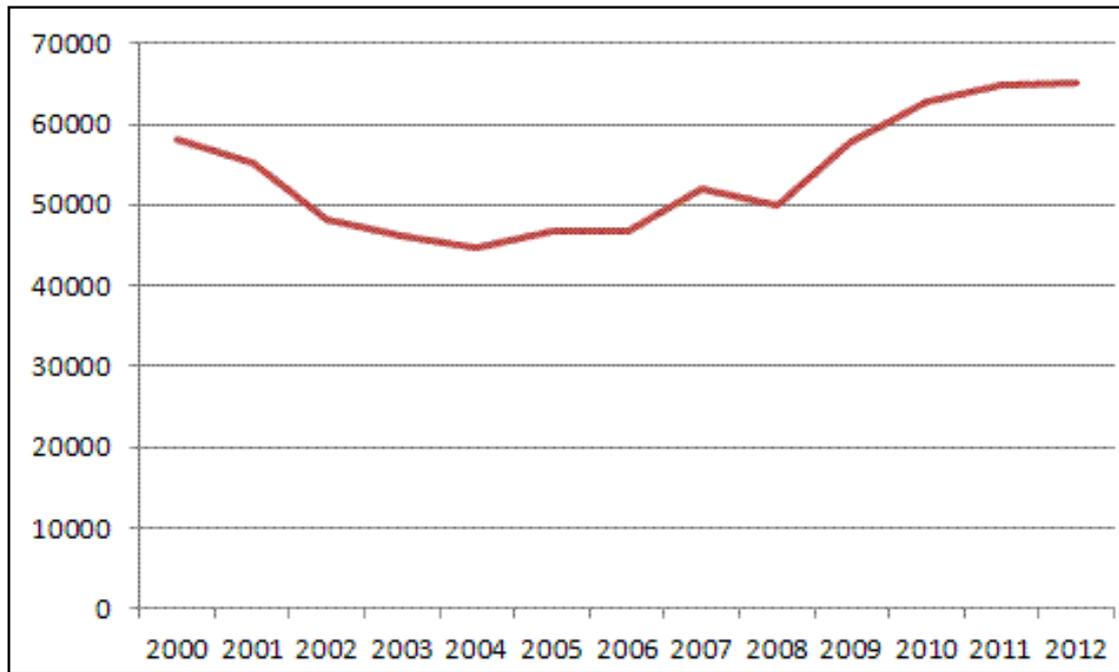


Figure 1.2 Annual incidence of Campylobacteriosis in England and Wales from 2000 to 2012 (PHE 2012).

Campylobacteriosis infections occur at all ages, although peaks are seen in children below the age of four and for patients aged between 15 and 55 years and increasing rates of Campylobacteriosis peak during the summer months, possibly due to an increase in barbeques and water sport activities, although this seasonality of *C. jejuni* infection is not fully understood (PHE 2012). Public Health England (PHE) data from 1989 to 2008 shows that there has also been a decline in infection in young children and an increase in Campylobacteriosis cases in the elderly (see Figure 1.3) (Nichols *et al.*, 2012). Infections have been attributed to the cause of deaths, typically in elderly or immunocompromised patients. (Rodgers and Kagnoff, 1987, Pacanowski *et al.*, 2008).

Campylobacter associated disease is also a problem in developing countries, but data from these sources is scarce (Butzler, 2004). Shared space with animals, lack of sanitation and poor hygiene are thought to be the main cause of Campylobacteriosis in developing countries (Coker *et al.*, 2002). In contrast to the disease presentation in developed countries, where a peak in prevalence is seen in young adults as bloody diarrhoea with mucus (Masanta *et al.*, 2013), *C. jejuni* in developing countries primarily affects children under the age of 5 years old and presents as a watery diarrhoea leading to electrolyte loss (Cooke, 2010), with mortality occurring frequently (Mshana *et al.*, 2009, Senok and Botta, 2009). Frequent exposure to *C. jejuni* in developing countries

provides a protective immunity, demonstrated by the rise in Immunoglobulin A (IgA) with asymptomatic presentation in older children and adults (Mshana *et al.*, 2009).

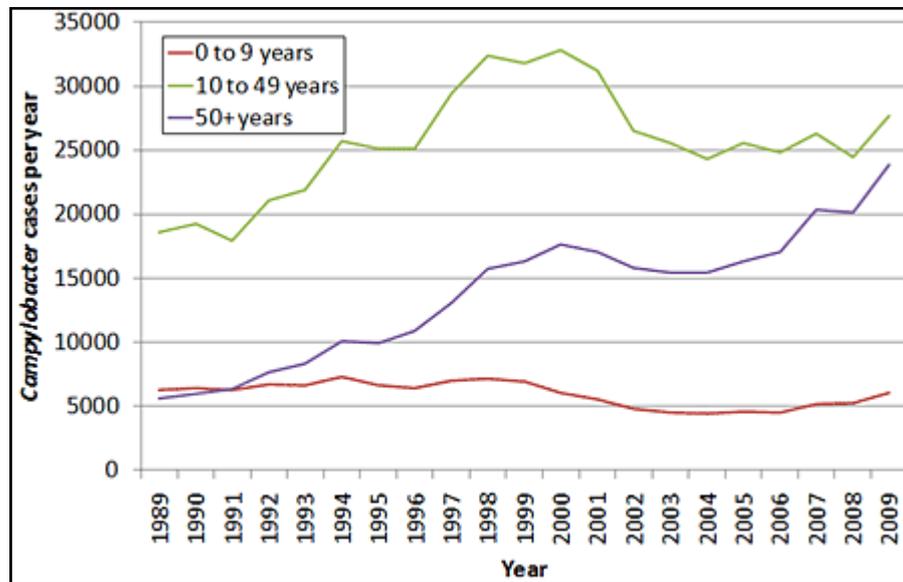


Figure 1.3 Campylobacteriosis incidence reports by age group in England and Wales 1989–2009 (PHE 2012)

1.1.4 Pathogenesis and Symptoms of *Campylobacter jejuni*

Human infection requires ingestion of as few as 100-500 colony forming units (cfu) of *C. jejuni* to cause gastroenteritis (Black *et al.*, 1988, Alter *et al.*, 2011), a low dose in comparison to the 10^3 - 10^8 cfu of *Vibrio cholerae* that are required to be ingested for successful human infection (Schmid-Hempel and Frank, 2007). Similarly, the infectious dose for both *Salmonella* and *E. coli* species is reported to be over 10^5 cfu (Kothary and Babu, 2001). Once the bacterium is ingested, there is a need to overcome host physiological and immunological barriers. A previous study hypothesised that the natural conditions in the human intestine may trigger *C. jejuni* to release virulence factors that could allow disease progression (Malik-Kale *et al.*, 2008). *C. jejuni* colonises the lower sections of the intestinal tract (ileum, jejunum and colon and is thought to adhere to and invade the small intestinal and colonic mucosae (Konkel *et al.*, 2001, Dasti *et al.*, 2010). In most symptomatic cases, Campylobacteriosis is a self-limiting disease and presents with an incubation period of 1–10 days with 1-3 days of prodromal symptoms of fever, vomiting and headaches. By 3-7 days, most people exhibit clinical symptoms (Dasti *et al.*, 2010). Infection with *C. jejuni* may result in a mild, watery, non-inflammatory diarrhoea and abdominal pain or severe bloody,

inflammatory diarrhoea with fever and vomiting (Blaser, 1997). Symptoms however are usually self-limiting and can be treated with antibiotics if required (Blaser, 1997). *C. jejuni* pathogenesis depends on both host- and pathogen-specific factors, including previous infection which provides specific humoral immunity to *C. jejuni* (Blaser, Sazie *et al.* 1987).

1.1.5 The human gastrointestinal tract

The human gastrointestinal tract (GIT) is a complex microbial ecosystem with over 400 bacterial species present at any given time (Hooper *et al.*, 1998). As well as providing a protective natural defence barrier (O'Hara and Shanahan, 2006), the interplay between the organisms encountered at the epithelium surface and the innate immune response protects the host from intestinal bacteria by distinguishing the resident flora from enteric pathogens that may penetrate the host intestinal epithelium and spread into the surrounding tissues (Cebra, 1999).

The human gut epithelium is protected from microbial pathogens by several mechanisms. These include, rapid cell turnover which removes colonised pathogens (Radtke and Clevers, 2005, Chichlowski and Hale, 2008), rapid cell exfoliation, presence of tight junctions, providing strong structural integrity of the epithelial cell barrier. The competitive colonisation of the epithelium by the commensal microbiota provides protection against potential bacterial pathogens and the innate defence mechanisms which maintain the host immune tolerance as well as the production of a mucus layer (Hoffmann, 2003, Leser and Molbak, 2009, Neish, 2009, Sansonetti and Medzhitov, 2009, Kim *et al.*, 2010). The importance of intact epithelial surfaces is as a highly effective barrier against invasion by pathogens (Webb and Kahler, 2008). The ability to breach these structures is an important characteristic for numerous bacterial pathogens (Hornef *et al.*, 2002).

The main aims of pathogenic bacteria is to gain access to the host, avoid or subvert the host innate defences, evade acquired specific immune responses, multiply and persist to cause tissue damage or disease and finally to transmit to new hosts (Falkow, 1996). Numerous bacterial pathogens have evolved highly specialised strategies to enable them to subvert the host cell, hijack the cellular machinery in order to gain access and possess

adaptive intracellular survival systems to avoid the host defence mechanisms (Kim *et al.*, 2010).

1.1.6 Post-infectious sequelae of *Campylobacter jejuni*

In most cases of Campylobacteriosis, the disease manifestations are self-limiting, however *C. jejuni*-mediated enteritis has been linked to bacteraemia which is rare in healthy individuals and mainly observed in patients that present with immunocompromised conditions such as liver cirrhosis or AIDS (Pigrau *et al.*, 1997). *Campylobacter* enteritis has also been associated with the development of inflammatory bowel disease (Garcia Rodriguez *et al.*, 2006). The bacterium has been linked to a number of more serious post-infectious sequelae that are rare but include polyarthralgia and a serious neurodegenerative disease Guillain-Barré Syndrome (GBS), which can cause axonal degeneration leading to paralysis (Nachamkin, Allos *et al.* 1998). It is hypothesised that molecular mimicry of human gangliosides in the peripheral nervous system by *C. jejuni* lipo-oligosaccharide (LOS) induces cross-reactive antibodies that lead to GBS in approximately 1-2 cases per 1000 infections (Prendergast *et al.*, 1998, Godschalk *et al.*, 2004, Heikema *et al.*, 2010). GBS and Miller Fisher Syndrome (which presents as a paralysis more localised in the nerves of the head and neck) are recognised post-infectious sequelae of *C. jejuni* infection (Rees *et al.*, 1995, Jacobs *et al.*, 1998, Nachamkin *et al.*, 2007, Islam *et al.*, 2010).

1.2 Models Used to Study *Campylobacter jejuni* Pathogenesis

Despite the prevalence of *C. jejuni* as a major cause of gastroenteritis, our knowledge of how this bacterium causes disease, interaction with intestinal epithelial cells (IECs), how the bacterium causes diarrhoea and survival in the environment is still limited and lagging far behind our knowledge of other enteropathogens. (Young *et al.*, 2007). One of the biggest hurdles in understanding the disease process is the lack of a convenient small animal model, if available, would allow us to accurately observe the interactions between *C. jejuni* and the host and efficiently reproduce human-like disease (Dorrell and Wren, 2007). Ethical considerations prevent human studies due to the risk of volunteers developing GBS and standard *in vitro* infection models are of limited use (Friis, Pin *et al.* 2005, Dorrell and Wren 2007). Current models used to study *C. jejuni* are described in the following sections.

1.2.1 *in vivo* models used to study *C. jejuni*

To gain a better understanding of *C. jejuni* interactions with the human host, numerous animal models have been employed with minimal success and often with major limitations (Chang and Miller, 2006). Chicks (Beery *et al.*, 1988), non-human primates (Russell *et al.*, 1989), canine pups (Macartney *et al.*, 1988), mice, ferrets (Bell and Manning, 1991, Yao *et al.*, 1997) and newborn piglets (Babakhani *et al.*, 1993) have all been challenged experimentally with *C. jejuni* in an attempt to replicate human disease (Newell, 2001). However the lack of reproducible data, as well as the high cost and maintenance of animals for such studies has hampered progress in this area (Chang and Miller, 2006). There has been some successful representation of disease using ferret and rhesus monkey experimental models, reproducing a human-like Campylobacteriosis disease (Russell *et al.*, 1989, Bell and Manning, 1991). Using these models, researchers observed penetration of the intestinal mucosa by intracellular invasion, with inflammation, bacteraemia and infiltration of the lamina propria by neutrophils, suggesting *C. jejuni* invasion to be an important pathogenic mechanism during infection (Harvey, Battle *et al.* 1999). However these models are not widely available, require special facilities and the unavailability of genetic manipulation procedures often makes these models unsuitable for *C. jejuni* pathogenesis studies (Newell, 2001, Mansfield *et al.*, 2008, Mills *et al.*, 2012).

The chick model is a relatively inexpensive model and has been used extensively in *C. jejuni* studies (Hendrixson and DiRita, 2004, Flanagan *et al.*, 2009). However experimental colonisation of the chick model with *C. jejuni* does not reproduce typical disease patterns as seen in humans, despite the fact that *C. jejuni* can colonise chicken caeca in numbers exceeding 10^{10} cfu per gram of caecal contents (Clench, 1995). Chicken intestinal mucus has been proposed to play a role in inhibition of the bacteria, indicating that the molecular makeup of the mucus may allow the high levels of colonisation but without infection in chickens (Nachamkin *et al.*, 1993). However these models do not inform us about the course of human disease. The most favourable model that mimics some aspects of human disease is the mouse model, where the small size, wide availability, relatively low costs and ease of maintenance has led to the use of this model in *C. jejuni* experimentation studies for well over 30 years (Blaser *et al.*, 1983a), providing some useful immunological data (Wassenaar and Blaser, 1999). Studies using

Nuclear Factor- κ B (NF- κ B) knockout mouse models reported that when the *C. jejuni* 81-176 wild-type strain and a Cytolethal Distending Toxin (CDT) mutant were inoculated into NF- κ B deficient and C57BL/129 (wild-type) mice, the wild-type strain produced severe gastritis and proximal duodenitis in C57BL/129 mice and with significantly more severe gastrointestinal lesions than those caused by the CDT mutant, whilst persistent colonisation of NF- κ B deficient mice with both the wild-type strain and the CDT mutant was associated with significantly impaired Immunoglobulin G (IgG) and the subclass Immunoglobulin G2a (IgG2a) humoral responses. This data correlates with defects in the innate or adaptive immune system, suggesting that the mechanism of clearance of *C. jejuni* is NF- κ B dependent and that CDT exhibits proinflammatory activity *in vivo* (Fox *et al.*, 2004). Subsequent studies found that *C. jejuni* was unable to colonise adult immunocompetent mice, but was able to colonise mice deficient in myeloid differentiation factor 88 (MyD88), an adaptor protein necessary for cell signalling through the Toll-like receptors (TLR). Virulence gene mutants were unable to colonise MyD88 mice and the researchers also reported that mouse models deficient in a key integral membrane protein termed Natural resistance-associated macrophage protein 1 (N-ramp1) exhibited increased susceptibility to *C. jejuni* (Watson *et al.*, 2007). N-ramp1 is expressed by professional phagocytes and is reported to play an important role in the capacity of the host cells ability to control intracellular invasion and survival of pathogens by limiting access to divalent cations in the host cell such as magnesium and calcium (Marshall and Piddock, 1994, Gruenheid and Gros, 2000).

Both mammalian and bacterial transporters may compete for these cations in the phagosomal space, so N-ramp1 is thought to function by interfering with intracellular microbial replication, therefore affecting the survival of intracellular pathogens (Gruenheid and Gros, 2000). Champion *et al.* reported a significant reduction in *C. jejuni* colonisation in N-ramp1 positive mice, whilst N-ramp1 negative mice showed no significant reduction in *C. jejuni* colonisation, however the mice exhibited chronic inflammatory responses and some histopathological lesions (Champion *et al.*, 2008). Collectively, MyD88 and N-ramp1 mouse models have been shown to be a useful tool in *C. jejuni* pathogenesis studies (Watson *et al.*, 2007, Champion *et al.*, 2008). Another mouse model is the interleukin-10 (IL-10) knock out mouse model (Mansfield *et al.*, 2007). IL-10 is secreted by Th2 cells (a sub-set of T cells) (Fiorentino *et al.*, 1989) and

is essential in the activation of B-cells for successful antibody-mediated responses against pathogens and acts as regulatory cytokine with important functions in macrophages or mononuclear cells (Moore *et al.*, 1993). Expression of IL-10 in both human and mouse cells is thought to be critical in the inflammatory response as IL-10 inhibits pro-inflammatory chemokines and is also thought to be of importance in the immune response to normal enteric flora antigens (Kuhn *et al.*, 1993, Moore *et al.*, 2001). Studies by Mansfield *et al.* reported that, the *C. jejuni* 11168 wild-type strain colonised the GIT tract of IL-10-deficient (IL-10-negative) mice at a higher level than the wild-type mouse (C57BL), with the IL-10-negative mice developing clinical signs and pathology such as a severe typhlocolitis. There was also a significant increase in circulating anti-*C. jejuni* plasma immunoglobulin levels in both wild-type and IL-10-negative mice. This increased *C. jejuni* wild-type colonisation of IL-10-negative mice, due to altered IL-10 immunity, indicates the use of C57BL/6 IL-10-positive and IL-10-negative mice as potential disease study models for *C. jejuni* pathogenesis in humans (Mansfield *et al.*, 2007).

However the results obtained using these models have been known to exhibit variability in experimental findings, due to the differences in the species of mice used, inoculation methods and variation in bacterial cfu loads have produced disease severity levels, ranging from asymptomatic to severe diarrhoea (Yrios and Balish, 1985, Stanfield *et al.*, 1987, Baqar *et al.*, 1996). Despite these issues, there has been significant progress with this model, however there are still many questions concerning *C. jejuni* pathogenesis yet to be answered and so the availability of a consistent, reliable, reproducible small animal model is still to be achieved.

1.2.2 *Galleria mellonella* model of *C. jejuni* infection

Due to the lack of a convenient small animal model to study *C. jejuni* pathogenesis and the potential virulence determinants involved in disease progression, researchers have used alternative models involving invertebrate hosts for infection (Champion *et al.*, 2010). *Caenorhabditis elegans* has been used as model for a variety of bacterial pathogens, including *Staphylococcus aureus*, *Burkholderia* species, *Salmonella enterica* Typhimurium, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Yersinia pestis* (Darby, 2005, Peleg *et al.*, 2009). However, one disadvantage of models such as *C. elegans* is that they are unable to survive at 37°C and also lack specific components of

the mammalian immune response such as phagocytic cells (Mylonakis *et al.*, 2007). One advantage of insect models such as *Galleria mellonella* larvae is that they can survive at 37°C. This insect model also possesses specialised structures called haemocytes that behave in a similar way to mammalian phagocytes (Mylonakis *et al.*, 2007, Champion *et al.*, 2010). These haemocytes have the ability to phagocytose microbial pathogens, are capable of releasing antimicrobial peptides and produce reactive oxygen species (Lavine and Strand, 2002, Bergin *et al.*, 2005). Studies by Champion *et al.* found that the *G. mellonella* insect larvae model could be successfully infected with *C. jejuni* to study potential virulence factors (Champion *et al.*, 2010). *C. jejuni* can survive within *G. mellonella* and cause damage to the gut of these larvae (Senior *et al.*, 2011). There are other advantages of using *G. mellonella* larvae as an infection model. The larvae are commercially available and bred as live food for domestic reptiles. The larvae are small, around 1–2 cm long, allowing ease of handling and if kept under the right conditions, can survive for up to 3 weeks before pupating. Upkeep is minimal, no feeding is required and the larvae require minimal maintenance. In contrast to other invertebrate models of infection, *G. mellonella* larvae can be injected with precise amounts of bacteria (Champion *et al.*, 2010). The *G. mellonella* response to infection produces a melanisation, changing the colour of the larvae from cream to dark brown or black. The exact cause of this discoloration is not fully understood but seems to be in response to the injection of the bacteria (Nappi and Christensen, 2005). The ease of use of *G. mellonella* larvae as a model of infection for *C. jejuni* virulence, with the ability to survive at 37°C, indicate that this is an ideal model for screening *C. jejuni* wild-type strains and mutants.

1.2.3 *in vitro* Models Used to Study *Campylobacter jejuni*

The lack of availability of a convenient small animal model has led researchers to use alternative means to study *C. jejuni* pathogenesis, particularly tissue culture cells which have provided most of the data with regards to *C. jejuni* interactions with host cells *in vitro* (Friis *et al.*, 2005). Tissue culture studies investigating host pathogen interactions often utilise cells that are not polarised, these cell lines have been used to study the interactions of bacteria, the triggering of host cell signalling pathways and the methods bacteria utilise to internalise into the host cell (Everest *et al.*, 1992). The mucosal epithelium in contrast is polarised with distinct apical and basolateral membranes. Both

membranes also display distinct transport roles (Mostov *et al.*, 2000, Snoeck *et al.*, 2005) and in addition, the presence of particular surface structures such as Toll-like receptors (Backhed and Hornef, 2003). Therefore, polarised cell lines are more suitable for studying bacterial interactions with host cells, cell invasion and host cell permeability (McCormick, 2003).

1.2.4 Cell Line Models

C. jejuni has been reported to attach to and invade a number of cell lines *in vitro*, both polarised and non-polarised intestinal epithelial cells (IECs) such as Caco-2 IECs (Everest *et al.*, 1992), T84 IECs (Monteville and Konkel, 2002) and INT407 IECs (Konkel *et al.*, 1992b). However, variation in the levels of *C. jejuni* adhesion and invasion reported for each individual cell line is high and the levels of invasion of *C. jejuni* strains are lower than those observed for other enteropathogenic bacteria (Friis *et al.*, 2005). A possible reason for the low numbers of interacting and invading *C. jejuni* is that *in vitro* tissue culture studies are usually performed in a CO₂ incubator under atmospheric O₂ conditions, due to the oxygen requirements of the IECs (Mills *et al.*, 2012). Despite a number of oxidative stress responses in *C. jejuni* including superoxide-dismutase B (SodB) and catalase A (KatA) (Purdy *et al.*, 1999, Day *et al.*, 2000) which enable *C. jejuni* survival under higher oxygen conditions, these experimental conditions probably alter the behaviour of the bacteria during interactions with tissue culture cells.

Microscopic observations have demonstrated translocation of *C. jejuni* both through and between cultured epithelial cells (Harvey, Battle *et al.* 1999) and in some cases identified *C. jejuni* localised in between junctional spaces (Konkel, Mead *et al.* 1992). *C. jejuni* invasion thus seems to employ transcellular (translocation through enterocytes / M cells) and paracellular routes (in between adjacent epithelial cells), however the relative importance of these different routes has yet to be elucidated (Kopecko, 2008).

Numerous cell culture systems, such as HEp-2 and INT407, have been employed widely to study disease mechanisms of *Campylobacter* species (De Melo, Gabbiani *et al.* 1989). In an effort to understand host-pathogen interactions, these different cell types are useful research tools, but as these cells of epithelial origin are non-polarised, the infection pattern may not be truly representative of enteric bacterial infection. However, important information has been obtained on bacterial virulence, cell signalling pathways

and ultimately internalisation of the pathogen into the host cell by the experimental use of non-polarised cell lines (Everest, Goossens *et al.* 1992, Elsinghorst 1994). The enterocyte-like Caco-2 (human colon adenocarcinoma) and T84 (human colonic carcinoma) cell lines are more appropriate to use in interaction (adhesion and invasion) and invasion assays with *C. jejuni* as these cell lines are more representative of the *in vivo* situation, in both makeup and function (Grant, Konkel *et al.* 1993). Polarised IECs produce distinct apical and basolateral surfaces through the process of differentiation; these cells possess microvilli, tight junctions, brush borders, organised actin cytoskeletons and distinct membrane domains which are involved in cell movement, division and transport of molecules in and out of the cell and are important characteristics of IECs (Grant *et al.*, 1993, Schuck and Simons, 2004, Snoeck *et al.*, 2005, Laukoetter *et al.*, 2008).

Polarised cell models are extremely useful for analysing the effects of microbes on the permeability of host cells, transcytosis and invasion of the host cell (McCormick 2003). These cells form polarised monolayers when grown *in vitro* in tissue culture flasks, thereby mimicking the epithelium and layout of the human intestine that is encountered by the enteric pathogens. Previous bacterial invasion studies including those on *Campylobacter* species have used Caco-2 cells grown on non-permeable tissue wells (Conte, Longhi *et al.* 1996). Despite previous studies demonstrating the ability of *C. jejuni* to translocate across Caco-2 cell monolayers (Ketley 1995), the mechanism *C. jejuni* employs to invade these cells, or the relevance of *C. jejuni* translocation paracellularly into host cells and the eventual epithelial penetration and invasion of host cells is not fully understood (O. Croinin and Backert, 2012). Previous studies have found that for *C. jejuni* to cause enteritis, the organism has to enter and invade host epithelial mucosa (Russell and Blake 1994, Allos 1997).

The numbers of invading *C. jejuni* into IECs can be quantified by using the aminoglycoside antibiotic gentamicin (Elsinghorst, 1994). IECs are co-cultured with bacteria for the desired time period, followed by washing of the cells to remove any unattached bacteria. The intestinal epithelial cells are then incubated with gentamicin. The gentamicin protection assay works on the premise that gentamicin has minimal ability to enter eukaryotic cells, but kills extracellular bacteria whilst the internalised bacteria are protected by the host cell (Backert and Hofreuter, 2013). Using this method,

the number of adhered and invading bacteria can be quantified and the same procedure can be used for studying the behaviour of the bacteria following extended incubation as with intracellular survival studies (Friis *et al.*, 2005). The gentamicin protection assay however has resulted in varying results for *C. jejuni* adherence and invasion studies, depending on the strain (Harvey *et al.*, 1999, Biswas *et al.*, 2000), the type and age of IECs and the length of incubation time (Fauchere *et al.*, 1986, De Melo *et al.*, 1989).

1.2.5 The Vertical Diffusion Chamber Model

The levels of interaction with and invasion of IECs for *C. jejuni* are considerably lower than those observed for other enteric pathogens (Friis *et al.*, 2005). With invasion levels of only around 1% of the co-cultured bacteria recovered following gentamicin treatment (Friis *et al.*, 2005). This low level is not a true reflection of *C. jejuni* invasion and fails to correlate with the clinical presentation of *C. jejuni* infection observed in humans (Friis *et al.*, 2005). A major limitation of *in vitro* models used to study gastrointestinal infections is that the culture conditions including high oxygen levels generally favour eukaryotic cell survival. However conditions in the intestinal lumen are almost anaerobic (Blaut and Clavel, 2007). Pathogens under very low oxygen conditions express virulence genes whose expression changes under aerobic conditions (Marteyn *et al.*, 2010). As such, data obtained using standard cell culture models may give an inaccurate indication of bacterial interactions with host cells. The use of a Vertical Diffusion Chamber (VDC) system (see Figure 1.4) has been developed which permits the co-culture of bacteria and host cells under different medium and gas conditions (Cottet *et al.*, 2002a, Schuller and Phillips, 2010, Mills *et al.*, 2012). The VDC system is advantageous as it closely mimics the conditions present in the human intestine, where bacteria will be under conditions of considerably low oxygen tension whilst the tissue will be supplied with oxygen from the blood.

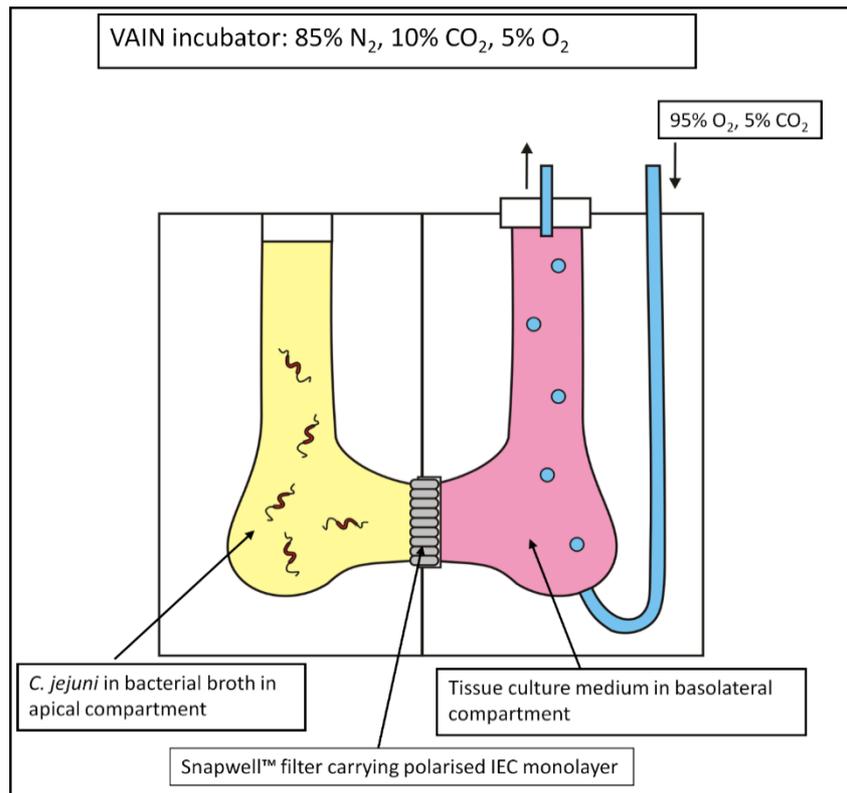


Figure 1.4 The Vertical Diffusion Chamber Model. Polarised IEC monolayers grown in Snapwell™ inserts were placed into a VDC creating an apical and basolateral compartment, which were individually filled with bacterial broth and cell culture medium respectively (Naz *et al.*, 2013).

The VDC has been successfully used previously with *Helicobacter pylori* and resulted in both increased bacterial adhesion and an increased expression of the virulence factor cytotoxin-associated gene-A antigen (CagA) (Covacci *et al.*, 1993, Cottet *et al.*, 2002b). Similar studies reported that co-culturing of enterohaemorrhagic *E. coli* (EHEC) with IECs displayed increased bacterial adhesion with IECs using the VDC system (Schuller and Phillips, 2010), indicating that the behaviour of non-microaerobic bacteria was also variable when co-cultured with IECs under either microaerobic or anaerobic conditions. Thus, the VDC model is an improved and very useful model for analysis of the host-pathogen interaction. Data obtained by Mills *et al.* using the VDC showed significantly increased interactions with and invasion of IECs by *C. jejuni* 11168H and 81-176 wild-type strains and this was mirrored with an equally significant increase in the induction of the pro-inflammatory cytokine Interleukin-8 (IL-8), indicating the importance of low oxygen conditions in the apical compartment of the VDC for *C. jejuni* interactions with host cells (Mills *et al.*, 2012).

1.3 Molecular basis of *Campylobacter jejuni* pathogenesis

Once ingested, *C. jejuni* utilises numerous bacterial virulence determinants which are thought to contribute to the pathogenesis of this microorganism. Numerous virulence determinants including, flagella and motility, have been shown to be required for the attachment to and colonisation of the host intestinal epithelium (Guerry, 2007, O. Croinin and Backert, 2012). Adherence mechanisms utilised by microorganisms typically consist of adhesins, often appendages such as the pili and/or flagella that are found on the surface of many Gram-negative and Gram-positive species (see Figure 1.5) (Pizarro-Cerda and Cossart, 2006). These will be discussed below.

Bacterial factor	Proposed function	Applied experimental methods	Strains used	Cell system used	Reference
AspA, AspB	Aspartate ammonia lyase and amino transferase	Infection <i>in vitro</i> , GPA, ISA	81-176	T84	Novik et al. (2010)
→ CadF	Adhesin to fibronectin	Infection <i>in vitro</i> and in chickens, GPA, FBA, CBA, ELISA	F38011, 11168, 81-176	INT-407, T84, LMH	Konkel et al. (1997), Ziprin et al. (1999), Monteville et al. (2003), Scott et al. (2010)
CapA	Adhesin/Invasin	Infection <i>in vitro</i> and chickens, GPA	11168, F38011	Caco-2, LMH	Ashgar et al. (2007), Flanagan et al. (2009)
CDT	Cytolethal distending toxin	Treatment of cells <i>in vitro</i> , MI, FACS, IFM, DNase assays	81-176	COS-1, REF52, Henle-407	Lara-Tejero and Galán (2000)
→ CiaB, CiaC	Invasin	Infection <i>in vitro</i> , GPA, MLA, T3SS assays	F38011, 11168	INT-407	Konkel et al. (1999), Christensen et al. (2009)
→ CiaI	Intracellular survival	Infection <i>in vitro</i> , GPA, MLA, IFM	F38011	INT-407, HeLa	Buelow et al. (2011)
CJ0977 ^a	Invasion	Infection <i>in vitro</i> and ferrets, EM, MA, GPA	81-176	INT-407	Goon et al. (2006)
CstII	LOS sialylation	Infection <i>in vitro</i> , GPA	GB2, GB11, GB19	Caco-2, T84	Louwen et al. (2008)
FlaC	Invasin	Infection <i>in vitro</i> , MA, EM, cell fractionation, GPA, IFM	TGH9011	HEp-2	Song et al. (2004)
→ FlpA	Adhesin to fibronectin	Infection <i>in vitro</i> and in chickens, FBA, ABB, GPA, IFM, ELISA	F38011	INT-407, HeLa, LMH	Flanagan et al. (2009), Konkel et al. (2010), Eucker and Konkel (2012)
FspA	Apoptosis	BRP, AA	81-176, CG8486	INT-407	Poly et al. (2007)
GGT	Gamma-glutamyl transpeptidase	Infection <i>in vitro</i> , mice and chickens, cell fractionation, MA, GPA, HPS, AA	RM1221, 81-176, 81116, 11168	INT-407, CCD841 CoN	Hofreuter et al. (2006, 2008), Barnes et al. (2007)
HtrA	Periplasmic protease and chaperone	Infection <i>in vitro</i> , GPA	11168	INT-407	Baek et al. (2011)
JlpA ^b	Adhesion to HSP90- α , proinflammatory responses	Infection <i>in vitro</i> , GPA, BRP, ABB, ligand overlays, geldanamycin inhibitor, p38/NF κ B activation using AABs	TGH9011	HEp-2	Jin et al. (2001), Jin et al. (2003)
KpsE, KpsM, KpsT	Capsule proteins, invasion	Infection <i>in vitro</i> , infection of chicken and ferrets, GPA	81-176, 81116	INT-407	Bacon et al. (2001), Bachtiar et al. (2007)
Peb1, Peb3, and Peb4 ^c	Transport proteins and chaperones	Infection <i>in vitro</i> and in mice, BRP, GPA	81-176, 11168	HeLa, INT-407	Leon-Kempis Mdel et al. (2006), Min et al. (2009), Asakura et al. (2007), Kale et al. (2011)
PfIA	Motility	Infection <i>in vitro</i> , GPA	81-176	INT-407	Yao et al. (1994)
PorA (MOMP)	Major outer membrane protein	Infection <i>in vitro</i> , BRP	K22, 1767	INT-407	Schröder and Moser (1997), Moser et al. (1997)
pVIR	Invasion	Infection <i>in vitro</i> and in ferrets, GPA	81-176, VC83	INT-407	Bacon et al. (2000)
SodB	Superoxide dismutase	Infection <i>in vitro</i> and in mice, GPA, ISA	81-176	T84	Novik et al. (2010)
VirK	Intracellular survival	Infection <i>in vitro</i> and in mice, GPA, IFM	81-176	T84, COS-7	Novik et al. (2009)

AA, apoptosis assay; AB, antibody; AAB, activation-specific antibodies; ABB, antibody blocking; BRP, binding assays using recombinant or purified protein; CBA, competitive binding assay; EM, electron microscopy; FACS, fluorescence-activated cell sorting; FBA, fibronectin binding assay; GPA, gentamicin protection assay; HPS, hydrogen peroxide susceptibility test; IFM, immunofluorescence microscopy; ISA, intracellular survival assay; MI, microinjection of proteins; MA, motility assay on agar; MLA, ³⁵S-methionine labeling assay; pVIR, mobilizable plasmid in some strains; T3SS assays, translocation assay using the *Yersinia flagellar type-III secretion apparatus*.

^aAnother report indicated that the CJ0097 mutant has a deficiency in motility in liquid broth (Novik et al., 2010).

^bIdentified as an adhesin in strain TGH9011, but no effect observed with *jlpA* mutants in either 11168 or 81-176 strains (van Alphen et al., 2008; Novik et al., 2010).

^cThese structural studies along with other assays suggest primary roles in protein transport. Originally, the Peb's were identified as putative adhesins (Pei et al., 1991).

Figure 1.5 Current list of potential *C. jejuni* virulence factors. *C. jejuni* bacterial virulence factors with arrows indicating possible roles of CadF, FlpA, CiaB, CiaC and CiaI in pathogenesis (O. Croinin and Backert, 2012).

1.3.1 *C. jejuni* Flagellum

C. jejuni contains one or two polar flagella which endow the bacterium with efficient motility and enable the bacterium to colonise a host by penetrating the mucous layer (Morooka *et al.*, 1985, Guerry, 2007, Young *et al.*, 2007). Furthermore, flagella play a role in secretion and in host-cell invasion where the bacteria require close contact to enter cells (Newell *et al.*, 1985, Black *et al.*, 1988, Wassenaar *et al.*, 1991) as has been demonstrated in the chick model (Barrero-Tobon and Hendrixson, 2012). Early studies on *C. jejuni* motility revealed not only that motility was required for attachment to and invasion of IECs (Malik-Kale *et al.*, 2008, Dasti *et al.*, 2010) but that the adhesion and invasive ability of *C. jejuni* was increased when mucosal viscosity was increased (Morooka *et al.*, 1985, Grant *et al.*, 1993, Szymanski *et al.*, 1995).

C. jejuni motility is regulated by chemotaxis, which allows the bacterium to swim towards favourable conditions and away from unfavourable ones (Lertsethtakarn *et al.*, 2011). A *C. jejuni* flagellum contains three distinct sections: the basal body, the hook and the filament. The basal body anchors the flagella to the bacterial cell envelope, the filament protrudes from the bacterial surface and the hook transfers energy between the two (Konkel *et al.*, 2004, Guerry, 2007). The filament section is made up of two proteins, a major flagellin FlaA and a minor flagellin FlaB, with an estimated size of ~59 kDa (Guerry *et al.*, 1991). The genes which encode the proteins responsible for the structural assembly of the flagella are transcribed in the order required for flagella assembly (Chevance and Hughes, 2008). This order is conserved in numerous species of bacteria including *P. aeruginosa*, *H. pylori* and *V. cholera* (Totten *et al.*, 1990, Klose and Mekalanos, 1998, Spohn and Scarlato, 1999). The two flagellin genes *flaA* and *flaB* present in *C. jejuni* are almost identical (Nuijten *et al.*, 1990). The regulation of transcription of *flaA* and *flaB* genes requires σ^{28} (FliA) and σ^{54} (RpoN) respectively. In *C. jejuni*, these two alternative σ factors control the expression of the flagellar genes that encode components of the flagellar organelle (Hendrixson *et al.*, 2001). σ^{54} is required for the expression of FlaB as well as the flagellar rod and hook genes, while σ^{28} is required for the expression of *flaA*, which encodes the major flagellin and other filament genes (Hendrixson and DiRita, 2003, Carrillo *et al.*, 2004, Wosten *et al.*, 2004). The Type III secretion (T3SS) components of the flagellar include FlhA, FlhB, FliP and FliR. FlgRS, is a two-component regulatory system (a sensor protein and a

response regulator protein) (Hendrixson and DiRita, 2003). The FlhF GTPase is another regulator that initiates transcription by σ^{54} RNA polymerase holo-enzyme (Hendrixson and DiRita, 2003, Balaban *et al.*, 2009, Boll and Hendrixson, 2011). Two component regulatory systems are essential for the detection and transduction of the signals that facilitate linking of external and internal stimuli to correct certain behavioural responses such as gene expression (Gao and Stock, 2009). Current models suggests that the cytoplasmic FlgS histidine kinase autophosphorylates due to a stimulatory signal from the flagellar T3SS which then leads to activation of the FlgR response regulator for σ^{54} -dependent expression of flagellar rod and hook genes (Joslin and Hendrixson, 2009). As with other motile bacteria, the activity of σ^{28} in *C. jejuni* is suppressed by FlgM (an anti- σ factor) until the flagellar rod and hook biosynthesis is complete (Wösten *et al.*, 2010). Following rod and hook formation, FlgM is secreted from the cytoplasm to release σ^{28} from transcriptional suppression, thereby resulting in *flaA* expression which is required for filament synthesis (Balaban *et al.*, 2009, Barrero-Tobon and Hendrixson, 2012). The FlgSR and σ^{54} are also required for expression of the Fed proteins, which are co-expressed with flagellar proteins but are not required for motility. Five Fed proteins have been identified as necessary for avian colonisation: FedA, FedB, FedC, FedD, and CiaI. Recently one of these proteins, CiaI, was shown to be required for invasion of human intestinal epithelial cells (Buelow *et al.*, 2011a, Barrero-Tobon and Hendrixson, 2012)

1.3.2 *C. jejuni* Glycan structures

Genome sequence analysis of the first *C. jejuni* strain NCTC11168 revealed the surprising ability of the pathogen to produce a diverse array of carbohydrates (Parkhill *et al.*, 2000). These carbohydrate structures include a capsule, LOS, *O*-linked glycosylation of the flagella and a *N*-linked general glycosylation system (Karlyshev *et al.*, 2005b). These glycan structures are found on all bacterial cells and are thought to play an important role in the interaction of bacteria with the host and the environment (Parker *et al.*, 2008, Nizet V, 2009).

1.3.3 Capsule

The capsular polysaccharide (CPS) is on the outer most part of the bacterial cell and CPS has been suggested to play a role in interaction with host cells and in pathogenesis,

avoiding phagocytosis and enhancing bacterial survival. (Szymanski *et al.*, 2003b, Guerry and Szymanski, 2008). CPS was first observed in 1996, but it was not until several years later that the biosynthetic mechanism of CPS was identified (Chart *et al.*, 1996, Karlyshev *et al.*, 2000). CPS is assembled by an ATP binding cassette (ABC) pathway, these comprise a large class of proteins with roles in nutrient uptake, cellular functions and polysaccharides (George and Jones, 2012). *C. jejuni* capsule biosynthesis genes are split into three regions (see Figure 1.6), the first and third regions encode the Kps proteins that are involved in capsular assembly, transport and are highly conserved whilst the second central region is involved in polysaccharide synthesis is variable. This variation forms the basis of the different *C. jejuni* Penner serotypes. This genetic ordering of *C. jejuni* capsular genes is similar to those found in *E. coli* K1, K5, *Haemophilus influenza* and *Neisseria meningitidis* (Guerry *et al.*, 2012).

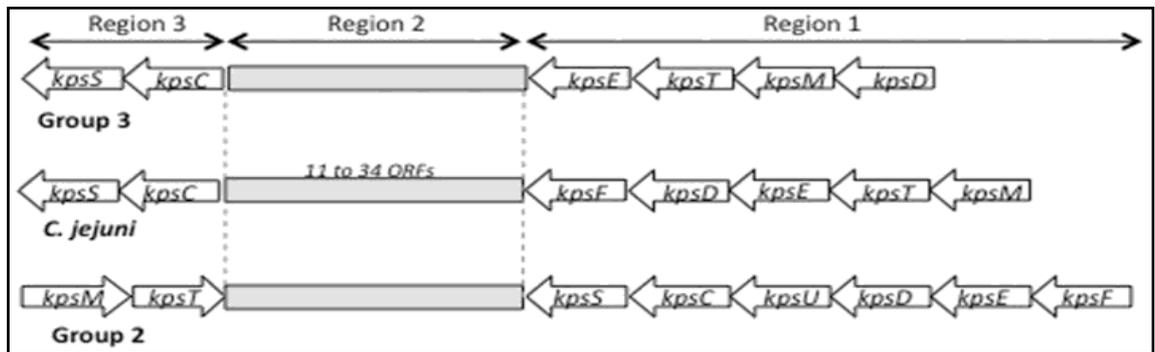


Figure 1.6 Comparison of bacterial capsule organisation genes of *E. coli* groups 2 and 3 and *C. jejuni* (Guerry *et al.*, 2012).

The presence of *C. jejuni* CPS is dependent on a functional *kps* gene cluster and determines the Penner serotype (Karlyshev *et al.*, 2000, Parkhill *et al.*, 2000). KpsE and KpsM are both involved in *C. jejuni* capsule synthesis and mutation of *kpsE* resulted in a complete absence of capsular polysaccharide and although this mutant was able to colonise the chick model, the number of bacteria isolated from the caecum was significantly lower than those observed with the wild-type strain (Bachtiar *et al.*, 2007). Similarly, mutation of *kpsM* also resulted in a loss of the capsule. Bacon *et al.* showed that a 81-176 *kpsM* mutant displayed a significant reduction in the ability to interact with and invade INT407 cells and displayed reduced virulence in the ferret model (Bacon *et al.*, 2001), similar data was obtained with other non-capsular mutants (Corcionivoschi *et al.*, 2009). CPS rather than LOS is the sero-determinant of the Penner serotyping system (Penner and Hennessy, 1980, Karlyshev *et al.*, 2005a). CPS

displays a high level of variation between strains and this variation has been attributed to the presence of different biosynthetic genes and also phase variation of some biosynthetic genes due to slip-strand mispairing (Parkhill *et al.*, 2000, Guerry *et al.*, 2002, Szymanski *et al.*, 2003b).

1.3.4 Lipooligosaccharide

C. jejuni also expresses a LOS core, with the genes encoding proteins involved in the biosynthesis of LOS, this is highly variable and current genotyping has identified 19 different classes (Parker *et al.*, 2005, Parker *et al.*, 2008). The genes involved in the synthesis of lipid A and the inner core are conserved and are the same in all strains (Houliston *et al.*, 2011). Hence, *C. jejuni* LOS displays high levels of variation in the outer core and is important for virulence, epithelial cell adherence and invasion, endows protection from antimicrobials and plays a role in serum resistance (Linton *et al.*, 2000, Parkhill *et al.*, 2000, Naito *et al.*, 2010).

LOS is made up of two distinct structural sections, a hydrophobic lipid A portion that is anchored in the bacterial outer membrane possessing endotoxic activity (Moran, 1997) and a non-repeating oligosaccharide which also has a conserved inner core and a variable outer core region (Parker *et al.*, 2008). *C. jejuni* is capable of endogenously synthesising sialic acid residues for attachment onto the ganglioside-like LOS cores (Haddad *et al.*, 2010). LOS displays a high level of variation due to phase variation of some LOS biosynthetic genes, in particular *cgtA* which encodes an *N*-acetylgalactosaminyltransferase and *wlaN* which encodes a β -1,3 galactosyltransferase (Linton *et al.*, 2000, Guerry *et al.*, 2002). The cluster of genes responsible for the biosynthesis of LOS show a great range of genetic diversity (Gilbert *et al.*, 2004). The LOS separates *C. jejuni* from other enteric Gram-negative bacterial pathogens due to the ability of the bacterium to mimic the structures of human gangliosides present on peripheral nerves. Due to this molecular mimicry, antibodies produced against *C. jejuni* LOS structures cross react with the host gangliosides (Yuki, 1997, Prendergast *et al.*, 1998) this is consistent with a role in immune avoidance and can give rise to autoimmune disorders such as GBS (see Section 1.1.6).

1.3.5 Glycosylation pathways

C. jejuni possesses both *N*- and *O*- linked glycosylation systems (Szymanski *et al.*, 2003a) and these pathways have been attributed to play a role in *C. jejuni* pathogenesis (Karlyshev *et al.*, 2005b, Mahdavi *et al.*, 2014).

1.3.5.1 *O*-linked protein glycosylation

Glycosylation of the *C. jejuni* flagella was first identified in 1989 (Logan *et al.*, 1989) (see Figure 1.7) and reported to be a prerequisite for filament assembly as mutants defective in flagellar glycan synthesis were unable to assemble a fully functional filament (Guerry and Szymanski, 2008). The *O*-linked glycosylation locus of NCTC11168 consists of around 50 genes, with variation in the genetic organisation of the *O*-linked locus between both species and strains. This is evident in a large cluster of genes *Cj1318–Cj1332* which are present in NCTC11168 but are absent in 81-176 (Parkhill *et al.*, 2000, Logan, 2006). *Campylobacter* flagellins are decorated with *O*-linked glycans which modify serine or threonine residues within flagellin and are essential for the production of a functional filament and flagella (Goon *et al.*, 2003, Logan, 2006). Studies have identified up to 19 sites for *O*-linked glycosylation which contributes ~10% of flagellin mass (Guerry and Szymanski, 2008). The 81-176 wild-type strain was found to be heavily glycosylated with pseudaminic acid (Thibault *et al.*, 2001).

The importance of the flagella in the role of motility and functioning as a possible flagellar export apparatus for the secretion of the Cia proteins indicates that the *O*-linked glycosylation pathway is directly related to the organism's motility, autoaggregation, interaction with and invasion of host cells (Guerry *et al.*, 2006). Early studies found that the flagella is the dominant immune antigen on the bacterial surface and mutations in *Cj1331* and *Cj1332* in *C. coli* VC167 (involved in post-translational modification of flagellin) resulted in reduced immunogenicity (Guerry *et al.*, 1996, Logan, 2006). Mutations introduced in the *O*-linked glycosylation locus genes which led to a loss of 5,7-diacetamido-3,5,7,9-tetra-deoxy-L-glycero-L-manno nonulosonic acid (or pseudaminic acid, Pse5Ac7Ac), a nine-carbon sugar that is structurally similar to sialic acid (Neu5Ac) or the acetamidino form of Pse5Ac7Ac (Pse5Am7Ac), led to a reduction in interaction, invasion and reduced virulence in the ferret model (Guerry *et*

al., 2006). This was further confirmed by an inability of non-flagellated mutants to colonise experimental animals (Guerry *et al.*, 1991, Yao *et al.*, 1994). Numerous genes in the flagellar glycosylation locus contain homopolymeric tracts, making these genes susceptible to slipped strand mispairing with subsequent phase variation, resulting in variation of the flagellin glycan structures. This variation in surface exposed flagellar glycan structures has been proposed to enable evasion of the host immune system response (Alm *et al.*, 1992, Karlyshev *et al.*, 2005a). However, the human immune system is unlikely to provide an influential evolutionary selective pressure in the life cycle of *C. jejuni* and more immediate environmental factors within the avian species such as the presence of bacteriophages are likely to be the evolutionary forces for instigating changes in the surface of the *C. jejuni* cell (Scott *et al.*, 2007). The exact role of the *O*-linked modifications on the flagella filament biosynthesis is unclear and requires further investigation (Gilbreath *et al.*, 2011).

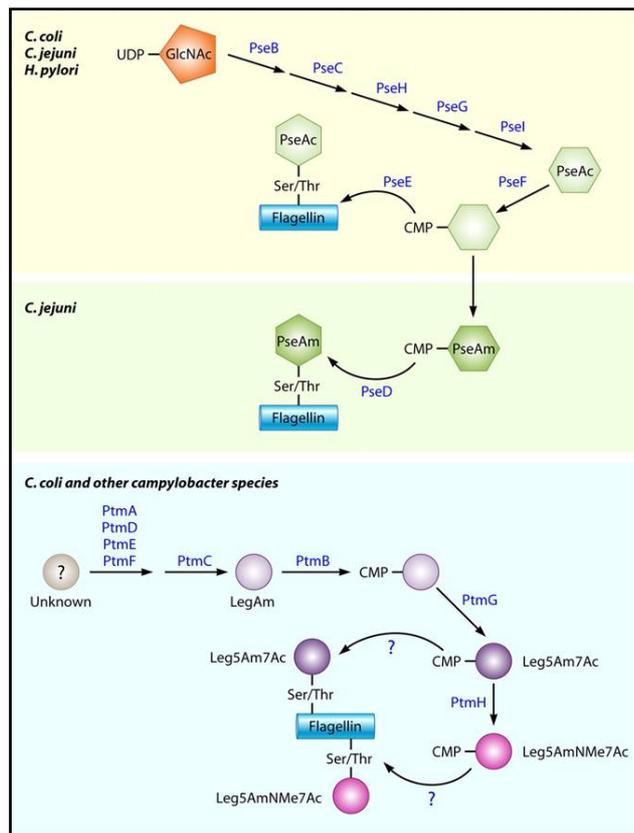


Figure 1.7 O-linked protein glycosylation system. The *O*-linked protein glycosylation systems of *Campylobacter* and *Helicobacter* species. The *pseB* gene encodes the enzyme which converts the starting compound UDP- α -d-GlcNAc to the first intermediate of the PseAc biosynthesis pathway resulting in the production of varying types of glycans linked to flagellin proteins (Gilbreath *et al.*, 2011).

1.3.5.2 *N*-linked protein glycosylation

Prior to the discovery of *N*-linked glycosylation in *C. jejuni*, this type of post-translational modification of proteins had only been previously observed in eukaryotes and archaea (Szymanski *et al.*, 1999). In contrast to *C. jejuni* surface structures such as the capsule, LOS and *O*-linked glycan, the *N*-linked glycan encoded by a 16 kb gene cluster termed the protein glycosylation locus (*pgl*) is highly conserved in all *C. jejuni* strains studied to date (Dorrell *et al.*, 2001, Szymanski *et al.*, 2003b). *N*-linked glycosylation involves an oligosaccharyltransferase (PglB) which enables coupling of a glycan to an asparagine residue forming a *N*-linked glycoprotein (Szymanski *et al.*, 2003a), this type of *N*-linked glycosylation is rare in most bacteria with the exception of *C. jejuni*, *H. influenza* and *H. pullorum* (Szymanski *et al.*, 1999, Linton *et al.*, 2002, Grass *et al.*, 2010, Jarvis *et al.*, 2010). The conserved sequence for *N*-linked glycosylation in *C. jejuni* consists of D/E-X-N-X-S/T (see Figure 1.8) (where N is the modified asparagine and X can be any amino acid except for proline) (Nita-Lazar *et al.*, 2005, Kowarik *et al.*, 2006).

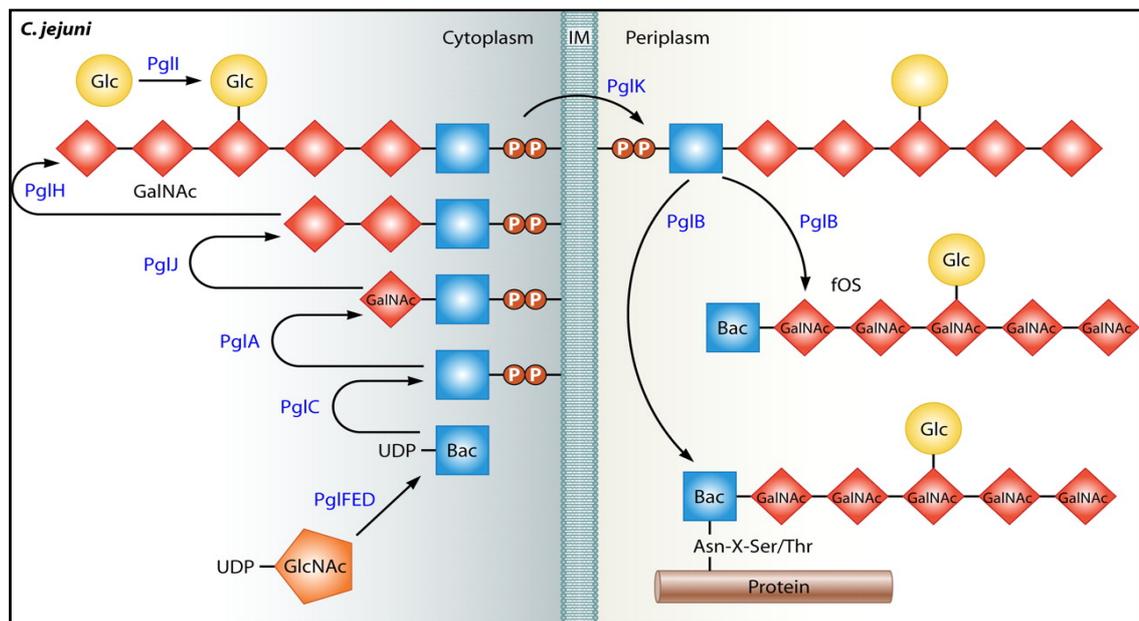


Figure 1.8 *N*-linked protein glycosylation. *N*-linked protein glycosylation pathway in *C. jejuni* begins with UDP-GlcNAc resulting in a heptasaccharide that is attached to a lipid carrier on the cytoplasmic side of the inner membrane (IM). The heptasaccharide is then flipped to the periplasm by PglK, removed from the carrier by PglB and attached to a protein (Gilbreath *et al.*, 2011).

The role of the *N*-linked glycosylation system is the modification of over 100 periplasmic proteins with diverse roles in colonisation, adhesion and invasion

(Szymanski *et al.*, 1999, Dasti *et al.*, 2010, Scott *et al.*, 2012, Alemka *et al.*, 2013). Disruption of *N*-linked glycosylation genes *pglB*, *pglD*, *pglE*, *pglF*, *pglH* or *pglK* has resulted in changes in levels of immune reactivity of specific glycosylated proteins, a reduced capacity of interaction with and invasion of IECs and a reduction in colonisation in the chick and mouse model (Szymanski *et al.*, 1999, Szymanski *et al.*, 2002, Hendrixson and DiRita, 2004, Jones *et al.*, 2004, Karlyshev *et al.*, 2004). In addition, *in vitro* studies by Karlyshev *et al.* and Szymanski *et al.* reported that for the successful adherence and invasion of IECs, the complete PgL system was essential, indicating that *N*-linked glycosylation is required for *C. jejuni* interactions with and invasion of human IEC and avian hosts, however the exact roles have yet to be revealed (Szymanski *et al.*, 2002, Karlyshev *et al.*, 2004).

1.3.6 Cytotoxic distending toxin

CDT is a type of bacterial toxin, that specifically interferes with the eukaryotic cell cycle, due to the presence of nuclease activity that damages the chromosomal DNA (Heywood *et al.*, 2005). CDT causes arrest at the growth period/synthesis (G₁/S) or pre-mitotic phase (G₂/M) transition of the cell cycle, depending on the cell type (Whitehouse, Balbo *et al.* 1998, Lara-Tejero and Galan 2000, Lara-Tejero and Galan 2001). The presence of CDT in *Campylobacter* species was first reported in 1988 where cell distension and cytotoxicity of mammalian cells was observed (Johnson and Lior, 1988). CDTs are produced by a number of Gram-negative bacteria such as *E. coli*, *Helicobacter* species, *Haemophilus ducreyi*, *Shigella dysenteriae*, *Aggregatibacter actinomycetemcomitans* and *Salmonella enterica* serotype Typhimurium (Guerra *et al.*, 2011). CDT is formed from three proteins CdtA, CdtB and CdtC, all required for toxin activity (Jinadasa *et al.*, 2011). The toxic component is CdtB as studies have shown this protein leads to similar cell damage as observed with the holotoxin (Lara-Tejero and Galan, 2000). The action of CdtB is that of a DNase and this subunit shares homology with DNase I-like proteins (Lara-Tejero and Galan, 2001). CdtB is thought to be delivered by the binding proteins CdtA and CdtC into the host cell where CdtB localises with the nucleus, resulting in DNA damage (Lara-Tejero and Galan, 2000). *C. jejuni* CDT has been found to trigger the release of IL-8, but IL-8 can also be triggered in the absence of CDT (Hickey *et al.*, 2000). CDT may possibly play a role in immune

modulation and invasiveness (Purdy *et al.*, 2000). The exact role of CDT in *C. jejuni* pathogenesis is not fully understood and further studies need to be undertaken.

1.4 Host response to *C. jejuni* infection

Despite many *in vitro* studies to investigate the interaction of *C. jejuni* with human immune cells, it is still unclear as to the exact mechanism by which the infection is cleared (Young *et al.*, 2007). Human infection with *C. jejuni* occurs via the oral route following ingestion of contaminated foods (Schildt *et al.*, 2006, Newell *et al.*, 2011). The acidic environment of the stomach kills a significant number of the bacteria, however those that remain able to attack the mechanical and immunological barriers of the GI tract. The mucus layer of the GI tract serves as a first line of defence against the bacteria. *C. jejuni* has specific traits such as motility and a cork screw like morphology that enable the bacterium to over-come this barrier (Young *et al.*, 2007). *C. jejuni* is able to then attach to IECs, colonising the lower intestinal tract (ileum, jejunum, and colon) and begin replication (Janssen *et al.*, 2008). However, the exact mechanisms of how *C. jejuni* causes disease are still poorly understood.

Studies have shown that *C. jejuni* is able to invade cell monolayers (Everest *et al.*, 1992, Fernandez and Trabulsi, 1995). Invasion leads to disruption of the epithelium and allows *C. jejuni* access to the host cell (Walker *et al.*, 1986, Wooldridge and Ketley, 1997). *in vitro* experiments with human IECs have shown that *C. jejuni* can induce the secretion of a range of cytokines and chemokines such as Interleukin-1 β , IL-8 and nitric oxide (NO) (Hickey *et al.*, 1999, Hickey *et al.*, 2000, Al-Salloom *et al.*, 2003, Jin *et al.*, 2003, Enocksson *et al.*, 2004). Following infection with *C. jejuni*, human monocytes produce a number of cytokines and chemokines, including IL-6, IL-8, IL-1 β and tumour necrosis factor alpha (TNF α) and it has been suggested that the activation and production of these host cytokines and chemokines could be responsible for the disease pathology observed (Jones *et al.*, 2003, Siegesmund *et al.*, 2004).

IECs are capable of microbial sensing and producing specific immune responses (Kagnoff and Eckmann, 1997, Eckmann, 2005). The innate immune system is activated when pathogen associated molecular pattern molecules (PAMPS) engage with the pattern recognition receptors (PRRs) found on IECs, dendritic cells (DCs) and macrophages (Lavelle *et al.*, 2009). Of the PRRs, the most widely studied are a group of

integral membrane proteins termed toll-like receptors (TLRs), which are central in recognising specific pathogen ligands such as flagella, LOS and DNA and initiating the immune defence against foreign invaders (Philpott and Girardin, 2004, Janssen *et al.*, 2008, Zilbauer *et al.*, 2008). The activation of TLRs leads to the production of pro-inflammatory cytokines (O'Hara and Shanahan, 2006). Watson and Galan reported that *Campylobacter* flagellin was not recognized by IEC TLR5 and did not play a role in the bacterial-mediated immune response, therefore the role of other TLRs in interaction with *C. jejuni* warranted further study (Watson and Galan, 2005). *C. jejuni* is capable of evading the immune stimulation of TLR5 due to the high level of differences in the amino acid sequences present in the flagellar structure compared to other bacteria (Andersen-Nissen *et al.*, 2005, Watson and Galan, 2008b). Studies on the involvement of *C. jejuni* with a second intracellular member of the PRR family termed nucleotide binding oligomerisation domain (NOD) proteins, showed that NOD1 was triggered and initiated an immune response including the induction of IL-8 and human- β defensin (H β D2). NOD1 knockouts in Caco-2 IECs produced a significant reduction in innate immune activity, with increased intracellular *C. jejuni* observed during this reduced innate immune response. In contrast, studies with NOD2 reported a minimal involvement of this PRR in intestinal epithelial innate immunity (Inohara *et al.*, 2005, Zilbauer *et al.*, 2007).

An important *C. jejuni* virulence factor is the diversity found in the LOS structure. A subdivision of *C. jejuni* strains that express an LOS class A, B, or C gene locus have been found to harbour genes involved in sialic acid biosynthesis and are therefore able to synthesise sialylated LOS (Guerry *et al.*, 2000, Gilbert *et al.*, 2000, Godschalk *et al.*, 2004). The presence of a *cst-II* gene in *C. jejuni* class A and B strains encodes a sialyltransferase which transfers sialic acid onto the LOS core and LOS sialylation of *C. jejuni* class C strains depends on the presence of the *cst-III* gene (Chiu *et al.*, 2004). Thus these *C. jejuni* possess genes encoding proteins involved in sialic acid biosynthesis and are capable of LOS sialylation (Louwen *et al.*, 2008).

LOS sialylation has been found to play an important role in microbial recognition and activation of the immune response (Angata and Varki, 2002, Varki and Gagneux, 2012) and LOS sialylated *C. jejuni* strains have been found to invade IEC at higher levels, produce a more aggressive form of gastroenteritis. In addition these strains were also

reduced in susceptibility to human serum and are an important risk factor in the development of GBS (Guerry *et al.*, 2000, van Belkum *et al.*, 2001, Louwen *et al.*, 2008, Mortensen *et al.*, 2009).

LOS sialylation has been reported to activate DCs and mouse myeloid cells (Hu *et al.*, 2006a, Kuijf *et al.*, 2010, Huizinga *et al.*, 2012). DCs are widely distributed in tissues including the intestinal mucosa and are involved in not only innate but also the adaptive immune responses to microbial pathogens due to the ability to present antigens to T-cells (Medzhitov and Janeway, 1997). DCs are antigen presenting cells and are important in both innate and adaptive immune defence activation (Balazs *et al.*, 2002, Craxton *et al.*, 2003). *C. jejuni* is susceptible to serum, which highlights the importance of complement mediated killing (Blaser *et al.*, 1985). The primary method by which bacteria are cleared by the body is via cell mediated immunity and includes the action of DCs and macrophages (Al-Salloom *et al.*, 2003, Fox *et al.*, 2004, Hu *et al.*, 2006a). *C. jejuni* infection of T84 IECs has been found to increase the expression of IL-8, which has been reported to be involved in the recruitment of DCs (Johanesen and Dwinell, 2006).

in vitro studies have found that *C. jejuni* is rapidly internalised by DCs (Hu *et al.*, 2006a), resulting in the activation of NF- κ b and the subsequent induction of numerous cytokines and TNF- α , a signature of DC activation (Jones *et al.*, 2003). Kuijf *et al.* reported that LOS stimulation of human DCs triggered the release of soluble immune factors which enhanced the proliferation of B cells (Kuijf *et al.*, 2010). Subsequent work by Stephenson *et al.* reported a direct correlation between the variations in TLR4 stimulation in cytokine induction and the degree of LOS sialylation (Stephenson *et al.*, 2013). Other components of the innate immune response found to play a key role in defence against *C. jejuni* are the antimicrobial human β -defensins, these molecules belong to a family of epithelial antimicrobial peptides and are a major component of the host innate defence at the gastrointestinal mucosal surface (Hamanaka *et al.*, 2001, Dhaliwal *et al.*, 2003, Dommert *et al.*, 2005). Members of the human β Defensin (h β D) family have been found to be secreted during gastrointestinal infection (Bevins *et al.*, 1999, Wehkamp *et al.*, 2003, Zaalouk *et al.*, 2004, Zilbauer *et al.*, 2005), previous studies have reported that the constitutive expression of h β D-1 was necessary for immune surveillance whilst h β D-2 and h β D-3 are up-regulated during gastrointestinal

infection (O'Neil *et al.*, 1999, Dhaliwal *et al.*, 2003, Lehrer, 2004, Zilbauer *et al.*, 2005). These h β Ds have been found to be secreted during *C. jejuni* infection of IEC, with an equal increase in the levels of peptide expression and bactericidal effects were also observed in the presence of recombinant- β -defensins, providing further evidence that these antimicrobial peptides produce a potent response against *C. jejuni* (Bevins *et al.*, 1999, Wehkamp *et al.*, 2003, Zaalouk *et al.*, 2004, Zilbauer *et al.*, 2005).

1.4.1 Humoral Immune Response to *C. jejuni*

Following natural or experimental infection with *C. jejuni*, most people develop antibodies to *C. jejuni* surface proteins and LOS (Wenman *et al.*, 1985, Nachamkin and Hart, 1985, Dunn *et al.*, 1987). Previous studies have reported that experimental infection of human volunteers and macaques causes antibodies to appear around 6 to 7 days after infection (Mills and Bradbury, 1984, Russell *et al.*, 1989). The suggestion that humoral immunity plays a key role in clearance of the bacteria is supported by the reduction in clinical symptoms of gastroenteritis observed along with the production of circulating anti-*C. jejuni* antibodies (Mills and Bradbury, 1984). *in vitro* studies showed that anti-*C. jejuni* antibodies were effective at killing the homologous strain used for infection (Black, 1992). However, when sera from volunteers infected with heterologous strains was used to kill *C. jejuni*, the bactericidal activity was significantly reduced indicating strain-specific antibody protection (Pennie *et al.*, 1986). The ability of *C. jejuni* to induce inflammatory cytokines and the interaction of *C. jejuni* with the host or the regulation of host cell responses may be crucial to the course of infection (Johanesen and Dwinell, 2006, Dasti *et al.*, 2010).

1.5 Adhesion to and invasion of host epithelial cells

Bacterial attachment to host cells is a key process in initiating the early stages required for successful colonisation (Finlay and Falkow, 1997). Binding to host cells enables cell invasion and thus protection from humoral immunity (Roberts, 1990, Isberg and Van Nhieu, 1994, Alruz and Isberg, 1998). A major theme is the ability of pathogenic microorganisms to hijack host cell molecules which enable binding and ultimately entry of the pathogen into the host cell (Watarai *et al.*, 1996). The bacterial factors involved in adhesion and invasion of host cells range from single monomeric proteins to complex multimeric macromolecules that are involved in highly developed functions (Pizarro-

Cerda and Cossart, 2006). Tissue culture studies have identified *C. jejuni* virulence determinants including CadF (*Campylobacter* Adhesion to Fibronectin) (Konkel, Garvis *et al.* 1997), a second fibronectin- like binding protein FlpA (Flanagan *et al.*, 2009), a periplasmic binding protein PEB1 (Pei and Blaser 1993) and the surface lipoprotein JlpA (Jin, Joe *et al.* 2001) as key molecules that facilitate bacterial adhesion and invasion. Also, secretion of *Campylobacter* invasion antigens (Cia) proteins into host IECs have been identified (Hu and Kopecko 1999, Konkel, Kim *et al.* 1999) and CiaB has been reported to be required for *C. jejuni* internalisation (Konkel, Kim *et al.* 1999).

1.5.1 *C. jejuni* Invasion

C. jejuni invasion is an essential process in the internalisation and translocation of the bacteria across the epithelial cell barrier of the intestine and is important for causing enteritis (van Spreeuwel, Duursma *et al.* 1985). However the understanding *C. jejuni* invasion of IECs is still incomplete (O. Croinin and Backert, 2012). Several *in vitro* models have shown that *C. jejuni* can cross cell monolayers but with only a small number of bacteria actually invading cells (Friis *et al.*, 2005). Translocation of *C. jejuni* by disruption of the tight cell junctions was observed in Caco-2 IECs (MacCallum, Hardy *et al.* 2005). There is also a particular inflammatory signature of invasion, as the production of IL-8 and the prostaglandin E2 by polarised human colonic HCA-7 cells was only induced upon infection with invasive *C. jejuni* strains and not with non-invasive strains (Beltinger, del Buono *et al.* 2008).

Pathogens interacting with the intestinal epithelium can modify the function of the epithelium and enable penetration across the epithelial barrier and to target mucosal host defences for survival (Lu and Walker 2001). Similar to other enteric pathogens, *C. jejuni* interacts with the host cytoskeleton, producing changes in the architecture of the host cell, leading to eventual uptake of the bacteria by the cell (Clerc and Sansonetti, 1987, Cossart and Sansonetti, 2004). The eukaryotic host cytoskeleton composition is made up of microtubules (MTs) and microfilaments (MFs), the latter of which provides rigidity to the cell (Granger *et al.*, 2014). This network of filaments provides the cell with shape, function and movement (Mundy *et al.*, 2002, Chang and Goldman, 2004). Extracellular stimuli can produce changes in the cytoskeleton (Rosenshine 1998) and many enteric pathogens can subvert the host cell cytoskeleton into uptake of the bacterium by the secretion of proteins into the host cell using a specialised T3SS. These

are complex macromolecular structures that allow pathogenic bacteria to secrete proteins across the inner and outer membranes (Pizarro-Cerda and Cossart, 2006). Through this mechanism, extracellular bacteria that are in close contact with eukaryotic cells can deliver bacterial proteins into the cytosol (Gauthier and Finlay, 1998, Cornelis, 2006). Many T3SS effector proteins have been identified in *Salmonella* species, *Shigella* species, enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) and *Yersinia* species which appear to act directly on intracellular host effector proteins (Finlay and Cossart, 1997, Kubori *et al.*, 1998, Goosney *et al.*, 1999). Upon secretion into host cells, bacterial effector proteins cause activation of the host cell tyrosine kinase phosphorylation and calcium movement from within intracellular stores or activation of small guanine-nucleotide binding proteins (GTPases) belonging to the *Ras* superfamily, such as Rac and Rho proteins, these small GTPases play highly organised roles in cell processes (Finlay and Cossart, 1997, Vetter and Wittinghofer, 2001). This process eventually leads to actin cytoskeleton rearrangements and internalisation of the bacteria into the cell (Neutra 1999).

Early studies showed that *C. jejuni* motility was essential for IEC invasion *in vitro* and that an increase in mucus viscosity increased the levels of motility, adherence and invasion (Szymanski *et al.*, 1995). This association between motility and invasion combined with the lack of an identifiable classical T3SS or type IV secretion system (T4SS) led researchers to conclude that the flagellum could be used as a secretory apparatus for injection of invasion-associated effector molecules (Desvaux *et al.*, 2006). The *C. jejuni* flagellum components are homologous to the classical T3SS and play an essential role in *C. jejuni* pathogenesis (Neal-McKinney and Konkel, 2012).

The flagellum of *C. jejuni* is composed of a basal body, hook and filament. The basal body spans both the inner and outer membranes. The hook is composed of the protein FlgE, and capped by the hook-filament proteins FlgK and FlgL. Mutation of *flgK*, *flgL* or *flgE* results in an inability to form a functional flagellar filament (Konkel *et al.*, 2004, Fernando *et al.*, 2007b). The flagellar filament is composed of FlaA and FlaB and is capped by FliD, which allows the flagellin subunits to polymerise into the extending flagellum (Yokoseki *et al.*, 1995). Previously, the flagellum transport machinery was thought only to be essential in organelle biogenesis. Studies performed with *Yersinia enterocolitica* found that this bacteria secreted flagellar outer proteins (FopS) via the

flagellum which are involved in host cell interactions, thus suggesting that the flagellum may be a general method for the secretion of bacterial proteins and therefore acts as a T3SS, additionally a *C. jejuni flhB* export mutant was found to be deficient in secretion of the Cia proteins (Young *et al.*, 1999, Christensen *et al.*, 2009, Neal-McKinney *et al.*, 2010).

The first non-flagellar *C. jejuni* secreted factor identified was CiaB, secretion of this protein seemed to require at least one of the flagellar subunits and mutations of *ciaB* resulted in a significant reduction in intracellular bacteria (Konkel *et al.*, 1999b, Konkel *et al.*, 2004). Other secreted proteins identified so far are CiaC (Christensen *et al.*, 2009), CiaI (Buelow *et al.*, 2011b) and CiaD (Samuelson *et al.*, 2013). Numerous studies have reported that *C. jejuni* proteins secreted via the flagellum have been found to play a role in chick colonisation and in the invasion of IECs (Konkel *et al.*, 1999b, Ziprin *et al.*, 2001, Fernando *et al.*, 2007b). However, the only potential T3SS in *C. jejuni* is the flagella (Parkhill *et al.*, 2000). The role of the flagella as a T3SS in *C. jejuni* is controversial, as another study reported no significant reduction in invasion by a 81-176 *ciaB* mutant suggesting that further work is required on CiaB (Novik *et al.*, 2010). In the absence of a “classical T3SS” in *C. jejuni*, other potential virulence factors have been investigated. The ability of *C. jejuni* to harbour plasmids and the involvement of plasmids in *Campylobacter* pathogenesis and antimicrobial resistance has been studied since the 1980s (Taylor *et al.*, 1983). The pVir plasmid found in some *C. jejuni* isolates is a ~37.5 kb plasmid conferring tetracycline resistance and has been implicated in virulence (Bacon *et al.*, 2000). Sequencing of pVir found that the plasmid harboured four genes which encoded homologs of a bacterial (T4SS), utilised by a number of pathogenic bacteria (Bacon *et al.*, 2002). Studies with *C. jejuni* isolates containing this plasmid have demonstrated pVir plays roles in cell invasion and pathogenicity in the ferret model (Hu and Kopecko, 1999, Bacon *et al.*, 2000, Desvaux *et al.*, 2006, Goni, 2014). Further to this, the presence of pVir was significantly associated with bloody diarrhoea indicating a role for pVir in the pathogenesis of more invasive strains and severe forms of *Campylobacter* infection (Louwen *et al.*, 2006). However, electroporation of the pVir plasmid into NCTC11168 did not produce an increase in invasion of IEC (Bacon *et al.*, 2000), indicating that other virulence determinants are also probably involved in severe forms of *C. jejuni* infections (Skirrow MB, 2000). The

possible mechanisms of *C. jejuni* secretion have been investigated but remain poorly understood in comparison to other bacterial pathogens (see Section 1.5.4).

Enteric pathogens have been shown to hijack the host cell by inducing changes in the host cytoskeleton by rearranging the vast actin network of MFs that leads to uptake of the invading pathogen (Sansonetti 2001). Enteric organisms such as *Salmonella* and *Shigella* (Finlay and Falkow 1988, Elsinghorst, Baron *et al.* 1989, Finlay and Falkow 1989, Sansonetti 2001) have been found to induce cytoskeletal changes leading to bacterial internalisation. Bacterial interactions with both MFs and MTs have been identified as key to invasion and intracellular survival (Yoshida and Sasakawa 2003, Abrahams and Hensel 2006). However the details on *C. jejuni* entry and invasion are unclear, for instance *C. jejuni* has been reported to gain entry using either MF interactions (De Melo, Gabbiani *et al.* 1989, Konkel and Joens 1989), occurring mainly at the basolateral surface of the host cell (Kopecko 2008), or using MT interactions, which is the most predominantly observed pathway occurring at the apical surface (Oelschlaeger, Guerry *et al.* 1993, Hu and Kopecko 1999). *C. jejuni* has also been reported to exploit both MF- and MT-dependent pathways simultaneously for invasion (Oelschlaeger *et al.*, 1993, Kopecko *et al.*, 2001b, Biswas *et al.*, 2003, Monteville *et al.*, 2003b) or neither (Russell and Blake 1994) (See Figure 1.9)

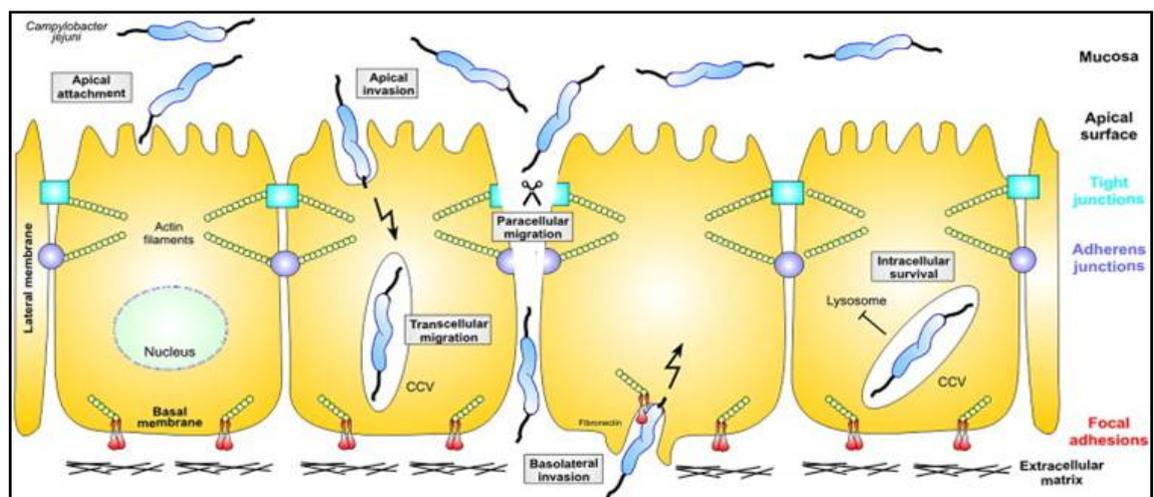


Figure 1.9 Hypothetical model of *C. jejuni*, interacting with and invasion of intracellular survival and transmigration mechanisms. (Backert and Hofreuter, 2013).

Studies have reported a reduction in the invasion levels of certain *C. jejuni* strains with the use of MT and MF inhibitors (Fauchere *et al.*, 1986, Konkel and Joens, 1989, Biswas *et al.*, 2003). However, *C. jejuni* 81-176 invasion was significantly enhanced using MF inhibitors (Oelschlaeger, Guerry *et al.* 1993). Previous studies have shown that some inhibitors can increase or decrease the invasiveness of bacteria depending on bacterial species and tissue type (Wells *et al.*, 1998). Also the passage number of tissue culture cells, the multiplicity of infection (MOI) and time-length of assay may all influence such data (Delie and Rubas, 1997).

In summary, there is conflicting data in the literature with regards to *C. jejuni* invasion. This can in part be attributed to differences with the bacterial strain used in experiments (Harvey, Battle *et al.* 1999, Biswas, Itoh *et al.* 2000), cell line and culturing methods and the assay methodology used which can all lead to variation in the results obtained (Friis *et al.* 2005). The ability of *C. jejuni* to adhere to and invade human IECs has been studied extensively using tissue culture methods (Konkel and Joens 1989). However, such studies suggest relatively low levels of invasion (~1%) and until recently there was little evidence to suggest that *C. jejuni* survives in IECs (Friis *et al.*, 2005, Watson and Galan, 2008b)

When engulfed by macrophages, *C. jejuni* is unable to avoid delivery to the lysosomes and is rapidly destroyed (Watson and Galan 2008). However, *C. jejuni* can survive within IECs by entering vacuoles and avoiding delivery to lysosomes, the bacteria appear to survive within a *Campylobacter* Containing Vacuole (CCV) in IECs which moves to a close proximity to the Golgi apparatus (Watson and Galan, 2008b). It is therefore clear that uptake via active invasion, rather than passively by phagocytosis, is crucial for virulence in *C. jejuni* infection (Watson and Galan, 2008b).

It is still unclear which route of invasion “zipper” or “trigger” is utilised by this bacteria (O. Croinin and Backert, 2012). However, recent studies using high resolution electron microscopy imaging of infected INT407 IECs showed that interestingly, *C. jejuni* may share certain features of both “trigger” and “zipper” entry (Krause-Gruszczynska *et al.*, 2011, Boehm *et al.*, 2012). The *C. jejuni* flagellum is able to transport bacterial effectors into the host cell and/ or the flagella can act as an adhesin by binding to host cell receptors, leading to signalling and invasion of *C. jejuni*. *C. jejuni* triggers membrane ruffling via direct contact with the host cell followed by entry with first the flagella tip,

then the opposite flagella end (Krause-Gruszczynska *et al.*, 2007a, Krause-Gruszczynska *et al.*, 2011, O. Croinin and Backert, 2012). Further studies are needed to more precisely understand this process.

1.5.2 The putative role of *C. jejuni* outer membrane vesicles in invasion of IEC

Both pathogenic and non-pathogenic Gram-negative bacteria release outer membrane vesicles (OMVs) from growing cells, spherical in shape with varying sizes of ~10 to 500 nm diameter (Mayrand and Grenier, 1989, Beveridge, 1999, Kuehn and Kesty, 2005, McBroom and Kuehn, 2007). OMVs are formed from sections of the bacterial membrane which bleb off to form vesicles (see Figure 1.10) (Chatterjee and Das, 1967, Ellis and Kuehn, 2010, Kulp and Kuehn, 2010). OMVs in non-pathogenic bacteria play a protective role by reducing the presence of toxic components, aiding bacterial survival (Loeb and Kilner, 1978, Kobayashi *et al.*, 2000). The secretion of OMVs has been found to be increased when the bacteria are under environmental stress (McBroom and Kuehn, 2007).

OMVs play a role in pathogenesis, mediating intercellular exchange events and by acting as vehicles for delivery of virulence factors, include toxins, adhesins, lipopolysaccharide (LPS) and immune-modulatory compounds (Beveridge, 1999, Horstman and Kuehn, 2000, Kesty *et al.*, 2004, Kuehn and Kesty, 2005). OMVs are also involved in bacterial quorum sensing, aggregation of bacteria thereby enabling biofilm formation (Mayrand and Grenier, 1989, Whitchurch *et al.*, 2002, Nakamura *et al.*, 2008, Ellis and Kuehn, 2010). In addition, OMVs have been reported to modulate the host immune response, as previous studies have found that OMVs secreted by *Moraxella catarrhalis* contain a super-antigen molecule which is able to induce a non-specific immune response that diverts from targeting the actual *Moraxella catarrhalis* bacterial cells, therefore playing an important role in the pathogenesis of this microorganism (Bomberger *et al.*, 2009, Vidakovics *et al.*, 2010). Numerous Gram-negative bacteria including *E. coli*, *P. aeruginosa*, *C. jejuni* and *H. pylori* have been shown to produce OMVs (Bomberger *et al.*, 2009, Lindmark *et al.*, 2009, Kulp and Kuehn, 2010, Vidakovics *et al.*, 2010, Elmi *et al.*, 2012). OMV production has also been reported to be important in the life cycle of intracellular pathogens as *Francisella* species, *Salmonella* species and *Legionella pneumophila* have been shown to release

OMVs directly into the phagosomal compartments the bacteria reside in (Anthony *et al.*, 1991, Garcia-del Portillo *et al.*, 1997, Fernandez-Moreira *et al.*, 2006).

Delivery or injection of bacterial virulence factors directly into the target cell is an important mechanism and requires intimate contact with the host cell via a T3SS or T4SS, utilised by both pathogenic and non-pathogenic bacteria, allowing the delivery of adhesins, toxins and immunomodulatory compounds, which can directly mediate bacterial binding and invasion, causing cell death and modulation of the host immune response (Mota and Cornelis, 2005, Cornelis, 2006, Grant *et al.*, 2006). *C. jejuni* lacks classical virulence factors identified in other Gram-negative pathogenic bacteria (Parkhill *et al.*, 2000). The *C. jejuni* flagella apparatus has been reported to function as a possible T3SS (Parkhill *et al.*, 2000) and also to be involved in the secretion of Cia proteins into the host cell (Konkel *et al.*, 2004). However the flagellar serving as a secretory apparatus for the delivery of *C. jejuni* Cia proteins is controversial (Novik *et al.*, 2010). The identification of *C. jejuni* OMVs was noted over 30 years ago, but has only recently become an active area of research due to the discovery that 81-176 OMVs contain CDT (Logan and Trust, 1982, Blaser *et al.*, 1983b, Lindmark *et al.*, 2009). Lindmark's study was the first evidence to show that *C. jejuni* was capable of delivering virulence factors into IECs and the surrounding environment using OMVs (Lindmark *et al.*, 2009). A recent study reported that *C. jejuni* OMVs alone were capable of inducing the production of significant levels of IL-8, IL-6, TNF- α , and hBD-3 from T84 IECs (Elmi *et al.*, 2012). Therefore, in the absence of classical virulence associated secretion systems in the *C. jejuni* cell, the hypothesis that *C. jejuni* may be utilising OMVs in order to deliver bacterial effector proteins which promotes the interactions with, invasion of IECs and immune modulation of the host response (Elmi *et al.*, 2012).

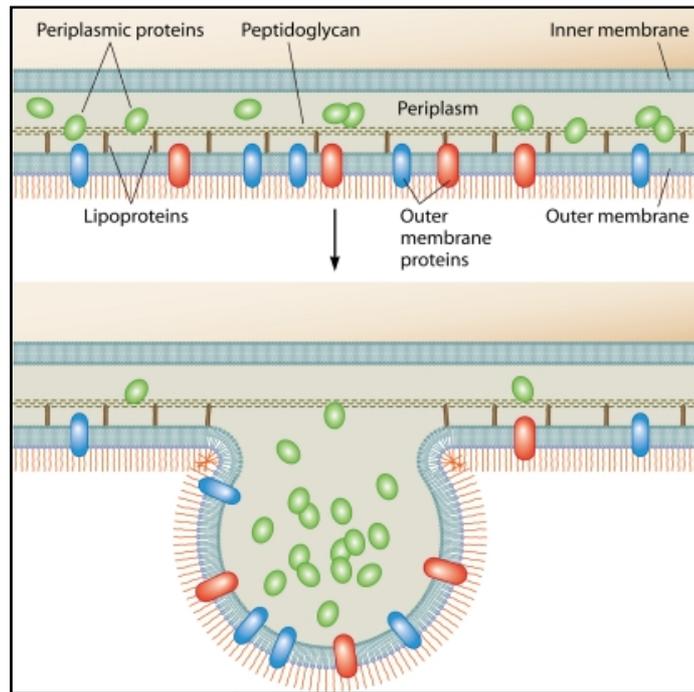


Figure 1.10 Outer membrane vesicle model. Bacterial outer membrane vesicle formation model. The OMVs contain periplasmic material and outer membrane proteins, lipids and other virulence factors (Ellis and Kuehn, 2010) This model shows the budding of the Gram-negative bacterial envelope.

1.5.3 Inhibition of *C. jejuni* adhesion to and invasion of intestinal epithelial cells

In order to gain access to the host, evade the host immune system and cause disease. Many enteropathogenic bacteria enter the host cell via the interaction of microbial surface determinants with host receptors that once engaged are capable of transmitting signals which disrupt the cytoskeleton and enable entry of the pathogen (Fasano, 1998). The cytoskeleton is made up of three components; microfilaments, intermediate filaments and microtubules which are the largest protein filaments (Cooper, 2000) . These cytoskeletal structures are required for normal cell function by playing a role in cell division, movement of organelles, as well as being involved in cell signalling and maintaining the cytoskeletal dynamics of the cell (Desai and Mitchison, 1997). By utilising the host cell actin and microtubules which are involved in the transportation of endosomes and lysosomes within the cells, the bacteria can use the host cells polymerised filamentous actin as a method of uptake, where the bacteria is the key initiator of internalisation. (Stahl and Schwartz, 1986, Swanson and Watts, 1995). These protein filaments allow movement of bacteria into the host cell (Finlay and Cossart, 1997). Many enteric invasive microorganisms have been found to enter the host cell via

the MF pathway such as *Salmonella*, *Shigella*, *Listeria* and *Yersinia* species (Clerc and Sansonetti, 1987, Finlay and Falkow, 1989, Falkow *et al.*, 1992, Isberg and Van Nhieu, 1994).

Different types of endocytosis have been described and are dependent on the size of the endocytic vesicle, the type of cargo and the mechanism of vesicle formation. These are; phagocytosis, caveolin-mediated endocytosis, macropinocytosis, clathrin-mediated endocytosis and clathrin- and caveolin-independent endocytosis (Conner and Schmid, 2003). These endocytic pathways have been targeted by microbial pathogens, enabling internalization into non-professional phagocytes via caveolae and clathrin-mediated endocytosis, macropinocytosis or by avoiding delivery to the lysosomes (Gruenberg and van der Goot, 2006). In order to understand the mechanisms of *C. jejuni* invasion, the actin filament polymerisation inhibitor Cytochalasin D has been used previously. This is a fungal metabolite and acts by binding to the fast growing ends of microfilaments and blocking the action of the actin monomers (MacLean-Fletcher and Pollard, 1980, Brown and Spudich, 1979, Pollard and Mooseker, 1981). Cytochalasin D has previously been found to both decrease and increase *C. jejuni* invasion of IECs (Hu and Kopecko, 1999, Hu *et al.*, 2008, Bouwman *et al.*, 2013).

The cholesterol depleting agent methyl-beta-cyclodextrin (M β CD) has been used to ascertain whether lipid rafts present in the host plasma membrane are involved in bacterial invasion. Membrane lipid rafts were first proposed in 1997 by Simons and Ikonen and are rich in sphingolipids and cholesterol, these areas of high lipid concentrations play a role in intracellular trafficking and possible cell signalling events (Simons and Ikonen, 1997). Lipid rafts have been found to be involved in membrane trafficking, construction of immunological synapses, involvement in cellular processes such as cell adhesion mechanisms and endocytic activity (Simons and Ikonen, 1997, Brown and London, 1998). Lipid rafts have also been found to provide a gateway for a number of pathogens including several species of *E. coli* (EPEC), *S. flexneri*, *S. Typhimurium*, *Mycobacterium* species, *Chlamydia* species, *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, *C. jejuni*, *L. monocytogenes* and *Mycoplasma fermentans* (Manes *et al.*, 2003, Pelkmans and Helenius, 2003, Lafont *et al.*, 2004, Seveau *et al.*, 2004, Yavlovich *et al.*, 2004). Endocytosis of the organism through lipid rafts allows translocation of the organism into the cell (Zaas *et al.*, 2005). Clathrin-mediated

endocytosis of *C. jejuni* has been observed (Oelschlaeger *et al.*, 1993, Biswas *et al.*, 2000) and recently Watson and Galan showed that *C. jejuni* was translocated into IECs via lipid rafts (Watson and Galan, 2008b).

Many studies have found that bacterial attachment to and invasion of the host cell leads to activation of host cell signalling cascades (Finlay and Falkow, 1997). Host cell effector proteins, such as phosphatidylinositol 3-kinase (PI3-kinase), are signal transducer enzymes and are involved in cell growth, motility differentiation, proliferation, survival and intracellular trafficking and once induced can lead to dramatic changes in the host cell, whereby triggering Rac1 downstream leads to massive cytoskeletal deformation, actin rearrangements and membrane ruffling (Hawkins *et al.*, 1995), with eventual internalisation of the bacteria into the cell (Finlay and Cossart, 1997). Wortmannin, a potent specific inhibitor of the PI3-Kinase pathway, this pathway has been found to be involved in the endocytosis of *C. jejuni* as previous studies have found inhibition of PI3-Kinase led to decreased invasion levels of *C. jejuni* (Arcaro and Wymann, 1993, Ninomiya *et al.*, 1994, Wooldridge *et al.*, 1996b, Krause-Gruszczynska *et al.*, 2011, Sun *et al.*, 2013).

Clathrin-mediated endocytosis involves the uptake of material from outside of the cell to the inside of the cell using clathrin coated vesicles which are pre-formed on the plasma membrane (Pearse, 1976). This cellular machinery has been found to be harnessed by many pathogenic organisms including, *S. aureus*, *Salmonella species*, *Shigella species*, uropathogenic *E. coli*, *Candida albicans* and *Yersinia pestis* as well as viruses and toxins (Oelschlaeger *et al.*, 1994, Almeida *et al.*, 1996, Pizarro-Cerda *et al.*, 1997, Cossart, 1997, Duncan *et al.*, 2002, Green and Brown, 2006, Nazli *et al.*, 2006, Veiga *et al.*, 2007). Previous work by Oelschlaeger *et al.* showed that *C. jejuni* 81-176 entry via clathrin-mediated endocytosis was inhibited by the use of specific inhibitors of coated pit formation g-Strophanthin and Monodansylcadaverine (Oelschlaeger *et al.*, 1993). These inhibitors of clathrin-mediated endocytosis have been used since the early 1980s in many different cell lines (Schlegel *et al.*, 1982, Mato *et al.*, 1983). Russell and Blake found that *C. jejuni* 81-176 bacterial entry into Caco-2 IECs was not inhibited by monodansylcadaverine, however Biswas *et al.* reported a decrease in *C. jejuni* 81-176 invasion into INT407 IECs in the presence of monodansylcadaverine (Russell and Blake, 1994, Biswas *et al.*, 2000), suggesting the importance of multiple cellular

pathways utilised by different strains of *C. jejuni* in different eukaryotic cell lines (Hu and Kopecko, 1999, Friis *et al.*, 2005, Young *et al.*, 2007, van Putten *et al.*, 2009, Bouwman LI 2012).

Some pathogenic organisms have been found to utilise the microtubule pathway to gain entry into host cells (Finlay and Falkow, 1989, Finlay and Falkow, 1997, Yoshida and Sasakawa, 2003). Previous work by Oelschlaeger *et al.* found that *C. jejuni* required intact microtubules for gaining entry into the INT407 IECs, as use of the microtubule inhibitor colchicine, a potent mitotic inhibitor and therefore results in microtubule formation arrest, reduced the number of intracellular bacteria (Dustin, 1978, Kang *et al.*, 1990, Oelschlaeger *et al.*, 1993). How *C. jejuni* uses the host cell cytoskeleton is still under investigation.

1.5.4 Role of CiaB

Previous studies on intestinal biopsies from infected patients showed that *C. jejuni* is an invasive organism due to the presence of internalised bacteria observed in the gut tissue (van Spreuwel *et al.*, 1985). This same level of invasion has also been observed during *in vitro* studies where *C. jejuni* isolated from individuals suffering from diarrhoea and fever were able to attach and invade IECs at a higher level than the strains isolated from patients with no reported symptoms (Fauchere *et al.*, 1986). *C. jejuni* secretes a set of proteins, termed *Campylobacter* invasion antigens (Cia) that are required for the invasion of cultured IECs (Konkel *et al.*, 1999b, Rivera-Amill *et al.*, 2001). The first Cia protein identified was termed *Campylobacter* invasion antigen B (CiaB), a 73 KDa protein with weak homology to other bacterial T3SS effector proteins. CiaB was thought to be translocated into the cytoplasm of host cells and was thus proposed to be a T3SS effector protein (Konkel *et al.*, 1999b). The presence of CiaB was also reported to be directly involved in the secretion of 18 other Cia proteins which were found to be secreted upon contact with host cells, calf serum or bile salts (Rivera-Amill and Konkel, 1999, Malik-Kale *et al.*, 2008). Mutants that lack a functional *ciaB* gene did not display any differences in binding to INT407 IECs but were significantly affected in invasion ability and also exhibited reduced chick colonisation levels (Konkel *et al.*, 1999b, Ziprin *et al.*, 2001). Currently, the characterised Cia proteins are designated CiaB, CiaC, CiaD, CiaI, FlaC and FspA (Konkel *et al.*, 1999a, Song *et al.*, 2004, Poly *et al.*, 2007, Christensen *et al.*, 2009, Buelow *et al.*, 2011b, Samuelson *et al.*, 2013). The most

widely studied is CiaB which has also been found to be secreted in the presence of mucus (Biswas *et al.*, 2007). However, the secretion of these Cia proteins does not occur via a classical T3SS but reported to be released via the flagellum which in the case of *C. jejuni* produces a mechanistic action similar to that of a T3SS (Konkel *et al.*, 2004).

in vitro studies with *C. jejuni* infection of IECs, showed an increase in the expression of the genes that are responsible for encoding the Cia proteins (Konkel and Cieplak, 1992, Konkel *et al.*, 1993). Once inside the host cell, in particular the action of one Cia protein (CiaC) has been proposed to interact and interfere with host cell signalling proteins such as Rac-1, leading to changes in the host cell structure and allowing the entry of *C. jejuni* (Krause-Gruszczynska *et al.*, 2007a, Eucker and Konkel, 2012a) although the exact role of CiaC in Rac-1 activation is not yet fully known (O. Croinin and Backert, 2012). These Cia proteins have been shown to play an important role in *C. jejuni* pathogenesis.(Konkel *et al.*, 1999b, Rivera-Amill *et al.*, 2001). The importance of CiaB was demonstrated using a piglet infection model, when inoculated with a *C. jejuni* wild-type strain, gastrointestinal and histological disturbances were observed including diarrhoea within 24 h of infection, in contrast when the piglets were inoculated with a *ciaB* mutant, no symptoms were observed until 3-4 days post inoculation, where only mild histological changes and some diarrhoea was observed. The *ciaB* mutant strain therefore demonstrated an attenuation in virulence (Konkel *et al.*, 2001, Raphael *et al.*, 2005).

Mutations in flagellar subunit genes such as *flgB*, *flgC* and *flgE2* that encode proteins that are involved in flagellar filament assembly resulted in the inability to secrete CiaB, indicating a possible function in flagellar assembly and export (Konkel *et al.*, 2004). For the secretion of CiaB, either FlaA or FlaB are required and *in vitro* studies with INT407 IECs found that invasion was dependent on the secretion of these Cia antigens (see Section 1.5.1) and motility (Konkel *et al.*, 2004). *in vitro* studies found that FlaC was secreted via the flagellar apparatus and displayed maximal adherence in binding to Hep-2 cells and *flaC* mutants were found to be significantly reduced in invasion of Hep-2 cells indicating that FlaC plays an important role in cell invasion (Song *et al.*, 2004). FspA was also found to be secreted via the flagellar and *in vitro* studies using INT407 IECs resulted in cell death, further to this a high level of heterogeneity was observed in

the *fspA* alleles, suggesting a varying virulence potential mechanism in *C. jejuni* strains (Poly *et al.*, 2007).

More recently further Cia proteins have been found to be involved in *C. jejuni* virulence. CiaC was reported to be required for the interaction with and invasion of INT407 IECs by modulating host cell proteins and inducing cytoskeletal rearrangements of the IECs leading to internalised *C. jejuni* and studies by Konkel *et al.* found that CiaC was delivered into the host cell cytosol by the presence of bound bacteria (Christensen *et al.*, 2009, Neal-McKinney and Konkel, 2012). CiaI was also found to be secreted through the flagella apparatus and was reported to play a role in *C. jejuni* intracellular survival (Buelow *et al.*, 2011b). CCVs enable *C. jejuni* intracellular survival (Watson and Galan, 2008a). The CCV was found to deviate from the typical endocytic pathway as increased numbers of CCVs surrounding the cell nucleus were observed (Watson and Galan, 2008a, Buelow *et al.*, 2011a). The mechanism of how the bacterium manipulates the vacuole to ensure survival and avoidance in delivery of the CCV to the lysosomes is not fully understood. However, the discovery of CiaI led to studies showing the importance of CiaI in the formation of the CCV, by avoidance of the delivery of the CCV to lysosomes and the in the intracellular survival of *C. jejuni* (Buelow *et al.*, 2011a). Both CiaC and CiaI were found to modulate the host cell signalling activities and further studies found that the expression of CiaC and CiaI was directly related to virulence (Konkel *et al.*, 2004, Christensen *et al.*, 2009). In 2013, CiaD was identified, this Cia protein was also reported to be delivered into host cell cytosol, altering the behaviour of the host cell and was involved in both the maximal invasion of INT407 IECs and triggering the secretion of IL-8 from IECs (Samuelson *et al.*, 2013). Studies by Samuelson *et al.* found that CiaD contained a possible nucleotidyltransferase domain and these domains have been found to also be present in other bacterial effector proteins and function by remodelling the host cell actin cytoskeleton via adenylation of RhoGTPase and inducing the release of IL-8, indicating that CiaD may also be involved in cell signalling events (Samuelson *et al.*, 2013). The same group also reported a significant reduction in invasion of INT407 IECs with both *ciaD* and *ciaC* mutants in comparison to the wild-type strain, a similar observation was made in *in vivo* mouse infection studies with a *ciaD* mutant which resulted in no gastrointestinal disease. Infection with the wild-type strain resulted in overt gastrointestinal disturbances including enlarged lymph nodes and blood present in the

caecum and colon. The wild-type phenotype was restored with complementation, indicating a role for the CiaD protein in development of disease in the mouse model (Samuelson *et al.*, 2013, Itzen *et al.*, 2011). CiaB and other secreted Cia proteins (CiaA–H) have been reported to require a functional flagellar export apparatus for their secretion (Konkel, Klena *et al.* 2004).

However *C. jejuni* does not possess a classical T3SS and evidence for the injection of bacterial virulence proteins into the host cell is not totally convincing as Novik *et al.* found that CiaB did not play a role in IEC invasion (Novik, Hofreuter *et al.* 2010). Therefore the role of flagellar in secretion of *C. jejuni* virulence proteins during IEC invasion is still controversial.

1.5.5 Role of CadF

In order for a microorganism to cause infection, for persistence and dissemination in the host and to avoid removal by the fluids that bathe the cells, attachment to host cells is vital (Boland *et al.*, 2000). Bacteria have evolved to possess an array of adhesion proteins (Wilson, 2002). Fibronectin is a large essential glycoprotein found in the extracellular matrix (ECM), body fluids and on the surface of eukaryotic cells, consisting of two 250 kDa monomers (Hynes 1973, Hynes 2004). Fibronectin is an essential component of the extracellular matrix (ECM), regulating cellular processes and acting as a scaffolding protein by maintaining tissue organisation (Ali *et al.*, 1977, Hynes, 1990, Midwood *et al.*, 2006). Fibronectin is a large dimeric glycoprotein and is composed of 12 Fibronectin type I repeats, two Fibronectin type II repeats and 15–17 fibronectin type III repeats (Xu *et al.*, 2009). Binding of pathogens to fibronectin has been reported to trigger the formation of integrin clusters and recruitment of host cell effector molecules (Talay *et al.*, 2000, Schwarz-Linek *et al.*, 2004, Marjenberg *et al.*, 2011). The focal complexes formed from the assembly of multiproteins act as a signalling platform and enables the linkage of fibronectin in the ECM to the cytoskeleton and the cell's signalling machinery, this cell signalling via the focal complexes enables cytoskeletal and membrane rearrangements by fibronectin and these cytoskeletal movements are involved in mitosis, motility and phagocytosis (Pankov and Yamada, 2002, Mao and Schwarzbauer, 2005, Broussard *et al.*, 2008, Sandig *et al.*, 2009). Proteins that are present at these focal complex sites include cytoskeletal elements such as α -actinin, vinculin, talin, scaffolding proteins such as paxillin and

signalling molecules such as focal adhesion kinase (FAK). These focal complexes serve several important functions by not only linking integrins to the cytoskeleton but with roles in the transmission of signalling inside the cell (Zamir and Geiger, 2001).

The presence of fibronectin not only plays a major role as a bacterial adhesion target enabling bacterial attachment but also in the stimulation of host cell pathways leading to signalling events that promote internalisation of the pathogen (Sandig *et al.*, 2009, Henderson *et al.*, 2011, Marjenberg *et al.*, 2011). For this purpose the presence of fibronectin allows attachment of the bacteria to the host cell surface. Fibronectin is an important target in initial bacterial attachment (Pankov and Yamada 2002). Fibronectin is also a common target for many pathogens such as *Mycobacterium avium*, *E. coli*, *Streptococcus pyogenes* and *S. aureus* (Kuusela, 1978, Myhre and Kuusela, 1983, Ryden *et al.*, 1983, Froman *et al.*, 1984, Visai *et al.*, 1991, Schorey *et al.*, 1996, Jaffe *et al.*, 1996). Bacterial proteins that bind to components of the ECM have been termed microbial surface components recognising adhesive matrix molecules (MSCRAMMs) (Schwarz-Linek, Hook *et al.* 2006). Over a hundred bacterial fibronectin binding proteins have been identified (Henderson, Nair *et al.* 2011). Despite the lack of a complete understanding of *C. jejuni* virulence, several proteins are known to contribute to adherence to eukaryotic cells and binding of *C. jejuni* to specific ligands on the host cell surface is suggested to play a pivotal role in colonisation of the host, thereby avoiding the removal of the organism from the intestines by the normal function of peristalsis and cell washing (Fauchere, Rosenau *et al.* 1986).

One of the most widely studied *C. jejuni* adhesins is a 37 kDa major outer membrane protein (MOMP) termed CadF (*Campylobacter* adhesion to Fibronectin), the action of this protein was confirmed by binding of recombinant CadF to fibronectin (Kuusela *et al.*, 1989, Konkkel *et al.*, 1997, Moser *et al.*, 1997). Due to the localisation of fibronectin on the basolateral side of confluent IEC monolayers (Kowalczyk *et al.*, 1990), previous studies have found that *C. jejuni* invasion occurs preferentially at the basolateral layer (Konkkel, Garvis *et al.* 1997). CadF is conserved among *C. jejuni* strains (Konkkel, Gray *et al.* 1999) and mutations in *cadF* prevent binding to fibronectin and a reduction in the level of invasion of IECs (Krause-Gruszczynska, van Alphen *et al.* 2007). *C. jejuni cadF* mutants were also found to exhibit reduced adhesion to INT407 IECs compared to

the wild-type strain (Monteville, Yoon *et al.* 2003) and reduced colonisation in chick IECs (Ziprin *et al.*, 1999, Monteville *et al.*, 2003b).

Further studies by Konkel's group identified the surface exposed amino acid binding sites of CadF and specific mutations in the respective amino acid residues led to a significant reduction in *C. jejuni* binding to INT407 IECs (Monteville *et al.*, 2003b). Gruszczynska *et al.* reported that *C. coli* possessed an extra 39 bp insertion in *cadF*, thereby increasing the size of CadF in *C. coli*, *in vitro* studies with these strains resulted in significantly reduced interaction and invasion of INT407 IECs (Krause-Gruszczynska *et al.*, 2007b), suggesting the importance of the presence of a conserved gene. Recent studies report that *C. jejuni* binds to fibronectin, triggering β -1 integrin and focal adhesion kinase (FAK) complexes, leading to phosphorylation of epidermal growth factor receptor (EGFR) / platelet derived growth factor receptor (PDGFR). Followed by Vav2 activation (guanine nucleotide exchange factor), Vav proteins then activate the Rho family GTPases. These GTPases control the organisation of the host cell cytoskeleton and cause PI3-K stimulation, finally leading to activation of cell division control protein (Cdc42). Cdc42 is involved in regulation of the cell cycle and leads to manipulation and rearrangements of actin or microtubules allowing the eventual entry of *C. jejuni* (see Figure 1.11) (Krause-Gruszczynska *et al.*, 2007a, Krause-Gruszczynska *et al.*, 2011, Eucker and Konkel, 2012b). However, other studies have reported that the fibronectin and integrin β 1 receptors are positioned basolaterally and it is unclear as to how *C. jejuni* is able to access these receptors (O. Croinin and Backert, 2012, Backert *et al.*, 2013)

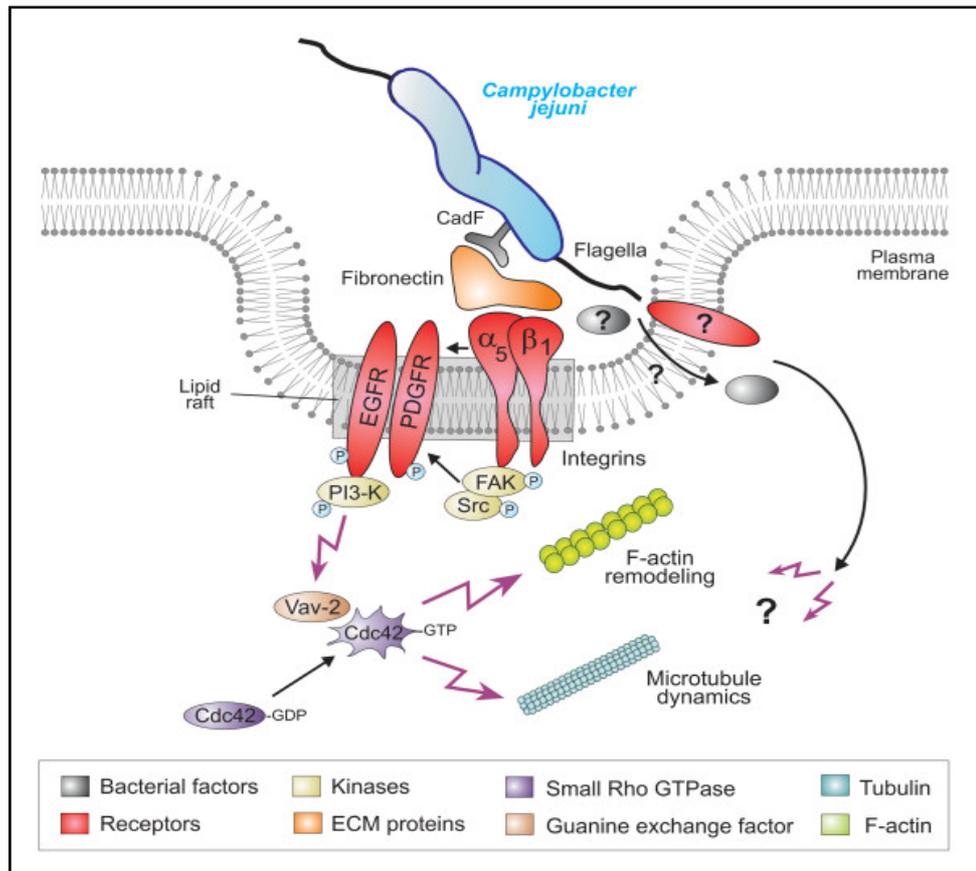


Figure 1.11 Activation of the host cell signalling pathway by CadF leading to *C. jejuni* invasion Hypothetical model for CadF-induced signalling and activation of Cdc42 leading to bacterial invasion. *C. jejuni* adheres to host cells via the fibronectin-binding protein CadF by engaging the integrin- β 1 receptor. Integrin occupancy and clustering in lipid rafts leads to activation of FAK. Phosphorylation of FAK and Src triggers a cascade of signalling leading to the formation of protein complexes which activate other signalling factors. Assembly of integrin- complexes leads to phosphorylation of PDGFR and EGFR, followed by activation of PI3-K and Vav2. Vav2 then induces the activation of Cdc42. This signalling leads to actin and microtubule rearrangements at the site of *C. jejuni* entry, resulting in *C. jejuni* uptake.

1.5.6 Role of FlpA

In addition to CadF, *C. jejuni* possesses a lipoprotein that also harbours fibronectin binding amino-acid domains termed fibronectin-like protein A (FlpA) and *flpA* is conserved in *C. jejuni* strains, indicating a possible role in pathogenesis (Larson *et al.*, 2013). Initially, FlpA was found to play a significant role in the colonisation of chickens, as mutation of *flpA* completely prevented the colonisation of chicks (Flanagan, Neal-McKinney *et al.* 2009). Further studies by Konkel's group reported FlpA to exhibit binding to fibronectin and a *C. jejuni flpA* mutant also displayed a reduction in interaction to INT407 IECs (Konkel, Larson *et al.* 2010). Recent studies have suggested that CadF and FlpA may act co-operatively to target fibronectin and

allow binding of *C. jejuni* and invasion of IECs by triggering integrin receptors α_5 and β_1 . Leading to the activation of host cellular proteins and the epidermal growth factor (EGF). In addition, inhibition of EGF receptor resulted in a significant drop in invading *C. jejuni* and the importance of integrin mediated cell adhesion and triggering of the EGF receptor has been confirmed previously (Moro *et al.*, 2002). Konkel's group also reported that for efficient *C. jejuni* host cell invasion, PI3-Kinase, tyrosine kinase protein (c-Src) (involved in cell growth) and FAK are required. As inhibition of these host cell proteins led to a significant reduction in *C. jejuni* invasion, indicating a role for these proteins in cytoskeletal rearrangements (Eucker and Konkel, 2012b).

A subsequent study showed that invasion of IECs with *C. jejuni cadF* or *flpA* mutants was significantly reduced and a *cadF flpA* double mutant did not activate the EGF receptor and RhoGTPases (Larson *et al.*, 2013). Further to this, the role of another host cell signalling molecule which is located downstream of EGF, the extracellular signal-regulated kinase (ERK) was investigated. ERKs are involved in the regulation of a number of cellular processes including differentiation, proliferation and survival (Deschenes-Simard *et al.*, 2014). INT407 IECs infected with a *C. jejuni flpA* mutant produced reduced levels of ERK phosphorylation whereas infection with a *C. jejuni capA* mutant (*Campylobacter* adhesion protein) actually produced an increase in the levels of ERK phosphorylation. This indicated that ERK was activated by FlpA-mediated binding to the IEC and that FlpA triggers downstream signalling events from the outside of the host cell (Larson *et al.*, 2013).

In addition, infection of INT407 IECs with a *flpA* mutant resulted in both reduced cell membrane ruffling and activation of Rac1, suggesting that the loss of FlpA disrupted activation of host cell signalling pathways (Krause-Gruszczynska *et al.*, 2011, Larson *et al.*, 2013). *In vivo* studies performed by Larson *et al.* also investigated the role of FlpA in disease development using an IL-10 negative mouse model. Mice infected with a *C. jejuni* wild-type strain and a *flpA* complement resulted in the presence of overt disease and mice showed signs of oedema and softening of stools in comparison to no disease observed with infection of mice with a *flpA* mutant. In addition, lower numbers of bacteria were found in the spleen of mice infected with a *flpA* mutant in comparison to those infected with *C. jejuni* wild-type and a *flpA* complement, further demonstrating the importance of this adhesin in disease development (Larson *et al.*, 2013).

Another pathway that may be utilised by *C. jejuni* is the activation of paxillin, a focal adhesion signalling molecule and component of the focal complex which produced an increase in tyrosine phosphorylation in response to *C. jejuni* infection (Monteville *et al.*, 2003b). The hypothetical pathway utilising paxillin involves a signalling cascade of; CadF/FlpA binding to fibronectin which activates integrin- β 1 receptor and stimulates focal adhesion kinase, this in turn activates paxillin (Monteville *et al.*, 2003a). Phosphorylation of paxillin occurred at the same time as intracellular bacterial numbers increased, indicating the importance of fibronectin binding by *C. jejuni* for mediating host cell internalisation (Monteville *et al.*, 2003b, Eucker and Konkel, 2012b). Further studies with a *C. jejuni flpA* mutant indicated that signalling from the outside of the host cell is triggered by binding of FlpA to fibronectin through integrin β ₁ receptors (Larson *et al.*, 2013). This could be providing a platform for *C. jejuni* invasion of IECs by activation of ERK signalling which ultimately leads to actin rearrangements and uptake of *C. jejuni* (Larson *et al.*, 2013). The exact mechanism and role of ERK activation by FlpA in *Campylobacter* pathogenesis requires further investigation.

1.5.7 Intracellular survival of *C. jejuni*

A number of microbial pathogens are capable of intracellular survival and replication within the harsh and constantly changing internal environment of mammalian cells (Mekalanos, 1992). Following phagocytosis by macrophages, bacteria are contained within a membrane bound vacuole and the trafficking of the vacuole containing the bacteria normally fuses with the lysosomes where it is rapidly destroyed due to the presence of reactive oxygen species released by the macrophages, however the survival, replication and escape of the pathogen varies greatly for those pathogens with intracellular adaptations (Hassett and Cohen, 1989, Garcia-del Portillo and Finlay, 1995, Mosser and Edwards, 2008). Some pathogens such as *Shigella flexneri* (Cossart and Sansonetti, 2004, Ogawa and Sasakawa, 2006), *Trypanosoma cruzi* (Brener, 1973), *Listeria monocytogenes* (Goebel and Kuhn, 2000) are able to break out of the phagocytic vacuole and replicate inside the infected cell. A pathogen with specific adaptations is *Leishmania* species, which is also able to survive in the very hostile, low oxygen, low (Alexander *et al.*, 1999). Another highly studied intracellular pathogen is *Salmonella typhimurium*, an organism which is also capable of surviving within a

vesicular compartment and avoiding delivery to lysosomes (Knodler and Steele-Mortimer, 2003).

Previous studies by Watson and Galan found that *C. jejuni* co-localises in a compartment within the host cytoplasm which is distinctly separate from the lysosomes and termed the CCV. The CCV has been reported to interact transiently with early endosomal marker proteins and RhoGTPase members Rab4 and Rab5, however the CCV did not progress via the normal endocytic pathway as CCVs were rarely observed associating with the lysosomal marker Cathepsin B and clustering of CCVs occurred around the nucleus (Konkel *et al.*, 1992b, Watson and Galan, 2008b). However, the exact mechanism of the formation of the CCV and how *C. jejuni* manipulates the internal environment of the CCV enabling deviation from the endocytic pathway is still unclear as the genes involved in *C. jejuni* intracellular survival and trafficking are unknown. A *C. jejuni cial* mutant is significantly reduced in intracellular survival ability within INT407 IECs compared to the wild-type strain (Buelow *et al.*, 2011a). Also, when INT407 IECs were infected with a *cial* mutant, the CCVs were found to more readily localise with the endosomal marker Cathepsin D (which is an endoprotease and is involved in proteolysis) in comparison to the CCVs in INT407 IECs infected with the wild-type strain, suggesting that the secretion of CiaI may in some way be involved in modifying the internal environment of the CCV (Boya *et al.*, 2003, Buelow *et al.*, 2011b, Bewley *et al.*, 2011). Novik *et al.* investigated a number of *C. jejuni* genes that were potentially required for intracellular survival and identified three genes *aspA* which encodes aspartate ammonia-lyase, *aspB* which encodes aspartate aminotransferase, these enzymes produce fumarate which acts both as a carbon source and electron acceptor in anaerobic respiration and *sodB* which encodes (superoxide dismutase) which is an important virulence factor in *C. jejuni* and *C. coli* (Pesci *et al.*, 1994, Purdy *et al.*, 1999). SodB detoxifies reactive oxygen intermediaries generated during oxidative stress by catalysing the conversion of superoxide (O_2^-) to less harmful substances, oxygen and hydrogen peroxide (H_2O_2) (Hassan, 1989, Miller and Britigan, 1997). Mutations in the above genes produced a significant reduction in intracellular survival in T84 IECs as well as reduction in colonisation levels in the mouse model but the results observed could not be solely attributed to the mutation as the phenotype of *aspA* and *aspB* mutants was reversed when the bacteria was supplied with fumarate (Novik *et al.*, 2010). Further studies on *aspA*, *aspB* and *sodB* mutants found that the

reduction in the amount of bacteria recovered was not due solely to the respective mutation but due to a combination of defects in both adhesion and intracellular survival (Novik *et al.*, 2010). Further studies are still required to dissect the molecular basis of *C. jejuni* intracellular survival.

1.5.8 Imaging of *C. jejuni* adhesion to and invasion of Caco-2 intestinal epithelial cells

In order to study the interactions of bacteria within the host cell, cell staining procedures have allowed investigators to study host-pathogen interactions using fluorescence microscopy, confocal microscopy, flow cytometry and fluorochrome labelling (Falk *et al.*, 1994). Laser and confocal fluorescence microscopy are powerful tools to observe and study molecular events occurring in cells (Takata *et al.*, 1997). The advantage of fluorescence microscopy is the detailed study of molecules in cells and tissues by multiple fluorescent staining and allows researchers to visualise the location of particular molecules of interest in the cell. Cellular structures such as the cell cytoskeleton, plasma membrane and nucleus can be observed with various fluorochromes that bind to these structures (Suzuki *et al.*, 2007). Staining particular cellular structures such as actin with Phalloidin, a toxic compound produced by the deathcap mushroom *Amanita phalloides*, specifically binds to F-actin in the cell (Suzuki *et al.*, 2007). Following labelling with the appropriate fluorochrome, the cellular structures of interest can be visualised by fluorescence microscopy.

Fluorescent labelling of cellular structures can provide high levels of sensitivity that are useful for a wide range of analytical applications. Conjugating antibodies with fluorescent labels is usually achieved by *in vitro* chemical conjugation of specific organic fluorophores (Hermanson, 1996). This requires large amounts of purified protein and leads to issues in optimisation of the level of conjugation and loss of activity. These techniques have proved to be time consuming and sometimes inefficient (Casey *et al.*, 2000). The Green fluorescent protein (GFP) which was originally isolated from the jellyfish *Aequorea victoria*, has been genetically conjugated to numerous proteins in a number of different species to produce stable chimeras capable of retaining both biological activity and fluorescent properties of the native GFP for visualising *in vivo* host-pathogen interactions. These GFPs are particularly useful in the study of bacteria within a host cell, using microscopy (Chalfie *et al.*, 1994, Stearns, 1995,

Valdivia and Falkow, 1997, Yrlid *et al.*, 2001, Mixter *et al.*, 2003). As a result, GFP is now widely used as a reporter for gene expression and as a fusion tag to monitor protein localisation within living cells (Misteli and Spector, 1997). GFP displays high levels of stability making GFP an important tool in *in vitro* studies. Previously, bacterial pathogens have been studied indirectly using fluorescent antibodies (Valdivia *et al.*, 1996). The problems encountered with fluorescent antibodies arise from the coupling reaction as there is a limit to the amount of fluorescence molecules that can be conjugated to an antibody. This has been reported to result in a population of antibodies where the number of fluorescence molecules per antibody is variable and so the fluorescence detected may result in large variations (The and Feltkamp, 1970). Multiple fluorophores being used in the same procedure can actually lead to a decrease in fluorescence via quenching mechanisms (Gruber *et al.*, 2000).

Optimisation studies performed previously have identified problems in under and over antibody labelling, leading to decreases in fluorescence due to either a lack of fluorophores or too many fluorophores. This has resulted in non-specific staining and loss of antibody-antigen specificity (Holmes and Lantz, 2001). In order to study the interactions of bacteria inside the host cell, fluorescent antibodies do not always provide efficient results due to time consuming procedures, bleaching and dilution of the fluorescence signal over time (Ellenberg, 1997). Whilst more recently, the utilisation of fluorescent marker proteins has enabled the visualisation of host-pathogen interaction on a whole new level. The increased use of techniques involving fluorescent protein (FP) labelling and computer-controlled systems for fluorescence and laser-confocal microscopes has enabled direct visualisation of the intricate structure of living cells (Miyawaki, 2003). Conventional fluorescent staining involves immunostaining using the fluorochrome-labelled specific antibodies and chemical staining that specifically react with the target molecules in the cell with fluorochrome-labelled chemical reagents (Suzuki *et al.*, 2007). Fluorescent labelling of molecules by genetic engineering do not require the time-consuming staining procedures necessary for microscopy, allowing the easy observation of molecules of interest in living cells.

1.6 Aims and Objectives

Given the uncertainty of the mechanisms of *C. jejuni* pathogenesis and the development of new methods such the VDC, the aim of my thesis was to reinvestigate the mechanisms of *C. jejuni* adhesion to, invasion of and survival within human intestinal epithelial cells (IECs).

The specific objectives were:-

1. Reinvestigation of *C. jejuni* invasion of intestinal epithelial cells by studying the effects of inhibition of microfilaments, microtubules, caveolae-mediated endocytosis, clathrin-mediated endocytosis and host protein tyrosine phosphorylation.
2. Reinvestigation of the role of CiaB in *C. jejuni* invasion of intestinal epithelial cells.
3. Reinvestigation of the roles of CadF and FlpA in *C. jejuni* adhesion to and invasion of intestinal epithelial cells.

Chapter 2: Materials and Methods

2.1 Bacterial strains and plasmids

In this study, two *C. jejuni* wild-type strains were used to investigate bacterial interactions with IECs. The 11168H wild-type strain is a hypermotile variant of NCTC11168 and readily colonises the GI tract of chicks and as such is considered a better strain to use to investigate host-pathogen interactions. (Parkhill *et al.*, 2000, Karlyshev *et al.*, 2002, Jones *et al.*, 2004). The 81-176 wild-type strain was isolated from a milk-borne outbreak in the USA and is probably the most widely studied laboratory strain. 81-176 harbours a putative virulence plasmid (pVir), which encodes a potential T4SS which has been reported to be involved in *C. jejuni* invasion of IECs (Korlath *et al.*, 1985b, Black *et al.*, 1988, Bacon *et al.*, 2000). All *C. jejuni* strains and mutants used in this study are listed in Table 2.1. All *E. coli* strains used in cloning studies are listed in Table 2.2 and all the plasmids used are listed in Table 2.3.

2.1.1 Chemicals and reagents

Chemicals used in this study were purchased from Invitrogen/Gibco (Paisley, UK), Sigma-Aldrich (Poole, UK), Fisher Scientific (Loughborough, UK) or VWR/BDH/Merck (Poole, UK), unless stated otherwise.

2.1.1.1 Sterilisation

All reagents, media and solutions were sterilised by autoclaving at 121°C for 20 minutes in a wet autoclave (LTE Scientific, Oldham, UK) or dry steam sterilisation was performed at 134°C for 15 minutes in an AAJ autoclave (Astell Scientific, Sidcup, UK). Filter sterilisation was performed using a 10 ml syringe (BD Plastipak, Oxford, UK) and a 0.2 µm (32 mm) Acrodisc[®] syringe filter with Stupor[®] membrane (Pall Life Sciences, Portsmouth, UK).

2.1.1.2 Reagent preparation

Unless otherwise stated, all reagents, solutions and media were prepared using either Ultra-pure Milli-Q water or Ultra-pure Milli-RO water (Millipore, Billerica, USA).

Table 2.1 *C. jejuni* strains used in this study

<i>C. jejuni</i> strains /mutants	Description	References
81-176	A strain isolated from a multi-state milk borne outbreak in the USA. Wild-type laboratory passaged strain.	(Blaser <i>et al.</i> , 1979, Korlath <i>et al.</i> , 1985b)
11168H	Hypermotile derivative of original sequenced strain NCTC11168, which produces higher levels of colonisation in chick model	(Karlyshev <i>et al.</i> , 2002, Jones <i>et al.</i> , 2004)
81-176+ <i>bsI</i>	<i>C. jejuni</i> strain expressing Evoglow® fluorescent protein	This study
11168H+ <i>ppl</i>	<i>C. jejuni</i> strain expressing Evoglow® fluorescent protein	This study
81-176+eGFP	<i>C. jejuni</i> expressing enhanced green fluorescent protein	This study
11168H+eGFP	<i>C. jejuni</i> expressing enhanced green fluorescent protein	This study
81-176 <i>ciaB</i> mutant	Isogenic mutant, <i>Cj0194</i> 81-176 (<i>ciaB</i>) gene function disrupted with insertion of 1.4 kb Km ^r cassette.	This study
11168H <i>ciaB</i> mutant	Isogenic mutant, <i>Cj0921</i> 11168H (<i>ciaB</i>) gene function disrupted with insertion of 1.4 kb Km ^r cassette.	This study
81-176 <i>ciaB</i> complement	Complemented mutant containing <i>Cj0194</i> 81-176 gene and chl _r inserted into one of three ribosomal genes pRRC vector	This study
11168H <i>ciaB</i> complement	Complemented mutant containing <i>Cj0921</i> 11168H gene and chl _r inserted into one of three ribosomal genes in pRRC vector	This study
81-176 <i>cadF</i> mutant	Isogenic mutant, <i>Cjj1471</i> 81-176 (<i>cadF</i>) gene function disrupted with insertion of 1.4kb Km ^r cassette.	This study
11168H <i>cadF</i> mutant	Isogenic mutant, <i>Cj1478</i> 11168H (<i>cadF</i>) gene function disrupted with insertion of 1.4kb Km ^r cassette.	This study
81-176 <i>flpA</i> mutant	Isogenic mutant, <i>Cjj1295</i> 81-176 (<i>flpA</i>) gene function disrupted with insertion of 1.4 kb Km ^r cassette.	This study
11168H <i>flpA</i> mutant	Isogenic mutant, <i>Cj1279c</i> 11168H (<i>flpA</i>) gene function disrupted with insertion of 1.4 kb Km ^r cassette.	This study

Table 2.2 *E. coli* strains used in this study

<i>E. coli</i> strains	Description	References
DH5 α competent cells	<i>F</i> ⁻ , ϕ 80 <i>deoR lacZ</i> Δ <i>M15 endA1 recA1 hsdR17(rK-mk⁺) supE44 thi-1 gyrA96 relA1</i> Δ (<i>lacZYA-argF</i>) U169	Invitrogen, Paisley UK
XL2- BLUE MRF high efficiency competent cells	<i>endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'</i> [:: <i>Tn10 proAB+ lacIq</i> Δ (<i>lacZ</i>) <i>M15 Amy CmR</i>] <i>hsdR17(Rk-mK⁺)</i>	Stratagene, Agilent Technologies, Santa Clara, CA, USA
SCS110 competent cells	<i>rpsL(Str^r) thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44</i> Δ (<i>lac-proAB</i>) [<i>FtraD36 proAB lac^f Z</i> Δ <i>M15</i>]	Stratagene, Agilent Technologies, Santa Clara, CA, USA

Table 2.3 Plasmids used in this study

Plasmid	Information	References
pGEMT®-T easy	Commercial TA cloning vector with ampicillin resistance cassette	Promega
pGEMT+ <i>Cj1478</i> 11168H	pGEMT-easy vector with inserted 0.96 kb 11168H <i>cadF</i> gene disrupted by insertion of Km ^r cassette	This study
pGEMT+ <i>Cjj1471</i> 81-176	pGEMT-easy vector with inserted 0.96 kb 81-176 <i>cadF</i> gene disrupted by insertion of Km ^r cassette	This study
pGEMT+ <i>Cj1279c</i> 11168H	pGEMT-easy vector with inserted 1.2 kb 11168H <i>flpA</i> gene disrupted by insertion Km ^r cassette	This study
pGEMT+ <i>Cjj1275</i> 81-176	pGEMT-easy vector with inserted 1.2 kb 81-176 <i>flpA</i> gene disrupted by insertion Km ^r cassette	This study
pGEMT+ <i>Cj0921</i> 11168H	pGEMT-easy vector with inserted 1.86 kb 11168H <i>ciaB</i> gene disrupted by insertion of Km ^r cassette	This study
pGEMT+ <i>Cjj0194</i> 81-176	pGEMT-easy vector with inserted 1.86 kb 81-176 <i>ciaB</i> gene disrupted by insertion of Km ^r cassette	This study
pRRC	<i>C. jejuni</i> complementation vector pRRC for studies	(Karlyshev and Wren, 2005)
eGFP	<i>E. coli</i> expressing enhanced green fluorescent protein (Received from Dr Dominic Mills, LSHTM)	(Karlyshev and Wren, 2005)
pGLOW- <i>bsI/ppI</i>	pGLOW plasmid containing genes encoding a fluorescent reporter protein (Evocatel, Dusseldorf)	Evoglow®
pRRC+ <i>bsI</i>	pRRC vector containing Evoglow® <i>bsI</i> gene encoding a fluorescent reporter protein	This study
pRRC+ <i>ppI</i>	pRRC vector containing Evoglow® <i>ppI</i> gene encoding a fluorescent reporter protein	This study
pJMK30	Plasmid containing a gene encoding for Kanamycin resistance (<i>aphA3</i>) (1.4 kb)	(Trieu-Cuot <i>et al.</i> , 1985)

2.1.2 Microbiological procedures

2.1.2.1 Bacterial strains, media and growth conditions

C. jejuni strains were routinely cultured on blood agar (BA) plates containing Columbia agar (Oxoid, Basingstoke, UK) supplemented with *Campylobacter* selective supplement (Oxoid) and 7% (v/v) horse blood (TCS Biosciences, Botolph Claydon, UK). *C. jejuni* strains were incubated in a Variable Atmosphere Incubator (VAIN) (Don Whitley Scientific, Sheffield, UK) filled with a mixture of 85% N₂, 10% CO₂ and 5% O₂ at 37°C. *C. jejuni* mutants and complements were grown on BA plates supplemented with kanamycin (50 µg/ml) or chloramphenicol (10 µg/ml) as required. All antibiotics were obtained from Sigma-Aldrich. Strains were routinely sub-cultured every 3-4 days up to a maximum of 10 passages. *C. jejuni* cultures were grown in pre-equilibrated Brucella broth (Oxoid) in 25 cm² tissue culture flasks (Corning NY, USA) with shaking at 75 revolutions per minute (rpm) on a SSM-1 platform orbital shaker (Stuart/Bibby Scientific, Stone, UK) in the VAIN.

2.1.2.2 Culturing conditions for *E. coli*

E. coli strains were routinely grown on Luria Bertani (LB) agar plates (Oxoid) and incubated at 37°C under aerobic conditions in a MIR-262 incubator (Sanyo, Loughborough, UK). *E. coli* cultures were grown in LB broth (Oxoid) at 37°C in a shaking incubator (Gallenkamp/Sanyo, Loughborough, UK) at 150 rpm. When required, antibiotics were added in the following concentrations; ampicillin (100 µg/ml), kanamycin (50 µg/ml) or chloramphenicol (50 µg/ml).

2.1.3 Examination of *C. jejuni* morphology

2.1.3.1 Gram staining

A single colony was taken from a 24 hour BA plate and resuspended in filter sterilised Milli-Q water. A drop of this suspension was placed onto a microscope slide (Fisher Scientific) and the slide briefly passed through a flame to heat fix. The slide was then flooded with 2 ml of crystal violet stain (0.02% w/v) for 1 minute, then 2 ml of Iodine solution (0.01% w/v) for 3 minutes, followed by decolourisation with alcohol for 20 seconds. The slide was then counterstained with 2 ml of safranin (0.025% w/v) for 2 minutes. Slides were examined using a Nikon Eclipse E600 microscope (Fisher Scientific).

2.1.4 Preparation of specific OD₆₀₀ suspension of *C. jejuni*

C. jejuni were grown on a BA plate for 24 h, bacteria were collected using a sterile 10 µl loop and re-suspended in 1 ml Brucella broth. The suspension was mixed either by pipetting or vortexing. The optical density at a wavelength of 600 nm (OD₆₀₀) was determined by diluting the bacterial suspension 1:10 with 900 µl of Brucella broth in a cuvette (10 mm path length, Kartell, Noviglio, Italy). 1 ml of Brucella broth was used as a blank. The OD₆₀₀ was recorded using a spectrophotometer (WPA Lightware®, Cambridge, UK).

The OD₆₀₀ inoculum required was modified depending on the experiment. The inoculum required to produce an OD₆₀₀ 0.1 was calculated as follows; Volume required =

$$\left[\frac{\text{Inoculum required at OD}_{600} 0.1}{(10 \times \text{OD}_{600} \text{ reading})} \right] \times \text{Final volume} \times 1000$$

2.1.5 Glycerol stock preparation of *C. jejuni* and *E. coli* strains

For preparation of glycerol stocks for *C. jejuni*, 900 µl of a log phase culture in Brucella broth supplemented with 10% (v/v) foetal calf serum (FCS) (Sigma-Aldrich) was mixed with 100 µl sterile glycerol (Sigma-Aldrich). 100 µl aliquots were transferred into 0.6 ml microcentrifuge tubes (Eppendorf, Histon, UK) and snap-frozen by immersion into an ethanol and dry ice filled container for 5 minutes. Frozen stocks were then stored in a -80°C freezer (New Brunswick Scientific, St Albans, UK). Frozen stocks of *E. coli* were prepared by mixing 800 µl of a 16 hour broth culture with 150 µl of sterile glycerol in a Nunc Cryovial (Fisher Scientific) and transferred directly into a -80°C freezer.

2.1.6 Preparation of electrocompetent *C. jejuni* cells

C. jejuni electrocompetent cells were prepared as described previously (Karlyshev and Wren, 2005). Briefly, *C. jejuni* was grown on a BA plate for 24 h, bacteria harvested with a sterile loop and resuspended in 10 ml of ice-cold sterile EBF buffer (272 mM sucrose, 15% (v/v) glycerol) (see Appendix). The suspension was centrifuged at 4,000 rpm for 15 min at 4°C and the supernatant discarded. The pellet was resuspended in 1 ml of ice-cold EBF wash buffer and transferred to a sterile 1.6 ml eppendorf. The

suspension was centrifuged at 13,000 rpm for 5 minutes at 4°C. The supernatant was removed and the pellet resuspended in another 1 ml of ice-cold EBF buffer, mixed by gently pipetting and the process repeated a further two times. After the final spin, the supernatant was discarded and the pellet was resuspended in 1 ml ice-cold EBF buffer. Aliquots of 100 µl were transferred into pre-labelled 0.6 ml eppendorf tubes and stored at -80°C until required.

2.1.7 Resuscitation of *C. jejuni* and *E. coli* frozen stocks.

C. jejuni frozen stocks were removed from the -80°C freezer and placed on ice and allowed to thaw. Once thawed, 100 µl was restreaked onto a BA plate and incubated for 48 h. Individual colonies were picked and sub-cultured onto a fresh BA plate. *E. coli* strains were resuscitated from glycerol stocks by plating onto LB agar plates and incubated at 37°C overnight. LB plates streaked with *E. coli* were parafilm sealed (Neenah, WI, USA) and maintained at 4°C for three weeks.

2.1.8 Motility assays

Motility assays were performed as described previously (Malik-Kale *et al.*, 2007). Briefly, a 24 h culture of *C. jejuni* was adjusted to OD₆₀₀ 1.0. 5 µl of this suspension was pipetted into the centre of a motility agar plate consisting of Brucella broth supplemented with 0.4% (w/v) bacteriological agar (Oxoid). Plates were incubated (without inverting) at 37°C under microaerobic conditions. Plates were checked at intervals of 24, 48 and 72 h and images were recorded on a gel doc system (GeneGenius Gel imager (UVP), Cambridge, UK). Motility was recorded by measuring the diameter of the halo around the centre of the plate.

2.1.9 *C. jejuni* in vitro growth kinetics assay

10 ml of Brucella broth was transferred into a 25 cm³ tissue culture flask and pre-incubated in the VAIN on a platform orbital shaker at 75 rpm for 18 h. A *C. jejuni* suspension was prepared and used to inoculate the 10 ml of pre-incubated Brucella broth to a starting OD₆₀₀ of 0.1. The culture was then incubated in the VAIN with shaking at 75 rpm. Samples were taken every 2 h from time 0 h to 32 h and OD₆₀₀ readings were recorded as an indication of bacterial growth.

2.1.10 Quantifying bacterial numbers by counting colony forming units

To quantify bacterial numbers in a culture at selected time points, 100 µl of culture was serially diluted 1:10 in Brucella broth up to a dilution factor of 10⁻⁷. 200 µl of each dilution was plated on BA plates in triplicate, spread out with L-shaped spreaders (VWR) and incubated for 3 days. Dilutions with counts between 100-300 cfus were selected for counting to calculate bacterial numbers in the initial culture.

2.1.11 *C. jejuni* autoaggregation studies

Autoaggregation (AAG) assays were performed as described previously (Howard *et al.*, 2009). *C. jejuni* was grown on a 24 h BA plate. Bacteria were harvested using a sterile cotton swab and resuspended in 10 mM PBS (pH 7.2) and the OD₆₀₀ adjusted to approximately 2.0. The suspension was transferred into 13 x 100 mm diameter sterilised glass test tubes (Fisher Scientific) and incubated for 24 h at 37°C under microaerobic conditions. After the 24 h incubation period, the top 1.0 ml was gently aspirated and the OD₆₀₀ reading recorded. The reduction in the OD₆₀₀ reading from the initial reading of 2.0 reflected the degree of autoagglutination.

$$\% \text{ AAG} = \left[\frac{\text{Pre-incubation value (OD}_{600}) - \text{Sample value (OD}_{600})}{\text{Pre-incubation value (OD}_{600})} \right] \times 100$$

2.1.12 *Galleria mellonella* model of *C. jejuni* infection

G. mellonella larvae were purchased from Livefoods UK Ltd (Rooks Bridge, Somerset, UK) and stored on wood chips at 16°C. Experiments were performed similar to the original published method (Champion *et al.*, 2009). Briefly, for each experiment, 10 *G. mellonella* larvae of similar weight were selected. *C. jejuni* grown on BA plates for 24 h was harvested, resuspended in PBS and the OD₆₀₀ adjusted to OD₆₀₀ of 0.1. The larvae were infected with ~10⁶ cfu in 10 µl inocula by micro-injection (Hamilton, Nevada, USA) into the right foremost leg. Controls of PBS only and no injection were also performed with each set of experiments. The larvae were incubated at 37°C and survival

was recorded at 24 h intervals for 72 h. Larvae were recorded as dead when the colouration changed from normal pale cream to dark brown and larvae were non-responsive to touch.

2.2 Mammalian cell culture

2.2.1 Tissue culture

The cell lines used in this study were human enterocyte-like Caco-2 IEC line (HTB37™), human T84 colonic IEC line (CCL-248™) IECs and the murine macrophage-like cell line (J774A.1), obtained from American type culture collection (ATCC) (LGC Standards, Teddington, England). Caco-2 IECs possess the ability to produce high trans-epithelial electrical resistance due to the formation of tight junctions (Pinto *et al.*, 1983, Everest *et al.*, 1992). Caco-2 IECs possess distinctive polarity due to the presence of apical microvilli and a basolaterally positioned nucleus (Hidalgo *et al.*, 1989). T84 IECs were derived from a lung metastasis of a colon carcinoma in a 72 year old male. These cells also express polarised characteristics such as tight junctions between cells (MacCallum *et al.*, 2006).

2.2.2 Maintenance and passaging of tissue culture cell lines

The Caco-2 human IECs and J774A.1 mouse macrophage cell lines were maintained using the following media:-

Dulbeccos Modified Eagles Medium (DMEM) (Sigma-Aldrich)	500 ml
FCS 10% (v/v) heat-inactivated	50 ml
Non-essential amino acids 1% (v/v) (Sigma-Aldrich)	5 ml
Penicillin 100 Units/ml) and Streptomycin (100 µg/ml) (Sigma-Aldrich)	5 ml

The T84 IECs were maintained using the following media:-

1:1 mixture of DMEM and Ham's F12 medium and GlutaMAX™ 2.5 mM, L-glutamine, 15 mM Hepes and 0.5 mM sodium Pyruvate (Gibco)	500 ml
FCS 10% (v/v) heat-inactivated.....	50 ml
Penicillin (100 U/ml) Streptomycin (100 µg/ml) solution	5 ml

These two media will henceforth be referred to as cell culture media in this study. Both cell lines were incubated in the appropriate cell culture medium at 37°C and 5% CO₂ incubator (Sanyo, Loughborough, UK). Cell culture medium was replaced every 2 days.

2.2.3 Passaging of tissue culture cells

Caco-2 or T84 IECs were passaged every 5-7 days and J774A.1 cells were passaged every 2 days when the cells reached a confluency level of around 90%. Cell culture media was removed and discarded. Then the monolayer was gently washed three times with 10 ml of pre-warmed PBS and gently tilting the flask to ensure even coverage. The PBS was removed and discarded followed by the addition of 5 ml of 0.05% (w/v) Trypsin / 0.02% (w/v) EDTA in Hanks' Balanced Salt Solution (Invitrogen). The flasks were placed back in the incubator for 10 minutes. Flasks were visually checked for detachment of cells. After the 10 minute incubation time, 8 ml of cell culture medium was added to the flasks to inhibit any further trypsin activity. The suspension was gently pipetted up and down to mix the cells. The suspension was transferred into a sterile 50 ml Falcon tube and centrifuged at 1300 rpm for 10 minutes. The supernatant was decanted and the pellet gently resuspended in fresh cell culture medium at a ratio of 1:4.

2.2.4 Enumerating the number of cells using a haemocytometer

Calculating the number of cells required for interaction, invasion, intracellular survival and macrophage survival assays, was performed by taking 100 µl of the cell suspension and adding this to a 5 ml sterile bijoux, followed by the addition of 800 µl of cell culture medium and 100 µl of Trypan Blue dye (Sigma-Aldrich). The suspension was mixed by pipetting and 10 µl of this suspension was loaded onto the haemocytometer (Weber Scientific, Teddington, UK) and covered with a slide (VWR). The average number of cells present in the suspension was determined by counting the number of cells per

square using an inverted microscope (Leica Microsystems, Milton Keynes, UK) and applying the following formula:-

$$\text{Total number of viable cells/ml} = N \times \text{dilution factor (1000)} \times \text{dilution (10)}$$

(Where N is the mean number of cells counted in 5 large squares).

Appropriate dilutions were performed to obtain the required concentration of cells for the relevant assay and 1 ml volumes were then seeded into 24 well plates (Corning) and incubated at 37°C in a CO₂ incubator.

2.2.5 Storage of tissue culture cells

Cells were grown for 5-7 days until confluent, followed by washing and detaching as described above. Cells were pelleted and resuspended in DMEM without antibiotics but supplemented with 5% (v/v) Dimethyl sulfoxide (DMSO) to act as a cryoprotectant. The suspension was aliquotted in 1 ml volumes into cryovials (Nunc) and allowed to cool slowly down to -80°C overnight using a Mr Frosty™ freezing box (Nunc). Cryovials containing IECs were placed in a liquid nitrogen storage tank (Statebourne, Washington Tyne & Wear, UK).

2.2.6 Resuscitation of tissue culture cells from frozen stocks

A cryovial of frozen cells was removed from liquid nitrogen storage and placed in a 37°C incubator. The vial was gently mixed to ensure quick and even thawing. Once the vial was thawed fully, the vial was wiped with 70% (v/v) ethanol before placing in the hood (Envair, Haslingden, U.K). The thawed cells from the vial were transferred to an Easy Flask® containing fresh pre-warmed culture medium and placed in the incubator. The cell medium was replaced with fresh cell medium 24 h later to prevent dead cells inhibiting the growth of the cells.

2.2.7 Bacterial interaction assay

1 x 10⁵ Caco-2 IECs or 5 x 10⁵ ml T84 IECs were seeded into each well of a 24 well tray. Cells were grown for 5-7 days until 80-90% confluency was reached. Once cells were confluent, the wells were washed three times with 1 ml of pre-warmed PBS. Interaction (adhesion and invasion) assays were performed using *C. jejuni* grown from a

24 h plate. Bacteria was collected with a sterile swab and resuspended in a 1.6 ml microcentrifuge tube containing 1 ml DMEM. The suspension was adjusted to an OD₆₀₀ of 0.1 (~1 x 10⁸ cfu/ml) in a final volume of 24 ml and 1 ml of this suspension was added to each well, equating to a multiplicity of infection (MOI) of approximately 100 bacteria to one eukaryotic cell. The infected monolayers were incubated for 3 h, 6 h or 24 h. After the desired incubation time, the supernatant was gently removed from the wells and the cells were washed three times with 1 ml of pre-warmed PBS, which removed the unadhered *C. jejuni* bacteria. The cells were then lysed in 1 ml of 0.2% (v/v) Triton X-100 (Sigma-Aldrich) in PBS at 37°C in a CO₂ incubator for 20 minutes. This step allowed the enumeration of adhered and invaded bacteria. 100 µl of the lysed suspension was serially diluted 10-fold up to 10⁻⁴ and 200 µl of the final suspension was spread plated out in triplicate onto BA plates. Plates were incubated for 72 h in the VAIN and cfu numbers counted.

2.2.8 Bacterial invasion assays

To enumerate the number of intracellular *C. jejuni* bacteria within the IECs, invasion assays were performed as described above, however before lysing the IECs with Triton X-100, the extracellular bacteria were killed using gentamicin. 1 ml of fresh DMEM supplemented with 1% (v/v) FCS and 150 µg/ml of gentamicin was added to each well and incubated at 37°C in a CO₂ incubator for 2 h. Monolayers were then washed three times with PBS and lysed as described previously.

2.2.9 Triton and gentamicin sensitivity assays

C. jejuni was grown on BA plates for 24 h at 37°C. Bacterial cells were harvested in 1 ml PBS and adjusted to an OD₆₀₀ of 0.1. Bacterial cells were exposed to 0.2% (v/v) Triton X-100 for 20 minutes or gentamicin (150 µg/ml) for 2 h. Sensitivity assays were performed on all the mutants and respective wild-type strains. Serial dilutions were prepared (10⁻¹ to 10⁻⁷) and 200 µl of the final dilution was plated onto BA plates in triplicate, incubated for 48 h and then colonies counted.

2.2.10 Immunofluorescence staining of Caco-2 IECs

Caco-2 IECs were seeded (~ 1 x 10⁵ cells) onto 17 mm cover slips in 24 well plates and grown to confluency. On the day of the experiment, cell culture media was removed and

the cells washed three times with PBS. The Caco-2 IECs were then fixed with 500 µl of 4% (v/v) paraformaldehyde (PFA) (Sigma-Aldrich) for 15 min at room temperature. IECs were washed three times with PBS, followed by permeabilisation with 0.1% (v/v) Triton X-100 (Sigma-Aldrich) for 5 min at room temperature and then blocked with 1% (w/v) FCS in PBS for 1 h at room temperature. For actin staining, IECs were incubated with Alexa Fluor 555-conjugated phalloidin (Invitrogen) (stock diluted 1:1,000 in PBS) for 1 h in the dark, coverslips were washed and mounted in Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; final concentration 1.5 µg/ml) (Vector Laboratories, Peterborough, United Kingdom) on a coverslip (Fisher Scientific) and examined with a Zeiss LSM510 confocal microscope (Carl Zeiss AG, Jena, Germany).

2.2.11 Anti- *C. jejuni* antibody staining of *C. jejuni* for confocal microscopy

Caco-2 cells were seeded ($\sim 1 \times 10^5$ cells) onto 17 mm cover slips in 24 well plates and grown to confluency. For infection, *C. jejuni* culture was added to the wells at an MOI 100:1 and incubated for 2 h and 6 h. Coverslips were washed three times with PBS and fixed in 4% (v/v) PFA for 15 minutes at room temperature. Cover slips were washed with PBS and stained with rabbit polyclonal IgG anti-*C. jejuni* antibody (10 µg/ml) in PBS and 5 % (v/v) FCS for 45 min at room temperature (Abcam, Cambridge, UK). After three washes with PBS for 10 minutes each, coverslips were stained with a secondary antibody (Alexa Fluor 488- conjugated goat anti-rabbit antibody) in the dark for 30 min at room temperature. To perform intracellular staining of internalised *C. jejuni*, cells were stained with anti-*C. jejuni* antibody in PBS with 5% (v/v) FCS and 0.05% (w/v) saponin (Sigma-Aldrich) (to permeabilise cells) for 45 min at room temperature, followed by Alexa Fluor 546-conjugated goat anti-rabbit antibody in the same buffer for 30 min at room temperature. Coverslips were washed three times in PBS. The coverslips were washed and mounted in Vectashield mounting medium containing (DAPI; final concentration 1.5 µg/ml) on to a clean glass slide and sealed with nail varnish. Slides were stored at 4°C in dark until imaging on a Zeiss LSM510 confocal microscope.

2.2.12 Quantification of mean fluorescence intensity (MFI)

A Zeiss LSM510 confocal microscope was used to capture images of eGFP or Evoglow®-expressing bacteria. Volocity software (PerkinElmer) was used to quantify confocal images following the manufacturer's instructions. The eGFP or Evoglow®-expressing bacteria were identified by volocity measurements (standard 'find' protocol) and the fluorescence intensities were quantified.

2.2.13 Pre-treatment of Caco-2 IECs with biochemical inhibitors

Stock inhibitors used in this study were prepared as follows:-

Inhibitor	Stock	Final concentration used in experiments per/ml
Cytochalasin D (CytD)	10 mM (in DMSO)	2 µM
Methy-β-cyclodextrin (MβCD)	10 mM (in MQ-H ₂ O)	5 µM
Colchicine	1 M (in DMSO)	10 µM
Wortmannin	10 mM (in DMSO)	1 µM
Monodansylcadaverine (MDC)	100 mM (in DMSO)	250 µM

Inhibition of invasion studies were performed basically as described previously (Oelschlaeger *et al.*, 1993, Yao *et al.*, 1997, Hu and Kopecko, 1999). Inhibitors of eukaryotic cell processes were added to the cell monolayer 1 h prior to the addition of bacteria. Monolayers were then washed three times with PBS. *C. jejuni* cells from a 24 h plate were collected using a sterile cotton swab and re-suspended in a 1.6 ml microcentrifuge tube containing 1 ml DMEM and adjusted to produce a final OD₆₀₀ of 0.1 (~ 1 x 10⁸ cfu/ml). 100 µl of this suspension was added to each well of a 24 well plate and incubated for 3 h, 6 h or 24 h. Following the incubation period, the infected

monolayer was washed three times with PBS and the numbers of interacting and invading bacteria were enumerated as described previously.

2.2.14 Cytotoxicity assays

To ascertain whether the use of chemical inhibitors had any detrimental effect on the integrity of the IECs, cytotoxicity assays were performed. Caco-2 IECs were seeded onto 24 well plates ($\sim 1 \times 10^5$ cells per well). IECs were grown for 5-7 days until confluent, the IECs were then washed three times with PBS followed by the addition of the appropriate concentration of each inhibitor as well as a control without inhibitor. The IECs were incubated for 1 h with the inhibitor, then the supernatants were removed and the IECs washed three times with pre-warmed PBS. This was followed by the addition of 1 ml fresh culture medium and the Caco-2 IECs were incubated for a further 24 h. After 24 h, the supernatants were collected and analysed for the release of lactate dehydrogenase (LDH) enzyme using the CytoTox 96[®] non-Radioactive Cytotoxicity Assay kit (Promega, Southampton, UK). Total lysis of IECs following treatment with 1% (v/v) Triton X-100 represented the 100% cytotoxicity positive control.

2.2.15 Intracellular survival assays

Intracellular survival assays were performed using the same protocol as that for the invasion assays, except that after incubating the cells with 150 $\mu\text{g/ml}$ gentamicin for 2 h, the monolayers were washed with 1 ml of PBS and then incubated in DMEM containing a reduced concentration of gentamicin (10 $\mu\text{g/ml}$) for 19 h (Naito *et al.*, 2010). The low concentration of gentamicin does not affect the internalised bacteria over the extended incubation period, but allows the killing of any extracellular bacteria. Following the incubation period, the monolayers were then washed three times with PBS and lysed and described previously.

2.2.16 Macrophage survival assays

Macrophage survival assays were performed using J774A.1 mouse macrophages. A bacterial suspension with an OD_{600} of 0.1 was prepared in DMEM. *C. jejuni* cells ($\sim 1 \times 10^8$ cfu) were added to a culture of $\sim 5 \times 10^5$ J774A.1 mouse macrophage cells (MOI 200:1) and incubated for 3 h. IECs were washed three times in PBS followed by incubation in DMEM containing gentamicin (150 $\mu\text{g/ml}$) for 2 h to allow killing of

extracellular bacteria. The macrophages were then incubated in DMEM containing a reduced concentration of gentamicin (10 µg/ml) and bacterial survival determined at 0 h, 4 h and 16 h. At each time point, the macrophages were washed three times with PBS and lysed by adding 0.2% (v/v) Triton X-100. Serial dilutions, plating and enumeration of bacterial numbers were performed as described previously.

2.2.17 Maintenance of IECs for Vertical Diffusion Chamber experiments

Caco-2 cells or T84 IECs were seeded ($\sim 1 \times 10^7$) into the upper compartment of a Snapwell filter (Corning Life sciences, Amsterdam, Netherlands). IECs were grown for a minimum of 21 days for Caco-2 IECs or 14 days for T84 IECs to allow for the formation of a polarised monolayer (Mills *et al.*, 2012). The growth medium was changed every 2 to 3 days.

2.2.18 Measurement of transepithelial electrical resistance of IEC monolayer for Vertical Diffusion Chamber experiments

The transepithelial electrical resistance (TEER) of a Caco-2 monolayer in a Vertical Diffusion Chamber (VDC) was measured by placing two voltage-sensing AgCl electrodes close to the cell monolayer on each side of the insert, passing a current through two further electrodes placed at the two distal ends of the insert and reading the voltage necessary to keep the current flowing. Resistance was calculated according to Ohm's law ($R = V/I$, where R = resistance, V = voltage, and I = current) and multiplied by the surface area of the monolayers (1.12 cm²).

2.2.19 Assembly of the Vertical Diffusion Chamber model system for *C. jejuni* Caco-2 and T84 IECs invasion studies.

Before each experiment, the two compartments of the VDC (Harvard Apparatus, Holliston, MA) were sterilised by immersion in Haz-Tabs (Guest Medical, Ltd., Aylesford, United Kingdom) solution for 2 h, followed by three washes with Milli-Q water. A Snapwell filter carrying a polarised monolayer of Caco-2 or T84 IECs was removed from the culture plate, washed three times with pre-warmed PBS and placed in between the two compartments of the VDC. For *C. jejuni* invasion studies, bacteria were harvested from a 24 h BA plate, resuspended in 1 ml brucella broth, adjusted to an OD₆₀₀ of 0.1 and added to the apical compartment. The basolateral compartment was

filled with tissue culture medium supplemented with 1% (v/v) FCS and 1% (v/v) non-essential amino acids. The VDC was maintained under microaerobic conditions in a VAIN to ensure the survival of *C. jejuni*. Whilst a gas mixture of 95% O₂ and 5% CO₂ was perfused through the basolateral compartment to maintain the IECs.

2.2.20 Disassembly of the VDC for enumeration of intracellular *C. jejuni*

At the desired time point of co-culturing, the basolateral supernatants were removed from the VDC and stored at -80°C for subsequent analysis. The Snapwell filter was removed from the VDC placed into a 6-well tissue culture dish. The Snapwells were washed three times with 400 µl PBS. The IECs were then incubated in DMEM containing 150 µg/ml gentamicin for 2 h at 37°C, followed by lysis with Triton X-100. All VDC experiments were performed with at least two technical replicates and at least three biological replicates per experimental data set.

2.2.21 Cytokine analysis of IEC supernatants following co-culture with *C. jejuni*

T84 IECs were infected with *C. jejuni* for 24 h as described previously. The IEC supernatants were probed for the presence of IL-8 or TNFα using a human IL-8 or human TNFα enzyme-linked immunosorbent assay (ELISA) development kit (eBioscience, Hatfield, UK), according to the manufacturers' instructions.

2.3 Molecular biology techniques

2.3.1. Isolation of *C. jejuni* genomic DNA

C. jejuni genomic DNA (gDNA) was isolated from a 24 hour BA plate using the 5 PRIME DNA extraction kit (5prime, GmbH, Germany) following the manufacturer's instructions.

2.3.2 Bacterial gDNA extraction for PCR screening

A single colony was transferred into a microcentrifuge tube containing 200 µl filter sterilised water and vortexed. The suspension was incubated at 95°C for 10 min, centrifuged at 13,000 rpm for 10 min then the resulting supernatant collected and used as a template for PCR. This supernatant was referred to as a boilate.

2.3.3 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated from overnight cultures of *E. coli* using the QIAprep[®] Miniprep Kit (Qiagen, Crawley, UK) following the manufacturer's instructions.

2.3.4 Oligonucleotide design

Primers used in this study are listed in Table 2.4. Primers were designed manually and checked (<http://www.oligoevaluator.com>) for the formation of secondary structures, such as hair-pins, dimers and melting temperature (T_m). The primers listed in Table 2.4 were synthesised by Invitrogen. Primers were reconstituted with sterilised Milli-Q H₂O to 100 μM, aliquotted and stored at -20°C.

Table 2.4 Primers used in this study

Primers	Sequence 5' to 3'	Restriction sites
pGLOW- <i>pp1</i> -Forward	TAATTCTAGAAAGCTTGCATGCCTGCAGTC	XbaI
pGLOW- <i>pp1</i> -Reverse	TAATTCTAGAGCATGCCTGCAGTCAGTG	XbaI
pGLOW- <i>bs1</i> -Forward	TAATTCTAGAGAATTCGAGCTCCATATG	XbaI
pGLOW- <i>bs1</i> -Reverse	GCAGGTCGACTCTAGAGGATC	XbaI
eGFP-Forward	GGACTAGTAGGAGATTTAAATGGTGAGCAAGGG CGAGGAGCTGTTTAC	XbaI
eGFP-Reverse	CTCTAGAAGGCCTTTACTTGTACAGCTCGTCCAT GCCGAGAGTG	XbaI
pRRC-Forward	GGATGAATTACAAGACTTGC	Vector specific
<i>ciaB</i> -Forward	AGAAATAGCTAAATTGGTTA	BclI
<i>ciaB</i> -Reverse	CTTATATCTTTCAAATTCTCC	BclI
<i>ciaB</i> comp-Forward	CCC TCTAGA GAGAATAAAAAGTATGAATAATTTTAAAG	XbaI
<i>ciaB</i> comp-Reverse	CCCTCTAGATTAATGATGATGATGATGATGTTTT TTCTTATATCTTTCAAATTC	XbaI
<i>cadF</i> - Forward	ATGAAAAAATATTCTTATGTTTAGGTTTG	BclI
<i>cadF</i> -Reverse	TTATCTTAAAATAAATTTAGCATCCACTCT	BclI
<i>cadF</i> comp-Forward	CCCTCTAGGTTTTTTCTATGAAAAAATATTATT ATG	XbaI
<i>cadF</i> comp-Reverse	CCCTCTAGATTAATGATGATGATGATGATGTCTT AAAATAAATTTAGCATCC	XbaI
<i>flpA</i> -Forward	ATGATGAAAAGATTTCGC	
<i>flpA</i> -Reverse	CTACTGAGCCGCCTTAAC	
<i>flpA</i> -IPCRM-Forward	ATCAGATCTGCAAGGCTTTACCTCCTCAAG	BglII
<i>flpA</i> -IPCRM-Reverse	ATCAGATCTACCCACTCTTTATCCCATT	BglII
pGEMT-SP6	GATTTAGGTGACACTATAG	Vector specific
pGEMT-T7	TAATACGACTCACTATAGGG.	Vector specific
Kan ^r - Forward	GTGGTATGACATTGCCTTCTGCG	
Kan ^r - Reverse	TGGGTTTCAAGCATTAGTCCATGCAAG	
<i>flpA</i> comp-Forward	AAATCTAGAGTTAAAGCACCTTTGATGGAAG	XbaI
<i>flpA</i> comp-Reverse	TCTTCTAGACTAATGATGATGATGATGATGCTGA GCCGCCTTAACCTTG	XbaI

2.3.5 Amplification of *C. jejuni* genes for cloning

Defined isogenic mutants were constructed by designing the appropriate primers (see Table 2.4) to amplify the gene of interest. The presence of a unique restriction site (BglII, BamHI or BclI sites) removed the need for IPCRM (see Section 2.3.17).

2.3.6 Polymerase chain reaction PCR

All PCR reactions were prepared in thin-walled 0.6 ml microcentrifuge tubes (Eppendorf) in a final volume of 25 μ l, containing 0.5 μ l of each forward and reverse primers (100 μ M), 1 μ l of gDNA (\sim 1 μ g), 23 μ l of PCR pre-mix (2 μ l dNTP mix (dNTPS at 10 mM), 5 μ l 10X PCR buffer and 1 μ l GoTaq DNA polymerase (10 U/ μ l)) (Promega). PCR reactions performed for complementation studies used a proof reading DNA polymerase *Pfx* (Invitrogen) according to the manufacturer's instructions. PCR reactions were performed using a Tetrad-2 thermal cycler (Biorad, Hemel Hempstead, UK). PCR conditions were modified depending on the Tms of the primers and the length of the size of the product to be amplified.

dNTPs mix was prepared:-

dNTP stock solution (1.25 mM of dATP, dTTP, dCTP and dGTP) prepared:-

dATP (100 mM)	12.5 μ l
dTTP (100 mM)	12.5 μ l
dGTP (100 mM)	12.5 μ l
dCTP (100 mM)	12.5 μ l
Sterilised Milli-Q water	to 1000 μ l

PCR premix was prepared:-

10 X buffer	100 μ l
dNTPs (1.25 mM)	168 μ l
MgCl ₂ (25 mM)	60 μ l
Taq polymerase (5 U/ μ l)	6 μ l
Sterilised Milli-Q water	to 1000 μ l

Premix was mixed and stored at -20°C until required.

PCR reactions were set up as follows:-

Forward primer (100 μ M)	0.5 μ l
Reverse primer (100 μ M.)	0.5 μ l
Genomic DNA (~ 1 μ g)	1 μ l
PCR Pre-mix	23 μ l

PCR program:-

Step 1: Denature at 94°C for 15 seconds

Step 2: Anneal at 55°C for 1 min

Step 3: Extension at 72°C for 1 min

Repeat Steps 1-3 34 times

Step 4: End cycle with 72°C for 4 min

Steps 2 and 3 were varied to optimise PCR amplification conditions / amplification of larger products (usually 1 minute per Kb). Modifications to PCR reaction conditions were also dependent on the T_m of the primers used.

2.3.7 Analysing PCR products by gel electrophoresis

Prior to cloning, the amplified PCR products were analysed on a 0.7% (w/v) agarose gel in 1X TAE electrophoresis buffer (see Appendix) supplemented with $0.5 \mu\text{g ml}^{-1}$ ethidium bromide (Fisher Scientific). $5 \mu\text{l}$ of each PCR reaction was mixed with $1.5 \mu\text{l}$ of 5 X DNA loading buffer (Bioline, London, UK) and loaded into a well of the agarose gel. $5 \mu\text{l}$ of hyperladder I marker (Bioline) was also added to a separate well. The gel was placed into an electrophoresis tank (AGE electrophoresis tank, Biorad, Hemel Hempstead, UK). The gel was run at 120 mV for 30-45 min. PCR products were visualised using a transilluminator (UVP, Cambridge, UK) and gel images recorded using a GeneGenius Gel imager (UVP).

2.3.8 Purification of PCR products

PCR products intended for further cloning studies were purified using the QIAquick[®] PCR purification kit (Qiagen). Briefly, $20 \mu\text{l}$ of the PCR product was transferred to a 1.6 ml eppendorf and $100 \mu\text{l}$ of binding buffer BP1 was added, mixed by gently pipetting, and then transferred to a QIAquick spin column in a 2 ml collection tube. The tube was centrifuged at 13,000 rpm for 1 minute and the flow-through discarded. The tube was placed back into the 2 ml collection tube, $750 \mu\text{l}$ of RPE buffer added to the tube, then centrifuged again at 13,000 rpm for 1 minute. The QIAquick column was placed into a fresh 1.6 ml eppendorf, $20 \mu\text{l}$ of pre-heated sterile Milli-Q water was added to the centre

of the QIAquick spin column and left to stand for 1 minute at room temperature, followed by centrifuging at 13,000 rpm for 1 minute to elute the DNA. The sample was incubated on ice and the purity of DNA was quantified using the Nano-drop. The purified PCR products were stored at -20°C.

2.3.9 Restriction enzyme digestion

Restriction enzyme digest reactions of PCR products or plasmid DNA were set up as follows. Briefly, 5 µl of 10 X reaction buffer and 2 µg of the PCR product or plasmid DNA were mixed in a 1.6 ml eppendorf and the volume adjusted to 48 µl with Milli-Q water and 1 µl (10 U/µl) of BglII, BamHI, XbaI (New England Biolabs, Hitchin, England) added. The reaction tubes were placed in a 37°C water bath for 60-90 min, followed by purification using the QIAquick[®] PCR purification kit. The purified products were quantified using the Nano-Drop. Purified products were stored at -20°C.

2.3.10 Dephosphorylation of plasmid DNA

Prior to use in subsequent cloning procedures, digested plasmid DNA was dephosphorylated using Antarctic phosphatase (New England Biolabs) to avoid the vector self-ligating. Briefly, 8 µl of plasmid DNA (~1 µg) was added to a 0.6 ml tube, followed by 1 µl of 10 X dephosphorylation buffer and finally 1 µl of Antarctic phosphatase enzyme (10 U/µl) was added. The reaction mixture was incubated at 37°C for 15 minutes, followed by a further incubation of the reaction mixture at 65°C in a heat block to inactivate the enzyme. The sample was then ready to use in further reactions.

2.3.11 Cloning of purified PCR products into a plasmid vector

0.6 ml eppendorf tubes were labelled as background control, test sample and positive control. Into each tube, 5 µl of 10 X ligation buffer, 1 µl of pGEMT[®]- easy vector (50 ng/µl), 0.4 µl of T4 DNA ligase (10 U/µl) (New England Biolabs) and 1 µl H₂O were added. Finally 2 µl of the PCR product (~ 1µg/ml) was added to the test sample tube and 2 µl of the control insert was added to the positive control tube. To the background control, a further 2 µl of H₂O was added. The reactions were mixed by pipetting and incubated overnight at 4°C. Ligation reactions were prepared with ratio of insert to vector of either 1:1 or 3:1:-

Amount of plasmid to insert was calculated using the following equation:

$$\left(\frac{\text{insert size (kb)}}{\text{vector size (kb)}} \right) \times \text{nanogram vector} = \text{nanogram insert.}$$

2.3.12 Transformation of *E. coli*

Tubes containing the ligation reaction mix were centrifuged to collect contents. 2 μ l of the ligation reaction was placed into a 1.6 ml eppendorf, pre-cooled on ice. 50 μ l of electrocompetent *E. coli* cells (see Table 2.2) were thawed on ice, gently tapped to mix the contents, 2 μ l β -mercaptoethanol (14.4 M) (Stratagene) was added to these cells, mixed by pipetting and incubated on ice for 10 minutes. The cells were then transferred to the pre-cooled eppendorf. As a control to check the transformation efficiency, 2 μ l of uncut plasmid was added to a separate pre-cooled tube containing 50 μ l of cells. The transformation mixtures were incubated on ice for 20 min. The samples were then heat shocked in a water bath at 42°C for ~ 45-50 seconds. The samples were placed immediately back on ice for a further 2 min. Then 950 μ l room temperature SOC broth (Bioline) was added and the reactions were incubated for 1.5 h at 37°C with shaking at 150 rpm. 200 μ l of the transformation reactions were plated onto LB amp plates supplemented with 0.5 mM Isopropyl- β -D-thio-galactoside (IPTG) (Promega) and 80 μ g/ml of X-Gal (Promega). The plates were then incubated at 37°C overnight.

2.3.13 PCR screening of transformants

Following incubation, individual colonies were selected from the transformation plates using a sterile loop for each colony, restreaked on fresh LB amp plates and incubated at 37°C overnight. Boilates were prepared from the restreaks, using a sterile loop, a small amount of growth was picked from each of the transformants and re-suspended in 100 μ l of Milli-Q water. The suspension was heated for 10 minutes at 95 °C and spun at 13,000 rpm for 5 minutes. 1 μ l of the supernatant was used as template DNA in a 20 μ l PCR reaction.

2.3.14 Insertion of a Kanamycin cassette

Prior to ligation with the kanamycin cassette, the plasmid DNA containing the gene to be mutated was digested and purified. Samples were checked for purity using the Nanodrop. A BamHI fragment of plasmid pJMK30 containing the Kan^r cassette was ligated into the digested plasmid DNA as follows:-

Plasmid DNA digested with unique restriction enzyme (~250 ng/μl)	2 μl
Digested Kanamycin cassette (~ 20 ng/μl)	5 μl
10 X Ligase buffer	1 μl
T4 DNA Ligase (3 Weiss units/μl)	2 μl

The ligation reaction was mixed by gentle pipetting, briefly spun down and incubated overnight at 4°C. Following incubation, the reaction mixture was transformed into DH5α cells and plated onto LB amp kan plates then incubated at 37°C overnight. Positive transformants selected on LB amp kan plates were restreaked onto fresh LB amp kan plates and incubated overnight at 37°C. Transformants were screened by PCR using gene specific primers to confirm the presence of a larger band due to the insertion of the Km^r cassette. Plasmid DNA was isolated from the positive colonies. Insertion and orientation of kanamycin cassette was confirmed using kan Forward/Gene specific Forward to check that the Km^r cassette was in the correct orientation. The Km^r cassette does not have a transcriptional terminator, which thereby allows formation of multicistronic mRNA allowing the Km^r cassette to be in the same position and direction as the gene of interest, thereby reducing the possibility of polar effects (van Vliet *et al.*, 1998).

2.3.15 DNA sequencing

To confirm that positive transformants contained the kanamycin cassette, sequencing was performed by adding 1 μl of the plasmid DNA (~ 0.5 μg) into two 0.6 ml eppendorfs, followed by 8 μl of ABI[®] prism terminator ready reaction mix (ABI[®], Carlsbad, CA, USA) and 1 μl (100 pmol) of either the forward or reverse primer into the respective tubes, in a final volume of 20 μl. Tubes were placed into a GeneAmp 9600 thermal cycler (Perkin Elmer, Beaconsfield, UK). The program used was as follows:

- 25 cycles of denaturation at 96°C for 10 seconds
- Annealing at 50°C for 5 seconds
- Extension at 60°C for 4 minutes.

After the run was complete the reaction mixture was transferred into a sterile 1.6 ml eppendorf containing 80 µl of 75% (v/v) isopropanol, vortexed briefly and incubated for 1 hour at -20°C, centrifuged at 13,000 rpm for 30 minutes and the supernatant removed and discarded. Next, 400 µl of 75% (v/v) ethanol was added, the tubes were vortexed briefly and spun at 13,000 rpm for 15 minutes. The supernatant was removed and discarded and the tubes were spun briefly to collect any residual ethanol, which was also discarded. Tubes were then allowed to air dry for approximately 20 minutes. DNA sequencing was performed by Dr Ozan Gundogdu or Ellie Thompson at the Faculty of Infectious & Tropical Diseases sequencing facility. DNA sequencing data was analysed with sequence Navigator software Chromas (Applied Biosystems, Warrington, UK).

2.3.16 Electroporation of *C. jejuni*

100 µl aliquots of *C. jejuni* electrocompetent cells (see section 2.1.6) were removed from the -80°C freezer and allowed to thaw on ice for 10 min. A 1.6 ml eppendorf was prepared with 2 µl of plasmid DNA (~0.5-1.0 µg), then 30 µl of the thawed electrocompetent cells were added. The contents were gently mixed and incubated on ice for a further 10 minutes. The mixture was then transferred to a pre-chilled electroporation (2 mm gap) cuvette (Gene Pulser, Bio-Rad, CA, USA). Electroporation was performed using a Gene Pulser Xcell electroporation system (Bio-Rad) with the following settings: 25 µFD, 2.5 Kv and 200 Ω. Following electroporation, 100 µl of room temperature SOC broth (Bioline) was added to the cuvette and mixed thoroughly by pipetting. The contents of the cuvette were then pipetted onto the centre of a BA plate and incubated for 24 h at 37°C in the VAIN. Bacterial growth was collected using a sterile cotton swab and resuspended in 1 ml of Brucella broth, then 200 µl of this suspension was plated onto a fresh BA plate supplemented with the appropriate antibiotic(s) and incubated for 2-3 days. The resulting colonies were restreaked and boilate PCR was performed on the colonies to confirm homologous recombination of the correct construct.

2.3.17 Inverse Polymerase Chain Reaction Mutagenesis

Where the gene to be mutated did not contain a unique BglII, BclI or BamHI site, inverse polymerase chain reaction mutagenesis (IPCRM) was performed. IPCRM primers were designed by selecting around two regions of 15-20 nucleotides in the centre of the gene to be mutated with an interspacing region of 20-25 nucleotides with the 5' primer downstream of the 3' primer. The 5' end of the IPCRM primer included ATC residues to allow efficient restriction enzyme digestion followed by the BglII sequence AGATCT.

IPCRM primers were resuspended in filter sterilised Milli-Q water according to manufacturer's instructions and then diluted down to a final concentration of 100 μ M.

IPCRM reactions were performed using cloned fragments of the gene to be mutated at a diluted concentration (~ 0.1-10 ng). The plasmid template concentration was reduced to minimise false positives following transformation into DH5 α cells.

IPCRM pre-mix was prepared:-

Buffer I (10X)	10 μ l
Accuprime Taq DNA Hi fi (5 U/ μ l)	0.4 μ l
dNTPS in buffer I concentration of	400 μ M
Milli-Q water	to 100 μ l

IPCRM reactions were set up:-

IPCRM premix -	98.4 μ l
DNA (0.1-10 ng)	1.0 μ l
IPCRM primers (100 pmol/ μ l)	0.6 μ l

IPCRM program:-

Step 1. 94°C for 2 min

Step 2. Denature at 94°C for 1 min

Step 3. Anneal at 45°C for 1 min

Step 4. Extension at 72°C for X mins (X= size of plasmid in KB x 1.5)

Repeat steps 2-4 40 times

Step 5. 72°C for 8 min

10-15 µl of the IPCRM reaction was loaded onto an agarose gel and the remainder of the reaction was purified with Qiagen QIAquick® PCR purification kit. Samples were checked on the Nanodrop ND-1000 spectrophotometer. Following purification, the IPCRM product was digested with BglII and DpnI for 3 hours at 37°C. DpnI was used to digest methylated template DNA that would produce false positives (Shenoy and Visweswariah, 2003).

Digestions were set up as follows:-

IPCRM product (10-500 ng/µl)	20 µl
BglII (20 units)	2 µl
DpnI (20 units)	2 µl
NEBuffer 2 (10X)	10 µl
Milli-Q water	to 100 µl

Restriction digested products were purified using the QIAquick® PCR purification kit. Purified samples were checked for purity using the Nanodrop. At this stage, the digested Kan^r cassette by BamHI was ligated with the digested IPCRM product:-

IPCRM product digested with BglII and DpnI (~250 ng/µl)	2 µl
Pre-digested Kanamycin cassette with BamHI (~20 ng/µl)	5 µl
10 X ligase buffer	1 µl
T4 DNA/NEB Ligase (3 Weiss units/µl)	2 µl

The reaction was incubated overnight at 4°C and then ligation reactions were transformed into DH5α cells.

2.3.18 Construction of complements

Complementation procedures were performed by inserting a copy of the functional gene into the mutant chromosome using the *C. jejuni* NCTC11168H & 81-176 complementation vector pRRC (Karlyshev and Wren, 2005).

2.4 Outer membrane vesicles assays

OMVs were isolated as described previously (Elmi *et al.*, 2012). Briefly, *C. jejuni* strains from 24 h BA plates were resuspended in 1 ml Brucella broth and inoculated in 75 cm² tissue culture flasks (NUNC™, Roskilde, Denmark) containing 25 ml of Brucella broth to an OD₆₀₀ of 0.1. Following a 14 h incubation with shaking, OD₆₀₀ measurements of the bacterial suspensions from the flasks were recorded and the bacterial suspension was transferred into sterile 50 ml centrifuge tubes (Corning, NY, USA). The bacterial cells were removed from the culture fluid by centrifugation at 4000 rpm for 1 h at 4°C in a Beckman Coulter (Allegra™ X-22R) centrifuge, (Beckman Instruments, Palo Alto, CA, USA) and the supernatant was filtered through a 0.22 μm membrane (Millipore) to remove broken cells and other debris collecting the cell-free supernatant into 50 ml centrifuge tubes. Absence of viable bacteria was confirmed by plating OMV samples on BA plates and incubating both aerobic and microaerobic conditions for 48 h. The filtrate was concentrated to 2 ml by ultra-filtration using an Amicon Ultra-15 Centrifugal Filter Devices with a nominal 10 kDa cutoff (Millipore) and centrifugation at 4000 rpm for 90 min at 4°C. The concentrated filtrate was transferred into an Ultra-clear centrifuge tube (Beckman Coulter) and centrifuged at 45,000 rpm for 3 hours at 4°C using a TLS 55 rotor (Beckman Instruments, Palo Alto, CA, USA). After ultracentrifugation, the resulting pellet was resuspended in 200 μl PBS and stored at -20°C.

2.4.1 Pre-treatment of *C. jejuni* OMVs with Caco-2 IECs

Caco-2 cells were seeded (~ 1 x 10⁵ cells per well) in a 24 well plate and incubated with 10 μg OMVs at 37°C in a CO₂ incubator 1 h. The cells were washed with PBS and then infected with live *C. jejuni* (~ 1 x 10⁸ cfu/ml). The monolayers were incubated for 24 h

at 37°C in a CO₂ incubator. After the desired incubation time was complete, the supernatant was gently removed from the wells and the cells were washed three times with 1 ml of pre-warmed PBS which removed the unadhered *C. jejuni* bacteria. The numbers of adhered and invaded bacteria were enumerated as described previously.

2.4.2 *C. jejuni* fibronectin binding assays

Fibronectin binding assays were performed as described previously (Konkel *et al.*, 2010). Briefly, the wells of 96-well flat-bottom plates (Costar, Corning, NY) were coated with a 1 mg/ml solution of fibronectin in 0.05 M Tris-buffered saline, pH 7.5 (Sigma) overnight at 4°C. For a control, wells were also coated with 1% (w/v) BSA in PBS. *C. jejuni* was harvested from 24 h BA plate and re-suspended in PBS at an OD₆₀₀ of 0.1 (~ 1 x 10⁷ cfu). Wells were rinsed with PBS and 100 µl of the bacterial suspension was added to each well and incubated at 37°C in a CO₂ incubator for 1 h. The wells were washed three times with PBS and adherent bacteria were removed by the addition of 0.05% (w/v) Trypsin / 0.02% (w/v) EDTA in Hanks' Balanced Salt Solution. To enumerate the number of adherent bacteria, serial dilutions of the trypsin suspension were plated on BA plates.

2.4.3 Statistical Analysis

Statistical analysis was performed using Prism software (GraphPad Software). Data are presented as mean ± standard deviation (SD). All experiments represent three biological replicates performed in triplicate for each experiment. Variables were compared using Student's *t* test, *p* values of *p* <0.05 were considered significant and *p* <0.001 were considered highly significant.

Chapter 3. Reinvestigation of *C. jejuni* adhesion to and invasion of intestinal epithelial cells

3.1 Introduction

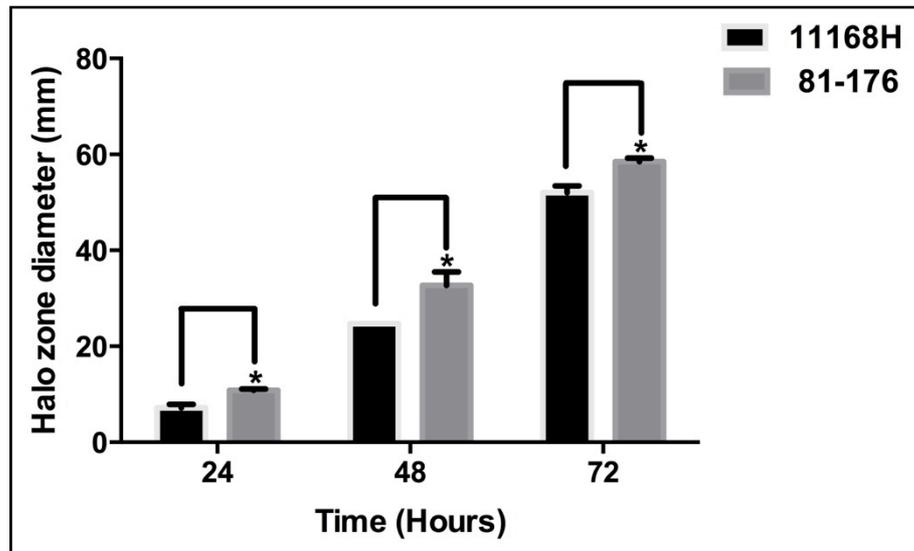
C. jejuni adhesion to, invasion of and survival within IECs has been widely studied using standard *in vitro* tissue culture assays (see Section 1.2.3). *C. jejuni* strains are known to exhibit differences in the ability to adhere and invade different cell lines. (Newell *et al.*, 1985, Konkel and Joens, 1989, Everest *et al.*, 1992, Friis *et al.*, 2005). Multiple mechanisms of invasion have been reported (see Section 1.5.1). As such, there is currently considerable confusion in the literature regarding *C. jejuni* invasion mechanisms and also the inhibition of *C. jejuni* invasion by modulating host cell pathways (see Section 1.5.3). The initial aim of this study was to reinvestigate *C. jejuni* adhesion to and invasion of intestinal epithelial cells using two independent widely studied wild-type strains. 11168H is a hypermotile derivative of the sequence strain NCTC11168 (Karlyshev *et al.*, 2004), that displays high levels of colonisation in a chick model (Jones *et al.*, 2004) and as such is considered a better strain to use to investigate host-pathogen interactions. *C. jejuni* 81-176 is a gastroenteritis isolate from a multistate outbreak from contaminated milk (Korlath *et al.*, 1985a) and one of the most invasive *C. jejuni* laboratory strains (Friis *et al.*, 2005).

3.2. Results

3.2.1 Motility

C. jejuni motility plays an important role in infection of humans and colonisation of other animals (Black, Levine *et al.* 1988). Prior to investigating adhesion to and invasion of IECs, comparative differences in the motility of the *C. jejuni* wild-type strains were investigated. Motility assays were performed as described in Section 2.1.8. Surprisingly, the 81-176 wild-type strain displayed a more motile phenotype than the 11168H wild-type strain (see Figure 3.1).

A)



(B)

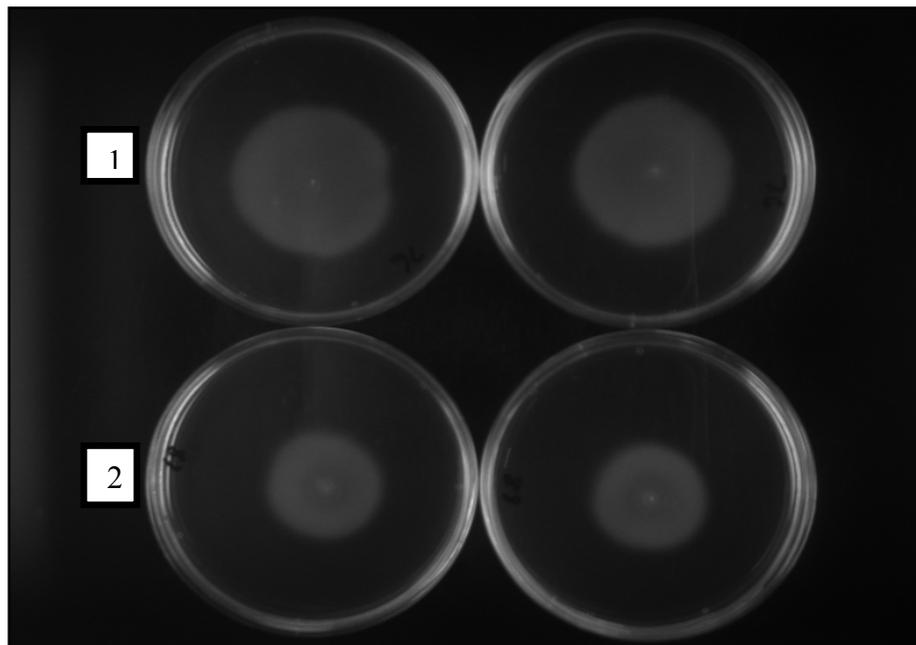


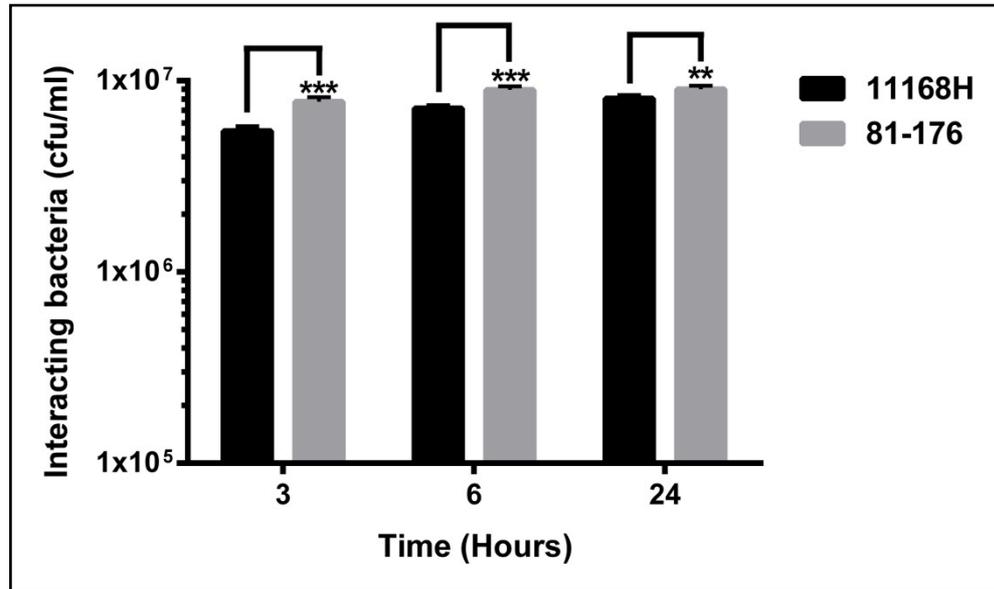
Figure 3.1 Motility assays. Bacteria were grown for 24 h on blood agar. A suspension was prepared and adjusted to an OD_{600} of 0.1. 2 μ l of this suspension was pipetted into the centre of soft agar plates and incubated at 37°C. The level of motility was assessed by measuring the diameter of growth at 24 h, 48 h and 72 h. (A) Differences in level of motility for the 11168H and 81-176 wild-type strains. (B) A representative image showing the motility of (1) 81-176 and (2) 11168H at 48 h. Data is representative of three independent experiments. Asterisks denote a statistically significant difference ($* = p < 0.05$) for the 81-176 wild-type compared to the 11168H wild-type.

3.2.2 The *C. jejuni* 81-176 wild-type strain exhibits higher levels of interaction with and invasion of Caco-2 IECs.

Comparison of the two 11168H and 81-176 wild-type strains after 3 h, 6 h and 24 h co-culture with Caco-2 IECs, revealed significant differences for both interaction and invasion (see Figure 3.2).

The 81-176 wild-type strain interacted and invaded at a significantly higher level than the 11168H wild-type strain. Previous studies have suggested that 81-176 is much more invasive in standard tissue culture assays (Oelschlaeger *et al.*, 1993, Hu and Kopecko, 1999). Therefore the 81-176 wild-type strain was selected for use in further experiments.

(A)



(B)

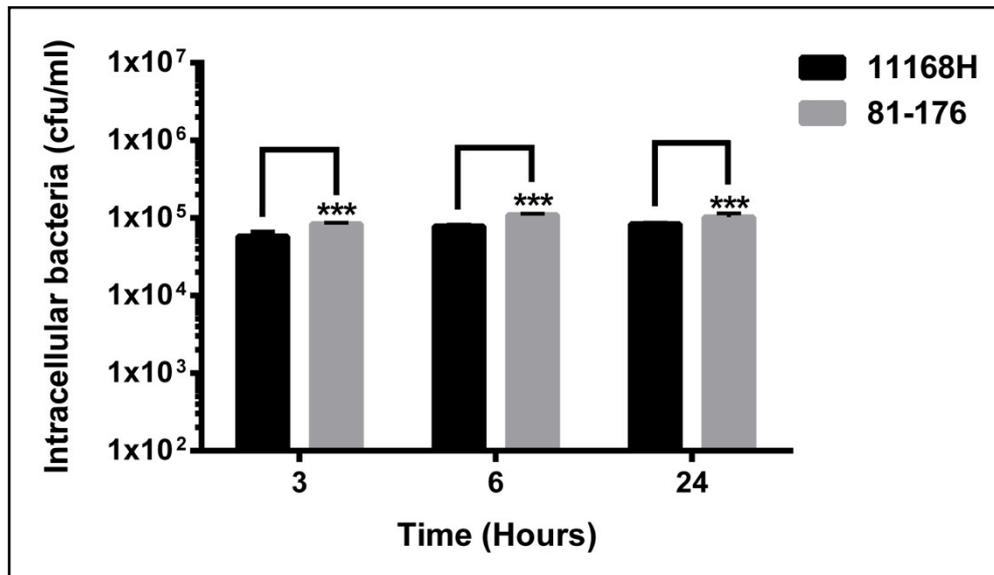


Figure 3.2 Interaction (adhesion and invasion) and invasion assays. *C. jejuni* wild-type strains 11168H & 81-176 were co-cultured with Caco-2 IECs over a period of 3 h, 6 h and 24 h. For interaction assays (A) Caco-2 IECs were lysed with 0.2% (v/v) Triton X-100 and interacting bacteria enumerated. For invasion assays (B) Caco-2 IECs were incubated for 2 h with gentamicin (150 µg/ml) to kill any extracellular bacteria, the IECs were then lysed with Triton X-100 and intracellular bacteria enumerated. Asterisks denote a statistically significant difference (** = $p < 0.01$, *** = $p < 0.001$) for the 81-176 wild-type compared to the 11168H wild-type. Data are representative of triplicate independent experiments.

3.2.3 Inhibitors of bacterial invasion of eukaryotic cells

The inhibitors of bacterial invasion used in this study were selected for the ability to target host cell structures or effector proteins which have been previously shown to be hijacked for bacterial invasion (Wells *et al.*, 1998, Yoshida and Sasakawa, 2003, Pizarro-Cerda and Cossart, 2006, Krachler *et al.*, 2011). The purpose of this study was to determine whether microfilaments, microtubules, caveolae-mediated endocytosis, clathrin-mediated endocytosis and host cell signalling pathways (PI3-Kinase) are targeted during *C. jejuni* invasion of IECs.

Table 3.1 List of inhibitors of bacterial invasion used in this study.

Inhibitor	Inhibitor action	Inhibitor mechanism
Cytochalasin D (CytD)	Microfilament disruption	Blocks actin polymerisation by binding to fast growing microfilament ends, blocking both assembly and disassembly of individual the actin monomers.
Methyl-beta-cyclodextrin (M β CD)	Inhibits caveolae mediated endocytosis	Depletes cholesterol from cell membrane/lipid rafts
Colchicine	Microtubule disruption	Disrupts cell division by interfering with the assembly and disassembly of tubulin into microtubule polymers
Wortmannin	Inhibits phosphatidylinositol 3-kinase (PI 3-kinase)	Covalently binds to the catalytic p110 subunit of mammalian PI-3 kinase
Monodansylcadaverine (MDC)	Blocks clathrin-mediated endocytosis	Inhibits clustering and internalisation of ligand-receptor complexes into clathrin-coated vesicles

The concentration of each inhibitor was selected for maximal inhibitory effect without affecting the integrity of the epithelial cell monolayer based on published studies (see Table 2.2.13).

3.2.4 Investigation of the cytotoxicity of individual inhibitors

Prior to incorporating pharmacological inhibitors into interaction and invasion assays, cytotoxicity assays and confocal microscopy was performed to ensure that the individual inhibitors had no effect on the viability of the Caco-2 IECs and that the results observed following co-culture were due to the interaction between *C. jejuni* and the Caco-2 IECs. The cytotoxic effect of the individual inhibitors was similar to that of DMEM with no inhibitor added and was not statistically different.

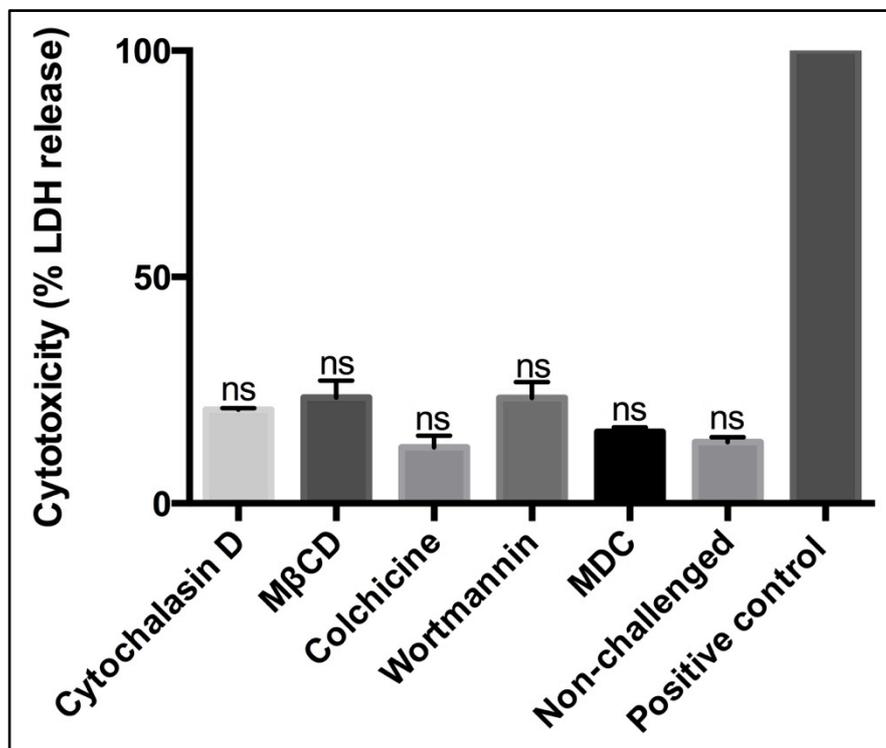
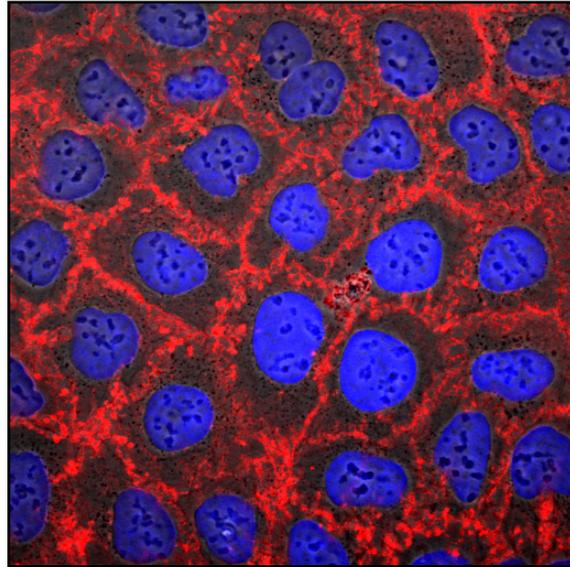


Figure 3.3 Cytotoxic effect of biochemical inhibitors on Caco-2 intestinal epithelial cells. Caco-2 IECs were pre-treated with each of the respective inhibitors for 1 h, supernatants removed and monolayers washed 3 times with PBS. Fresh media was added and the IECs incubated for a further 24 h. The cytotoxic effect on the Caco-2 IECs was measured by quantifying the release of cytosolic lactate dehydrogenase (LDH) as a measure of cell damage. Total lysis of cells following treatment with 1% (v/v) Triton X-100 represented the 100% cytotoxicity control. (ns = no significant difference). Data are representative of triplicate independent experiments.

In addition to performing cytotoxicity assays, the integrity of the cells following treatment with individual inhibitors and DMSO was investigated using confocal microscopy to visually ensure cell integrity. No significant differences between the pre-treated and untreated Caco-2 IECs was observed. A representative image comparing CytD pre-treated Caco-2 IECs with untreated Caco-2 IECs is shown (see Figure 3.4)

(A)



(B)

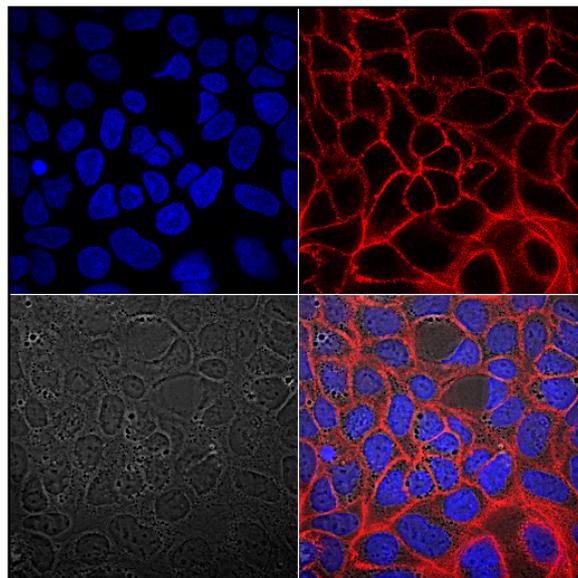


Figure 3.4 Confocal imaging of Caco-2 IECs following pre-treatment with selected inhibitors. Caco-2 IECs were grown on cover slips in 24 well plates for 7 days and pre-treated with each of the respective inhibitors for 1 h, supernatants removed and IECs washed 3 times with PBS. The cover slips were fixed and stained with Phalloidin for actin (red) and DAPI for nuclein (blue) (A) control Caco-2 IECs with no inhibitor pre-treatment and (B) Caco-2 IECs following pre-treatment with 2 μ M CytD.

As none of the inhibitors of bacterial invasion had a significant negative effect on IEC viability, the effect of the inhibition of eukaryotic cell processes on bacterial adhesion and invasion was investigated.

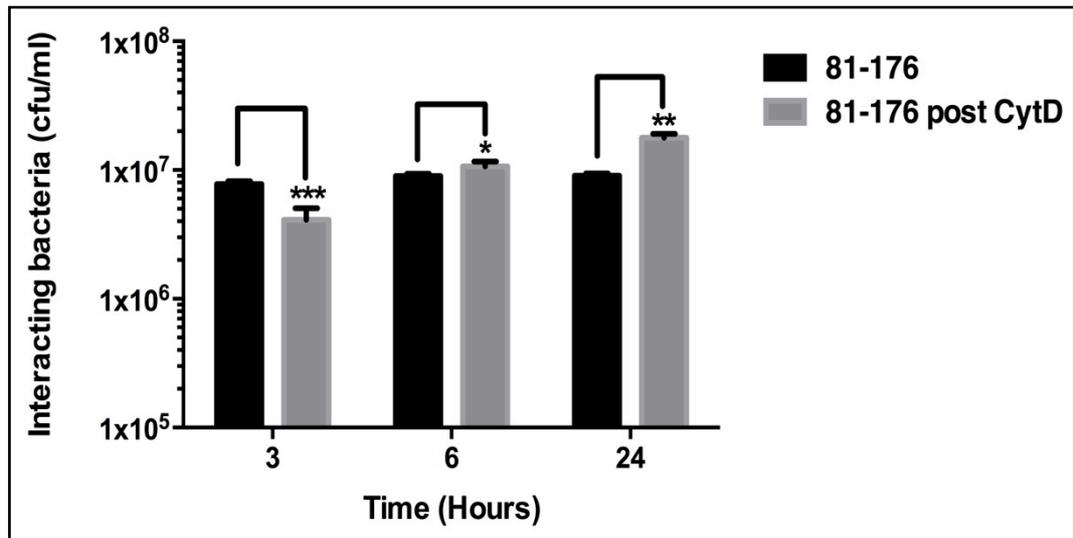
3.2.5 Disruption of microfilaments by inhibition of actin polymerisation using Cytochalasin D enhances *C. jejuni* invasion

Microorganisms have been found to utilise the host cell cytoskeleton to allow uptake into the host cell or to promote movement from within the cell (Rosenshine *et al.*, 1994). Invasion assays are a functional way of ascertaining whether pathogens are exploiting the host cell machinery. Cytochalasin D (CytD) has been shown to inhibit the entry into host cells of *Listeria monocytogenes* (Cossart, 1995), *Shigella flexneri* (Mounier *et al.*, 1997) and enteropathogenic *E. coli* (Sanger *et al.*, 1996). Differing results have been reported with the use of CytD on *C. jejuni* invasion where some researchers have shown a decrease in invasion (Monteville *et al.*, 2003b), whilst others have reported an increase (Bouwman *et al.*, 2013).

Interaction and invasion assays were performed in order to study the effect of CytD on *C. jejuni* interaction and invasion of Caco-2 IECs. CytD (2 μ M) was added 1 h prior to infection with live bacterial cells. The 81-176 wild-type strain displayed an increase in both interaction and invasion levels upon co-culture with Caco-2 IECs pre-treated with CytD compared to the untreated Caco-2 IECs.

Bacterial interactions with Caco-2 IECs pre-treated with CytD were initially reduced at the 3 h time-point, followed by a significant increase in interacting bacteria observed at the 6 h and 24 h time points. *C. jejuni* invasion was significantly increased in Caco-2 IECs pre-treated with CytD at the 3 h, 6 h and 24 h time-points, with numbers of invading bacteria reaching over (10^6 cfu) compared to (10^5 cfu) observed in the untreated Caco-2 IECs.

(A)



(B)

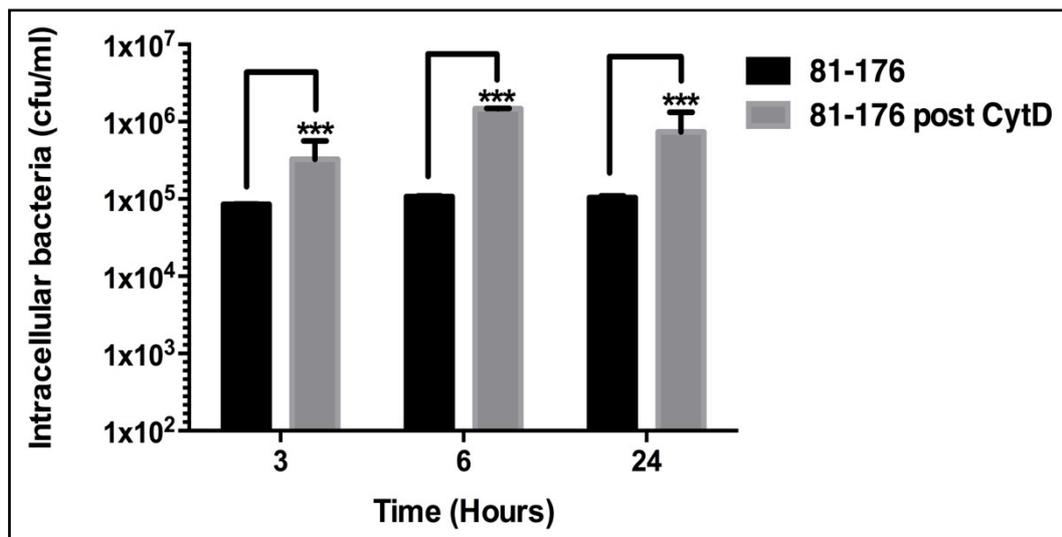


Figure 3.5. Inhibition of Interaction (adhesion and invasion) assays. Caco-2 IECs were pre-treated with CytD (2 μ M) for 1 h prior to infection. *C. jejuni* 81-176 were co-cultured with Caco-2 IECs that were pre-treated with CytD or a control with no inhibitor treatment, over a period of 3 h, 6 h and 24 h. For interaction assays (A), Caco-2 IECs were lysed with 0.2% (v/v) Triton X-100 and interacting bacteria enumerated. For invasion assays (B), Caco-2 IECs were incubated for 2 h with gentamicin (150 μ g/ml) to kill any extracellular bacteria, IECs were then lysed with Triton X-100 and intracellular bacteria enumerated. Asterisks denote a statistically significant difference (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$) for 81-176 post CytD compared to 81-176 with no pre-treatment. Data are representative of triplicate independent experiments.

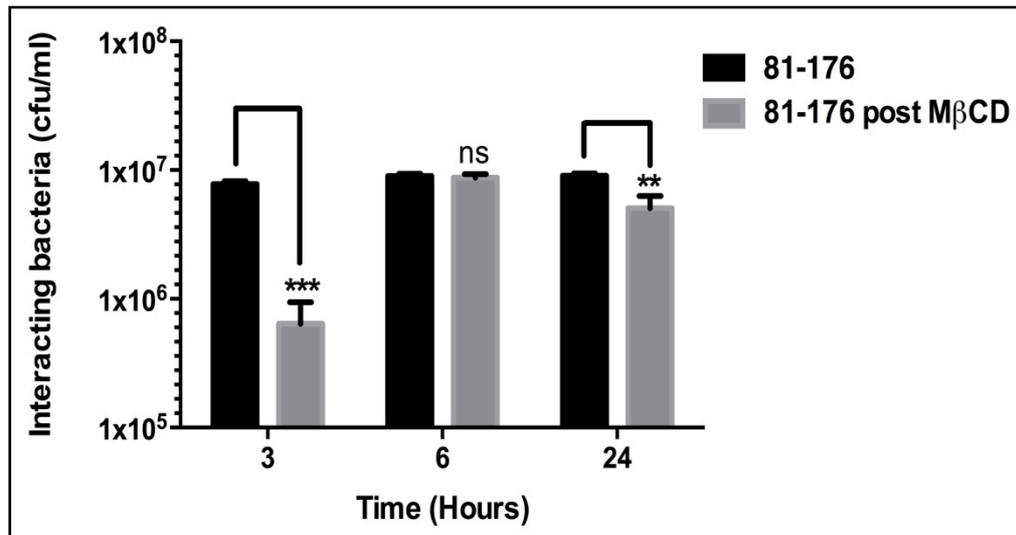
3.2.6 Disruption of lipid rafts by removal of cholesterol from the cell plasma membrane using methyl-beta-cyclodextrin reduces *C. jejuni* invasion

Cyclodextrins are capable of depleting cholesterol from the cell membrane and therefore prevent endocytosis involving caveolae entry (Rohde *et al.*, 2003). The use of M β CD has been found to be more efficient in comparison to other cyclodextrins (Klein *et al.*, 1995). Bacterial internalisation has been found to be reduced by sequestering of cholesterol (Lafont, 2012). M β CD has been shown to block internalisation of *C. jejuni* into Caco-2, T84 and INT407 IECs, suggesting that caveolae-mediated endocytosis may play a role in internalisation (Wooldridge *et al.*, 1996b, Hu *et al.*, 2006c, Krause-Gruszczynska *et al.*, 2007a, Watson and Galan, 2008b).

The ability of *C. jejuni* to interact with and invade Caco-2 IECs pre-treated with M β CD (5 μ M) was investigated at 3 h, 6 h and 24 h time-points.

Pre-treatment with M β CD significantly reduced the number of interacting bacteria with Caco-2 IECs at the 3 h time point to 10^6 cfu compared to 10^7 cfu observed in the untreated Caco-2 IECs. By the 6 h time point, there was no difference observed in the numbers of interacting bacteria. At the 24 h time point, a significant decrease in interacting bacteria was observed with numbers dropping below 10^7 cfu compared to the untreated Caco-2 IECs. Pre-treatment of Caco-2 IECs with M β CD had a more significant effect on the number of intracellular bacteria with a severe reduction over the infection period, with only a slight increase from the 3 h time point (10^3 cfu) to the 6 h and 24 h time-points (10^4 cfu) in comparison to no M β CD pre-treatment (10^5 cfu). This data suggests that caveolae-mediated endocytosis plays a role in both *C. jejuni* interactions with and invasion of Caco-2 IECs.

(A)



(B)

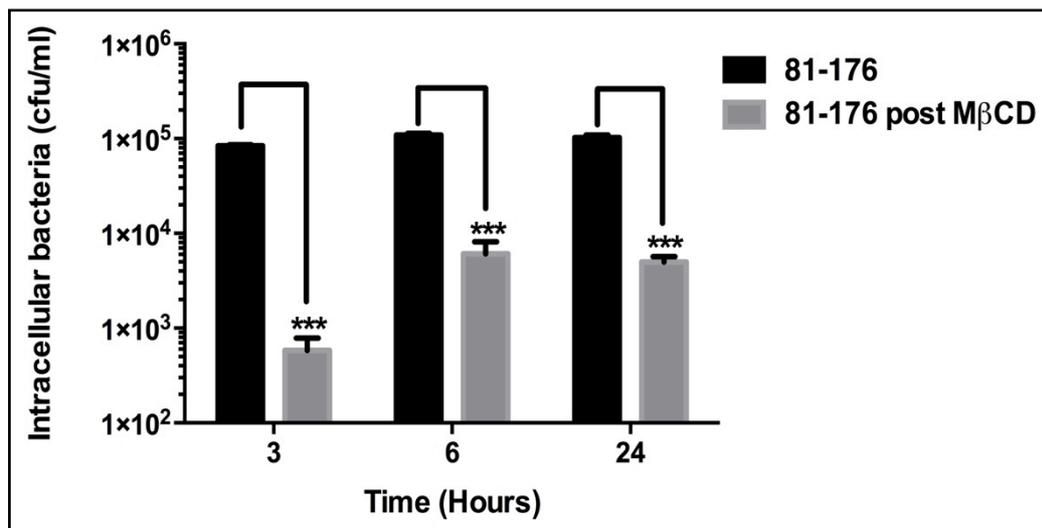


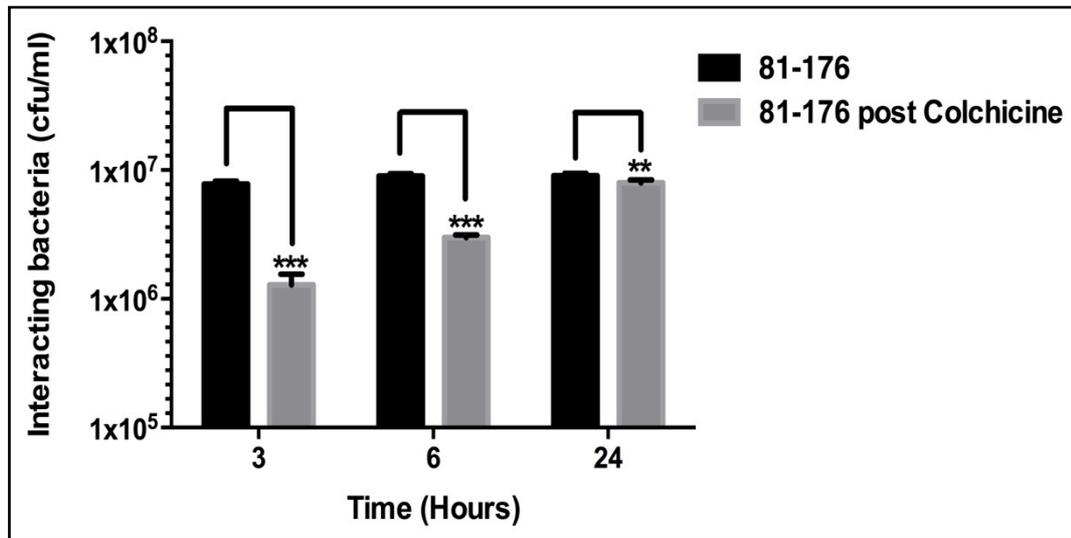
Figure 3.6 Inhibition of Interaction (adhesion and invasion) and invasion assays. Caco-2 IECs were pre-treated with MβCD (5 μM) for 1 h prior to infection. *C. jejuni* 81-176 were co-cultured with Caco-2 IECs that were pre-treated with MβCD or a control with no inhibitor treatment, over a period of 3 h, 6 h and 24 h. For interaction assays (A), Caco-2 IECs were lysed with 0.2% (v/v) Triton X-100 and interacting bacteria enumerated. For invasion assays (B), Caco-2 IECs were incubated for 2 h with gentamicin (150 μg/ml) to kill any extracellular bacteria, IECs were then lysed with Triton X-100 and intracellular bacteria enumerated. Asterisks denote a statistically significant difference (** = $p < 0.01$; *** = $p < 0.001$; ns = no significant difference) for 81-176 post MβCD compared to 81-176 with no pre-treatment. Data are representative of triplicate independent experiments.

3.2.7 Disruption of microtubules using colchicine reduces *C. jejuni* invasion.

Microtubules have been implicated in the invasion mechanisms of *C. jejuni*, *Citrobacter freundii* and enteropathogenic *E. coli* (EPEC) (Donnenberg *et al.*, 1990, Oelschlaeger *et al.*, 1993, Hu and Kopecko, 1999, Biswas *et al.*, 2003). *C. jejuni* invasion of INT407 IECs was blocked in the presence of colchicine (Biswas *et al.*, 2003) but the same effect was not observed in T84 IECs (Wine *et al.*, 2008). Tubulin is essential for cell division and previous studies have found that colchicine is able to bind to the subunit of tubulin heterodimers forming a tubulin–colchicine complex which inhibits the polymerisation of tubulin and therefore affected cytoskeletal changes (Wei *et al.*, 2013). In this study, inhibition of microtubules reduced the numbers of *C. jejuni* interacting with and invading Caco-2 IECs.

The ability of *C. jejuni* to interact with and invade Caco-2 IECs in the presence of colchicine (5 µg) was investigated at 3 h, 6 h and 24 h time-points. Pre-treatment with colchicine significantly reduced *C. jejuni* interaction with Caco-2 IECs at the 3 h time-point, with numbers of interacting bacteria recovering by the 24 h time-point. Pre-treatment with colchicine significantly reduced the number of intracellular *C. jejuni* (~ 10³ cfu) compared to no colchicine pre-treatment (~ 10⁵ cfu) at all time points.

(A)



(B)

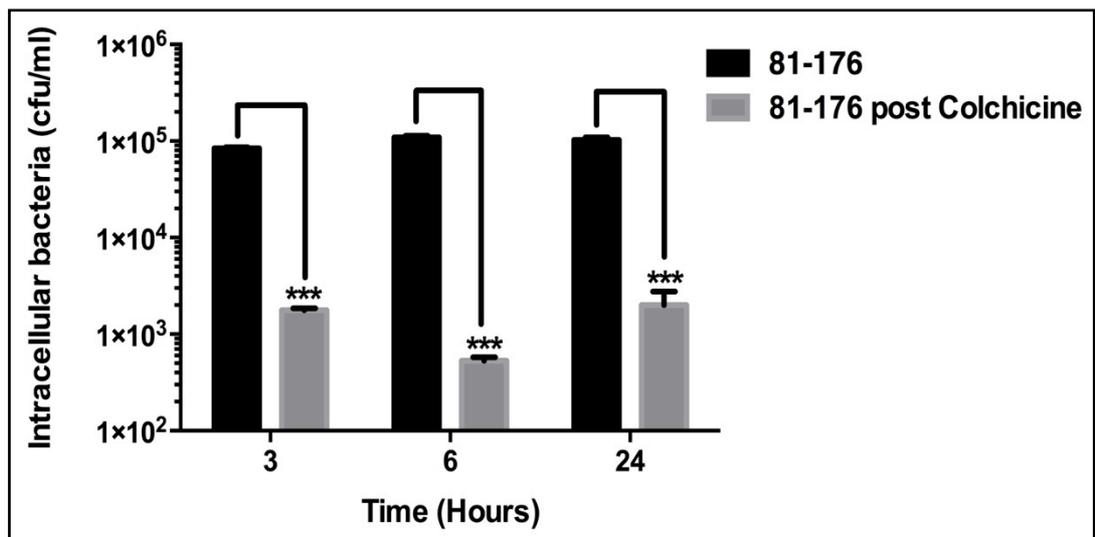


Figure 3.7 Inhibition of Interaction (adhesion and invasion) and invasion assays. Caco-2 IECs were pre-treated with colchicine ($5 \mu\text{g}$) for 1 h prior to infection. *C. jejuni* 81-176 were co-cultured with Caco-2 IECs that were pre-treated with colchicine or a control with no inhibitor treatment, over a period of 3 h, 6 h and 24 h. For interaction assays (A), Caco-2 IECs were lysed with 0.2% (v/v) Triton X-100 and interacting bacteria enumerated. For invasion assays (B), Caco-2 IECs were incubated for 2 h with gentamicin ($150 \mu\text{g/ml}$) to kill any extracellular bacteria, IECs were then lysed with Triton X-100 and intracellular bacteria enumerated. Asterisks denote a statistically significant difference ($** = p < 0.01$; $*** = p < 0.001$) for 81-176 post colchicine compared to 81-176 with no pre-treatment. Data are representative of triplicate independent experiments.

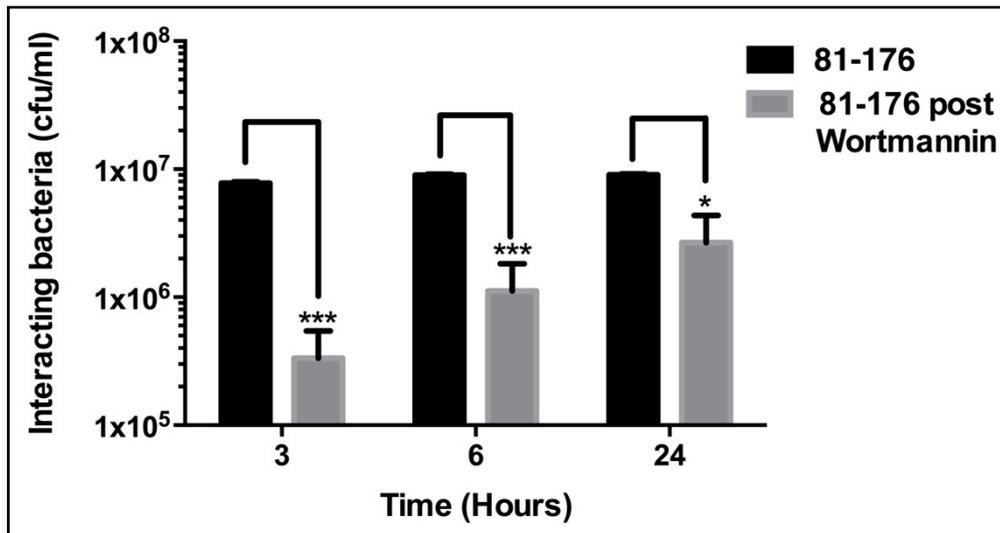
3.2.8 Disruption of the Phosphatidylinositol 3-kinase pathway with wortmannin reduces *C. jejuni* invasion

Bacteria can interact with host cells via engagement of receptors on the host surface with those on the microbial surface. This process enables cross-talk to take place, triggering cell signalling events which in turn disrupt or subvert numerous eukaryotic cell activities, manipulate the host cell cytoskeleton and trigger bacterial entry, promoting survival and replication (Fasano, 1998).

PI3-kinases are signal transducer enzymes, involved in cell growth, motility, differentiation, proliferation, survival and intracellular trafficking (Fry, 1994, Rameh and Cantley, 1999, Katso *et al.*, 2001). Microbial activation of PI3-Kinase leads to dramatic changes in the host cell, triggering of Rac1 downstream leads to massive cytoskeletal deformation, actin rearrangements and membrane ruffling (Hawkins *et al.*, 1995, Pizarro-Cerda and Cossart, 2004). In the presence of wortmannin, both *C. jejuni* invasion and host cell GTPase activation were inhibited in INT407 cells (Krause-Gruszczynska *et al.*, 2011).

The ability of *C. jejuni* to interact with and invade Caco-2 IECs pre-treated with wortmannin (1 μ M) was investigated at 3 h, 6 h and 24 h time-points. Pre-treatment with wortmannin initially produced a reduction in the number of interacting bacteria at the 3 h time point ($<10^6$ cfu) compared to the control with no inhibitor added (10^7 cfu). The number of interacting bacteria recovered at the 6 h and 24 h to levels still lower than those observed with no inhibitor. Pre-treatment with wortmannin significantly reduced the number of intracellular *C. jejuni* to ($\sim 10^3$ cfu) at 3 h, 6 h and 24 h time-points compared to the numbers observed with no inhibitor ($\sim 10^5$ cfu).

(A)



(B)

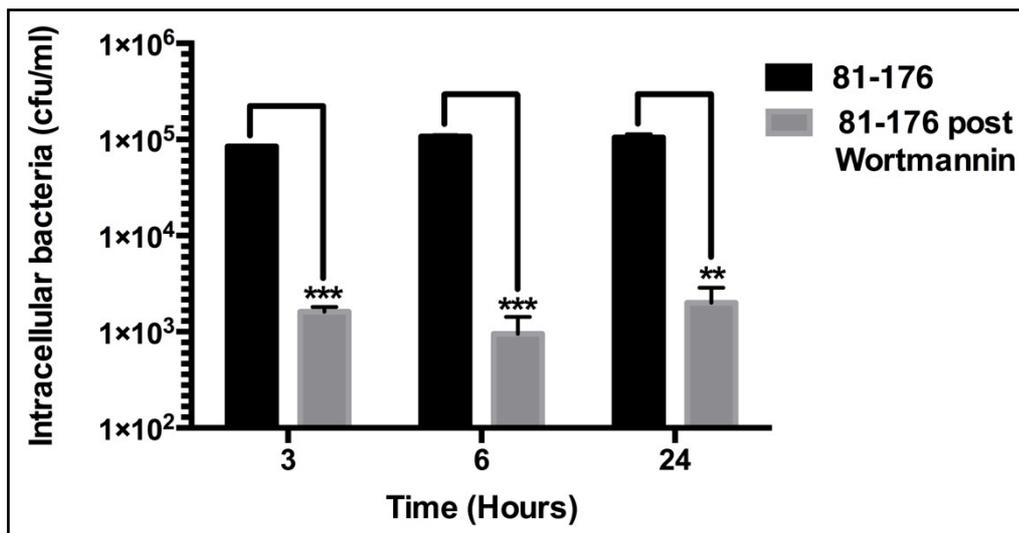


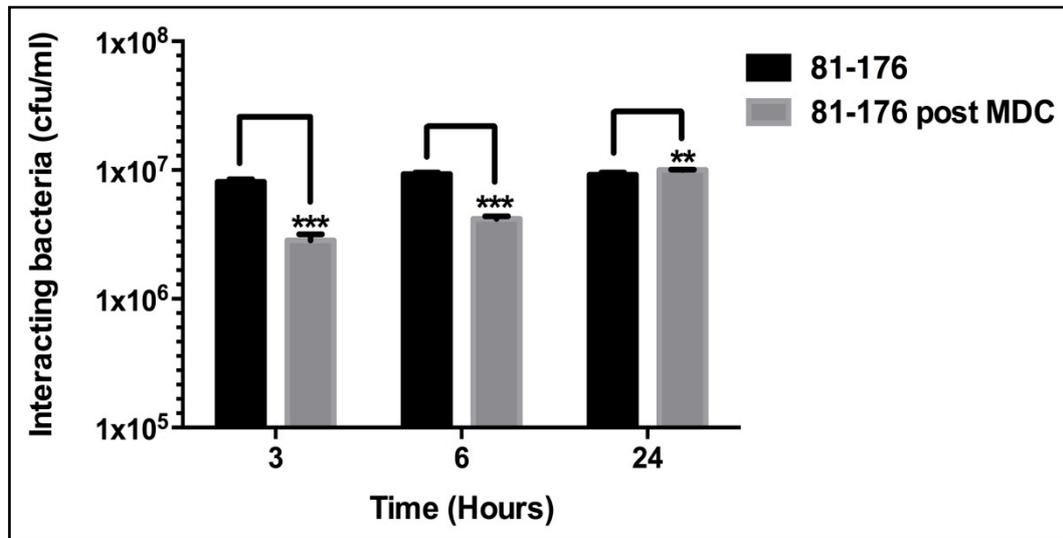
Figure 3.8 Inhibition of Interaction (adhesion and invasion) and invasion assays. Caco-2 IECs were pre-treated with wortmannin (1 μ M) for 1 h prior to infection. *C. jejuni* 81-176 were co-cultured with Caco-2 IECs that were pre-treated with wortmannin or a control with no inhibitor treatment, over a period of 3 h, 6 h and 24 h. For interaction assays (A), Caco-2 IECs were lysed with 0.2% (v/v) Triton X-100 and interacting bacteria enumerated. For invasion assays (B), Caco-2 IECs were incubated for 2 h with gentamicin (150 μ g/ml) to kill any extracellular bacteria, IECs were then lysed with Triton X-100 and intracellular bacteria enumerated. Asterisks denote a statistically significant difference (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$) for 81-176 post wortmannin compared to 81-176 with no pre-treatment. Data are representative of triplicate independent experiments.

3.2.9 Disruption of clathrin-mediated endocytosis with monodansylcadaverine reduces *C. jejuni* invasion.

Monodansylcadaverine (MDC) is a pharmacological inhibitor of clathrin-mediated endocytosis and has been used extensively in numerous mammalian cell lines (Davies *et al.*, 1980, Bradley *et al.*, 1993, Wang and Liu, 2003, Panicker *et al.*, 2006). MDC acts by directly inhibiting enzymes of the transglutaminase family which are involved in the post-transcriptional transamidation of a number of proteins (Lorand and Graham, 2003, Mishra and Murphy, 2004). In particular, the transglutaminases play key roles in the activation of RhoGTPase proteins, which are involved in regulation and control of the cell actin and cytoskeletal mechanics (Etienne-Manneville and Hall, 2002, Singh *et al.*, 2003). Disruption of transglutaminase with MDC was found to interfere with the actin dynamics of the cell (Heuser and Anderson, 1989, Kang *et al.*, 1995, Singh *et al.*, 2003). MDC was also shown to inhibit the numbers of intracellular *C. jejuni* in numerous cell lines such as INT407, T84, Hep2 and Caco-2 IEC's (Konkel *et al.*, 1992b, Oelschlaeger *et al.*, 1993, Wooldridge *et al.*, 1996b, Kopecko, 2000, Biswas *et al.*, 2000).

The ability of *C. jejuni* to interact with and invade Caco-2 IECs in the presence of MDC (0.5 mM) was investigated at 3 h, 6 h and 24 h time-points. Pre-treatment with MDC reduced the number of interacting bacteria at the 3 h and 6 h time points with the numbers recovering by 24 h compared to the controls with no inhibitor added. Pre-treatment with MDC significantly reduced the numbers of intracellular bacteria at 3 h ($<10^4$ cfu), at 6 h ($>10^4$ cfu) and at 24 h time-points ($<10^4$ cfu) compared to no MDC treatment ($\sim 10^5$ cfu).

(A)



(B)

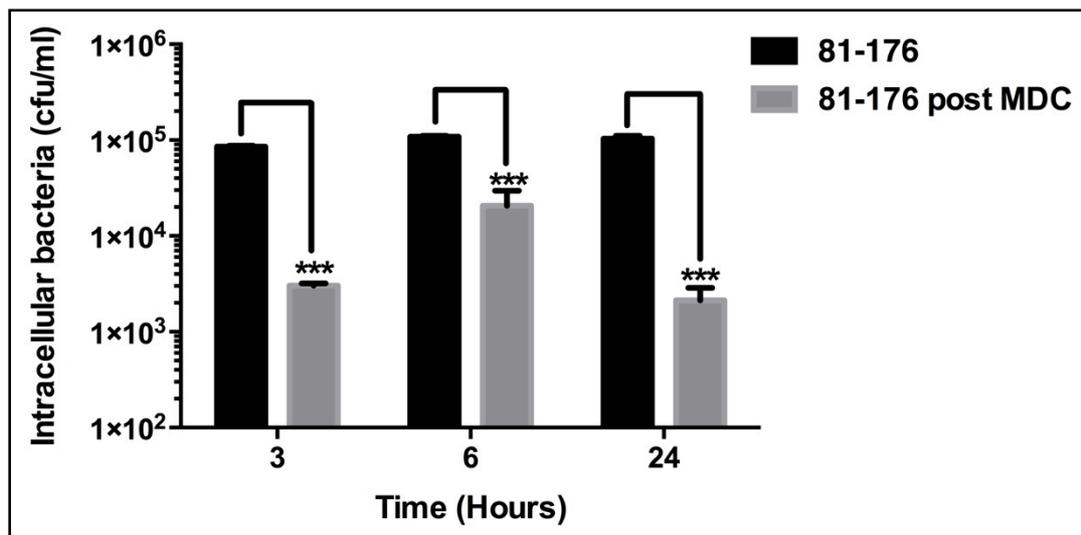


Figure 3.9 Inhibition of Interaction (adhesion and invasion) and invasion assays. Caco-2 IECs were pre-treated with MDC (0.5 mM) for 1 h prior to infection. *C. jejuni* 81-176 were co-cultured with Caco-2 IECs that were pre-treated with MDC or a control with no inhibitor treatment, over a period of 3 h, 6 h and 24 h. For interaction assays (A), Caco-2 IECs were lysed with 0.2% (v/v) Triton X-100 and interacting bacteria enumerated. For invasion assays (B), Caco-2 IECs were incubated for 2 h with gentamicin (150 µg/ml) to kill any extracellular bacteria, IECs were then lysed with Triton X-100 and intracellular bacteria enumerated. Asterisks denote a statistically significant difference (** = $p < 0.01$; *** = $p < 0.001$) for 81-176 post MDC compared to 81-176 with no pre-treatment. Data are representative of triplicate independent experiments.

3.2.10 Comparison of the inhibition of *C. jejuni* 81-176 wild-type strain interactions with and invasion of Caco-2 intestinal epithelial cells.

The inhibitors employed in this study produced either an increase or a decrease in the numbers of interacting and invading bacteria. Table 3.2 and Table 3.3 show the comparative effect of each inhibitor on *C. jejuni* interaction and invasion respectively.

Table 3.2 Effect of inhibitors on *C. jejuni* ability to interact with Caco-2 intestinal epithelial cells

Inhibitor	Effect on <i>C. jejuni</i> interaction with Caco-2 IECs
Cytochalasin D	↑ Interaction
Methyl-beta-cyclodextrin	↓ Interaction, then ↑ Interaction
Colchicine	↓ Interaction
Wortmannin	↓ Interaction, then ↑ Interaction
Monodansylcadaverine	↓ Interaction, then ↑ Interaction

Table 3.3 Effect of inhibitors on *C. jejuni* ability to invade Caco-2 intestinal epithelial cells

Inhibitor	Effect on <i>C. jejuni</i> invasion of Caco-2 IECs
Cytochalasin D	↑ Invasion
Methyl-beta-cyclodextrin	↓ Invasion
Colchicine	↓ Invasion
Wortmannin	↓ Invasion
Monodansylcadaverine	↓ Invasion

The number of *C. jejuni* interacting with or invading Caco-2 IECs was calculated as a percentage of the initial inoculum (see Table 3.4 and Table 3.5)

Table 3.4 Percentage of the initial inoculum of *C. jejuni* interacting with Caco-2 IECs following pre-treatment with individual inhibitors.

Inhibitor	3 h	6 h	24 h
Cytochalasin D	51.7	115.6	146.1
Methylbetacyclodextrin	8.8	93.0	60.8
Colchicine	0.2	32.7	88.0
Wortmannin	13.7	33.1	88.6
Monodansylcadaverine	34.9	45.1	109.3

Table 3.5 Percentage of the initial inoculum of *C. jejuni* invading Caco-2 IECs following pre-treatment with individual inhibitors.

Inhibitor	3 h	6 h	24 h
Cytochalasin D	314.3	764.1	574.1
Methylbetacyclodextrin	0.7	4.7	4.5
Colchicine	2.1	0.5	2.2
Wortmannin	2.0	47.4	1.8
Monodansylcadaverine	3.5	19.2	2.1

3.3 Imaging of *C. jejuni* adhesion to and invasion of Caco-2 intestinal epithelial cells

In order to visualize *C. jejuni* within IECs, a number of different strategies were attempted. Expression of the Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is a useful method for visualising *in vivo* host-pathogen interactions, in particular the study of bacteria within a host cell (Prasher *et al.*, 1992, Chalfie *et al.*, 1994, Inouye and Tsuji, 1994). GFP-tagged bacteria have previously been used in

ecological studies monitoring bacterial populations in activated sludge communities, in symbiotic relationships with plant cells, infection of macrophages and in survival studies of *E. coli* (Kremer *et al.*, 1995, Gage *et al.*, 1996, Eberl *et al.*, 1997, Yrlid *et al.*, 2001). The use of GFP has many advantages over conventional antibody staining, including a visual check for viability of the bacteria and a confirmation of the presence of bacteria. In addition expression of GFP in bacteria is less disruptive to the biomechanics of the bacterial cell than staining (Errampalli *et al.*, 1999). The major disadvantage of existing GFP-like reporter proteins (GFP or eGFP) is that these GFP proteins require oxygen as a co-factor to enable post-translational folding of the GFP protein and allow the expression of the fluorescence chromophores, although this can be remedied with further genetic mutagenesis procedures (Cubitt *et al.*, 1995). Due to the strict microaerobic growth requirements of *C. jejuni*, GFP expressing *C. jejuni* produced very low levels of fluorescent signal following infection of Caco-2 IECs (Labigne-Roussel *et al.*, 1987, Guerry *et al.*, 1994, Miller *et al.*, 2000), therefore an alternative fluorescence reporter system was investigated. The flavin mononucleotide (FMN)-based fluorescent proteins from the Evoglow® series were developed to overcome the above restrictions. These proteins can be used as fluorescent reporters in both aerobic and anaerobic biological systems.

In order to study the localisation of *C. jejuni* within intestinal epithelial cells, *C. jejuni* expressing eGFP and Evoglow® were constructed to investigate the behaviour of the bacteria once inside the host cell.

3.3.1 Construction of Evoglow® expressing *C. jejuni* strains

Evoglow genes *pp1* (441 bp) and *bs1* (411 bp) were inserted into either *C. jejuni* 81-176 or 11168H wild-type strains using the *C. jejuni* complementation vector pRRC (Karlyshev and Wren, 2005). The coding regions for *pp1* and *bs1* were amplified from the respective Evoglow® plasmid (pGLOW) by PCR using primers pGLOW-*pp1*-Forward/pGLOW-*pp1*-Reverse and pGLOW-*bs1*-Forward/pGLOW-*bs1*-Reverse which also introduced an XbaI site at the 5' end and at the 3' end. Following digestion with XbaI, each PCR product was ligated into the pRRC vector. The pRRC-*pp1/bs1* constructs were transformed into *E. coli* DH5 α cells and plated out on LB chl agar plates. Positive colonies selected by growth on LB chl agar were screened using boilate PCR to check for the presence of *pp1/bs1*. Each construct was checked by PCR,

sequenced and electroporated into either the 81-176 or 11168H wild-type strain. Positive clones were selected on blood agar plates containing chloramphenicol. Confirmation of the presence of *pp1* and *bs1* was performed by PCR using the pRRC-Forward and pGLOW-*pp1/bs1*-Reverse primers (see Figure 3.10). The amplified size of *pp1* is 441 bp, the amplified size of *bs1* is 411 bp and successful insertion of each gene fragment into the pRRC vector produced a total amplified size for pRRC-*pp1* construct of 566 bp and for pRRC-*bs1* construct of 536 bp.

Eg: (pRRC-Forward primer amplified (125 bp) + *pp1* gene specific reverse (441 bp) = 566 bp) and (pRRC- Forward primer amplified (125 bp) + *bs1* gene specific reverse (411 bp) = 536 bp).

3.3.2 Construction of eGFP construct expressing *C. jejuni* strains

The eGFP plasmids were obtained from Dr Dominic Mills (LSHTM). *C. jejuni* competent cells were prepared and 1 µg of the purified eGFP plasmid DNA was used to transform the *C. jejuni* 81-176 or 11168H wild-type strains. Positive clones were selected on blood agar plates containing chloramphenicol. Plates were incubated for 24 h under microaerobic conditions. Positive colonies were tested by PCR using eGFP-Forward and eGFP-Reverse primers for the presence of eGFP gene (720 bp) and confocal microscopy for the expression of the eGFP.

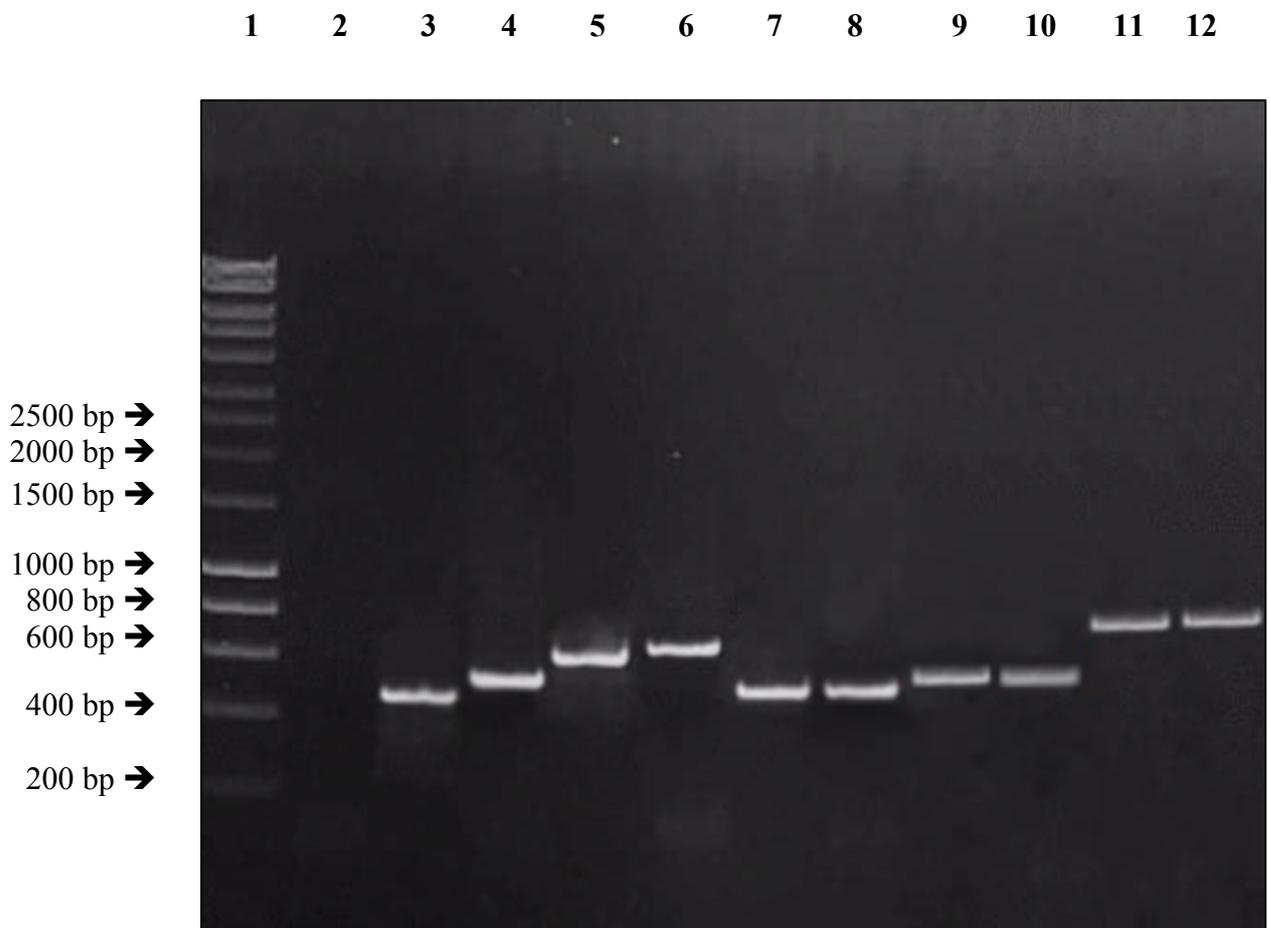


Figure 3.10. PCR verification of Evoglow® genes and *C. jejuni* expressing Evoglow® and eGFP genes. Lane 1: 1 Kb ladder (Bioline). Lane 2: Negative control. Lane 3: Evoglow® *bsl*. Lane 4: Evoglow® *ppl*. Lane 5: pRRC-*bsl*-construct. Lane 6: pRRC-*ppl*-construct. Lane 7: 11168H Evoglow® *bsl*. Lane 8: 81-176 Evoglow® *bsl*. Lane 9: 11168H Evoglow® *ppl*. Lane 10: 81-176 Evoglow® *ppl*. Lane 11: eGFP 11168H. Lane 12: eGFP 81-176

3.3.3 Comparison of eGFP versus Evoglow® expressing *C. jejuni* strains

Following construction of eGFP and Evoglow® expressing *C. jejuni* wild-type strains, mean fluorescence intensity (MFI) was analysed and compared using confocal microscopy. The eGFP expressing strains displayed higher levels of fluorescence compared to the Evoglow® expressing *C. jejuni* strains (see Figure 3.11).

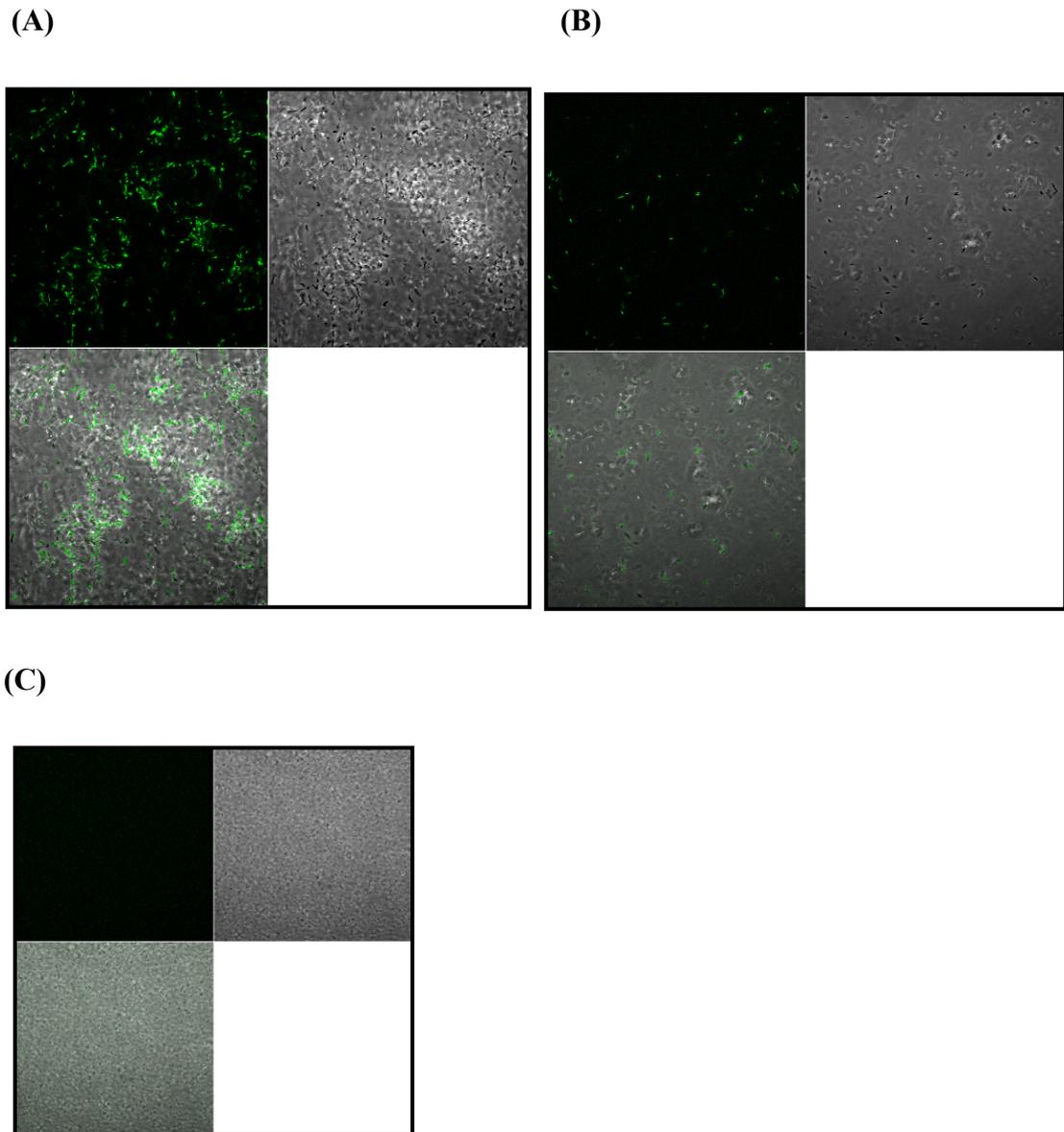
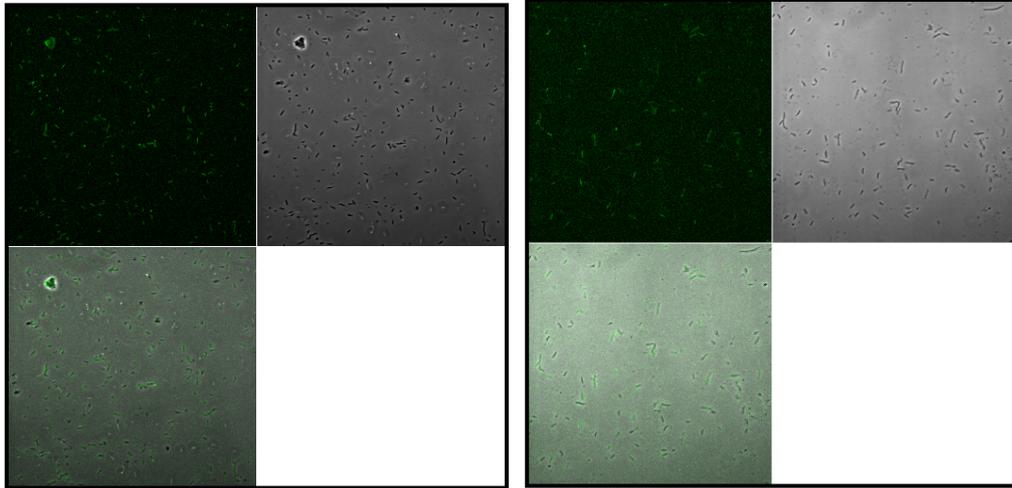


Figure 3.11 Confocal images of *C. jejuni* strains expressing eGFP. *C. jejuni* strains expressing eGFP were harvested from overnight broth cultures and samples were prepared for confocal microscopy by placing a suspension of the bacterial culture onto a sterile slide. A cover slip was placed on top, sealed with nail varnish and visualised using a confocal microscope. (A) 81-176 expressing eGFP (B) 11168H expressing eGFP (C) 81-176 wild-type control.

C. jejuni strains expressing Evoglow® (*pp1* or *bs1*) were also investigated using confocal microscopy and displayed significantly lower levels of fluorescence compared to the eGFP expressing *C. jejuni* 81-176 and 11168H strains (see Figure 3.12).

(A)

(B)



(C)

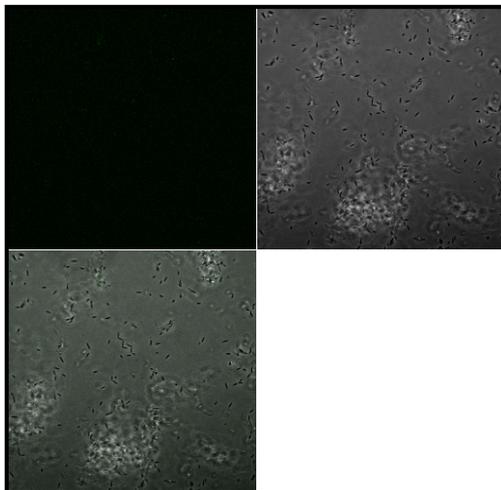


Figure 3.12 Confocal microscopy images of *C. jejuni* expressing Evoglow. *C. jejuni* strains expressing Evoglow® were harvested from overnight cultures and samples were prepared for confocal microscopy by placing a suspension of the bacterial culture onto a sterile slide. A cover slip was placed on top, sealed with nail varnish and visualised using a confocal microscope. (A) *C. jejuni* 11168H expressing Evoglow® *pp1* and (B) *C. jejuni* 81-176 expressing Evoglow® *bs1* (C) 81-176 wild-type control.

3.3.4 Comparison of the fluorescence intensity of *C. jejuni* expressing eGFP and Evoglow® using Volocity software.

In order to quantify the levels of fluorescence emitted from *C. jejuni* strains expressing either eGFP or Evoglow®, volocity software was used to measure the mean fluorescence intensity. A Zeiss LSM510 confocal microscope was used to capture images of eGFP or Evoglow® expressing *C. jejuni* strains. Image capture settings were not altered between samples to ensure the fluorescent intensities of the different samples were comparable. Images were imported into Volocity software and the fluorescence intensities of the bacteria were quantified. The results were calculated using Volocity software and statistical analysis was performed using GraphPad InStat statistical software.

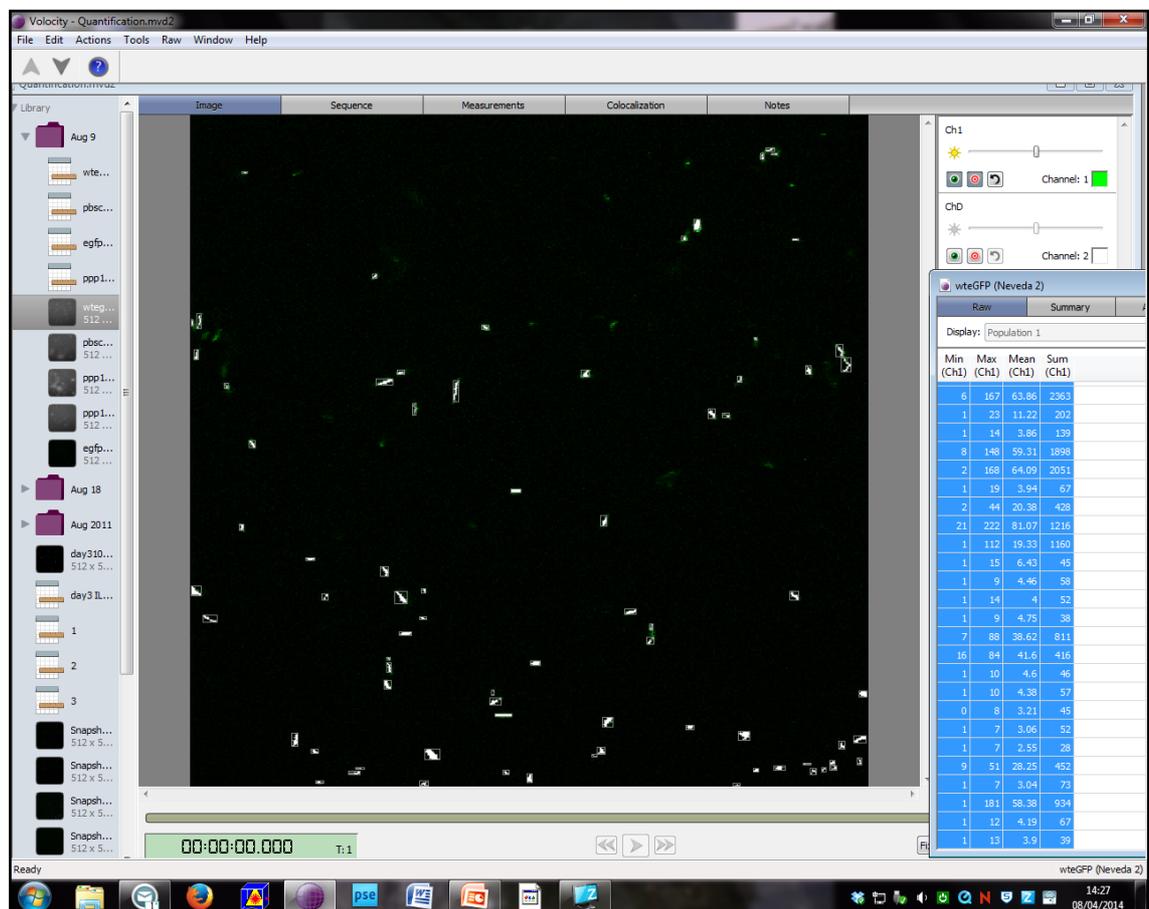


Figure 3.13 Volocity software screen grab displaying the quantification of fluorescence from confocal images. Confocal image results were loaded into Volocity software and mean fluorescence was calculated for eGFP and Evoglow® expressing *C. jejuni* strains.

Quantification of the fluorescence intensity produced by eGFP and Evoglow® expressing *C. jejuni* strains was plotted in Graphpad. The eGFP expressing *C. jejuni* strains produced a significantly higher level of fluorescence compared to the Evoglow®. *E. coli* expressing eGFP was grown aerobically and included as a control.

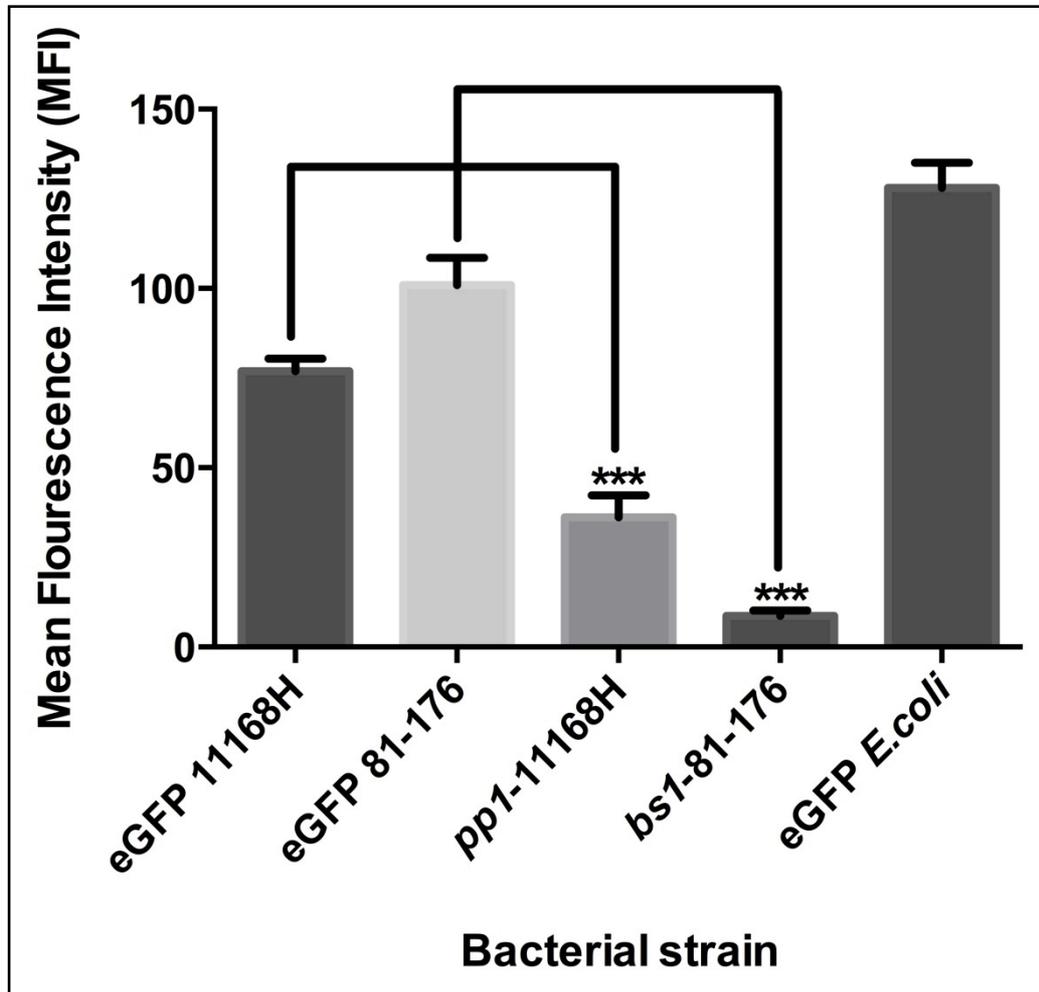


Figure 3.14 Comparison of the mean fluorescence intensity of eGFP and Evoglow® expressing *C. jejuni* strains. Mean fluorescence intensity of *C. jejuni* expressing eGFP or Evoglow® was quantified using Volocity software. Asterisks denote a statistically significant difference (***) = $p < 0.001$). Data is representative of three independent experiments.

3.3.5 Infection of Caco-2 intestinal epithelial cells with Evoglow® expressing *C. jejuni*

In order to view the interaction of Evoglow® expressing *C. jejuni* with Caco-2 IECs, following infection, the IECs were stained with DAPI and Phalloidin and viewed with the confocal microscope. eGFP expressing *C. jejuni* was also tested but resulted in only a few bacteria been visualised (data not shown).

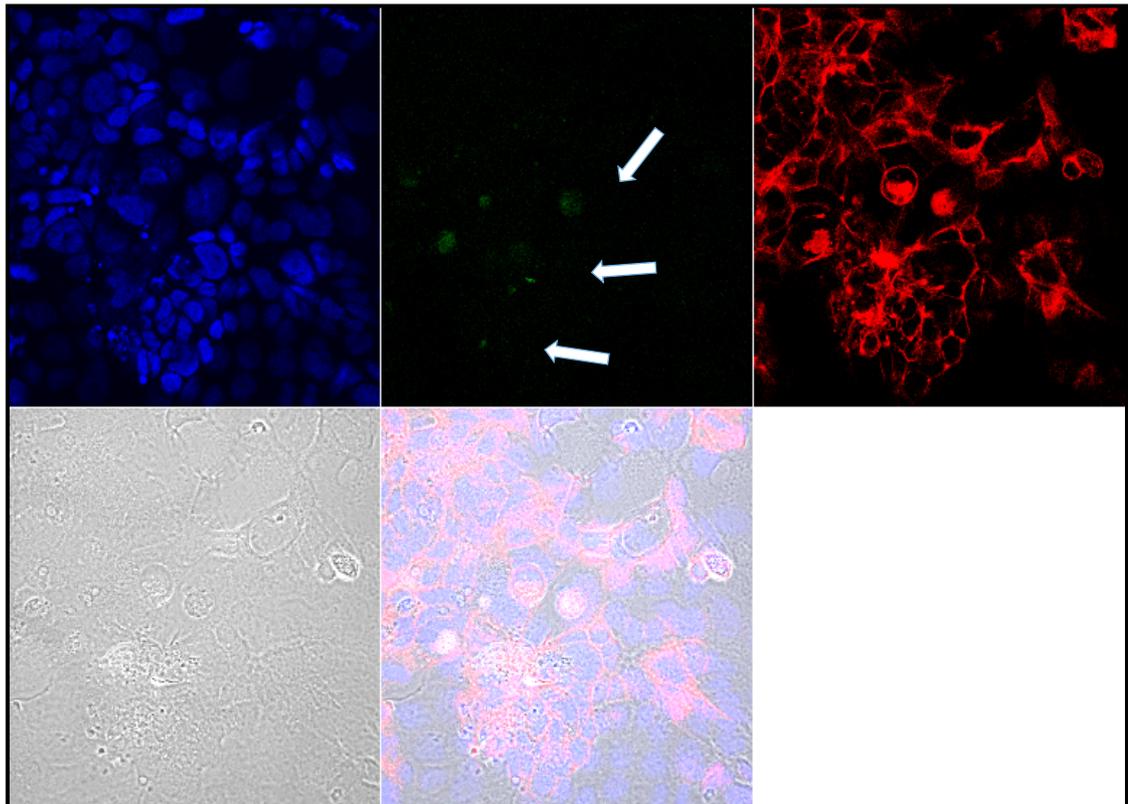
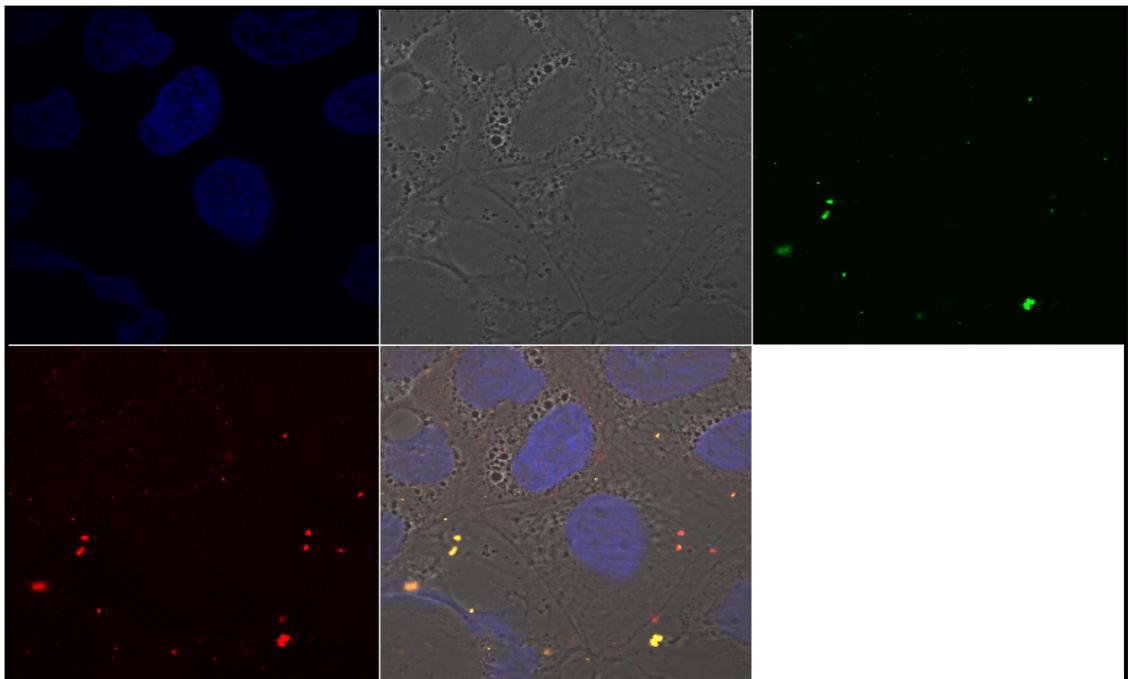


Figure 3.15 Confocal images of *C. jejuni* expressing Evoglow® co-cultured with Caco-2 intestinal epithelial cells. *C. jejuni* 81-176 expressing Evoglow® were co-cultured with Caco-2 IECs for 3 h. The slides were fixed and stained with Phalloidin for actin (red) and nuclein for DNA (blue), then analysed for green fluorescent bacteria (indicated by white arrows) with the confocal microscope.

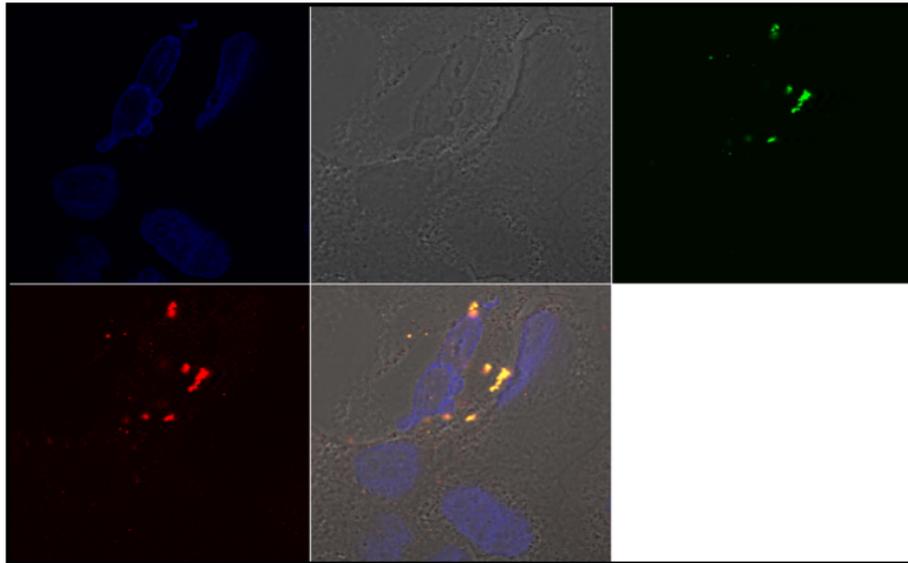
3.3.6 Anti-*C. jejuni* antibody staining to distinguish between adhered and invaded bacteria using Caco-2 IECs

Following the unsuccessful attempts at visualising Evoglow® expressing *C. jejuni* 81-176 or 11168H, antibody staining was utilised to study the localisation of both extracellular and intracellular *C. jejuni* following co-culture with Caco-2 IECs. To distinguish between extracellular and intracellular bacterial localisation, non-permeabilised cells were stained with rabbit anti-*C. jejuni* antibody (Abcam) followed by Alexa Fluor 488 conjugated Goat anti-rabbit IgG (Molecular Probes). Cells were then stained with anti *C. jejuni* antibody in the presence of saponin (Sigma, 10% stock diluted 1:20 in PBS+5% FCS) to permeabilise cells, followed by Alexa fluor 568 labelled goat anti-rabbit IgG (Molecular Probes). The latter treatment allowed for additional staining of extracellular Alexa fluor 488-labelled bacteria leading to a yellow appearance (merging of green Alexa fluor 488 and red Alexa fluor-568). In contrast, intracellular bacteria remained red, only acquiring the Alexa fluor 568 stain. Cell nuclei were counterstained with DAPI. Coverslips were then mounted in Vectashield (Vector Laboratories) and imaged on a Zeiss LSM 510 confocal microscope.

(A)



(B)



(C)

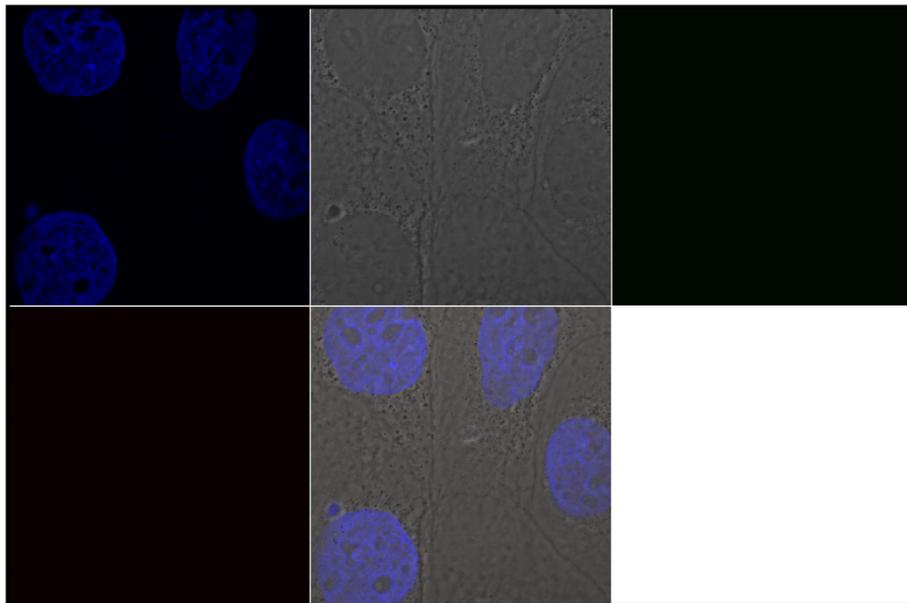
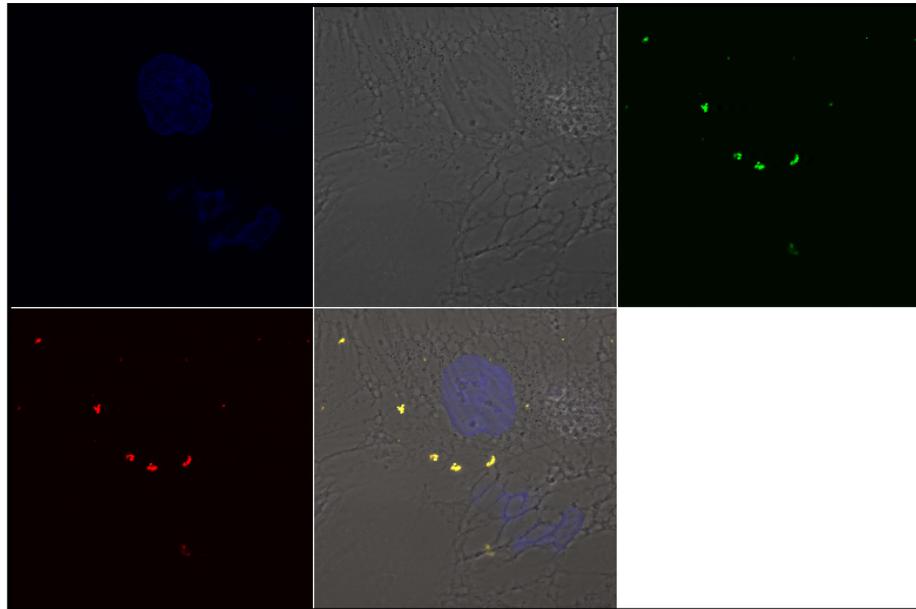


Figure 3.16 Demonstration of *C. jejuni* interaction with and invasion of Caco-2 IECs by confocal microscopy. Bacteria were co-cultured with Caco-2 IECs grown on cover slips for 2 h. IECs were fixed and then stained with anti-*C. jejuni* antibody. (A) 2 h extracellular (yellow) and intracellular (red) *C. jejuni* 81-176 (B) 2 h extracellular (yellow) and intracellular (red) *C. jejuni* 11168H. (C) Caco-2 IECs control. Representative images from two experiments (performed in triplicate) are shown.

Demonstration of both extracellular (yellow) *C. jejuni* and intracellular (red) *C. jejuni* 81-176 wild-type in Caco-2 IECs after 2 h infection (see Figure 3.17).

(A)



(B)

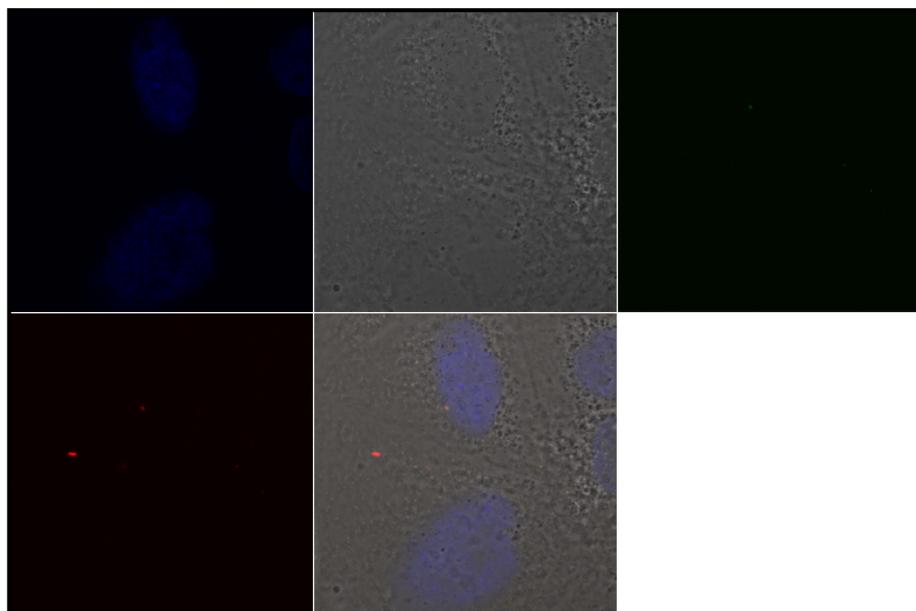


Figure 3.17 Demonstration of extracellular and invading *C. jejuni* of Caco-2 IECs by confocal microscopy. (A) Extracellular (yellow) *C. jejuni* 81-176 after 2 h infection, (B) Intracellular (red) *C. jejuni* 81-176 after 2 h infection.

3.4 Discussion

The aim of this chapter was to reinvestigate *C. jejuni* adhesion to and invasion of IECs. Initial work was carried out to choose a suitable strain for the above inhibition of invasion studies. *C. jejuni* displays varying levels of invasion depending on the cell line being utilised (see Section 1.2.4). Phenotypic characterisation showed that 81-176 displayed marginally increased levels of motility compared to 11168H. The 81-176 wild-type strain displayed higher numbers of bacteria interacting with and invading both Caco-2 and T84 IECs than the 11168H wild-type strain. Due to the 81-176 wild-type strain exhibiting higher levels of invasion, this strain was chosen to use in further studies for a better indication of inhibitor activity.

3.4.1 Inhibition of invasion studies

Tissue culture studies with *C. jejuni* using IECs have shown that *C. jejuni* is capable of utilising a number of host cellular pathways to gain access to the host cell (Oelschlaeger *et al.*, 1993, Russell and Blake, 1994, Hu and Kopecko, 1999, Kopecko *et al.*, 2001b, Monteville *et al.*, 2003b). Such studies have revealed that *C. jejuni* invasion routes may not be distinct, but require different cellular factors such as microtubules, microfilaments or lipid rafts (Young *et al.*, 2007, van Putten *et al.*, 2009, Bouwman LI 2012). These cellular pathways have previously been reported to be disrupted using different biochemical inhibitors, but the effect on *C. jejuni* interaction and invasion of IECs has produced varying results creating confusion in the literature.

The host cell structure is composed of numerous building blocks of complex proteins such as actin which make up the microfilaments and tubulin that make up the microtubules (Granger *et al.*, 2014). These structures have been shown to play pivotal roles in the mechanics of the host cell (Stossel, 1993, Small, 1994, Lauffenburger and Horwitz, 1996, Mitchison and Cramer, 1996). The disruption of microfilaments using CytD, a potent inhibitor of actin polymerisation, resulted in significant increases in invading bacteria. This increase in the numbers of intracellular *C. jejuni* within IECs pre-treated with CytD could be a result of the actin disruption and retractions, allowing *C. jejuni* bacteria to gain access to the subcellular space of the IECs via the base of the IEC. A previous study reported that some strains of *C. jejuni* were capable of efficiently gaining access to the subcellular space possibly by entering through M cells, with

migrating bacteria moving underneath the IEC and then entering, thereby avoiding the potentially hostile environment (Everest *et al.*, 1993, Walker *et al.*, 1988, van Alphen *et al.*, 2008). Disruption of actin could possibly allow more access to these subspaces.

Other researchers have reported that pre-treatment of IECs with CytD enhances *C. jejuni* internalisation, possibly by removal or damage of the actin structure, therefore actin depolymerisation and subsequent polymerisation are probably required for *C. jejuni* entry (Hu and Kopecko, 1999, Watson and Galan, 2008b). However, other researchers reported a decrease in *C. jejuni* invasion in the presence of CytD and the mixed involvement of both microtubules and microfilaments during 81-176 invasion (Monteville *et al.*, 2003b, Schlaepfer *et al.*, 1999). These differences could be attributed to the different methods employed such as centrifugation of monolayers in order to aid contact between bacteria and the IEC (Flanagan *et al.*, 2009, Novik *et al.*, 2010). The gentamicin protection assay (GPA) step can also be variable in both concentration and time-length, as some researchers use 250 µg/ml for 3 h (Buelow *et al.*, 2011a), whereas another group reported 150 µg/ml for 2 h to be sufficient to kill extracellular bacteria (Novik *et al.*, 2010). In this study 150 µg/ml of gentamicin for 2 h was found to be sufficient in killing extracellular bacteria.

The infection period of IECs is another variable, a minimum of 30 minutes to 3 h incubation periods have been utilised (Monteville *et al.*, 2003a). Different strains of *C. jejuni* and different IEC lines have been used in *C. jejuni* invasion studies which may explain some of the differences reported (Friis *et al.*, 2005, O. Croinin and Backert, 2012). In some inhibition of invasion studies with *C. jejuni*, inhibitors have been kept in the media throughout the infection period, whereas in this study Caco-2 IECs were pre-treated with the inhibitor and then washed prior to addition of bacteria (Oelschlaeger *et al.*, 1993, Watson and Galan, 2005).

The next pathway to reinvestigate was the role of caveola-mediated endocytosis in *C. jejuni* internalisation. These microdomains are found in the host cell membrane and contain cholesterol, proteins, sphingolipids and can act as surface platforms that allow bacterial interactions, binding and even internalisation (Pike, 2003, Lafont and van der Goot, 2005). Bacteria have been shown to utilise these microdomains and an advantage of this pathway is the avoidance of fusing with lysosomes (Zaas *et al.*, 2005). In this study, the use of M β CD as a cholesterol depleting agent showed that interactions and

invasion of *C. jejuni* with Caco-2 IECs were significantly reduced. A slight increase in the numbers of interacting bacteria was observed at the 6 h time-point, possibly due the bacteria making use of other cellular pathways, hence the increase in the number of interacting bacteria at later time-points. It has also been reported that infection of IECs with *C. jejuni* can cause an accumulation of lipid rafts localised to one area, whereby interaction between *C. jejuni* and lipid rafts is therefore increased, leading directly to interactions with more of the cholesterol enriched domains (Lafont and van der Goot, 2005, Kalischuk *et al.*, 2009).

Similar results published using M β CD and other cholesterol depleting agents are consistent in that *C. jejuni* cellular invasion requires cholesterol to be present in the cell. Bacteria such as *Salmonella enterica* serotype *Typhimurium* and *C. jejuni* have been found to utilise caveolae for internalisation as treatment of IECs with Fillipin III (which also binds to cholesterol) or M β CD, caused inhibition of bacterial internalization (Lim *et al.*, 2010). Previous studies have also shown that disruption of lipid rafts using Fillipin III or M β CD produced a reduction in 81-176 and N82 wild-type invasion of Caco-2, T84 and INT407 IECs (Wooldridge *et al.*, 1996b, Hu *et al.*, 2006c, Watson and Galan, 2008b, Lim *et al.*, 2010).

However, a study by Watson and Galan theorised that *C. jejuni* internalisation was not strictly due to the removal of cholesterol from lipid rafts, but the presence of certain proteins, such as caveolae or caveolin-1 playing a role in the host cell signalling events required for *C. jejuni* bacterial internalisation (Watson and Galan, 2008a). Studies by Konkel *et al.* indicate that *C. jejuni* internalisation occurs via a caveolae-independent manner (Konkel *et al.*, 2013). To add to the confusion, previous work has supported the hypothesis that *C. jejuni* internalisation occurs in a caveolae-dependent manner, this is because caveolin-1 is a cholesterol-binding protein and treating cells with a cholesterol-depleting agent would also therefore disrupt the levels of caveolin-1 present (Watson and Galan, 2008b, Konkel *et al.*, 2013). Disruption and/ or removal of caveolin-1 in HeLa cells by treatment with caveolin-1 siRNA had no effect on *C. jejuni* invasion and treatment of Caco-2 IECs with M β CD inhibited *C. jejuni* internalisation. Further to this, the results showed that the action of M β CD is not specifically to target and disrupt the caveolae, but to cause the overall disruption of all lipid rafts and hence the removal of all cholesterol in the cell. Therefore, blocking the potential action of *C. jejuni* in

triggering the signalling cascade inside the cell by close interactions with fibronectin on the outside of the cell (Konkel *et al.*, 2013).

In eukaryotes, microtubules are important structural components of cells (Valiron *et al.*, 2001). MTs serve as tracks within the cell cytoplasm allowing vesicles and proteins to be trafficked via motor-driven transport (Watson and Galan, 2008b, Granger *et al.*, 2014). MTs have been found to play vital roles in the maintenance of cell shape and chromosome segregation in mitosis and as such microtubules are dynamic structure and function (Winsor and Schiebel, 1997, Granger *et al.*, 2014). To investigate the potential involvement of MTs in internalisation of *C. jejuni*, disruption of MTs in Caco-2 IECs with the inhibitor colchicine resulted in a significant reduction in the levels of interacting and invading bacteria. The results obtained in this study show that over the infection period, the interaction with and invasion of Caco-2 IECs by *C. jejuni* changed dramatically and the results were similar to previous reports, showing that *C. jejuni* exhibited a reduction in invasion in the presence of MT inhibitors (Oelschlaeger *et al.*, 1993, Kopecko *et al.*, 2001a, Biswas *et al.*, 2003).

The exact role of MTs in *C. jejuni* invasion is not fully understood and the reduction in invasion is thought to be due to the MTs role in stimulating focal adhesion contacts and modulation of the cells adhesion ability to the ECM, as the use of MT inhibitors increases this adhesion to the ECM (Ballestrem *et al.*, 2000). Another possible explanation for the role of MTs is the involvement of these structures in trafficking proteins into the cell, following uptake of *C. jejuni* by MTs, the bacteria could also be transported to the perinuclear region at the centre of the cell allowing direct interaction with the host cell machinery (Konkel and Cieplak, 1992, Clausen *et al.*, 1997, Hu and Kopecko, 1999). A similar process has been observed in *C. freundii* whereby the invading bacteria replicates in an actin-dependent lamellipodia (Badger *et al.*, 1999).

The inhibitory effect of microtubule-depolymerising agents on the entry of *C. jejuni* strain 81-176 has been noted previously (Oelschlaeger *et al.*, 1993, Hu and Kopecko, 1999, Bacon *et al.*, 2000, Kopecko *et al.*, 2001a). This data is comparable with the results observed in this study with colchicine, as the number of interacting and invading *C. jejuni* were significantly reduced in comparison with the no colchicine controls. However, the results reported in the literature with regard to *C. jejuni* invasion and the involvement of MTs is varied. Some researchers report a reduction in *C. jejuni* invasion

in the presence of MT inhibitors and *C. jejuni* 81-176 is the only strain that has been found to be internalised via the microtubule-dependent pathway (Oelschlaeger *et al.*, 1993, Hu and Kopecko, 1999, Kopecko *et al.*, 2001a). In contrast to these results, previous work has shown that there was no reduction in *C. jejuni* invasion of Caco-2 IECs following inhibition of MTs and other studies have reported the involvement of both MFs and MTs in internalisation of *C. jejuni* 81-176 (Russell and Blake, 1994, Monteville *et al.*, 2003b). The differences observed between the above two studies could be due to the fact that Kopecko's group experiments involved maintaining the inhibitor in the medium, the cell lines used were Caco-2 and INT407 IECs, grown for 24 h to partial confluency, an MOI of 20 was used and monolayers were not centrifuged. Incubations were performed for 2 h and the lysis step was performed at room temperature (Hu and Kopecko, 1999). In contrast, Konkel's group pre-incubated the INT407 IECs with inhibitors for 45 minutes, prior to the addition of 5×10^7 cfu of F38011 wild-type strain, followed by centrifugation of the monolayers. The inhibitors were maintained throughout the infection period. Adhesion assays were performed after a 2 h incubation and invasion assays were performed after a 3 h incubation (Monteville *et al.*, 2003b). Russell and Blake reported no involvement of MFs or MTs with the 81-176 wild-type in Caco-2 IECs. In Russell and Blakes study, Caco-2 IECs were grown for around 72 h, pre-treatment of IECs was performed 30 minutes prior to the addition of 3×10^9 cfu of the 81-176 wild-type and incubations were performed for 30 min to 2.5 h. This group reported that centrifugation of the bacteria onto the monolayers for 10 minutes at 500 or 1000 rpm resulted in a significant increase (42% and 83%) in the number of cell-associated bacteria, compared to no centrifugation step (Russell and Blake, 1994). It is clear that there is no standardised technique and a wide variety of experimental variations have been reported, resulting in the significant levels of confusion in the literature with regard to *C. jejuni* invasion mechanisms.

The main reason for the diarrhoeal symptoms of *C. jejuni* infection is presumed to be due to the tissue damage incurred during the course of infection, as previous studies on intestinal biopsies from infected patients have shown that *C. jejuni* is capable of invading the gut and the same observation has also been made using *in vitro* tissue culture studies (van Spreeuwel *et al.*, 1985, Oelschlaeger *et al.*, 1993). Despite this evidence, our understanding of the molecular mechanisms involved in tissue damage are in their infancy compared to our knowledge of other enteropathogens such as

Escherichia coli, *Shigella* species, *Salmonella* species and *Yersinia* species (Young *et al.*, 2007). Recent studies have showed that Rho GTPases Rac1 and Cdc42 play a role in the membrane ruffling observed prior to *C. jejuni* internalisation (Krause-Gruszczynska *et al.*, 2007a). Previous studies have found that *C. jejuni* invasion of INT407 IECs requires a functional bacterial CadF, leading to activation of host cell proteins, such as Paxillin (an integrin associated protein), resulting in cytoskeletal changes and allowing internalization of *C. jejuni* (Monteville and Konkel, 2002, Monteville *et al.*, 2003a). Recent data has shown that FAK and protein kinases such as EGFR and platelet derived growth factor receptor (PDGFR) are stimulated by *C. jejuni* and are involved in the uptake of cells (Krause-Gruszczynska *et al.*, 2007a, Krause-Gruszczynska *et al.*, 2011, Boehm *et al.*, 2012, Eucker and Konkel, 2012a).

In this study, the use of wortmannin, a potent inhibitor of PI3-kinase was used to assess the possible role of host cell kinases in *C. jejuni* internalisation. The results obtained using this inhibitor produced a significant reduction in the number of interacting and invading *C. jejuni*. Previous studies with wortmannin have produced a significant reduction in the number of intracellular *C. jejuni* N82 in Caco-2 IECs following an incubation period of 3 h (Wooldridge *et al.*, 1996b). Another group reported that the pre-treatment of Caco-2 IECs with Staurosporine (a general inhibitor of tyrosine phosphorylation) significantly reduced the invasion ability of a number of *C. jejuni* strains, indicating that tyrosine protein kinase activity in host cells may be essential for *C. jejuni* invasion (Biswas *et al.*, 2004). This study used a large number of strains and inhibition of invasion studies were performed similar to Konkel's method with inhibitors maintained throughout the 3 h infection period (Biswas *et al.*, 2004).

These results indicate that *C. jejuni* is capable of activating down-stream signalling pathways by activating integrin receptors, as blocking the activity of host proteins, such as PI3-kinase and MAP kinases, inhibits *C. jejuni* bacterial invasion. (Hu *et al.*, 2006b). The results observed here are similar to those reported by other researchers, as pharmacological studies using specific inhibitors of host cell proteins have found that EGF, PDGFR, PI3-kinase, protein kinase C (PKC) and MAPKs are utilised by *C. jejuni* and have been reported to play key roles in the events leading to *C. jejuni* internalisation (Wooldridge *et al.*, 1996b, MacCallum *et al.*, 2005, Hu *et al.*, 2006b, Watson and Galan, 2008b). However, some reports contradict the effect of wortmannin on *C. jejuni* invasion as treatment of INT407 IECs with wortmannin had no significant effects on *C.*

jejuni invasion (Biswas *et al.*, 2000). Biswas *et al.* reported that wortmannin (1 mM) exhibited an insignificant effect (<10%) on the internalisation of *C. jejuni* into INT407 IECs following an infection period of 3 h. Again, the bacterial strain used, the cell line, type and concentration of inhibitor, monolayers being centrifuged and infection period are variables that could result in disparate results.

In order to ascertain whether clathrin-mediated endocytosis is required for *C. jejuni* internalisation, Caco-2 IECs were pre-treated with MDC which resulted in a significant reduction in the number of interacting and invading bacteria compared to no pre-treatment controls. This indicates that some receptors required for *C. jejuni* interactions are located in coated pits in the cell membrane (Ketley and Konkel, 2005, Hu *et al.*, 2006c). MDC has previously been found to block internalisation of numerous *C. jejuni* strains including 81-176 (Konkel *et al.*, 1992b, Oelschlaeger *et al.*, 1993, Wooldridge *et al.*, 1996a, Kopecko, 2000), indicating a likely role for clathrin-mediated endocytosis in *C. jejuni* entry.

A number of other pathogens have also been found to utilise the clathrin-mediated pathway. *S. aureus* entry into host cells requires several host mechanisms including clathrin-coated pits and receptor-mediated endocytosis (Ellington *et al.*, 1999). Previous studies showed that cell invasion by *C. jejuni* and *C. freundii* may involve clathrin-receptor-mediated endocytosis (Oelschlaeger *et al.*, 1993). Contrasting data to this reported that no inhibition of *C. jejuni* internalisation occurred in Caco-2 IECs when the inhibitor MDC was used, suggesting that this pathway was not an important entry route in *C. jejuni* invasion (Russell and Blake, 1994). The differences reported could possibly be due to differences in experimental technique, as Oelschlaeger *et al.* used *C. jejuni* 81-176 wild-type strain and the INT407 cell line or the human bladder cell line T24, with an OD₆₀₀ of ~ 0.4-0.6 bacteria added to the IECs and monolayers were then centrifuged at 200 rpm to aid contact. The Inhibitor MDC (250 mM) was maintained throughout the incubation period (2 h) (Oelschlaeger *et al.*, 1993). In contrast, Russell and Blake used Caco-2 IECs, the MDC concentration used was 0.5 mM to 1 mM and maintained throughout the 2.5 h infection period. An OD₆₀₀ 0.8 was used to infect the Caco-2 IECs and monolayers were centrifuged for 10 minutes to aid contact between bacteria and IECs.

An important and protective feature of the gut epithelium *in vivo* is cell polarity. Polarised intestinal epithelial cells display distinct apical and basolateral surfaces separated by tight junctions and also possess microvilli structures with a well-defined brush border expressing several defined marker proteins (Backert *et al.*, 2013). Therefore polarised cell models are particularly useful for studying microbial interactions, disruption of TEER and route of invasion. Many important gut pathogens such as *Listeria*, *Salmonella* and *Shigella* have developed strategies to cross this epithelial barrier, gain access to submucosal tissues, triggering tissue damage and causing disease in humans. For example, *Salmonella* invades differentiated Caco-2 cells via the apical surface (Finlay and Falkow, 1989) whereas *Shigella* invades differentiated Caco-2 IECs via the basolateral surface (Mounier *et al.*, 1997). *Y. pseudotuberculosis* invades undifferentiated cells that express large amounts of the β_1 integrin receptor for invasin, (a bacterial effector protein associated with uptake by epithelial cells) (Isberg and Van Nhieu, 1994). In contrast, *L. monocytogenes* is capable of entering through the entire surface of non-polarised Caco-2 IECs but only through the basolateral surface of polarised Caco-2 monolayers (Gaillard and Finlay, 1996). TEM studies have revealed that *C. jejuni* invasion proceeds from the apical to the basolateral surface of Caco-2 IECs by passing both through and between cells (Konkel *et al.*, 1992c).

C. jejuni has previously been reported to traverse intact monolayers without causing significant disruption to the TEER (Konkel *et al.*, 1992c). In contrast, infection of cell monolayers with *S. typhimurium* has been found to cause significant disruption to the TEER and changes in cell morphology. *C. jejuni* invasion of IECs was performed in this study using polarised intact monolayers, thereby more closely mimicking the *in vivo* conditions present in the human gut. *C. jejuni* does not efficiently invade polarised Caco-2 IECs apically or induce membrane ruffling in this cell line. This could be due to the fact that the ECM components of Caco-2 and T84 IECs are basolaterally located and since the basolateral membrane is rich in ECM components such as fibronectin, laminin and collagen, thus a polarised monolayer would prove difficult to invade by *C. jejuni*. Ideally, *C. jejuni* basolateral invasion of Caco-2 and T84 IECs could also have been investigated using IECs grown on Transwells filters, such studies should enhance the data obtained from this study.

The literature reports surrounding *C. jejuni* invasion of IECs is varied and confusing (O. Croinin and Backert, 2012). The interactions appear to be complex in comparison to other enteric pathogens such as *S. flexneri*, *S. typhimurium* and *Yersinia* species which enter host cells via a MF-dependent process (Clerc and Sansonetti, 1987, Finlay *et al.*, 1991, Jones *et al.*, 1993). The literature seems to show that the specificity of cell line and the presence or absence of specific receptors may be involved in the preferential mechanisms of endocytosis of the bacteria into the different cell lines (O. Croinin and Backert, 2012). In summary, the current data seems to suggest that different strains of *C. jejuni* invasion strategies may require MTs or MFs, both or neither. After decades of research the *C. jejuni* mediated triggers that lead to MT or MF rearrangements and eventual internalization are yet to be fully elucidated (O. Croinin and Backert, 2012). The percentage of the initial inoculum of interacting *C. jejuni* recovered following inhibition of interaction at the 24 h time point showed the number of interacting *C. jejuni* recovered was highest for the IECs pre-treated with CytD, followed by MDC. The biggest difference observed was in the percentage of the initial inoculum of intracellular *C. jejuni* recovered where pre-treatment of IECs with CytD at the 24 h time-point showed a higher number of intracellular bacteria being recovered compared to between 1% and 4% of intracellular bacteria recovered for the remaining inhibitors, indicating a possible role for the actin cytoskeleton in *C. jejuni* internalisation.

Inhibitors of bacterial invasion produced varying results in the numbers of interacting with and invading bacteria, indicating that *C. jejuni* is able to invade more when the actin structure was disrupted, equally the disruption of MTs indicated that this is a pathway utilised by *C. jejuni* 81-176 strain and this reflects data from previous studies that have reported this strain invades IECs apically, by a strong MT dependent pathway (Hu and Kopecko, 1999, Bacon *et al.*, 2000). In addition, 81-176 was significantly reduced in invasion after M β DC treatment and PI3-kinase inhibition, an indication that *C. jejuni* is capable of the stimulation of putative receptor(s) located in these caveola sites which in turn could activate the PI3-kinase and MAP kinases, eventually leading to cytoskeletal rearrangements (Oelschlaeger *et al.*, 1993, Wooldridge *et al.*, 1996b, Hu and Kopecko, 1999, Biswas *et al.*, 2000, Hu *et al.*, 2006c).

3.4.2 Visualisation of *C. jejuni* within IECs

The next part of this study was to visually determine the co-localisation of *C. jejuni* within Caco-2 IECs, using either eGFP or Evoglow® expressing *C. jejuni* strains and anti-*C. jejuni* antibody labelling to determine the location of both intracellular and extracellular bacteria. Evoglow® was chosen on the basis that the expression of these fluorescent proteins did not require oxygen as a co-factor and therefore would be more suitable for studies performed under low oxygen conditions. Prior to testing the strains in confocal studies, the levels of mean fluorescence intensity were compared to ascertain whether there were any significant differences in fluorescence. To determine the mean fluorescence intensity of eGFP and Evoglow® expressing *C. jejuni* strains, the levels of fluorescence emitted was measured using Volocity software. The analysis of the confocal data was imported in to the Volocity software showed that eGFP expressing *C. jejuni* produced significantly higher levels of fluorescence compared to the Evoglow® expressing *C. jejuni*. Therefore, both eGFP and Evoglow® expressing *C. jejuni* were tested in confocal microscopy studies.

Interaction and invasion assays were performed using the eGFP and Evoglow® expressing *C. jejuni* strains to ensure the presence of either the eGFP or Evoglow® genes did not affect the interaction and invasion ability of these strains. No significant differences were observed in interactions with and invasion of Caco-2 IECs compared to the control 81-176 and 11168H wild-type strains (see Section 3.2.2).

Following infection of Caco-2 IECs with Evoglow® expressing *C. jejuni*, little or no bacteria were observed either extracellularly or intracellularly. The experiments were repeated with the same results observed on each occasion. The Evoglow® expressing *C. jejuni* was effective in fluorescence intensity during growth in the VAIN. However, the fluorescence signal seemed to reduce significantly once co-cultured with Caco-2 IECs. eGFP expressing *C. jejuni* were also tested to measure the fluorescence signal. The eGFP expressing *C. jejuni* produced sufficient fluorescence to warrant testing this strain during invasion of Caco-2 IECs. The eGFP expressing *C. jejuni* was used to infect Caco-2 IECs, however following the required incubation period, only a few bacteria were visible and the fluorescent signal reduced significantly whilst being observed. The same result was observed on each occasion.

Due to the difficulties encountered with the use of eGFP and Evoglow® fluorescence in microscopy studies, anti-*C. jejuni* antibody staining was performed to distinguish between extracellular and intracellular *C. jejuni*, to track the route, course and co-localisation of the bacteria within the IECs. Caco-2 IECs were infected with the 81-176 wild-type strain and incubated for 2 h, 3 h and 6 h. After 2 h of infection, both extracellular and intracellular *C. jejuni* were observed. Few bacteria were found to be internalised, whilst the majority were extracellular. Previous studies have reported the efficiency of internalisation was the highest at the lower end of the MOI (0.02) and decreased with higher MOIs due to AAG of *C. jejuni* at higher MOIs and the maximum MOI reported for INT407 IECs was 200 with invasion occurring at the 4 h time-point (Hu and Kopecko, 1999). A large contrast, in comparison to *S. typhi* which was capable of invading within 1 h of infection of INT407 IECs (Hu and Kopecko, 1999). The numbers of *C. jejuni* internalised depend on the growth phase of the bacteria and the age of the cell line being utilised, as the highest efficiency of invasion of Caco-2 IECs has previously been observed at lower MOI (0.02) in 1 day old Caco-2 IECs. *C. jejuni* was found to enter 7 to 15 day old differentiated Caco-2 IECs at substantially lower invasion efficiencies (Hu *et al.*, 2008). The low levels of internalised bacteria under standard tissue culture conditions in this study reflect the results reported in the literature (Friis *et al.*, 2005).

Previous studies on *C. jejuni* in host cell association assays showed that *C. jejuni* was capable of interacting with around 80% of INT407 IECs, in contrast, only 20% of Caco-2 IECs were reported to interact with by *C. jejuni* (Hu *et al.*, 2008). *C. jejuni* invasion of INT407 IECs was rapid and occurred within 10 min, increasing gradually over the infection period with around 2 bacterial cells being internalised. In contrast, Caco-2 IECs internalised far fewer bacterial cells (1-20 *C. jejuni*) after a 2 h infection period (MacCallum *et al.*, 2005, Hu *et al.*, 2008). The researchers suggested that these large variations in numbers of *C. jejuni* being internalised was possibly due the Caco-2 IECs undergoing differentiation, resulting in the lack of availability of a putative invasion receptor (Levy *et al.*, 1994). The presence of certain M-like Caco-2 IECs containing internalised *C. jejuni* were found to collapse during the infection period suggested that a small number of the differentiated IECs were still susceptible to *C. jejuni* invasion (MacCallum *et al.*, 2005, Hu *et al.*, 2008). *C. jejuni* 81-176 intracellular survival studies using T84 IECs reported that the following internalisation the bacteria were shown to be

contained within CCVs and that the bacteria were capable of survival within IECs and this intracellular survival decreased over the 24 h period. Internalisation of the bacteria into macrophages resulted in rapid killing (Russell and Blake, 1994, Watson and Galan, 2008b).

3.4.3 Limitations of the study

Due to time constraints in this study, one cell line was utilised for inhibition of invasion studies. The use of other cell lines would provide further data in characterising the possible cellular pathways this bacterium utilises for entry. In addition, different strains of *C. jejuni* could be used, different combinations of inhibitors with the use of the *C. jejuni* mutants constructed in this study to further characterise the possible cellular pathways. Variations on assay time length as in this study, the dynamics of *C. jejuni* interactions with and invasion of Caco-2 IECs showed clear differences in numbers of bacteria interacting with and invading IECs. In addition, the tissue culture studies performed did not involve centrifuging the monolayers to aid contact between bacteria and IECs so as to study the realistic behaviour of the interactions between *C. jejuni* and the IEC. eGFP and Evoglow® expressing *C. jejuni* strains failed to provide data that would allow the tracking, visualisation and localisation of the wild-type and the mutants. The problems encountered involved a lack of fluorescence signal once *C. jejuni* was internalised into IECs, this procedure was time consuming and despite fluorescence being detectable under microaerobic conditions the results were non reproducible with IEC infection studies under standard tissue culture conditions.

Chapter 4. Reinvestigation of the role of CiaB in *C. jejuni* invasion of intestinal epithelial cells

4.1 Introduction

The adhesion of *C. jejuni* to host IECs, activation of host cytoskeletal rearrangements and eventual bacterial entry into the host cell has been found to be an essential early process in the course of *C. jejuni* pathogenesis and was first evident from human intestinal biopsy samples (van Spreeuwel *et al.*, 1985). The method by which *C. jejuni* achieves this is still unclear, yet a multitude of bacterial factors have been reported to be involved in *C. jejuni* invasion of IECs, such as motility, involvement of flagella, glycosylation, capsule and adherence proteins on the surface of *C. jejuni* (Grant *et al.*, 1993, Pei *et al.*, 1998, Bacon *et al.*, 2001, Konkel *et al.*, 2001, Szymanski *et al.*, 2002). Research has shown that mutation of genes encoding proteins involved in these processes have resulted in a reduction in attachment and invasion of human cell lines, mice or chicken cell lines (Hendrixson and DiRita, 2004, Watson and Galan, 2008b, Novik *et al.*, 2010). *C. jejuni* was reported to trigger membrane ruffling via direct contact with the host cell followed by insertion with first the flagella tip, then with the opposite flagella end (Krause-Gruszczynska *et al.*, 2007a, Krause-Gruszczynska *et al.*, 2011, O. Croinin and Backert, 2012). Work by Konkel's group found that *C. jejuni* synthesised a set of proteins upon co-culturing with INT407 IECs, termed *Campylobacter* invasion antigens (Cia proteins) (Konkel *et al.*, 1999c). *Campylobacter* invasion antigen B (CiaB) is the most widely studied and was found to exhibit a weak amino acid similarity to T3SS effector systems present in other pathogens, CiaB has been found to exhibit 45% similarity to *Salmonella* invasion protein B (SipB) from *Salmonella typhimurium*. CiaB also showed 40.6% amino acid similarity to *Shigella flexneri* invasion plasmid antigen B (IpaB) and *Yersinia* outer protein B (YopB) from *Yersinia pseudotuberculosis* (Konkel *et al.*, 1999b).

Mutation of *ciaB* did not affect the adherence of *C. jejuni* to INT407 IECs, but the numbers of intracellular bacteria were significantly reduced (Monteville and Konkel, 2002). CiaB was also found to be important in the secretion of other Cia proteins (Konkel *et al.*, 1999b). CiaB was reported to be directly translocated into the host cell, indicating a role as a potential bacterial effector molecule (Konkel *et al.*, 1999b).

Further studies found CiaB secretion to be affected when genes encoding proteins involved in flagella biosynthesis were mutated, suggesting that secretion of CiaB required the presence of a fully functional flagella (Konkel *et al.*, 2004). Little is known about the bacterial virulence factors that are potentially involved in mediating *C. jejuni* entry and intracellular survival. Non-motile mutants are significantly decreased in ability to invade IECs (Wassenaar *et al.*, 1991, Grant *et al.*, 1993, Yao *et al.*, 1994). However, this data still fails to answer the question of whether the flagellar structure has a direct role in enabling *C. jejuni* internalisation or whether the non-motile mutants are severely hampered in entering cells, possibly suggesting that it is motility that is required for invasion (Szymanski *et al.*, 1995). To date, four Cia proteins have been identified CiaB, (Konkel *et al.*, 1999b). CiaI (Buelow *et al.*, 2011b), CiaC (Neal-McKinney and Konkel, 2012) and CiaD (Samuelson *et al.*, 2013). However, other researchers have cast doubt over the role of CiaB as a 81-176 *ciaB* mutant showed no difference in invasion ability compared to the wild-type strain (Novik *et al.*, 2010). The aim of this chapter was to reinvestigate the role of CiaB in *C. jejuni* interactions with and invasion of IECs.

Results 4.2

4.2.1 Chromosomal location of *ciaB*

The chromosomal location of the *ciaB* gene in *C. jejuni* 81-176 and 11168H strains was viewed in Artemis software, with adjacent genes listed below.

C. jejuni 81-176 *ciaB* upstream genes are Cjj81-176_0923/*cstA* (probable MCP protein methyltransferase/chemotaxis and motility), *prsA* (ribose-phosphate pyrophosphokinase) and 81-176 *ciaB* downstream genes are *hup* (DNA binding protein), *cysK* (cysteine synthase A), Cjj81-176_0915 (putative hydrolase), Cjj81-176_0913 (DNA binding protein).

C. jejuni 11168H *ciaB* upstream genes are Cj0916c (conserved hypothetical protein), *cstA* (carbon starvation protein A), *prsA* (ribose-phosphate pyrophosphokinase), *Cj0919c* (putative ABC-type amino-acid transporter permease) and 11168H *ciaB* downstream are *hupB* (DNA binding protein), *cysM* (cysteine synthase B), *Cj0906* (putative periplasmic protein). In both 81-176 and 11168H strains, the upstream genes

are similar except in 81-176 the presence of a gene encoding a putative hydrolase is different to the putative periplasmic protein present in 11168H. The downstream genes are the same in both 81-176 and 11168H respectively.

(A)



(B)

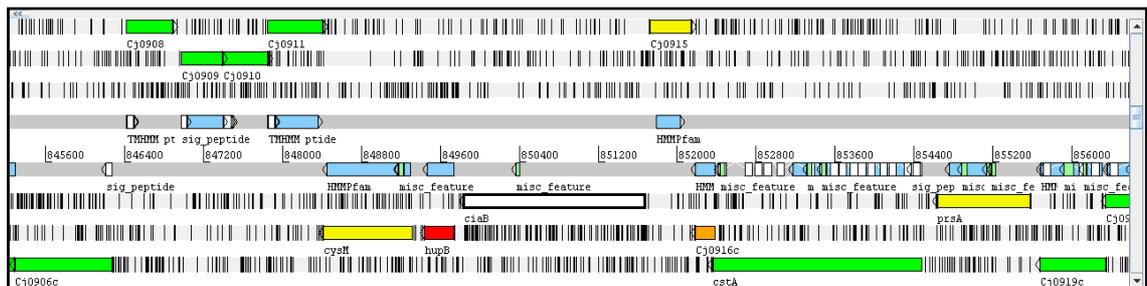


Figure 4.1 Artemis Images displaying the positions of *ciaB* and the location of both the up and downstream genes in *C. jejuni* 81-176 (A) & 11168H (B)

4.2.2 Construction of 81-176 & 11168H *ciaB* mutants

Defined isogenic *ciaB* mutants were constructed in both 81-176 and 11168H wild-type strains. The primers *ciaB*-Forward-81-176/*ciaB*-Reverse-81-176 and *ciaB*-Forward-11168H/*ciaB*-Reverse-11168H were designed for the amplification of *ciaB* from 81-176 & 11168H genomic DNA (gDNA) respectively. PCR reactions were set up using the designed primers; PCR products analysed by agarose gel electrophoresis and purified using the Qiagen PCR clean up kit. Each purified PCR product was ligated into the pGEMT-easy vector, transformed into XL-2 Blue MRF^r competent cells and plated out on LB amp agar plates. Colonies selected by growth on LB amp agar were screened by boilate PCR to check for the presence of *ciaB*. Plasmid DNA from positive colonies was sequenced to confirm the presence of the correct gene. The cloned *ciaB* gene fragment was disrupted by insertion of a kanamycin cassette (Km^r) (Trieu-Cuot *et al.*, 1985). The

Km^r cassette was excised by BamHI from the plasmid pJMK30 (van Vliet *et al.*, 1998). The pGEMT-easy-*ciaB*-81-176/11168H constructs were digested with BglII and ligated with the Km^r cassette to form pGEMT-easy-*ciaB*-81-176/11168H-Km^r. These constructs were then transformed into XL-2 Blue MRF['] competent cells, plated onto LB amp kan agar plates and incubated at 37°C for 24 h. Transformants were screened by PCR using *ciaB*-gene specific and Km^r-specific primers. Constructs with the Km^r cassette in the same orientation as *ciaB* were selected and electroporated into the 81-176 or 11168H wild-type strains. Electroporated bacteria were plated onto blood agar plates and incubated at 37°C under microaerobic conditions for 2 to 3 days. Cells were harvested and resuspended in 0.5 ml PBS. Two hundred microliters of this suspension was spread onto blood agar plates containing kanamycin. Plates were incubated at 37°C under microaerobic conditions for 2 to 3 days. Putative *ciaB* mutants were screened by PCR and sequencing using gene specific primers to confirm insertion and correct orientation of the kanamycin cassette.

4.2.3 Construction 81-176 & 11168H *ciaB* complements

As this study used both *C. jejuni* wild-type strains, complementation procedures were also performed in both strains to confirm that the phenotypes observed were due to the mutation and not due to random DNA changes or polar effects. This was performed by inserting a copy of the functional *ciaB* gene and 20 extra bases of the upstream sequence into the chromosomes of the mutants. Complementation in 81-176 and 11168H was performed using a *C. jejuni* complementation vector pRRC (Karlyshev and Wren, 2005). The coding region for *ciaB* was amplified by PCR using primers *ciaB*comp-Forward-81-176/*ciaB*comp-Reverse-81-176 and *ciaB*comp-Forward-11168H/*ciaB*comp-Reverse-11168H which introduced an XbaI site at both the 5' and 3' ends, as well as the native ribosome binding site for *ciaB*. Following digestion with XbaI, each PCR product was ligated into the pRRC vector which contains a chloramphenicol resistance cassette, transformed into DH5 α competent cells and plated onto LB chl agar, then incubated at 37°C for 24 hours. Transformants selected on LB chl agar were screened by PCR using *ciaB*comp-Forward-81-176/*ciaB*comp-Reverse-81-176 and *ciaB*comp-Forward-11168H/*ciaB*comp-Reverse-11168H primers and sequencing to ensure no mutations had been introduced into the functional *ciaB* gene. These constructs were then electroporated into the respective *ciaB* mutant. Positive clones were selected

on blood agar plates containing chloramphenicol and kanamycin. Confirmation of the presence of both the mutated *ciaB* gene and the functional *ciaB* was performed by PCR using the *ciaB*comp-Forward/*ciaB*comp-Reverse and *ciaB* gene-specific Forward/Reverse primers and also by sequencing.

The size of *ciaB* is 1.86 kb, the amplified size of the *ciaB* gene fragment is 1.8 kb and successful insertion of the 1.4 kb kanamycin cassette produced a amplified size of 3.2 kb.

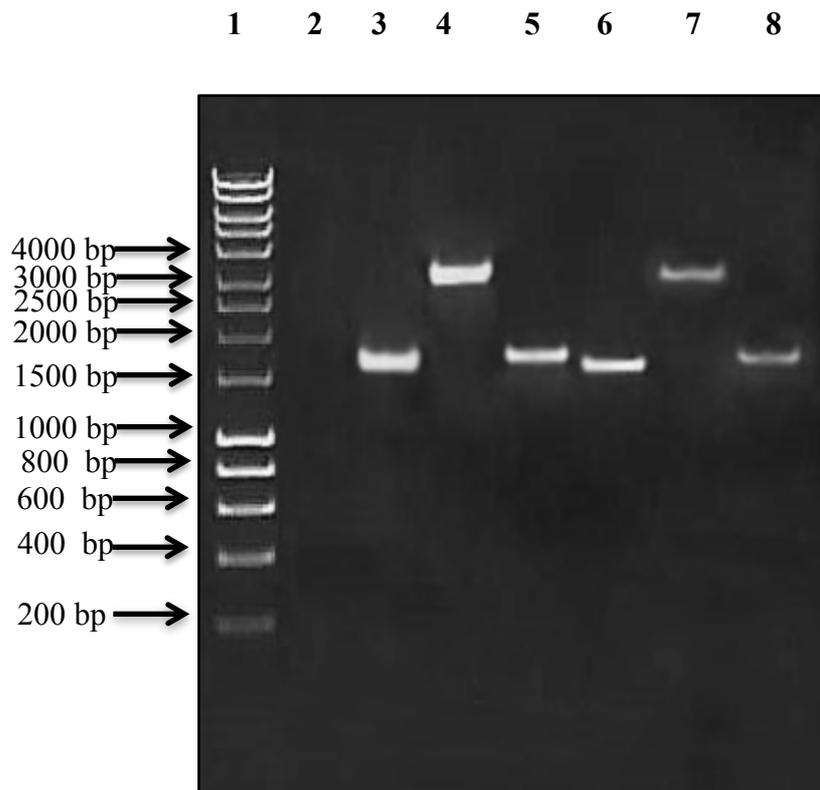


Figure 4.2. PCR verification of *C. jejuni* wild-types, *ciaB* mutants and *ciaB* complements. Lane 1: 1 Kb ladder (Bioline). Lane 2: Negative control. Lane 3: 11168H *ciaB*. Lane 4: 11168H *ciaB* mutant. Lane 5: 11168H *ciaB* complement. Lane 6: 81-176 *ciaB*. Lane 7: 81-176 *ciaB* mutant. Lane 8: 81-176 *ciaB* complement.

4.2.4 Characterisation of 81-176 and 11168H *ciaB* mutants

Following the construction of the 81-176 and 11168H *ciaB* mutants, initial phenotypic characterisation studies for growth rate, motility and autoaggregation were performed.

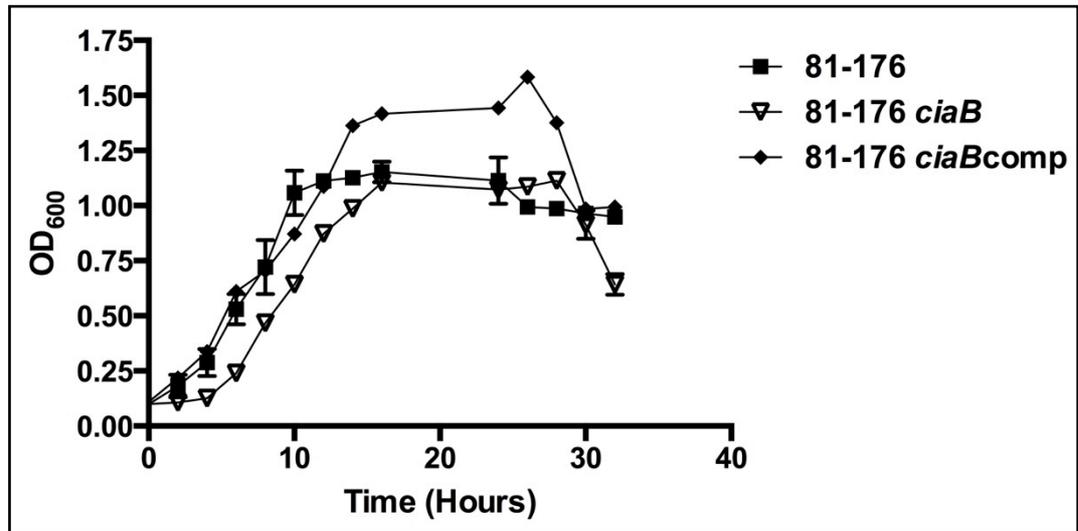
4.2.4.1 Growth rate assays and cfu counts

To assess the effect of the mutation in *ciaB*, growth rate experiments were performed. OD₆₀₀ readings were recorded every 2 h over a 32 h growth period as an indication of bacterial growth.

Both the 81-176 and 11168H *ciaB* mutants displayed similar growth rates compared to the respective wild-type strain (see Figure 4.3). The 81-176 and 11168H *ciaB* complements displayed a higher growth rate levels at the 24 h time point compared to both the respective wild-type strain and mutant.

The numbers of colony forming units (cfu) at different time-points were also investigated for the 81-176 and 11168H wild-type strains, *ciaB* mutants and complements (see Figure 4.4). The 81-176 *ciaB* mutant displayed slightly reduced growth rate levels at 12 h, 18 h and 24 h compared to the wild-type strain. Similarly, the 11168H *ciaB* mutant displayed slightly reduced cfu counts, specifically at the 18 h and 24 h time-points compared to the wild-type strain. The *ciaB* complements exhibited higher cfu numbers at 24 h.

(A)



(B)

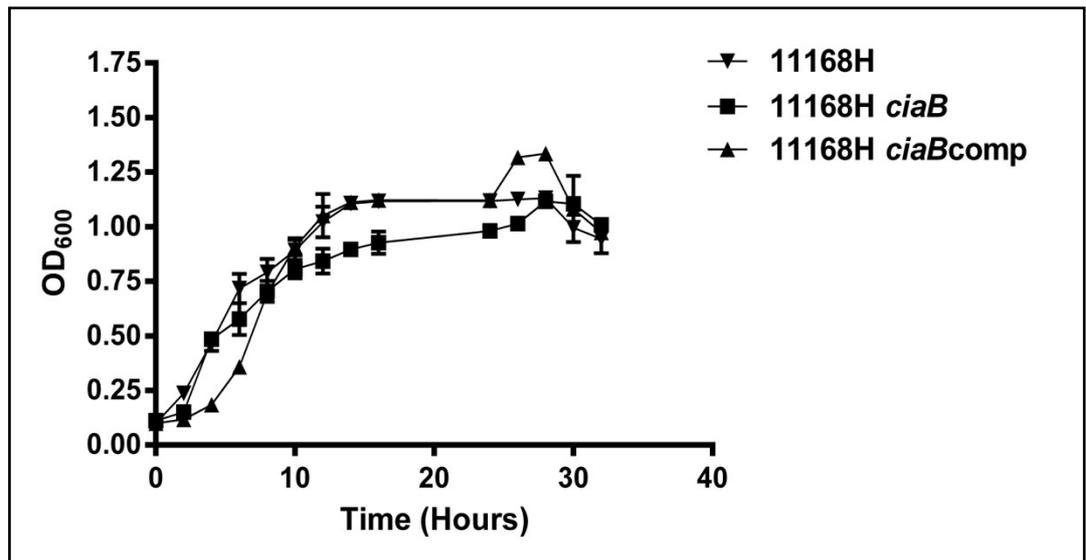
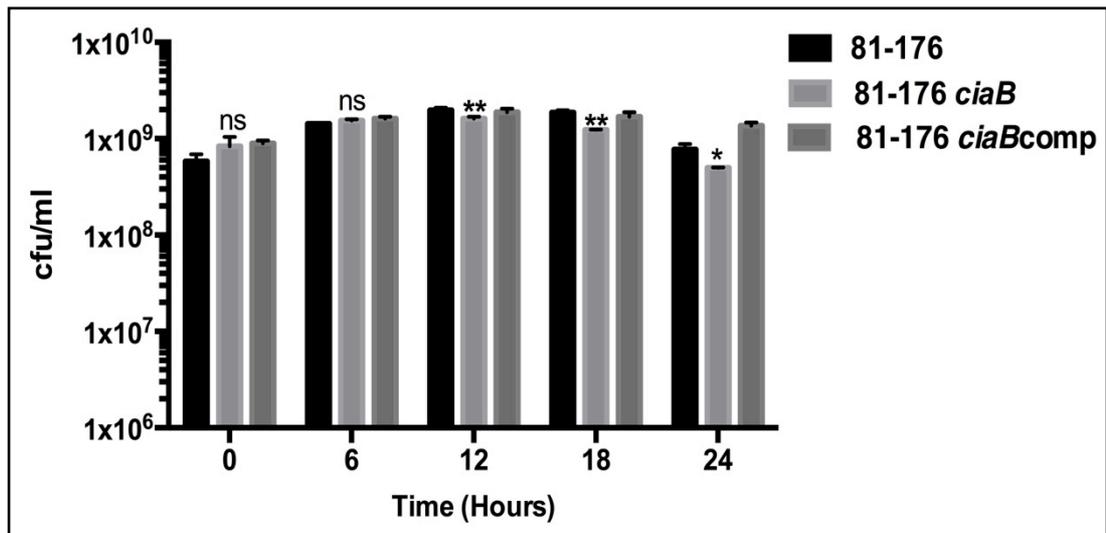


Figure 4.3 Growth rates of 81-176 and 11168H wild-type strains, *ciaB* mutants and *ciaB* complements. *C. jejuni* strains from 24 h plates were inoculated at OD₆₀₀ 0.1 into 10 ml of pre-incubated brucella broth and grown at 37°C microaerobically with shaking at 75 rpm. (A) 81-176 wild-type, *ciaB* mutant and complement (B) 11168H wild-type *ciaB* mutant and complement. Growth was assessed by recording OD₆₀₀ readings of the cultures every 2 h. The data represents the mean of triplicate independent experiments.

(A)



(B)

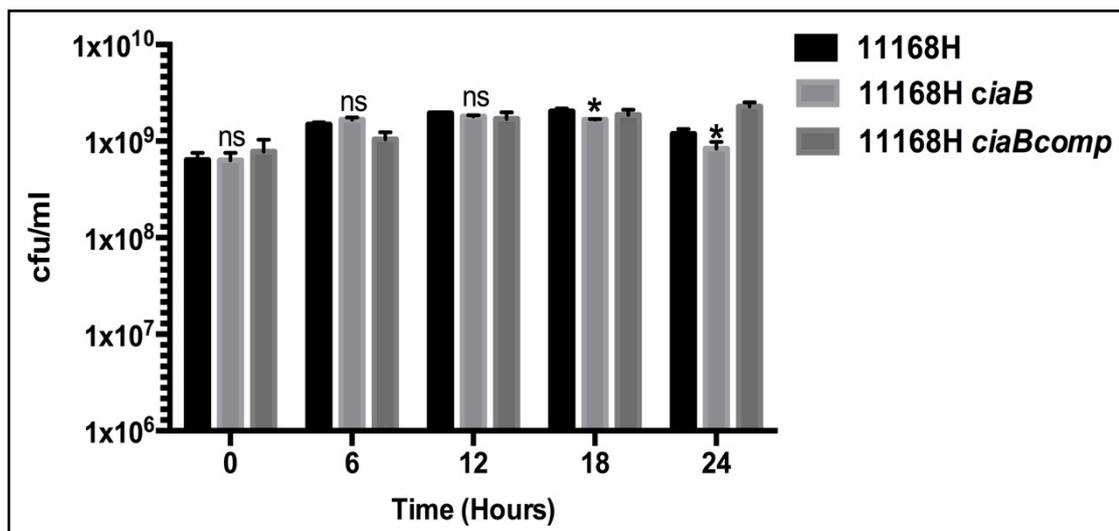
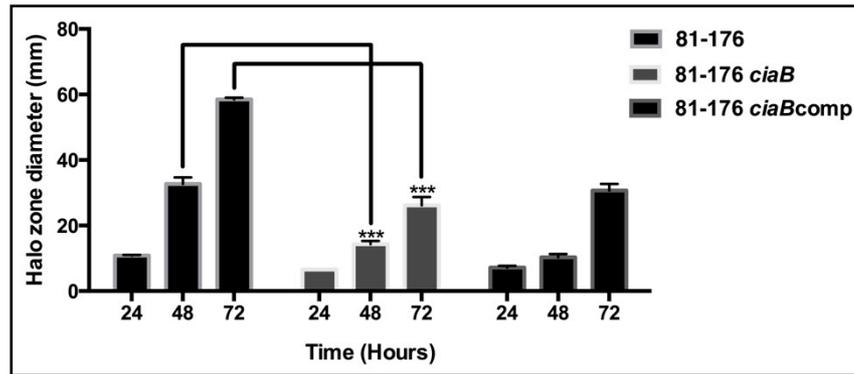


Figure 4.4 Growth curve cfu counts for 81-176 and 11168H wild-type strains, *ciaB* mutants and complements. *C. jejuni* strains from 24 h plates were inoculated at OD₆₀₀ 0.1 into 10 ml of pre-incubated brucella broth and grown at 37°C microaerobically with shaking at 75 rpm. At the desired time-point, 1 ml of brucella broth was removed and serial dilutions were performed from 10⁻¹ to 10⁻⁷ and 200 µl of the final dilution was plated out onto blood agar plates. Colonies were enumerated following incubation at 37°C for 2 to 3 days. (A) 81-176 wild-type, *ciaB* mutant and complement (B) 11168H wild-type, *ciaB* mutant and complement. Asterisks denote a statistically significant difference (* = $p < 0.05$, ** = $p < 0.01$, ns = no significant difference). The data represents triplicate independent experiments.

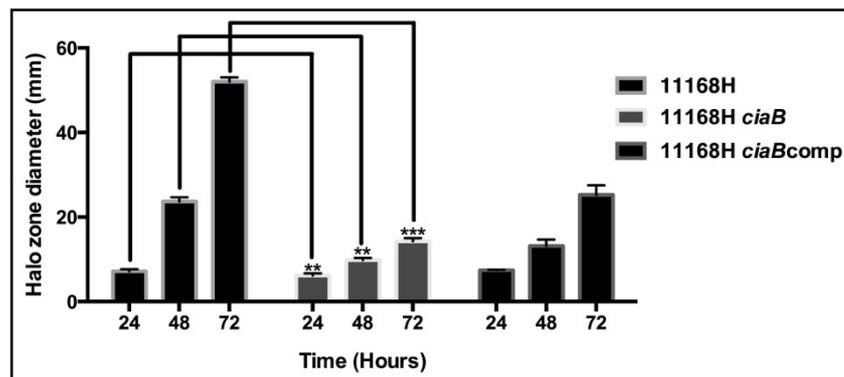
4.2.4.2 Motility

C. jejuni displays rapid and darting motility, enabling the organism to colonise the intestines of humans and animals. (Morooka *et al.*, 1985, Black *et al.*, 1988, Wassenaar *et al.*, 1991, Poly and Guerry, 2008). To study the effects of the mutation of *ciaB* on *C. jejuni* motility, motility assays were performed. The 81-176 *ciaB* mutant was significantly reduced in motility compared to 81-176 wild-type strain. The 11168H *ciaB* mutant also displays significantly reduced motility compared to the 11168H wild-type strain. Both 81-176 and 11168H *ciaB* complements did not restore the wild-type phenotype.

(A)



(B)



(C)

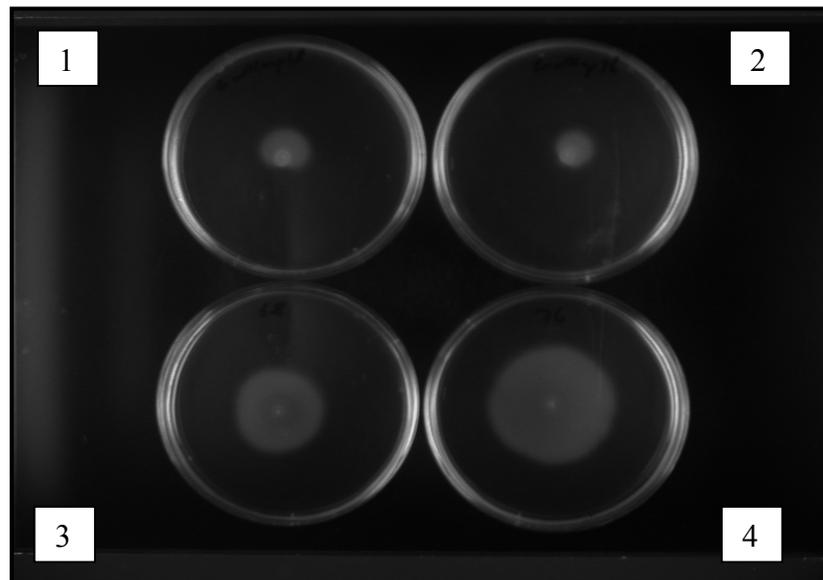


Figure 4.5 Motility assays. Bacteria were grown for 24 h on blood agar under microaerobic conditions. A suspension was prepared and adjusted to an OD_{600} of 0.1. 2 μ l of this suspension was pipetted into the centre of soft agar plates and incubated at 37°C under microaerobic conditions. The level of motility was assessed by measuring the diameter of growth at 24 h, 48 h and 72 h. **(A)** Differences in motility levels of *C. jejuni* 81-176 *ciaB* mutant, *ciaB* complement and 81-176 wild-type. **(B)** Differences in motility levels of *C. jejuni* 11168H *ciaB* mutant, *ciaB* complement and 11168H wild-type. **(C)** Representative image showing the motility of *ciaB* mutants and wild-types at 48 h. **(C):1** 11168H *ciaB* mutant **(C):2** 81-176 *ciaB* mutant **(C):3** 11168H wild-type and **(C):4** 81-176 wild-type. Asterisks denote a statistically significant difference (** = $p < 0.01$, *** = $p < 0.001$). Data are representative of three independent experiments.

4.2.4.3 Autoaggregation Assays

Autoaggregation (AAG) of *C. jejuni* plays a role during interactions with and invasion of host cells (Guerry *et al.*, 2006). However, the role of AAG in *C. jejuni* pathogenesis is yet to be fully determined. The amount of AAG also varies between *C. jejuni* strains (Misawa and Blaser, 2000). AAG has been found to play a role in resistance to host defences such as phagocytosis, in biofilm formation and in adherence to host cells (Skurnik, Bolin *et al.* 1984, Galdiero, Romano Carratelli *et al.* 1988, Chiang, Taylor *et al.* 1995, Frick, Morgelin *et al.* 2000, Sherlock, Vejborg *et al.* 2005).

In order to investigate the effect of the *ciaB* mutations on *C. jejuni* AAG, *ciaB* mutants were tested and compared to the 81-176 and 11168H wild-type strains. RpoN is an alternative sigma factor involved in the transcription of genes encoding proteins involved in flagella biosynthesis (Hendrixson *et al.*, 2001). 81-176 and 11168H *rpoN* mutants which lack a functional flagella and do not autoaggregate were included as AAG negative controls.

The 81-176 *ciaB* mutant was significantly increased in AAG compared to the 81-176 wild-type strain. The 81-176 wild-type AAG phenotype was restored in the 81-176 *ciaB* complement. There was no significant difference in the levels of AAG observed between the 11168H *ciaB* mutant and 11168H wild-type strain. The 11168H *ciaB* complement was significantly reduced in AAG.

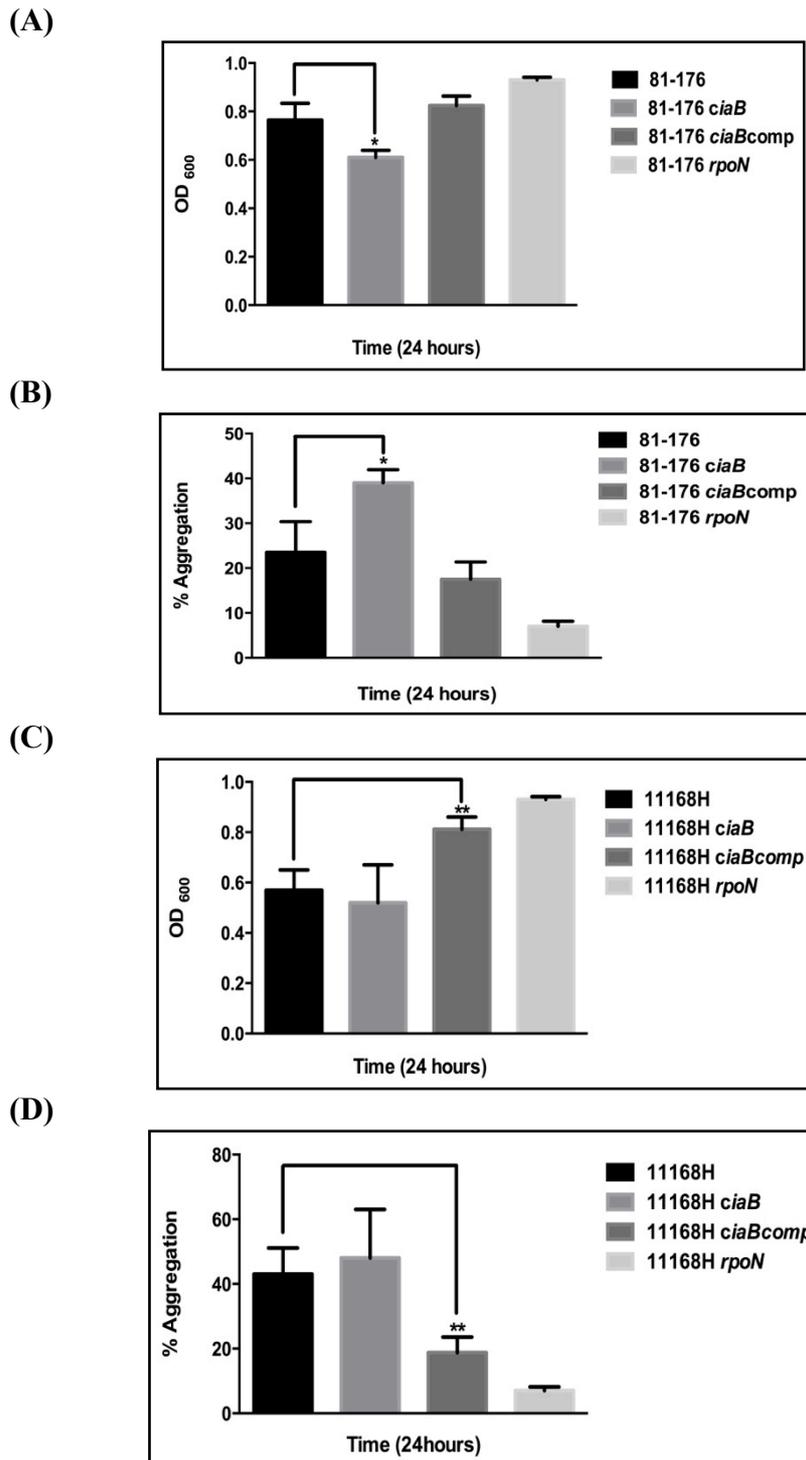


Figure 4.6 Autoaggregation assays for the 81-176 & 11168H wild-type strains, *ciaB* mutants and *ciaB* complements. Bacteria were grown for 24 h on blood agar under microaerobic conditions. A suspension was prepared and adjusted to an OD₆₀₀ of 1.0 in PBS. Suspensions (2 ml) were incubated for 24 h at 37°C under microaerobic conditions. AAG was analysed by measuring the decrease in optical density of the upper 1 ml of suspended cells after 24 h. An OD₆₀₀ of 1.0 was considered 0% AAG and an OD₆₀₀ of 0 considered 100% AAG. (A) & (C): OD₆₀₀ readings after 24 hour incubation. (B) & (D): Percentage of AAG of suspended cells. Asterisks denote a statistically significant difference (* = $p < 0.05$, ** = $p < 0.01$). Data are representative of triplicate individual experiments.

4.2.4.4 *Galleria mellonella* infection model

G. mellonella larvae have been widely used as a non-mammalian model for bacterial infection studies (Champion *et al.*, 2009). Larvae can be infected at 37°C and possess specialised phagocytic cells called haemocytes (Bergin, Reeves *et al.* 2005). These cells mimic mammalian phagocytic cells and produce bactericidal compounds such as superoxide (Bergin, Reeves *et al.* 2005).

Infection with both 81-176 and 11168H *ciaB* mutants resulted in reduced cytotoxicity compared to the respective wild-type strain over 72 h. The 11168H *ciaB* complement exhibited slightly higher cytotoxicity than the 81-176 *ciaB* complement, which exhibited only a partial restoration of the wild-type phenotype.

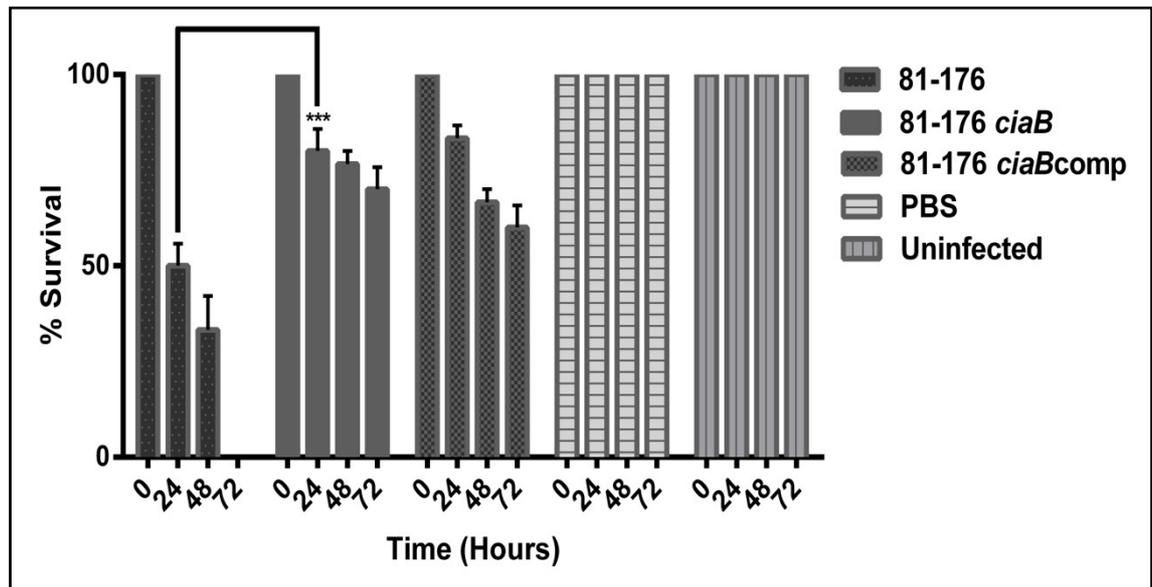


Figure 4.7 Effect of *C. jejuni* 81-176 wild-type strain, *ciaB* mutant and *ciaB* complement in the *Galleria mellonella* model of infection. *G. mellonella* larvae were injected with a 10 μ l inoculum of 24 h *C. jejuni* culture diluted to an OD₆₀₀ of 0.1 by microinjection in the right foremost leg, giving an infectious dose of approximately 10⁶ cfu. The larvae were incubated at 37°C with survival recorded at 24 h intervals. PBS and no injection controls were also included. For each experiment, 10 *G. mellonella* larvae were infected and the experiments were repeated in triplicate. The asterisks denote a statistically significant difference (***) = $p < 0.001$.

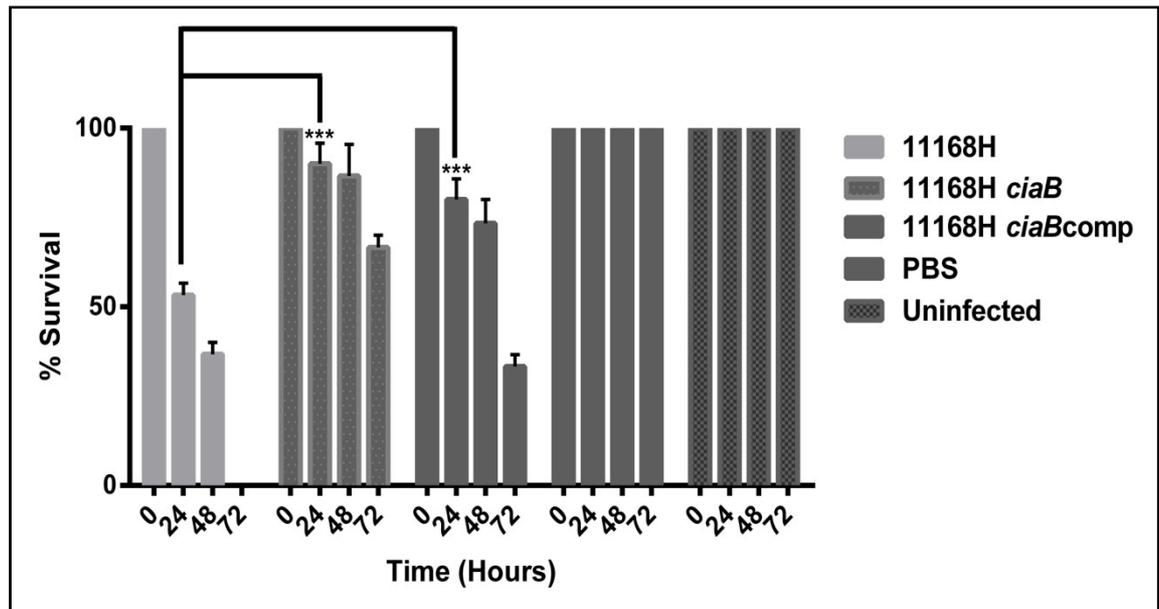


Figure 4.8 Effect of *C. jejuni* 11168H wild-type strain, *ciaB* mutant and *ciaB* complement in the *Galleria mellonella* model of infection. *G. mellonella* larvae were injected with a 10 μ l inoculum of 24 h *C. jejuni* culture diluted to an OD₆₀₀ of 0.1 by microinjection in the right foremost leg, giving an infectious dose of approximately 10⁶ cfu. The larvae were incubated at 37°C with survival recorded at 24 h intervals. PBS and no injection controls were also included. For each experiment, 10 *G. mellonella* larvae were infected and the experiments were repeated in triplicate. The asterisks denote a statistically significant difference (***) = $p < 0.001$.

4.2.5 Interaction and invasion assays

The effect of the mutation in *ciaB* on *C. jejuni* interactions with IECs was investigated.

4.2.5.1 The role of CiaB of *C. jejuni* interactions with and invasion of Caco-2 IECs

There was a significant reduction in the ability of the 81-176 *ciaB* mutant to interact with and invade Caco-2 IECs in comparison to the 81-176 wild-type strain. The 81-176 *ciaB* mutant exhibited a significantly reduced ability to interact with Caco-2 IECs compared to the wild-type strain with numbers of interacting bacteria increasing over the infection period (see Figure 4.9). The 81-176 *ciaB* complement exhibited a delayed ability to interact with Caco-2 IECs but at the 6 h and 24 h time-point, the levels were similar to the 81-176 wild-type strain. The 81-176 *ciaB* mutant also displayed a significant reduction in invasion with numbers of intracellular bacteria gradually decreasing by 24 h (see Figure 4.10). The 81-176 *ciaB* complement did not restore the wild-type phenotype and displayed an even lower number of intracellular bacteria at all time-points.

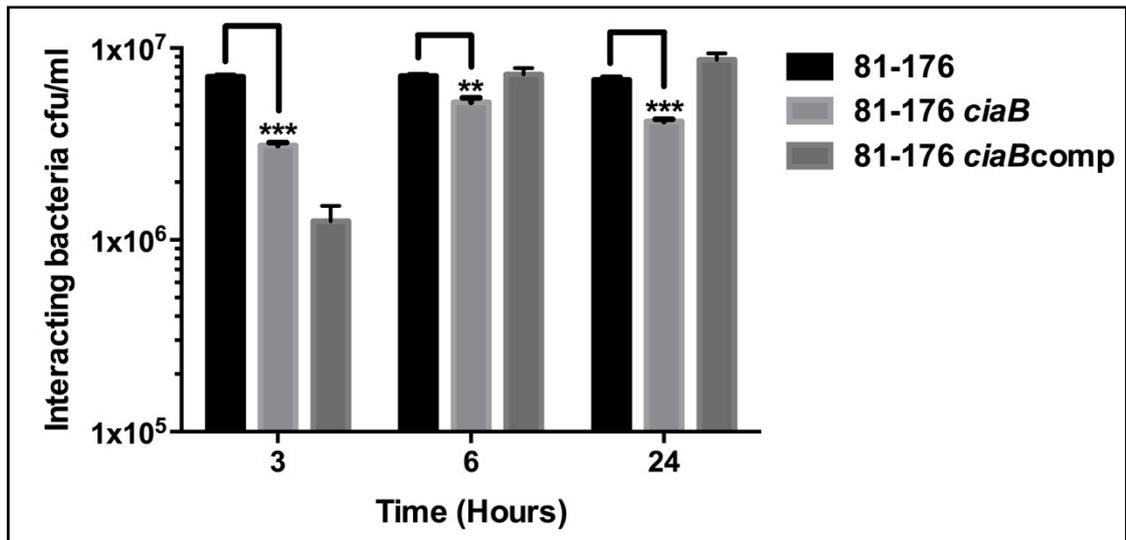


Figure 4.9 Interaction (adhesion and invasion) assays Bacteria were co-cultured with Caco-2 IECs for 3 h, 6 h and 24 h. Caco-2 IECs were lysed with Triton X-100 and interacting bacteria were enumerated. Asterisks denote a statistically significant difference (** = $p < 0.01$, *** = $p < 0.001$) for the 81-176 *ciaB* mutant compared to the wild-type. Data are representative of triplicate independent experiments.

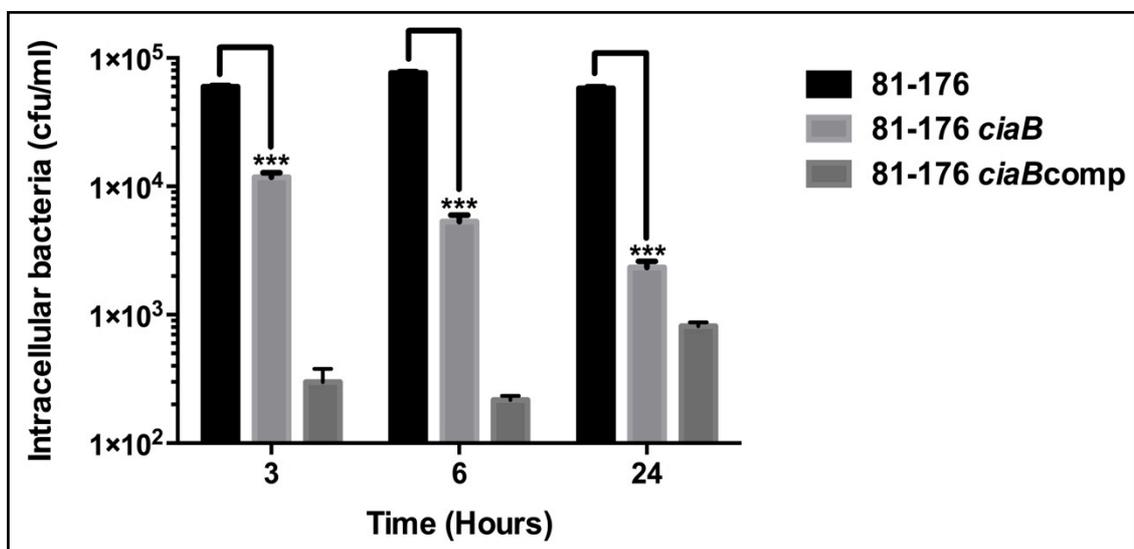


Figure 4.10 Invasion assays. Bacteria were co-cultured with Caco-2 IECs for 3 h, 6 h and 24 h. Caco-2 IECs were incubated with gentamicin (150 $\mu\text{g}/\text{ml}$) for 2 h to kill extracellular bacteria, then lysed and numbers of intracellular bacteria assessed. Asterisks denote a statistically significant difference (***) = $p < 0.001$) for the 81-176 *ciaB* mutant compared to the wild-type strain. Data are representative of triplicate independent experiments.

4.2.5.2 The role of CiaB in *C. jejuni* interactions with and invasion of T84 IECs

There was also a significant difference between the ability of the *ciaB* mutant to interact with and invade T84 IECs in comparison to the 81-176 wild-type strain. The 81-176 *ciaB* mutant exhibited a significantly reduced ability to interact with T84 IECs compared to the wild-type strain (see Figure 4.11). The wild-type phenotype was partially restored by the 81-176 *ciaB* complement. The 81-176 *ciaB* mutant also displayed a significant reduction in invasion, (see Figure 4.12). The 81-176 *ciaB* complement partially restored the wild-type phenotype.

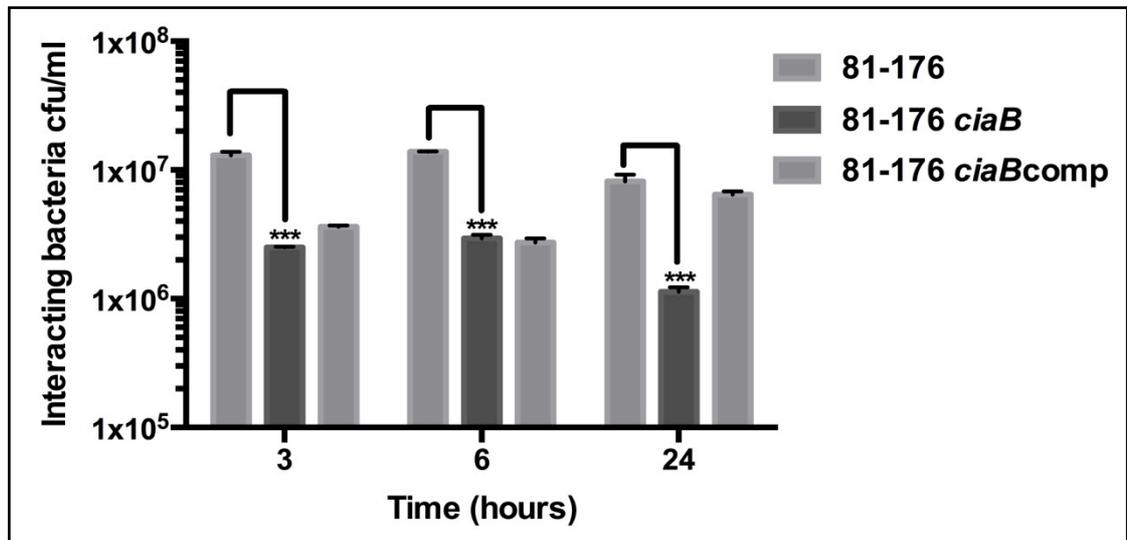


Figure 4.11 Interaction (adhesion and invasion) assays. Bacteria were co-cultured with T84 IECs for 3 h, 6 h and 24 h. T84 IECs were lysed with Triton X-100 and interacting bacteria were enumerated. Asterisks denote a statistically significant difference (***) = $p < 0.001$) for the 81-176 *ciaB* mutant compared to the wild-type strain. Data are representative of triplicate independent experiments.

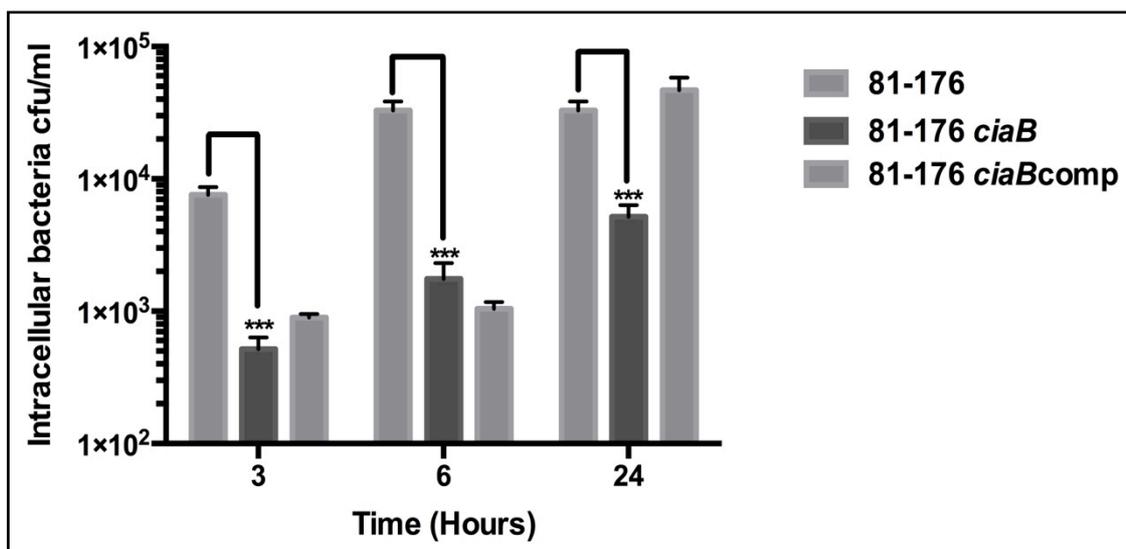


Figure 4.12 Invasion assays. Bacteria were co-cultured with T84 IECs for 3 h, 6 h and 24 h. T84 IECs were incubated with gentamicin (150 µg/ml) for 2 h to kill extracellular bacteria, then lysed and numbers of intracellular bacteria assessed. Asterisks denote a statistically significant difference (***) = $p < 0.001$) for the 81-176 *ciaB* compared to the wild-type strain. Data are representative of triplicate independent experiments.

Table 4.1 Summary of the effect of the mutation in *ciaB* on *C. jejuni* interactions with Caco-2 IECs.

Bacterial strain	Effect of interaction with Caco-2 IECs (3 h)	Effect of interaction with Caco-2 IECs (6 h)	Effect of interaction with Caco-2 IECs (24 h)
81-176 wild-type	< 10 ⁷ cfu	< 10 ⁷ cfu	< 10 ⁷ cfu
<i>ciaB</i> mutant	> 10 ⁶ cfu	> 10 ⁶ cfu	> 10 ⁶ cfu
<i>ciaB</i> complement	> 10 ⁶ cfu	> 10 ⁶ cfu	> 10 ⁷ cfu

Table 4.2 Summary of the effect of the mutation in *ciaB* on *C. jejuni* invasion of Caco-2 IECs.

Bacterial strain	Effect of invasion of Caco-2 IECs (3 h)	Effect of invasion of Caco-2 IECs (6 h)	Effect of invasion of Caco-2 IECs (24 h)
81-176 wild-type	< 10 ⁵ cfu	< 10 ⁵ cfu	< 10 ⁵ cfu
<i>ciaB</i> mutant	> 10 ⁴ cfu	> 10 ³ cfu	> 10 ³ cfu
<i>ciaB</i> complement	> 10 ² cfu	> 10 ² cfu	> 10 ³ cfu

Table 4.3 Summary of the effect of the mutation in *ciaB* on *C. jejuni* interactions with T84 IECs.

Bacterial strain	Effect of interaction with T84 IECs (3 h)	Effect of interaction with T84 IECs (6 h)	Effect of interaction with T84 IECs (24 h)
81-176 wild-type	>10 ⁷ cfu	>10 ⁷ cfu	< 10 ⁷ cfu
<i>ciaB</i> mutant	> 10 ⁶ cfu	> 10 ⁶ cfu	> 10 ⁶ cfu
<i>ciaB</i> complement	> 10 ⁶ cfu	> 10 ⁶ cfu	> 10 ⁶ cfu

Table 4.4 Summary of the effect of the mutation in *ciaB* on *C. jejuni* invasion of T84 IECs.

Bacterial strain	Effect of interaction with T84 IECs (3 h)	Effect of interaction with T84 IECs (6 h)	Effect of interaction with T84 IECs (24 h)
81-176 wild-type	< 10 ⁴ cfu	> 10 ⁴ cfu	> 10 ⁴ cfu
<i>ciaB</i> mutant	> 10 ² cfu	> 10 ³ cfu	> 10 ³ cfu
<i>ciaB</i> complement	> 10 ³ cfu	> 10 ³ cfu	> 10 ⁴ cfu

4.2.6 Intracellular survival assays

Our knowledge of *C. jejuni* interactions with, invasion of and survival within intestinal epithelial cells is limited compared to other enteropathogenic bacteria (Watson and Galan, 2008b, Bouwman *et al.*, 2013). However studies have revealed that *C. jejuni* can evade phagocytosis and survive for a certain period intracellularly and also trigger the release of host cytokines (Kiehlbauch *et al.*, 1985, Jones *et al.*, 2003, Watson and Galan, 2008b). The exact process of how this invasion and intracellular survival occurs is still unclear (Backert and Hofreuter, 2013). *C. jejuni* avoids delivery to the lysosomes and resides within *Campylobacter* containing vacuoles (CCV) (Watson and Galan, 2008b, Pryjma *et al.*, 2012).

To study the effect of the mutation of *ciaB* on the ability of *C. jejuni* to survive intracellularly in Caco-2 IECs, intracellular survival assays were performed. The 81-176 *ciaB* mutant exhibited significantly reduced intracellular survival levels compared to the wild-type strain (see Figure 4.13). The 81-176 *ciaB* complement did not fully restore the wild-type phenotype. The percentage survival of 81-176 wild-type, *ciaB* mutant and

complement was also calculated (see Table 4.5) which indicated reduced intracellular survival of the *ciaB* mutant.

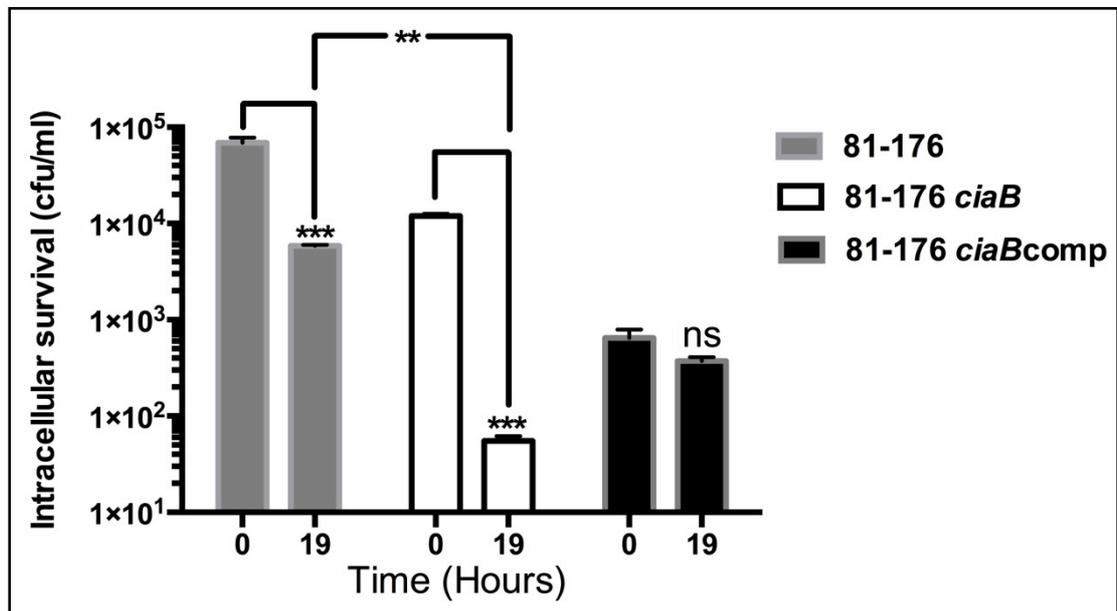


Figure 4.13 Intracellular survival assay. The 81-176 wild-type, *ciaB* mutant and complement were co-cultured with Caco-2 IECs for 3 h, washed three times with PBS, then incubated with gentamicin (150 µg/ml) for 2 h to kill extracellular bacteria, followed by further incubation with gentamicin for 19 h at a reduced concentration (10 µg/ml). The monolayers were then washed three times in PBS, lysed with Triton X-100 and numbers of intracellular bacteria assessed. Asterisks denote a statistically significant difference (** = $p < 0.01$, *** = $p < 0.001$, ns = no significant difference). Data represents triplicate independent experiments.

Table 4.5 Percentage survival of intracellular of 81-176 wild-type strain, *ciaB* mutant and complement after 19 h within Caco-2 IECs.

Bacterial Strain	% survival after 19 h
81-176 wild-type	10.10
81-176 <i>ciaB</i> mutant	0.72
81-176 <i>ciaB</i> complement	11.70

4.2.7 Macrophage survival assays

Once inside the host relatively little is known about the intracellular life cycle of *C. jejuni*, although it is presumed that *C. jejuni* behaves in a similar way to other enteropathogens with well characterised intracellular lifestyles and that *C. jejuni* may have evolved specific mechanisms to enable the survival of the bacteria in the harsh intracellular environment of the host cell (Watson and Galan, 2008b, Backert and Hofreuter, 2013). Macrophage survival assays were performed as described previously (Watson and Galan, 2008b) using J774A.1 mouse macrophages (Sikic Pogacar *et al.*, 2009). There was no significant difference in macrophage survival ability between the *ciaB* mutant and wild-type strain at 4 h (see Figure 4.14). However a significantly reduced intracellular survival level was observed at the 16 h time-point for the *ciaB* mutant compared to the wild-type strain. The *ciaB* complement displayed similar levels of survival to the wild-type at time 0 h and displayed significantly increased survival levels at the 4 h time point, however at the 16 h time point no bacteria were recovered.

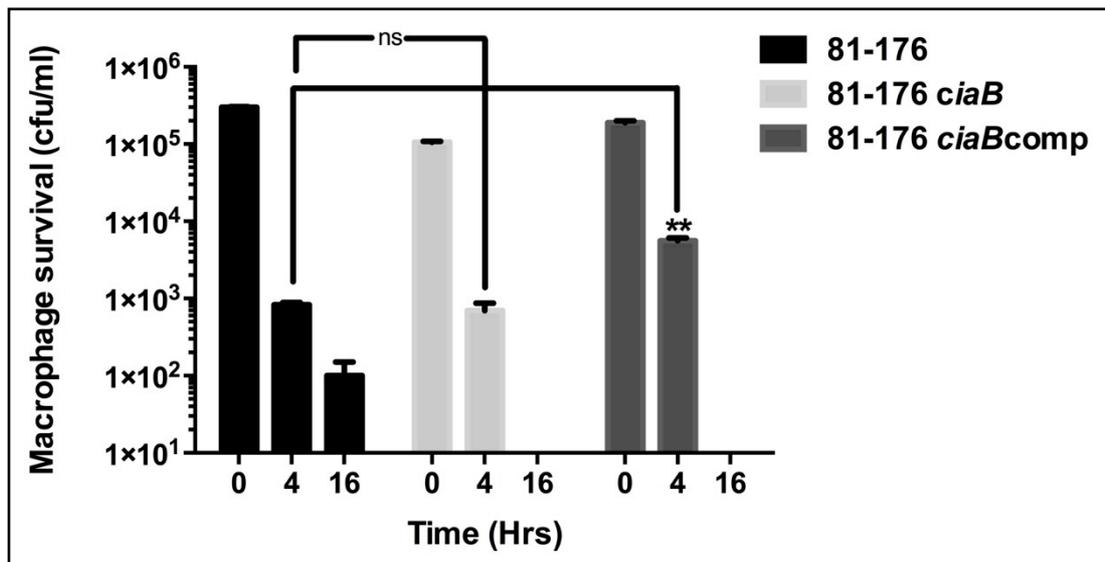
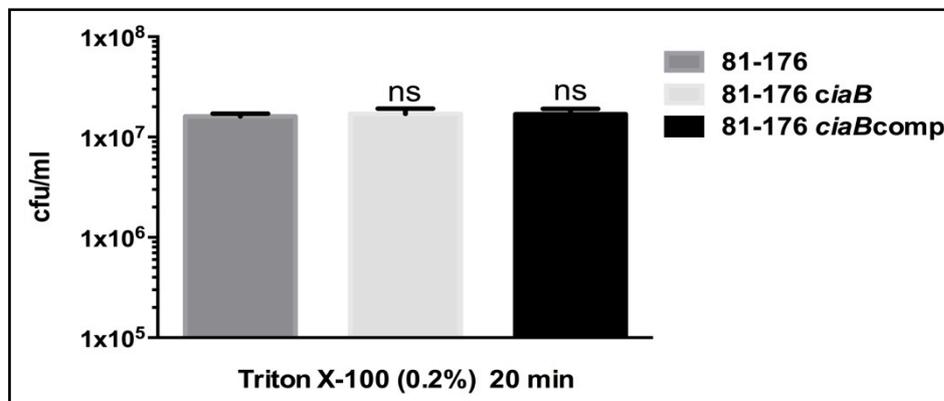


Figure 4.14 Macrophage survival assays. The 81-176 wild-type strain, *ciaB* mutant and *ciaB* complement were co-cultured with J774A.1 mouse macrophages for 3 h. The cells were washed three times in PBS, followed by incubation in DMEM containing gentamicin (150 µg/ml) for 2 h to allow killing of extracellular bacteria. The macrophages were then incubated in DMEM containing a reduced concentration of gentamicin (10 µg/ml) and bacterial survival determined at 0 h, 4 h and 16 h. At each time point, the macrophages were washed three times with PBS and lysed by adding 0.2% (v/v) Triton X-100 in PBS and the number of intracellular bacteria were assessed. The asterisks denote a statistically significant difference (** = $p < 0.01$, ns = no significant difference). Data represents triplicate independent experiments.

4.2.8. Sensitivity of *C. jejuni* to Triton X-100

To ascertain whether the above results were due to a genuine *ciaB* mutant phenotype and not due to increased sensitivity to Triton X-100 or gentamicin, sensitivity assays were performed on all the mutants and wild-type strains with 0.2% (v/v) Triton X-100 or gentamicin (150 µg/ml). No significant differences were observed between the levels of sensitivity to Triton X-100 (see Figure 4.15) or gentamicin (data not shown) between the respective wild-type strains and *ciaB* mutants, though the 11168H *ciaB* complement exhibited slightly decreased levels of sensitivity to Triton X-100.

(A)



(B)

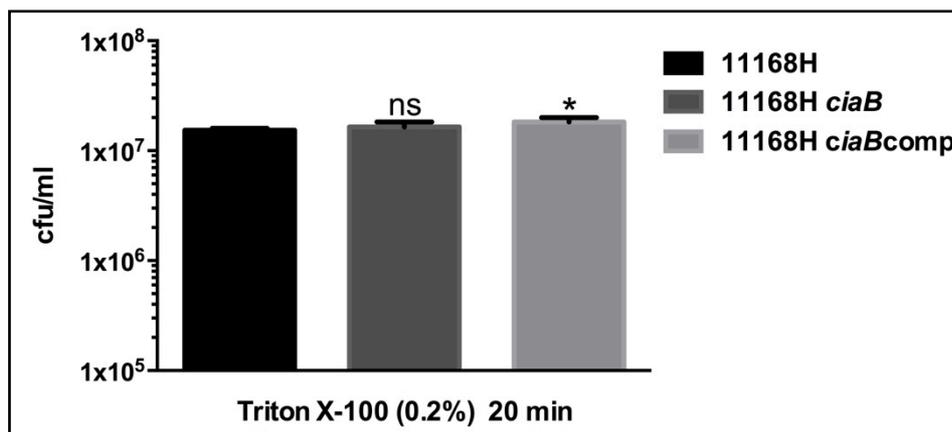


Figure 4.15. Sensitivity of *C. jejuni* strains to Triton X-100. Bacteria was grown for 24 h on blood agar. The bacteria were resuspended in PBS and adjusted to an OD₆₀₀ of 0.1. Triton X-100 was added to a final concentration of 0.2% (v/v) and after incubation at 37°C with 5% CO₂ for 20 min the number of viable bacteria was determined. (A) 81-176 wild-type strain, *ciaB* and *ciaB* complement (B) 11168H wild-type strain, *ciaB* and *ciaB* complement. Asterisks denote a statistically significant difference (* = $p < 0.05$, ns = no significant difference). Data represents triplicate independent experiments.

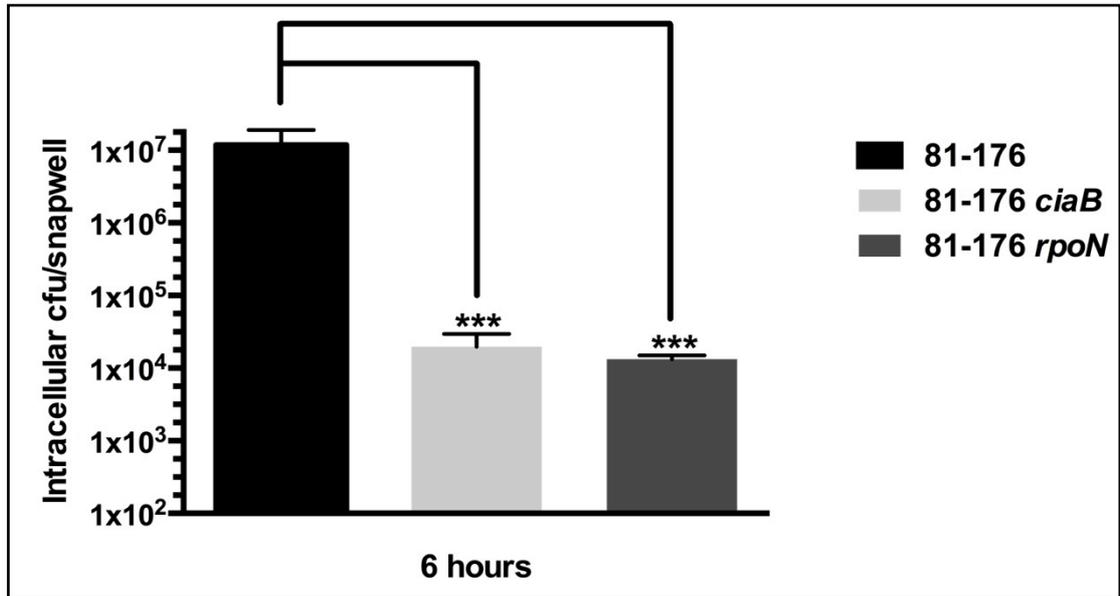
4.2.9 Vertical Diffusion Chamber assays

A major limitation of *in vitro* models used to study gastrointestinal infections is that standard tissue culture conditions include high oxygen levels which generally favour eukaryotic cell survival and do not allow the true observation of the biological interactions of the bacteria with the host cell (Mills *et al.*, 2012). In the case of *C. jejuni*, a number of different cell culture assays have been used to investigate the interactions of *C. jejuni* with host cells. Caco-2 (Everest *et al.*, 1992), INT407 (Konkel *et al.*, 1992b) and T84 (Monteville and Konkel, 2002) IEC lines have all been used extensively to study the adhesion and invasion capabilities of different *C. jejuni* strains. However, it has been reported that the levels of bacterial adhesion and invasion for *C. jejuni* with IECs are dramatically lower than for other enteric pathogens (Friis *et al.*, 2005).

The use of a Vertical Diffusion Chamber (VDC) system allows the co-culture of bacteria and host cells under different medium and gas conditions (Cottet *et al.*, 2002a, Schuller and Phillips, 2010, Mills *et al.*, 2012).

The 81-176 *ciaB* mutant exhibited significantly reduced invasion of both Caco-2 and T84 IECs compared to the wild-type strain (see Figure 4.16), however the numbers of intracellular bacteria were slightly higher when using T84 IECs. The *C. jejuni rpoN* mutant is non-motile and aflagellate and therefore unable to secrete CiaB and was included as a non-motile control (Mills *et al.*, 2012).

(A)



(B)

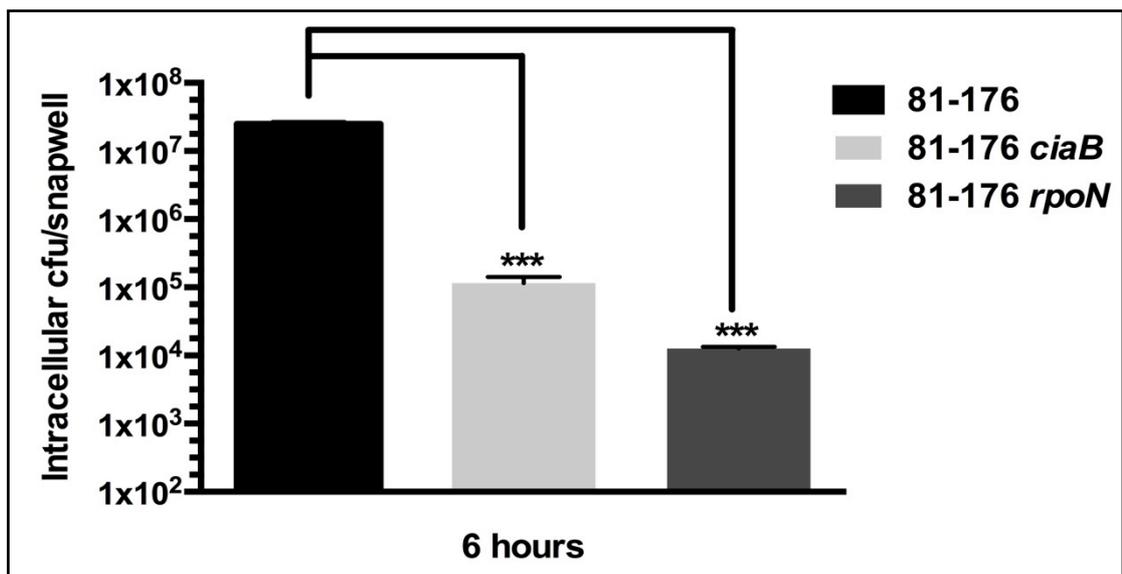


Figure 4.16 Vertical Diffusion Chambers assays. Bacteria were co-cultured with Caco-2 or T84 IECs in a Vertical Diffusion chamber at a multiplicity of infection (MOI) of approximately 100:1 under microaerobic conditions for 6 h and the numbers of intracellular bacteria were assessed. Caco-2 or T84 IECs were incubated with gentamicin (150 μ g/ml) for 2 h to kill extracellular bacteria, then lysed and numbers of intracellular bacteria assessed. 81-176 wild-type strain, *ciaB* and *rpoN* mutants co-cultured with (A) Caco-2 or (B) T84 IECs. The asterisks denote a statistically significant difference (***) = $p < 0.001$). Data are representative of triplicate independent experiments.

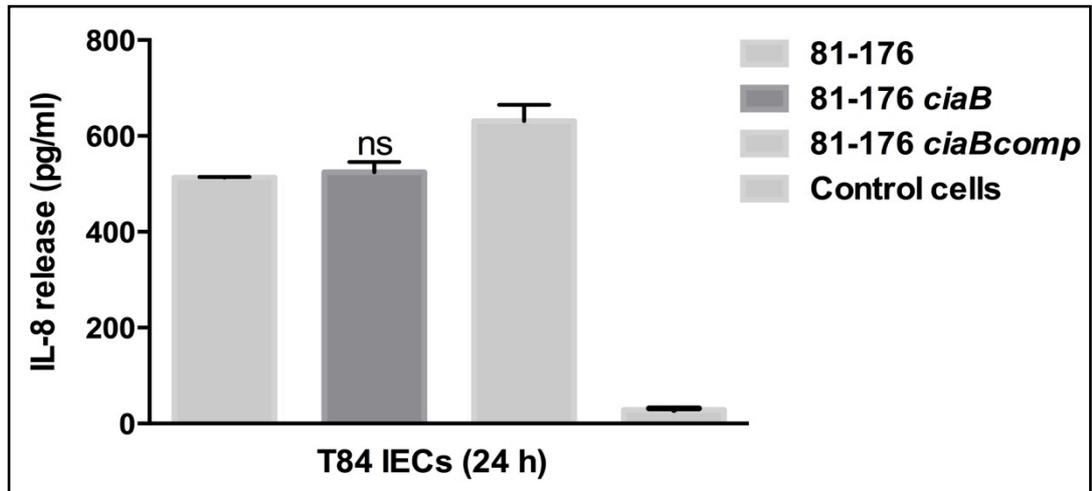
4.2.10 ELISA assays

IL-8 is a well characterised marker of the host immune response to pathogens (Oppenheim *et al.*, 1991) and interaction with and invasion of IECs by *C. jejuni* triggers both innate and adaptive immune responses (Chen *et al.*, 2006, Rathinam *et al.*, 2008). Cytokines of the TNF α family have been found to play central roles in the development of a stable immune system and protection from pathogens (Pfeffer, 2003).

Supernatants from uninfected T84 cells and T84 cells infected with 81-176 or 11168H wild-type strains, *ciaB* mutants and *ciaB* complement at an MOI of 100:1 for 24 h were collected. The levels of IL-8 and TNF α secretion were assessed.

No significant differences were observed in the induction of IL-8 between the 81-176, 11168H wild-type strains, *ciaB* mutants and *ciaB* complement (see Figure 4.17).

(A)



(B)

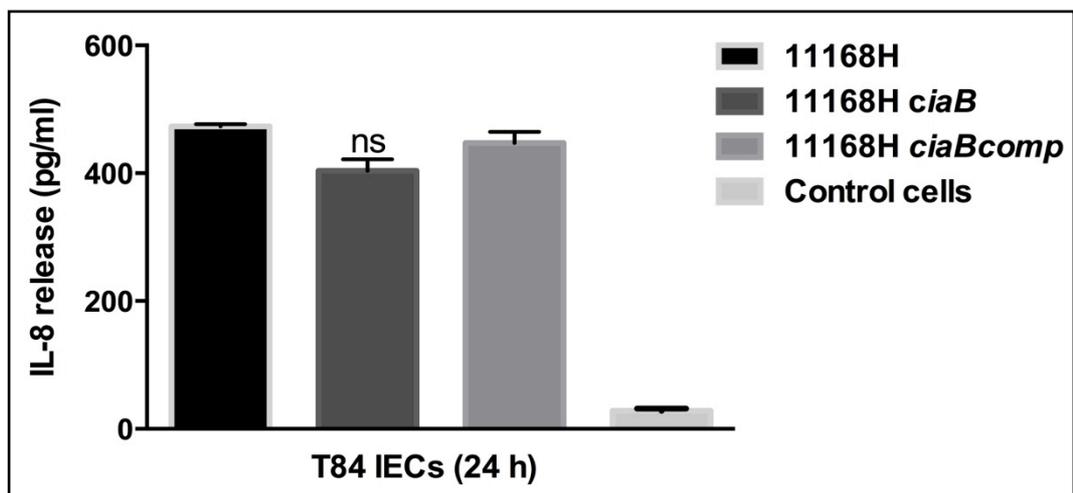
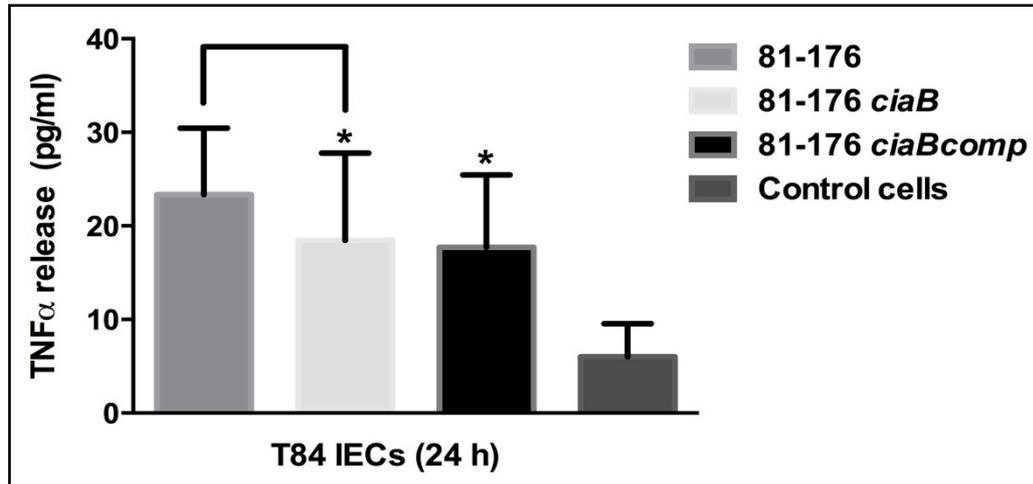


Figure 4.17. The role of CiaB in *C. jejuni* induction of IL-8 from T84 IECs. The 81-176 and 11168H wild type strains, *ciaB* mutants and *ciaB* complements were assessed. The levels of IL-8 secreted after 24 h *C. jejuni* interaction (MOI 100:1) with T84 IECs were quantified using a human IL-8 ELISA. (ns = no significant difference). Data are representative of triplicate independent experiments.

Both 81-176 and 11168H *ciaB* mutants induced reduced TNF α in comparison to the respective wild-type strains. The 81-176 *ciaB* complement did not restore the wild-type phenotype (see Figure 4.18).

(A)



(B)

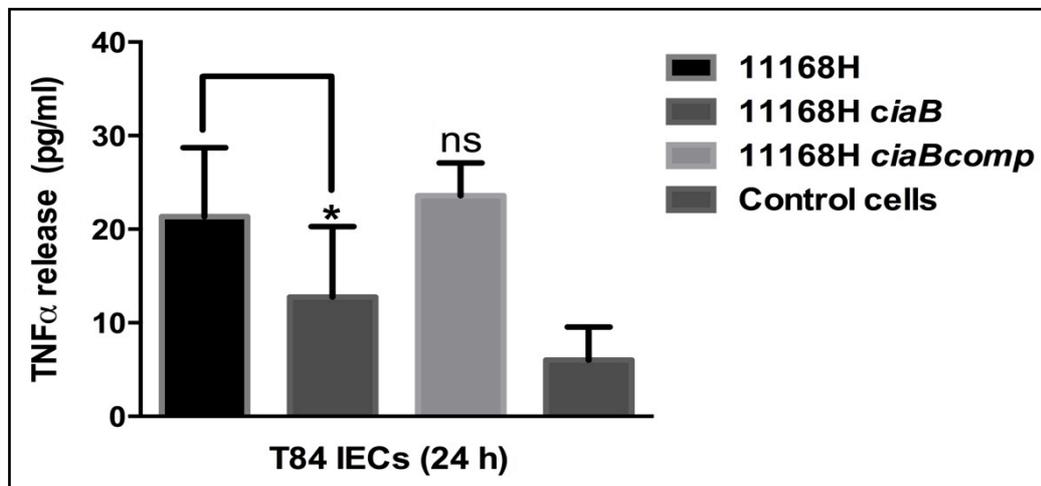
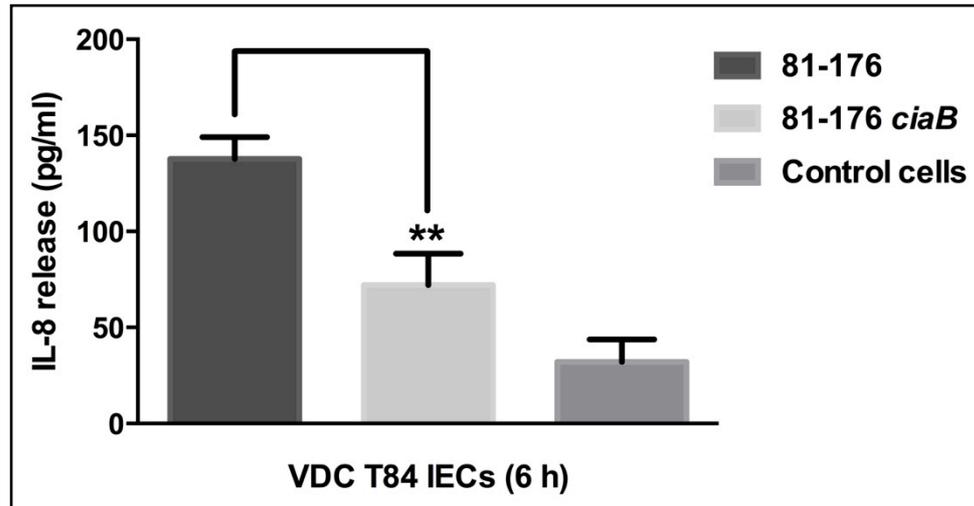


Figure 4.18. The role of CiaB in TNF α induction from T84 IECs. The 81-176 and 11168H wild type strains, *ciaB* mutants and *ciaB* complements strains were assessed. The levels of TNF α secreted after 24 h *C. jejuni* interaction (MOI 100:1) with T84 IECs were quantified using a human TNF α ELISA. The asterisks denote a statistically significant difference (* = $p < 0.05$, ns = no significant difference). Data are representative of triplicate independent experiments.

Following 6 h infection of T84 IECs in the VDC system, the levels of IL-8 and TNF α were assessed in the basolateral compartment. The 81-176 *ciaB* mutant induced a reduced IL-8 response from T84 IECs compared to the wild-type and only a slight reduction was observed in TNF α induction from T84 IECs by the *ciaB* mutant compared to the wild-type (see Figure 4.19).

(A)



(B)

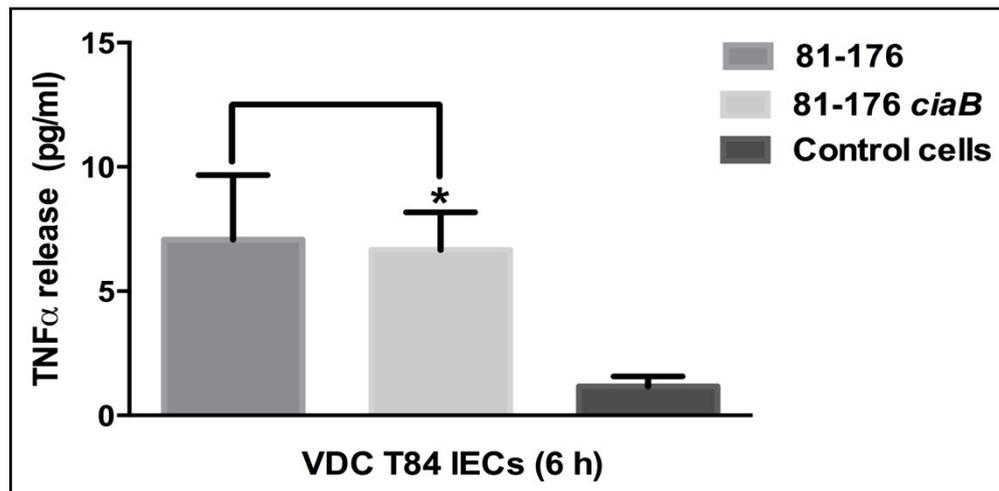


Figure 4.19. The role of CiaB in IL-8 and TNF α induction from T84 cells in the VDC system. T84 IEC responses to 6 h co-incubation with *C. jejuni* 81-176 wild type strain and *ciaB* mutant (MOI 100:1). The levels of IL-8 and TNF α secreted during *C. jejuni* interaction with T84 cells in the VDC were quantified using a human IL-8 or human TNF α ELISA. (A) IL-8 and (B) TNF α . Uninfected IECs were used included as controls. The asterisks denote a statistically significant difference (* = $p < 0.05$, ** = $p < 0.01$). Data are representative of triplicate independent experiments.

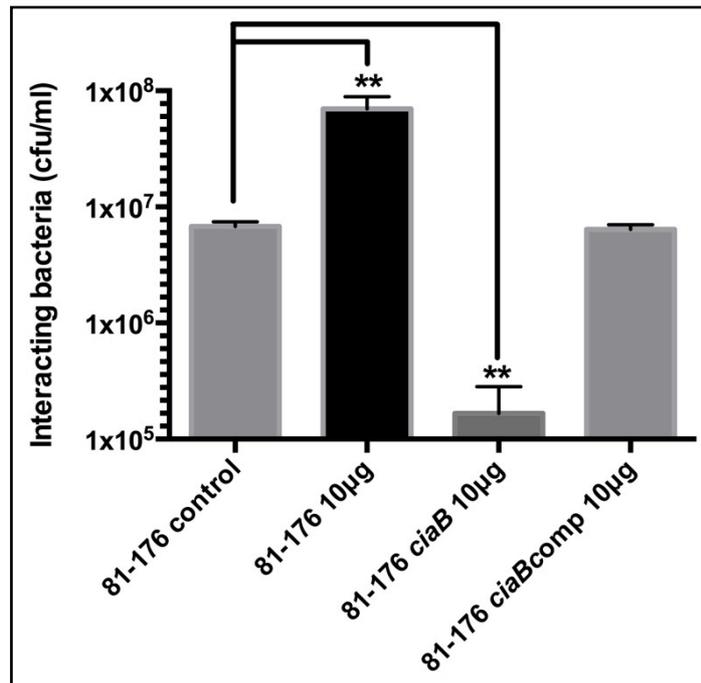
4.2.11 Role of outer membrane vesicles in *C. jejuni* interactions with IECs

Many Gram-negative pathogenic and non-pathogenic bacteria have been found to produce outer membrane vesicles (OMVs) (Kuehn and Kesty, 2005, Ellis and Kuehn, 2010). OMVs have also been found to contain virulence factors which play a role in pathogenesis (Ellis and Kuehn, 2010). Numerous Gram-negative bacteria including *E. coli*, *P. aeruginosa*, *C. jejuni* and *H. pylori* have been shown to produce OMVs (Horstman and Kuehn, 2000, Kesty *et al.*, 2004, Kuehn and Kesty, 2005, McBroom and Kuehn, 2007, Ellis and Kuehn, 2010, Kulp and Kuehn, 2010).

Caco-2 IECs were pre-incubated with OMVs for 24 h to ascertain whether OMVs would affect *C. jejuni* interactions with and invasion IECs

Pre-incubation with OMVs (10 µg) isolated from the 81-176 produced a significant increase in the number of interacting bacteria compared to the control with no treatment (see Figure 4.20). Pre-incubation with OMVs (10 µg) isolated from the 81-176 *ciaB* mutant produced a significant reduction in interacting bacteria compared to the control with no treatment. Pre-incubation with OMVs isolated from the *ciaB* complement restored the wild-type phenotype (see Figure 4.20). Pre-incubation with OMVs isolated from the *ciaB* mutant or *ciaB* complement (10 µg) resulted in a significant reduction in intracellular bacteria.

(A)



(B)

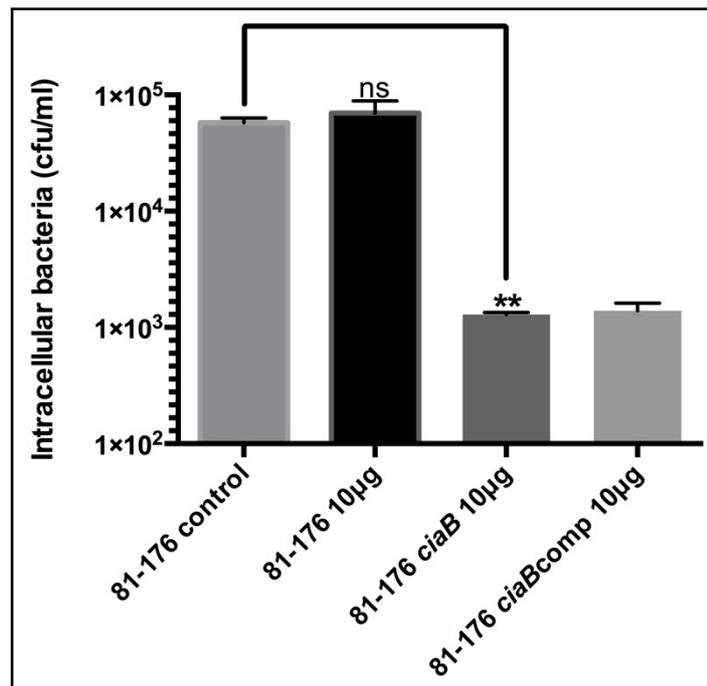


Figure 4.20 Effect of pre-incubation of *C. jejuni* OMVs on bacterial interactions with and invasion of Caco-2 IECs. OMVs (10 µg) isolated from *C. jejuni* 81-176 wild-type strain, *ciaB* mutant and *ciaB* complement were pre-incubated with Caco-2 IEC for 24 h. Monolayers were washed three times with PBS. Bacteria was harvested from 24 h plates, resuspended in PBS and adjusted to an OD₆₀₀ of 0.1. Bacteria was co-cultured with Caco-2 IECs for 24 h. (A) Caco-2 IECs were lysed with Triton X-100 and interacting bacteria were enumerated. (B) For invasion, Caco-2 IEC cells were incubated with gentamicin (150 µg/ml) for 2 h to kill extracellular bacteria, then lysed and numbers of intracellular bacteria assessed. Asterisks denote a statistically significant difference (* = $p < 0.05$; ** = $p < 0.01$, ns = no significant difference). Data are representative of triplicate independent experiments.

4.3 Discussion

Numerous reported studies using a variety of different cells lines and animal models have shown that mutations in *C. jejuni* genes encoding proteins involved with adherence, glycosylation, motility and capsule synthesis, result in reduced interactions with and invasion levels of IECs (Grant *et al.*, 1993, Pei *et al.*, 1998, Bacon *et al.*, 2001, Konkel *et al.*, 2001, Szymanski *et al.*, 2002, Hendrixson and DiRita, 2004, Kakuda and DiRita, 2006, Watson and Galan, 2008b). However there is no direct correlation that any specific virulence factor is involved in the invasion process (O. Croinin and Backert, 2012). One such virulence factor is CiaB and the role of this protein in *C. jejuni* invasion is contentious as CiaB is reported to be delivered directly into the host cell cytoplasm via the flagellar (Konkel *et al.*, 2004). In addition the same group reported that a F38011 *ciaB* mutant was capable of interacting with INT407 IECs following a 2 h incubation period to levels similar to the wild-type strain, however internalisation of the *ciaB* mutant was severely affected after a 3 h incubation period (Konkel *et al.*, 1999b). However, a 81-176 *ciaB* mutant was reported to display no differences in invasion, bringing into question the role of CiaB in *C. jejuni* invasion (Novik *et al.*, 2010).

In this study, *ciaB* mutants and complements were constructed in both the 81-176 and 11168H wild-type strains. Phenotypic characterisation of *ciaB* mutants was performed and no significant differences were observed in growth rates compared to the respective wild-type strains. Both 81-176 and 11168H *ciaB* complements exhibited increased growth rates in comparison to the wild-type strains and *ciaB* mutants and this was also reflected in the number of cfu counts enumerated at selected time-points points. *C. jejuni* motility has been linked to the ability of the bacteria to invade and is essential for colonisation of both human and animal models (Guerry, 2007). Both 81-176 and 11168H *ciaB* mutants were significantly reduced in motility compared to the wild-type strains, but still displayed some motility. The wild-type phenotype was not restored by the *ciaB* complement. It is not clear from the literature whether other research groups also showed a reduction in motility for their *ciaB* mutants studied.

Both 81-176 and 11168H *ciaB* mutants and complements were constructed. The role of CiaB in interactions with and invasion of Caco-2 and T84 IECs was investigated over a 3 h, 6 h and 24 h infection period. Initially, the 81-176 *ciaB* mutant exhibited reduced

interactions with Caco-2 IECs compared to the wild-type strain, with the number of interacting bacteria increasing over the 6 h and 24 h infection time-points. The 81-176 *ciaB* complement initially exhibited a reduction in interacting bacteria at 3 h and recovered to the wild-type levels by 6 h. In the invasion assay, the 81-176 *ciaB* mutant exhibited a significant reduction in the numbers of invading bacteria. The 81-176 *ciaB* complement also exhibited reduced numbers of intracellular bacteria, with a slight increase observed at the later time-points.

The interaction and invasion assays were also performed using T84 IECs to study the role of CiaB in interactions with and invasion of a second cell line. The number of 81-176 *ciaB* mutant bacteria interacting with T84 IECs was significantly reduced compared to the wild-type. The 81-176 *ciaB* complement was initially reduced but then recovered over the 6 h and 24 h infection period to wild-type levels. Previous work reported similar levels of interactions with and invasion of T84 IECs with the 81-176 wild-type strain after a 3 h infection period, however, the same study also reported that the F38011 *ciaB* mutant was not reduced in interactions with T84 IECs compared to the wild-type (Monteville and Konkel, 2002). In contrast another study reported no significant reduction in invasion of T84 IECs with a 81-176 *ciaB* mutant after a 2 h infection period and a similar study also failed to report any significant reductions in the *ciaB* mutant invasion of INT407 IECs (Goon *et al.*, 2006, Novik *et al.*, 2010). Furthermore, there have been no published studies on the invasion of Caco-2 IECs by a *ciaB* mutant which makes it difficult to compare these findings to the existing body of knowledge. The reduced motility of the 81-176 *ciaB* mutant may have affected the initial delay in interactions with Caco-2 IECs and as the 81-176 *ciaB* mutant exhibited increased levels of AAG, this may account for the increased interactions with and invasion of Caco-2 and T84 IECs observed over the infection time-points. The *ciaB* complement restored the wild-type phenotype in interactions with Caco-2 IECs, but failed to restore the wild-type invasion phenotype. However the *ciaB* complement restored the wild-type phenotype in both interactions with and invasion of T84 IECs.

The results obtained in this study using a 81-176 *ciaB* mutant are very different to those reported in the literature; a possible reason could be due to the variation in experimental procedures. For example, Konkel's work used a *C. jejuni* F38011 wild-type strain, the IEC line used was INT407 and in contrast to this study, higher numbers of bacterial

numbers (5×10^7 cfu) were used to infect the cells, followed by centrifuging the monolayers for 10 minutes to increase contact of the bacteria with the IECs and incubation times were between 30 minutes to 2 h (Konkel *et al.*, 1999b). In contrast Novik *et al.* used the 81-176 wild-type strain, the T84 IEC line and infected with an OD₆₀₀ of 0.1, monolayers were centrifuged for 5 minutes and incubated for 2 h (Novik *et al.*, 2010).

A key virulence determinant is the ability of *C. jejuni* to enter and survive intracellularly within IECs by residing in membrane-bound compartments (CCVs), modifying the internal conditions of the CCV which provides protection from the host immune system and avoiding delivery to the lysosomes and in addition intracellular replication of *C. jejuni* 81-176 has also been observed (Hickey *et al.*, 2005, Watson and Galan, 2008b, Buelow *et al.*, 2011a). Exactly how *C. jejuni* is able to modify the CCV environment is still unknown (Day *et al.*, 2000, Watson and Galan, 2008b, Backert and Hofreuter, 2013). This study showed that intracellular survival of the 81-176 *ciaB* mutant was significantly reduced compared to the wild-type strain and the wild-type phenotype was not restored by the 81-176 *ciaB* complement. As CiaB expression has also been reported to be required for the secretion of other Cia proteins (see Section 1.5.4), specifically a deficiency in the secretion of CiaI reported to play a role in intracellular survival by modification of the CCV environment, mutation of *ciaB* may result in the absence of CiaI and may affect *C. jejuni* intracellular survival. A *C. jejuni* *ciaI* mutant exhibited reduced intracellular survival in INT407 IECs (Novik *et al.*, 2010, Buelow *et al.*, 2011b). Previous studies report that *C. jejuni* loses viability within IECs over extended incubation periods and no intracellular replication is thought to occur (Konkel *et al.*, 1992b).

AAG is a well known virulence marker in a number of pathogenic Gram-negative bacteria, including *C. jejuni* (Misawa and Blaser, 2000). The 81-176 *ciaB* mutant exhibited significantly increased AAG. The 11168H *ciaB* mutant displayed no significant differences in AAG and the 11168H *ciaB* complement was significantly reduced in AAG compared to the wild-type strain. The role of CiaB was investigated in the *G. mellonella* model where the 81-176 *ciaB* mutant exhibited significantly reduced cytotoxicity in the larvae with over 70% of the larvae surviving at the 72 h infection period. The 81-176 *ciaB* complement partially restored wild-type levels of cytotoxicity

but with levels considerably lower than that of the wild-type strain. The 11168H *ciaB* mutant also exhibited a reduced cytotoxicity compared to the wild-type with 60% of the larvae surviving at 72 h but only 20% of the larvae survived after infection with the 11168H *ciaB* complement at 72 h.

To further study the intracellular survival ability of *C. jejuni ciaB* mutants, macrophage survival assays were performed. Previous studies have shown that macrophages are capable of killing *C. jejuni* within 24 h of internalisation (Watson and Galan, 2008a). In this study the survival of the 81-176 *ciaB* mutant was significantly reduced at the 16 h time-point, with no survival of bacteria observed compared to the wild-type. The 81-176 *ciaB* complement exhibited significantly increased levels of intracellular survival within macrophages at the 4 h time point compared to the wild-type and no bacteria were recovered at the 16 h time-point.

To further investigate the role of CiaB in invasion of Caco-2 and T84 IECs, VDC assays were performed. This system allowed a more accurate investigation of the interaction between *C. jejuni* and the IECs in an environment that more closely mimics the *in vivo* conditions encountered in the human gut. The 81-176 *ciaB* mutant was significantly reduced in invasion of both Caco-2 IECs and T84 IECs after a 6 h infection period, compared to the wild-type strain. A 81-176 *rpoN* mutant was included as a non-motile control with no Cia secretion (Fernando *et al.*, 2007b). In the complete absence of motility, the 81-176 *rpoN* mutant was still able to interact with and invade IECs, though at significantly reduced levels compared to the wild-type strain and the *ciaB* mutant, exhibited reduced motility and no secretion of CiaB yet was still capable of invasion. As motility is reported to be required for *C. jejuni* invasion (Poly and Guerry, 2008) yet in this study a non-motile mutant is still capable of invasion of IECs.

The VDC data showed higher levels of interactions with and invasion of Caco-2 and T84 IECs compared to the results obtained from standard tissue culture assays. The number of intracellular *ciaB* mutant recovered after a 6 h infection period of T84 IECs in the VDC was significantly higher than the numbers of intracellular bacteria enumerated under standard tissue culture conditions. These results show that *C. jejuni* invasion ability is dynamic depending on the experimental conditions and increased especially when co-cultured with IECs in the VDC (Mills *et al.*, 2012). In line with the reduced intracellular survival ability of the *ciaB* mutant and reduced levels of invasion

of Caco-2 and T84 IECs, CiaB could be playing a role in intracellular survival of *C. jejuni*. However, similarly to the results obtained from the interaction and invasion assays with the *ciaB* mutant, although significantly reduced, the *ciaB* mutant was still capable of surviving and invading IECs over extended periods of infection and this data is in contrast to a previous study reporting that *C. jejuni* was not capable of survival within the IECs for extended periods (De Melo *et al.*, 1989).

The role of *C. jejuni* in triggering the host immune response has been previously investigated and the results have been varied, dependent on the cell line and bacterial strain used (Backert and Hofreuter, 2013). Induction of IL-8 is reported to be an early signature of infection by enteric bacteria and also thought to be a signal of the acute inflammatory response to *C. jejuni* (Jung *et al.*, 1995, Hickey *et al.*, 1999). The levels of IL-8 induction from T84 IECs following infection with the 81-176 and 11168H *ciaB* mutants were not significantly different compared to the levels induced by the respective wild-type strains and similar levels of IL-8 induction have been shown following infection of INT407 IECs with 81-176. In addition, invasive *C. jejuni* strains have been shown to induce higher levels of IL-8 (Oelschlaeger *et al.*, 1993, Hickey *et al.*, 1999). The 81-176 *ciaB* complement induced slightly higher levels of IL-8 compared to the wild-type and the 11168H *ciaB* complement. Both 81-176 and 11168H wild-type strains induced similar levels of IL-8 secretion from T84 IECs.

In the induction of TNF α , both wild-type strains induced similar levels of TNF α . In contrast, both 81-176 and 11168H *ciaB* mutants induced reduced levels of TNF α compared to the respective wild-type strain. The 81-176 *ciaB* complement did not restore the wild-type phenotype. The supernatants from the VDC were probed for IL-8 and TNF α following a 6 h infection with the 81-176 wild-type and *ciaB* mutant. Infection of T84 IECs with the *ciaB* mutant in the VDC resulted in a significant reduction in IL-8 and TNF α induction compared to the wild-type at 6 h which concurs with the hypothesis that *C. jejuni* displays dynamic interactions with IECs when under microaerobic conditions in the VDC than when in standard tissue culture. In contrast to no significant differences in IL-8 induction observed following infection with the *ciaB* mutant under standard tissue culture conditions, the IL-8 levels induced were still significantly higher under standard *in vitro* assay culture conditions than those induced following co-culture of the *ciaB* mutant in the VDC. The differences observed could

potentially be due to the incubation period, as the standard *in vitro* tissue culture assays were incubated for 24 h, whilst the VDC infection period was 6 h. Whether this is reflective of the real *in vivo* interactions of *C. jejuni* with IECs would require further study.

The role of OMVs isolated from the *ciaB* mutant in invasion was investigated as the hypothesis proposed was that pre-incubation of Caco-2 IECs with *C. jejuni* OMVs prior to infection with live bacterial cells could exhibit effects on the bacterial interactions with and invasion of Caco-2 IECs. Pre-incubation with OMVs 10 µg isolated from *ciaB* mutant resulted in a significant reduction in interacting bacteria compared to the control with no pre-incubation. Equally, a significant reduction in intracellular bacteria was also observed after pre-incubation with OMVs 10 µg isolated from *ciaB* mutant compared to the control with no pre-incubation. Pre-incubation of Caco-2 IECs with OMVs 10 µg isolated from 81-176 wild-type significantly increased bacterial interactions with Caco-2 IECs compared with the no pre-treatment control. Previous studies at LSHTM found that proteomic analysis of OMVs isolated from the 11168H wild-type found that neither CiaB, CiaC or CiaI were associated with OMVs and that the production of OMVs by *C. jejuni* was not a route for secretion for these Cia proteins (Elmi *et al.*, 2012).

In summary, the mutation of *ciaB* clearly affects *C. jejuni*, with varying results in both 81-176 and 11168H strains including reduced interactions with and invasion of IECs, This supports Konkel's work, in addition it is difficult to compare the *ciaB* T84 IEC invasion data from this study to that reported by Novik *et al.* Although the report suggests no differences in invasion of the 81-176 *ciaB* mutant compared to the wild-type, there is clearly a reduction observed, as the *ciaB* mutant was ~ 80% reduced compared to the wild-type. However, the data is difficult to compare with complete accuracy due to the ways the data were measured (Novik *et al.*, 2010).

Chapter 5. Reinvestigation of the roles of CadF and FlpA in *C. jejuni* adhesion to and invasion of intestinal epithelial cells

5.1 Introduction

The ability of pathogenic bacteria to adhere to host tissues is an important early event in colonisation (Roberts, 1990, Isberg and Van Nhieu, 1994, Alruz and Isberg, 1998). *C. jejuni* virulence factors that contribute to pathogenesis are multifactorial and include motility, host cell adherence, invasion, protein secretion, manipulation of host cell signalling and evasion of host immune defences (Konkel *et al.*, 2001, Wilson, 2002). When microbial pathogens gain access to the host and colonise tissues, these pathogens can utilise components of the host cell extracellular matrix (ECM) to adhere to and enter host cells. The ECM can be subdivided into two families, collagens such as laminin and glycoproteins such as fibronectin (Mecham, 2011). The host ECM components are a network of proteins each with a role in building the architecture of the cell and in cell adhesion (Carsons, 1989, Hynes, 1990, Yamada, 2012). Fibronectin is a large glycoprotein found on the surface of mammalian cells (McKeown-Longo, 1987), with a number of domains that exhibit specific binding properties with other host cell proteins and ECM proteins (Romberger, 1997, Kleba *et al.*, 2002). Fibronectin is a component of the ECM, present in plasma and connective tissue, serving many *in vivo* functions such as maintenance of the cell, organisation of tissues, substrate binding and a role in cell motility (Romberger, 1997). Many pathogens have evolved ways to target and utilise this large glycoprotein, by expressing fibronectin binding proteins on the bacterial surface (Signas *et al.*, 1989, Kreikemeyer *et al.*, 1995).

Invasion of the epithelial cells lining the intestinal tract is hypothesised to be essential for the development of *C. jejuni*-mediated enteritis (Young *et al.*, 2007, Dasti *et al.*, 2010). Studies have indicated that bacterial ability to bind to fibronectin is a key feature in successful cell invasion (Monteville and Konkel, 2002, Krause-Gruszczynska *et al.*, 2007b, Krause-Gruszczynska *et al.*, 2011). Previous studies have identified a number of adhesins expressed by *C. jejuni* that allow binding to cultured epithelial cells (Konkel *et al.*, 1992a). *C. jejuni* expresses a number of outer membrane proteins that have been reported to act as adhesins by binding to specific receptors on the host cell surface and allowing interaction with host cells (Young *et al.*, 2007). One of the most well

characterised *C. jejuni* adhesins is CadF, a 37 kDa protein with binding affinity to fibronectin (Konkel *et al.*, 1997). CadF was shown to bind to INT407 membrane fractions (Moser *et al.*, 1997). Further studies highlighted the importance of CadF as *cadF* mutants were unable to colonise chickens (Ziprin *et al.*, 1999). In addition, *C. jejuni* possesses another adhesin FlpA that also contains fibronectin binding amino-acid domains. FlpA was found to play a significant role in the colonisation of chickens, as mutations in *flpA* completely prevented the colonisation of chicks (Flanagan, Neal-McKinney *et al.* 2009). A *flpA* mutant also displayed a reduction in adherence to INT407 cells (Konkel, Larson *et al.* 2010). Studies have shown that CadF and FlpA also play a role in activation of host cell proteins such as small Rho GTPases Rac1 and Cdc42, leading to membrane ruffling and dynamic changes in the host cell and are thus required for bacterial entry into the host cell (Krause-Gruszczynska *et al.*, 2007b, Krause-Gruszczynska *et al.*, 2011). This part of the study will reinvestigate the role of CadF and FlpA in *C. jejuni* adhesion to and invasion of intestinal epithelial cells. In particular the use of the Galleria infection models, VDC and OMVs will provide new data on these reported *C. jejuni* determinants.

5.2 Results

5.2.1 Chromosomal location of *cadF* and *flpA*

The Chromosomal location of *cadF* and *flpA* in 81-176 and 11168H strains was viewed in Artemis software, with up and downstream genes listed below.

C. jejuni 81-176 *cadF* upstream genes are *rpsI* (ribosomal protein), *Cjj81-176_1474 rpIM*, (Type II protein secretion system D protein/ ribosomal protein S13), *Cjj81-176_1475* (unknown function) and the downstream genes are: *Cjj81-176_1470 ctsF* (pseudo/general secretory pathway protein), *Cjj81-176_1469* (putative pyruvate-flavodoxin oxidoreductase) (see Figure 5.1).

C. jejuni 11168H *cadF* upstream genes are *rpsI* (ribosomal protein) *Cj1481c rpIM*, (ribosomal protein S13), *Cj1482c* (unknown function). The 11168H *cadF* downstream genes are: *Cj1477c* (hydrolase), *Cj1476c* (putative pyruvate-flavodoxin oxidoreductase) and *ctsR* (orf) (see Figure 5.1).

In both 81-176 and 11168H strains, the genes upstream and downstream of *cadF* are the same except that the *ctsF* gene in 81-176 is different to *Cj1477c* gene encoding a hydrolase.

(A)



(B)

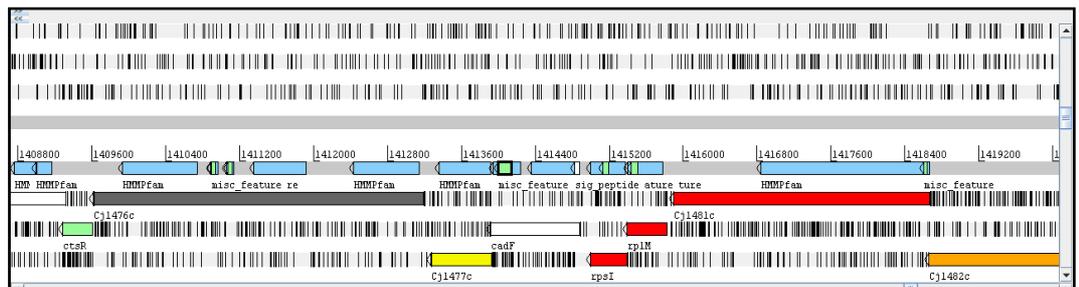


Figure 5.1 Artemis Images displaying the positions of *cadF* genes in *C. jejuni* 81-176 (A) and 11168H (B)

C. jejuni 81-176 *flpA* upstream genes are *Cjj81-176_1296* ribosomal large subunit pseudouridine synthase A / (putative antimicrobial efflux pump). The downstream genes are *Cjj81176_1294 trmB* (tRNA (guanine-N7-)-methyltransferase activity), *lolD* (putative ABC transporter ATP-binding protein), *Cjj81-176_1292* (deoxycytidine triphosphate deaminase/putative cell division protein), *Cjj81-176_1291* (putative biotin carboxyl carrier protein of acetyl-CoA carboxylase/fatty acid biosynthesis/family peptidase M24/M37), *pyrH* (enzymatic reaction of uridylylate kinase), *rpoZ* (promotes RNA assembly).

C. jejuni 11168H *flpA* upstream genes are *Cj1280* (putative ribosomal protein synthesis and modification/ribosomal pseudouridine synthase) and *Cj1285c* (conserved hypothetical protein/putative periplasmic solute binding protein). The downstream

genes are, *trmB* (tRNA (guanine-N7-)-methyltransferase activity), *Cj1227c* (two component regulator/ABC transporter ATP binding), *Cj1276c* (putative integral membrane protein), *Cj1275c* (putative periplasmic protein), *pyrH* (enzymatic reaction of uridylylate kinase), *rpoZ* (promotes RNA assembly).

In both 81-176 and 11168H, the genes adjacent to *flpA* are the same except that the genes downstream of *flpA* in 81-176 contain a *Cjj_1292* which encodes a putative cell division protein not present in 11168H.

(A)



(B)

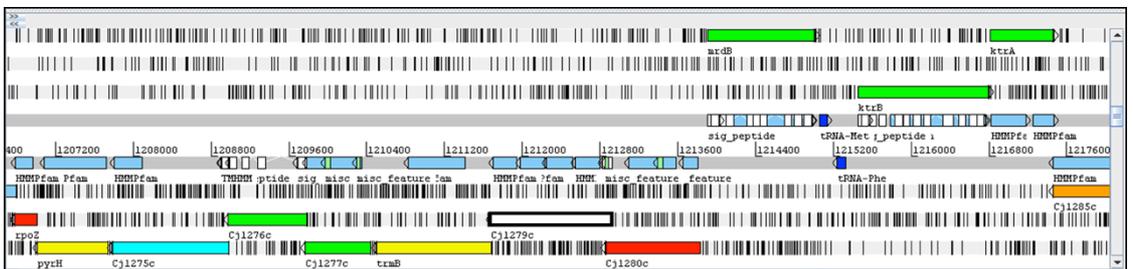


Figure 5.2 Artemis Images displaying the positions of *flpA* genes in *C. jejuni* 81-176 (A) and 11168H (B)

5.2.2 Construction of 81-176 and 11168H *cadF* mutants

C. jejuni genomic DNA (gDNA) was extracted and primers *cadF*-Forward-81-176/*cadF*-Reverse-81-176 and *cadF*-Forward-11168H/*cadF*-Reverse-11168H were designed for the amplification of the *cadF* genes from 81-176 and 11168H gDNA respectively. PCR reactions were set up using the appropriate primers. Each PCR product was analysed by agarose gel electrophoresis and purified using the Qiagen PCR clean up kit. Each purified PCR product was ligated into the pGEMT-easy vector (Promega). Each pGEMT-easy-*cadF* construct was transformed into *E. coli* SCS110

competent cells and plated out on LB amp agar plates. Positive colonies selected by growth on LB amp agar were screened using boilate PCR to check for the presence of *cadF* then sequenced to confirm cloning of the correct gene fragment. Each cloned gene fragment was disrupted by insertion of a Km^r cassette (Trieu-Cuot *et al.*, 1985). The Km^r cassette was excised using BamHI from plasmid pJMK30 (van Vliet *et al.*, 1998). pGEMT-easy-*cadF* plasmid DNA was digested with BclI and ligated with the Km^r cassette to form pGEMT-easy-*cadF*-Km^r. These constructs were then transformed into *E. coli* SCS110 competent cells and transformants selected on LB amp kan agar. Transformants were screened by boilate PCR using *cadF*-gene specific and Km^r-specific primers. pGEMT-easy-*cadF*-Km^r constructs with the Km^r cassette in the same orientation as the *cadF* gene were selected and electroporated into either the 81-176 or 11168H wild-type strain. Electroporated bacteria were plated onto blood agar plates and incubated at 37°C under microaerobic conditions for 2 to 3 days. Cells were harvested and resuspended in 0.5 ml PBS. Two hundred microliters of this suspension was spread onto blood agar plates containing kanamycin and incubated for 3 to 5 days. Putative *cadF* mutants were screened using PCR and sequencing. Gene specific primers were used to confirm presence of *cadF* and to confirm insertion and correct orientation of the kanamycin cassette.

5.2.3 Construction of 81-176 and 11168H *flpA* mutants using Inverse PCR

Mutagenesis

Inverse PCR mutagenesis (IPCRM) can be performed when the gene to be mutated does not contain a BamHI, BclI or BglIII restriction site. The *Cjj81-176_1295* and *Cj1279c* genes were amplified by PCR using *flpA*-Forward-81-176/*flpA*-Reverse-81-176 and *flpA*-Forward-11168H/*flpA*-Reverse-11168H from *C. jejuni* and 11168H gDNA respectively (see Figure 5.3). Each PCR product was analysed by agarose gel electrophoresis and purified using the Qiagen PCR clean up kit. Each purified PCR product was ligated into the pGEMT-easy vector (Promega). Each pGEMT-easy-*flpA* construct was transformed into XL-2 Blue MRF⁷ competent cells and plated out on LB amp agar plates. Positive colonies selected by growth on LB amp agar were screened using boilate PCR to check for the presence of *flpA*, then sequenced to confirm cloning of the correct gene fragment. A BglIII restriction site was introduced using primers *flpA*-IPCRM-Forward and *flpA*-IPCRM-Reverse. IPCRM was performed on cloned

pGEMT-easy-*flpA* fragments at a diluted concentration of approximately 0.1-10 ng. The reduced concentration of template DNA was used to minimise the number of false positives after transformation into XL-2 Blue MRF' competent cells. IPCRM products were analysed on an agarose gel. The amplified IPCRM product was purified using the QIAquick PCR Purification kit. The amplified IPCRM product was then digested with BglIII and DpnI for 3 h at 37°C, then purified using the Qiagen PCR clean up kit. Digestion using DpnI was performed to ensure the reduction/elimination of methylated template DNA that could lead to false positive transformation (Shenoy and Visweswariah, 2003). Each cloned IPCRM-*flpA* gene fragment was disrupted by insertion of Km^r (Trieu-Cuot *et al.*, 1985). The Km^r cassette was excised using BamHI from plasmid pJMK30 (van Vliet *et al.*, 1998). The Km^r cassette was ligated with the IPCRM-*flpA* to form pGEMT-easy-*flpA*-Km^r. These constructs were then transformed into XL-2 Blue MRF' competent cells and transformants selected on LB amp kan agar. Transformants were screened by boilate PCR using *flpA*-gene specific and Km^r-specific primers. The pGEMT-easy-*flpA*-Km^r constructs with the Km^r cassette in the same orientation as the *flpA* gene were selected and electroporated into either the 81-176 or 11168H wild-type strain. Electroporated bacteria were plated onto blood agar plates and incubated at 37°C under microaerobic conditions for 2 to 3 days. Cells were harvested and resuspended in 0.5 ml PBS. Two hundred microliters of this suspension was spread onto blood agar plates containing kanamycin and incubated for 3 to 5 days. Putative *flpA* mutants were screened using PCR and sequencing. Gene specific primers were used to confirm presence of *flpA* and to confirm insertion and correct orientation of the kanamycin cassette.

ATGATGAAAAGATTTTCGCTTGAGTTTTTACTTGAGTTTTTTAACTCTTTTGCT
 TAGCGCTTGTAGTGTTCGCAAATGAATTCGCTAGCTTCAAGTAAAGAGCCT
 GCTGTAAATGAAAGCTTGCCAAAGGTTGAAAGTTTAAAAAGTCTTAGTGAT
 ATGAGTAATATGCTTTTGAATGGGAGCCTTTATATAATGAAAATATTAAGG
 ATTTTATTTGTATCGTTCTAGTGATGAAAATCCTGATTTTAACTTGTAGGCA
 CTATTAAGATAAGTTTCAAACCTCATTATGTAGATACTAAATTAGAGCCTGG
 TACTAAGTATCGTTATATGATGAAAAGCTTTAATGAGCAAGGACAAATTC
 AGAAGATGGCAAGGTTATAGAAGTGAGCACAGCTCCAAGACTTGAAGCTGT
 TCCTTTTGTTC AAGCTGTGACTAATTTGCCTAATCGTATTA AACTTATTTGGC
 GTCCGCATCCTGATTTTAGGGTTGATTCTTATATTATTGAAAGAACCAAAGG
 TGATGATAAAGAATTTAAAAAATTGCAGAAGTAAAAAATCGTTTAAACGC
 TGAATACATCGATAGTGATTTAAAGCCTAATGAAAATTCAAGTTATAGAATT
 ATTGCTGTGAGTTTT **AATGGGATAAAGAGTGGGT**CAAGTCAAGTTGTAAGTT
 CTACA **GCAAGGCTTTACCTCCTCAAG**TTGAGCATTTAAGTGCTAGCACAGA
 TGGTTCTAGTAAAATCATTTTAACTTGGGATGCTCCTACGTATGAAGATTTT
 TCTTATTATAAAGTTTATTCTACGAGCTCAAGCTTCCTTCCTTTTAGTGTTTT
 GGCAAAGACTGATAAAAATTCTTATGAGGATATAGTAGAAGGAGCAGGTAA
 AAGCAAGTATTATAAAGTAACAATGGTGGATAAAGATGGTCTTGAAAGTCC
 TATGCCAAAAGATGGTGTAGAAGGTAAAACCTTTAGGCAACCCTTTGGCTCC
 TAGTATTATTTTGGCTCAAAGTACAAGCGAAGGGATAAATTTAGAATGGAG
 TGATAATGATACTAGAGCTGTTGAGTATGAAGTAAGACGCTATGGCGGGGA
 GCAAATGCAGTTTTTAAAGGCATTAAGAAAAGCGATTA AAGATGTAAA
 AGCTTTGCCAGGGGTGGAATATAGTTATGAAGTTATTGCTATTGATTCAGCC
 GGGCTTCGTT CAGAACCTTCAAGCAAAG **GTTAAGGCGGCTCAGTA**

flpA-IPCRM F - ATCAGATCT **GCAAGGCTTTACCTCCTCAAG**

flpA-IPCRM R - ATCAGATCT **ACCCACTCTTTATCCCATT**

Figure 5.3. PCR and IPCRM primers designed for construction of the *flpA* mutant. The 5'-end of the IPCRM primers contained three extra residues to allow efficient functionality of the BglII restriction site. This was followed by the BglII complementary sequence – AGATCT.

5.2.4 Construction of 81-176 and 11168H *cadF* and *flpA* complements

As this study used both *C. jejuni* wild-type strains, complementation procedures were also performed in both strains to confirm that the phenotypes observed were due to the mutation and not due to random DNA changes or polar effects. Complementation was performed by inserting a copy of the functional *cadF* or *flpA* gene into the respective mutant chromosome using a *C. jejuni* complementation vector pRRC (Karlyshev and Wren, 2005). The coding region for *cadF* or *flpA* was amplified by PCR using primer pairs *cadF*comp-Forward-81-176/*cadF*comp-Reverse-81-176, *cadF*comp-Forward-11168H/*cadF*comp-Reverse-11168H, *flpA*comp-Forward-81-176/*flpA*comp-Reverse-81-176 or *flpA*comp-Forward-11168H/*flpA*comp-Reverse-11168H which introduced an XbaI site at both the 5' and 3' ends, as well as including the native ribosomal binding site. Each PCR product was analysed by agarose gel electrophoresis and purified using the Qiagen PCR clean up kit. Each purified PCR product and the pRRC vector were digested with XbaI. The digested products were purified using the Qiagen PCR clean up kit then ligated with the pRRC vector. Each pRRC-*cadF*/*flpA*-comp construct was transformed into *E. coli* DH5 α cells and plated out LB chl agar plates. Positive colonies selected by growth on LB chl agar were screened using boilate PCR to check for the presence of *cadF* or *flpA*. Plasmid DNA was extracted from positive colonies. Sequencing the pRRC-*cadF*-comp construct showed that the *cadF*comp gene was in the incorrect orientation and also the *Pfx* high quality polymerase amplification of *cadF* had introduced numerous base changes into the amplified *cadF* gene. Despite numerous attempts, the same result was always observed. The pRRC-*flpA*-comp construct was more successful as the presence of the correct gene and orientation was confirmed by PCR and sequencing. However, following electroporation into either the 81-176 or 11168H *flpA* mutants, no growth was observed. The procedure was repeated with the same result on each occasion.

cadF is 960 bp, the amplified *cadF* gene fragment is 900 bp and successful insertion of the 1400 bp kanamycin cassette produced a total amplified size of 2300 bp. *flpA* is 1200 bp, the amplified *flpA* gene fragment is 1200 bp and insertion of 1400 bp kanamycin cassette produced a total amplified size of 2600 bp.

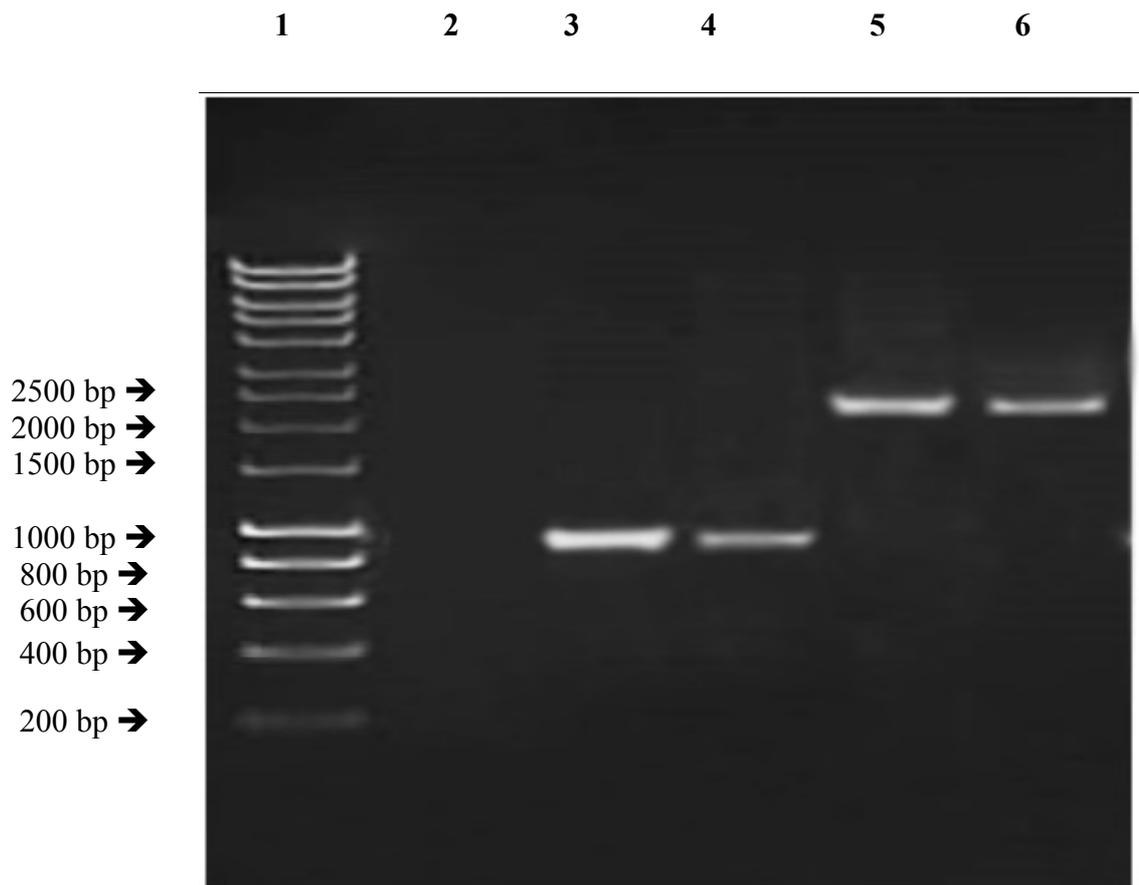


Figure 5.4 PCR verification of *C. jejuni* wild-type and *cadF* mutants. **Lane 1:** 1 Kb ladder (Bioline). **Lane 2:** Negative control. **Lane 3:** 11168H *cadF*. **Lane 4:** 81-176 *cadF*. **Lane 5:** 11168H *cadF* mutant. **Lane 6:** 81-176 *cadF* mutant.

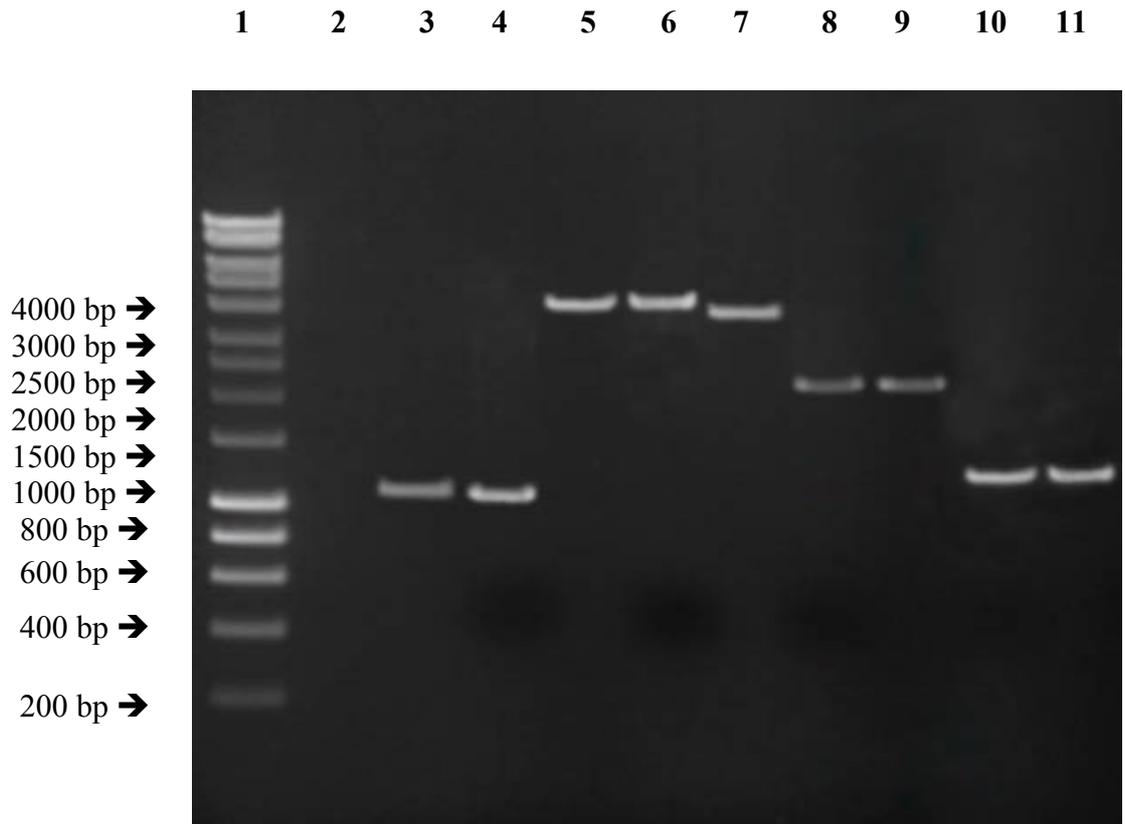


Figure 5.5 PCR verification of *C. jejuni* wild-type *flpA* mutant and *flpA* complement: Lane 1: 1 Kb ladder (Bioline). Lane 2: Negative control. Lane 3: 11168H *flpA* Lane 4: 81-176 *flpA* Lane 5: 11168H-*flpA*-IPCRM. Lane 6: 81-176-*flpA*-IPCRM. Lane 7: Digested IPCRM product. Lane 8:11168H *flpA* mutant. Lane 9: 81-176 *flpA* mutant. Lane 10: 11168H *flpA* complement. Lane 11: 81-176 *flpA* complement

5.2.4. Characterisation of 81-176 and 11168H *cadF* and *flpA* mutants

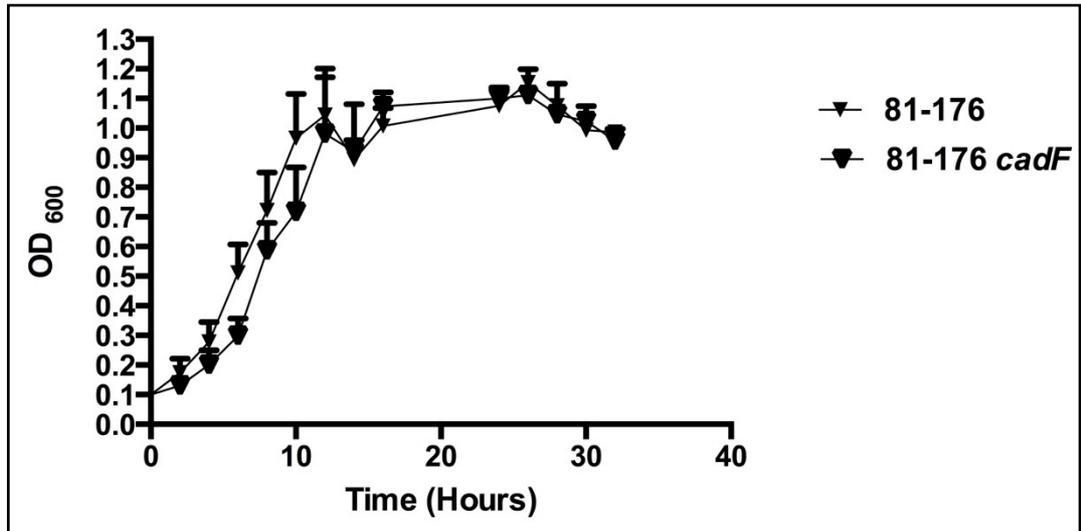
Following the construction of the 81-176 and 11168H *cadF* and *flpA* mutants, initial phenotypic characterisation studies were performed.

5.2.4.1 Growth rate assays and cfu counts

To assess the effect of the mutations of *cadF* and *flpA*, growth rate experiments were performed. OD₆₀₀ readings were recorded every 2 hours over a 32 h growth period as an indication of bacterial growth.

Both the 81-176 and 11168H *cadF* mutants exhibited similar growth rates compared to the respective wild-type strain (see Figure 5.6). The numbers of colony forming units (cfu) at different time-point were also investigated for the 81-176 and 11168H wild-type strains and *cadF* mutants (see Figure 5.7). The 11168H *cadF* displayed a slightly reduced cfu count in comparison to the wild-type strain at most time-points.

(A)



(B)

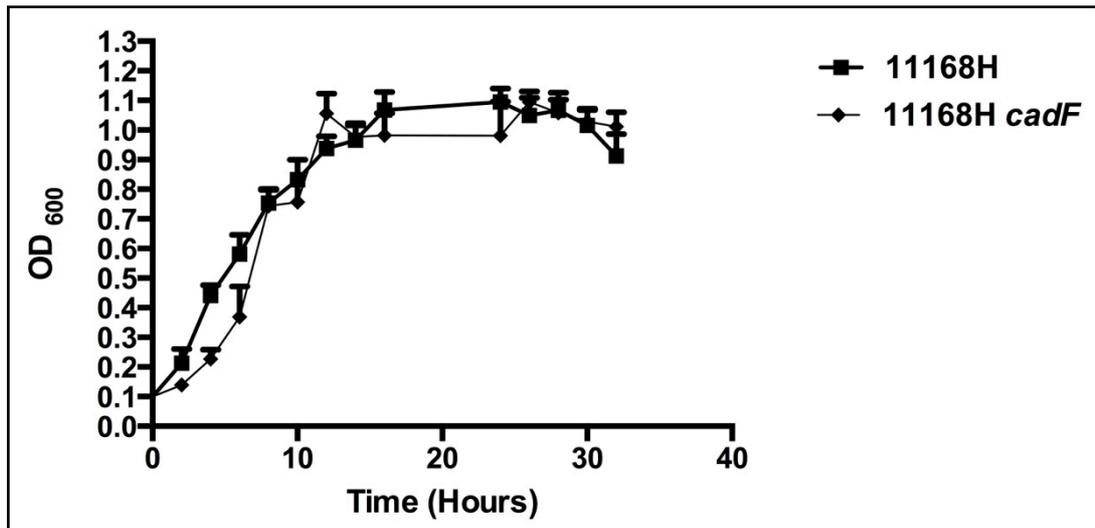
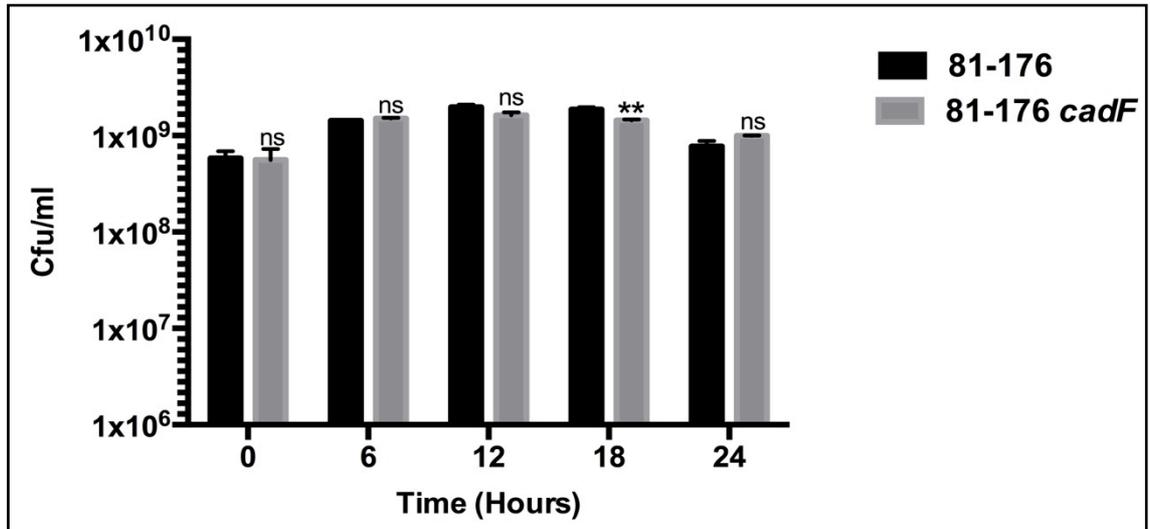


Figure 5.6. Growth rate counts for 81-176 and 11168H wild-type strains and *cadF* mutants. *C. jejuni* strains from 24 h plates were inoculated at OD₆₀₀ 0.1 into 10 ml of pre-incubated brucella broth and grown at 37°C microaerobically with shaking at 75 rpm. (A) 81-176 wild-type and *cadF* mutant (B) 11168H wild-type and *cadF* mutant. Growth was assessed by recording OD₆₀₀ readings of the cultures every 2 h. The data represents the mean of triplicate independent experiments.

(A)



(B)

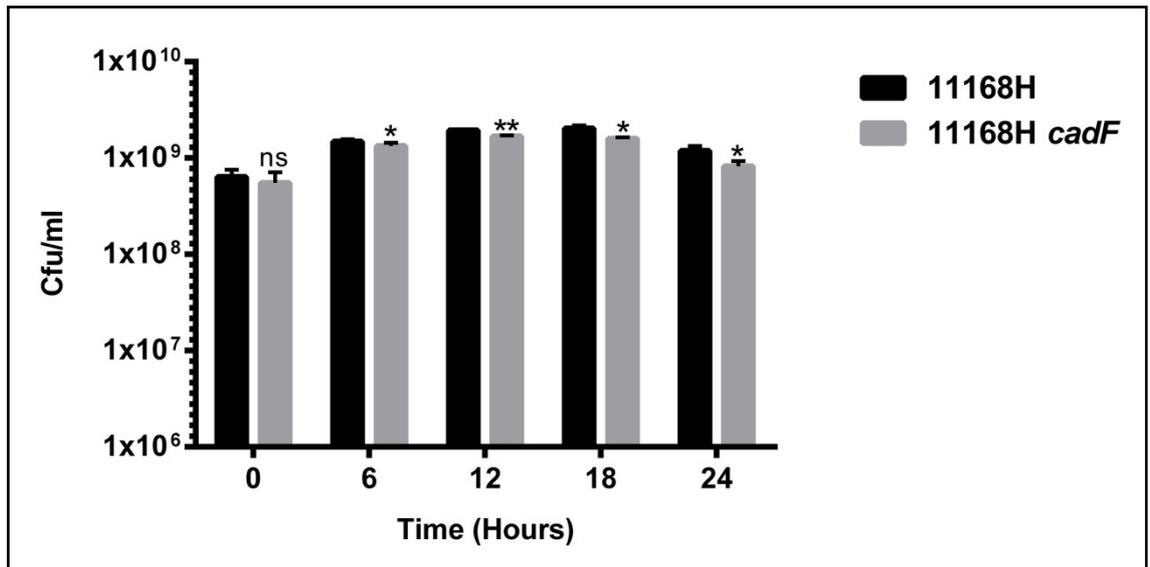
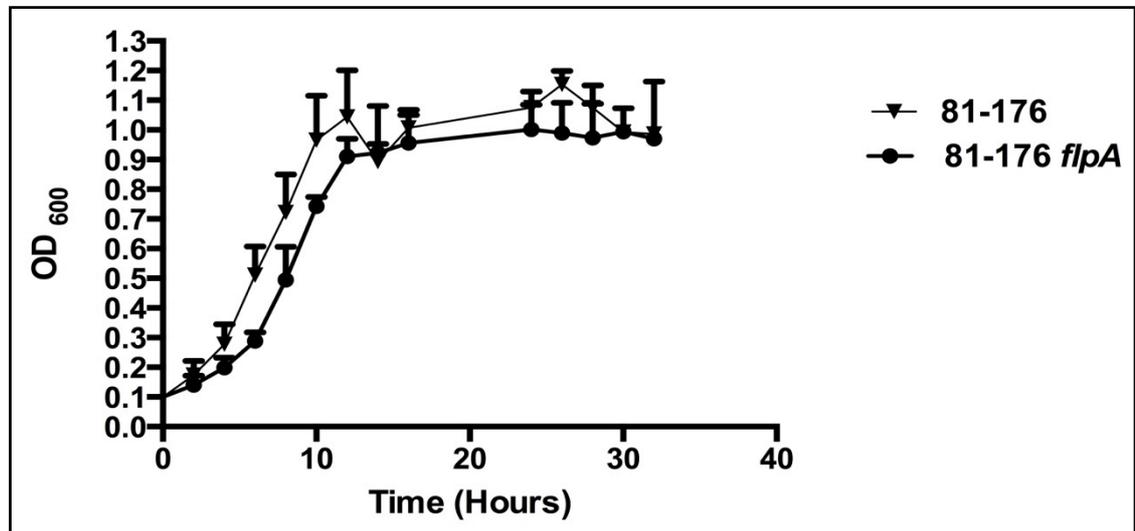


Figure 5.7 Growth curve cfu counts for 81-176 and 11168H wild-type strains and *cadF* mutants. *C. jejuni* strains from 24 h plates were inoculated at OD₆₀₀ 0.1 into 10 ml of pre-incubated brucella broth and grown at 37°C microaerobically with shaking at 75 rpm. At the desired time-point, 1 ml of brucella broth was removed and serial dilutions were performed from 10⁻¹ to 10⁻⁷ and 200 µl of the final dilution was plated onto blood agar plates. Colonies were enumerated following incubation at 37°C under microaerobic conditions for 2 to 3 days. **(A)** 81-176 wild-type and *cadF* mutant **(B)** 11168H wild-type and *cadF* mutant. Asterisks denote a statistically significant difference (* = $p < 0.05$; ** = $p < 0.01$; ns = not significant). The data represents triplicate independent experiments.

The *flpA* mutants also displayed similar growth rates compared to the respective wild-type strain (see Figure 5.8 and Figure 5.9).

(A)



(B)

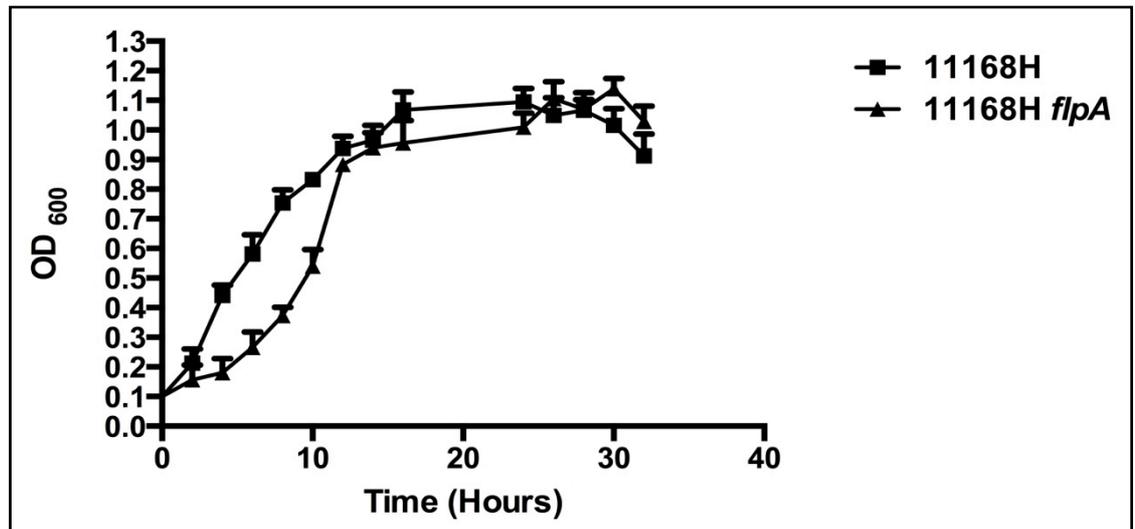
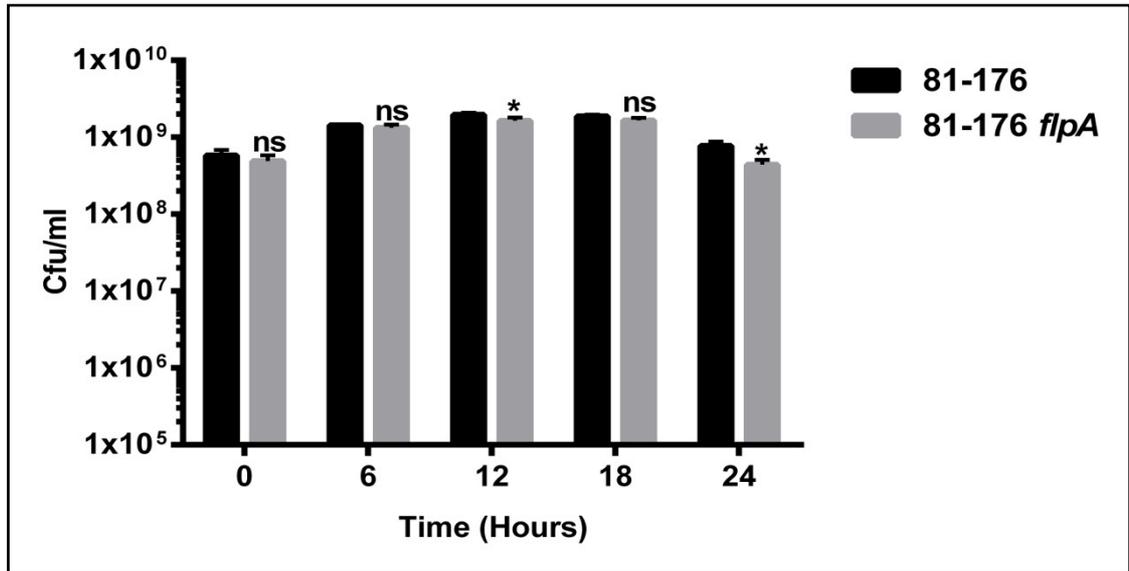


Figure 5.8 Growth rate counts for 81-176 and 11168H wild-type strains and *flpA* mutants. *C. jejuni* strains from 24 h plates were inoculated at OD₆₀₀ 0.1 into 10 ml of pre-incubated brucella broth and grown at 37°C microaerobically with shaking at 75 rpm. (A) 81-176 wild-type and *flpA* mutant (B) 11168H wild-type and *flpA* mutant. Growth was assessed by recording OD₆₀₀ readings of the cultures every 2 h. The data represents the mean of triplicate independent experiments.

(A)



(B)

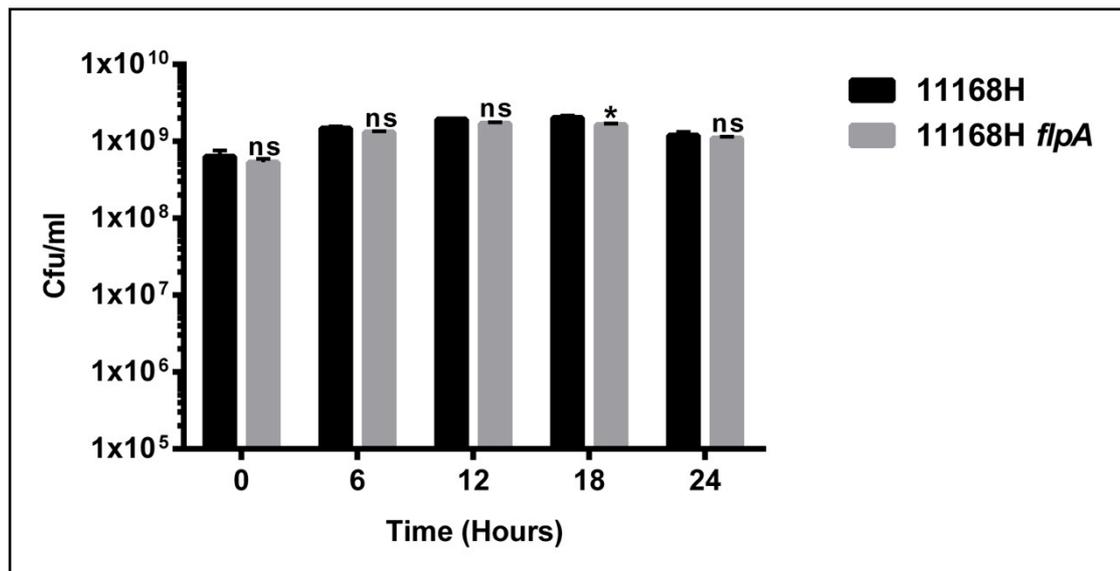


Figure 5.9 Growth curve cfu counts for 81-176 and 11168H wild-type strains and *flpA* mutants. *C. jejuni* strains from 24 h plates were inoculated at OD_{600} 0.1 into 10 ml of pre-incubated brucella broth and grown at 37°C microaerobically with shaking at 75 rpm. At the desired time point, 1 ml of brucella broth was removed and serial dilutions were performed from 10^{-1} to 10^{-7} and 200 μ l of the final dilution was plated onto blood agar plates. Colonies were enumerated following incubation at 37°C under microaerobic conditions for 2 to 3 days. (A) 11168H wild-type and *flpA* mutant (B) 81-176 wild-type and *flpA* mutant. Asterisks denote a statistically significant difference (* = $p < 0.05$; ns = not significant). The data represents triplicate independent experiments.

5.2.4.2 Motility assays

C. jejuni motility plays an important role in infection of humans and colonisation of other animals (Black, Levine *et al.* 1988). To study the effects of the mutations in *cadF* and *flpA* on *C. jejuni* motility, motility assays were performed.

Both 81-176 and 11168H *cadF* mutants displayed a reduction in motility compared to the wild-type. The 81-176 *cadF* mutant was not as motile as the 11168H *cadF* mutant at the 72 h time-point. Both 81-176 and 11168H *flpA* mutants appeared to be non-motile.

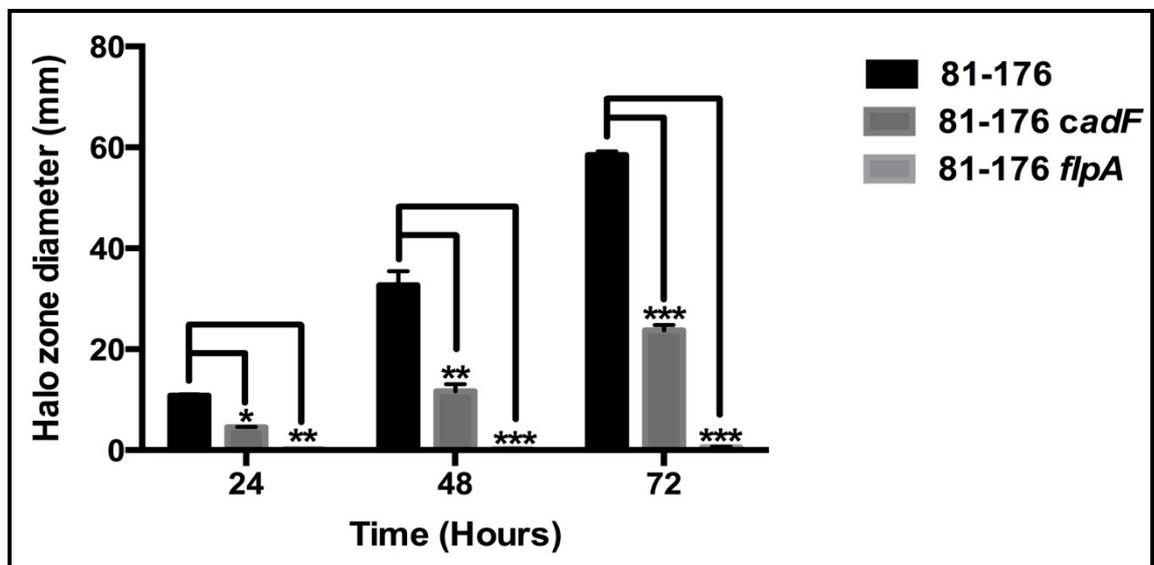


Figure 5.10. Comparison of motility of 81-176 wild-type strain compared with *cadF* and *flpA* mutants. Bacteria were grown for 24 h on blood agar. A suspension was prepared and adjusted to an OD₆₀₀ of 0.1. 2 μ l of this suspension was pipetted into the centre of soft agar plates and incubated at 37°C. The level of motility was assessed by measuring the diameter of the growth from the centre of the inoculation point at 24 h, 48 h and 72 h. Asterisks denote a statistically significant difference (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). Data is representative of three independent experiments.

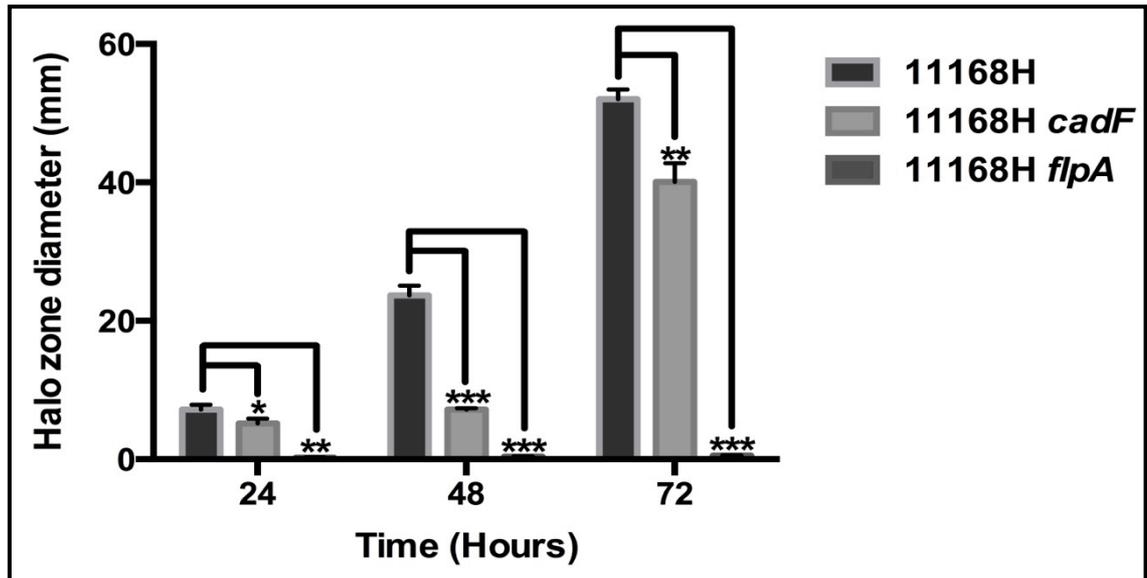


Figure 5.11 Comparison of motility of 11168H wild-type strain with *cadF* and *flpA* mutants. Bacteria were grown for 24 hours on blood agar. A suspension was prepared and adjusted to an OD₆₀₀ of 0.1. 2µl of this suspension was pipetted into the centre of soft agar plates and incubated at 37°C. The level of motility was assessed by measuring the diameter of the growth from the centre of the inoculation point at 24 h, 48 h and 72 h. Asterisks denote a statistically significant difference (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$). Data is representative of three independent experiments.

5.2.4.3 Autoaggregation

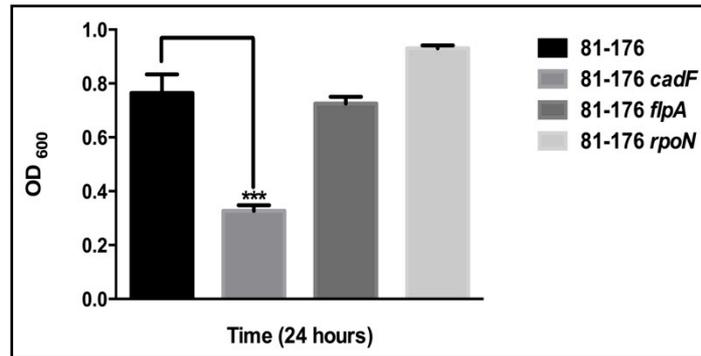
Autoaggregation (AAG) of *C. jejuni* plays a role during interactions with and invasion of host cells (Guerry *et al.*, 2006). However the role of AAG in *C. jejuni* pathogenesis is yet to be fully determined. The amount of AAG also varies between *C. jejuni* strains (Misawa and Blaser, 2000). AAG has been found to play a role in resistance to host defences such as phagocytosis, in biofilm formation and in adherence to host cells (Skurnik, Bolin *et al.* 1984, Galdiero, Romano Carratelli *et al.* 1988, Chiang, Taylor *et al.* 1995, Frick, Morgelin *et al.* 2000, Sherlock, Vejborg *et al.* 2005).

In order to investigate the effect of the *cadF* or *flpA* mutations on *C. jejuni* AAG, *cadF* and *flpA* mutants were tested and compared to the 81-176 and 11168H wild-type strains. RpoN is an alternative sigma factor involved in the transcription of genes encoding proteins involved in flagella biosynthesis (Hendrixson *et al.*, 2001). 81-176 and 11168H *rpoN* mutants which lack a functional flagella and do not autoaggregate were included as AAG negative controls.

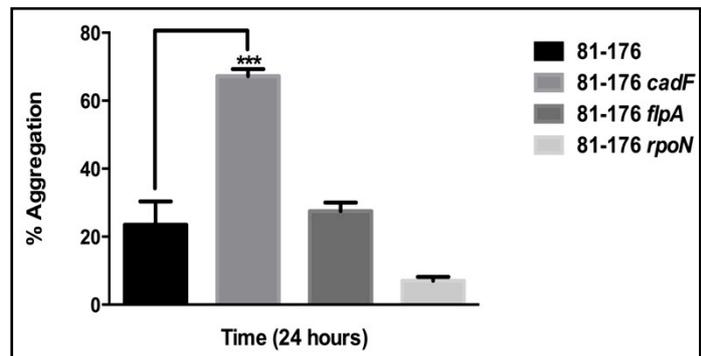
The 81-176 *cadF* mutant exhibited significantly increased AAG levels whilst the 81-176 *flpA* mutant did not exhibit any differences in AAG levels compared to the 81-176

wild-type. The 11168H *cadF* mutant was also significantly increased in AAG levels whilst the 11168H *flpA* mutant was significantly reduced in AAG levels compared to the 11168H wild-type.

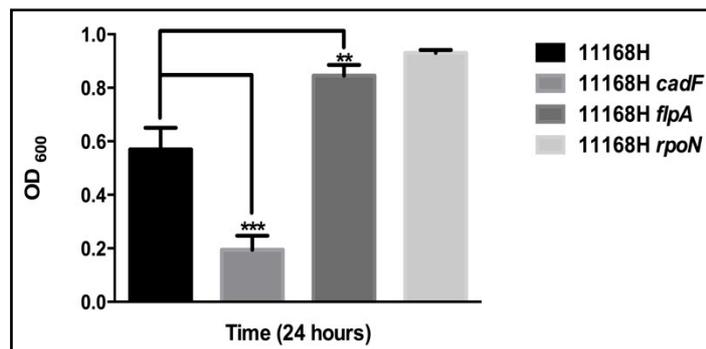
(A)



(B)



(C)



(D)

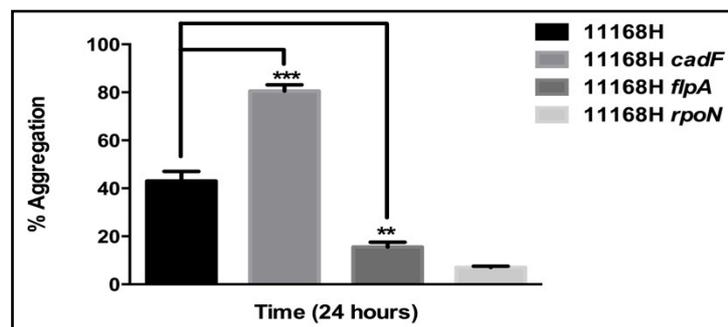


Figure 5.12. Autoaggregation assays for the 81-176 and 11168H wild-type strains, *cadF* and *flpA* mutants. Bacteria were grown for 24 h on blood agar. A suspension was prepared and adjusted to an OD₆₀₀ of 1.0 in PBS. Suspensions (2 ml) were incubated for 24 h at 37°C. AAG was analysed by measuring the decrease in optical density of the upper 1 ml of suspended cells after 24 h. An OD₆₀₀ of 1.0 was considered 0% AAG and an OD₆₀₀ of 0 considered 100% AAG. **(A) and (C):** OD₆₀₀ readings after 24 h incubation. **(B) and (D):** Percentage of AAG of suspended cells. Asterisks denote a statistically significant difference (** = $p < 0.01$, *** = $p < 0.001$). Data are representative of triplicate individual experiments.

5.2.4.4 *Galleria mellonella* infection model

G. mellonella larvae have been widely used as a non-mammalian model for bacterial infection studies (Champion *et al.*, 2009). Larvae can be infected at 37°C and possess specialised phagocytic cells called haemocytes (Bergin, Reeves *et al.* 2005). These cells mimic mammalian phagocytic cells and produce bactericidal compounds such as superoxide (Bergin, Reeves *et al.* 2005). Infection with the *cadF* or *flpA* mutants resulted in reduced cytotoxicity compared to the respective wild-type strain over 72 h.

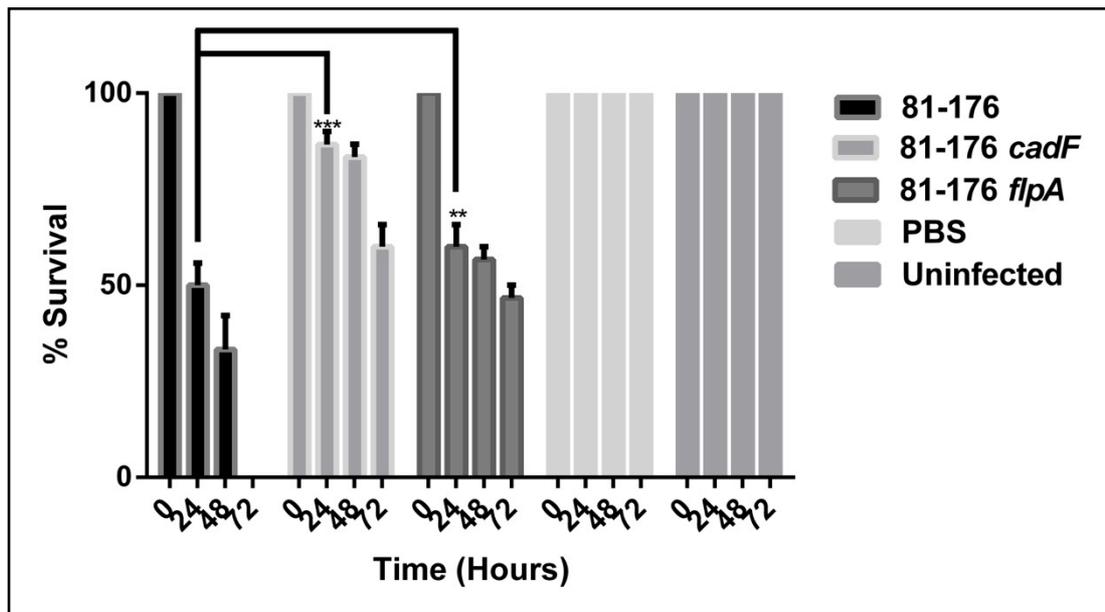


Figure 5.13. Effect of *C. jejuni* 81-176 wild-type strain, *cadF* and *flpA* mutants in the *Galleria mellonella* model of infection. *G. mellonella* larvae were injected with a 10 μ l inoculum of 24 h *C. jejuni* culture diluted to an OD₆₀₀ of 0.1 by microinjection in the right foremost leg, giving an infectious dose of approximately 10⁶ cfu. The larvae were incubated at 37°C with survival recorded at 24 h intervals. PBS and no injection controls were also included. For each experiment, 10 *G. mellonella* larvae were infected and the experiments were repeated in triplicate. The asterisks denote a statistically significant difference (***) = $p < 0.001$.

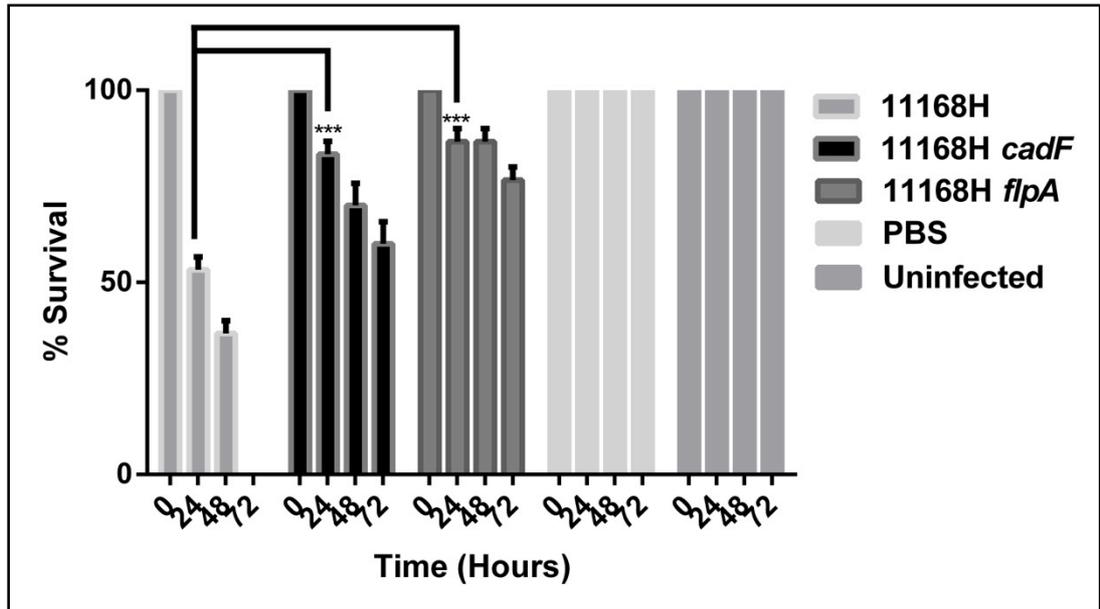


Figure 5.14. Effect of *C. jejuni* 11168H wild-type strain, 11168H *cadF* and *flpA* mutants in the *Galleria mellonella* model of infection. *G. mellonella* larvae were injected with a 10 μ l inoculum of 24 h *C. jejuni* culture diluted to an OD₆₀₀ of 0.1 by microinjection in the right foremost leg, giving an infectious dose of approximately 10⁶ cfu. The larvae were incubated at 37°C with survival recorded at 24 h intervals. PBS and no injection controls were also included. For each experiment, 10 *G. mellonella* larvae were infected and the experiments were repeated in triplicate. The asterisks denote a statistically significant difference of (***) = $p < 0.001$.

5.2.5 81-176 *cadF* and *flpA* mutants exhibit reduced binding to fibronectin

Previous studies have shown that *C. jejuni* CadF and FlpA are both microbial surface components recognising adhesive matrix molecules (Konkel *et al.*, 2005, Flanagan *et al.*, 2009). The binding of these outer membrane proteins to fibronectin on the host cell surface allows for bacterial attachment (Monteville and Konkel, 2002, Schwarz-Linek *et al.*, 2004, Konkel *et al.*, 2005, Krause-Gruszczynska *et al.*, 2007a). The *C. jejuni* FlpA amino acid sequence harbours fibronectin type III domains and studies with *C. jejuni* strain F38011 showed that *flpA* mutants exhibited a reduction in ability to bind to chicken epithelial cells (Flanagan *et al.*, 2009). Fibronectin binding assays demonstrated that both 81-176 *cadF* and *flpA* mutants exhibited significantly reduced ability to bind to fibronectin compared to the wild-type strain. The 81-176 *rpoN* mutant was included as a control.

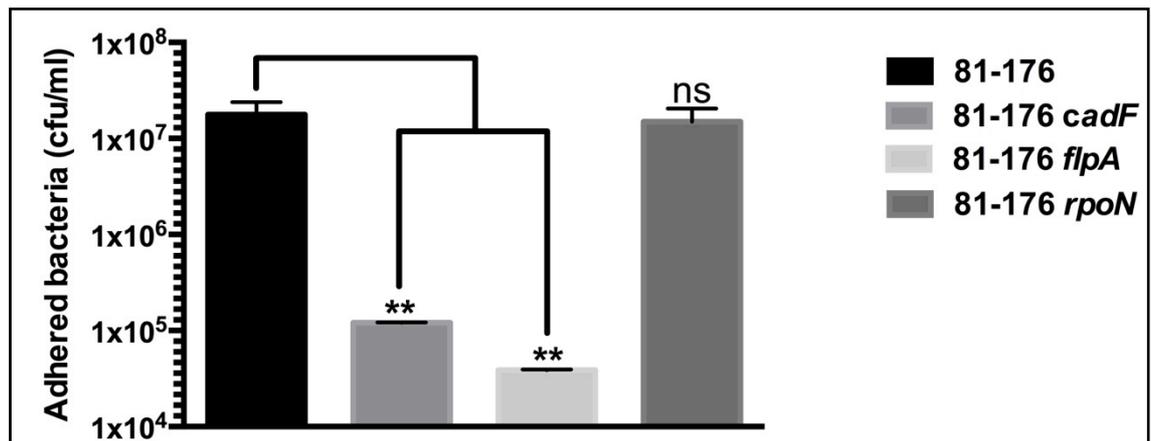


Figure 5.15. Fibronectin binding assays. The wells of 96-well flat-bottom plates were coated with a 1 mg/ml solution of fibronectin in 0.05 M Tris-buffered saline, pH 7.5 and incubated overnight at 4°C. *C. jejuni* 81-176 wild-type strain, *cadF* and *flpA* mutants were harvested from overnight plate cultures and resuspended in PBS at an OD₆₀₀ of 0.1. Wells were rinsed with PBS, then 100 µl aliquots of the bacterial suspensions were added to each well and incubated at 37°C in a CO₂ incubator for 1 h. The wells were washed three times with PBS, then adherent bacteria were removed by the addition of 0.05% (w/v) Trypsin. To enumerate the number of adherent bacteria, serial dilutions of the trypsin suspension were plated on blood agar plates. Asterisks denote a statistically significant difference (** = $p < 0.01$; ns = not significant). The data are representative of triplicate independent experiments

5.2.6 Interaction and invasion assays

The effect of the mutations in *cadF* and *flpA* on *C. jejuni* interactions with IECs was investigated.

5.2.6.1 The role of CadF in *C. jejuni* interactions with and invasion of Caco-2 IECs

There was a significant reduction in the ability of the 81-176 *cadF* mutant to interact with and invade Caco-2 IECs in comparison to the 81-176 wild-type strain. The 81-176 *cadF* mutant exhibited a significantly reduced ability to interact with Caco-2 IECs compared to the wild-type strain (see Figure 5.16). The 81-176 *cadF* mutant also displayed a significant reduction in invasion (see Figure 5.17).

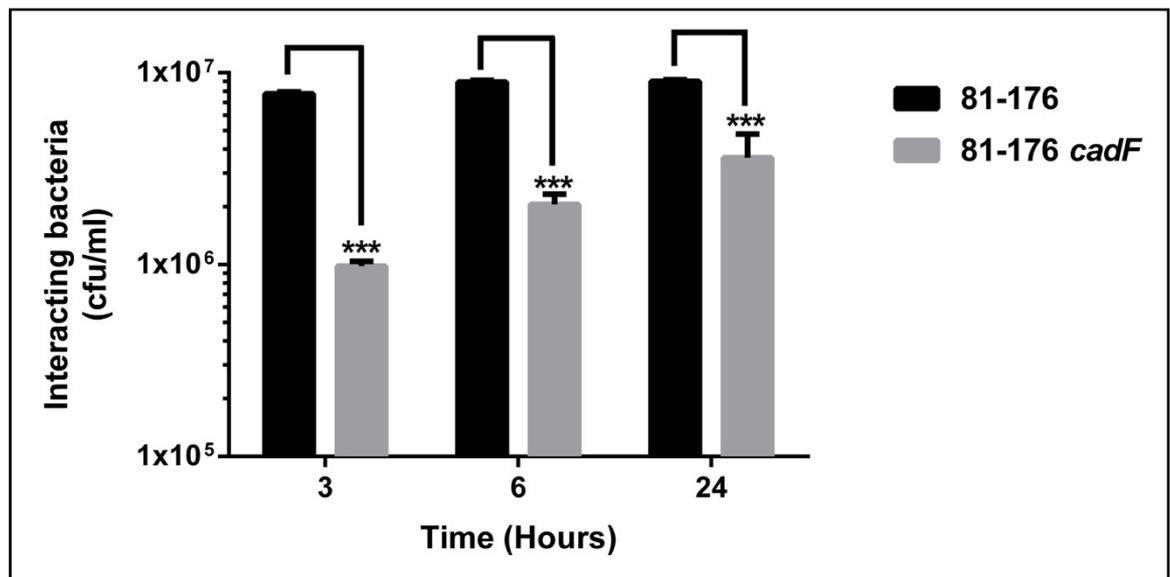


Figure 5.16. Interaction (adhesion and invasion) assays. Bacteria were co-cultured with Caco-2 IECs for 3 h, 6 h and 24 h. Caco-2 IECs were lysed with Triton X-100 and interacting bacteria were enumerated. Asterisks denote a statistically significant difference (***) = $p < 0.001$) for the 81-176 *cadF* mutant compared to the wild-type strain. Data are representative of triplicate independent experiments.

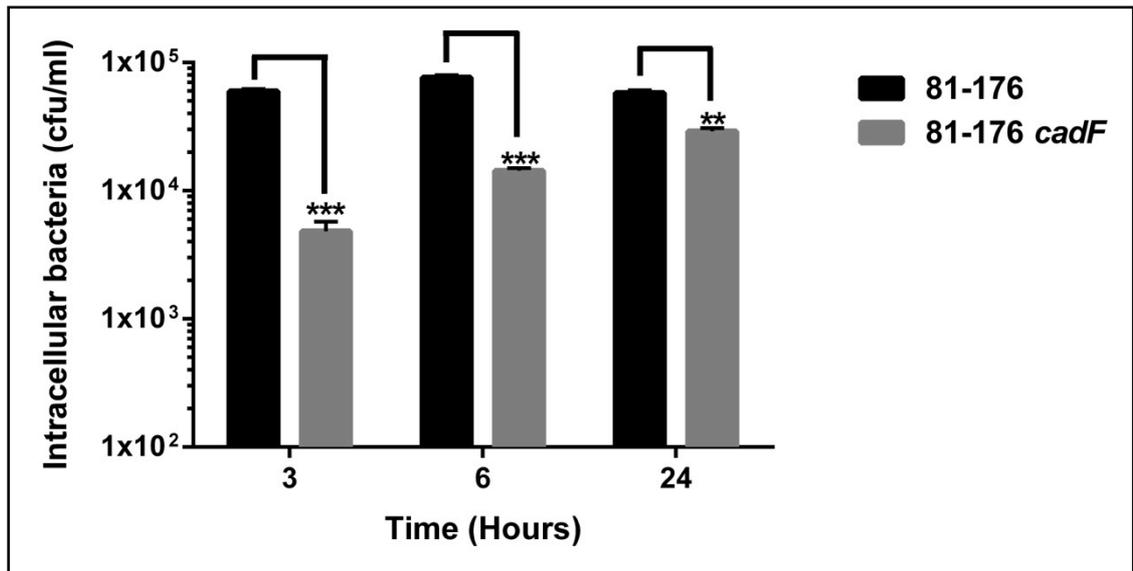


Figure 5.17 Invasion assays. Bacteria were co-cultured with Caco-2 IECs for 3 h, 6 h and 24 h. Caco-2 IECs were incubated with gentamicin (150 µg/ml) for 2 h to kill extracellular bacteria, then lysed and numbers of intracellular bacteria assessed. Asterisks denote a statistically significant difference (** = $p < 0.01$, *** = $p < 0.001$) for the 81-176 *cadF* mutant compared to the wild-type strain. Data are representative of triplicate independent experiments.

5.2.6.2 The role of CadF in *C. jejuni* interactions with and invasion of T84 IECs

There was also a significant difference between the ability of the 81-176 *cadF* mutant to interact with and invade T84 IECs in comparison to the 81-176 wild-type strain. The 81-176 *cadF* mutant exhibited a significantly reduced ability to interact with T84 IECs (see Figure 5.18). The 81-176 *cadF* mutant also displayed a significant reduction in invasion (see Figure 5.19).

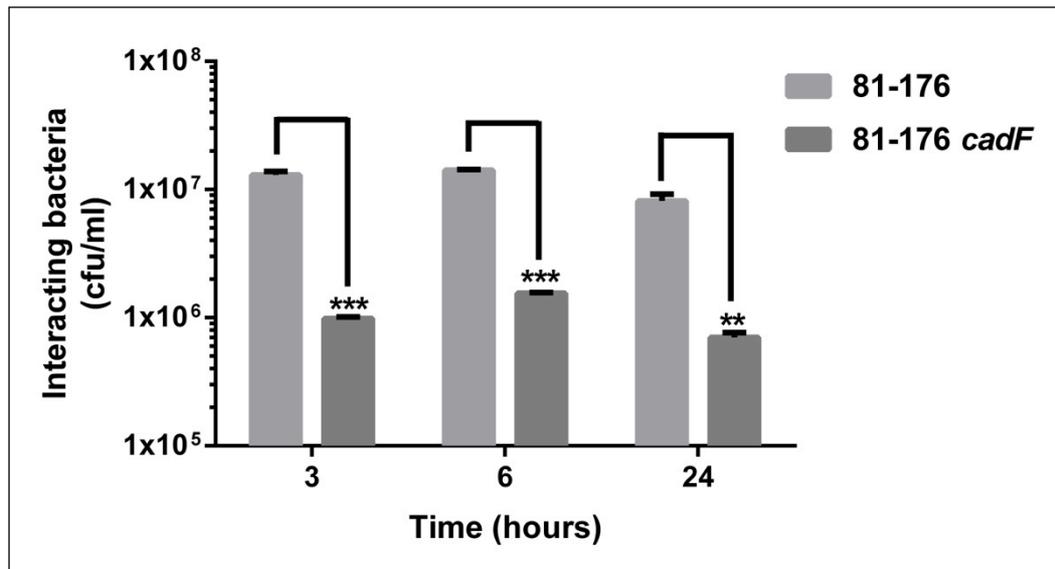


Figure 5.18 Interaction (adhesion and invasion) assays. Bacteria were co-cultured with T84 IECs for 3 h, 6 h and 24 h. T84 IECs were lysed with Triton X-100 and interacting bacteria were enumerated. Asterisks denote a statistically significant difference (** = $p < 0.01$, *** = $p < 0.001$) for the 81-176 *cadF* mutant compared to the wild-type strain. Data are representative of triplicate independent experiments.

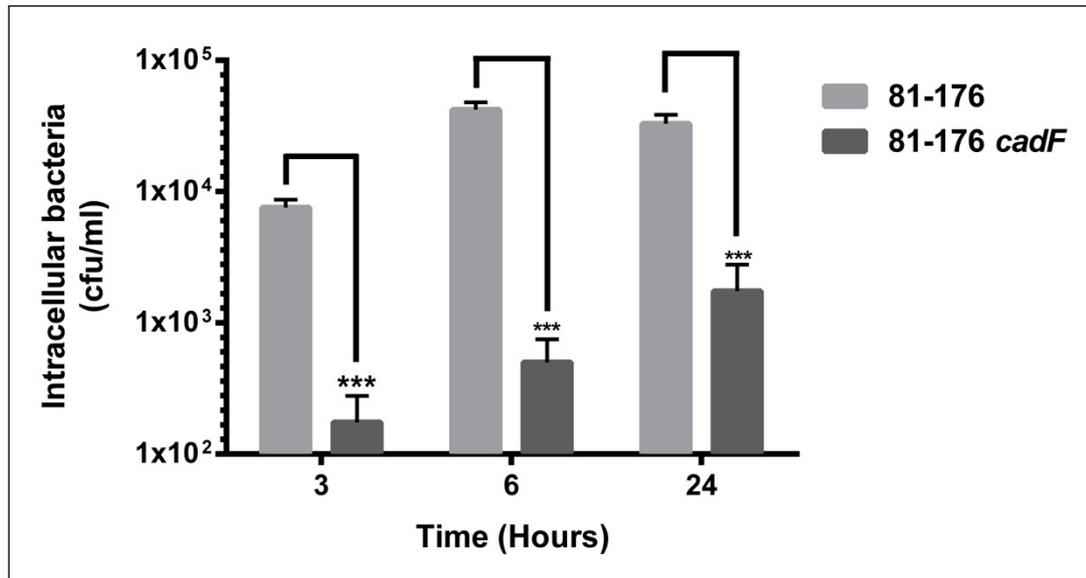


Figure 5.19 Invasion assays. Bacteria were co-cultured with T84 IECs for 3 h, 6 h and 24 h. T84 IECs were incubated with gentamicin (150 $\mu\text{g/ml}$) for 2 h to kill extracellular bacteria, then lysed and numbers of intracellular bacteria assessed. Asterisks denote a statistically significant difference (***) = $p < 0.001$) for the 81-176 *cadF* mutant compared to the wild-type strain. Data are representative of triplicate independent experiments.

5.2.6.3 The role of FlpA in *C. jejuni* interactions with and invasion of Caco-2 IECs

The 81-176 *flpA* mutant also exhibited a significantly reduced ability to interact with Caco-2 cells compared to the wild-type strain (see Figure 5.20). A significant reduction in invasion levels by the *flpA* mutant was also observed (see Figure 5.21).

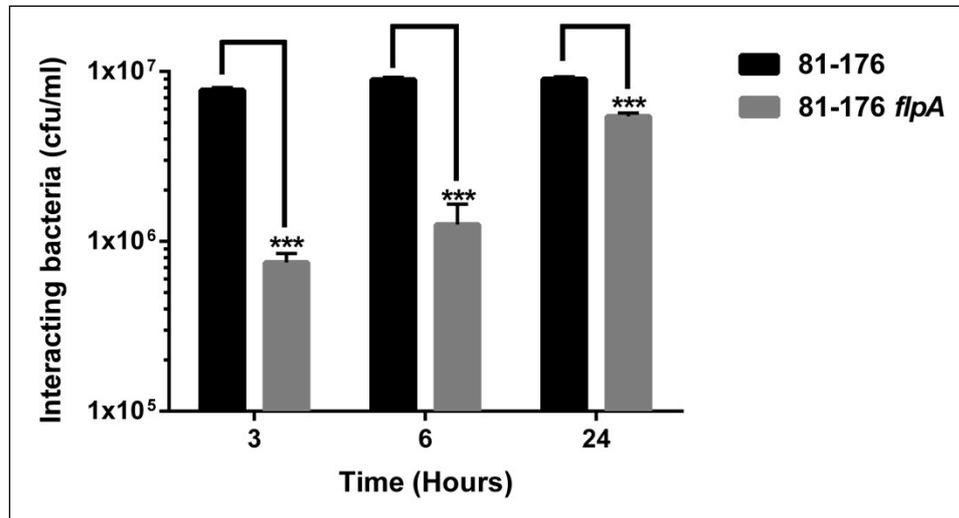


Figure 5.20 Interaction (adhesion and invasion). Bacteria were co-cultured with Caco-2 IECs for 3 h, 6 h and 24 h. Caco-2 IECs were lysed with Triton X-100 and interacting bacteria were enumerated. Asterisks denote a statistically significant difference (***) = $p < 0.001$) for the 81-176 *flpA* mutant compared to the wild-type strain. Data are representative of triplicate independent experiments.

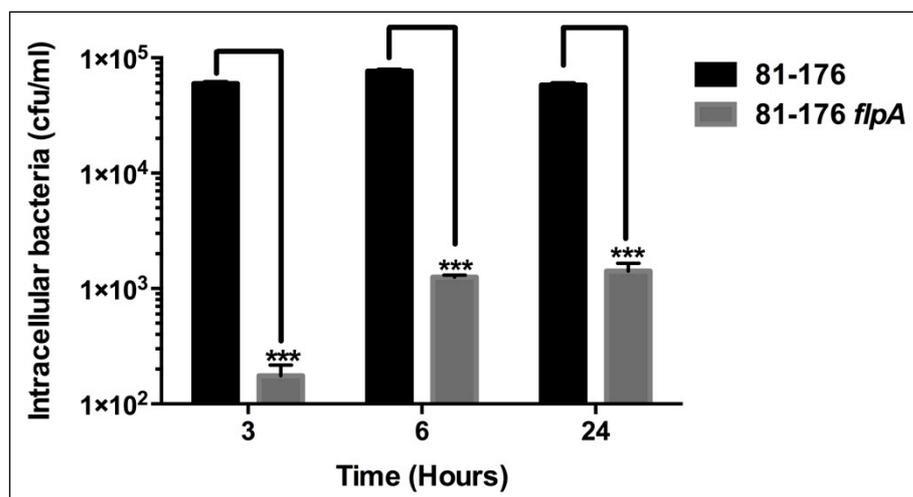


Figure 5.21 Invasion assays. Bacteria were co-cultured with Caco-2 IECs for 3 h, 6 h and 24 h. Caco-2 IECs were incubated with gentamicin (150 µg/ml) for 2 h to kill extracellular bacteria, then lysed and numbers of intracellular bacteria assessed. Asterisks denote a statistically significant difference (***) = $p < 0.001$) for the 81-176 *flpA* mutants compared to the wild-type strain. Data are representative of triplicate independent experiments.

5.2.6.4 The role of FlpA in *C. jejuni* interactions with and invasion of T84 IECs

The 81-176 *flpA* mutant was also significantly reduced in interactions with and invasion of T84 IECs compared to the 81-176 wild-type strain (see Figure 5.22 and Figure 5.23).

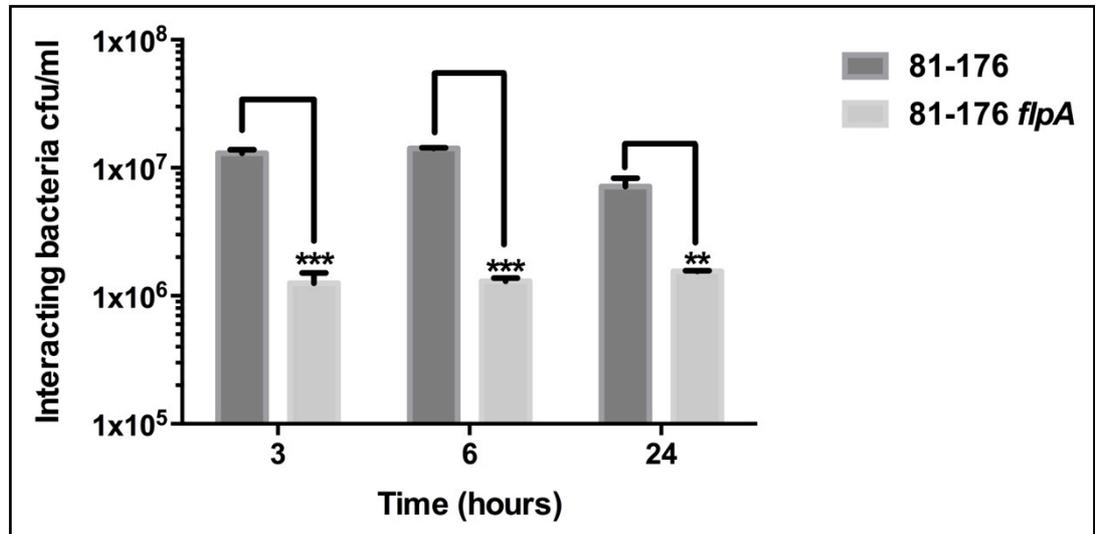


Figure 5.22 Interaction (adhesion and invasion) assays. Bacteria were co-cultured with T84 intestinal epithelial cells for 3 h, 6 h and 24 h. T84 IECs were lysed with Triton X-100 and interacting bacteria were enumerated. Asterisks denote a statistically significant difference (** = $p < 0.01$, *** = $p < 0.001$) for the 81-176 *flpA* mutant compared to the wild-type strain. Data are representative of triplicate independent experiments.

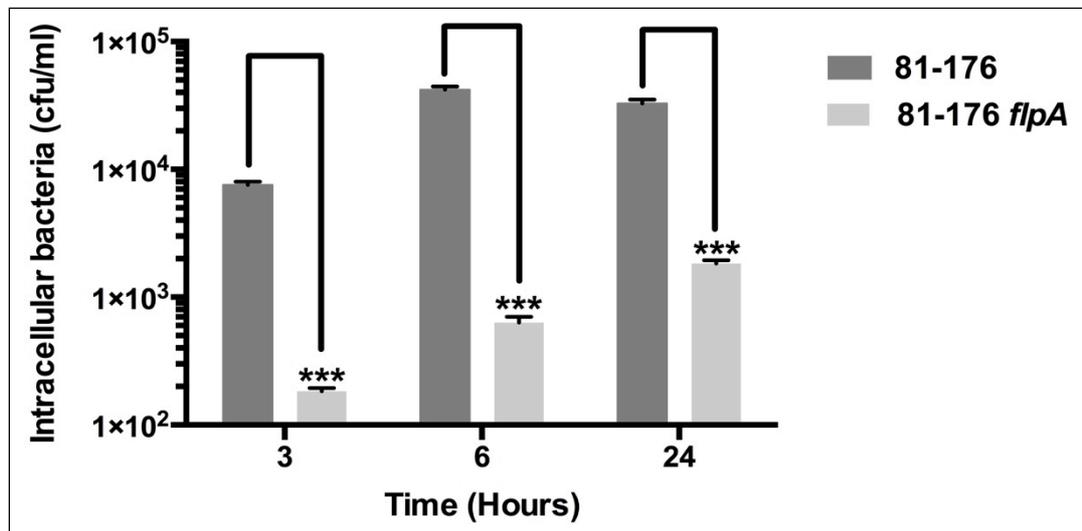


Figure 5.23 Invasion assays. Bacteria were co-cultured with T84 intestinal epithelial cells for 3 h, 6 h and 24 h. T84 IECs were incubated with gentamicin (150 $\mu\text{g/ml}$) for 2 h to kill extracellular bacteria, then lysed and numbers of intracellular bacteria assessed. Asterisks denote a statistically significant difference (***) = $p < 0.001$) for the 81-176 *flpA* mutant compared to the wild-type strain. Data are representative of triplicate independent experiments.

Table 5.1 Summary of the effect of the mutation in *cadF* and *flpA* on *C. jejuni* interactions with Caco-2 IECs.

Bacterial strain	Effect of interaction with Caco-2 IECs (3 h)	Effect of interaction with Caco-2 IECs (6 h)	Effect of interaction with Caco-2 IECs (24 h)
81-176 wild-type	< 10 ⁷ cfu	< 10 ⁷ cfu	< 10 ⁷ cfu
<i>cadF</i> mutant	> 10 ⁶ cfu	> 10 ⁶ cfu	> 10 ⁶ cfu
<i>flpA</i> mutant	> 10 ⁶ cfu	> 10 ⁶ cfu	> 10 ⁶ cfu

Table 5.2 Summary of the effect of the mutation in *cadF* and *flpA* on *C. jejuni* invasion of Caco-2 IECs.

Bacterial strain	Effect of invasion of Caco-2 IECs (3 h)	Effect of invasion of Caco-2 IECs (6 h)	Effect of invasion of Caco-2 IECs (24 h)
81-176 wild-type	<10 ⁵ cfu	< 10 ⁵ cfu	< 10 ⁵ cfu
<i>cadF</i> mutant	> 10 ³ cfu	> 10 ⁴ cfu	> 10 ⁴ cfu
<i>flpA</i> mutant	> 10 ² cfu	> 10 ³ cfu	> 10 ³ cfu

Table 5.3 Summary of the effect of the mutation in *cadF* and *flpA* on *C. jejuni* interactions with T84 IECs.

Bacterial strain	Effect of interaction with T84 IECs (3 h)	Effect of interaction with T84 IECs (6 h)	Effect of interaction with T84 IECs (24 h)
81-176 wild-type	>10 ⁷ cfu	>10 ⁷ cfu	>10 ⁷ cfu
<i>cadF</i> mutant	<10 ⁶ cfu	> 10 ⁶ cfu	< 10 ⁶ cfu
<i>flpA</i> mutant	> 10 ⁶ cfu	> 10 ⁶ cfu	> 10 ⁶ cfu

Table 5.4 Summary of the effect of the mutation in *cadF* and *flpA* on *C. jejuni* invasion of T84 IECs.

Bacterial strain	Effect of invasion of T84 IECs (3 h)	Effect of invasion of T84 IECs (6 h)	Effect of invasion of T84 IECs (24 h)
81-176 wild-type	<10 ⁴ cfu	>10 ⁴ cfu	>10 ⁴ cfu
<i>cadF</i> mutant	>10 ² cfu	> 10 ² cfu	> 10 ³ cfu
<i>flpA</i> mutant	>10 ² cfu	>10 ² cfu	> 10 ³ cfu

5.2.7 Intracellular survival assays

Our knowledge of *C. jejuni* interactions with, invasion of and survival within intestinal epithelial cells is limited compared to other enteropathogenic bacteria. However, studies have revealed that *C. jejuni* can evade phagocytosis and survive for a certain period intracellularly (Kiehlbauch *et al.*, 1985, Watson and Galan, 2008b) and also trigger the release of host cytokines (Jones *et al.*, 2003). The exact process of how this invasion and intracellular survival occurs is still unclear. *C. jejuni* avoids delivery to the lysosomes and resides within *Campylobacter* containing vacuoles (CCV) (Watson and Galan, 2008b, Pryjma *et al.*, 2012). To study the effect of mutation of *cadF* or *flpA* on the ability of *C. jejuni* to survive intracellularly within Caco-2 cells, intracellular survival assays were performed.

The 81-176 *cadF* mutant exhibited significantly reduced intracellular survival compared to the wild-type strain (see Figure 5.24). The 81-176 *flpA* mutant exhibited a reduced level of invasion, however no decrease in intracellular survival was observed. The percentage survival of 81-176 wild-type, *cadF* and *flpA* mutants was also calculated and the *cadF* mutant displayed a reduction in survival similar to the wild-type strain, whereas the *flpA* mutant showed no decrease in survival levels (see Table 5.5).

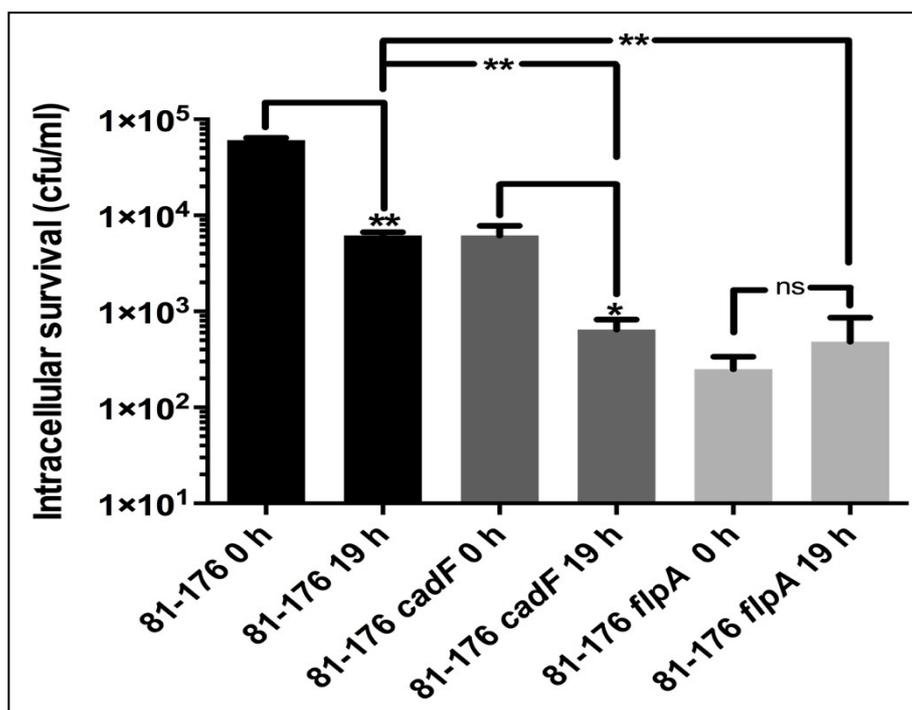


Figure 5.24 Intracellular survival assay. The 81-176 wild-type strain, *cadF* and *flpA* mutants were co-cultured with Caco-2 IECs for 3 h, washed three times with PBS, then incubated with gentamicin (150 µg/ml) for 2 h to kill extracellular bacteria, followed by further incubation with gentamicin for 19 h at a reduced concentration (10 µg/ml). The monolayers were then washed three times in PBS, lysed with Triton X-100 and numbers of intracellular bacteria assessed. Asterisks denote a statistically significant difference (* = $p < 0.05$; ** = $p < 0.01$, ns = no significant difference). Data represents triplicate independent experiments.

Table 5.5 Percentage survival of intracellular 81-176 wild-type strain, *cadF* and *flpA* mutants after 19 h within Caco-2 IECs

Bacterial Strain	% survival after 19 h
81-176 wild-type	10.10
81-176 <i>cadF</i> mutant	10.54
81-176 <i>flpA</i> mutant	100.0

5.2.8 Macrophage survival assays

Once inside the host, relatively little is known about the intracellular life cycle of *C. jejuni*, although it is possible that *C. jejuni* behaves in a similar way to other enteropathogens with well characterised intracellular survival strategies and that *C. jejuni* may have evolved specific mechanisms to survive intracellularly (Watson and Galan, 2008b). Macrophage survival assays were performed as described previously (Watson and Galan, 2008b) using J774A.1 mouse macrophages (Sikic Pogacar *et al.*, 2009). The 81-176 *cadF* mutant exhibited a significant reduction in macrophage survival, with no bacteria observed after 16 h compared to the wild-type strain (see Figure 5.25). The 81-176 *flpA* mutant also exhibited a significant reduction in macrophage survival after 4 h, but at 16 h, macrophage survival was similar to the wild-type strain.

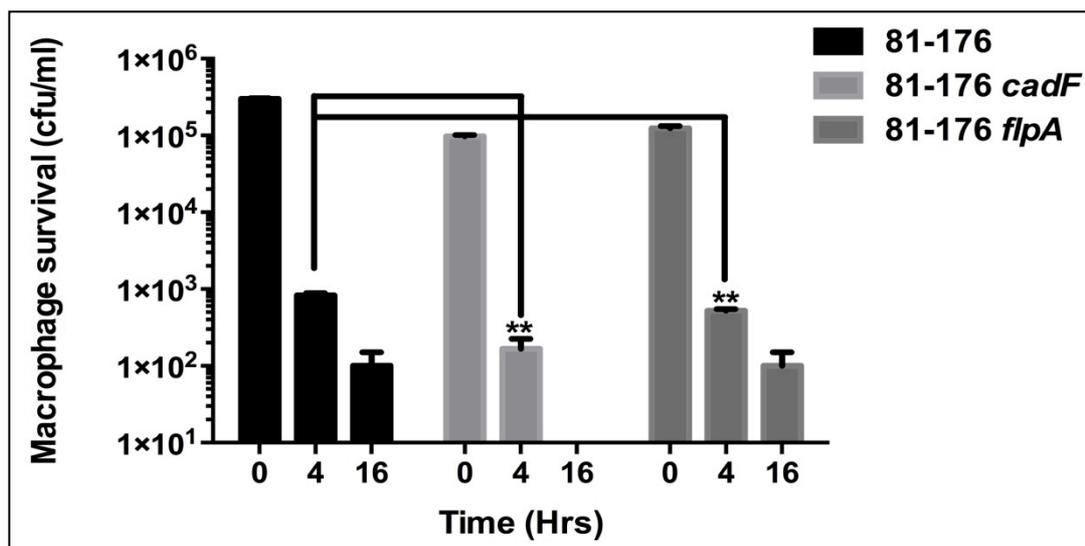
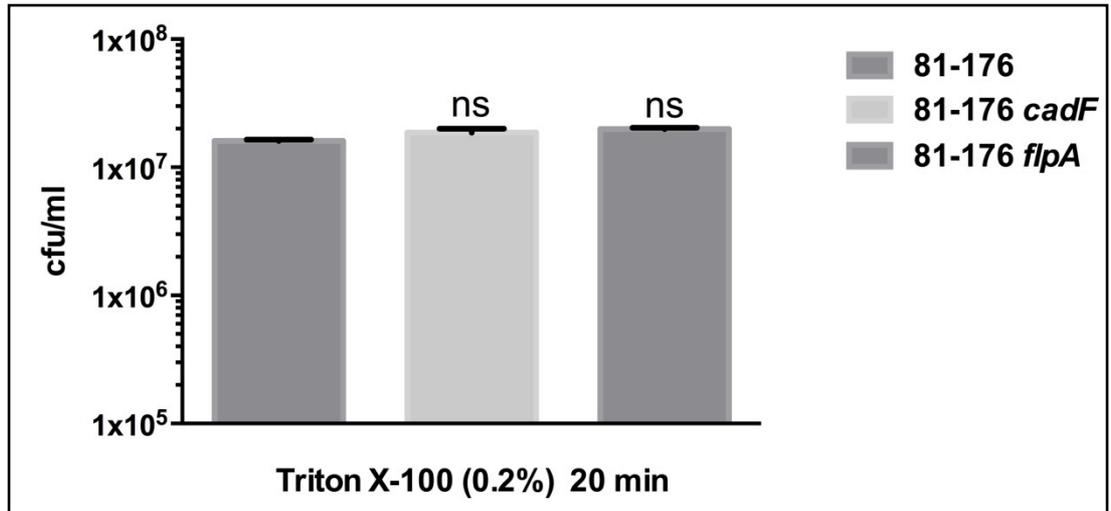


Figure 5.25 Macrophage survival assays. The 81-176 wild-type strain, *cadF* and *flpA* mutants were co-cultured with J774A.1 mouse macrophages for 3 h. The cells were washed three times in PBS, followed by incubation in DMEM containing gentamicin (150 $\mu\text{g/ml}$) for 2 h to allow killing of extracellular bacteria. The macrophages were then incubated in DMEM containing a reduced concentration of gentamicin (10 $\mu\text{g/ml}$) and bacterial survival determined at 0 h, 4 h and 16 h. At each time point, the macrophages were washed three times with PBS and lysed by adding 0.2% (v/v) Triton X-100 in PBS and the number of intracellular bacteria were assessed. The asterisks denote a statistically significant difference (** = $p < 0.01$). Data represents triplicate independent experiments.

5.2.9 Sensitivity of *C. jejuni* to Triton X-100 and gentamicin

To ascertain whether the above results were due to genuine *cadF* or *flpA* mutant phenotypes and not due to increased sensitivity to Triton X-100 or gentamicin, sensitivity assays were performed on all the mutants and respective wild-type strains with 0.2% (v/v) Triton X-100 or gentamicin (150 $\mu\text{g/ml}$). No significant differences were observed between the levels of sensitivity to Triton X-100 (see Figure 5.26) or gentamicin (data not shown) between the 81-176 and 11168H wild-type strains and the respective *cadF* or *flpA* mutants.

(A)



(B)

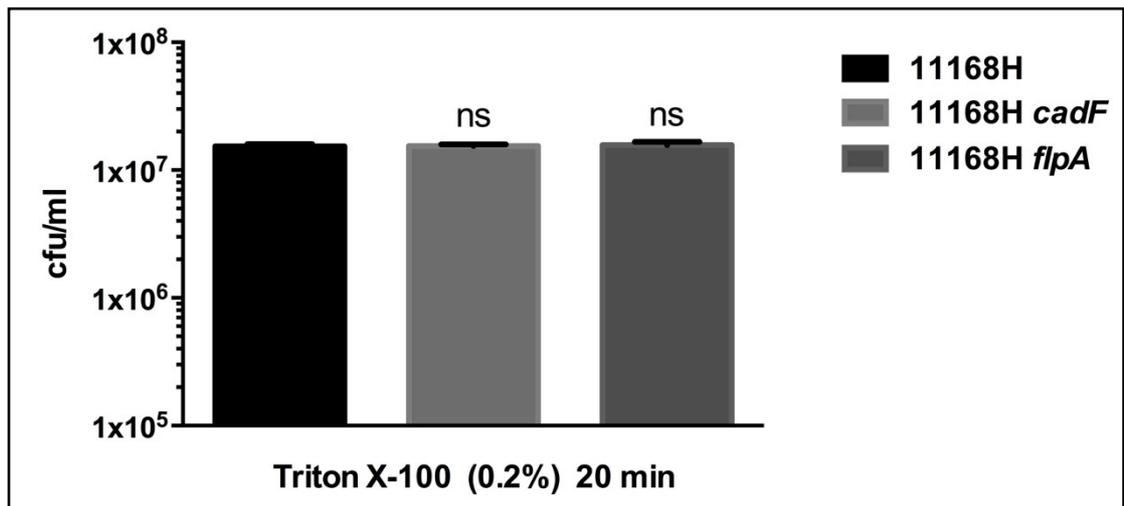


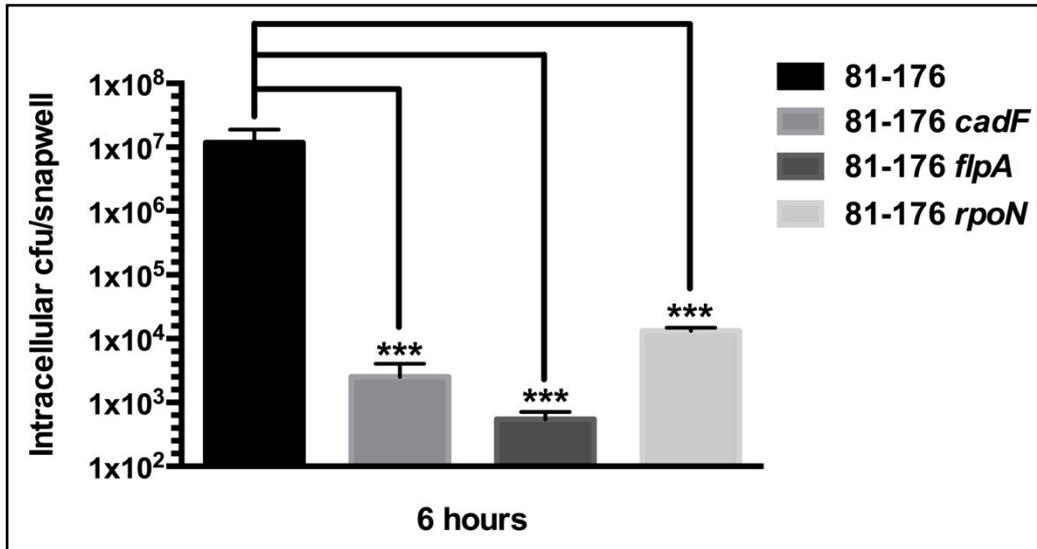
Figure 5.26 Sensitivity of *C. jejuni* strains to Triton X-100. Bacteria was grown for 24 h on blood agar. The bacteria were resuspended in PBS and adjusted to an OD₆₀₀ of 0.1. Triton X-100 was added to a final concentration of 0.2% (v/v) and after incubation at 37°C with 5% CO₂ for 20 minutes the number of viable bacteria was determined. **(A)** 11168H wild-type strain, *cadF* and *flpA* mutants **(B)** 81-176 wild-type strain, *cadF* and *flpA* mutants. (ns = no significant difference). Data represents triplicate independent experiments.

5.2.10 Vertical Diffusion Chamber Assays

A major limitation of *in vitro* models used to study gastrointestinal infections is that standard tissue culture conditions include high oxygen levels which generally favour eukaryotic cell survival and do not allow the true observation of the biological interactions of the bacteria with the host cell (Mills *et al.*, 2012). In the case of *C. jejuni*, a number of different cell culture assays have been used to investigate the interactions of *C. jejuni* with host cells. Caco-2 (Everest *et al.*, 1992), INT407 (Konkel *et al.*, 1992b) and T84 (Monteville and Konkel, 2002) cell lines have all been used extensively to study the adhesion and invasion capabilities of different *C. jejuni* strains. However, the levels of bacterial adhesion and invasion for *C. jejuni* with IECs are dramatically lower than for other enteric pathogens (Friis *et al.*, 2005).

The use of a Vertical Diffusion Chamber (VDC) system allows the co-culture of bacteria and host cells under different medium and gas conditions (Cottet *et al.*, 2002a, Schuller and Phillips, 2010, Mills *et al.*, 2012). The 81-176 *cadF* and *flpA* mutants both exhibited significantly reduced invasion of Caco-2 IECs compared to the wild-type strain and a reduced invasion ability of *cadF* and *flpA* mutants was also observed using T84 IECs, however the numbers of intracellular bacteria were slightly higher than when using Caco-2 IECs. A *C. jejuni* 81-176 *rpoN* mutant, was included as a non-motile control (Fernando *et al.*, 2007a). Infection of both Caco-2 and T84 IECs with the *rpoN* mutant, resulted in significantly reduced numbers of intracellular bacteria compared to the wild-type.

(A)



(B)

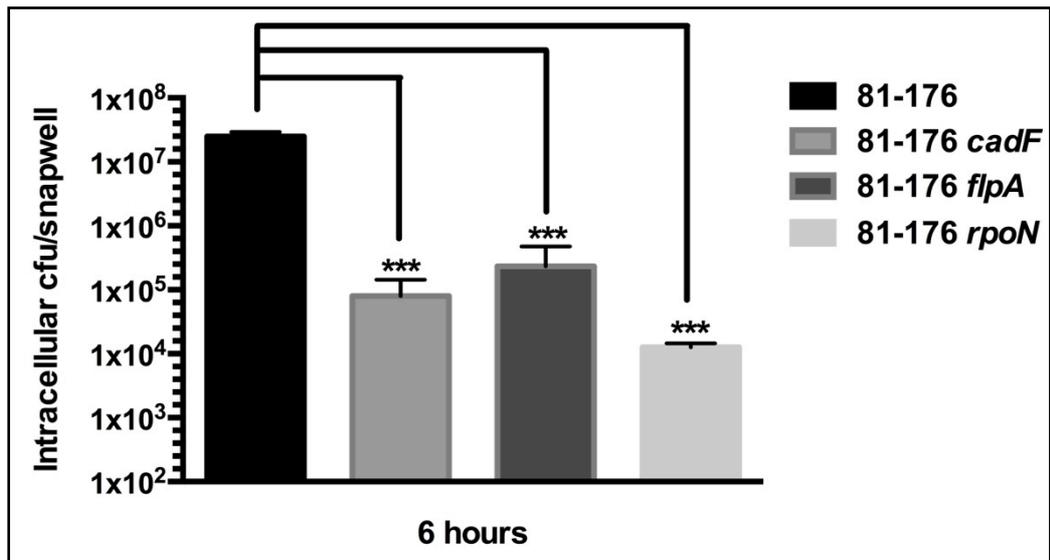


Figure 5.27 Vertical Diffusion Chambers assays. 81-176 wild-type strain, *cadF*, *flpA* and *rpoN* mutants co-cultured with (A) Caco-2 or (B) T84 IECs in a Vertical Diffusion chamber at a multiplicity of infection (MOI) of approximately 100:1 under microaerobic conditions for 6 h and the numbers of intracellular bacteria were assessed. Caco-2 or T84 IECs were incubated with gentamicin (150 µg/ml) for 2 h to kill extracellular bacteria, then lysed and numbers of intracellular bacteria assessed. The asterisks denote a statistically significant difference (***) = $p < 0.001$). Data are representative of triplicate independent experiments.

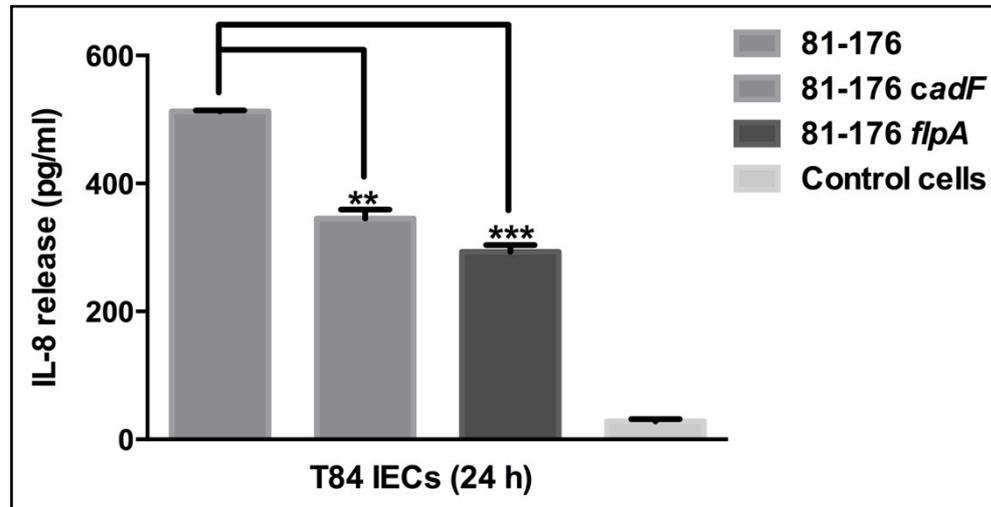
5.2.11 ELISA assays

IL-8 is a well characterised marker of the host immune response to pathogens (Oppenheim *et al.*, 1991) and interaction with and invasion of IECs by *C. jejuni* triggers both innate and adaptive immune responses (Chen *et al.*, 2006, Rathinam *et al.*, 2008). Cytokines of the TNF α family have been found to play central roles in the development of a stable immune system and protection from pathogens (Pfeffer, 2003).

Supernatants from uninfected and T84 IECs infected with 81-176 or 11168H wild-type strains, *cadF* and *flpA* mutants at an MOI of 100:1 for 24 h were collected. The levels of IL-8 and TNF α secretion were assessed.

Both the 81-176 and 11168H *cadF* mutants induced significantly reduced IL-8 levels in comparison to the respective wild-type strains. Both the 81-176 and 11168H *flpA* mutants also induced significantly reduced levels of IL-8 compared to the respective wild-type strains (see Figure 5.28).

(A)



(B)

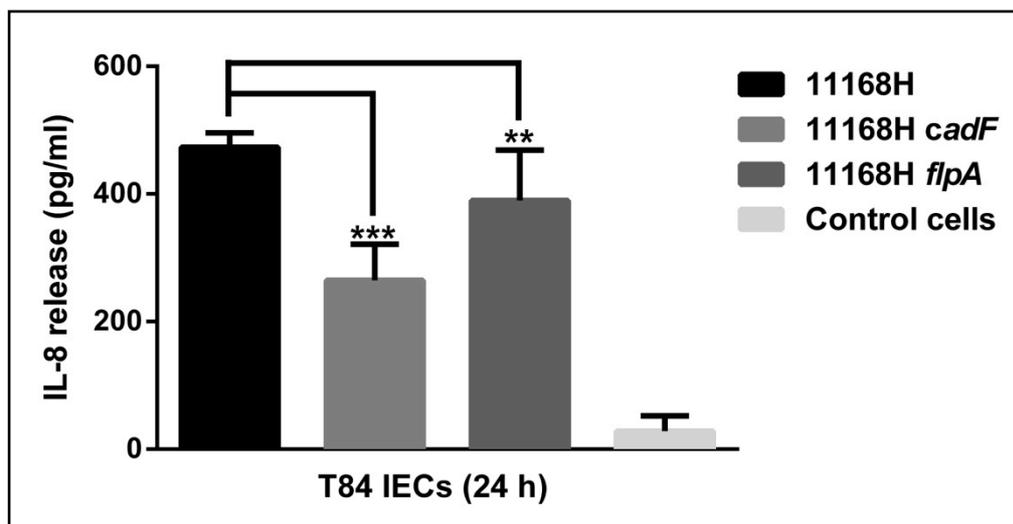
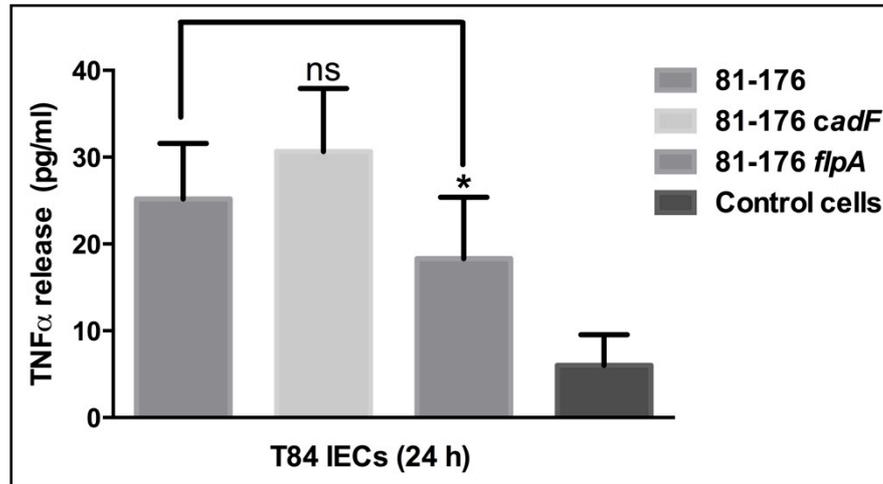


Figure 5.28 The role of CadF and FlpA in *C. jejuni* induction of IL-8 from T84 IECs. The 81-176 and 11168H wild type strains, *cadF* and *flpA* mutants were assessed. The levels of IL-8 secreted after 24 h *C. jejuni* interaction (MOI 100:1) with T84 IECs were quantified using a human IL-8 ELISA. The asterisks denote a statistically significant difference (** = $p < 0.01$, *** = $p < 0.001$). Data are representative of triplicate independent experiments.

The 11168H *cadF* mutant induced significantly increased levels of TNF α compared to the wild-type strain and whilst the 81-176 *cadF* mutant also induced increased TNF α levels compared to the wild-type strain, this was not significant. Both 11168H and 81-176 *flpA* mutants induced significantly reduced levels of TNF α compared to the wild-type strain.

(A)



(B)

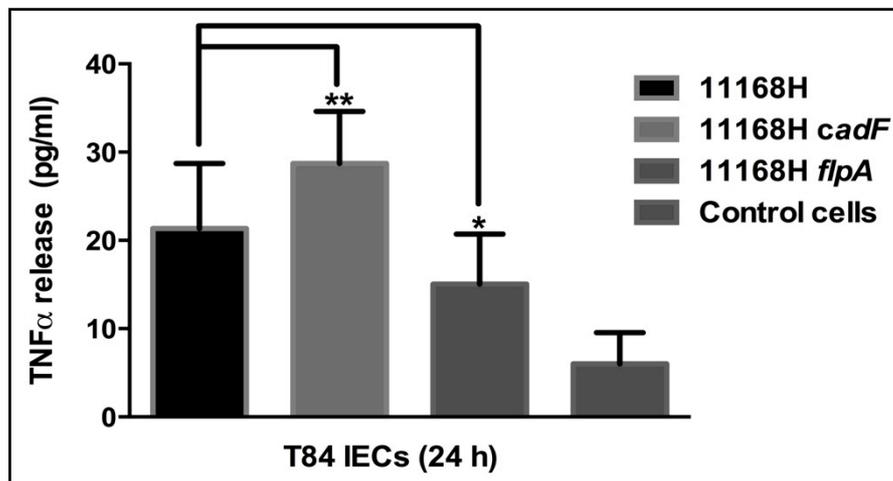
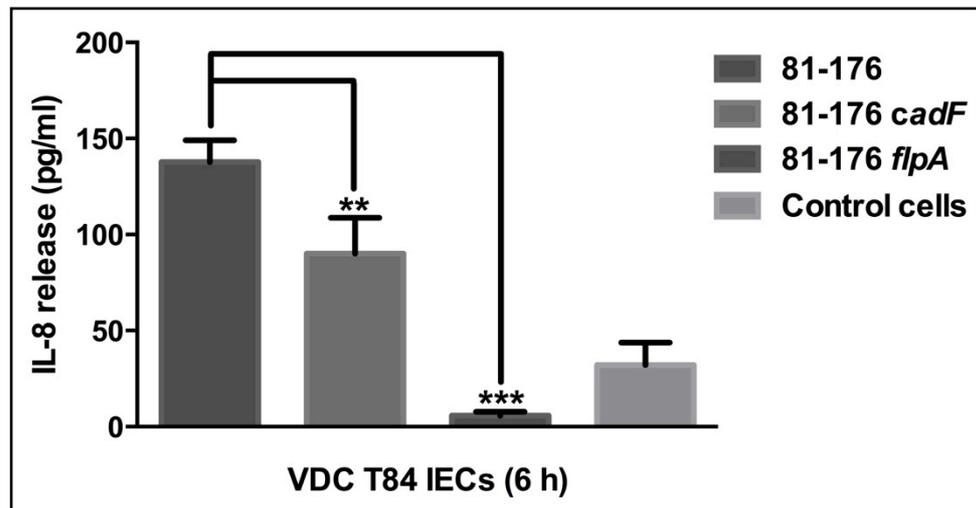


Figure 5.29 The role of CadF and FlpA in *C. jejuni* TNF α induction from T84 IECs. The 81-176 and 11168H wild type strains, *cadF* and *flpA* mutants were assessed. The levels of TNF α secreted after 24 h *C. jejuni* interaction (MOI 100:1) with T84 IECs were quantified using a human TNF α ELISA. The asterisks denote a statistically significant difference (* = $p < 0.05$, ** = $p < 0.01$, ns = no significant difference). Data are representative of triplicate independent experiments.

Following 6 h infection of T84 IECs in the VDC system, the levels of IL-8 and TNF α were assessed in the basolateral compartment. The 81-176 *cadF* and *flpA* mutants induced a reduced IL-8 and TNF α response from T84 IECs compared to the wild-type (see Figure 5.30).

(A)



(B)

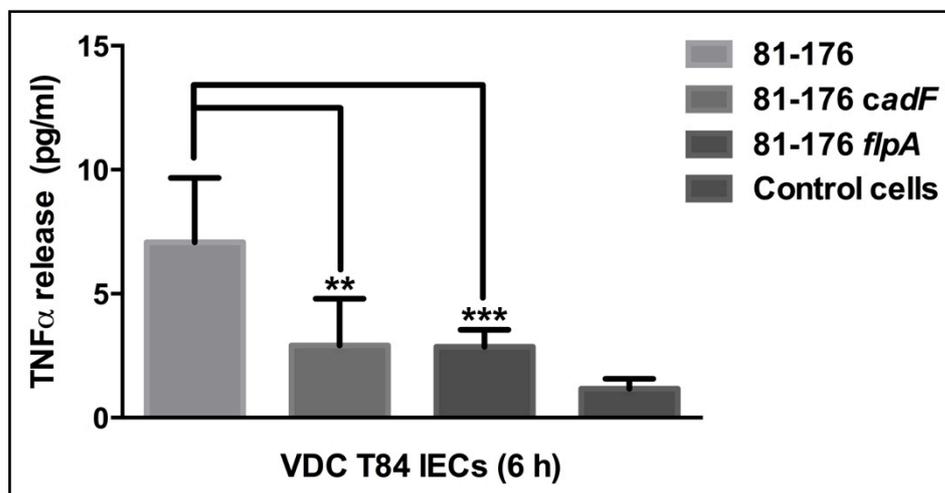


Figure 5.30 The role of CadF and FlpA in *C. jejuni* induction of IL-8 and TNF α from T84 IECs in the VDC system. T84 IEC responses to 6 h co-incubation with *C. jejuni* 81-176 wild type strain, *cadF* and *flpA* mutants (MOI 100:1). The levels of IL-8 and TNF α secreted during *C. jejuni* interaction with T84 IECs in the VDC were quantified using a human IL-8 (A) or human TNF α (B) ELISA. Uninfected IECs were used included as controls. The asterisks denote a statistically significant difference (** = $p < 0.01$, *** = $p < 0.001$). Data are representative of triplicate independent experiments.

5.2.12 Role of outer membrane vesicles in *C. jejuni* interactions with IECs

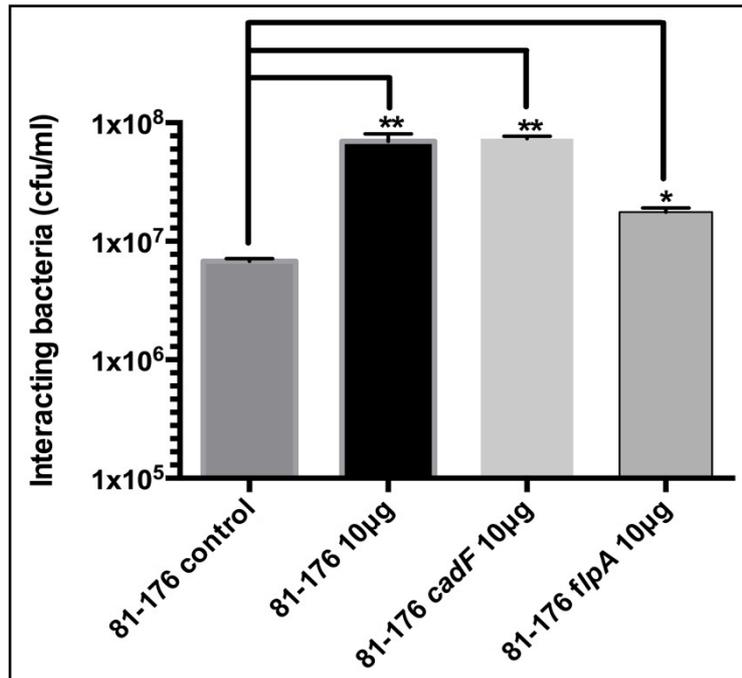
Many Gram-negative pathogenic and non-pathogenic bacteria have been found to produce outer membrane vesicles (OMVs) (Kuehn and Kesty, 2005, Ellis and Kuehn, 2010). OMVs have also been found to contain virulence factors which play a role in pathogenesis (Ellis and Kuehn, 2010). Numerous Gram-negative bacteria including *E. coli*, *P. aeruginosa*, *C. jejuni* and *H. pylori* have been shown to produce OMVs (Hoekstra *et al.*, 1976, Gankema *et al.*, 1980, Logan and Trust, 1982, Blaser *et al.*, 1983b, Kadurugamuwa and Beveridge, 1995, Fiocca *et al.*, 1999, Bauman and Kuehn, 2006).

Caco-2 IECs were pre-incubated with OMVs for 24 h to ascertain whether OMVs would affect *C. jejuni* interactions with and invasion of IECs.

Pre-incubation with OMVs (10 µg) isolated from 81-176 wild-type and the *cadF* mutant resulted in a significant increase in the number of interacting bacteria whilst pre-incubation with OMVs (10 µg) isolated from the *flpA* mutant resulted in only a marginal increase compared to the control with no OMV pre-incubation (see Figure 5.31).

Pre-incubation with OMVs (10 µg) isolated from 81-176 wild-type, the *cadF* and *flpA* mutants produced a significant reduction in the number of intracellular bacteria (10^3 cfu) compared to the control with no OMV pre-incubation (10^5 cfu). No significant differences were observed in the numbers of intracellular bacteria after pre-incubation with OMVs (10 µg) from 81-176 wild-type compared to the control with no pre-incubation (see Figure 5.31).

(A)



(B)

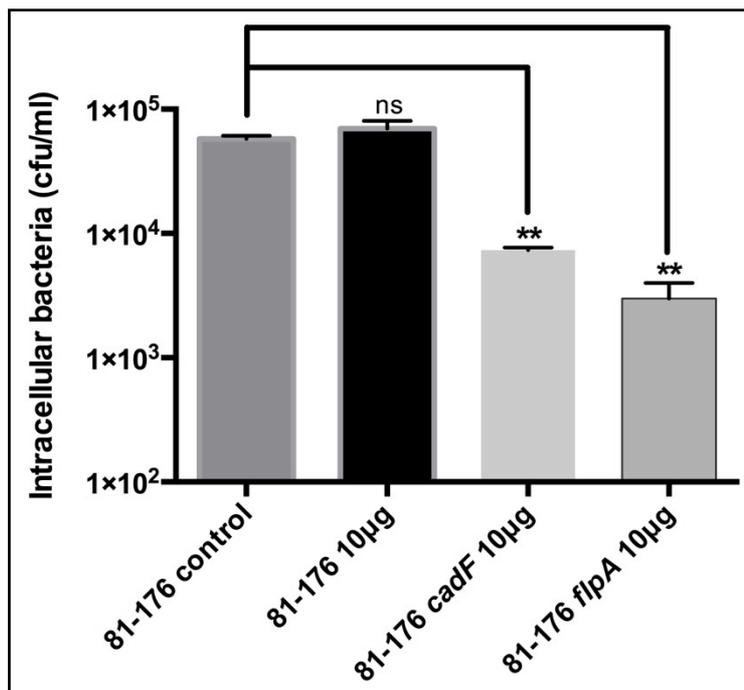


Figure 5.31 Effect of pre-incubation of *C. jejuni* OMVs on bacterial interactions with and invasion of Caco-2 IECs OMVs (10 µg) isolated from *C. jejuni* 81-176 wild-type strain, *cadF* and *flpA* mutants were pre-incubated with Caco-2 IEC for 24 h. Monolayers were washed three times with PBS. Bacteria was harvested from 24 h plates, resuspended in PBS and adjusted to an OD₆₀₀ of 0.1. Bacteria was co-cultured with Caco-2 IECs for 24 h. (A) Caco-2 IECs were lysed with Triton X-100 and interacting bacteria were enumerated. (B) For invasion, Caco-2 IEC cells were incubated with gentamicin (150 µg/ml) for 2 h to kill extracellular bacteria, then lysed and numbers of intracellular bacteria assessed. Asterisks denote a statistically significant difference (* = $p < 0.05$; ** = $p < 0.01$, ns = no significant difference). Data are representative of triplicate independent experiments.

5.3 Discussion

The attachment of pathogenic bacteria to structures on the surface of IECs is critical for colonisation of the host and for infection (Pizarro-Cerda and Cossart, 2006). There are over a hundred documented bacterial adhesins that have been shown to bind to fibronectin, highlighting the importance of this glycoprotein as an essential target for pathogenic bacteria (Henderson *et al.*, 2011, Larson *et al.*, 2013). Two *C. jejuni* outer membrane proteins CadF and FlpA have been shown to bind to fibronectin (Konkel *et al.*, 1997, Flanagan *et al.*, 2009). Currently, the most well characterised fibronectin binding protein is CadF (O. Croinin and Backert, 2012). The binding of this adhesin to fibronectin is proposed to trigger an integrin signalling cascade within the host cell which promotes *C. jejuni* internalisation and recent studies have suggested that CadF and FlpA function co-operatively in binding to fibronectin and inducing uptake of the bacteria (Eucker and Konkel, 2012a, Backert and Hofreuter, 2013).

For this chapter, *cadF* and *flpA* mutants were constructed in both 81-176 and 11168H wild-type strains. Initial phenotypic characterisation studies were performed on both 81-176 and 11168H *cadF* mutants. Growth rate studies showed that *cadF* and *flpA* displayed a slight reduction in growth in comparison to the respective wild-type strains. At selected time-points, growth curve cfu counts were also performed and the results of these reflected the growth rates observed. The 81-176 and 11168H *cadF* mutant displayed significantly reduced growth rates at the 12 h and 18 h time-points compared to the wild-type, similarly the 81-176 and 11168H *flpA* mutant exhibited slightly reduced growth rates compared to the respective wild-type strains.

Clearly, CadF and FlpA are important for adhesion, as interaction and invasion assays performed with *cadF* and *flpA* mutants, resulted in a significantly reduced ability of *C. jejuni* to interact with and invade Caco-2 and T84 IECs. However, an interesting finding observed in these experiments was the ability of the *cadF* and *flpA* mutants to recover over the 6 h and 24 h infection period that would indicate that other adhesins or bacterial virulence factors may be enabling the attachment of the bacteria over longer time periods. Previous studies have shown that *cadF* and *flpA* mutants, displayed significant reductions in interactions with INT407 IECs after a 30 min incubation period, the differences could have been due to experimental procedures as the INT407 IECs were grown for 18 h, followed by the addition of F38011 wild-type strain, *cadF* or

flpA mutants (5×10^7 cfu) and monolayers were centrifuged to enhance bacterial attachment to IECs. Bacterial numbers recovered after the 30 minute incubation period were $>10^6$ cfu for the wild-type, 10^5 cfu for the *cadF* mutant and $<10^6$ cfu for the *flpA* mutant (Monteville *et al.*, 2003b, Konkel *et al.*, 2010). In contrast to Konkel's data and the results of this study, another group reported neither *cadF*, *flpA* or any *cia* mutants were detected in a screen for invasion-related genes in *C. jejuni* 81-176 (Novik *et al.*, 2010). However more recent reports of the role of both CadF and FlpA in host cell invasion has made the study of these fibronectin binding proteins an important area of research.

Both *cadF* and *flpA* mutants displayed changes in the interaction with and invasion of Caco-2 or T84 IECs at 3 h, 6 h and 24 h time-points used in this study. However, this study found that although there is an initial reduction in attachment ability of both *cadF* and *flpA* mutants, which correlates with Konkel's work, there are changes clearly occurring over the longer periods in which the assays in this study were performed in. One of the reasons for this could be due to the increased AAG levels observed for the *cadF* mutant, which may allow higher numbers of the *cadF* mutant to adhere over time, (although, the increase for the *cadF* mutant was only observed in the Caco-2 IECs). *C. jejuni* is reported to translocate across Caco-2 IECs by entering through the IECs (transcellular entry) (Konkel *et al.*, 1992c, Grant *et al.*, 1993, Bras and Ketley, 1999). In the T84 IECs *C. jejuni* translocates between cells (paracellular entry) exiting at the basal surface and then re-attaching to the host receptors located basolaterally (Monteville and Konkel, 2002, Backert *et al.*, 2013). However, exactly how the bacteria accomplishes this is unknown. The adhesins CadF and FlpA which can bind to the basolaterally located fibronectin on T84 IECs, have been reported to cause triggering of integrin complex and thus invasion can occur from the bottom of epithelial cells (van Alphen *et al.*, 2008). Both *cadF* and *flpA* mutants were not only reduced in invasion of T84 IECs, but the numbers of internalized bacteria recovered were significantly lower than those recovered from Caco-2 IECs. Similarly, a previous study also reported a significant reduction for the F38011 *cadF* mutant interactions with T84 IECs after 2 h and 4 h (Monteville and Konkel, 2002).

The Caco-2 IEC line has been reported to lack detectable expression of fibronectin, EGFR and $\alpha 5$ and $\beta 1$ receptors (Kuwada and Li, 2000) and Caco-2 IEC differentiation

has been shown to lead to dramatic reductions specifically in the expression of fibronectin and laminin due to down regulation of gene expression (Levy *et al.*, 1994). This differentiation and possible lack of expression of fibronectin may affect *C. jejuni* interactions with Caco-2 IEC in both *in vitro* and *in vivo* conditions (Simo *et al.*, 1991, Levy *et al.*, 1994). Hence the variations not only observed in this study but also in previous reports in interactions with and invasion of IECs by *C. jejuni* may be due the presence or absence of specific receptor sites. However, in this study the interactions of *cadF* and *flpA* were the same for both Caco-2 and T84 IECs, but the numbers of internalised bacteria recovered from T84 IECs were less than those recovered from the Caco-2 IECs.

The intracellular survival of the *cadF* mutant was significantly reduced at time 0 h and further decreased at the 19 h time-point compared to the wild type. The *flpA* mutant, although significantly decreased in intracellular numbers compared to the wild-type, actually displayed an increase in the number of viable bacteria surviving intracellularly at the 19 h time-point, this may be due to lower numbers of the *flpA* mutant at the start may have had an effect on the numbers of bacteria surviving intracellularly. In addition the, *flpA* mutant still possesses a functioning CadF, perhaps the presence of this adhesin is allowing the bacteria to activate host cell signalling pathways and enabling intracellular survival.

The dynamic bacterial interactions observed for the *cadF* and *flpA* mutant in the interactions and invasion assays and intracellular survival studies may be due to the presence of one fully functioning fibronectin binding adhesin, that may still be capable of attaching to fibronectin triggering host cell signalling, leading to FAK activation of Cdc42 and internalization of the bacteria (Krause-Gruszczynska *et al.*, 2011).

There is still a need to use an optimum incubation time which would allow the study of realistic interactions of *C. jejuni* with IECs to be observed, but these timings have been found to vary for each cell line. Fauchere *et al* reported a 1 hour incubation time to be sufficient for *C. jejuni* to be internalised using Hela IECs (Fauchere *et al.*, 1986). Infection of INT407 IECs with *C. jejuni* strain 81116 was reported to require a maximal invasion time of 2 h (Wassenaar *et al.*, 1991, Gaynor *et al.*, 2004). In another study using Caco-2 IECs, Russell and Blake found that 1 h to 2.5 h was adequate for maximal invasion by *C. jejuni* (Russell *et al.*, 1989). Although for this cell line, incubation

periods of around 5 h are now more commonly used (Szymanski *et al.*, 1995). Incubation times exceeding 5 h is advised to be avoided as *C. jejuni* is reported to be unable to survive and or replicate for extended periods within the vacuole (De Melo *et al.*, 1989, Hu and Kopecko, 1999). Contrasting data was found in this study showing that *C. jejuni* was capable of interacting with and surviving within IECs over longer periods and a previous study also showed that other bacterial pathogens can efficiently utilise the vacuole for survival, for replication and spread to other tissues (O'Callaghan *et al.*, 1999). *C. jejuni* is able survive in the intracellular environment within CCVs avoiding delivery to lysozymes (Watson and Galan, 2008b). In line with extended survival times, *C. fetus* has been found to replicate for up to 24 h in INT407 IECs (Graham, 2002). In addition, *C. jejuni* 81-176 has been reported to internalise more efficiently into INT407 and Caco-2 IECs and that the levels of internalisation decreased in older monolayers (Hu and Kopecko, 1999).

Macrophages rapidly internalise and destroy *C. jejuni* within 24 h due to the presence of reactive oxygen species (Watson and Galan, 2008b). In contrast there have been reports regarding the ability of *C. jejuni* to survive within macrophages, depending on the *C. jejuni* strain and/ or macrophage cell type (Wassenaar *et al.*, 1997, Day *et al.*, 2000). In this part of the study, both *cadF* and *flpA* mutants were significantly reduced in macrophage survival, with the *cadF* mutant decreased in survival at 4 h and no survival at 16 h was observed compared to the wild-type. The *flpA* mutant exhibited reduced survival at 4 h but exhibited similar survival rates at 16 h to those observed for the wild-type.

In order to further study the interaction with and invasion of IECs, a Vertical Diffusion Chamber (VDC) system was used which allowed the co-culture of bacteria and IECs under different medium and gas conditions (Cottet *et al.*, 2002a, Schuller and Phillips, 2010, Mills *et al.*, 2012). The co-culture of the *cadF* and *flpA* mutants with Caco-2 IECs in the VDC resulted in severely reduced invasion levels of the *cadF* mutant and even further reduced levels for the *flpA* mutant compared to the wild-type strain. Equally, severely reduced invasion levels of T84 IECs was observed after infection with the *cadF* or *flpA* mutant, however the numbers of intracellular bacteria recovered were slightly higher than when using Caco-2 IECs. The use of the VDC system emphasises the importance of culture conditions, by mimicking the *in vivo* environment, the

conditions could be allowing access of the bacteria to the basolaterally-located fibronectin, thus allowing higher numbers of bacteria to be internalised. In addition, the importance of bacterial and strain differences when studying *C. jejuni* interactions with IECs are also shown to be important using the VDC. Using standard tissue culture assays, both the *cadF* and *flpA* mutant invaded T84 IECs at lower levels than the Caco-2 IECs. In the VDC, both mutants invaded the T84 IECs at slightly higher levels than Caco-2 IECs. A *rpoN* mutant that is completely aflagellate and non-motile was also included as a control and despite a complete absence of motility, was still capable of being internalised into Caco-2 IECs with the numbers recovered higher than those observed for the *cadF* and *flpA* mutant. However, the number of *cadF* and *flpA* mutant bacteria internalised into T84 IECs was significantly higher compared to the *rpoN* mutant. Perhaps a combination of bacterial virulence factors including motility, the presence of at least one functional fibronectin binding protein, under the right *in vivo* conditions still allows *C. jejuni* internalisation. The interactions of CadF and FlpA mutants with Caco-2 or T84 IECs in the VDC showed that these proteins are involved in *C. jejuni* invasion, however there are clearly other bacterial factors involved as *C. jejuni* were still able to invade the IECs.

The role of CadF and FlpA in induction of IL-8 and TNF α was also investigated following infection. Previous studies have reported that Caco-2 IECs secrete minimal levels of IL-8 in response to *C. jejuni* infection, whilst using the T84 IECs resulted in much higher levels of IL-8 secretion (MacCallum *et al.*, 2006). In addition, infection of Caco-2 and HEp-2 IECs with the 81-176 wild-type strain was reported to induce a higher IL-8 response than when infected with 11168H wild-type (Zilbauer *et al.*, 2005). This variation is indicative of the differences in strains producing differing levels of virulence and thereby triggering varying levels of cytokine release. IL-8 is a well-characterised marker of the host immune response against pathogens and been shown to be involved in the host innate immune response to *C. jejuni* (Hu and Hickey, 2005). TNF α , a cytokine primarily produced macrophages in response to stimuli activating toll-like receptors (Old, 1988, Oppenheim *et al.*, 1991, Hobbie *et al.*, 1997). TNF α is also expressed by activated natural killer cells, B cells and T cells, playing a role in cell recruitment, apoptosis, cell activation and differentiation (Old, 1988, Sedgwick *et al.*, 2000). Therefore, to study host cell response the T84 IEC line was used to study the induction of IL-8 and TNF α following co-culture with *C. jejuni*.

The *cadF* and *flpA* mutants induced significantly reduced levels of IL-8 compared to the wild-type strain. In the induction of TNF α , the 81-176 *cadF* mutant displayed no differences compared to the wild-type, whilst the 81-176 *flpA* mutant was significantly reduced in TNF α induction. The 11168H *cadF* was significantly increased in TNF α induction, whilst the *flpA* mutant induced a reduced TNF α response compared to the wild-type strain. The supernatants from the VDC were also probed following co-culture with *C. jejuni* for the presence of IL-8 and TNF α . Both *cadF* and *flpA* mutants produced a significant reduction in IL-8 induction, with the *flpA* mutant exhibiting a severe reduction in comparison to both the *cadF* mutant and the wild-type strain, in induction of IL-8. Both 81-176 and 11168H *flpA* mutants induced reduced levels of TNF α compared to the wild-type strain. Equally, the induction of TNF α was also significantly reduced compared to the wild-type following co-culture with T84 IECs in the VDC. Previous data showed higher levels of IL-8 induction in T84 IECs following 24 h infection time-points using the VDC compared to the results obtained in this study (Mills *et al.*, 2012). A possible explanation for this discrepancy could be due the fact that the VDC assays performed in this study were performed for a 6 h infection period. Both 81-176 and 11168H *flpA* mutants induced reduced levels of TNF α compared to the wild-type strain. Suggesting that these adhesins may play a role in triggering the pro-inflammatory cytokine TNF α .

The precise bacterial factors and host cell signalling events involved in the induction of the pro-inflammatory cytokines upon co-infection with *C. jejuni* are still unknown but it is thought to be caused by the attachment to and invasion of the IECs by the bacteria and by the production of CDT (Hickey *et al.*, 1999, Hickey *et al.*, 2000). The production of IL-8 is central in the host mucosal inflammatory response, this is an important response for not only inducing diarrhoea but also to clear the infection (Watson and Galan, 2005).

The first evidence that *C. jejuni* delivered virulence factors in OMVs was the discovery of OMVs containing CDT toxin, highlighting the biological significance of *C. jejuni* OMVs (Lindmark *et al.*, 2009). In this study, the effect of pre-treatment of Caco-2 IECs with OMVs on bacterial interactions with and invasion of Caco-2 IECs was investigated. Pre-incubation with OMVs 10 μ g isolated from 81-176 wild-type, *cadF* or *flpA* mutants significantly increased bacterial interactions compared to the control IECs

with no pre-incubation. Pre-incubation with OMVs isolated from *cadF* and *flpA* resulted in a significant reduction in intracellular bacteria compared to pre-incubation of IECs with the OMVs isolated from the 81-176 wild-type and the control. A previous study at LSHTM found that proteomic analysis of OMVs isolated from 11168H wild-type found 151 proteins and virulence-associated proteins identified included the fibronectin binding proteins CadF and FlpA. The study found that virulence-associated *C. jejuni* proteins are delivered via OMVs (Elmi *et al.*, 2012). Indicating that these adhesins may play a role in OMV production and enable or play a part in internalisation of *C. jejuni*.

Motility is essential for *C. jejuni* colonisation of the host and to determine the effect of the mutations in both *cadF* and *flpA*, motility assays were performed. The results showed that 81-176 and 11168H *cadF* were significantly reduced in motility compared to the wild-type strain, although the 11168H *cadF* seemed to exhibit some recovery of motility by the 72 h time-point. In contrast, the 81-176 and 11168H *flpA* mutants were severely affected in motility and displayed a non-motile phenotype compared to the respective wild type strain. As motility has been attributed to *C. jejuni* interactions with and invasion of IECs, the data produced in this study found that although a significant reduction in motility was observed for both *cadF* and *flpA* mutants which correlated with an initial reduction in interactions with and invasion of IECs at the 3 h time-point. Both *cadF* and *flpA* mutants were still capable of interaction with and invasion of IECs especially after extended periods of infection (6 h and 24 h). Motility studies were performed on F38011 *cadF* and *flpA* mutants were reported not to exhibit any differences in motility over a 48 h period (Flanagan *et al.*, 2009). However, the results were not shown which makes the comparison with the motility impossible.

Motility has been reported to play a role in AAG, a virulence trait of *C. jejuni* (Guerry *et al.*, 2006). The AAG results obtained with the 81-176 wild-type, *cadF* and *flpA* mutants showed that increased levels of AAG were observed with the *cadF* mutants compared to the wild-type AAG in both 81-176 and 11168H, whilst the 81-176 *flpA* mutant showed no difference in AAG ability in comparison to the wild-type strain, whilst displaying a non-motile phenotype. The 11168H *flpA* mutant displayed a reduction in AAG compared to the wild-type AAG levels. Previous studies have reported that different strains of *C. jejuni* exhibit varying levels of AAG (Misawa and Blaser, 2000).

Both the 81-176 *cadF* and *flpA* mutants exhibited reduced cytotoxicity in the *G. mellonella* model and the cytotoxicity levels were higher than those observed for the 11168H *cadF* and *flpA* mutants. The initial lack of motility of the *cadF* mutant may have affected the cytotoxicity in the larvae as the effect was increased after 72 h (correlating with lower survival levels of the larvae at these time-points). The AAG was increased for the *cadF* mutant in both strains, possibly increasing the interaction of the bacteria with the larval cells. In contrast, the *flpA* mutant was severely attenuated in motility in both strains, yet was still cytotoxic in the larvae at levels higher than those observed with the 81-176 *cadF* mutant. The AAG levels observed for the 81-176 *flpA* mutant were not significantly different to the wild-type strain but were significantly lower than those observed for the *cadF* mutant. These results indicate that a lack of motility may result in reduced cytotoxicity in the larvae and although the direct injection of bacteria into the larvae removes the need for motility and adhesion to the larval cells, there was still an attenuation in cytotoxicity of the larvae.

As both CadF and FlpA are fibronectin binding proteins (Konkel *et al.*, 1997, Flanagan *et al.*, 2009), fibronectin binding assays were performed to study the interaction of the *cadF* and *flpA* mutants with fibronectin. Binding to fibronectin coated wells was significantly reduced for the *cadF* mutant and the effect was more pronounced for the *flpA* mutant compared to the wild-type. Previous studies have similarly reported a reduced fibronectin binding ability for both *cadF* and *flpA* mutant (Konkel *et al.*, 2010). In addition, a *cadF/flpA* double mutant showed a similar reduction to the individual mutants in binding to INT407 IECs, showing that *cadF* and *flpA* are both required for binding to fibronectin and a *flpA* complement was shown to restore fibronectin binding ability (Konkel *et al.*, 2010). In this study, the construction of a *cadF* complement was unsuccessful despite numerous attempts. Konkel's group also reported difficulty in construction of the *cadF* complement (Derrick Samuelson, personal communication). The *flpA* complement was successful in construction, yet failed to grow; further work would involve remedying these problems.

In summary, *cadF* and *flpA* mutants were reduced in their interaction with and invasion of Caco-2 and T84 IECs, yet exhibited increasing levels of interactions with and invasion of IECs over the infection time-points.

Chapter 6: Final Discussion

6.1 Invasion

For invasion studies, the 81-176 wild-type displayed both a microfilament and microtubule-dependent mechanism of entry as invasion was enhanced in the presence of CytD and significantly reduced in the presence of colchicine. Also caveolae-mediated endocytosis, clathrin-mediated endocytosis and host cell signalling pathways are also involved, suggesting that *C. jejuni* modulates a number of host cell pathways during invasion. The limitations of the invasion study were that due to time constraints only one *C. jejuni* strain and one cell line were utilised for inhibition of invasion studies. Future work should focus on the use of different cell lines, a comparison of a number of fully sequenced *C. jejuni* wild-type strains, variations in the MOI, the use of different inhibitors and variation of the concentration of the inhibitors employed.

6.2 Visualisation of *C. jejuni*

In an attempt to track the course of *C. jejuni* invasion of and survival within IECs, studies were conducted using eGFP or Evoglow® expressing *C. jejuni* strains which were constructed for use in confocal studies but the results were only partially successful. The bacteria produced high levels of fluorescent signal when grown in broth cultures in the VAIN. Studies were attempted at visualising *C. jejuni* or *C. jejuni* expressing eGFP or Evoglow® within *G. mellonella* larvae in an attempt to locate CCVs within the larvae. The aim was to formalin fix the larvae and after a few days embed the larvae in wax to produce thin slices for subsequent staining and visualisation. The larvae were extremely difficult to slice thinly, different fixing times were applied, with the same disappointing results. The next step was to visualise the bacteria expressing eGFP or Evoglow® following infection of IECs. The main problem encountered was that once IECs were infected with these strains, the levels of fluorescence depleted rapidly. As the aim was to study the course of the bacteria during invasion, incubation times were extended to >2 h based on the results of the interaction and invasion assays. However, very few bacteria were observed within the IECs, possibly due to the presence of a polarised monolayer, as *C. jejuni* has been reported to traverse polarised IECs or the harsh intracellular environment of the IEC (Konkel *et al.*, 1992c, Bras and Ketley, 1999). The lack of detectable signal could also have been due

to the fact that certain *E. coli* promoters are unable to function in *C. jejuni* (Taylor, 1992, Wosten *et al.*, 1998). The use of an anti-*C. jejuni* antibody for visualisation of both extracellular and intracellular bacteria was also only partially successful, data produced using the anti-*C. jejuni* antibody showed internalised and non internalised bacteria. However, the numbers visualised were very low. This could be due to non-specific binding of the antibody utilised and the use of intact monolayers in an attempt to ascertain the real *in vivo* interactions of the bacteria with the IECs. Future work would require optimisation of the constructs, including the use of a stronger *C. jejuni* promoter upstream of the eGFP/Evoglow®. Modifications in the MOI used for infection studies and the use of different mutants as eGFP and Evoglow® expressing *cadF*, *flpA* and *ciaB* mutants were also constructed but not tested due to the lack of fluorescence signal observed with the wild-type strain. In addition, this work could be modified using monolayers that have not reached confluency as fully confluent and intact monolayers are less well invaded by *C. jejuni* (Konkel *et al.*, 1992c, Monteville and Konkel, 2002, van Alphen *et al.*, 2008).

6.3 *ciaB* mutant

The interaction and invasion data produced in this study supports Konkel's observations. The 81-176 *ciaB* mutant was significantly reduced in invasion compared to the wild-type strain. However, the levels of invasion for the *ciaB* mutant did increase over longer incubation time-points, suggesting a role for other virulence factors in internalisation of the bacteria. The *ciaB* mutant also exhibited reduced intracellular survival and reduced cytotoxicity in the *G. mellonella* model of infection. The role of CiaB is thus important in virulence of *C. jejuni* and future studies should aim to dissect this further, by including *ciaB* mutants in inhibition of invasion studies. A combination of inhibitor(s) alongside a *ciaB* mutant in interaction and invasion assays at the time-points used in this study could reveal more detail regarding the mechanism of entry and the role of CiaB. This could be further extended to use in the VDC models with treatment of Snapwells prior to infection with wild-type or *ciaB* mutant bacteria which should provide novel data. Due to time constraints and extensive optimisation studies required, this was not possible in this study.

6.4 *cadF* mutant

Studies with the 81-176 *cadF* mutant indicated the requirement of this adhesin for *C. jejuni* invasion. The 81-176 *cadF* mutant exhibited significant reductions in interactions with, invasion of IECs and reduced cytotoxicity in the *G. mellonella* model of infection. Although, over longer time-points the interactions of the *cadF* mutant increased. One issue with this part of the study was in the problems encountered in construction of a *cadF* complement. Following numerous attempts and the use of an alternative complementation vector, there were significant issues in the construction of the *cadF* complement. The availability of a *cadF* complement could be tested alongside the *cadF* mutant to study the effects. It should be noted that Konkel's group also admitted to having significant problems with regard to complementation of the *cadF* mutant and are yet to produce this (Derrick Samuelson-personal communication). Future studies could involve the use of a reliable proof reading enzyme as the use *pxf* and *pfu* polymerases introduced numerous errors into the amplified *cadF* gene. Further work should aim to test the role of this bacterial adhesin by using a combination of inhibitor(s) alongside a *cadF* mutant and a *cadF* complement in interaction and invasion assays at the time-points used in this study to reveal more detail regarding the mechanism of entry and the role of CadF in *C. jejuni* invasion of IECs.

6.5 *flpA* mutant

The 81-176 *flpA* mutant exhibited a severe reduction in motility in comparison to the wild-type strain. An initial reduction in interaction and invasion of IECs was observed and despite this severe lack of motility, the *flpA* mutant displayed increased interactions with and invasion of IEC over longer time-points. The numbers of intracellular *flpA* mutant recovered from Caco-2 IECs following co-culture in the VDC resulted in significantly lower numbers of bacteria recovered compared to the wild-type and *cadF* mutants, suggesting that this adhesin may be playing a more significant role in initial interactions with IECs and possibly in disrupting host cell signalling. One limitation of this part of the study was although a *flpA* complement was constructed, electroporation of the *flpA* construct into the *flpA* mutant resulted in no growth. Controls were set up to ensure the results were not due to the electroporation procedure. The problem remained with the *flpA* complement construct. In addition, attempts were made to construct a

cadF flpA double mutant, however following electroporation of the constructs, no bacterial growth was observed. Future work would involve problem solving this issue to ensure the availability of a viable double mutant and a complement and further dissecting the role of FlpA by using inhibitors that specifically disrupt the host cell signalling pathways.

6.6 Final comments

C. jejuni invasion mechanisms are still yet to be accurately elucidated as it is not exactly clear which routes *C. jejuni* utilises and various studies have reported multiple uptake pathways. There are differences reported in which pathways are important for *C. jejuni* invasion and the use of different *C. jejuni* strains, different cell lines, the fact that standard tissue culture assays are performed under normal atmospheric conditions are all contributory causes as to the differences in *C. jejuni* invasion which have been reported. The ways in which tissue culture assays are performed are highly varied as many variations of this method have been reported. There are no studies reporting the use of *cadF*, *flpA* or *ciaB* mutants in interaction and invasion assays with IECs over longer time-points, making it difficult to compare the data produced here. *C. jejuni* infection studies using the VDC system under microaerobic conditions were the first to be performed at LSHTM and resulted in novel data (Mills *et al.*, 2012).

This study provided further confirmation of the action of *C. jejuni* CadF, FlpA and CiaB virulence proteins through studying interaction and invasion, survival within IECs and macrophages, cytotoxic effect in the *G. mellonella* infection model, role in OMV production and roles in induction of IL-8 and TNF α . The study could be further improved by introducing additional studies in a number of areas. Firstly, with the use of *Galleria mellonella* as an infection model, the advantages of this model are ease of use, cheap to maintain, the larvae can be studied at 37°C and also the ability to screen large numbers of different mutants. This study found that *cadF*, *flpA* and *ciaB* mutants exhibited a reduced cytotoxic effect in the larvae compared to the wild-type strain. This model could have been further utilised by enumeration of bacteria from within the larvae at different time-points by dissecting the larvae that were inoculated with either the wild-type or the mutants and removing the haemolymph. This would provide additional data alongside the cytotoxicity data. Studies performed during this study but not reported in this thesis, attempted to visualise *C. jejuni* wild-type or mutants within

the *G. Mellonella* larvae, wax embedding was not very successful, however this could have been remedied by freezing the samples and then thin slicing for staining with hematoxylin and eosin (H&E). Also, in order to view CCVs within the *G. mellonella* larvae, the haemolymph from larvae infected with *C. jejuni* wild-type, *cadF*, *flpA* or *ciaB* mutants within CCVs could have been visualised by isolating the haemocytes and then analysing these samples by TEM for the presence of CCVs. The interaction and invasion studies performed in this study showed that the mutants were significantly reduced in interactions with and invasion of Caco-2 and T84 IECs over a 3 h, 6 h and 24 h infection period. The extended infection times were used due to the fact that there is a lack of studies reporting *C. jejuni* interaction with and invasion of IECs over 3 h. For this reason, *cadF*, *flpA* and *ciaB* mutants were tested at 3, 6 and 24 h. The use of a polarised cell model provided a relevant model for studying *C. jejuni* interactions by mimicking more closely the *in vivo* conditions present in the human gut, where the mucosal epithelium is highly differentiated and provides strong structural integrity. It has been reported that *C. jejuni* can translocate across Caco-2 and other polarised cell monolayers without causing significant disruption to the integrity of the cell monolayer (Konkel *et al.*, 1992c, Everest *et al.*, 1992).

However this integrity can be disrupted over time as the tight junctions can become leaky due to the damage of the proteins involved in the maintenance of these junctions such as occludin, claudins and a number of other proteins which provide important structural factors in the establishment of cell polarity. These proteins are necessary for the tight sealing of the cellular sheets, and maintain tissue homeostasis by enabling the control and movement of ions in the cell (Backert *et al.*, 2013). Inoculation of high doses of *C. jejuni* ($> 10^8$ - 10^9 cfu) onto monolayers was found to reduce the TEER at the 24 h time-point (Bras and Ketley, 1999), albeit much slower than the timings observed for *S. typhimurium* (< 6 h) (Jepson *et al.*, 1995). The results of this study showed an initial decrease in interactions and invasion of IECs by *cadF*, *flpA* and *ciaB* mutants but with numbers of bacteria increasing over the infection period. Many important bacterial pathogens are able to cross polarised IECs using different mechanisms: paracellular and transcellular routes. Previous studies have shown that *C. jejuni* was only able to invade polarised IECs following disruption of the tight junctions with EDTA, thereby providing access to the subcellular space (Monteville and Konkel, 2002, van Alphen *et al.*, 2008). However the question as to how *C. jejuni* reaches the subcellular space and

invades when a polarised monolayer is present has yet to be answered. The mechanism of this process may involve disruption of tight junctions or transcytosis enabling entry into the IECs. This has been observed in *in vitro* using IECs grown on Transwell supports (Hoy *et al.*, 2012, MacCallum *et al.*, 2005). Invasion may also occur due to the growth of *C. jejuni* in the intestinal crypts which would inevitably damage the epithelial cells, leading to a lack of a viable protective barrier thereby allowing *C. jejuni* access to the subcellular space (Bouwman *et al.*, 2013). In addition, similar to *Shigella* invasion, *C. jejuni* may utilise and gain entry through M-cells, these cells function by sampling the intestinal lumen and delivering the contents to underlying immune cells.

Previous studies have shown that *C. jejuni* can translocate across Caco-2 and other polarised cell monolayers without disruption to TEER. In contrast, other studies reported a time-dependent decrease of TEER in response to *C. jejuni* infection, however the actual bacterial factors involved in triggering a reduction in TEER were not reported on (Chen *et al.*, 2006). Thus, there is once again conflicting data in the literature and a consensus is yet to be reached among investigators as to the invasion mechanism of *C. jejuni*. Whether the results observed in this study were directly due to the specific mutation would require further studies as *cadF*, *flpA* and *ciaB* mutants exhibited slight increases in interaction and invasion of Caco-2 and T84 IECs over the 24 h infection period, possibly suggesting that *C. jejuni* is able to translocate more easily once cell junctions have been disrupted, especially over a prolonged period of infection. Another reason could be due to the accumulation of bacterial toxins or other bacterial products. Therefore the *cadF*, *flpA* and *ciaB* mutants were still able to enter between cells due to the disruption of the tight junctions/adherens and the presence of a viable adhesin present on each mutant (eg: The *cadF* mutant - possesses a viable FlpA adhesin and vice versa) and was therefore still capable of reaching the basolateral surface of the IECs and attach to fibronectin. Complementation of *cadF* and *flpA* mutants would also have provided further insights into the data collected in this study.

With regards to the intracellular survival assays and macrophage survival assays, firstly *C. jejuni* is able to penetrate the mucus layer in humans, interact with the IECs inducing IL-8 production. Induction of IL-8 leads to the recruitment of DC, macrophages and neutrophils, which interact with *C. jejuni*. These interactions result in a massive pro-inflammatory response (Young *et al.*, 2007). Future studies should involve the use of

neutrophils rather than Caco-2 IECs as a mechanism of studying host cell-bacterial interactions and therefore provide a more relevant model to answer the questions posed. This work is currently underway with our collaborators, Mona Bajaj-Elliott's group at the Institute of Child health (ICH). The macrophage cell line used in this study was J774A.1 (a murine macrophage line) as previous studies have reported that *C. jejuni* is killed rapidly by macrophages derived from human monocytes (Kiehlbauch *et al.*, 1985). Human macrophages would be a more appropriate model to study the survival of *C. jejuni* wild-type and the *cadF*, *flpA* and *ciaB* mutants.

The main drawbacks of the VDC system were the time-consuming preparation of the chambers prior to infection studies. The VDC had to be placed in the VAIN to provide microaerobic conditions for the bacteria and also attached via a gas manifold to a separate oxygen supply providing the IECs with oxygen. The regular use of the VAIN by other researchers inevitably resulted in gas and pressure alterations, affecting the chambers and the results. Another major drawback was the risk of contamination was high using the VDC, despite a 3 h soaking and washing step in Haz-tab the chambers were extremely difficult to sterilise. Future work could involve using the VDC system used to study the interactions of the *cadF*, *flpA* and *ciaB* mutants over longer time-points and compare the results with those observed in the standard tissue culture. The bacterial supernatants could be used to analyse gene expression of the wild-type and the mutants under the microaerobic conditions of the experiment. In addition, other *C. jejuni* mutants could be also be tested in the VDC system, different cell lines could be utilised. Pre-treatment of the Snapwells with inhibitors prior to infection with wild-type bacteria and the mutants could provide interesting results and the data could be compared to the results of the inhibition of invasion assays performed under standard tissue culture conditions. Staining of the Snapwell following infection with *C. jejuni* expressing eGFP or Evoglow® constructs to locate the bacteria once inside the IEC could provide further information on the behaviour of the wild-type strain, *cadF*, *flpA* and *ciaB* mutants.

7 Appendices

7.1 Appendix 1

Columbia blood agar plates

16 g of Columbia agar base was dissolved in 400 ml of Milli-RO water. The solution was mixed and autoclaved and allowed to cool. 24 ml of horse blood (7%) was also added to the agar. Skirrow was added and antibiotics.

Brucella broth

Brucella broth was prepared by dissolving 20 g of broth to 465 ml of Milli-RO water, the solution was mixed by shaking then autoclaved. When antibiotics were required, Brucella broth was cooled and supplemented with either individual or a combination of antibiotics; chloramphenicol (10 µg/ml), ampicillin (100 µg/ml), kanamycin (30 µg/ml).

Luria-Bertani LB agar plates

LB agar plates were prepared by dissolving 15 g of LB agar in 400 ml of Mill-RO water, the solution was mixed by shaking then autoclaved. For preparation of antibiotic plates, the medium was allowed to cool and supplemented with either individual or a combination of antibiotics; chloramphenicol (10 µg/ml), ampicillin (100 µg/ml), kanamycin (30 µg/ml). Plates were stored for at 4 °C for 3 weeks.

LB broth

LB broth was prepared by dissolving 18.4 g of LB broth in 400 ml of Milli-RO water, the solution was mixed by shaking then autoclaved. For preparation of overnight cultures, LB broth was supplemented with either individual or a combination of antibiotics; chloramphenicol (10 µg/ml), ampicillin (100 µg/ml), kanamycin (30 µg/ml). LB broth was stored at room temperature for up to 1 month.

Preparation of Motility Plates

C. jejuni motility media was prepared by dissolving 6 g of Brucella broth (Oxoid) and 1g of bacto agar (Oxoid) in 250 ml of DH₂O and autoclaved at 121°C for 15 min. For

motility plates, agar melted in microwave, cooled and poured into sterile petri dishes and stored at 4°C until required (appendices).

Inactivation of Fetal calf serum

FCS was heat inactivated at 56°C for 30 minutes in a water bath and then filter sterilised using 0.2 µM syringe prior to use in tissue culture experiments. The FCS was aliquoted into 50 ml sterile falcon tubes (Corning) and stored at -20°C.

Dulbeccos Modified Eagle Medium

For tissue culture studies Dulbeccos Modified Eagle Medium (DMEM) was prepared by adding 5 ml of penicillin and streptomycin (100x) and 50 ml of heat inactivated FCS. The solution was mixed and then filter sterilised using a 0.2 µM syringe filter and aliquoted into 50 ml sterile falcon tubes and stored at -20°C for up to 1 month.

Cell freezing medium

Cell freezing medium was prepared prior to freezing cells by mixing 10 % DMSO with DMEM (prepared as above).

Preparation of antibiotic concentrations.

Stock concentrations of ampicillin (20 mg/ml) were prepared by dissolving 1 g of ampicillin in 20 ml of sterilised water and filtered with a 0.22 µm filter. Aliquots of 1 ml were prepared and stored at -20°C for up to 1 year.

Stock concentrations of chloramphenicol (20 mg/ml) were prepared by dissolving 1 g of chloramphenicol in 50 ml of ethanol. Aliquots of 1 ml were prepared and stored at -20°C for up to 3 months.

Stock concentrations of kanamycin (25 mg/ml) were prepared by dissolving 500 mg of kanamycin sulphate powder in 10 ml sterile water and filtered with a 0.22 µm filter. Aliquots of 1 ml were prepared and stored at -20°C until required.

0.2 % Triton X-100 solution

200 µl of Triton X-100 was added to 100 ml of PBS

Preparation of oligonucleotide primers

100 μM stock solutions of primers were prepared using sterile DNase free water as described below:

Eg: $5.6 \text{ OD}_{260} = 37.18 \text{ nmol}$ ($37.18 \times 10 = 371.8$)

371.8 μl of water was added to the appropriate primer giving a 100 μM stock. 10 μM of working solutions prepared by diluting 1 in 10 and storing aliquots at -20°C .

Preparation of 10 mM dNTPS

25 μl of each dATP, dCTP, dGTP and dTTP (at stock concentrations of 100 mM) were added to 900 μl of sterile water, briefly vortexed. Final mixture contained 10 mM dNTPs (2.5 mM of each dNTP) and stored at -20°C .

Preparation of running buffer for agarose gel electrophoresis

50 x TAE

242 g of Tris base, 57.1 ml of Acetic acid and 18.6 g of EDTA (0.5M pH 8.0). The solution was mixed and the volume adjusted to 1 litre with Milli-RO water

Ethidium Bromide working solution

Ethidium bromide Stock (10 mg ml^{-1}) was diluted with 20 mM MOPS-NaOH (pH 7) and used at $0.5 \mu\text{g ml}^{-1}$

Agarose gel

1.5 g of agarose was added to an Erlenmeyer flask for a 1 % agarose gel. 150 ml of 1X TAE was added. Solution was mixed and heated in a microwave on a medium setting for 3 minutes or until the agarose completely melted. With safety glove the flask was inspected and swirled to ensure mixing and allowed to cool. 4 μl of ethidium bromide was added, mixed again by swirling and poured into a pre-prepared clean casting plate with an appropriate comb in place. The gel was then left to set for 30 minutes before use.

Ethanol

70% ethanol was prepared by adding 350 ml of 95% ethanol to 150 ml sterilised water and stored in Duran bottles until required (Duran, Mainz, Germany)

Primary culture

15 ml of Brucella Broth was pre- incubated in T75 flask, 37 °C under microaerobic conditions for 24hrs, with shaking. At the same time a 24 hour plate of *C. jejuni* was set up. From this a bacterial suspension was made and the OD₆₀₀ was adjusted to OD₆₀₀ 0.1. This was made using previously pre-incubated BB to produce an OD₆₀₀ of 0.1. This was then added to the pre-incubated flask and incubated at 37°C, microaerobic conditions with shaking. This method was used for taking measurements for GK, motility and autoaggregation studies.

Mini- prep purification

Mini preps were set up in 10 ml LB broth with the required antibiotic and a single colony of the restreaked transformants was added to the universal containing the LB broth and antibiotic. This was incubated at 37°C for 12-16 h in a shaking incubator at 150 rpm. The overnight bacterial suspensions were then centrifuged at 4,000k rpm for 10 min and supernatant discarded. The overnight were purified using QIAgen plasmid mini prep kit according to manufacturer's instructions, briefly. Bacterial cells were resuspended in 250 µl of Buffer P1 and transferred to clean 1.6 ml eppendorf tube. 250 µl of buffer P2 was added and the tube was inverted 4-6 times, followed by the addition of 350 µl buffer N3 and mixed immediately by inverting 4-6 times. The tube was then centrifuged at 13,000k rpm for 10 min. Supernatant was transferred to a QIAquick column and then centrifuged for 1 minute at 13,000k rpm. Flow through was discarded and 0.5 ml buffer PB was added to the column and centrifuged for 1 min. Flow through was discarded and 750 µl of buffer PE added to the column. Column was centrifuged at 13,000k rpm for 1 minute. Flow through was discarded and tube was centrifuged again to remove residual wash buffer. The QIAprep column was transferred to clean 1.5 ml tube and 30 µl of MQ sterilised water was applied to the column, allowed to stand for 1 minute and then centrifuged for 1 minute at 13k rpm. The purified plasmid DNA concentrations were quantified using Nano drop ND-1000 spectrophotometer, and samples were stored at -20C until required (appendices)

7.2 Appendix 2

dATP -	Promega, Southampton, UK
dGTP -	Promega, Southampton, UK
dCTP -	Promega, Southampton, UK
dTTP -	Promega, Southampton, UK
MgCl ₂ -	Promega, Southampton, UK
10X buffer, Taq DNA polymerase M166F (2,500 U)	Promega, Southampton, UK
ABI prism terminator ready reaction mix UK	Applied Biosystems, Warrington, UK
Agarose	Sigma-Aldrich, Poole, UK
Ampicillin	Sigma-Aldrich, Poole, UK
Bacto agar	BD, Oxford, UK
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Poole, UK
Brucella broth	Fluka, Gillingham, UK
Campylobacter selective supplement (Skirrow)	Oxoid Basingstoke, UK
Columbia agar	Fluka, Gillingham, UK
Dulbeccos Modified Eagles Medium (DMEM)	Sigma-Aldrich, Poole, UK
DH5- Alpha cells	Invitrogen, Paisley UK
Ethidium Bromide	Promega, Southampton, UK
Foetal Bovine serum	Sigma-Aldrich, Poole, UK
Genelute gel extraction kit	Sigma-Aldrich, Poole, UK
Glycerol	VWR, Lutterworth, UK
Horse blood in Alsevers	TCS Biosciences, Botolph, Claydon, UK

1kb ladder	Invitrogen, Paisley, UK
Kanamycin	Gibco/Invitrogen, Paisley, UK
Luria Bertani agar	Merck, Hoddesdon, UK
Luria Bertani broth	Difco, Northampton, UK
Lysozyme	Sigma-Aldrich, Poole, UK
β 2 Mercaptoethanol	Stratagene, Agilent Technologies, Santa Clara, CA, USA
Minelute PCR purification kit	Qiagen, Crawley, UK
Non-essential amino acids	Sigma-Aldrich, Poole, UK
Oligonucleotide primers	Sigma-Aldrich, Poole, UK
Pen-strep solution	Sigma-Aldrich, Poole, UK
Phosphate buffered saline	Sigma-Aldrich, Poole, UK
pGEM-T easy vector	Promega, Southampton, UK
Restriction endonucleases	Promega, Southampton, UK
Soc broth	Fluka, Gillingham, UK
SCS110 competent cells	Stratagene, Agilent Technologies, Santa Clara, CA, USA
T4 DNA ligase	Promega, Southampton, UK
T4 DNA ligase buffer	Promega, Southampton, UK
Triton x-100	Sigma-Aldrich, Poole, UK
0.25% Trypsin-EDTA	Sigma-Aldrich, Poole, UK
Qiaquick PCR purification kit	Qiagen, Crawley, UK
Qiaquick gel extraction kit	Qiagen, Crawley, UK
Qiaprep spin miniprep kit	Qiagen, Crawley, UK
Puregene DNA purification system	Gentra systems Inc. Flowgene, Lichfield, UK
XL2-Blue MRF cells	Stratagene, Agilent Technologies, Santa Clara, CA, USA

Gel loading buffer

100% (v/v) glycerol	10 ml
20% (v/v) SDS	500 µl
5% 9w/v) Bromothenol blue	100 µl
EDTA (0.5M)	100 µl
Sterilised Milli Q water	to 20 ml

dNTP 1.25mM stock (1ml total volume)

dATP (100mM)	12.5 µl
dTTP (100mM)	12.5 µl
dGTP (100mM)	12.5 µl
dCTP (100mM)	12.5 µl
Sterilised Milli-Q water	to 950 µl

PCR pre-mix (1ml total volume)

10X buffer	100 µl
dNTPS (1.25mM)	168 µl
MgCl ₂ (25mM)	60 µl
Taq polymerase (5 U/µl)	6 µl
Sterilised Milli Q water	to 1 ml

Ice cold wash buffer 100ml

100% (v/v) Glycerol	15 ml
10% (w/v) Sucrose	10 ml
Sterilised Milli Q water	to 100 ml

Soc broth

Tryptone (20g/l)

Sodium chloride (10nM)

Potassium chloride (2.5mM)

Yeast extract (5g/l)

Magnesium chloride (10mM)

Magnesium sulphate (10mM)

Glucose (20mM)

To a final pH 7

8. References

- AL-SALLOOM, F. S., AL MAHMEED, A., ISMAEEL, A., BOTTA, G. A. & BAKHIET, M. 2003. Campylobacter-stimulated INT407 cells produce dissociated cytokine profiles. *J Infect*, 47, 217-24.
- ALEMKA, A., NOTHAFT, H., ZHENG, J. & SZYMANSKI, C. M. 2013. N-glycosylation of Campylobacter jejuni surface proteins promotes bacterial fitness. *Infect Immun*, 81, 1674-82.
- ALEXANDER, J., SATOSKAR, A. R. & RUSSELL, D. G. 1999. Leishmania species: models of intracellular parasitism. *J Cell Sci*, 112 Pt 18, 2993-3002.
- ALI, I. U., MAUTNER, V., LANZA, R. & HYNES, R. O. 1977. Restoration of normal morphology, adhesion and cytoskeleton in transformed cells by addition of a transformation-sensitive surface protein. *Cell*, 11, 115-26.
- ALM, R. A., GUERRY, P., POWER, M. E., LIOR, H. & TRUST, T. J. 1991. Analysis of the role of flagella in the heat-labile Lior serotyping scheme of thermophilic Campylobacters by mutant allele exchange. *J Clin Microbiol*, 29, 2438-45.
- ALM, R. A., GUERRY, P., POWER, M. E. & TRUST, T. J. 1992. Variation in antigenicity and molecular weight of Campylobacter coli VC167 flagellin in different genetic backgrounds. *J Bacteriol*, 174, 4230-8.
- ALMEIDA, R. A., MATTHEWS, K. R., CIFRIAN, E., GUIDRY, A. J. & OLIVER, S. P. 1996. Staphylococcus aureus invasion of bovine mammary epithelial cells. *J Dairy Sci*, 79, 1021-6.
- ALRUTZ, M. A. & ISBERG, R. R. 1998. Involvement of focal adhesion kinase in invasin-mediated uptake. *Proc Natl Acad Sci U S A*, 95, 13658-63.
- ALTER, T., BERESWILL, S., GLUNDER, G., HAAG, L. M., HANEL, I., HEIMESAAT, M. M., LUGERT, R., RAUTENSCHLEIN, S., WEBER, R. M., ZAUTNER, A. E. & GROSS, U. 2011. [Campylobacteriosis of man : livestock as reservoir for Campylobacter species]. *Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz*, 54, 728-34.
- ANDERSEN-NISSEN, E., SMITH, K. D., STROBE, K. L., BARRETT, S. L., COOKSON, B. T., LOGAN, S. M. & ADEREM, A. 2005. Evasion of Toll-like receptor 5 by flagellated bacteria. *Proc Natl Acad Sci U S A*, 102, 9247-52.
- ANGATA, T. & VARKI, A. 2002. Chemical diversity in the sialic acids and related alpha-keto acids: an evolutionary perspective. *Chem Rev*, 102, 439-69.
- ANTHONY, L. D., BURKE, R. D. & NANO, F. E. 1991. Growth of Francisella spp. in rodent macrophages. *Infect Immun*, 59, 3291-6.
- ARCARO, A. & WYMANN, M. P. 1993. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem J*, 296 (Pt 2), 297-301.
- BABAKHANI, F. K., BRADLEY, G. A. & JOENS, L. A. 1993. Newborn piglet model for campylobacteriosis. *Infect Immun*, 61, 3466-75.
- BACHTIAR, B. M., COLOE, P. J. & FRY, B. N. 2007. Knockout mutagenesis of the kpsE gene of Campylobacter jejuni 81116 and its involvement in bacterium-host interactions. *FEMS Immunol Med Microbiol*, 49, 149-54.
- BACKERT, S., BOEHM, M., WESSLER, S. & TEGTMEYER, N. 2013. Transmigration route of Campylobacter jejuni across polarized intestinal epithelial cells: paracellular, transcellular or both? *Cell Commun Signal*, 11, 72.

- BACKERT, S. & HOFREUTER, D. 2013. Molecular methods to investigate adhesion, transmigration, invasion and intracellular survival of the foodborne pathogen *Campylobacter jejuni*. *Journal of Microbiological Methods*, 95, 8-23.
- BACKHED, F. & HORNEF, M. 2003. Toll-like receptor 4-mediated signaling by epithelial surfaces: necessity or threat? *Microbes Infect*, 5, 951-9.
- BACON, D. J., ALM, R. A., BURR, D. H., HU, L., KOPECKO, D. J., EWING, C. P., TRUST, T. J. & GUERRY, P. 2000. Involvement of a plasmid in virulence of *Campylobacter jejuni* 81-176. *Infect Immun*, 68, 4384-90.
- BACON, D. J., ALM, R. A., HU, L., HICKEY, T. E., EWING, C. P., BATCHELOR, R. A., TRUST, T. J. & GUERRY, P. 2002. DNA sequence and mutational analyses of the pVir plasmid of *Campylobacter jejuni* 81-176. *Infect Immun*, 70, 6242-50.
- BACON, D. J., SZYMANSKI, C. M., BURR, D. H., SILVER, R. P., ALM, R. A. & GUERRY, P. 2001. A phase-variable capsule is involved in virulence of *Campylobacter jejuni* 81-176. *Mol Microbiol*, 40, 769-77.
- BADGER, J. L., STINS, M. F. & KIM, K. S. 1999. *Citrobacter freundii* invades and replicates in human brain microvascular endothelial cells. *Infect Immun*, 67, 4208-15.
- BALABAN, M., JOSLIN, S. N. & HENDRIXSON, D. R. 2009. FlhF and its GTPase activity are required for distinct processes in flagellar gene regulation and biosynthesis in *Campylobacter jejuni*. *J Bacteriol*, 191, 6602-11.
- BALAZS, M., MARTIN, F., ZHOU, T. & KEARNEY, J. 2002. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. *Immunity*, 17, 341-52.
- BALLESTREM, C., WEHRLE-HALLER, B., HINZ, B. & IMHOF, B. A. 2000. Actin-dependent lamellipodia formation and microtubule-dependent tail retraction control-directed cell migration. *Mol Biol Cell*, 11, 2999-3012.
- BAQAR, S., BOURGEOIS, A. L., APPLEBEE, L. A., MOURAD, A. S., KLEINOSKY, M. T., MOHRAN, Z. & MURPHY, J. R. 1996. Murine intranasal challenge model for the study of *Campylobacter* pathogenesis and immunity. *Infect Immun*, 64, 4933-9.
- BARRERO-TOBON, A. M. & HENDRIXSON, D. R. 2012. Identification and analysis of flagellar coexpressed determinants (Feds) of *Campylobacter jejuni* involved in colonization. *Mol Microbiol*, 84, 352-69.
- BAUMAN, S. J. & KUEHN, M. J. 2006. Purification of outer membrane vesicles from *Pseudomonas aeruginosa* and their activation of an IL-8 response. *Microbes Infect*, 8, 2400-8.
- BEERY, J. T., HUGDAHL, M. B. & DOYLE, M. P. 1988. Colonization of gastrointestinal tracts of chicks by *Campylobacter jejuni*. *Appl Environ Microbiol*, 54, 2365-70.
- BELL, J. A. & MANNING, D. D. 1991. Evaluation of *Campylobacter jejuni* colonization of the domestic ferret intestine as a model of proliferative colitis. *Am J Vet Res*, 52, 826-32.
- BERGIN, D., REEVES, E. P., RENWICK, J., WIENTJES, F. B. & KAVANAGH, K. 2005. Superoxide production in *Galleria mellonella* hemocytes: identification of proteins homologous to the NADPH oxidase complex of human neutrophils. *Infect Immun*, 73, 4161-70.
- BEVERIDGE, T. J. 1999. Structures of gram-negative cell walls and their derived membrane vesicles. *J Bacteriol*, 181, 4725-33.

- BEVINS, C. L., MARTIN-PORTER, E. & GANZ, T. 1999. Defensins and innate host defence of the gastrointestinal tract. *Gut*, 45, 911-5.
- BEWLEY, M. A., MARRIOTT, H. M., TULONE, C., FRANCIS, S. E., MITCHELL, T. J., READ, R. C., CHAIN, B., KROEMER, G., WHYTE, M. K. & DOCKRELL, D. H. 2011. A cardinal role for cathepsin d in co-ordinating the host-mediated apoptosis of macrophages and killing of pneumococci. *PLoS Pathog*, 7, e1001262.
- BISWAS, D., ITOH, K. & SASAKAWA, C. 2000. Uptake pathways of clinical and healthy animal isolates of *Campylobacter jejuni* into INT-407 cells. *FEMS Immunol Med Microbiol*, 29, 203-11.
- BISWAS, D., ITOH, K. & SASAKAWA, C. 2003. Role of microfilaments and microtubules in the invasion of INT-407 cells by *Campylobacter jejuni*. *Microbiol Immunol*, 47, 469-73.
- BISWAS, D., NIWA, H. & ITOH, K. 2004. Infection with *Campylobacter jejuni* induces tyrosine-phosphorylated proteins into INT-407 cells. *Microbiol Immunol*, 48, 221-8.
- BLACK, R. E., LEVINE, M. M., CLEMENTS, M. L., HUGHES, T. P. & BLASER, M. J. 1988. Experimental *Campylobacter jejuni* infection in humans. *J Infect Dis*, 157, 472-9.
- BLACK, R. E., PERLMAN, D., CLEMENTS, M. L., LEVINE, M. M., BLASER, M. J. 1992. Human volunteer studies with *Campylobacter jejuni*. In: NACHAMKIN, I., BLASER, M. J. AND TOMPKINS, L. S. (ed.) *In Campylobacter jejuni: Current Status and Future Trends* Washington: ASM Press.
- BLASER, M. J. 1997. Epidemiologic and clinical features of *Campylobacter jejuni* infections. *J Infect Dis*, 176 Suppl 2, S103-5.
- BLASER, M. J., BERKOWITZ, I. D., LAFORCE, F. M., CRAVENS, J., RELLER, L. B. & WANG, W. L. 1979. *Campylobacter* enteritis: clinical and epidemiologic features. *Ann Intern Med*, 91, 179-85.
- BLASER, M. J., DUNCAN, D. J., WARREN, G. H. & WANG, W. L. 1983a. Experimental *Campylobacter jejuni* infection of adult mice. *Infect Immun*, 39, 908-16.
- BLASER, M. J., HOPKINS, J. A., BERKA, R. M., VASIL, M. L. & WANG, W. L. 1983b. Identification and characterization of *Campylobacter jejuni* outer membrane proteins. *Infect Immun*, 42, 276-84.
- BLASER, M. J., SMITH, P. F. & KOHLER, P. F. 1985. Susceptibility of *Campylobacter* isolates to the bactericidal activity of human serum. *J Infect Dis*, 151, 227-35.
- BLAUT, M. & CLAVEL, T. 2007. Metabolic diversity of the intestinal microbiota: implications for health and disease. *J Nutr*, 137, 751s-5s.
- BOEHM, M., HOY, B., ROHDE, M., TEGTMEYER, N., BAEK, K. T., OYARZABAL, O. A., BRONDSTED, L., WESSLER, S. & BACKERT, S. 2012. Rapid paracellular transmigration of *Campylobacter jejuni* across polarized epithelial cells without affecting TER: role of proteolytic-active HtrA cleaving E-cadherin but not fibronectin. *Gut Pathog*, 4, 3.
- BOKKENHEUSER, V. 1970. *Vibrio fetus* infection in man. I. Ten new cases and some epidemiologic observations. *Am J Epidemiol*, 91, 400-9.
- BOLAND, T., LATOUR, R. & STUTZENBERGER, F. 2000. Molecular Basis of Bacterial Adhesion. In: AN, Y. & FRIEDMAN, R. (eds.) *Handbook of Bacterial Adhesion*. Humana Press.

- BOLL, J. M. & HENDRIXSON, D. R. 2011. A specificity determinant for phosphorylation in a response regulator prevents in vivo cross-talk and modification by acetyl phosphate. *Proc Natl Acad Sci U S A*, 108, 20160-5.
- BOMBERGER, J. M., MACEACHRAN, D. P., COUTERMARSH, B. A., YE, S., O'TOOLE, G. A. & STANTON, B. A. 2009. Long-distance delivery of bacterial virulence factors by *Pseudomonas aeruginosa* outer membrane vesicles. *PLoS Pathog*, 5, e1000382.
- BOUWMAN, L. I., NIEWOLD, P. & VAN PUTTEN, J. P. 2013. Basolateral invasion and trafficking of *Campylobacter jejuni* in polarized epithelial cells. *PLoS One*, 8, e54759.
- BOUWMAN LI, V. P. J. 2012. Biology of *Campylobacter* Infection. In: SM, F. (ed.) *Foodborne and Waterborne Bacterial Pathogens*. Norfolk, UK: Caister Academic Press.
- BOYA, P., ANDREAU, K., PONCET, D., ZAMZAMI, N., PERFETTINI, J. L., METIVIER, D., OJCIUS, D. M., JAATTELA, M. & KROEMER, G. 2003. Lysosomal membrane permeabilization induces cell death in a mitochondrion-dependent fashion. *J Exp Med*, 197, 1323-34.
- BRADLEY, J. R., JOHNSON, D. R. & POBER, J. S. 1993. Four different classes of inhibitors of receptor-mediated endocytosis decrease tumor necrosis factor-induced gene expression in human endothelial cells. *J Immunol*, 150, 5544-55.
- BRAS, A. M. & KETLEY, J. M. 1999. Transcellular translocation of *Campylobacter jejuni* across human polarised epithelial monolayers. *FEMS Microbiol Lett*, 179, 209-15.
- BRENER, Z. 1973. Biology of *Trypanosoma cruzi*. *Annu Rev Microbiol*, 27, 347-82.
- BROUSSARD, J. A., WEBB, D. J. & KAVERINA, I. 2008. Asymmetric focal adhesion disassembly in motile cells. *Curr Opin Cell Biol*, 20, 85-90.
- BROWN, D. A. & LONDON, E. 1998. Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol*, 14, 111-36.
- BROWN, S. S. & SPUDICH, J. A. 1979. Cytochalasin inhibits the rate of elongation of actin filament fragments. *J Cell Biol*, 83, 657-62.
- BUELOW, D., CHRISTENSEN, J., NEAL-MCKINNEY, J. & KONKEL, M. 2011a. *Campylobacter jejuni* survival within human epithelial cells is enhanced by the secreted protein CiaI. *Mol Microbiol*, 80, 1296 - 1312.
- BUELOW, D. R., CHRISTENSEN, J. E., NEAL-MCKINNEY, J. M. & KONKEL, M. E. 2011b. *Campylobacter jejuni* survival within human epithelial cells is enhanced by the secreted protein CiaI. *Mol Microbiol*, 80, 1296-312.
- BUTZLER, J. P. 2004. *Campylobacter*, from obscurity to celebrity. *Clin Microbiol Infect*, 10, 868-76.
- BUTZLER, J. P., DEKEYSER, P., DETRAIN, M. & DEHAEN, F. 1973. Related vibrio in stools. *J Pediatr*, 82, 493-5.
- CARRILLO, C. D., TABOADA, E., NASH, J. H., LANTHIER, P., KELLY, J., LAU, P. C., VERHULP, R., MYKYTCZUK, O., SY, J., FINDLAY, W. A., AMOAKO, K., GOMIS, S., WILLSON, P., AUSTIN, J. W., POTTER, A., BABIUK, L., ALLAN, B. & SZYMANSKI, C. M. 2004. Genome-wide expression analyses of *Campylobacter jejuni* NCTC11168 reveals coordinate regulation of motility and virulence by *flhA*. *J Biol Chem*, 279, 20327-38.
- CARSONS, S. E. 1989. *Fibronectin in Health and Disease*, Florida, CRC Press Inc.
- CASEY, J. L., COLEY, A. M., TILLEY, L. M. & FOLEY, M. 2000. Green fluorescent antibodies: novel in vitro tools. *Protein Engineering*, 13, 445-452.

- CEBRA, J. J. 1999. Influences of microbiota on intestinal immune system development. *Am J Clin Nutr*, 69, 1046S-1051S.
- CHALFIE, M., TU, Y., EUSKIRCHEN, G., WARD, W. W. & PRASHER, D. C. 1994. Green fluorescent protein as a marker for gene expression. *Science*, 263, 802-5.
- CHAMPION, O. L., COOPER, I. A., JAMES, S. L., FORD, D., KARLYSHEV, A., WREN, B. W., DUFFIELD, M., OYSTON, P. C. & TITBALL, R. W. 2009. *Galleria mellonella* as an alternative infection model for *Yersinia pseudotuberculosis*. *Microbiology*, 155, 1516-22.
- CHAMPION, O. L., KARLYSHEV, A. V., SENIOR, N. J., WOODWARD, M., LA RAGIONE, R., HOWARD, S. L., WREN, B. W. & TITBALL, R. W. 2010. Insect infection model for *Campylobacter jejuni* reveals that O-methyl phosphoramidate has insecticidal activity. *J Infect Dis*, 201, 776-82.
- CHAMPION, O. L., VALDEZ, Y., THORSON, L., GUTTMAN, J. A., MENENDEZ, A., GAYNOR, E. C. & FINLAY, B. B. 2008. A murine intraperitoneal infection model reveals that host resistance to *Campylobacter jejuni* is Nrp1 dependent. *Microbes Infect*, 10, 922-7.
- CHANG, C. & MILLER, J. F. 2006. *Campylobacter jejuni* colonization of mice with limited enteric flora. *Infect Immun*, 74, 5261-71.
- CHANG, L. & GOLDMAN, R. D. 2004. Intermediate filaments mediate cytoskeletal crosstalk. *Nat Rev Mol Cell Biol*, 5, 601-13.
- CHART, H., FROST, J. A., OZA, A., THWAITES, R., GILLANDERS, S. & ROWE, B. 1996. Heat-stable serotyping antigens expressed by strains of *Campylobacter jejuni* are probably capsular and not long-chain lipopolysaccharide. *J Appl Bacteriol*, 81, 635-40.
- CHATTERJEE, S. N. & DAS, J. 1967. Electron microscopic observations on the excretion of cell-wall material by *Vibrio cholerae*. *J Gen Microbiol*, 49, 1-11.
- CHEN, M. L., GE, Z., FOX, J. G. & SCHAUER, D. B. 2006. Disruption of tight junctions and induction of proinflammatory cytokine responses in colonic epithelial cells by *Campylobacter jejuni*. *Infect Immun*, 74, 6581-9.
- CHEVANCE, F. F. & HUGHES, K. T. 2008. Coordinating assembly of a bacterial macromolecular machine. *Nat Rev Microbiol*, 6, 455-65.
- CHICHLOWSKI, M. & HALE, L. P. 2008. Bacterial-mucosal interactions in inflammatory bowel disease: an alliance gone bad. *Am J Physiol Gastrointest Liver Physiol*, 295, G1139-49.
- CHIU, C. P. C., WATTS, A. G., LAIRSON, L. L., GILBERT, M., LIM, D., WAKARCHUK, W. W., WITHERS, S. G. & STRYNADKA, N. C. J. 2004. Structural analysis of the sialyltransferase CstII from *Campylobacter jejuni* in complex with a substrate analog. *Nat Struct Mol Biol*, 11, 163-170.
- CHRISTENSEN, J. E., PACHECO, S. A. & KONKEL, M. E. 2009. Identification of a *Campylobacter jejuni*-secreted protein required for maximal invasion of host cells. *Mol Microbiol*, 73, 650-62.
- CLAUSEN, J. D., CHRISTIANSEN, G., HOLST, H. U. & BIRKELUND, S. 1997. *Chlamydia trachomatis* utilizes the host cell microtubule network during early events of infection. *Mol Microbiol*, 25, 441-9.
- CLENCH, M. H. M., J. R. 1995. The avian cecum: a review. *The Wilson Bulletin*, 107, 93-121.
- CLERC, P. & SANSONETTI, P. J. 1987. Entry of *Shigella flexneri* into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. *Infect Immun*, 55, 2681-8.

- COKER, A. O., ISOKPEHI, R. D., THOMAS, B. N., AMISU, K. O. & OBI, C. L. 2002. Human campylobacteriosis in developing countries. *Emerg Infect Dis*, 8, 237-44.
- CONNER, S. D. & SCHMID, S. L. 2003. Regulated portals of entry into the cell. *Nature*, 422, 37-44.
- COOKE, M. L. 2010. Causes and Management of Diarrhoea in Children in a Clinical Setting. *The South African Journal of Clinical Nutrition*.
- COOPER, G. 2000. Structure and Organization of Actin Filaments. *The Cell: A Molecular Approach. 2nd edition*. 2nd edition ed. Sunderland (MA): Sinauer Associates.
- COOPER, I. A. & SLEE, K. J. 1971. Human infection by *Vibrio fetus*. *Med J Aust*, 1, 1263-7.
- CORCIONIVOSCHI, N., CLYNE, M., LYONS, A., ELMI, A., GUNDOGDU, O., WREN, B. W., DORRELL, N., KARLYSHEV, A. V. & BOURKE, B. 2009. *Campylobacter jejuni* cocultured with epithelial cells reduces surface capsular polysaccharide expression. *Infect Immun*, 77, 1959-67.
- CORNELIS, G. R. 2006. The type III secretion injectisome. *Nat Rev Microbiol*, 4, 811-25.
- COSSART, P. 1995. Actin-based bacterial motility. *Curr Opin Cell Biol*, 7, 94-101.
- COSSART, P. 1997. Host/pathogen interactions. Subversion of the mammalian cell cytoskeleton by invasive bacteria. *J Clin Invest*, 99, 2307-11.
- COSSART, P. & SANSONETTI, P. J. 2004. Bacterial invasion: the paradigms of enteroinvasive pathogens. *Science*, 304, 242-8.
- COTTET, S., CORTHESEY-THEULAZ, I., SPERTINI, F. & CORTHESEY, B. 2002a. Microaerophilic conditions permit to mimic in vitro events occurring during in vivo *Helicobacter pylori* infection and to identify Rho/Ras-associated proteins in cellular signaling. *J Biol Chem*, 277, 33978-86.
- COTTET, S., CORTHESEY-THEULAZ, I., SPERTINI, F. & CORTHESEY, B. 2002b. Microaerophilic Conditions Permit to Mimic in Vitro Events Occurring during in Vivo *Helicobacter pylori* Infection and to Identify Rho/Ras-associated Proteins in Cellular Signaling. *Journal of Biological Chemistry*, 277, 33978-33986.
- COVACCI, A., CENSINI, S., BUGNOLI, M., PETRACCA, R., BURRONI, D., MACCHIA, G., MASSONE, A., PAPINI, E., XIANG, Z., FIGURA, N. & ET AL. 1993. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. *Proc Natl Acad Sci U S A*, 90, 5791-5.
- CRAXTON, A., MAGALETTI, D., RYAN, E. J. & CLARK, E. A. 2003. Macrophage- and dendritic cell-dependent regulation of human B-cell proliferation requires the TNF family ligand BAFF. *Blood*, 101, 4464-71.
- CUBITT, A. B., HEIM, R., ADAMS, S. R., BOYD, A. E., GROSS, L. A. & TSIEN, R. Y. 1995. Understanding, improving and using green fluorescent proteins. *Trends Biochem Sci*, 20, 448-55.
- DARBY, C. 2005. Interactions with microbial pathogens. *WormBook*, 1-15.
- DASTI, J. I., TAREEN, A. M., LUGERT, R., ZAUTNER, A. E. & GROSS, U. 2010. *Campylobacter jejuni*: a brief overview on pathogenicity-associated factors and disease-mediating mechanisms. *Int J Med Microbiol*, 300, 205-11.
- DAVIES, P. J., DAVIES, D. R., LEVITZKI, A., MAXFIELD, F. R., MILHAUD, P., WILLINGHAM, M. C. & PASTAN, I. H. 1980. Transglutaminase is essential in receptor-mediated endocytosis of alpha 2-macroglobulin and polypeptide hormones. *Nature*, 283, 162-7.

- DAY, W. A., JR., SAJECKI, J. L., PITTS, T. M. & JOENS, L. A. 2000. Role of catalase in *Campylobacter jejuni* intracellular survival. *Infect Immun*, 68, 6337-45.
- DE MELO, M. A., GABBIANI, G. & PECHERE, J. C. 1989. Cellular events and intracellular survival of *Campylobacter jejuni* during infection of HEP-2 cells. *Infect Immun*, 57, 2214-22.
- DELIE, F. & RUBAS, W. 1997. A human colonic cell line sharing similarities with enterocytes as a model to examine oral absorption: advantages and limitations of the Caco-2 model. *Crit Rev Ther Drug Carrier Syst*, 14, 221-86.
- DESAI, A. & MITCHISON, T. J. 1997. Microtubule polymerization dynamics. *Annu Rev Cell Dev Biol*, 13, 83-117.
- DESCHENES-SIMARD, X., KOTTAKIS, F., MELOCHE, S. & FERBEYRE, G. 2014. ERKs in cancer: friends or foes? *Cancer Res*, 74, 412-9.
- DESVAUX, M., HEBRAUD, M., HENDERSON, I. R. & PALLEN, M. J. 2006. Type III secretion: what's in a name? *Trends Microbiol*, 14, 157-60.
- DHALIWAL, W., BAJAJ-ELLIOTT, M. & KELLY, P. 2003. Intestinal defensin gene expression in human populations. *Mol Immunol*, 40, 469-75.
- DOMMETT, R., ZILBAUER, M., GEORGE, J. T. & BAJAJ-ELLIOTT, M. 2005. Innate immune defence in the human gastrointestinal tract. *Mol Immunol*, 42, 903-12.
- DONNENBERG, M. S., DONOHUE-ROLFE, A. & KEUSCH, G. T. 1990. A comparison of HEP-2 cell invasion by enteropathogenic and enteroinvasive *Escherichia coli*. *FEMS Microbiol Lett*, 57, 83-6.
- DORRELL, N., MANGAN, J. A., LAING, K. G., HINDS, J., LINTON, D., ALGHUSEIN, H., BARRELL, B. G., PARKHILL, J., STOKER, N. G., KARLYSHEV, A. V., BUTCHER, P. D. & WREN, B. W. 2001. Whole genome comparison of *Campylobacter jejuni* human isolates using a low-cost microarray reveals extensive genetic diversity. *Genome Res*, 11, 1706-15.
- DORRELL, N. & WREN, B. W. 2007. The second century of *Campylobacter* research: recent advances, new opportunities and old problems. *Curr Opin Infect Dis*, 20, 514-8.
- DUNCAN, M. J., SHIN, J. S. & ABRAHAM, S. N. 2002. Microbial entry through caveolae: variations on a theme. *Cell Microbiol*, 4, 783-91.
- DUNN, B. E., BLASER, M. J. & SNYDER, E. L. 1987. Two-dimensional gel electrophoresis and immunoblotting of *Campylobacter* outer membrane proteins. *Infect Immun*, 55, 1564-72.
- DUSTIN, P. 1978. Microtubules. Berlin: Springer-Verlag.
- EBERL, L., SCHULZE, R., AMMENDOLA, A., GEISENBERGER, O., ERHART, R., STERNBERG, C., MOLIN, S. & AMANN, R. 1997. Use of green fluorescent protein as a marker for ecological studies of activated sludge communities. *FEMS Microbiology Letters*, 149, 77-83.
- ECKMANN, L. 2005. Defence molecules in intestinal innate immunity against bacterial infections. *Curr Opin Gastroenterol*, 21, 147-51.
- ELLENBERG, J. L.-S., J 1997. Fluorescence photobleaching techniques In: SPECTOR, D., GOLDMAN, R. & LEINWAND, L. (ed.) *Cells: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- ELLINGTON, J. K., REILLY, S. S., RAMP, W. K., SMELTZER, M. S., KELLAM, J. F. & HUDSON, M. C. 1999. Mechanisms of *Staphylococcus aureus* invasion of cultured osteoblasts. *Microb Pathog*, 26, 317-23.

- ELLIS, T. N. & KUEHN, M. J. 2010. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol Mol Biol Rev*, 74, 81-94.
- ELMI, A., WATSON, E., SANDU, P., GUNDOGDU, O., MILLS, D. C., INGLIS, N. F., MANSON, E., IMRIE, L., BAJAJ-ELLIOTT, M., WREN, B. W., SMITH, D. G. & DORRELL, N. 2012. Campylobacter jejuni outer membrane vesicles play an important role in bacterial interactions with human intestinal epithelial cells. *Infect Immun*, 80, 4089-98.
- ELSINGHORST, E. A. 1994. Measurement of invasion by gentamicin resistance. *Methods Enzymol*, 236, 405-20.
- ENOCKSSON, A., LUNDBERG, J., WEITZBERG, E., NORRBY-TEGLUND, A. & SVENUNGSSON, B. 2004. Rectal nitric oxide gas and stool cytokine levels during the course of infectious gastroenteritis. *Clin Diagn Lab Immunol*, 11, 250-4.
- ERRAMPALLI, D., LEUNG, K., CASSIDY, M. B., KOSTRZYNSKA, M., BLEARS, M., LEE, H. & TREVORS, J. T. 1999. Applications of the green fluorescent protein as a molecular marker in environmental microorganisms. *J Microbiol Methods*, 35, 187-99.
- ETIENNE-MANNEVILLE, S. & HALL, A. 2002. Rho GTPases in cell biology. *Nature*, 420, 629-35.
- EUCKER, T. & KONKEL, M. 2012a. The cooperative action of bacterial fibronectin-binding proteins and secreted proteins promote maximal Campylobacter jejuni invasion of host cells by stimulating membrane ruffling. *Cell Microbiol*, 14, 226 - 238.
- EUCKER, T. P. & KONKEL, M. E. 2012b. The cooperative action of bacterial fibronectin-binding proteins and secreted proteins promote maximal Campylobacter jejuni invasion of host cells by stimulating membrane ruffling. *Cell Microbiol*, 14, 226-38.
- EVEREST, P. H., GOOSSENS, H., BUTZLER, J. P., LLOYD, D., KNUTTON, S., KETLEY, J. M. & WILLIAMS, P. H. 1992. Differentiated Caco-2 cells as a model for enteric invasion by Campylobacter jejuni and C. coli. *J Med Microbiol*, 37, 319-25.
- EVEREST, P. H., GOOSSENS, H., SIBBONS, P., LLOYD, D. R., KNUTTON, S., LEECE, R., KETLEY, J. M. & WILLIAMS, P. H. 1993. Pathological changes in the rabbit ileal loop model caused by Campylobacter jejuni from human colitis. *J Med Microbiol*, 38, 316-21.
- FALK, P., BOREN, T., HASLAM, D. & CAPARON, M. 1994. Bacterial adhesion and colonization assays. *Methods Cell Biol*, 45, 165-92.
- FALKOW, S. 1996. *The evolution of pathogenicity in Escherichia, Shigella, and Salmonella*, Washington DC, ASM press.
- FALKOW, S., ISBERG, R. R. & PORTNOY, D. A. 1992. The interaction of bacteria with mammalian cells. *Annu Rev Cell Biol*, 8, 333-63.
- FASANO, A. 1998. Cellular microbiology: how enteric pathogens socialize with their intestinal host. *J Pediatr Gastroenterol Nutr*, 26, 520-32.
- FAUCHERE, J. L., ROSENAU, A., VERON, M., MOYEN, E. N., RICHARD, S. & PFISTER, A. 1986. Association with HeLa cells of Campylobacter jejuni and Campylobacter coli isolated from human feces. *Infect Immun*, 54, 283-7.
- FERNANDEZ-MOREIRA, E., HELBIG, J. H. & SWANSON, M. S. 2006. Membrane vesicles shed by Legionella pneumophila inhibit fusion of phagosomes with lysosomes. *Infect Immun*, 74, 3285-95.

- FERNANDEZ, H. & TRABULSI, L. R. 1995. Invasive and enterotoxic properties in *Campylobacter jejuni* and *Campylobacter coli* strains isolated from humans and animals. *Biol Res*, 28, 205-10.
- FERNANDO, U., BISWAS, D., ALLAN, B., WILLSON, P. & POTTER, A. A. 2007a. Influence of *Campylobacter jejuni* *fliA*, *rpoN* and *flgK* genes on colonization of the chicken gut. *Int J Food Microbiol*, 118, 194-200.
- FERNANDO, U., BISWAS, D., ALLAN, B., WILLSON, P. & POTTER, A. A. 2007b. Influence of *Campylobacter jejuni* *fliA*, *rpoN* and *flgK* genes on colonization of the chicken gut. *International Journal of Food Microbiology*, 118, 194-200.
- FINLAY, B. B. & COSSART, P. 1997. Exploitation of mammalian host cell functions by bacterial pathogens. *Science*, 276, 718-25.
- FINLAY, B. B. & FALKOW, S. 1989. Common themes in microbial pathogenicity. *Microbiol Rev*, 53, 210-30.
- FINLAY, B. B. & FALKOW, S. 1997. Common themes in microbial pathogenicity revisited. *Microbiol Mol Biol Rev*, 61, 136-69.
- FINLAY, B. B., RUSCHKOWSKI, S. & DEDHAR, S. 1991. Cytoskeletal rearrangements accompanying salmonella entry into epithelial cells. *J Cell Sci*, 99 (Pt 2), 283-96.
- FIOCCA, R., NECCHI, V., SOMMI, P., RICCI, V., TELFORD, J., COVER, T. L. & SOLCIA, E. 1999. Release of *Helicobacter pylori* vacuolating cytotoxin by both a specific secretion pathway and budding of outer membrane vesicles. Uptake of released toxin and vesicles by gastric epithelium. *J Pathol*, 188, 220-6.
- FIORENTINO, D. F., BOND, M. W. & MOSMANN, T. R. 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J Exp Med*, 170, 2081-95.
- FLANAGAN, R. C., NEAL-MCKINNEY, J. M., DHILLON, A. S., MILLER, W. G. & KONKEL, M. E. 2009. Examination of *Campylobacter jejuni* putative adhesins leads to the identification of a new protein, designated FlpA, required for chicken colonization. *Infect Immun*, 77, 2399-407.
- FOX, J. G., ROGERS, A. B., WHARY, M. T., GE, Z., TAYLOR, N. S., XU, S., HORWITZ, B. H. & ERDMAN, S. E. 2004. Gastroenteritis in NF-kappaB-deficient mice is produced with wild-type *Campylobacter jejuni* but not with *C. jejuni* lacking cytolethal distending toxin despite persistent colonization with both strains. *Infect Immun*, 72, 1116-25.
- FRIEDMAN C. R., N. J., WEGENER H. C., TAUXE R. V. 2000. "Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations," in *Campylobacter*, Washington, DC, ASM Press.
- FRIIS, L. M., PIN, C., PEARSON, B. M. & WELLS, J. M. 2005. In vitro cell culture methods for investigating *Campylobacter* invasion mechanisms. *J Microbiol Methods*, 61, 145-60.
- FROMAN, G., SWITALSKI, L. M., FARIS, A., WADSTROM, T. & HOOK, M. 1984. Binding of *Escherichia coli* to fibronectin. A mechanism of tissue adherence. *J Biol Chem*, 259, 14899-905.
- FRY, M. J. 1994. Structure, regulation and function of phosphoinositide 3-kinases. *Biochim Biophys Acta*, 1226, 237-68.
- GAGE, D. J., BOBO, T. & LONG, S. R. 1996. Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). *J Bacteriol*, 178, 7159-66.

- GAILLARD, J. L. & FINLAY, B. B. 1996. Effect of cell polarization and differentiation on entry of *Listeria monocytogenes* into the enterocyte-like Caco-2 cell line. *Infect Immun*, 64, 1299-308.
- GANKEMA, H., WENSINK, J., GUINEE, P. A., JANSEN, W. H. & WITHOLT, B. 1980. Some characteristics of the outer membrane material released by growing enterotoxigenic *Escherichia coli*. *Infect Immun*, 29, 704-13.
- GAO, R. & STOCK, A. M. 2009. Biological insights from structures of two-component proteins. *Annu Rev Microbiol*, 63, 133-54.
- GARCIA-DEL PORTILLO, F. & FINLAY, B. B. 1995. The varied lifestyles of intracellular pathogens within eukaryotic vacuolar compartments. *Trends Microbiol*, 3, 373-80.
- GARCIA-DEL PORTILLO, F., STEIN, M. A. & FINLAY, B. B. 1997. Release of lipopolysaccharide from intracellular compartments containing *Salmonella typhimurium* to vesicles of the host epithelial cell. *Infect Immun*, 65, 24-34.
- GARCIA RODRIGUEZ, L. A., RUIGOMEZ, A. & PANES, J. 2006. Acute gastroenteritis is followed by an increased risk of inflammatory bowel disease. *Gastroenterology*, 130, 1588-94.
- GAUTHIER, A. & FINLAY, B. B. 1998. Protein translocation: delivering virulence into the host cell. *Curr Biol*, 8, R768-70.
- GAYNOR, E. C., CAWTHRAW, S., MANNING, G., MACKICHAN, J. K., FALKOW, S. & NEWELL, D. G. 2004. The genome-sequenced variant of *Campylobacter jejuni* NCTC 11168 and the original clonal clinical isolate differ markedly in colonization, gene expression, and virulence-associated phenotypes. *J Bacteriol*, 186, 503-17.
- GEORGE, A. M. & JONES, P. M. 2012. Perspectives on the structure-function of ABC transporters: the Switch and Constant Contact models. *Prog Biophys Mol Biol*, 109, 95-107.
- GILBERT, M., BRISSON, J. R., KARWASKI, M. F., MICHNIEWICZ, J., CUNNINGHAM, A. M., WU, Y., YOUNG, N. M. & WAKARCHUK, W. W. 2000. Biosynthesis of ganglioside mimics in *Campylobacter jejuni* OH4384. Identification of the glycosyltransferase genes, enzymatic synthesis of model compounds, and characterization of nanomole amounts by 600-mhz (1)h and (13)c NMR analysis. *J Biol Chem*, 275, 3896-906.
- GILBERT, M., GODSCHALK, P. C., KARWASKI, M. F., ANG, C. W., VAN BELKUM, A., LI, J., WAKARCHUK, W. W. & ENDTZ, H. P. 2004. Evidence for acquisition of the lipooligosaccharide biosynthesis locus in *Campylobacter jejuni* GB11, a strain isolated from a patient with Guillain-Barre syndrome, by horizontal exchange. *Infect Immun*, 72, 1162-5.
- GILBREATH, J. J., CODY, W. L., MERRELL, D. S. & HENDRIXSON, D. R. 2011. Change is good: variations in common biological mechanisms in the epsilonproteobacterial genera *Campylobacter* and *Helicobacter*. *Microbiol Mol Biol Rev*, 75, 84-132.
- GODSCHALK, P. C., HEIKEMA, A. P., GILBERT, M., KOMAGAMINE, T., ANG, C. W., GLERUM, J., BROCHU, D., LI, J., YUKI, N., JACOBS, B. C., VAN BELKUM, A. & ENDTZ, H. P. 2004. The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain-Barre syndrome. *J Clin Invest*, 114, 1659-65.
- GOEBEL, W. & KUHN, M. 2000. Bacterial replication in the host cell cytosol. *Curr Opin Microbiol*, 3, 49-53.

- GONI, F. M. 2014. The basic structure and dynamics of cell membranes: An update of the Singer-Nicolson model. *Biochim Biophys Acta*.
- GOON, S., EWING, C. P., LORENZO, M., PATTARINI, D., MAJAM, G. & GUERRY, P. 2006. A sigma28-regulated nonflagella gene contributes to virulence of *Campylobacter jejuni* 81-176. *Infect Immun*, 74, 769-72.
- GOON, S., KELLY, J. F., LOGAN, S. M., EWING, C. P. & GUERRY, P. 2003. Pseudaminic acid, the major modification on *Campylobacter* flagellin, is synthesized via the Cj1293 gene. *Mol Microbiol*, 50, 659-71.
- GOOSNEY, D. L., KNOEHEL, D. G. & FINLAY, B. B. 1999. Enteropathogenic *E. coli*, *Salmonella*, and *Shigella*: masters of host cell cytoskeletal exploitation. *Emerg Infect Dis*, 5, 216-23.
- GRAHAM, L. L. 2002. *Campylobacter fetus* adheres to and enters INT 407 cells. *Can J Microbiol*, 48, 995-1007.
- GRANGER, E., MCNEE, G., ALLAN, V. & WOODMAN, P. 2014. The role of the cytoskeleton and molecular motors in endosomal dynamics. *Seminars in Cell & Developmental Biology*, 31, 20-29.
- GRANT, C. C., KONKEL, M. E., CIEPLAK, W., JR. & TOMPKINS, L. S. 1993. Role of flagella in adherence, internalization, and translocation of *Campylobacter jejuni* in nonpolarized and polarized epithelial cell cultures. *Infect Immun*, 61, 1764-71.
- GRANT, S. R., FISHER, E. J., CHANG, J. H., MOLE, B. M. & DANGL, J. L. 2006. Subterfuge and manipulation: type III effector proteins of phytopathogenic bacteria. *Annu Rev Microbiol*, 60, 425-49.
- GRASS, S., LICHTI, C. F., TOWNSEND, R. R., GROSS, J. & ST GEME, J. W., 3RD 2010. The *Haemophilus influenzae* HMW1C protein is a glycosyltransferase that transfers hexose residues to asparagine sites in the HMW1 adhesin. *PLoS Pathog*, 6, e1000919.
- GREEN, B. T. & BROWN, D. R. 2006. Differential effects of clathrin and actin inhibitors on internalization of *Escherichia coli* and *Salmonella choleraesuis* in porcine jejunal Peyer's patches. *Vet Microbiol*, 113, 117-22.
- GRUBER, H. J., HAHN, C. D., KADA, G., RIENER, C. K., HARMS, G. S., AHRER, W., DAX, T. G. & KNAUS, H. G. 2000. Anomalous fluorescence enhancement of Cy3 and cy3.5 versus anomalous fluorescence loss of Cy5 and Cy7 upon covalent linking to IgG and noncovalent binding to avidin. *Bioconjug Chem*, 11, 696-704.
- GRUENBERG, J. & VAN DER GOOT, F. G. 2006. Mechanisms of pathogen entry through the endosomal compartments. *Nat Rev Mol Cell Biol*, 7, 495-504.
- GRUENHEID, S. & GROS, P. 2000. Genetic susceptibility to intracellular infections: Nramp1, macrophage function and divalent cations transport. *Curr Opin Microbiol*, 3, 43-8.
- GUERRA, L., CORTES-BRATTI, X., GUIDI, R. & FRISAN, T. 2011. The biology of the cytolethal distending toxins. *Toxins (Basel)*, 3, 172-90.
- GUERRY, P. 2007. *Campylobacter* flagella: not just for motility. *Trends Microbiol*, 15, 456-61.
- GUERRY, P., ALM, R. A., POWER, M. E., LOGAN, S. M. & TRUST, T. J. 1991. Role of two flagellin genes in *Campylobacter* motility. *J Bacteriol*, 173, 4757-64.
- GUERRY, P., DOIG, P., ALM, R. A., BURR, D. H., KINSELLA, N. & TRUST, T. J. 1996. Identification and characterization of genes required for post-translational

- modification of *Campylobacter coli* VC167 flagellin. *Mol Microbiol*, 19, 369-78.
- GUERRY, P., EWING, C. P., HICKEY, T. E., PRENDERGAST, M. M. & MORAN, A. P. 2000. Sialylation of lipooligosaccharide cores affects immunogenicity and serum resistance of *Campylobacter jejuni*. *Infect Immun*, 68, 6656-62.
- GUERRY, P., EWING, C. P., SCHIRM, M., LORENZO, M., KELLY, J., PATTARINI, D., MAJAM, G., THIBAUT, P. & LOGAN, S. 2006. Changes in flagellin glycosylation affect *Campylobacter* autoagglutination and virulence. *Mol Microbiol*, 60, 299-311.
- GUERRY, P., POLY, F., RIDDLE, M., MAUE, A. C., CHEN, Y. H. & MONTEIRO, M. A. 2012. *Campylobacter* polysaccharide capsules: virulence and vaccines. *Front Cell Infect Microbiol*, 2, 7.
- GUERRY, P. & SZYMANSKI, C. M. 2008. *Campylobacter* sugars sticking out. *Trends Microbiol*, 16, 428-35.
- GUERRY, P., SZYMANSKI, C. M., PRENDERGAST, M. M., HICKEY, T. E., EWING, C. P., PATTARINI, D. L. & MORAN, A. P. 2002. Phase variation of *Campylobacter jejuni* 81-176 lipooligosaccharide affects ganglioside mimicry and invasiveness in vitro. *Infect Immun*, 70, 787-93.
- GUERRY, P., YAO, R., ALM, R. A., BURR, D. H. & TRUST, T. J. 1994. Systems of experimental genetics for *Campylobacter* species. *Methods Enzymol*, 235, 474-81.
- HADDAD, N., MAILLART, G., GARENAUX, A., JUGIAU, F., FEDERIGHI, M. & CAPPELIER, J. M. 2010. Adhesion ability of *Campylobacter jejuni* to Ht-29 cells increases with the augmentation of oxidant agent concentration. *Curr Microbiol*, 61, 500-5.
- HAMANAKA, Y., NAKASHIMA, M., WADA, A., ITO, M., KURAZONO, H., HOJO, H., NAKAHARA, Y., KOHNO, S., HIRAYAMA, T. & SEKINE, I. 2001. Expression of human beta-defensin 2 (hBD-2) in *Helicobacter pylori* induced gastritis: antibacterial effect of hBD-2 against *Helicobacter pylori*. *Gut*, 49, 481-7.
- HARVEY, P., BATTLE, T. & LEACH, S. 1999. Different invasion phenotypes of *Campylobacter* isolates in Caco-2 cell monolayers. *J Med Microbiol*, 48, 461-9.
- HASSAN, H. M. 1989. Microbial superoxide dismutases. *Adv Genet*, 26, 65-97.
- HASSETT, D. J. & COHEN, M. S. 1989. Bacterial adaptation to oxidative stress: implications for pathogenesis and interaction with phagocytic cells. *Faseb j*, 3, 2574-82.
- HAWKINS, P. T., EGUINO, A., QIU, R. G., STOKOE, D., COOKE, F. T., WALTERS, R., WENNSTROM, S., CLAEISSON-WELSH, L., EVANS, T., SYMONS, M. & ET AL. 1995. PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase. *Curr Biol*, 5, 393-403.
- HEIKEMA, A. P., BERGMAN, M. P., RICHARDS, H., CROCKER, P. R., GILBERT, M., SAMSOM, J. N., VAN WAMEL, W. J., ENDTZ, H. P. & VAN BELKUM, A. 2010. Characterization of the specific interaction between sialoadhesin and sialylated *Campylobacter jejuni* lipooligosaccharides. *Infect Immun*, 78, 3237-46.
- HENDERSON, B., NAIR, S., PALLAS, J. & WILLIAMS, M. A. 2011. Fibronectin: a multidomain host adhesin targeted by bacterial fibronectin-binding proteins. *FEMS Microbiol Rev*, 35, 147-200.

- HENDRIXSON, D. R., AKERLEY, B. J. & DIRITA, V. J. 2001. Transposon mutagenesis of *Campylobacter jejuni* identifies a bipartite energy taxis system required for motility. *Mol Microbiol*, 40, 214-24.
- HENDRIXSON, D. R. & DIRITA, V. J. 2003. Transcription of sigma54-dependent but not sigma28-dependent flagellar genes in *Campylobacter jejuni* is associated with formation of the flagellar secretory apparatus. *Mol Microbiol*, 50, 687-702.
- HENDRIXSON, D. R. & DIRITA, V. J. 2004. Identification of *Campylobacter jejuni* genes involved in commensal colonization of the chick gastrointestinal tract. *Mol Microbiol*, 52, 471-84.
- HERMANSON, G. T. 1996. Bioconjugate Techniques. San Diego: Academic Press.
- HEUSER, J. E. & ANDERSON, R. G. 1989. Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J Cell Biol*, 108, 389-400.
- HEYWOOD, W., HENDERSON, B. & NAIR, S. P. 2005. Cytolethal distending toxin: creating a gap in the cell cycle. *J Med Microbiol*, 54, 207-16.
- HICKEY, T. E., BAQAR, S., BOURGEOIS, A. L., EWING, C. P. & GUERRY, P. 1999. *Campylobacter jejuni*-stimulated secretion of interleukin-8 by INT407 cells. *Infect Immun*, 67, 88-93.
- HICKEY, T. E., MAJAM, G. & GUERRY, P. 2005. Intracellular survival of *Campylobacter jejuni* in human monocytic cells and induction of apoptotic death by cytolethal distending toxin. *Infect Immun*, 73, 5194-7.
- HICKEY, T. E., MCVEIGH, A. L., SCOTT, D. A., MICHIELUTTI, R. E., BIXBY, A., CARROLL, S. A., BOURGEOIS, A. L. & GUERRY, P. 2000. *Campylobacter jejuni* cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. *Infect Immun*, 68, 6535-41.
- HIDALGO, I. J., RAUB, T. J. & BORCHARDT, R. T. 1989. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology*, 96, 736-49.
- HOBBIE, S., CHEN, L. M., DAVIS, R. J. & GALAN, J. E. 1997. Involvement of mitogen-activated protein kinase pathways in the nuclear responses and cytokine production induced by *Salmonella typhimurium* in cultured intestinal epithelial cells. *J Immunol*, 159, 5550-9.
- HOEKSTRA, D., VAN DER LAAN, J. W., DE LEIJ, L. & WITHOLT, B. 1976. Release of outer membrane fragments from normally growing *Escherichia coli*. *Biochim Biophys Acta*, 455, 889-99.
- HOFFMANN, J. A. 2003. The immune response of *Drosophila*. *Nature*, 426, 33-8.
- HOLMES, K. L. & LANTZ, L. M. 2001. Protein labeling with fluorescent probes. *Methods Cell Biol*, 63, 185-204.
- HOOPER, L. V., BRY, L., FALK, P. G. & GORDON, J. I. 1998. Host-microbial symbiosis in the mammalian intestine: exploring an internal ecosystem. *Bioessays*, 20, 336-43.
- HOPKINS, R. S., OLMSTED, R. & ISTRE, G. R. 1984. Endemic *Campylobacter jejuni* infection in Colorado: identified risk factors. *Am J Public Health*, 74, 249-50.
- HORNEF, M. W., WICK, M. J., RHEN, M. & NORMARK, S. 2002. Bacterial strategies for overcoming host innate and adaptive immune responses. *Nat Immunol*, 3, 1033-40.
- HORSTMAN, A. L. & KUEHN, M. J. 2000. Enterotoxigenic *Escherichia coli* secretes active heat-labile enterotoxin via outer membrane vesicles. *J Biol Chem*, 275, 12489-96.

- HOULISTON, R. S., VINOGRADOV, E., DZIECIATKOWSKA, M., LI, J., ST MICHAEL, F., KARWASKI, M. F., BROCHU, D., JARRELL, H. C., PARKER, C. T., YUKI, N., MANDRELL, R. E. & GILBERT, M. 2011. Lipooligosaccharide of *Campylobacter jejuni*: similarity with multiple types of mammalian glycans beyond gangliosides. *J Biol Chem*, 286, 12361-70.
- HOWARD, S. L., JAGANNATHAN, A., SOO, E. C., HUI, J. P., AUBRY, A. J., AHMED, I., KARLYSHEV, A., KELLY, J. F., JONES, M. A., STEVENS, M. P., LOGAN, S. M. & WREN, B. W. 2009. *Campylobacter jejuni* glycosylation island important in cell charge, legionaminic acid biosynthesis, and colonization of chickens. *Infect Immun*, 77, 2544-56.
- HOY, B., GEPPERT, T., BOEHM, M., REISEN, F., PLATTNER, P., GADERMAIER, G., SEWALD, N., FERREIRA, F., BRIZA, P., SCHNEIDER, G., BACKERT, S. & WESSLER, S. 2012. Distinct roles of secreted HtrA proteases from gram-negative pathogens in cleaving the junctional protein and tumor suppressor E-cadherin. *J Biol Chem*, 287, 10115-20.
- HU, L., BRAY, M. D., OSORIO, M. & KOPECKO, D. J. 2006a. *Campylobacter jejuni* induces maturation and cytokine production in human dendritic cells. *Infect Immun*, 74, 2697-705.
- HU, L. & HICKEY, T. E. 2005. *Campylobacter jejuni* induces secretion of proinflammatory chemokines from human intestinal epithelial cells. *Infect Immun*, 73, 4437-40.
- HU, L. & KOPECKO, D. J. 1999. *Campylobacter jejuni* 81-176 associates with microtubules and dynein during invasion of human intestinal cells. *Infect Immun*, 67, 4171-82.
- HU, L., MCDANIEL, J. & KOPECKO, D. 2006b. Signal transduction events involved in human epithelial cell invasion by *Campylobacter jejuni* 81-176. *Microb Pathog*, 40, 91 - 100.
- HU, L., MCDANIEL, J. P. & KOPECKO, D. J. 2006c. Signal transduction events involved in human epithelial cell invasion by *Campylobacter jejuni* 81-176. *Microb Pathog*, 40, 91-100.
- HU, L., TALL, B. D., CURTIS, S. K. & KOPECKO, D. J. 2008. Enhanced microscopic definition of *Campylobacter jejuni* 81-176 adherence to, invasion of, translocation across, and exocytosis from polarized human intestinal Caco-2 cells. *Infect Immun*, 76, 5294-304.
- HUIZINGA, R., EASTON, A. S., DONACHIE, A. M., GUTHRIE, J., VAN RIJS, W., HEIKEMA, A., BOON, L., SAMSOM, J. N., JACOBS, B. C., WILLISON, H. J. & GOODYEAR, C. S. 2012. Sialylation of *Campylobacter jejuni* lipooligosaccharides: impact on phagocytosis and cytokine production in mice. *PLoS One*, 7, e34416.
- HYNES, R. O. 1990. *Fibronectins*, New York, Springer-Verlag.
- INOHARA, CHAMAILLARD, MCDONALD, C. & NUNEZ, G. 2005. NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. *Annu Rev Biochem*, 74, 355-83.
- INOUE, S. & TSUJI, F. I. 1994. Aequorea green fluorescent protein. Expression of the gene and fluorescence characteristics of the recombinant protein. *FEBS Lett*, 341, 277-80.
- ISBERG, R. R. & VAN NHIEU, G. T. 1994. Two mammalian cell internalization strategies used by pathogenic bacteria. *Annu Rev Genet*, 28, 395-422.
- ISLAM, Z., JACOBS, B. C., VAN BELKUM, A., MOHAMMAD, Q. D., ISLAM, M. B., HERBRINK, P., DIORDITSA, S., LUBY, S. P., TALUKDER, K. A. &

- ENDTZ, H. P. 2010. Axonal variant of Guillain-Barre syndrome associated with *Campylobacter* infection in Bangladesh. *Neurology*, 74, 581-7.
- ITZEN, A., BLANKENFELDT, W. & GOODY, R. S. 2011. Adenylation: renaissance of a forgotten post-translational modification. *Trends Biochem Sci*, 36, 221-8.
- JACOBS, B. C., ROTHBARTH, P. H., VAN DER MECHE, F. G., HERBRINK, P., SCHMITZ, P. I., DE KLERK, M. A. & VAN DOORN, P. A. 1998. The spectrum of antecedent infections in Guillain-Barre syndrome: a case-control study. *Neurology*, 51, 1110-5.
- JAFFE, J., NATANSON-YARON, S., CAPARON, M. G. & HANSKI, E. 1996. Protein F2, a novel fibronectin-binding protein from *Streptococcus pyogenes*, possesses two binding domains. *Mol Microbiol*, 21, 373-84.
- JANSSEN, R., KROGFELT, K. A., CAWTHRAW, S. A., VAN PELT, W., WAGENAAR, J. A. & OWEN, R. J. 2008. Host-pathogen interactions in *Campylobacter* infections: the host perspective. *Clin Microbiol Rev*, 21, 505-18.
- JEPSON, M. A., COLLARES-BUZATO, C. B., CLARK, M. A., HIRST, B. H. & SIMMONS, N. L. 1995. Rapid disruption of epithelial barrier function by *Salmonella typhimurium* is associated with structural modification of intercellular junctions. *Infection and Immunity*, 63, 356-359.
- JERVIS, A. J., LANGDON, R., HITCHEN, P., LAWSON, A. J., WOOD, A., FOTHERGILL, J. L., MORRIS, H. R., DELL, A., WREN, B. & LINTON, D. 2010. Characterization of N-linked protein glycosylation in *Helicobacter pullorum*. *J Bacteriol*, 192, 5228-36.
- JIN, S., SONG, Y. C., EMILI, A., SHERMAN, P. M. & CHAN, V. L. 2003. JlpA of *Campylobacter jejuni* interacts with surface-exposed heat shock protein 90alpha and triggers signalling pathways leading to the activation of NF-kappaB and p38 MAP kinase in epithelial cells. *Cell Microbiol*, 5, 165-74.
- JINADASA, R. N., BLOOM, S. E., WEISS, R. S. & DUHAMEL, G. E. 2011. Cytotoxic distending toxin: a conserved bacterial genotoxin that blocks cell cycle progression, leading to apoptosis of a broad range of mammalian cell lineages. *Microbiology*, 157, 1851-75.
- JOHANESSEN, P. A. & DWINELL, M. B. 2006. Flagellin-independent regulation of chemokine host defense in *Campylobacter jejuni*-infected intestinal epithelium. *Infect Immun*, 74, 3437-47.
- JOHNSON, W. M. & LIOR, H. 1988. A new heat-labile cytotoxic distending toxin (CLDT) produced by *Campylobacter* spp. *Microb Pathog*, 4, 115-26.
- JONES, B. D., PATERSON, H. F., HALL, A. & FALKOW, S. 1993. *Salmonella typhimurium* induces membrane ruffling by a growth factor-receptor-independent mechanism. *Proc Natl Acad Sci U S A*, 90, 10390-4.
- JONES, M. A., MARSTON, K. L., WOODALL, C. A., MASKELL, D. J., LINTON, D., KARLYSHEV, A. V., DORRELL, N., WREN, B. W. & BARROW, P. A. 2004. Adaptation of *Campylobacter jejuni* NCTC11168 to high-level colonization of the avian gastrointestinal tract. *Infect Immun*, 72, 3769-76.
- JONES, M. A., TOTEMEYER, S., MASKELL, D. J., BRYANT, C. E. & BARROW, P. A. 2003. Induction of proinflammatory responses in the human monocytic cell line THP-1 by *Campylobacter jejuni*. *Infect Immun*, 71, 2626-33.
- JOSLIN, S. N. & HENDRIXSON, D. R. 2009. Activation of the *Campylobacter jejuni* FlgSR two-component system is linked to the flagellar export apparatus. *J Bacteriol*, 191, 2656-67.
- JUNG, H. C., ECKMANN, L., YANG, S. K., PANJA, A., FIERER, J., MORZYCKA-WROBLEWSKA, E. & KAGNOFF, M. F. 1995. A distinct array of

- proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *J Clin Invest*, 95, 55-65.
- KADURUGAMUWA, J. L. & BEVERIDGE, T. J. 1995. Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. *J Bacteriol*, 177, 3998-4008.
- KAGNOFF, M. F. & ECKMANN, L. 1997. Epithelial cells as sensors for microbial infection. *J Clin Invest*, 100, 6-10.
- KAKUDA, T. & DIRITA, V. J. 2006. Cj1496c encodes a *Campylobacter jejuni* glycoprotein that influences invasion of human epithelial cells and colonization of the chick gastrointestinal tract. *Infect Immun*, 74, 4715-23.
- KALISCHUK, L. D., INGLIS, G. D. & BURET, A. G. 2009. *Campylobacter jejuni* induces transcellular translocation of commensal bacteria via lipid rafts. *Gut Pathog*, 1, 2.
- KANG, G. J., GETAHUN, Z., MUZAFFAR, A., BROSSI, A. & HAMEL, E. 1990. N-acetylcolchicinol O-methyl ether and thiocolchicine, potent analogs of colchicine modified in the C ring. Evaluation of the mechanistic basis for their enhanced biological properties. *J Biol Chem*, 265, 10255-9.
- KANG, S. J., SHIN, K. S., SONG, W. K., HA, D. B., CHUNG, C. H. & KANG, M. S. 1995. Involvement of transglutaminase in myofibril assembly of chick embryonic myoblasts in culture. *J Cell Biol*, 130, 1127-36.
- KAPPERUD, G., SKJERVE, E., BEAN, N. H., OSTROFF, S. M. & LASSEN, J. 1992. Risk factors for sporadic *Campylobacter* infections: results of a case-control study in southeastern Norway. *J Clin Microbiol*, 30, 3117-21.
- KARLYSHEV, A. V., CHAMPION, O. L., CHURCHER, C., BRISSON, J. R., JARRELL, H. C., GILBERT, M., BROCHU, D., ST MICHAEL, F., LI, J., WAKARCHUK, W. W., GOODHEAD, I., SANDERS, M., STEVENS, K., WHITE, B., PARKHILL, J., WREN, B. W. & SZYMANSKI, C. M. 2005a. Analysis of *Campylobacter jejuni* capsular loci reveals multiple mechanisms for the generation of structural diversity and the ability to form complex heptoses. *Mol Microbiol*, 55, 90-103.
- KARLYSHEV, A. V., EVEREST, P., LINTON, D., CAWTHRAW, S., NEWELL, D. G. & WREN, B. W. 2004. The *Campylobacter jejuni* general glycosylation system is important for attachment to human epithelial cells and in the colonization of chicks. *Microbiology*, 150, 1957-64.
- KARLYSHEV, A. V., KETLEY, J. M. & WREN, B. W. 2005b. The *Campylobacter jejuni* glycome. *FEMS Microbiol Rev*, 29, 377-90.
- KARLYSHEV, A. V., LINTON, D., GREGSON, N. A., LASTOVICA, A. J. & WREN, B. W. 2000. Genetic and biochemical evidence of a *Campylobacter jejuni* capsular polysaccharide that accounts for Penner serotype specificity. *Mol Microbiol*, 35, 529-41.
- KARLYSHEV, A. V., LINTON, D., GREGSON, N. A. & WREN, B. W. 2002. A novel paralogous gene family involved in phase-variable flagella-mediated motility in *Campylobacter jejuni*. *Microbiology*, 148, 473-80.
- KARLYSHEV, A. V. & WREN, B. W. 2005. Development and application of an insertional system for gene delivery and expression in *Campylobacter jejuni*. *Appl Environ Microbiol*, 71, 4004-13.
- KATSO, R., OKKENHAUG, K., AHMADI, K., WHITE, S., TIMMS, J. & WATERFIELD, M. D. 2001. Cellular function of phosphoinositide 3-kinases:

- implications for development, homeostasis, and cancer. *Annu Rev Cell Dev Biol*, 17, 615-75.
- KESTY, N. C., MASON, K. M., REEDY, M., MILLER, S. E. & KUEHN, M. J. 2004. Enterotoxigenic *Escherichia coli* vesicles target toxin delivery into mammalian cells. *Embo j*, 23, 4538-49.
- KETLEY, J. M. & KONKEL, M. E. 2005. *Campylobacter: Molecular and Cellular Biology*, Horizon Bioscience.
- KIEHLBAUCH, J. A., ALBACH, R. A., BAUM, L. L. & CHANG, K. P. 1985. Phagocytosis of *Campylobacter jejuni* and its intracellular survival in mononuclear phagocytes. *Infect Immun*, 48, 446-51.
- KIM, M., ASHIDA, H., OGAWA, M., YOSHIKAWA, Y., MIMURO, H. & SASAKAWA, C. 2010. Bacterial interactions with the host epithelium. *Cell Host Microbe*, 8, 20-35.
- KLEBA, B. J., BANTA, E., LINDQUIST, E. A. & STEPHENS, R. S. 2002. Recruitment of mammalian cell fibronectin to the surface of *Chlamydia trachomatis*. *Infect Immun*, 70, 3935-8.
- KLEIN, U., GIMPL, G. & FAHRENHOLZ, F. 1995. Alteration of the myometrial plasma membrane cholesterol content with beta-cyclodextrin modulates the binding affinity of the oxytocin receptor. *Biochemistry*, 34, 13784-93.
- KLOSE, K. E. & MEKALANOS, J. J. 1998. Differential regulation of multiple flagellins in *Vibrio cholerae*. *J Bacteriol*, 180, 303-16.
- KNODLER, L. A. & STEELE-MORTIMER, O. 2003. Taking possession: biogenesis of the *Salmonella*-containing vacuole. *Traffic*, 4, 587-99.
- KOBAYASHI, H., UEMATSU, K., HIRAYAMA, H. & HORIKOSHI, K. 2000. Novel toluene elimination system in a toluene-tolerant microorganism. *J Bacteriol*, 182, 6451-5.
- KONKEL, M., SAMUELSON, D., EUCKER, T., SHELDEN, E. & O'LOUGHLIN, J. 2013. Invasion of epithelial cells by *Campylobacter jejuni* is independent of caveolae. *Cell Communication and Signaling*, 11, 100.
- KONKEL, M. E., CHRISTENSEN, J. E., KEECH, A. M., MONTEVILLE, M. R., KLENA, J. D. & GARVIS, S. G. 2005. Identification of a fibronectin-binding domain within the *Campylobacter jejuni* CadF protein. *Mol Microbiol*, 57, 1022-35.
- KONKEL, M. E. & CIEPLAK, W., JR. 1992. Altered synthetic response of *Campylobacter jejuni* to cocultivation with human epithelial cells is associated with enhanced internalization. *Infect Immun*, 60, 4945-9.
- KONKEL, M. E., CORWIN, M. D., JOENS, L. A. & CIEPLAK, W. 1992a. Factors that influence the interaction of *Campylobacter jejuni* with cultured mammalian cells. *J Med Microbiol*, 37, 30-7.
- KONKEL, M. E., GARVIS, S. G., TIPTON, S. L., ANDERSON, D. E., JR. & CIEPLAK, W., JR. 1997. Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. *Mol Microbiol*, 24, 953-63.
- KONKEL, M. E., GRAY, S. A., KIM, B. J., GARVIS, S. G. & YOON, J. 1999a. Identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* based on the cadF virulence gene and its product. *J Clin Microbiol*, 37, 510-7.
- KONKEL, M. E., HAYES, S. F., JOENS, L. A. & CIEPLAK, W., JR. 1992b. Characteristics of the internalization and intracellular survival of *Campylobacter jejuni* in human epithelial cell cultures. *Microb Pathog*, 13, 357-70.

- KONKEL, M. E. & JOENS, L. A. 1989. Adhesion to and invasion of HEp-2 cells by *Campylobacter* spp. *Infect Immun*, 57, 2984-90.
- KONKEL, M. E., KIM, B. J., RIVERA-AMILL, V. & GARVIS, S. G. 1999b. Bacterial secreted proteins are required for the internalization of *Campylobacter jejuni* into cultured mammalian cells. *Mol Microbiol*, 32, 691-701.
- KONKEL, M. E., KIM, B. J., RIVERA-AMILL, V. & GARVIS, S. G. 1999c. Identification of proteins required for the internalization of *Campylobacter jejuni* into cultured mammalian cells. *Adv Exp Med Biol*, 473, 215-24.
- KONKEL, M. E., KLENA, J. D., RIVERA-AMILL, V., MONTEVILLE, M. R., BISWAS, D., RAPHAEL, B. & MICKELSON, J. 2004. Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus. *J Bacteriol*, 186, 3296-303.
- KONKEL, M. E., LARSON, C. L. & FLANAGAN, R. C. 2010. *Campylobacter jejuni* FlpA binds fibronectin and is required for maximal host cell adherence. *J Bacteriol*, 192, 68-76.
- KONKEL, M. E., MEAD, D. J. & CIEPLAK, W., JR. 1993. Kinetic and antigenic characterization of altered protein synthesis by *Campylobacter jejuni* during cultivation with human epithelial cells. *J Infect Dis*, 168, 948-54.
- KONKEL, M. E., MEAD, D. J., HAYES, S. F. & CIEPLAK, W., JR. 1992c. Translocation of *Campylobacter jejuni* across human polarized epithelial cell monolayer cultures. *J Infect Dis*, 166, 308-15.
- KONKEL, M. E., MONTEVILLE, M. R., RIVERA-AMILL, V. & JOENS, L. A. 2001. The pathogenesis of *Campylobacter jejuni*-mediated enteritis. *Curr Issues Intest Microbiol*, 2, 55-71.
- KOPECKO, D., HU, L. & ZAAL, K. 2001a. *Campylobacter jejuni*-microtubule-dependent invasion. *Trends Microbiol*, 9, 389 - 396.
- KOPECKO, D. J., HU, L. & ZAAL, K. J. 2001b. *Campylobacter jejuni*--microtubule-dependent invasion. *Trends Microbiol*, 9, 389-96.
- KOPECKO, L. H. A. D. J. 2000. Interactions of *Campylobacter* with eukaryotic cells: gut luminal colonization and mucosal invasion mechanisms. In: I. NACHAMKIN, M. J. B. (ed.) *Campylobacter*. ASM Press.
- KOPECKO, L. H. A. D. J. 2008. *Cell Biology of Human Host Cell Entry by Campylobacter jejuni*, 3rd Ed, 297-309.
- KORLATH, J. A., OSTERHOLM, M. T., JUDY, L. A., FORFANG, J. C. & ROBINSON, R. A. 1985a. A point-source outbreak of campylobacteriosis associated with consumption of raw milk. *J Infect Dis*, 152, 592-6.
- KORLATH, J. A., OSTERHOLM, M. T., JUDY, L. A., FORFANG, J. C. & ROBINSON, R. A. 1985b. A Point-Source Outbreak of Campylobacteriosis Associated with Consumption of Raw Milk. *Journal of Infectious Diseases*, 152, 592-596.
- KOTHARY, M. H. & BABU, U. S. 2001. INFECTIVE DOSE OF FOODBORNE PATHOGENS IN VOLUNTEERS: A REVIEW. *Journal of Food Safety*, 21, 49-68.
- KOWALCZYK, A. P., TULLOH, R. H. & MCKEOWN-LONGO, P. J. 1990. Polarized fibronectin secretion and localized matrix assembly sites correlate with subendothelial matrix formation. *Blood*, 75, 2335-42.
- KOWARIK, M., YOUNG, N. M., NUMAO, S., SCHULZ, B. L., HUG, I., CALLEWAERT, N., MILLS, D. C., WATSON, D. C., HERNANDEZ, M., KELLY, J. F., WACKER, M. & AEBI, M. 2006. Definition of the bacterial N-glycosylation site consensus sequence. *Embo j*, 25, 1957-66.

- KRACHLER, A. M., WOOLERY, A. R. & ORTH, K. 2011. Manipulation of kinase signaling by bacterial pathogens. *J Cell Biol*, 195, 1083-92.
- KRAUSE-GRUSZCZYNSKA, M., BOEHM, M., ROHDE, M., TEGTMEYER, N., TAKAHASHI, S., BUDAY, L., OYARZABAL, O. A. & BACKERT, S. 2011. The signaling pathway of *Campylobacter jejuni*-induced Cdc42 activation: Role of fibronectin, integrin beta1, tyrosine kinases and guanine exchange factor Vav2. *Cell Commun Signal*, 9, 32.
- KRAUSE-GRUSZCZYNSKA, M., ROHDE, M., HARTIG, R., GENTH, H., SCHMIDT, G., KEO, T., KONIG, W., MILLER, W. G., KONKEL, M. E. & BACKERT, S. 2007a. Role of the small Rho GTPases Rac1 and Cdc42 in host cell invasion of *Campylobacter jejuni*. *Cell Microbiol*, 9, 2431-44.
- KRAUSE-GRUSZCZYNSKA, M., VAN ALPHEN, L. B., OYARZABAL, O. A., ALTER, T., HANEL, I., SCHLIEPHAKE, A., KONIG, W., VAN PUTTEN, J. P., KONKEL, M. E. & BACKERT, S. 2007b. Expression patterns and role of the CadF protein in *Campylobacter jejuni* and *Campylobacter coli*. *FEMS Microbiol Lett*, 274, 9-16.
- KREIKEMEYER, B., TALAY, S. R. & CHHATWAL, G. S. 1995. Characterization of a novel fibronectin-binding surface protein in group A streptococci. *Mol Microbiol*, 17, 137-45.
- KREMER, L., BAULARD, A., ESTAQUIER, J., POULAIN-GODEFROY, O. & LOCHT, C. 1995. Green fluorescent protein as a new expression marker in mycobacteria. *Molecular Microbiology*, 17, 913-922.
- KUBORI, T., MATSUSHIMA, Y., NAKAMURA, D., URALIL, J., LARA-TEJERO, M., SUKHAN, A., GALÁN, J. E. & AIZAWA, S. I. 1998. Supramolecular structure of the salmonella typhimurium type III protein secretion system. *Science*, 280, 602-605.
- KUEHN, M. J. & KESTY, N. C. 2005. Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes Dev*, 19, 2645-55.
- KUHN, R., LOHLER, J., RENNICK, D., RAJEWSKY, K. & MULLER, W. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*, 75, 263-74.
- KUIJF, M. L., SAMSOM, J. N., VAN RIJS, W., BAX, M., HUIZINGA, R., HEIKEMA, A. P., VAN DOORN, P. A., VAN BELKUM, A., VAN KOOYK, Y., BURGERS, P. C., LUIDER, T. M., ENDTZ, H. P., NIEUWENHUIS, E. E. & JACOBS, B. C. 2010. TLR4-mediated sensing of *Campylobacter jejuni* by dendritic cells is determined by sialylation. *J Immunol*, 185, 748-55.
- KULP, A. & KUEHN, M. J. 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu Rev Microbiol*, 64, 163-84.
- KUUSELA, P. 1978. Fibronectin binds to *Staphylococcus aureus*. *Nature*, 276, 718-20.
- KUUSELA, P., MORAN, A. P., VARTIO, T. & KOSUNEN, T. U. 1989. Interaction of *Campylobacter jejuni* with extracellular matrix components. *Biochim Biophys Acta*, 993, 297-300.
- KUWADA, S. K. & LI, X. 2000. Integrin alpha5/beta1 mediates fibronectin-dependent epithelial cell proliferation through epidermal growth factor receptor activation. *Mol Biol Cell*, 11, 2485-96.
- LABIGNE-ROUSSEL, A., HAREL, J. & TOMPKINS, L. 1987. Gene transfer from *Escherichia coli* to *Campylobacter* species: development of shuttle vectors for genetic analysis of *Campylobacter jejuni*. *J Bacteriol*, 169, 5320-3.
- LAFONT, F., ABRAMI, L. & VAN DER GOOT, F. G. 2004. Bacterial subversion of lipid rafts. *Curr Opin Microbiol*, 7, 4-10.

- LAFONT, F. & VAN DER GOOT, F. G. 2005. Bacterial invasion via lipid rafts. *Cell Microbiol*, 7, 613-20.
- LAFONT, K. M. A. F. 2012. Mechanisms Of Bacterial Entry into Host Cells. In: SIMONET, C. L. A. M. (ed.) *Bacterial Pathogenesis: Molecular and Cellular Mechanisms*. Norfolk, UK.: Caister Academic Press.
- LARA-TEJERO, M. & GALAN, J. E. 2000. A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. *Science*, 290, 354-7.
- LARA-TEJERO, M. & GALAN, J. E. 2001. CdtA, CdtB, and CdtC form a tripartite complex that is required for cytolethal distending toxin activity. *Infect Immun*, 69, 4358-65.
- LARSON, C. L., SAMUELSON, D. R., EUCKER, T. P., O'LOUGHLIN, J. L. & KONKEL, M. E. 2013. The fibronectin-binding motif within FliA facilitates *Campylobacter jejuni* adherence to host cell and activation of host cell signaling. *Emerg Microbes Infect*, 2, e65.
- LAUFFENBURGER, D. A. & HORWITZ, A. F. 1996. Cell migration: a physically integrated molecular process. *Cell*, 84, 359-69.
- LAUKOETTER, M. G., NAVA, P. & NUSRAT, A. 2008. Role of the intestinal barrier in inflammatory bowel disease. *World J Gastroenterol*, 14, 401-7.
- LAVELLE, E. C., MURPHY, C., O'NEILL, L. A. J. & CREAGH, E. M. 2009. The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis. *Mucosal Immunol*, 3, 17-28.
- LAVINE, M. D. & STRAND, M. R. 2002. Insect hemocytes and their role in immunity. *Insect Biochem Mol Biol*, 32, 1295-309.
- LEHRER, R. I. 2004. Primate defensins. *Nat Rev Microbiol*, 2, 727-38.
- LERTSEHTAKARN, P., OTTEMANN, K. M. & HENDRIXSON, D. R. 2011. Motility and chemotaxis in *Campylobacter* and *Helicobacter*. *Annu Rev Microbiol*, 65, 389-410.
- LESER, T. D. & MOLBAK, L. 2009. Better living through microbial action: the benefits of the mammalian gastrointestinal microbiota on the host. *Environ Microbiol*, 11, 2194-206.
- LEVY, P., LOREAL, O., MUNIER, A., YAMADA, Y., PICARD, J., CHERQUI, G., CLEMENT, B. & CAPEAU, J. 1994. Enterocytic differentiation of the human Caco-2 cell line is correlated with down-regulation of fibronectin and laminin. *FEBS Letters*, 338, 272-276.
- LIM, J. S., CHOY, H. E., PARK, S. C., HAN, J. M., JANG, I. S. & CHO, K. A. 2010. Caveolae-mediated entry of *Salmonella typhimurium* into senescent nonphagocytotic host cells. *Aging Cell*, 9, 243-51.
- LINDMARK, B., ROMPIKUNTAL, P. K., VAITKEVICIUS, K., SONG, T., MIZUNOE, Y., UHLIN, B. E., GUERRY, P. & WAI, S. N. 2009. Outer membrane vesicle-mediated release of cytolethal distending toxin (CDT) from *Campylobacter jejuni*. *BMC Microbiol*, 9, 220.
- LINTON, D., ALLAN, E., KARLYSHEV, A. V., CRONSHAW, A. D. & WREN, B. W. 2002. Identification of N-acetylgalactosamine-containing glycoproteins PEB3 and CgpA in *Campylobacter jejuni*. *Mol Microbiol*, 43, 497-508.
- LINTON, D., GILBERT, M., HITCHEN, P. G., DELL, A., MORRIS, H. R., WAKARCHUK, W. W., GREGSON, N. A. & WREN, B. W. 2000. Phase variation of a beta-1,3 galactosyltransferase involved in generation of the ganglioside GM1-like lipo-oligosaccharide of *Campylobacter jejuni*. *Mol Microbiol*, 37, 501-14.

- LOEB, M. R. & KILNER, J. 1978. Release of a special fraction of the outer membrane from both growing and phage T4-infected *Escherichia coli* B. *Biochim Biophys Acta*, 514, 117-27.
- LOGAN, S. M. 2006. Flagellar glycosylation - a new component of the motility repertoire? *Microbiology*, 152, 1249-62.
- LOGAN, S. M. & TRUST, T. J. 1982. Outer membrane characteristics of *Campylobacter jejuni*. *Infect Immun*, 38, 898-906.
- LOGAN, S. M., TRUST, T. J. & GUERRY, P. 1989. Evidence for posttranslational modification and gene duplication of *Campylobacter* flagellin. *J Bacteriol*, 171, 3031-8.
- LORAND, L. & GRAHAM, R. M. 2003. Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nat Rev Mol Cell Biol*, 4, 140-156.
- LOUWEN, R., HEIKEMA, A., VAN BELKUM, A., OTT, A., GILBERT, M., ANG, W., ENDTZ, H. P., BERGMAN, M. P. & NIEUWENHUIS, E. E. 2008. The sialylated lipooligosaccharide outer core in *Campylobacter jejuni* is an important determinant for epithelial cell invasion. *Infect Immun*, 76, 4431-8.
- LOUWEN, R. P., VAN BELKUM, A., WAGENAAR, J. A., DOORDUYN, Y., ACHTERBERG, R. & ENDTZ, H. P. 2006. Lack of association between the presence of the pVir plasmid and bloody diarrhea in *Campylobacter jejuni* enteritis. *J Clin Microbiol*, 44, 1867-8.
- MACARTNEY, L., AL-MASHAT, R. R., TAYLOR, D. J. & MCCANDLISH, I. A. 1988. Experimental infection of dogs with *Campylobacter jejuni*. *Vet Rec*, 122, 245-9.
- MACCALLUM, A., HARDY, S. P. & EVEREST, P. H. 2005. *Campylobacter jejuni* inhibits the absorptive transport functions of Caco-2 cells and disrupts cellular tight junctions. *Microbiology*, 151, 2451-8.
- MACCALLUM, A. J., HARRIS, D., HADDOCK, G. & EVEREST, P. H. 2006. *Campylobacter jejuni*-infected human epithelial cell lines vary in their ability to secrete interleukin-8 compared to in vitro-infected primary human intestinal tissue. *Microbiology*, 152, 3661-5.
- MACLEAN-FLETCHER, S. & POLLARD, T. D. 1980. Identification of a factor in conventional muscle actin preparations which inhibits actin filament self-association. *Biochem Biophys Res Commun*, 96, 18-27.
- MAHDAVI, J., PIRINCCIOGLU, N., OLDFIELD, N. J., CARLSOHN, E., STOOF, J., ASLAM, A., SELF, T., CAWTHRAW, S. A., PETROVSKA, L., COLBORNE, N., SIHLBOM, C., BOREN, T., WOOLDRIDGE, K. G. & ALA'ALDEEN, D. A. 2014. A novel O-linked glycan modulates *Campylobacter jejuni* major outer membrane protein-mediated adhesion to human histo-blood group antigens and chicken colonization. *Open Biol*, 4, 130202.
- MALIK-KALE, P., PARKER, C. T. & KONKEL, M. E. 2008. Culture of *Campylobacter jejuni* with sodium deoxycholate induces virulence gene expression. *J Bacteriol*, 190, 2286-97.
- MALIK-KALE, P., RAPHAEL, B. H., PARKER, C. T., JOENS, L. A., KLENA, J. D., QUINONES, B., KEECH, A. M. & KONKEL, M. E. 2007. Characterization of genetically matched isolates of *Campylobacter jejuni* reveals that mutations in genes involved in flagellar biosynthesis alter the organism's virulence potential. *Appl Environ Microbiol*, 73, 3123-36.
- MANES, S., DEL REAL, G. & MARTINEZ, A. C. 2003. Pathogens: raft hijackers. *Nat Rev Immunol*, 3, 557-68.

- MANSFIELD, L. S., BELL, J. A., WILSON, D. L., MURPHY, A. J., ELSHEIKHA, H. M., RATHINAM, V. A., FIERRO, B. R., LINZ, J. E. & YOUNG, V. B. 2007. C57BL/6 and congenic interleukin-10-deficient mice can serve as models of *Campylobacter jejuni* colonization and enteritis. *Infect Immun*, 75, 1099-115.
- MANSFIELD, L. S., PATTERSON, J. S., FIERRO, B. R., MURPHY, A. J., RATHINAM, V. A., KOPPER, J. J., BARBU, N. I., ONIFADE, T. J. & BELL, J. A. 2008. Genetic background of IL-10(-/-) mice alters host-pathogen interactions with *Campylobacter jejuni* and influences disease phenotype. *Microb Pathog*, 45, 241-57.
- MAO, Y. & SCHWARZBAUER, J. E. 2005. Fibronectin fibrillogenesis, a cell-mediated matrix assembly process. *Matrix Biol*, 24, 389-99.
- MARJENBERG, Z. R., ELLIS, I. R., HAGAN, R. M., PRABHAKARAN, S., HOOK, M., TALAY, S. R., POTTS, J. R., STAUNTON, D. & SCHWARZ-LINEK, U. 2011. Cooperative binding and activation of fibronectin by a bacterial surface protein. *J Biol Chem*, 286, 1884-94.
- MARSHALL, A. J. & PIDDOCK, L. J. 1994. Interaction of divalent cations, quinolones and bacteria. *J Antimicrob Chemother*, 34, 465-83.
- MARTEYN, B., WEST, N. P., BROWNING, D. F., COLE, J. A., SHAW, J. G., PALM, F., MOUNIER, J., PREVOST, M. C., SANSONETTI, P. & TANG, C. M. 2010. Modulation of *Shigella* virulence in response to available oxygen in vivo. *Nature*, 465, 355-8.
- MASANTA, W. O., HEIMESAAT, M. M., BERESWILL, S., TAREEN, A. M., LUGERT, R., GROSS, U. & ZAUTNER, A. E. 2013. Modification of intestinal microbiota and its consequences for innate immune response in the pathogenesis of campylobacteriosis. *Clin Dev Immunol*, 2013, 526860.
- MATO, J. M., PENCEV, D., VASANTHAKUMAR, G., SCHIFFMANN, E. & PASTAN, I. 1983. Inhibitors of endocytosis perturb phospholipid metabolism in rabbit neutrophils and other cells. *Proc Natl Acad Sci U S A*, 80, 1929-32.
- MAYRAND, D. & GRENIER, D. 1989. Biological activities of outer membrane vesicles. *Can J Microbiol*, 35, 607-13.
- MCBROOM, A. J. & KUEHN, M. J. 2007. Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. *Mol Microbiol*, 63, 545-58.
- MCCORMICK, B. A. 2003. The use of transepithelial models to examine host-pathogen interactions. *Curr Opin Microbiol*, 6, 77-81.
- MCFADYEAN, J. S., S. 1913. Report of the departmental committee appointed by the Board of Agriculture and Fisheries to inquire into epizootic abortion. Part III. Abortion in sheep. London:HMSO. *Biochem Biophys Res Commun*.
- MCKEOWN-LONGO, P. J. 1987. Fibronectin-cell surface interactions. *Rev Infect Dis*, 9 Suppl 4, S322-34.
- MECHAM, R. P. 2011. The Extracellular Matrix: An overview. In: MECHAM, R. (ed.) *Biology of Extracellular Matrix*. Berlin, Germany: Springer-Verlag.
- MEDZHITOV, R. & JANEWAY, C. A., JR. 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell*, 91, 295-8.
- MEKALANOS, J. J. 1992. Environmental signals controlling expression of virulence determinants in bacteria. *J Bacteriol*, 174, 1-7.
- MIDWOOD, K. S., MAO, Y., HSIA, H. C., VALENICK, L. V. & SCHWARZBAUER, J. E. 2006. Modulation of cell-fibronectin matrix interactions during tissue repair. *J Invest Dermatol Symp Proc*, 11, 73-8.

- MILLER, R. A. & BRITIGAN, B. E. 1997. Role of oxidants in microbial pathophysiology. *Clin Microbiol Rev*, 10, 1-18.
- MILLER, W. G., BATES, A. H., HORN, S. T., BRANDL, M. T., WACHTEL, M. R. & MANDRELL, R. E. 2000. Detection on surfaces and in Caco-2 cells of *Campylobacter jejuni* cells transformed with new gfp, yfp, and cfp marker plasmids. *Appl Environ Microbiol*, 66, 5426-36.
- MILLS, D. C., GUNDOGDU, O., ELMİ, A., BAJAJ-ELLIOTT, M., TAYLOR, P. W., WREN, B. W. & DORRELL, N. 2012. Increase in *Campylobacter jejuni* invasion of intestinal epithelial cells under low-oxygen coculture conditions that reflect the in vivo environment. *Infect Immun*, 80, 1690-8.
- MILLS, S. D. & BRADBURY, W. C. 1984. Human antibody response to outer membrane proteins of *Campylobacter jejuni* during infection. *Infect Immun*, 43, 739-43.
- MISAWA, N. & BLASER, M. J. 2000. Detection and characterization of autoagglutination activity by *Campylobacter jejuni*. *Infect Immun*, 68, 6168-75.
- MISHRA, S. & MURPHY, L. J. 2004. Tissue transglutaminase has intrinsic kinase activity: identification of transglutaminase 2 as an insulin-like growth factor-binding protein-3 kinase. *J Biol Chem*, 279, 23863-8.
- MISTELI, T. & SPECTOR, D. L. 1997. Applications of the green fluorescent protein in cell biology and biotechnology. *Nat Biotechnol*, 15, 961-4.
- MITCHISON, T. J. & CRAMER, L. P. 1996. Actin-based cell motility and cell locomotion. *Cell*, 84, 371-9.
- MIXTER, P. F., KLENA, J. D., FLOM, G. A., SIEGESMUND, A. M. & KONKEL, M. E. 2003. In vivo tracking of *Campylobacter jejuni* by using a novel recombinant expressing green fluorescent protein. *Appl Environ Microbiol*, 69, 2864-74.
- MIYAWAKI, A. 2003. Visualization of the spatial and temporal dynamics of intracellular signaling. *Dev Cell*, 4, 295-305.
- MONTEVILLE, M., YOON, J. & KONKEL, M. 2003a. Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer-membrane protein and microfilament reorganization. *Microbiology*, 149, 153 - 165.
- MONTEVILLE, M. R. & KONKEL, M. E. 2002. Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. *Infect Immun*, 70, 6665-71.
- MONTEVILLE, M. R., YOON, J. E. & KONKEL, M. E. 2003b. Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer-membrane protein and microfilament reorganization. *Microbiology*, 149, 153-65.
- MOORE, K. W., DE WAAL MALEFYT, R., COFFMAN, R. L. & O'GARRA, A. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*, 19, 683-765.
- MOORE, K. W., O'GARRA, A., DE WAAL MALEFYT, R., VIEIRA, P. & MOSMANN, T. R. 1993. Interleukin-10. *Annu Rev Immunol*, 11, 165-90.
- MORAN, A. P. 1997. Structure and conserved characteristics of *Campylobacter jejuni* lipopolysaccharides. *J Infect Dis*, 176 Suppl 2, S115-21.
- MORO, L., DOLCE, L., CABODI, S., BERGATTO, E., BOERI ERBA, E., SMERIGLIO, M., TURCO, E., RETTA, S. F., GIUFFRIDA, M. G., VENTURINO, M., GODOVAC-ZIMMERMANN, J., CONTI, A., SCHAEFER, E., BEGUINOT, L., TACCHETTI, C., GAGGINI, P., SILENGO, L., TARONE, G. & DEFILIPPI, P. 2002. Integrin-induced epidermal growth factor (EGF)

- receptor activation requires c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosines. *J Biol Chem*, 277, 9405-14.
- MOROOKA, T., UMEDA, A. & AMAKO, K. 1985. Motility as an intestinal colonization factor for *Campylobacter jejuni*. *J Gen Microbiol*, 131, 1973-80.
- MORTENSEN, N. P., KUIJF, M. L., ANG, C. W., SCHIELLERUP, P., KROGFELT, K. A., JACOBS, B. C., VAN BELKUM, A., ENDTZ, H. P. & BERGMAN, M. P. 2009. Sialylation of *Campylobacter jejuni* lipo-oligosaccharides is associated with severe gastro-enteritis and reactive arthritis. *Microbes Infect*, 11, 988-94.
- MOSER, I., SCHROEDER, W. & SALNIKOW, J. 1997. *Campylobacter jejuni* major outer membrane protein and a 59-kDa protein are involved in binding to fibronectin and INT 407 cell membranes. *FEMS Microbiol Lett*, 157, 233-8.
- MOSSER, D. M. & EDWARDS, J. P. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*, 8, 958-69.
- MOSTOV, K. E., VERGES, M. & ALTSCHULER, Y. 2000. Membrane traffic in polarized epithelial cells. *Curr Opin Cell Biol*, 12, 483-90.
- MOTA, L. J. & CORNELIS, G. R. 2005. The bacterial injection kit: type III secretion systems. *Ann Med*, 37, 234-49.
- MOUNIER, J., BAHRANI, F. K. & SANSONETTI, P. J. 1997. Secretion of *Shigella flexneri* Ipa invasins on contact with epithelial cells and subsequent entry of the bacterium into cells are growth stage dependent. *Infect Immun*, 65, 774-82.
- MSHANA, S. E., JOLOBA, M., KAKOOZA, A. & KADDU-MULINDWA, D. 2009. *Campylobacter* spp among children with acute diarrhea attending Mulago hospital in Kampala--Uganda. *Afr Health Sci*, 9, 201-5.
- MUNDY, D. I., MACHLEIDT, T., YING, Y. S., ANDERSON, R. G. & BLOOM, G. S. 2002. Dual control of caveolar membrane traffic by microtubules and the actin cytoskeleton. *J Cell Sci*, 115, 4327-39.
- MYHRE, E. B. & KUUSELA, P. 1983. Binding of human fibronectin to group A, C, and G streptococci. *Infect Immun*, 40, 29-34.
- MYLONAKIS, E., CASADEVALL, A. & AUSUBEL, F. M. 2007. Exploiting amoeboid and non-vertebrate animal model systems to study the virulence of human pathogenic fungi. *PLoS Pathog*, 3, e101.
- NACHAMKIN, I., ARZARTE BARBOSA, P., UNG, H., LOBATO, C., GONZALEZ RIVERA, A., RODRIGUEZ, P., GARCIA BRISENO, A., CORDERO, L. M., GARCIA PEREA, L., PEREZ, J. C., RIBERA, M., ALDAMA, P. C., GUITERREZ, G. D., SARNAT, L. F., GARCIA, M. R., VEITCH, J., FITZGERALD, C., CORNBLATH, D. R., RODRIGUEZ PINTO, M., GRIFFIN, J. W., WILLISON, H. J., ASBURY, A. K. & MCKHANN, G. M. 2007. Patterns of Guillain-Barre syndrome in children: results from a Mexican population. *Neurology*, 69, 1665-71.
- NACHAMKIN, I. & HART, A. M. 1985. Western blot analysis of the human antibody response to *Campylobacter jejuni* cellular antigens during gastrointestinal infection. *J Clin Microbiol*, 21, 33-8.
- NACHAMKIN, I., YANG, X. H. & STERN, N. J. 1993. Role of *Campylobacter jejuni* flagella as colonization factors for three-day-old chicks: analysis with flagellar mutants. *Appl Environ Microbiol*, 59, 1269-73.
- NAITO, M., FRIRDICH, E., FIELDS, J. A., PRYJMA, M., LI, J., CAMERON, A., GILBERT, M., THOMPSON, S. A. & GAYNOR, E. C. 2010. Effects of sequential *Campylobacter jejuni* 81-176 lipooligosaccharide core truncations on biofilm formation, stress survival, and pathogenesis. *J Bacteriol*, 192, 2182-92.

- NAKAMURA, S., HIGASHIYAMA, Y., IZUMIKAWA, K., SEKI, M., KAKEYA, H., YAMAMOTO, Y., YANAGIHARA, K., MIYAZAKI, Y., MIZUTA, Y. & KOHNO, S. 2008. The roles of the quorum-sensing system in the release of extracellular DNA, lipopolysaccharide, and membrane vesicles from *Pseudomonas aeruginosa*. *Jpn J Infect Dis*, 61, 375-8.
- NAPPI, A. J. & CHRISTENSEN, B. M. 2005. Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity. *Insect Biochem Mol Biol*, 35, 443-59.
- NAZ, N., MILLS, D. C., WREN, B. W. & DORRELL, N. 2013. Enteric bacterial invasion of intestinal epithelial cells in vitro is dramatically enhanced using a vertical diffusion chamber model. *J Vis Exp*, e50741.
- NAZLI, A., WANG, A., STEEN, O., PRESCOTT, D., LU, J., PERDUE, M. H., SODERHOLM, J. D., SHERMAN, P. M. & MCKAY, D. M. 2006. Enterocyte cytoskeleton changes are crucial for enhanced translocation of nonpathogenic *Escherichia coli* across metabolically stressed gut epithelia. *Infect Immun*, 74, 192-201.
- NEAL-MCKINNEY, J. M., CHRISTENSEN, J. E. & KONKEL, M. E. 2010. Amino-terminal residues dictate the export efficiency of the *Campylobacter jejuni* filament proteins via the flagellum. *Mol Microbiol*, 76, 918-31.
- NEAL-MCKINNEY, J. M. & KONKEL, M. E. 2012. The *Campylobacter jejuni* CiaC virulence protein is secreted from the flagellum and delivered to the cytosol of host cells. *Front Cell Infect Microbiol*, 2, 31.
- NEISH, A. S. 2009. Microbes in gastrointestinal health and disease. *Gastroenterology*, 136, 65-80.
- NEWELL, D. G. 2001. Animal models of *Campylobacter jejuni* colonization and disease and the lessons to be learned from similar *Helicobacter pylori* models. *Journal of Applied Microbiology*, 90, 57S-67S.
- NEWELL, D. G., ELVERS, K. T., DOPFER, D., HANSSON, I., JONES, P., JAMES, S., GITTINS, J., STERN, N. J., DAVIES, R., CONNERTON, I., PEARSON, D., SALVAT, G. & ALLEN, V. M. 2011. Biosecurity-based interventions and strategies to reduce *Campylobacter* spp. on poultry farms. *Appl Environ Microbiol*, 77, 8605-14.
- NEWELL, D. G., MCBRIDE, H. & DOLBY, J. M. 1985. Investigations on the role of flagella in the colonization of infant mice with *Campylobacter jejuni* and attachment of *Campylobacter jejuni* to human epithelial cell lines. *J Hyg (Lond)*, 95, 217-27.
- NICHOLS, G. L., RICHARDSON, J. F., SHEPPARD, S. K., LANE, C. & SARRAN, C. 2012. *Campylobacter* epidemiology: a descriptive study reviewing 1 million cases in England and Wales between 1989 and 2011. *BMJ Open*, 2.
- NINOMIYA, N., HAZEKI, K., FUKUI, Y., SEYA, T., OKADA, T., HAZEKI, O. & UI, M. 1994. Involvement of phosphatidylinositol 3-kinase in Fc gamma receptor signaling. *J Biol Chem*, 269, 22732-7.
- NITA-LAZAR, M., WACKER, M., SCHEGG, B., AMBER, S. & AEBI, M. 2005. The N-X-S/T consensus sequence is required but not sufficient for bacterial N-linked protein glycosylation. *Glycobiology*, 15, 361-7.
- NIZET V, E. J. 2009. Bacterial and Viral Infections. In: VARKI A, C. R., ESKO JD, ET AL. (ed.) *Essentials of Glycobiology*. 2nd edition ed.: Cold Spring Harbor Laboratory Press.

- NOVIK, V., HOFREUTER, D. & GALAN, J. E. 2010. Identification of *Campylobacter jejuni* genes involved in its interaction with epithelial cells. *Infect Immun*, 78, 3540-53.
- NUIJTEN, P. J., VAN ASTEN, F. J., GAASTRA, W. & VAN DER ZEIJST, B. A. 1990. Structural and functional analysis of two *Campylobacter jejuni* flagellin genes. *J Biol Chem*, 265, 17798-804.
- O'CALLAGHAN, D., CAZEVIEILLE, C., ALLARDET-SERVENT, A., BOSCHIROLI, M. L., BOURG, G., FOULONGNE, V., FRUTOS, P., KULAKOV, Y. & RAMUZ, M. 1999. A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. *Mol Microbiol*, 33, 1210-20.
- O'HARA, A. M. & SHANAHAN, F. 2006. The gut flora as a forgotten organ. *EMBO Rep*, 7, 688-93.
- O'NEIL, D. A., PORTER, E. M., ELEWAUT, D., ANDERSON, G. M., ECKMANN, L., GANZ, T. & KAGNOFF, M. F. 1999. Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium. *J Immunol*, 163, 6718-24.
- O. CROININ AND BACKERT, S. 2012. Host epithelial cell invasion by *Campylobacter jejuni*: trigger or zipper mechanism? *Front Cell Infect Microbiol*, 2, 25.
- OELSCHLAEGER, T. A., BARRETT, T. J. & KOPECKO, D. J. 1994. Some structures and processes of human epithelial cells involved in uptake of enterohemorrhagic *Escherichia coli* O157:H7 strains. *Infect Immun*, 62, 5142-50.
- OELSCHLAEGER, T. A., GUERRY, P. & KOPECKO, D. J. 1993. Unusual microtubule-dependent endocytosis mechanisms triggered by *Campylobacter jejuni* and *Citrobacter freundii*. *Proc Natl Acad Sci U S A*, 90, 6884-8.
- OGAWA, M. & SASAKAWA, C. 2006. Intracellular survival of *Shigella*. *Cell Microbiol*, 8, 177-84.
- OLD, L. J. 1988. Tumor necrosis factor. *Sci Am*, 258, 59-60, 69-75.
- OPPENHEIM, J. J., ZACHARIAE, C. O., MUKAIDA, N. & MATSUSHIMA, K. 1991. Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu Rev Immunol*, 9, 617-48.
- PACANOWSKI, J., LALANDE, V., LACOMBE, K., BOUDRAA, C., LESPRIT, P., LEGRAND, P., TRYSTRAM, D., KASSIS, N., ARLET, G., MAINARDI, J. L., DOUCET-POPULAIRE, F., GIRARD, P. M., MEYNARD, J. L. & GROUP, C. S. 2008. *Campylobacter* bacteremia: clinical features and factors associated with fatal outcome. *Clin Infect Dis*, 47, 790-6.
- PANICKER, A. K., BUHUSI, M., ERICKSON, A. & MANESS, P. F. 2006. Endocytosis of beta1 integrins is an early event in migration promoted by the cell adhesion molecule L1. *Exp Cell Res*, 312, 299-307.
- PANKOV, R. & YAMADA, K. M. 2002. Fibronectin at a glance. *J Cell Sci*, 115, 3861-3.
- PARKER, C. T., GILBERT, M., YUKI, N., ENDTZ, H. P. & MANDRELL, R. E. 2008. Characterization of lipooligosaccharide-biosynthetic loci of *Campylobacter jejuni* reveals new lipooligosaccharide classes: evidence of mosaic organizations. *J Bacteriol*, 190, 5681-9.
- PARKER, C. T., HORN, S. T., GILBERT, M., MILLER, W. G., WOODWARD, D. L. & MANDRELL, R. E. 2005. Comparison of *Campylobacter jejuni* lipooligosaccharide biosynthesis loci from a variety of sources. *J Clin Microbiol*, 43, 2771-81.

- PARKHILL, J., WREN, B. W., MUNGALL, K., KETLEY, J. M., CHURCHER, C., BASHAM, D., CHILLINGWORTH, T., DAVIES, R. M., FELTWELL, T., HOLROYD, S., JAGELS, K., KARLYSHEV, A. V., MOULE, S., PALLAN, M. J., PENN, C. W., QUAIL, M. A., RAJANDREAM, M. A., RUTHERFORD, K. M., VAN VLIET, A. H., WHITEHEAD, S. & BARRELL, B. G. 2000. The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature*, 403, 665-8.
- PEARSE, B. M. 1976. Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles. *Proc Natl Acad Sci U S A*, 73, 1255-9.
- PEI, Z., BURUCOA, C., GRIGNON, B., BAQAR, S., HUANG, X. Z., KOPECKO, D. J., BOURGEOIS, A. L., FAUCHERE, J. L. & BLASER, M. J. 1998. Mutation in the *peb1A* locus of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. *Infect Immun*, 66, 938-43.
- PELEG, A. Y., JARA, S., MONGA, D., ELIOPOULOS, G. M., MOELLERING, R. C., JR. & MYLONAKIS, E. 2009. *Galleria mellonella* as a model system to study *Acinetobacter baumannii* pathogenesis and therapeutics. *Antimicrob Agents Chemother*, 53, 2605-9.
- PELKMANS, L. & HELENIUS, A. 2003. Insider information: what viruses tell us about endocytosis. *Curr Opin Cell Biol*, 15, 414-22.
- PENNER, J. L. & HENNESSY, J. N. 1980. Passive hemagglutination technique for serotyping *Campylobacter fetus* subsp. *jejuni* on the basis of soluble heat-stable antigens. *J Clin Microbiol*, 12, 732-7.
- PENNIE, R. A., PEARSON, R. D., BARRETT, L. J., LIOR, H. & GUERRANT, R. L. 1986. Susceptibility of *Campylobacter jejuni* to strain-specific bactericidal activity in sera of infected patients. *Infect Immun*, 52, 702-6.
- PESCI, E. C., COTTLE, D. L. & PICKETT, C. L. 1994. Genetic, enzymatic, and pathogenic studies of the iron superoxide dismutase of *Campylobacter jejuni*. *Infect Immun*, 62, 2687-94.
- PFEFFER, K. 2003. Biological functions of tumor necrosis factor cytokines and their receptors. *Cytokine Growth Factor Rev*, 14, 185-91.
- PHILPOTT, D. J. & GIRARDIN, S. E. 2004. The role of Toll-like receptors and Nod proteins in bacterial infection. *Mol Immunol*, 41, 1099-108.
- PIGRAU, C., BARTOLOME, R., ALMIRANTE, B., PLANES, A. M., GAVALDA, J. & PAHISSA, A. 1997. Bacteremia due to *Campylobacter* species: clinical findings and antimicrobial susceptibility patterns. *Clin Infect Dis*, 25, 1414-20.
- PIKE, L. J. 2003. Lipid rafts: bringing order to chaos. *J Lipid Res*, 44, 655-67.
- PINTO, M., ROBINE LEON, S. & APPAY, M. D. 1983. Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biology of the Cell*, 47, 323-330.
- PIZARRO-CERDA, J. & COSSART, P. 2004. Subversion of phosphoinositide metabolism by intracellular bacterial pathogens. *Nat Cell Biol*, 6, 1026-1033.
- PIZARRO-CERDA, J. & COSSART, P. 2006. Bacterial adhesion and entry into host cells. *Cell*, 124, 715-27.
- PIZARRO-CERDA, J., MORENO, E., DESJARDINS, M. & GORVEL, J. P. 1997. When intracellular pathogens invade the frontiers of cell biology and immunology. *Histol Histopathol*, 12, 1027-38.
- POLLARD, T. D. & MOOSEKER, M. S. 1981. Direct measurement of actin polymerization rate constants by electron microscopy of actin filaments nucleated by isolated microvillus cores. *J Cell Biol*, 88, 654-9.

- POLY, F., EWING, C., GOON, S., HICKEY, T. E., ROCKABRAND, D., MAJAM, G., LEE, L., PHAN, J., SAVARINO, N. J. & GUERRY, P. 2007. Heterogeneity of a *Campylobacter jejuni* protein that is secreted through the flagellar filament. *Infect Immun*, 75, 3859-67.
- POLY, F. & GUERRY, P. 2008. Pathogenesis of *Campylobacter*. *Curr Opin Gastroenterol*, 24, 27-31.
- PRASHER, D. C., ECKENRODE, V. K., WARD, W. W., PRENDERGAST, F. G. & CORMIER, M. J. 1992. Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene*, 111, 229-33.
- PRENDERGAST, M. M., LASTOVICA, A. J. & MORAN, A. P. 1998. Lipopolysaccharides from *Campylobacter jejuni* O:41 strains associated with Guillain-Barre syndrome exhibit mimicry of GM1 ganglioside. *Infect Immun*, 66, 3649-55.
- PRYJMA, M., APEL, D., HUYNH, S., PARKER, C. T. & GAYNOR, E. C. 2012. FdhTU-modulated formate dehydrogenase expression and electron donor availability enhance recovery of *Campylobacter jejuni* following host cell infection. *J Bacteriol*, 194, 3803-13.
- PURDY, D., BUSWELL, C. M., HODGSON, A. E., MCALPINE, K., HENDERSON, I. & LEACH, S. A. 2000. Characterisation of cytolethal distending toxin (CDT) mutants of *Campylobacter jejuni*. *J Med Microbiol*, 49, 473-9.
- PURDY, D., CAWTHRAW, S., DICKINSON, J. H., NEWELL, D. G. & PARK, S. F. 1999. Generation of a superoxide dismutase (SOD)-deficient mutant of *Campylobacter coli*: evidence for the significance of SOD in *Campylobacter* survival and colonization. *Appl Environ Microbiol*, 65, 2540-6.
- RADTKE, F. & CLEVERS, H. 2005. Self-renewal and cancer of the gut: two sides of a coin. *Science*, 307, 1904-9.
- RAMEH, L. E. & CANTLEY, L. C. 1999. The role of phosphoinositide 3-kinase lipid products in cell function. *J Biol Chem*, 274, 8347-50.
- RAPHAEL, B. H., MONTEVILLE, M. R., KLENA, J. D., JOENS, L. A. & KONKEL, M. E. 2005. Interactions of *Campylobacter jejuni* with non-professional phagocytic cells. *Campylobacter: Molecular and Cellular Biology*, 397-413.
- RATHINAM, V. A., HOAG, K. A. & MANSFIELD, L. S. 2008. Dendritic cells from C57BL/6 mice undergo activation and induce Th1-effector cell responses against *Campylobacter jejuni*. *Microbes Infect*, 10, 1316-24.
- REES, J. H., SOUDAIN, S. E., GREGSON, N. A. & HUGHES, R. A. 1995. *Campylobacter jejuni* infection and Guillain-Barre syndrome. *N Engl J Med*, 333, 1374-9.
- RIVERA-AMILL, V., KIM, B. J., SESHU, J. & KONKEL, M. E. 2001. Secretion of the virulence-associated *Campylobacter* invasion antigens from *Campylobacter jejuni* requires a stimulatory signal. *J Infect Dis*, 183, 1607-16.
- RIVERA-AMILL, V. & KONKEL, M. E. 1999. Secretion of *Campylobacter jejuni* Cia proteins is contact dependent. *Adv Exp Med Biol*, 473, 225-9.
- ROBERTS, D. D. 1990. Interactions of respiratory pathogens with host cell surface and extracellular matrix components. *Am J Respir Cell Mol Biol*, 3, 181-6.
- RODGERS, V. D. & KAGNOFF, M. F. 1987. Gastrointestinal manifestations of the acquired immunodeficiency syndrome. *West J Med*, 146, 57-67.
- ROHDE, M., MÜLLER, E., CHHATWAL, G. S. & TALAY, S. R. 2003. Host cell caveolae act as an entry-port for Group A streptococci. *Cellular Microbiology*, 5, 323-342.
- ROMBERGER, D. J. 1997. Fibronectin. *Int J Biochem Cell Biol*, 29, 939-43.

- ROSENSHINE, I., RUSCHKOWSKI, S., FOUBISTER, V. & FINLAY, B. B. 1994. Salmonella typhimurium invasion of epithelial cells: role of induced host cell tyrosine protein phosphorylation. *Infect Immun*, 62, 4969-74.
- RUSSELL, R. G. & BLAKE, D. C., JR. 1994. Cell association and invasion of Caco-2 cells by Campylobacter jejuni. *Infect Immun*, 62, 3773-9.
- RUSSELL, R. G., BLASER, M. J., SARMIENTO, J. I. & FOX, J. 1989. Experimental Campylobacter jejuni infection in Macaca nemestrina. *Infect Immun*, 57, 1438-44.
- RYDEN, C., RUBIN, K., SPEZIALE, P., HOOK, M., LINDBERG, M. & WADSTROM, T. 1983. Fibronectin receptors from Staphylococcus aureus. *J Biol Chem*, 258, 3396-401.
- SAMUELSON, D. R., EUCKER, T. P., BELL, J. A., DYBAS, L., MANSFIELD, L. S. & KONKEL, M. E. 2013. The Campylobacter jejuni CiaD effector protein activates MAP kinase signaling pathways and is required for the development of disease. *Cell Commun Signal*, 11, 79.
- SANDIG, H., MCDONALD, J., GILMOUR, J., ARNO, M., LEE, T. H. & COUSINS, D. J. 2009. Fibronectin is a TH1-specific molecule in human subjects. *J Allergy Clin Immunol*, 124, 528-35, 535 e1-5.
- SANGER, J. M., CHANG, R., ASHTON, F., KAPER, J. B. & SANGER, J. W. 1996. Novel form of actin-based motility transports bacteria on the surfaces of infected cells. *Cell Motil Cytoskeleton*, 34, 279-87.
- SANSONETTI, P. J. & MEDZHITOV, R. 2009. Learning tolerance while fighting ignorance. *Cell*, 138, 416-20.
- SCHILDT, M., SAVOLAINEN, S. & HANNINEN, M. L. 2006. Long-lasting Campylobacter jejuni contamination of milk associated with gastrointestinal illness in a farming family. *Epidemiol Infect*, 134, 401-5.
- SCHLAEPFER, D. D., HAUCK, C. R. & SIEG, D. J. 1999. Signaling through focal adhesion kinase. *Prog Biophys Mol Biol*, 71, 435-78.
- SCHLEGEL, R., DICKSON, R. B., WILLINGHAM, M. C. & PASTAN, I. H. 1982. Amantadine and dansylcadaverine inhibit vesicular stomatitis virus uptake and receptor-mediated endocytosis of alpha 2-macroglobulin. *Proc Natl Acad Sci U S A*, 79, 2291-5.
- SCHMID-HEMPEL, P. & FRANK, S. A. 2007. Pathogenesis, virulence, and infective dose. *PLoS Pathog*, 3, 1372-3.
- SCHMID, G. P., SCHAEFER, R. E., PLIKAYTIS, B. D., SCHAEFER, J. R., BRYNER, J. H., WINTERMEYER, L. A. & KAUFMANN, A. F. 1987. A one-year study of endemic campylobacteriosis in a midwestern city: association with consumption of raw milk. *J Infect Dis*, 156, 218-22.
- SCHOREY, J. S., HOLSTI, M. A., RATLIFF, T. L., ALLEN, P. M. & BROWN, E. J. 1996. Characterization of the fibronectin-attachment protein of Mycobacterium avium reveals a fibronectin-binding motif conserved among mycobacteria. *Mol Microbiol*, 21, 321-9.
- SCHUCK, S. & SIMONS, K. 2004. Polarized sorting in epithelial cells: raft clustering and the biogenesis of the apical membrane. *J Cell Sci*, 117, 5955-64.
- SCHULLER, S. & PHILLIPS, A. D. 2010. Microaerobic conditions enhance type III secretion and adherence of enterohaemorrhagic Escherichia coli to polarized human intestinal epithelial cells. *Environ Microbiol*, Epub.
- SCHWARZ-LINEK, U., HOOK, M. & POTTS, J. R. 2004. The molecular basis of fibronectin-mediated bacterial adherence to host cells. *Mol Microbiol*, 52, 631-41.

- SCOTT, A. E., TIMMS, A. R., CONNERTON, P. L., LOC CARRILLO, C., ADZFA RADZUM, K. & CONNERTON, I. F. 2007. Genome dynamics of *Campylobacter jejuni* in response to bacteriophage predation. *PLoS Pathog*, 3, e119.
- SCOTT, N. E., NOTHAFT, H., EDWARDS, A. V. G., LABBATE, M., DJORDJEVIC, S. P., LARSEN, M. R., SZYMANSKI, C. M. & CORDWELL, S. J. 2012. Modification of the *Campylobacter jejuni* N-Linked Glycan by EptC Protein-mediated Addition of Phosphoethanolamine. *Journal of Biological Chemistry*, 287, 29384-29396.
- SEBALD, M. & VERON, M. 1963. [Base DNA Content and Classification of Vibrios]. *Ann Inst Pasteur (Paris)*, 105, 897-910.
- SEDGWICK, J. D., RIMINTON, D. S., CYSTER, J. G. & KORNER, H. 2000. Tumor necrosis factor: a master-regulator of leukocyte movement. *Immunol Today*, 21, 110-3.
- SENIOR, N. J., BAGNALL, M. C., CHAMPION, O. L., REYNOLDS, S. E., LA RAGIONE, R. M., WOODWARD, M. J., SALGUERO, F. J. & TITBALL, R. W. 2011. *Galleria mellonella* as an infection model for *Campylobacter jejuni* virulence. *J Med Microbiol*, 60, 661-9.
- SENOK, A. C. & BOTTA, G. A. 2009. *Campylobacter* enteritis in the Arabian Gulf. *J Infect Dev Ctries*, 3, 74-82.
- SEVEAU, S., BIERNE, H., GIROUX, S., PREVOST, M. C. & COSSART, P. 2004. Role of lipid rafts in E-cadherin-- and HGF-R/Met--mediated entry of *Listeria monocytogenes* into host cells. *J Cell Biol*, 166, 743-53.
- SHENOY, A. R. & VISWESWARIAH, S. S. 2003. Site-directed mutagenesis using a single mutagenic oligonucleotide and DpnI digestion of template DNA. *Anal Biochem*, 319, 335-6.
- SIEGISMUND, A. M., KONKEL, M. E., KLENA, J. D. & MIXTER, P. F. 2004. *Campylobacter jejuni* infection of differentiated THP-1 macrophages results in interleukin 1 beta release and caspase-1-independent apoptosis. *Microbiology*, 150, 561-9.
- SIGNAS, C., RAUCCI, G., JONSSON, K., LINDGREN, P. E., ANANTHARAMAIAH, G. M., HOOK, M. & LINDBERG, M. 1989. Nucleotide sequence of the gene for a fibronectin-binding protein from *Staphylococcus aureus*: use of this peptide sequence in the synthesis of biologically active peptides. *Proc Natl Acad Sci U S A*, 86, 699-703.
- SIKIC POGACAR, M., RUBESA MIHALJEVIC, R., KLANCNIK, A., BRUMINI, G., ABRAM, M. & SMOLE MOZINA, S. 2009. Survival of stress exposed *Campylobacter jejuni* in the murine macrophage J774 cell line. *Int J Food Microbiol*, 129, 68-73.
- SIMO, P., SIMON-ASSMANN, P., BOUZIGES, F., LEBERQUIER, C., KEDINGER, M., EKBLUM, P. & SOROKIN, L. 1991. Changes in the expression of laminin during intestinal development. *Development*, 112, 477-87.
- SIMONS, K. & IKONEN, E. 1997. Functional rafts in cell membranes. *Nature*, 387, 569-72.
- SINGH, U. S., PAN, J., KAO, Y. L., JOSHI, S., YOUNG, K. L. & BAKER, K. M. 2003. Tissue transglutaminase mediates activation of RhoA and MAP kinase pathways during retinoic acid-induced neuronal differentiation of SH-SY5Y cells. *J Biol Chem*, 278, 391-9.
- SKIRROW, M. B. 2006. John McFadyean and the centenary of the first isolation of *Campylobacter* species. *Clin Infect Dis*, 43, 1213-7.

- SKIRROW MB, B. M. 2000. Clinical aspects of Campylobacter infection. *In:* NACHAMKIN I, B. M. (ed.) *Campylobacter*. Washington: ASM Press.
- SLEE, K. 1972. Human vibriosis and endogenous infection? *Australian Journal Of Medical Technology*, 3, 7.
- SMALL, J. V. 1994. Lamellipodia architecture: actin filament turnover and the lateral flow of actin filaments during motility. *Semin Cell Biol*, 5, 157-63.
- SNOECK, V., GODDEERIS, B. & COX, E. 2005. The role of enterocytes in the intestinal barrier function and antigen uptake. *Microbes Infect*, 7, 997-1004.
- SONG, Y. C., JIN, S., LOUIE, H., NG, D., LAU, R., ZHANG, Y., WEERASEKERA, R., AL RASHID, S., WARD, L. A., DER, S. D. & CHAN, V. L. 2004. FlaC, a protein of Campylobacter jejuni TGH9011 (ATCC43431) secreted through the flagellar apparatus, binds epithelial cells and influences cell invasion. *Molecular Microbiology*, 53, 541-553.
- SPOHN, G. & SCARLATO, V. 1999. Motility of Helicobacter pylori is coordinately regulated by the transcriptional activator FlgR, an NtrC homolog. *J Bacteriol*, 181, 593-9.
- STAHL, P. & SCHWARTZ, A. L. 1986. Receptor-mediated endocytosis. *J Clin Invest*, 77, 657-62.
- STANFIELD, J. T., MCCARDELL, B. A. & MADDEN, J. M. 1987. Campylobacter diarrhea in an adult mouse model. *Microb Pathog*, 3, 155-65.
- STEARNS, T. 1995. Green fluorescent protein. The green revolution. *Curr Biol*, 5, 262-4.
- STEPHENSON, H. N., JOHN, C. M., NAZ, N., GUNDOGDU, O., DORRELL, N., WREN, B. W., JARVIS, G. A. & BAJAJ-ELLIOTT, M. 2013. Campylobacter jejuni lipooligosaccharide sialylation, phosphorylation, and amide/ester linkage modifications fine-tune human Toll-like receptor 4 activation. *J Biol Chem*, 288, 19661-72.
- STOSSEL, T. P. 1993. On the crawling of animal cells. *Science*, 260, 1086-94.
- SUN, X., LIU, B., SARTOR, R. B. & JOBIN, C. 2013. Phosphatidylinositol 3-kinase-gamma signaling promotes Campylobacter jejuni-induced colitis through neutrophil recruitment in mice. *J Immunol*, 190, 357-65.
- SUZUKI, T., MATSUZAKI, T., HAGIWARA, H., AOKI, T. & TAKATA, K. 2007. Recent advances in fluorescent labeling techniques for fluorescence microscopy. *Acta Histochem Cytochem*, 40, 131-7.
- SWANSON, J. A. & WATTS, C. 1995. Macropinocytosis. *Trends Cell Biol*, 5, 424-8.
- SZYMANSKI, C. M., BURR, D. H. & GUERRY, P. 2002. Campylobacter protein glycosylation affects host cell interactions. *Infect Immun*, 70, 2242-4.
- SZYMANSKI, C. M., KING, M., HAARDT, M. & ARMSTRONG, G. D. 1995. Campylobacter jejuni motility and invasion of Caco-2 cells. *Infect Immun*, 63, 4295-300.
- SZYMANSKI, C. M., LOGAN, S. M., LINTON, D. & WREN, B. W. 2003a. Campylobacter--a tale of two protein glycosylation systems. *Trends Microbiol*, 11, 233-8.
- SZYMANSKI, C. M., MICHAEL, F. S., JARRELL, H. C., LI, J., GILBERT, M., LAROCQUE, S., VINOGRADOV, E. & BRISSON, J. R. 2003b. Detection of conserved N-linked glycans and phase-variable lipooligosaccharides and capsules from campylobacter cells by mass spectrometry and high resolution magic angle spinning NMR spectroscopy. *J Biol Chem*, 278, 24509-20.

- SZYMANSKI, C. M., YAO, R., EWING, C. P., TRUST, T. J. & GUERRY, P. 1999. Evidence for a system of general protein glycosylation in *Campylobacter jejuni*. *Mol Microbiol*, 32, 1022-30.
- TAKATA, K., HIRANO, H. & KASAHARA, M. 1997. Transport of glucose across the blood-tissue barriers. *Int Rev Cytol*, 172, 1-53.
- TALAY, S. R., ZOCK, A., ROHDE, M., MOLINARI, G., OGGIONI, M., POZZI, G., GUZMAN, C. A. & CHHATWAL, G. S. 2000. Co-operative binding of human fibronectin to SfbI protein triggers streptococcal invasion into respiratory epithelial cells. *Cell Microbiol*, 2, 521-35.
- TAYLOR, D. E. 1992. Genetics of *Campylobacter* and *Helicobacter*. *Annu Rev Microbiol*, 46, 35-64.
- TAYLOR, D. E., NEWELL, D. G. & PEARSON, A. D. 1983. Incidence of plasmid DNA in strains of *Campylobacter jejuni* isolated from stool specimens at 37 C and 43 C. *J Infect Dis*, 147, 965-6.
- THE, T. H. & FELTKAMP, T. E. 1970. Conjugation of fluorescein isothiocyanate to antibodies. I. Experiments on the conditions of conjugation. *Immunology*, 18, 865-73.
- THIBAUT, P., LOGAN, S. M., KELLY, J. F., BRISSON, J.-R., EWING, C. P., TRUST, T. J. & GUERRY, P. 2001. Identification of the Carbohydrate Moieties and Glycosylation Motifs in *Campylobacter jejuni* Flagellin. *Journal of Biological Chemistry*, 276, 34862-34870.
- TOTTEN, P. A., LARA, J. C. & LORY, S. 1990. The rpoN gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. *J Bacteriol*, 172, 389-96.
- TRIEU-CUOT, P., GERBAUD, G., LAMBERT, T. & COURVALIN, P. 1985. In vivo transfer of genetic information between gram-positive and gram-negative bacteria. *EMBO J*, 4, 3583-7.
- VALDIVIA, R. H. & FALKOW, S. 1997. Fluorescence-Based Isolation of Bacterial Genes Expressed Within Host Cells. *Science*, 277, 2007-2011.
- VALDIVIA, R. H., HROMOCKYJ, A. E., MONACK, D., RAMAKRISHNAN, L. & FALKOW, S. 1996. Applications for green fluorescent protein (GFP) in the study of host-pathogen interactions. *Gene*, 173, 47-52.
- VALIRON, O., CAUDRON, N. & JOB, D. 2001. Microtubule dynamics. *Cell Mol Life Sci*, 58, 2069-84.
- VAN ALPHEN, L. B., BLEUMINK-PLUYM, N. M., ROCHAT, K. D., VAN BALKOM, B. W., WOSTEN, M. M. & VAN PUTTEN, J. P. 2008. Active migration into the subcellular space precedes *Campylobacter jejuni* invasion of epithelial cells. *Cell Microbiol*, 10, 53-66.
- VAN BELKUM, A., VAN DEN BRAAK, N., GODSCHALK, P., ANG, W., JACOBS, B., GILBERT, M., WAKARCHUK, W., VERBRUGH, H. & ENDTZ, H. 2001. A *Campylobacter jejuni* gene associated with immune-mediated neuropathy. *Nat Med*, 7, 752-753.
- VAN PUTTEN, J. P., VAN ALPHEN, L. B., WOSTEN, M. M. & DE ZOETE, M. R. 2009. Molecular mechanisms of campylobacter infection. *Curr Top Microbiol Immunol*, 337, 197-229.
- VAN SPREEUWEL, J. P., DUURSMA, G. C., MEIJER, C. J., BAX, R., ROSEKRANS, P. C. & LINDEMAN, J. 1985. *Campylobacter colitis*: histological immunohistochemical and ultrastructural findings. *Gut*, 26, 945-51.
- VAN VLIET, A. H., WOOLDRIDGE, K. G. & KETLEY, J. M. 1998. Iron-responsive gene regulation in a campylobacter *jejuni* fur mutant. *J Bacteriol*, 180, 5291-8.

- VARKI, A. & GAGNEUX, P. 2012. Multifarious roles of sialic acids in immunity. *Ann NY Acad Sci*, 1253, 16-36.
- VEIGA, E., GUTTMAN, J. A., BONAZZI, M., BOUCROT, E., TOLEDO-ARANA, A., LIN, A. E., ENNINGA, J., PIZARRO-CERDA, J., FINLAY, B. B., KIRCHHAUSEN, T. & COSSART, P. 2007. Invasive and adherent bacterial pathogens co-Opt host clathrin for infection. *Cell Host Microbe*, 2, 340-51.
- VETTER, I. R. & WITTINGHOFER, A. 2001. The guanine nucleotide-binding switch in three dimensions. *Science*, 294, 1299-304.
- VIDAKOVICS, M. L., JENDHOLM, J., MORGELIN, M., MANSSON, A., LARSSON, C., CARDELL, L. O. & RIESBECK, K. 2010. B cell activation by outer membrane vesicles--a novel virulence mechanism. *PLoS Pathog*, 6, e1000724.
- VINZENT, R., J.DUMAS, N.PICARD 1947. Septicemie grave au cours de la grossesse due a un vibron: abortement consecutif. *Bull. Acad. Natl. Med. Paris*, 131, 90-92.
- VISAI, L., BOZZINI, S., PETERSEN, T. E., SPECIALE, L. & SPEZIALE, P. 1991. Binding sites in fibronectin for an enterotoxigenic strain of E. coli B342289c. *FEBS Lett*, 290, 111-4.
- WALKER, R. I., CALDWELL, M. B., LEE, E. C., GUERRY, P., TRUST, T. J. & RUIZ-PALACIOS, G. M. 1986. Pathophysiology of Campylobacter enteritis. *Microbiol Rev*, 50, 81-94.
- WALKER, R. I., SCHMAUDER-CHOCK, E. A., PARKER, J. L. & BURR, D. 1988. Selective association and transport of Campylobacter jejuni through M cells of rabbit Peyer's patches. *Can J Microbiol*, 34, 1142-7.
- WANG, J. & LIU, X. J. 2003. A G protein-coupled receptor kinase induces Xenopus oocyte maturation. *J Biol Chem*, 278, 15809-14.
- WASSENAAR, T. M. & BLASER, M. J. 1999. Pathophysiology of Campylobacter jejuni infections of humans. *Microbes Infect*, 1, 1023-33.
- WASSENAAR, T. M., BLEUMINK-PLUYM, N. M. & VAN DER ZEIJST, B. A. 1991. Inactivation of Campylobacter jejuni flagellin genes by homologous recombination demonstrates that flaA but not flaB is required for invasion. *EMBO J*, 10, 2055-61.
- WASSENAAR, T. M., ENGELSKIRCHEN, M., PARK, S. & LASTOVICA, A. 1997. Differential uptake and killing potential of Campylobacter jejuni by human peripheral monocytes/macrophages. *Med Microbiol Immunol*, 186, 139-44.
- WATARAI, M., FUNATO, S. & SASAKAWA, C. 1996. Interaction of Ipa proteins of Shigella flexneri with alpha5beta1 integrin promotes entry of the bacteria into mammalian cells. *J Exp Med*, 183, 991-9.
- WATSON, R. & GALAN, J. 2008a. Campylobacter jejuni survives within epithelial cells by avoiding delivery to lysosomes. *PLoS Pathog*, 4, e14.
- WATSON, R. O. & GALAN, J. E. 2005. Signal transduction in Campylobacter jejuni-induced cytokine production. *Cell Microbiol*, 7, 655-65.
- WATSON, R. O. & GALAN, J. E. 2008b. Campylobacter jejuni survives within epithelial cells by avoiding delivery to lysosomes. *PLoS Pathog*, 4, e14.
- WATSON, R. O., NOVIK, V., HOFREUTER, D., LARA-TEJERO, M. & GALAN, J. E. 2007. A MyD88-deficient mouse model reveals a role for Nramp1 in Campylobacter jejuni infection. *Infect Immun*, 75, 1994-2003.
- WEBB, S. A. & KAHLER, C. M. 2008. Bench-to-bedside review: Bacterial virulence and subversion of host defences. *Crit Care*, 12, 234.

- WEHKAMP, J., HARDER, J., WEICHENTHAL, M., MUELLER, O., HERRLINGER, K. R., FELLERMANN, K., SCHROEDER, J. M. & STANGE, E. F. 2003. Inducible and constitutive beta-defensins are differentially expressed in Crohn's disease and ulcerative colitis. *Inflamm Bowel Dis*, 9, 215-23.
- WEI, H., ZHANG, Y., FAN, Z.-Z., GE, H.-Y., ARENDT-NIELSEN, L., JIANG, H., YAO, W. & YUE, S.-W. 2013. Effects of colchicine-induced microtubule depolymerization on TRPV4 in rats with chronic compression of the dorsal root ganglion. *Neuroscience Letters*, 534, 344-350.
- WELLS, C. L., VAN DE WESTERLO, E. M., JECHOREK, R. P., HAINES, H. M. & ERLANDSEN, S. L. 1998. Cytochalasin-induced actin disruption of polarized enterocytes can augment internalization of bacteria. *Infect Immun*, 66, 2410-9.
- WENMAN, W. M., CHAI, J., LOUIE, T. J., GOUDREAU, C., LIOR, H., NEWELL, D. G., PEARSON, A. D. & TAYLOR, D. E. 1985. Antigenic analysis of Campylobacter flagellar protein and other proteins. *J Clin Microbiol*, 21, 108-12.
- WHITCHURCH, C. B., TOLKER-NIELSEN, T., RAGAS, P. C. & MATTICK, J. S. 2002. Extracellular DNA required for bacterial biofilm formation. *Science*, 295, 1487.
- WILSON, D. J., GABRIEL, E., LEATHERBARROW, A. J., CHEESBROUGH, J., GEE, S., BOLTON, E., FOX, A., FEARNHEAD, P., HART, C. A. & DIGGLE, P. J. 2008. Tracing the source of campylobacteriosis. *PLoS Genet*, 4, e1000203.
- WILSON, M. 2002. *Bacterial adhesion to host tissues: mechanisms and consequences*, United States, Cambridge University Press.
- WINE, E., CHAN, V. L. & SHERMAN, P. M. 2008. Campylobacter jejuni mediated disruption of polarized epithelial monolayers is cell-type specific, time dependent, and correlates with bacterial invasion. *Pediatr Res*, 64, 599-604.
- WINSOR, B. & SCHIEBEL, E. 1997. Review: an overview of the Saccharomyces cerevisiae microtubule and microfilament cytoskeleton. *Yeast*, 13, 399-434.
- WOOLDRIDGE, K., WILLIAMS, P. & KETLEY, J. 1996a. Host signal transduction and endocytosis of Campylobacter jejuni. *Microb Pathog*, 21, 299 - 305.
- WOOLDRIDGE, K. G. & KETLEY, J. M. 1997. Campylobacter-host cell interactions. *Trends Microbiol*, 5, 96-102.
- WOOLDRIDGE, K. G., WILLIAMS, P. H. & KETLEY, J. M. 1996b. Host signal transduction and endocytosis of Campylobacter jejuni. *Microb Pathog*, 21, 299-305.
- WOSTEN, M. M., BOEVE, M., KOOT, M. G., VAN NUENEN, A. C. & VAN DER ZEIJST, B. A. 1998. Identification of Campylobacter jejuni promoter sequences. *J Bacteriol*, 180, 594-9.
- WOSTEN, M. M., WAGENAAR, J. A. & VAN PUTTEN, J. P. 2004. The FlgS/FlgR two-component signal transduction system regulates the fla regulon in Campylobacter jejuni. *J Biol Chem*, 279, 16214-22.
- WÖSTEN, M. M. S. M., VAN DIJK, L., VEENENDAAL, A. K. J., DE ZOETE, M. R., BLEUMINK-PLUIJM, N. M. C. & VAN PUTTEN, J. P. M. 2010. Temperature-dependent FlgM/FliA complex formation regulates Campylobacter jejuni flagella length. *Molecular Microbiology*, 75, 1577-1591.
- XU, J., BAE, E., ZHANG, Q., ANNIS, D. S., ERICKSON, H. P. & MOSHER, D. F. 2009. Display of cell surface sites for fibronectin assembly is modulated by cell adherence to (1)F3 and C-terminal modules of fibronectin. *PLoS One*, 4, e4113.
- YAMADA, R. O. H. A. K. M. 2012. *Extracellular Matrix Biology*. Cold Spring Harbour, NY: CSHL Press.

- YAO, R., BURR, D. H., DOIG, P., TRUST, T. J., NIU, H. & GUERRY, P. 1994. Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni*: the role of motility in adherence and invasion of eukaryotic cells. *Mol Microbiol*, 14, 883-93.
- YAO, R., BURR, D. H. & GUERRY, P. 1997. CheY-mediated modulation of *Campylobacter jejuni* virulence. *Mol Microbiol*, 23, 1021-31.
- YAVLOVICH, A., KATZENELL, A., TARSHIS, M., HIGAZI, A. A. & ROTTEM, S. 2004. Mycoplasma fermentans binds to and invades HeLa cells: involvement of plasminogen and urokinase. *Infect Immun*, 72, 5004-11.
- YOKOSEKI, T., KUTSUKAKE, K., OHNISHI, K. & IINO, T. 1995. Functional analysis of the flagellar genes in the *fliD* operon of *Salmonella typhimurium*. *Microbiology*, 141 (Pt 7), 1715-22.
- YOSHIDA, S. & SASAKAWA, C. 2003. Exploiting host microtubule dynamics: a new aspect of bacterial invasion. *Trends Microbiol*, 11, 139-43.
- YOUNG, G. M., SCHMIEL, D. H. & MILLER, V. L. 1999. A new pathway for the secretion of virulence factors by bacteria: The flagellar export apparatus functions as a protein-secretion system. *Proceedings of the National Academy of Sciences*, 96, 6456-6461.
- YOUNG, K. T., DAVIS, L. M. & DIRITA, V. J. 2007. *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat Rev Microbiol*, 5, 665-79.
- YRIOS, J. W. & BALISH, E. 1985. Colonization and pathogenesis of *Campylobacter* spp. in athymic and euthymic germfree mice. *Prog Clin Biol Res*, 181, 199-202.
- YRLID, U., SVENSSON, M., HAKANSSON, A., CHAMBERS, B. J., LJUNGGREN, H. G. & WICK, M. J. 2001. In vivo activation of dendritic cells and T cells during *Salmonella enterica* serovar Typhimurium infection. *Infect Immun*, 69, 5726-35.
- YUKI, N. 1997. Molecular mimicry between gangliosides and lipopolysaccharides of *Campylobacter jejuni* isolated from patients with Guillain-Barre syndrome and Miller Fisher syndrome. *J Infect Dis*, 176 Suppl 2, S150-3.
- ZAALOUK, T. K., BAJAJ-ELLIOTT, M., GEORGE, J. T. & MCDONALD, V. 2004. Differential regulation of beta-defensin gene expression during *Cryptosporidium parvum* infection. *Infect Immun*, 72, 2772-9.
- ZAAS, D. W., DUNCAN, M., RAE WRIGHT, J. & ABRAHAM, S. N. 2005. The role of lipid rafts in the pathogenesis of bacterial infections. *Biochim Biophys Acta*, 1746, 305-13.
- ZAMIR, E. & GEIGER, B. 2001. Components of cell-matrix adhesions. *J Cell Sci*, 114, 3577-9.
- ZILBAUER, M., DORRELL, N., BOUGHAN, P. K., HARRIS, A., WREN, B. W., KLEIN, N. J. & BAJAJ-ELLIOTT, M. 2005. Intestinal innate immunity to *Campylobacter jejuni* results in induction of bactericidal human beta-defensins 2 and 3. *Infect Immun*, 73, 7281-9.
- ZILBAUER, M., DORRELL, N., ELMI, A., LINDLEY, K. J., SCHULLER, S., JONES, H. E., KLEIN, N. J., NUNEZ, G., WREN, B. W. & BAJAJ-ELLIOTT, M. 2007. A major role for intestinal epithelial nucleotide oligomerization domain 1 (NOD1) in eliciting host bactericidal immune responses to *Campylobacter jejuni*. *Cell Microbiol*, 9, 2404-16.
- ZILBAUER, M., DORRELL, N., WREN, B. W. & BAJAJ-ELLIOTT, M. 2008. *Campylobacter jejuni*-mediated disease pathogenesis: an update. *Trans R Soc Trop Med Hyg*, 102, 123-9.

- ZIPRIN, R. L., YOUNG, C. R., BYRD, J. A., STANKER, L. H., HUME, M. E., GRAY, S. A., KIM, B. J. & KONKEL, M. E. 2001. Role of *Campylobacter jejuni* potential virulence genes in cecal colonization. *Avian Dis*, 45, 549-57.
- ZIPRIN, R. L., YOUNG, C. R., STANKER, L. H., HUME, M. E. & KONKEL, M. E. 1999. The absence of cecal colonization of chicks by a mutant of *Campylobacter jejuni* not expressing bacterial fibronectin-binding protein. *Avian Dis*, 43, 586-9.