Biogenesis of the outer membrane of *Campylobacter jejuni*

A thesis submitted in part fulfilment for the degree of Doctor of Philosophy.

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September 2013

Summary

The food-borne pathogen *Campylobacter jejuni* has a complex outer membrane (OM) that acts as the primary interface with the host and has diverse functions in cell adhesion, interaction with host immune system, cell signalling and as a barrier against antimicrobial compounds. The periplasmic translocation and OM assembly mechanism of the OM proteins (OMPs) is not completely understood, but depends on periplasmic chaperones. This study was focused on the pathway of OMPs biogenesis in the periplasm of *C. jejuni*. The function of two periplasmic proteins; Cj1289 and Cj0694 that are proposed to have a chaperone role in *C. jejuni* NCTC 11168 was investigated based on phenotypic and biochemical evidence. The Cj1289 and Cj0694 dependency on OMPs is shown by the defective growth and reduced membrane integrity against antimicrobial peptides and detergents in the absence of these proteins. Evidence that Cj1289 and Cj0694 play a periplasmic chaperone role in *C. jejuni* was suggested by an altered abundance of OMPs in the *cj0694* and *cj1289* null mutants. Proteomic analysis of OM and periplasmic preparations was used to identify client proteins.

Biochemical characterisation of Cj0694 based on sequence similarity to PpiD, an inner membrane-anchored protein in *Escherichia coli* that has peptydyl-prolyl *cis/trans* isomerase (PPIase) function, revealed that purified recombinant Cj0694 is an active PPIase as shown by a significant Cj0694-dependent acceleration of the refolding rate of ribonuclease T_1. Also, Cj0694 was found to be an active holdase-type chaperone that can inhibit protein aggregation, indicating that it has a more general role in the periplasm for both periplasmic and OMPs, consistent with previous findings of PpiD. Attempts to obtain crystals of Cj0694 for structure determination were not successful. However, cloning, over-production and purification using different expression systems are reported and will be useful for future studies.

Lipid asymmetry in the OM of Gram-negative bacteria increases its barrier function, and is known to be maintained by the Mla system. The role of the Mla was shown by the defective growth and reduced membrane integrity of *mla* null mutants under stress conditions. Moreover, loss of membrane integrity was suggested by significant reduction in biofilm formation and increased motility in the absence of Mla proteins. These results provide a clearer understanding of the nature of the periplasmic mechanism involved in OM biogenesis and maintenance of its integrity and barrier function.

Acknowledgement

With the grace and mercy of Allah, this work is completed.

Firstly, I would like to thank my supervisor Professor David Kelly for his invaluable support, guidance and encouragements during my study under his supervision. Thank you Dave for being not only a supervisor, but a real friend whose advices and enthusiasm will remain with me as guidance for my future. It was an honour to have the opportunity to work for you, and the completion of this research would not be possible without your support.

I would like to thank my wife Sara for her kind patience and honest support during my entire study. Her unforgettable enthusiasm and emotional care which usually makes me smile and happy were a big part of the completion of my study. Also I would like to thank my lovely son Faris whose smile and happiness usually add so much fun to my life, and were great support during hard times, and also for been patient during busy times in this study.

Above all, I would like to express my great thanks to my parents for their guidance during my entire life, and for their honest prays to complete and succeed. My life could not be perfect without your support. I would not be where I am today without you all.

To all my past and present friends in the lab, Dr Ed Guccione, Dr Chatchawal Pansopa, Dr John Smart, Dr Andy Hichcock, Dr Rob Howlett, Mr John Kendall, Mr Yang-Wei Liu, Mr Rob Salmon, Ms Hala Alhaidari, Mrs Reem Farsi, Mrs Abrar Akbar and Mr Mike White. Thank you all for your kind assistance and cooperation. It has been a pleasure to work with you all. You are a great example of successful team.

To everyone in the department who collaborated and helped me during my work. In particular, Dr John Rafferty, Dr Svetlana Sedelnikova for the crystallography assistance and Dr Arthur Moir for protein sequencing. To the collaborators from the Institute of Food Research (IFR) in Norwich, Dr Francis Mulholland for proteomics analysis and Dr Arnoud van Vliet for providing mutants constructs.

Finally, I would like to thank my brothers Haytham and Ghassan and my sister Ghadah for their prays, guidance and support during my life before and during my study.

This thesis is dedicated to my father who passed away during this study, may Allah bless him and grant his soul the paradise. Also it is dedicated to my expected baby Qusai, who I cannot wait to see him.

Shadi Zakai
September 2013

Presentations

S. Zakai and D. J. Kelly (2011). Biogenesis of the outer membrane of *Campyobacter jejuni. CampylobacterUK meeting 2011, London School of Hyagene and Tropical Medicine, London, UK.* Poster presentation.

S. Zakai and D. J. Kelly (2013). *Campylobacter jejuni* at the host pathogen interface: The role of periplasmic chaperones in the biogenesis of outer membrane. *Society for General Microbiology, Manchester, UK.* Poster presentation.

S. Zakai and D. J. Kelly (2013). Involvement of the Mla transport system in the maintanence of outer membrane function in *Campylobacter jejuni. CHRO-2013, The University of Aberdeen, Aberdeen, UK.* Poster presentation. (Poster prize awarded).

S. Zakai, F. Mulholland and D. J. Kelly (2013). *Campylobacter jejuni* at the host pathogen interface: The role of periplasmic chaperones in the biogenesis of outer membrane. *CHRO-2013, The University of Aberdeen, Aberdeen, UK.* Oral presentation.

List of abbreviations

Ab^R	antibiotic resistance
ABC	ATP-binding cassette
AhpC	alkyl hydroperoxide reductase
Amp	ampicillin
AMPs	antimicrobial peptides
ANOVA	analysis of variance
APS	ammonium persulphate
APS	ammonium persulphate
ATP	adenine triphosphate
BAM	β-barrel proteins Assembly Machinery
bp	base pairs
BHI	brain Heart Infusion
BSA	bovine serum albumin
CAMPs	cationic antimicrobial peptides
cat	chloramphenicol acetyl transferase
CDSs	coding sequences
CDT	cytolethal distending toxin
CFE	cell tree extract
CFU	colony forming units
CgpA	*Campylobacter* glycoprotein A
Cia	*Campylobacter* invasion antigen
CO_2	carbon dioxide
CspA	cold shock protein
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemi-luminescence
EDTA	ethylenediamine tetra-acetic acid
FeEnt	ferric-enterobactin
FhuA	ferric hydroxamate uptake
FKBP	FK-binding proteins
Fur	ferric uptake regulator
GBS	Guillain-Barré syndrome
GdnHCl	guanidine-hydrochloride
GPDs	glycerophosphodiesters
H_2O_2	hydrogen peroxide
hCAP	human cationic antimicrobial protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIC	hydrophobic interaction chromatography
IM	inner membrane
IPTG	isopropyl β-D-1-thiogalactopyranoside

ISA	isothermal assembly
JlpA	jejuni lipoprotein A
kan	kanamycin
KatA	catalase
kb	kilobase
kDa	kiloDalton
LB	Luria-Bertani
LegAm	ligionamenic acid
LOS	lipooligosaccharides
LPS	lipopolysaccharides
lyso-PLs	lysophospholipids
MEM	minimum Essential Media
Mfr	methylmenaquinol:fumarate reductase
μg	microgram
MH	Muller-Hinton
MHS	Muller-Hinton with 20 mM serine
μL	microliter
Mla	maintenance of lipid asymmetry
μM	micromolar
mM	millimolar
MOMP	major outer membrane protein
MW	molecular weight
MWCO	molecular weight cut off
O_2	oxygen
OM	outer membrane
OMPs	outer membrane proteins
OMVs	outer membrane vesicles
ORF	open reading frame
PAGE	polyacrylamide electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PerR	peroxide stress regulator
pH	hydrogen potential
Pi	isoelectric point
PldA	phospholipase A
PLs	phospholipids
PPIase	peptydyl-prolyl-*cis/trans* isomerase
POTRA	polypeptide transport-associated
PseAm	pseudaminic acid
RBS	ribosome binding site
RCM-RNase T_1	reduced, carboxy-methylated ribonuclease T_1
RHB	rehydration lysis buffer
RNR	ribonucleotide reductase
ROS	reactive oxygen species

RPM	revolutions per minute
RT	room temperature
RT-PCR	real time polymerase chain reaction
SodB	superoxide dismutase
O_2^-	superoxide radicals
SDS	sodium dodecyl sulphate
SILAC	stable isotope labelling by amino acids in cell culture
SOE	splicing by overlapping extension
SP	signal peptidase
T_3S	type-III secretion
TAE	tris acetate EDTA solution
TCA	tricarboxylic acid
TEMED	N,N,N',N'-tetramethyl- ethane-1,2-diamine
TF	trigger factor
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
UDP	uridine diphosphate
UV	ultraviolet
v/v	concentration, volume/volume
w/v	concentration, weight/volume
xg	multiplied by gravitational force

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1 Introduction

1.1 Taxonomy and general microbiology of *Campylobacter jejuni*

Campylobacter jejuni is a member of the family *Campylobacteraceae* which belongs to the ε-proteobacteria, which includes the genera *Campylobacter*, *Arcobacter* and *Sulfurospirillum*. Typically, these genera are Gram-negative bacteria which require microaerobic conditions to grow, an optimum growth temperature of 30 – 37 °C, and have a low G+C content (29 – 47 mol%) (Selbald and Véron, 1963). *Campylobacter* and *Arcobacter* occur as commensals or parasites in human and domestic animals whereas *Sulfurospirillum* appears as free-living environmental organisms. *Campylobacter* and *Arcobacter* cells have a respiratory and chemoorganotrophic type of metabolism. They acquire energy from amino acids or tricarboxylic acid (TCA) cycle intermediates, rather than carbohydrates, which are neither fermented nor oxidised. Biochemically, they reduce fumarate to succinate, do not react with methyl red, and produce acetoin and indole (Debruyne *et al.*, 2008). Members belonging to the family *Campylobacteraceae* have the following characteristics. Cells are spiral bacilli or curved, S-shaped, of 0.5 – 5 µm long and 0.2 – 0.8 µm wide. They are non-spore forming cells with a typical screw-like motility generated from a single polar flagellum at one or both ends of the cell (Debruyne *et al.*, 2008). The NCTC 11168 strain used first in the *C. jejuni* genome sequencing (Parkhill *et al.*, 2000) possesses different characteristics of the original *C. jejuni* isolates (Figure 1.1). Its motility is less than the original isolate due to loss of spiral shape (Gaynor *et al.*, 2004). When *C. jejuni* is cultured several days *in vitro*, cells undergo morphological alteration from spiral rods to coccoid shape. This morphological alteration occurs in the stationary phase and characterised by loss of culturability (Kelly, 2001). Although these forms of cells are not culturable, they can be viable (Bode *et al.*, 1993, Rollins and Colwell, 1986, Shahamat *et al.*, 1993). However, evidence for the ability of these cells to return to normal spiral shape is still weak. Alternatively, it has been shown that the formation of this coccoid form of cells is a result of a degenerative process resulting from stress damage (Harvey and Leach, 1998).

Up to date, there are 17 species belonging to the genus *Campylobacter* which are mainly commensals in animals and poultry but may be pathogenic to humans

(Debruyne *et al.*, 2008). Among these species, *C. jejuni* and *C. coli* are the most important human enteropathogens so far. They only differ by their ability to hydrolyse hippurate, where *C. coli* is unable to do so. Nevertheless, hippurate-negative *C. jejuni* subsp. *jejuni* have also been reported, making it difficult to distinguish the two species (Debruyne *et al.*, 2008). Alternatively, genotypic methods have been developed to differentiate between the two subspecies (Jensen *et al.*, 2005, Mandrell *et al.*, 2005, Nayak *et al.*, 2005, Sails *et al.*, 2001).

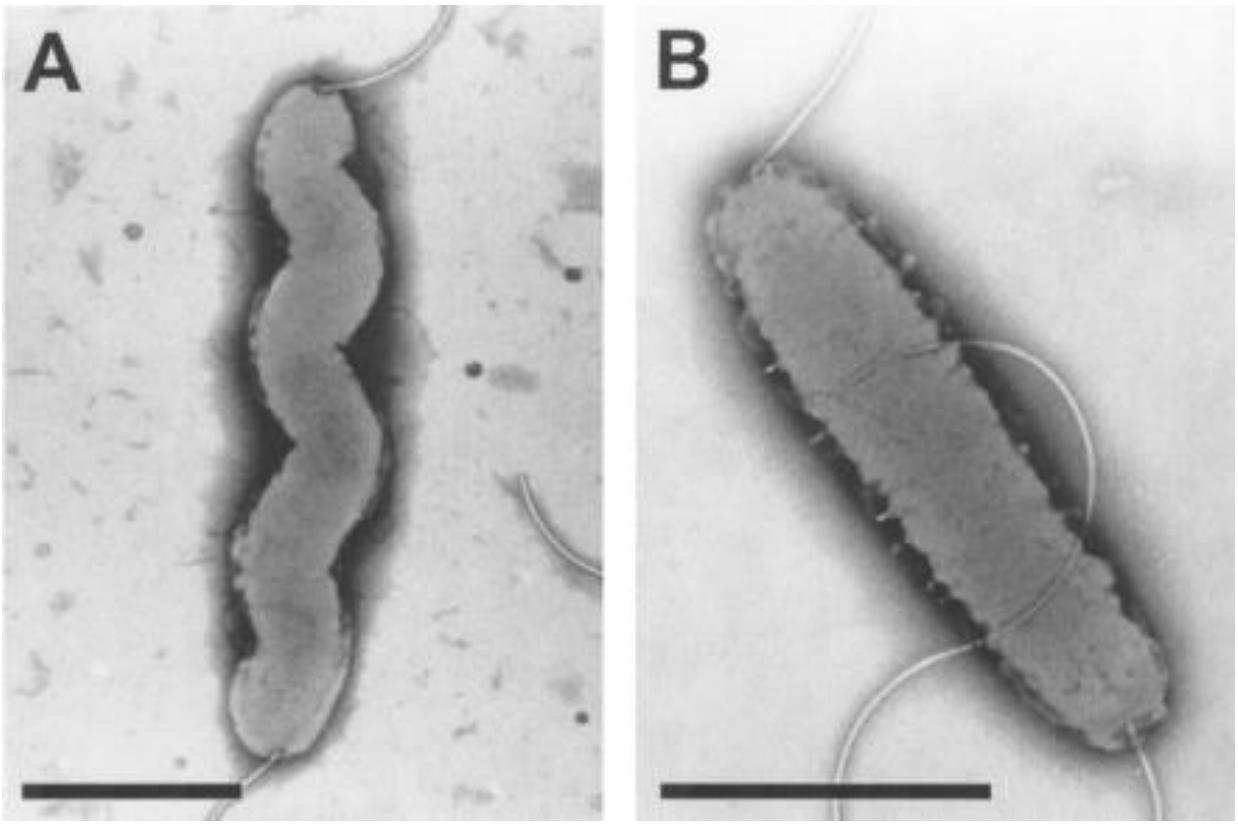


Figure 1.1 *C. jejuni* different morphologies. A) The original *C. jejuni* 11168 clinical isolate with spiral shape. **B)** The reference strain NCTC 11168 used in genome sequencing project with loss of spiral shape. Both strains were stained with negative stain and bars represent 1 µm. picture from Gaynor *et al.*, 2004.

1.2 Genomic analysis of *Campylobacter jejuni* strains

The complete genome sequence of *Campylobacter jejuni* was first published in 2000 by Parkhill *et al* and has then been re-annotated and re-analysed by Gundogdu *et al* in 2007, and since that time, numerous biological and chemical mechanisms have been understood. The published genome sequence of *C. jejuni* strain NCTC 11168, which is considered as wild-type strain in most research of *C. jejuni* shows a relatively small genome. It consists of a circular chromosome of 1,641,481 base pairs (bp) with a low G+C content (30.6 %), and it has 1,643 predicted coding sequences (CDSs) in which at least 20 represents pseudogenes, as well as 54 stable RNA species (Parkhill *et al.*, 2000). The average gene length is approximately 948 bp which represents a typical size of bacterial gene. However, when considering the size of the *C. jejuni* chromosome and the number of its CDSs in which 94.3 % of the genome codes for proteins, this makes it the most condensed bacterial genome sequence so far. Interestingly, the genome of *C. jejuni* NCTC 11168 consists of hypervariable sequences. These are short homopolymeric runs of nucleotides usually involved in genes encoding the biosynthesis or modification of surface structures, or in closely linked genes of unknown function. The high rate of variation of these homopolymerics can be of importance in the survival of *C. jejuni* (Parkhill *et al.*, 2000). Gene organisation into operons/clusters is uncommon in *C. jejuni* NCTC 11168 strain and there is no repetition in DNA sequences. Among the entire genome, there are only four repeated sequences, three of which are copies of ribosomal DNA operons and three duplicates or replicates of CDSs (Parkhill *et al.*, 2000).

In addition to *C. jejuni* NCTC 11168 strain, other strains have been sequenced including RM1221 (Fouts *et al.*, 2005), 81-176 (Hofreuter *et al.*, 2006), 81116 (Pearson *et al.*, 2007) and CG8486 (Poly *et al.*, 2007). *C. jejuni* 81-176 is highly used in research and is considered as a highly pathogenic strain (Hofreuter *et al.*, 2006). It was first isolated from a patient in an outbreak of campylobacteriosis (Hofreuter *et al.*, 2006, Korlath *et al.*, 1985). It possesses high levels of invasion to tissue culture cells which is directly proportional to virulence (Hu and Kopecko, 1999, Oelschlaeger *et al.*, 1993). The genome sequence of *C. jejuni* 81-176 consists of 1,594,651 bp. It contains additional 35 genes that are absent in the 11168 reference strain. Examples of these genes include a dimethyl sulphoxide reductase, cytochrome *c*, two homologues of the succinate efflux

protein DcuC, a glycerol-3-phosphate transporter (GlpT), and γ-glutamyltranspeptidase (*ggt*). (Gundogdu *et al.*, 2007, Hofreuter *et al.*, 2006). In contrast, 51 genes of NCTC 11168 strain are absent in 81-176, including those for an L-fucose permease and putative periplasmic/lipoproteins (Hofreuter *et al.*, 2006). In addition to the chromosome, *C. jejuni* 81-176 strain carries two extra resident plasmids, namely pVir and pTet which have been known as virulence factors (Bacon *et al.*, 2000).

1.3 Physiology of *C. jejuni*

1.3.1 Microaerophily and oxidative stress

In general, most *Campylobacter* species are microaerophilic, which means they depend on oxygen (O_2) for growth but in limited amount. *C. jejuni* grows best in complex media with atmospheric conditions containing 5 – 10 % *(v/v)* O_2 and 5 – 10 % *(v/v)* carbon dioxide (CO_2). Despite the fact that *C. jejuni* encodes several mechanisms for anaerobic respiration with alternative electron acceptors to oxygen, it is unable to grow in the absence of oxygen. One possible explanation for this phenomenon is because of the lack of the oxygen-independent ribonucleotide reductase (RNR) which makes *C. jejuni* fail to synthesise deoxynucleotides under such conditions (Parkhill *et al.*, 2000, Sellars *et al.*, 2002). Inhibition of *C. jejuni* by oxygen has not been completely understood. This is maybe due to the existence of oxygen sensitive enzymes such as pyruvate:flavodoxin oxidoreductase (Hughes *et al.*, 1995), 2-oxoglutarate oxidoreductase (Hughes *et al.*, 1998) and L-serine dehydratase (Velayudhan *et al.*, 2004). Although intracellular life provides the organism protection from the host immune system, *C. jejuni* also needs a self defence mechanism that works against oxidative stress factors. Typically, these enzymes include superoxide dismutase (SodB), catalase (KatA), alkyl hydroperoxide reductase (AhpC) and ferritin. For instance, when iron reacts with oxygen, reactive oxygen species (ROS) are generated such as superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2), which all damage DNA, protein and lipid (Kelly, 2001). To inactivate ROS and allow the organism to persist in host cells and tissues, *C. jejuni* utilises superoxide dismutase (SodB) and catalase (KatA) (Konkel *et al.*, 2001).

1.3.2 Iron acquisition

As a pathogenic organism, *C. jejuni* requires sufficient amounts of iron for essential metabolic mechanisms in order to establish infection. However, the amount of free iron in host fluid is extremely low due to the presence of iron-binding glycoproteins such as transferrin in serum and lactoferrin in mucosal secretion that bind to extracellular iron (Debruyne *et al.*, 2008). The concentration of free iron in the mammalian body is only around 10^{-18} M, which is far below the level required for the growth of bacteria (10^{-7} – 10^{-5} M) (Andrews *et al.*, 2003). Although some *C. jejuni* strains are shown to develop low molecular mass compounds that have high affinity for iron known as siderophores (Miller *et al.*, 2009), which can compete with the iron-binding proteins in the host (Field *et al.*, 1986), many strains use specific mechanisms for scavenging iron from surrounding environment in the host for survival and proliferation (Naikare *et al.*, 2006, Palyada *et al.*, 2004), such as exogenous siderophores; enterochelin and ferrichrome (Baig *et al.*, 1986) and rhodotorulic acid (Stintzi *et al.*, 2008). Comparing to the small genome size of *C. jejuni*, many genes are involved in iron uptake, regulation and homeostasis, which reflects the importance of iron for the survival of *C. jejuni*. It is crucial for *C. jejuni* to equilibrate and maintain the level of iron inside the cell to avoid excess accumulation of iron which can result in production of toxic ROS. During iron-starvation, genes that encode for production of proteins responsible for iron uptake must be switched on to allow sufficient iron-uptake for survival. However, these genes must be suppressed when the iron level becomes elevated to avoid accumulation of iron that may lead to production of damaging ROS (Miller *et al.*, 2009). Two regulatory proteins have been identified to be responsible for maintaining iron homeostasis in *C. jejuni*; the ferric uptake regulator (Fur) homologues Fur (Cj0400) and PerR (peroxide stress regulator; Cj0322) (van Vliet *et al.*, 1999). In *C. jejuni*, iron loaded siderophores are too large to cross the bacterial outer membrane barrier, and therefore, require specific bacterial outer membrane receptors to facilitate entry. CfrA is a Ferric-enterobactin (FeEnt) outermembrane receptor, and is responsible for iron acquisition (Palyada *et al.*, 2004). Recently, a new FeEnt receptor (CfrB) has been identified and characterised in *Campylobacter* (Xu *et al.*, 2010). CfrB is homologous to *C. jejuni* NCTC 11168 Cj0444, and 34% of its amino acids are identical to CfrA. In addition, they require the inner membrane ABC transport system CeuBCDE to be transferred to the cytosol (Konkel *et al.*, 2001). Genomic analysis of *C. jejuni* NCTC 11168 has revealed other

iron acquisition systems such as the hemin uptake operon which includes four proteins *Chu*ABCD (van Vliet *et al.*, 1998). Also, NCTC 11168 genome revealed other genes that encode for the production of putative siderophore receptors and ferrous uptake protein, similar to FeoB in *E. coli* (van Vliet and Ketley, 2001).

1.4 Pathogenecity of *C. jejuni*

1.4.1 Epidemiology, clinical aspects and transmission of *C. jejuni* infection

C. jejuni is considered to be one of the major food borne pathogens that cause bacterial gastroenteritis in humans worldwide (Nauta and Havelaar, 2008). The primary host of *Campylobacter* species is poultry, where it naturally colonises the gastrointestinal tract of these organisms. However, it is considered as an important human enteric pathogen. Humans can acquire infection by ingestion of contaminated poultry. In addition, direct contact with these organisms including consumption of raw meat, unpasteurised milk, or even food contaminated with human faeces can lead to infection (Miller and Mandrell, 2005, Tauxe, 1992). An infective dose as small as 500 cells is adequate to cause a disease (Olson *et al*., 2008). Although human infection can lead to severe but self-limiting diarrhoea (Blaser and Engberg, 2008), in very rare cases (0.1 – 1 %) serious consequences can develop such as Guillain-Barré syndrome (GBS), a neuro-muscular paralysis resulting from an autoimmune reaction against neuronal gangliosides (Jacobs *et al*., 2008).

1.4.2 Virulence factors

1.4.2.1 Motility and chemotaxis

Flagellar motility in *C. jejuni* and other motile bacteria is generated by the rotatory nanomachinery system to allow physical movement of the organism. Chemotactic motility is defined as a specific migration of a bacterium toward or away from objects in the microenvironment. Both are required for initiating and maintaining interactions of *C. jejuni* with hosts (Gilbreath *et al.*, 2011). Motility by the means of flagella and chemotaxis are considered as the best studied factors in the pathogenesis of *C. jejuni.*

Flagella are thought to play a crucial role in colonisation of mucosal cells of the gastrointestinal tract and also in invasion of host epithelial cells (Golden and Acheson, 2002, Yao *et al.*, 1994).The flagellar filament in *C. jejuni* contains two main proteins, flaA and flaB, which are regulated by σ28 and σ54 respectively (Hendrixson *et al.*, 2001, Nuijten *et al.*, 1991). However, *flaA* is expressed at higher levels than *flaB*. It has been reported that mutation in *flaA* results in production of truncated flagellar filament with severe deficiency in motility (Guerry *et al.*, 1991). In contrast, *flaB* mutation resulted in production of a flagellar filament that appears normal and has shown no significant changes in motility (Guerry *et al.*, 1991, Nuijten *et al.*, 1990, Wassenaar *et al.*, 1991). Interestingly, *C. jejuni* motility increases in viscous media and reaches a speed around 75 µm/sec (Lee *et al.*, 1986). This fast motility is suggested to facilitate the movement through the thick mucosa in the intestine (Ferrero and Lee, 1988). This motility allows *C. jejuni* to reach a specific niche in the host intestine, whereas non-motile mutants are unable to reach this niche to initiate long colonisation (Hendrixson and DiRita, 2004).

Chemotaxis in *C. jejuni* is of equal importance as flagellar motility, by which *C. jejuni* can sense the temporal changes of the concentration of carbon source in the microenvironment. Although chemotaxis has been studied in *Campylobacter* (Hugdahl *et al.*, 1988, Takata *et al.*, 1992), the sensory control of motility has not been fully understood. Examples of chemoattractants in *C. jejuni* include L-fucose and L- serine as well as pyruvate and succinate (Hugdahl *et al.*, 1988). The chemotaxis system *in C. jejuni* is similar to that of *E. coli*. Homologues of the histidine kinsase CheA (Cj0284), scaffold protein CheW (Cj0283), and response regulator CheY (Cj1118) are found in *C. jejuni* (Marchant *et al.*, 2002, Parkhill *et al.*, 2000), along with the receptor adaptation proteins CheR (Cj0923) and CheB (Cj0924) (Kanungpean *et al.*, 2011), and a putative CheW-like sensory protein CheV (Cj0285) (Hartley-Tassell *et al.*, 2010). Cj0700 may functional substitute for the absent phosphatase CheZ (Korolik and Ketley, 2008). Deletion of *cheA* resulted in a mutant unable to colonise mice intestinal mucosa (Chang and Miller, 2006), and also shown inability to invade tissue culture cells (Golden and Acheson, 2002). On the other hand, mutation in CheY resulted in decreased chicken colonisation (Hendrixson and DiRita, 2004), but shows increase invasion (Golden and Acheson, 2002, Yao *et al.*, 1997).

1.4.2.2 Adhesion, invasion and colonisation

In poultry, *C. jejuni* lives in the mucosa of deep caecum crypts, without developing intracellular proliferation in the intestinal cells (Van Deun *et al.*, 2008). This is why it acts as a commensal in avian hosts. In campylobacter-mediated gastroenteritis, penetration and translocation of the bacteria across the intestinal cell barrier reveals the mechanism of virulence which is used by this organism to gain access to intestinal mucosa, causing tissue damage, inflammation and then gastroenteritis. Adhesins are bacterial cell-surface molecules that enable *C. jejuni* to attach to the host cell receptors (Konkel *et al.*, 2001). The most common adhesins are flagella, outer membrane proteins (OMPs) and lipooligosaccharides (LOS) (Asakura *et al.*, 2007, Grant *et al.*, 1993). The role of flagella in the adherence to human intestinal cells was demonstrated by Grant *et al* (1993). They constructed a mutant of *C. jejuni* flagellar gene (*flaA*), and examined the ability of mutants to invade human epithelial cells. Mutants were able to show the same adhesion capacity with the parent wild-type but fail to cross the cell barrier of epithelial cells. The cell-binding protein PEB1a (Pei *et al.*, 1991), a major antigenic protein in *C. jejuni* that mediates interaction with epithelial cells (Pei *et al.*, 1998) and the OMP CadF which mediates binding to the extracellular fibronectin matrix (Konkel *et al.*, 1997) are known to be involved in adhesion in conjunction with the host cell processes such as endocytosis (van Vliet and Ketley, 2001). The major outer membrane protein (MOMP) encoded by *porA*, also known as OmpE (Moser *et al.*, 1997, Schroder and Moser, 1997), the surface-exposed lipoprotein JlpA (*j*ejuni *l*ipoprotein *A*) (Jin *et al.*, 2001), the autotransporter protein CapA (Ashgar *et al.*, 2007) and FlpA (Flanagan *et al.*, 2009) are thought to behave as adhesins. *C. jejuni* gains access to intestinal epithelial cells by a microtubule-dependent, actin-independent mechanism (Oelschlaeger *et al.*, 1993), making this organism unique in the entry process among other bacterial pathogens. The intracellular life of *C. jejuni* enables it to evolve specific mechanisms to adapt within the host cellular environment. Thus, *C. jejuni* undertakes physiological changes in oxygen sensitivity and metabolism that make it able to survive and replicate within a modified membrane bound compartment (Watson and Galan, 2008). The *Campylobacter* invasion antigen (Cia) is a newly identified protein that is secreted by the flagellar type-III secretion (T_3S) system, and involved in intracellular survival of *C. jejuni* (Buelow *et al.*, 2011, Christensen *et al.*, 2009).

1.4.2.3 Protein glycosylation

Glycosylation is the most abundant co-translational modification of protein with sugar. Two types of glycosylation have been known; the *O*-linked glycosylation, in which glycan is added to protein through the hydroxyl groups of serines or threonines, and the *N*-linked glycosylation, in which glycan is attached to the amide nitrogen of an asparagine residue. Generally, glycosylation has been considered only for eukaryotes for decades. However, it is now apparent that it is abundant in prokaryotes (Benz and Schmidt, 2002, Schaffer *et al.*, 2001), and has been found in several bacteria such as *Clostridium*, *Campylobacter* and *Helicobacter* species (Wacker *et al.*, 2002). Protein glycosylation systems are found in *Campylobacter* species for a variety of biological activities and influence host-cells interactions [reviewed in (Gilbreath *et al.*, 2011)]. In *C. jejuni*, the *pgl* gene cluster (*cj1119c-cj1130c*) encodes for biosynthesis of *N*-linked glycans (Linton *et al.*, 2002, Szymanski *et al.*, 1999) and the biosynthesis and link of the *N*-acetylgalactosamine-containing heptasaccharide in the periplasm (Linton *et al.*, 2005, Szymanski *et al.*, 1999). Linton *et al*. (2002) identified two glycoproteins that were modified by this system, namely PEB3 (a major antigenic protein) and CgpA (*Campylobacter* glycoprotein A; a putative periplasmic protein). Subsequently, Young *et al*. (2002) identified around 30 glycoproteins including PEB3 and CgpA, and showed that the linkage between glycan and protein was in the same way to that found in eukaryotes. The biological role of *N*-linked glycosylation in *C. jejuni* has not been entirely understood. However, mutation in *pgl* gene demonstrated defects in colonisation of chicken intestinal tract and adherence to and invasion of human intestinal epithelial cells (Hendrixson and DiRita, 2004, Karlyshev *et al.*, 2004).

On the other hand, the *O*-linked protein glycosylation in *C. jejuni* are either pseudaminic acid (PseAm) derivatives synthesized by the *pse* genes or ligionamenic acid (LegAm) derivatives synthesized by the *leg* gene (McNally *et al.*, 2006). This glycosylation is mainly utilised for the modification of flagellins, which are important for motility and filament formation (Doig *et al.*, 1996, Goon *et al.*, 2003). Characterisation of the biological role of the *O*-linked glycosylation in *C. jejuni* seems to be challenging. This is because glycan is required for production of flagella. Therefore, depletion of glycan results in an aflagellated mutant, which will complicate clarification of results as many interactions between bacterium and host cells require

flagellar motility. Nevertheless, the role of specific glycans was revealed by studying mutants with predominantly one glycan. PseAm was found to be essential for optimal adherence to and invasion of human intestinal epithelial (Guerry *et al.*, 2006). Moreover, depletion of LegAm derivatives resulted in decreased commensal chicken colonisation (Howard *et al.*, 2009). Also, because *O*-linked glycosylation takes place in the exposed region of the filament, glycans are suggested to be immunogenic and may play an important role in evading host immune response (Power *et al.*, 1994, Thibault *et al.*, 2001).

1.4.2.4 Toxin production

After the availability of the *C. jejuni* genome sequence (Parkhill *et al*., 2000), research progression has improved in identifying several genes encoded for the production of toxins. The well-studied toxin in *C. jejuni* is the cytolethal distending toxin (CDT) which irreversibly blocks the eukaryotic cell cycle (Whitehouse *et al.*, 1998). The CDT holotoxin comprises of A, B and C subunits encoded by three neighbouring genes *cdt*A, *cdt*B and *cdt*C (Ge *et al.*, 2008, Lara-Tejero and Galan, 2001, Pickett *et al.*, 1996, Smith and Bayles, 2006). The active toxic subunit A is the CdtB protein, whereas the B subunit, CdtA and CdtC are responsible for binding of the CDT and delivery of CdtB to cell (Lara-Tejero and Galan, 2001, Lee *et al.*, 2003, Pickett *et al.*, 1996). Lindmark *et al* (2009) found that all CDT subunits are released from the cells via outer membrane vesicles (OMVs) as detected by immunogold labelling and electron microscopy. The role of CDT in virulence remains unclear. However, it is suggested to play roles in immune suppression during pathogenesis (Pickett and Lee, 2005). Other genes detected in the *C. jejuni* genome encoding for the production of toxins include *pldA* (*cj1351*), a phospholipase (Ziprin *et al.*, 2001); *tlyA* (*cj0588*), a putative contact dependent haemolysin and (*cj0183*), a putative integral membrane protein with a haemolysin domain (Salamaszynska-Guz and Klimuszko, 2008).

1.5 Cell wall and outer membrane

As a typical Gram-negative bacterium, the cell envelope in *C. jejuni* is composed of two membranes, the inner membrane (IM) and outer membrane (OM), separated by a periplasmic space. The inner membrane is composed of phospholipids (PLs) and proteins which can be found in two forms; integral proteins, which cross the IM with α-helical transmembrane domains and lipoproteins which are anchored to the outer leaflet of the IM (Ruiz *et al.*, 2006). The periplasm occupies around 10 % of the cell volume and is characterised by having a high viscosity due to the presence of soluble proteins and peptidoglycan layer (Vanwielink and Duine, 1990). Many central molecular events for cell survival take place in the periplasm, such as translocation and refolding of proteins that build and maintain the cell envelope even in the event of stress. Moreover, sensing domains for many IM receptor proteins (e.g., histidine kinase) read environmental changes from the periplasmic face (Duguay and Silhavy, 2004, Mogensen and Otzen, 2005). Due to the lack of ATP in the periplasm, protein folding, trafficking and proteolysis occur in the absence of noticeable energy (Ruiz *et al.*, 2006). The peptidoglycan layer in the periplasm acts as an extracytoplasmic skeleton. It maintains bacterial cell shape and integrity. It contains glycan chains cross-linked with oligopeptides. The outer membrane consists of phospholipids (PLs) directed toward the inner side, and lipopolysaccharides (LPSs) directed to the outer side. The PLs in the OM slightly differs from that of the IM, as in the OM the PLs are enriched in saturated fatty acids and phosphatidyl ethanolamine (Lugtenberg and Peters, 1976, Poos *et al.*, 1972). In addition to LPS, OM also consists of lipoproteins and integral outer membrane proteins (OMPs), which differ from integral IM proteins by having amphipathic antiparallel β-strands instead of having α-helical transmembrane domains, which form a barrel-shape to serve as channels. Both IM and OM contain lipoproteins that are attached to them by *N*-terminal *N*-acyl-diacylglycerylcysteine (Figure 1.2). The outer membrane is a critical part of the bacterial cell because it acts as a protective barrier against external agents such as detergents and antibiotics (Bos *et al.*, 2007). Also, it has diverse functions in adhesion, cell signalling and interaction with the immune system, as well as a nutrient barrier.

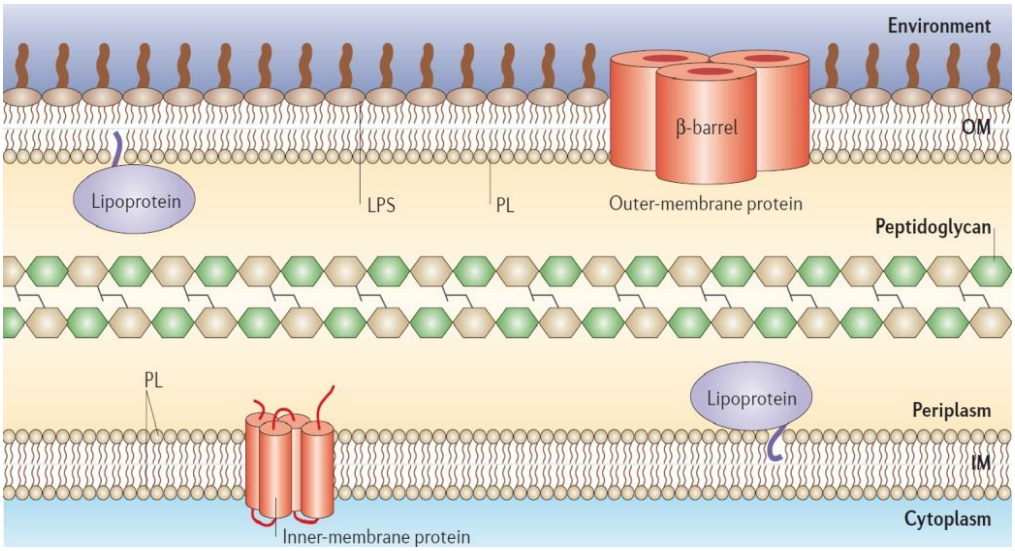


Figure 1.2 General structure of cell envelope in most Gram-negative bacterial including *C. jejuni*. Image from (Ruiz *et al*., 2006).

15.1 Entry of outer membrane proteins to the periplasm via the SecYEG translocon

Despite the progress made in understanding the structure and function of the OM, the mechanism required for OMPs to be translocated from the cytoplasm through the inner membrane and in the periplasm in order to be correctly assembled in the OM still requires more investigation. OMPs are synthesised in the cell cytoplasm as precursor proteins with an *N*-terminal signal sequence, a short hydrophobic amino acid segment contains 7 to 12 amino acids, and they require being transported and assembled to the outer membrane by a specific mechanism. Two systems have been shown to be responsible for transporting OMPs across the IM in *E. coli*, the Sec and Tat systems for the transport of unfolded and folded proteins respectively (de Keyzer *et al.*, 2003, Lee *et al.*, 2006). Outer membrane proteins are translocated across the IM by protein-conducting channels that have a protein complex known as the SecYEG translocon (Figure 1.3) (Or and Rapoport, 2007). Structural evidence had shown that the bacterial SecYEG complex is formed only by the 10 transmembrane parts of SecY, with the smaller SecE and SecG subunits on the periphery (Breyton *et al.*, 2002). The *N*-terminal signal sequence used to direct the OMPs toward the Sec translocon is cleaved by signal peptidase in the outer leaflet of the IM (Dalbey, 1991, Tokuda and Matsuyama, 2004).

1.5.2 Periplasmic chaperones stimulate outer membrane protein folding in the outer membrane model of *E. coli*

In *E. coli*, it has been described that when OMPs enter the periplasm, they gain tertiary structure before they assemble in the OM (Eppens *et al.*, 1997). Many periplasmic proteins have been reported to participate in the maturation of OMPs by helping precursor proteins in gaining the correct structural conformation, or leading them to the target destination in the OM. After gaining access to the periplasm through the Sec machinery system in an unfolded status, OMPs require enzymatic assistance to be correctly folded prior being translocated into the OM. This assistance can be gained from peptydyl-prolyl-*cis/trans* isomerase (PPIase); an enzyme required for the *cis/trans* isomerisation of proline residues. Therefore, unfolded OMPs are attached to chaperones located in the periplasmic layer (Figure 1.3) (Eppens *et al.*, 1997). Two of these periplasmic chaperones have been well studied and identified as Skp (Seventeen-Kilodalton Protein) and SurA (initially known as a protein required for survival during the stationary phase in *E. coli*) (Bos *et al.*, 2007). Typically, unfolded OMPs bind to the SurA chaperone, but if these substrate proteins fail to interact with SurA, then Skp can bind to these proteins (Bitto and McKay, 2003, Bulieris *et al.*, 2003, Sklar *et al.*, 2007, Walton and Sousa, 2004). The function of these chaperones is to assist in protein folding and maturation in order to translocate the unfolded OMPs to the outer membrane. Both a *skp* mutant and *surA* mutant are viable, however, when a double mutation of *surA* and *skp* (*skp/surA*) is attempted, the result is a synthetically lethal phenotype (Rizzitello *et al.*, 2001). This suggests that Skp and SurA are functionally related and they work by similar mechanisms for chaperone activity (Rizzitello *et al.*, 2001). Others favoured the possibility that both Skp and SurA function in the same pathway, where Skp may work as a holding chaperone to prevent aggregation of OMPs in the periplasm and SurA works as a folding chaperone (Bos *et al.*, 2007). Other periplasmic components in *E. coli* including PpiA, PpiD and FkpA are also involved in OMPs biogenesis. Nevertheless, direct interaction of these three proteins with OMPs is still not fully investigated (Bos *et al.*, 2007). Matern *et al.* (2010) suggested that PpiD works as a chaperone in the periplasm, and not only involved in OMPs maturation and the chaperone role of PpiD assists in early folding of newly translocated proteins. SurA and PpiD have been shown to have high sequence similarity to the catalytic domain of the small peptydyl-prolyl-*cis/trans* isomerase (PPIase) parvulin; an enzyme required for

the *cis/trans* isomerisation of proline residue (Stymest and Klappa, 2008). Despite these overlapping specificities, they might fulfil different functions in the periplasm. More recent studies proved that PpiD plays a role in the periplasmic chaperones network. They suggest that PpiD is used to aid in the early periplasmic folding of many newly translocated proteins, but is not specifically involved in the maturation of the OMPs (Matern *et al.*, 2010, Obi *et al.*, 2011). DegP is a periplasmic proteolytic enzyme that degrades unfolded proteins in the periplasm (Strauch and Beckwith, 1987, 1988). It has been reported that when *SurA* is mutated, production of DegP increases by six-fold. This suggests that accumulation of unfolded proteins in the periplasm leads to increase in the expression of *degP* (Lazar and Kolter, 1996).

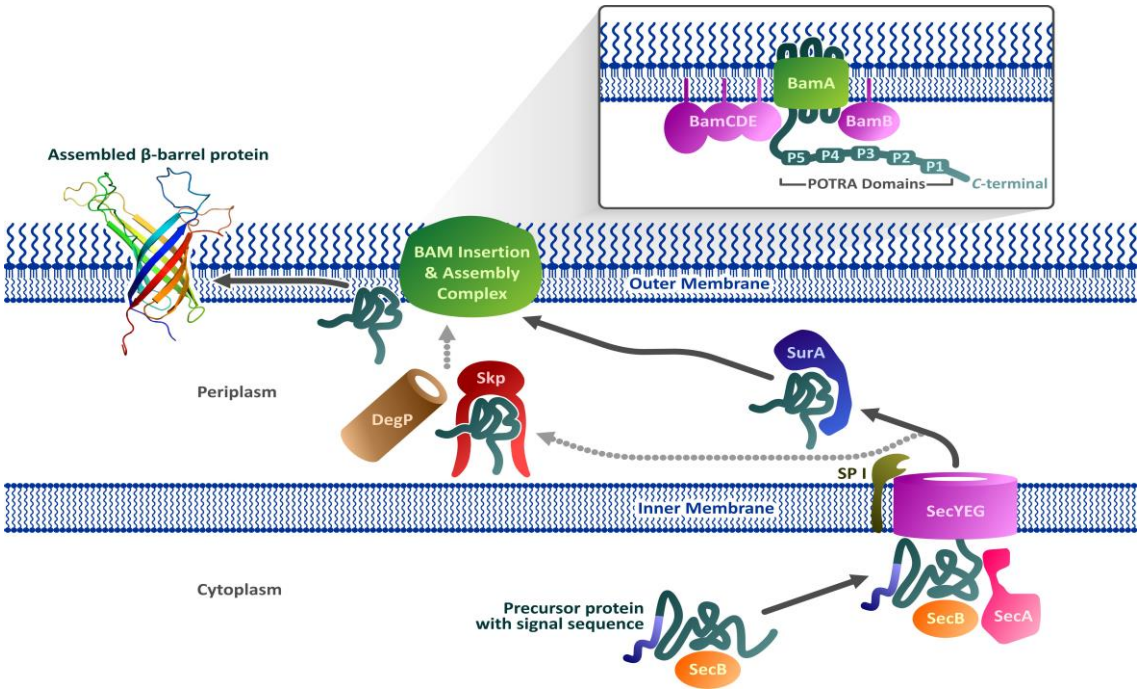


Figure 1.3 Outer membrane biogenesis in *E. coli*. OMPs are synthesised in the cytoplasm as precursors with N-terminal signal sequence and detected by SecA and SecB chaperones. SecA assists in translocating OMPs into the SecYEG machinery and the SP I processes the signal sequence. Then, the substrate protein is attached by the Chaperone SurA or Skp in the periplasm in order to be delivered to the BAM complex to catalyse insertion into the OM. Image from (Gatsos *et al.*, 2008).

1.6 The biological characteristics of peptydyl-prolyl *cis/trans* isomerisation

In all proteins, the partial double bond character of a peptide bond can create two structural conformations; *cis* and *trans* with ω = 0° and 180°, respectively. (Pauling, 1960, Ramachandran and Sasisekharan, 1968). This isomerisation is known to slow the rate of protein folding. However, this isomerisation can be catalysed by ubiquitous enzymes known as peptydyl-prolyl *cis*/*trans* isomerases (PPIases) (Rudd *et al.*, 1995). PPIases are also known as immunophilins due to their binding to immunosuppressor drugs such as cyclosporins, FK506 and rapamycin. They have been classified into three main families according to their binding sites; cyclophilins which bind cyclosporins, FK-binding proteins (FKBP) which bind FK506 (Fischer, 1994, Galat, 1993), and parvulins which bind juglone (Rudd *et al.*, 1995). PPIases are involved in large number of physiological mechanisms such as protein folding (Schmid, 1993), heat shock response (Sykes *et al.*, 1993, Weisman *et al.*, 1996), transcription and translation (Shaw, 2002), channel gating (Lehnart *et al.*, 2003), virus assembly (Luban *et al.*, 1993), signal transduction (Duina *et al.*, 1996, Freeman *et al.*, 1996), tumour metastasis (Lu, 2003), cell surface recognition (Anderson *et al.*, 1993) and cell cycle control (Schreiber, 1991).

1.6.1 Parvulin-type PPIase

Parvulin-type PPIases are characterised as globular protein domains with a length of around 100 residues. They are composed of a four-stranded antiparallel β-sheet core surrounded by four α-helices (βα3βαβ2 parvulin-fold) (Figure 1.4A) (Fanghanel and Fischer, 2004). Parvulin-type PPIases structure differs from that of FKBPs and cyclophilins, as the FKBPs consist of a four to six stranded β-sheet enfolded around a short amphipathic α-helix (Figure 1.4B) (Saul *et al.*, 2004), while cyclophilin is composed of eight-stranded antiparallel β-barrel capped at either end by two α-helices, forming a binding cavity lined with hydrophobic residues (Figure 1.4C) (Clubb *et al.*, 1994, Edwards *et al.*, 1997). Parvulin-type PPIases have been reported both in prokaryotic and in eukaryotic cells. Until present, structures of 7 different parvulins have been revealed. These include; human Pin1 (Bayer *et al.*, 2003, Ranganathan *et al.*, 1997) and Par14 (Sekerina *et al.*, 2000), Pin1 from *Arabidopsis thaliana* (Landrieu *et al.*, 2002), Par10 (Kuehlewein *et al.*, 2004) and SurA (Bitto and McKay, 2002) from

Escherichia coli, Ess1 from *Candida albicans* (Li *et al.*, 2005) and PrsA-PPIase from *Bacillus subtilis* (Tossavainen *et al.*, 2006).

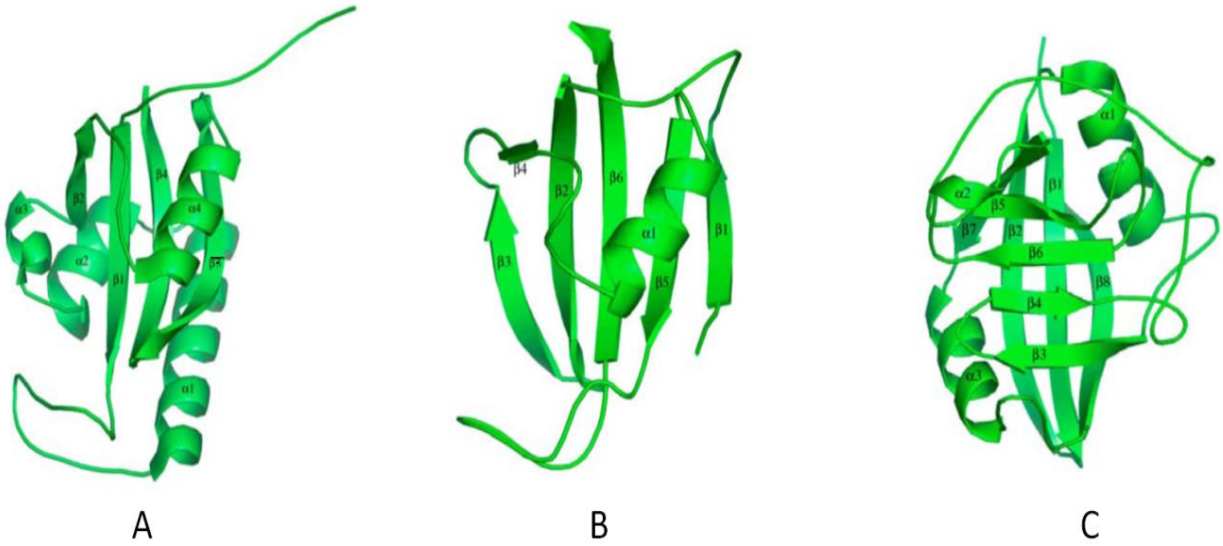


Figure 1.4 Ribbon representations of the globular folds of PPIase domains. A) Parvulin domain, represented by human Pin1. The domain consists of four β-strands and four α-helices. B) FKBP domain represented by human FKBP12. The domain consists of six antiparallel β-sheets capped with an amphiphilic α-helix. C) Cyclophilin domain represented by Cyp18. The domain consists of a β-barrel formed by eight antiparallel β-sheets and two amphipathic α-helices. Image from (Fanghänel and Fischer, 2004).

1.6.1.1 SurA is a periplasmic chaperone that is related to the parvulin family of PPIases

SurA was first identified in *E. coli* as a protein required for survival during the stationary phase (Tormo *et al.*, 1990). To date, there is no clear evidence that explains the reason of loss of viability by inactivation of *surA* gene. However, SurA is now among the most structurally and functionally characterised proteins that play essential periplasmic chaperone roles in Gram-negative bacteria. Rahfeld *et al*. (1994) had isolated and characterised SurA as a novel, low molecular weight PPIase from *E. coli*, and later, its biochemical function was suggested by amino acid sequence comparison and identified as a parvulin (Rudd *et al.*, 1995). From the phenotypic properties, mutation in *surA* results in a phenotype sensitive to bacitracin, vancomycin and bile salts (Lazar and Kolter, 1996). Given the fact that *E. coli* is normally resistant to these

antibiotics due to the presence of impermeable outer membrane barrier, these findings suggested that SurA plays a key role in the maintenance of the OM. Moreover, cells of a *surA* mutant cultured in enriched medium lysed in the stationary phase and formed mucoid colonies that reflect a severe membrane defect (Lazar and Kolter, 1996).

As a result of being a periplasmic protein that possesses parvulin characteristics, Lazar and Kolter (1996) hypothesised that SurA can be a periplasmic chaperone that assists in folding of periplasmic and OMPs in *E. coli*. They performed a trypsin sensitivity assay for different OMPs include; LamB, OmpA and OmpF. All the three OMPs were sensitive to trypsin in *surA* mutant, while they were all resistant in the wild-type strain. These findings suggested that the absence of SurA results in accumulation of outer membrane proteins in the periplasm, leading to severe defect in cell envelope.

1.6.1.2 The crystal structure of SurA reveals *N*-terminal chaperone domain and two PPIase domains

The crystal structure of SurA (Figure 1.5) is composed mainly of two parvulin-type PPIase domains and one *N*-terminal peptide binding region (Behrens-Kneip, 2010, Behrens *et al.*, 2001, Bitto and McKay, 2002, Stymest and Klappa, 2008). A SurA variant lacking the parvulin domain was shown to exhibit the ability to stop aggregation of denatured citrate synthase, which suggests that SurA is characterised by having a PPIase-independent chaperone-like activity (Behrens *et al.*, 2001). Moreover, SurA interacts with *in vitro* synthesised porins with about 50-fold higher efficiency than the non-porins proteins. These results strongly suggest that SurA is a molecular chaperone that assists in maturation of OMPs (Behrens *et al*., 2001). On the other hand, the PPIase activity of SurA was confirmed by *in vitro* proline-limited refolding of reduced, carboxy-methylated ribonuclease T_1 (RCM-RNase T_1) (Behrens *et al*., 2001).

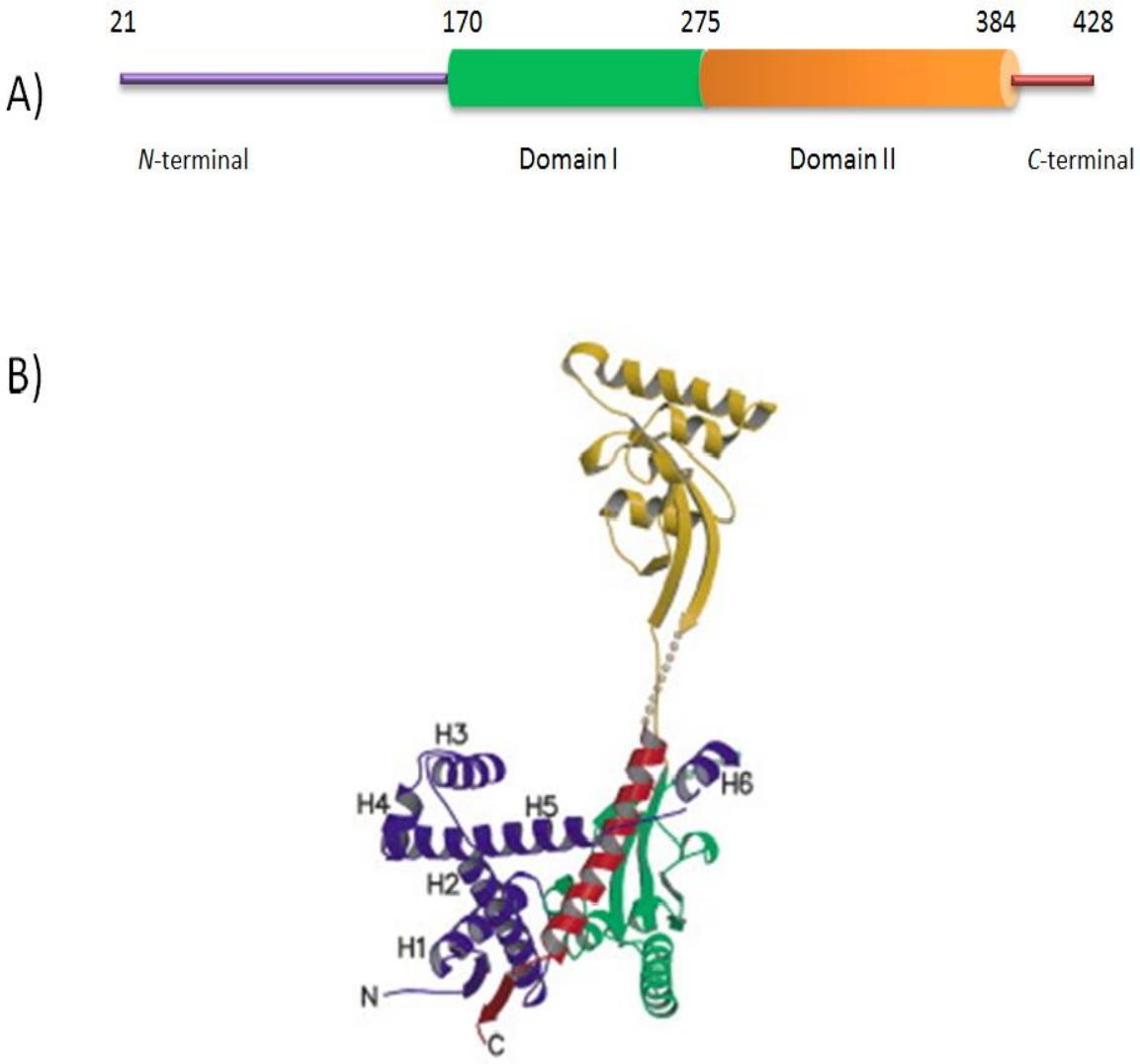


Figure 1.5 The periplasmic chaperone and PPIase, SurA. A) Schematic diagram shows the SurA protein excluding signal sequence (amino acids 1 – 20). Numbers indicate position of amino acids. The *N*-terminal chaperone domain is shown in purple, two PPIase domains, I & II in green and orange respectively, and the *C*-terminal chaperone domain in red. **B)** Ribbon drawing of SurA shows all domains in the same colour codes as in A. Images adapted from (Behrens-Kneip, 2010, Bitto and McKay, 2002).

1.6.1.3 SurA is a major molecular chaperone that possesses PPIase activity

Given the fact that SurA is composed of an *N*-terminal chaperone domain and two parvulin domains (I & II), it is noteworthy that only domain II of the parvulin-like domains has a significant PPIase activity and both domains can be deleted completely without affecting the function of SurA (Behrens *et al.*, 2001, Rouviere and Gross, 1996). However, the *N*-terminal chaperone domain and *C*-terminal tail cannot be deleted as they are essential for SurA to function as a molecular chaperone *in vivo* (Behrens-Kneip, 2010). It was reported that the PPIase domain I has no detectable activity whereas domain II has less PPIase activity than the one of intact SurA (Rouviere and Gross, 1996). Behrens *et al*. (2001) demonstrated these characteristics of SurA PPIase domains by conducting prolyl isomerisation experiments of an intact SurA protein and SurA variant in enhancing the folding of RCM-RNase T_1. They found that $SurA^{Domain\ I}$ has no PPIase activity while $SurA^{Domain\ II}$ has only about 1 % of the wild-type SurA PPIase activity. Also they found that $SurA^{Domain\ I + II}$ showed around 50 % of the intact SurA. Therefore, even though domain I has no detectable activity, it has some effect on domain II to interact with RCM-RNase T_1. In another experiment, $SurA^{N\text{-}terminal}$ had no PPIase activity whereas $SurA^{N\text{-terminal + Domain II}}$ shows a significant increase in PPIase activity compared to the intact SurA. From these experiments, they concluded that the presence of the parvulin domain I and the *N*-terminal peptide binding domain have some effect on the activity of domain II to interact with RCM-RNase T_1, although they have no detectable PPIase activity on their own. The *N*-terminal peptide binding domain was named as a chaperone domain, as it prevented the heat-denatured citrate synthase to form aggregates (Behrens *et al.*, 2001). The inactive parvulin domain (domain I) has been demonstrated to have a role in the recognition of *C*-terminal aromatic residue of the targeted protein (Stymest and Klappa, 2008).

1.6.2 Periplasmic chaperones and PPIases in *E. coli* other than SurA

1.6.2.1 PpiD

PpiD was first discovered as a multi-copy suppressor of a *surA* mutant (Dartigalongue and Raina, 1998). It is characterised as a periplasmic PPIase of *E. coli* that belongs to parvulin family. It is composed of a membrane-anchored *N*-terminal α-helix (residues 10 – 34) and three periplasmic domains facing the periplasm (Antonoaea *et al.*, 2008, Dartigalongue and Raina, 1998). The first and third domains (residues 35 – 263 and 358 – 623 respectively) are rich in α-helices and are proposed to be chaperone domains, while the middle domain (residues 264 – 357) is a parvulin-like domain (Stymest and Klappa, 2008). It was proposed that deletion of *ppiD* and *surA* is synthetically lethal to *E. coli* (Rizzitello *et al.*, 2001). However, this was disputed by Justice *et al.* (2005) who had shown that deletion of both *ppiD* and *surA* results in a viable mutant. The solution structure of PpiD revealed by NMR spectroscopy contains four α-helices and four stranded β-sheets, which is consistent with the general parvulin fold. Overall PpiD shows high structural similarity to the first parvulin-like domain of SurA (Weininger *et al.*, 2010). In the same study, the catalytic activity of PpiD was examined using the PPIase assay, and acceleration of prolyl isomerisation could not be detected, even when using high concentration of full-length PpiD. This can be explained by the fact that PpiD is structurally similar to the first parvulin-like domain of SurA and this domain is known to be inactive (Behrens *et al.*, 2001). Evidence suggested that PpiD has lower substrate specificity than SurA (Stymest and Klappa, 2008), and because it is anchored to the inner membrane, it acts as a periplasmic guard for the SecYEG translocon (Antonoaea *et al.*, 2008). Later, it was found that elevated levels of PpiD production serves as a rescuer for *surA skp* cells from lethality by increasing the folding stress in the cell envelope as a result of loss of periplasmic chaperone activity (Matern *et al.*, 2010). Although PpiD exhibits a rescue role in the network of periplasmic chaperones, it has no major role in the maturation of OMPs and cannot compensate for the lack of SurA in the periplasm (Matern *et al.*, 2010). It was recently established that deletion of *ppiD* and *degP* resulted it a temperature-sensitive mutant, which has a reduced growth at 37 °C and failed to grow at 42 °C, whereas single *ppiD* showed a reduced growth at both temperatures. Moreover *ppiD degP* double mutant has shown an extremely decreased production level of FhuA (ferric hydroxamate uptake); a monomeric porin that acts as a receptor for ferrichrome iron (Weski and Ehrmann, 2012).

1.6.2.2 Skp

In the periplasm of most Gram-negative bacteria, periplasmic chaperones hold unfolded proteins in their cavities to protect them from misfolding of aggregation. This mechanism occurs in the absence of ATP or other chemical source of energy. The Skp (seventeen kilo-dalton protein) is one of these periplasmic chaperones which facilitate the folding and insertion of proteins in the OM (Chen and Henning, 1996). The periplasmic localisation of Skp was first determined by Thome and Muller, (1991), and since that time its function was described as a periplasmic protein that binds in a highly selective manner to outer membrane proteins (Chen and Henning, 1996, Thome and Muller, 1991). The crystal structure of Skp reveals that it is a trimeric protein with a jellyfish-like architecture that contains α-helical tentacles protruding from a β-barrel body, providing a central cavity (Walton and Sousa, 2004). Interestingly, its structure was found to have structural similarity to that of prefoldin/GimC, a cytoplasmic molecular chaperone found in eukaryotes and archaea (Lundin *et al.*, 2004). It has been widely recognised that Skp is a periplasmic chaperone that also helps in folding of soluble proteins in the periplasm. An example of Skp-dependent OMPs is the OmpA, a model OMP with a membrane embedded β-barrel domain and a periplasmic αβ domain. OmpA requires simultaneous folding of its soluble αβ domain outside the cavity of Skp, while keeping the β-barrel in an unfolded phase as it is transported in the periplasm (Walton *et al.*, 2009). The α-helical tentacles bind to the β-barrel domain of the OmpA and keep it unfolded, while the soluble periplasmic domain folds into its native conformation outside the Skp cavity (Walton *et al.*, 2009). The *skp* mutants have been shown to be viable, however, when a double mutant (*skp/surA* mutant) is generated, the result is a synthetically lethal phenotype (Rizzitello *et al.*, 2001). This suggests that Skp and SurA are functionally related and they work by similar mechanisms for chaperone activity (Rizzitello *et al.*, 2001). Others favoured the possibility that both Skp and SurA function in the same pathway, where Skp may work as a holding chaperone to prevent aggregation of OMPs in the periplasm and SurA works as a folding chaperone (Bos *et al.*, 2007). Moreover, evidences have demonstrated that the absence of Skp resulted in a severe reduction in levels of porins, PorA and PorB in *Neisseria meningitides* (Volokhina *et al.*, 2011).

1.6.2.3 DegP

DegP protein of *E. coli* is another periplasmic protein that is not only considered to function as a chaperone, but also works as a periplasmic protease. It can switch from chaperone to protease status in a temperature-dependent manner, especially in elevated temperatures (Zumbrunn and Trueb, 1996). The structure of DegP consists mainly of three functional domains, a protease domain (residues 1–259) and two PDZ domains (PDZ1, residues 260–358; PDZ2, residues 359–448) (Krojer *et al.*, 2002). The antagonistic activity of DegP makes it a unique periplasmic protein that requires distinguishing between the proteins need to be degraded and that can be protected and refolded to ensure their safe transit across the periplasm (Krojer *et al.*, 2008). The chaperone activity of DegP was first investigated by Spiess *et al,* (1999) when it catalysed the folding of the periplasmic protein MalS *in vivo* and *in vitro*. Overproduction of DegP was reported in *surA* mutant, in which misfolded OMPs were accumulated in the periplasm, stimulating DegP over-production to serve as a typical periplasmic protease (Lazar and Kolter, 1996). In addition, recent evidence have revealed its effect on FhuA (ferric hydroxamate uptake), where mutation in *degP* and *ppiD* resulted in an extremely decreased production level of FhuA (Weski and Ehrmann, 2012).

1.6.2.4 FkpA

The periplasmic protein FkpA of *E. coli* possesses both PPIase and chaperone activities (Ramm and Pluckthun, 2001). The overall structure of FkpA reveals a V-shaped dimeric molecule in which the 245-residue subunit is divided into two domains. The *N*-terminal domain includes three helices that are intertwined with those of the other subunits to maintain the dimeric shape. The *C*-terminal contains a PPIase domain and belongs to the FK506-binding protein (FKBP) family (Saul *et al.*, 2004). As a member of the FKBP family, FkpA is inhibited by the immunosuppressor FK506 (Ramm and Pluckthun, 2001), and overproduced under stress conditions (Helbig *et al.*, 2011). FkpA has found utility in restoration of solubility and functionality of recombinant protein production (Bothmann and Pluckthun, 2000). The over-production of scFv antibody in the periplasm of *E. coli* usually results in an extensive protein misfolding and severe loss of cell viability (Gasser *et al.*, 2008). The co-expression of FkpA and Skp increases

the solubility of scFv and therefore increase cell viability (Ow *et al.*, 2010). It has been reported that deletion of *fkp* stimulates the transcription of *degP*, which encodes the heat-shock inducible periplasmic protease (Danese and Silhavy, 1997). FkpA exhibits a high level of PPIase activity as tested by the refolding of ribonuclease T_1 and prolyl *cis-trans* isomerization of oligopeptides (Ramm and Pluckthun, 2001). It has been shown *in vitro* that an FkpA variant lacking the *N*-terminal domain exhibits a PPIase activity similar to that of the intact wild-type protein but with no chaperone activity. Also the same study has shown that the chaperone activity was independent of the PPIase activity (Ramm and Pluckthun, 2001).

1.6.3 SurA-like chaperones in *C. jejuni*

In *C. jejuni*, a 31 kDa periplasmic protein, PEB4 has been implicated in the assembly of OMPs in *C. jejuni* (Asakura *et al.*, 2007, Rathbun *et al.*, 2009, Rathbun and Thompson, 2009). Recently, Kale *et al.* (2011) elucidated the crystal structure of PEB4. At a resolution of 2.2 Å, its structure reveals a dimeric organisation with SurA-like chaperone and PPIase domains (Figure 1.6). However, unlike SurA, PEB4 is formed of two domains; the first has *N*- and *C*-terminal regions of the protein and second has a standard PPIase fold. The chaperone domain is closely related to that of SurA but is different in the way helices from both domains interlock to form a domain-swapped structure. It has been shown by Kale *et al.*, 2011 that the PPIase domain in PEB4 is active when its proline-isomerisation limited protein folding was tested using ribonuclease T_1 which showed a significant PEB4-dependent refolding acceleration. Also, the chaperone activity of PEB4 was tested by refolding studies using the standard model protein rhodanese, which revealed that the yield of active refolded protein is decreased by PEB4. In addition, increasing concentration of PEB4 strongly inhibits the aggregation of renaturing rhodanese, as measured by light scattering kinetics. It has been suggested that PEB4 is a holdase-type chaperone that can inhibit protein folding and aggregation (Kale *et al.*, 2011).

Furthermore, bioinformatics using the SurA chaperone domain in structure prediction searches identified a second SurA-like periplasmic chaperone in *C. jejuni*, namely Cj1289. In the same study, the structure of Cj1289 was also reported (Kale *et al.*, 2011). Unlike PEB4, the Cj1289 crystal structure at 2.4 Å does not show the domain-swapped

structure, which makes it more similar to SurA (Figure 1.6). However, it has one parvulin-like PPIase domain instead of two in SurA. This domain is active in increasing the refolding rate of ribonuclease T_1, but its activity is lower than PEB4. Nevertheless, its chaperone domain is not active in preventing rhodanese refolding and aggregation (Kale *et al.*, 2011), suggesting that if Cj1289 has a chaperone role, it may be for specific substrates.

The third SurA-like periplasmic protein discovered in the same study was Cj0694 (Kale *et al.*, 2011). BLAST searching using Cj0694 as a query protein revealed weak sequence similarity to the PpiD protein, a membrane-anchored protein by an *N*-terminal uncleaved signal sequence in *E. coli* (Dartigalongue and Raina, 1998). As discussed above, PpiD is thought to be a general chaperone that interacts with periplasmic proteins in addition to OMPs by the fact that its over-production can help survival when *surA* is deleted (Dartigalongue and Raina, 1998). This finding was disputed by a more recent study which shows that PpiD has no specific role in the maturation of OMPs (Matern *et al.*, 2010). Although PpiD has a parvulin-like domain, it is catalytically inactive (Weininger *et al.*, 2010). Unfortunately, Kale *et al*, (2011) could not obtain a soluble form of recombinant Cj0694 for structural studies. However, the sequence similarity to PpiD and the predicted *N*-terminal membrane anchored region suggest a similar role of this protein in *C. jejuni* as in *E. coli*.

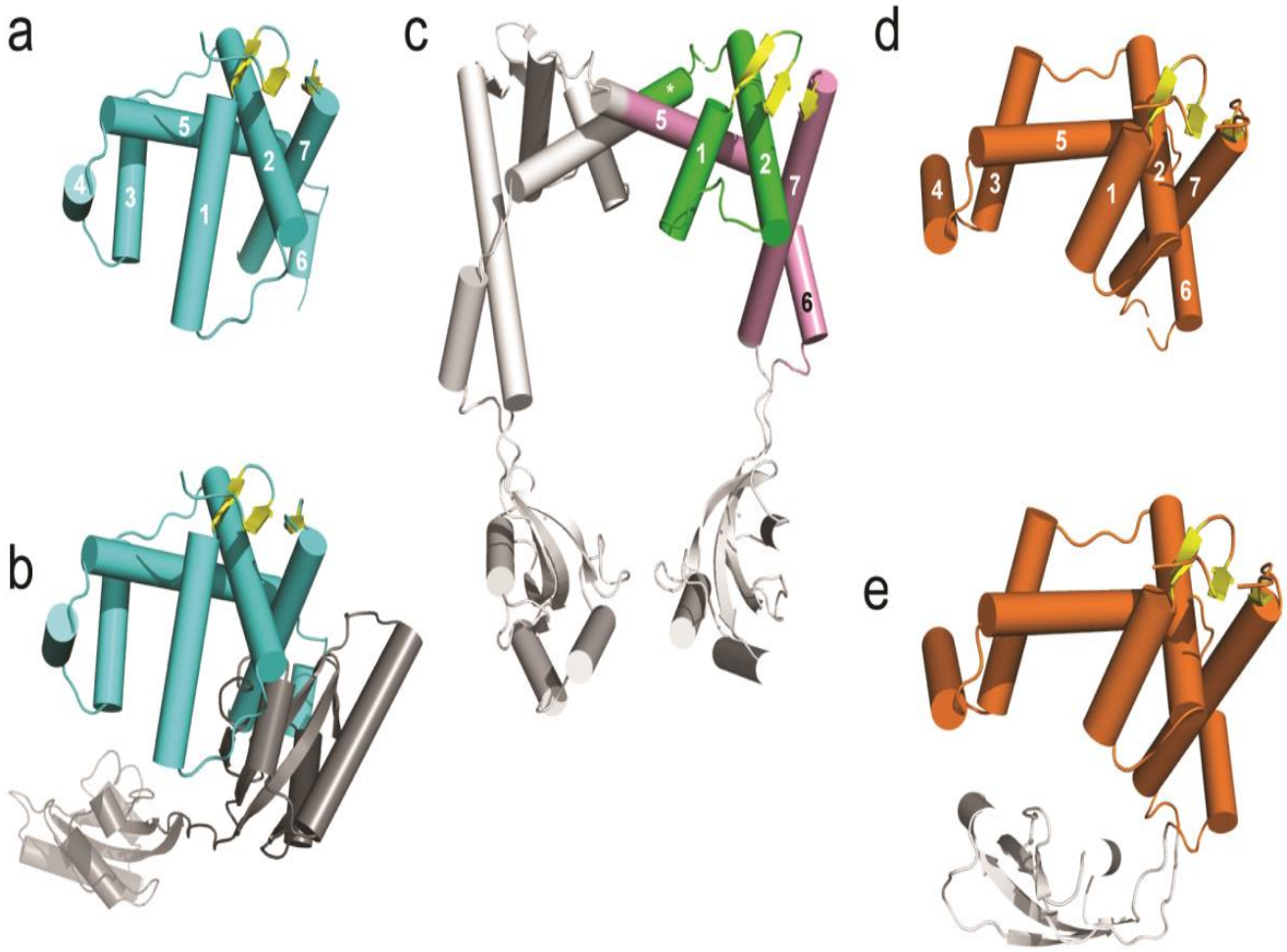


Figure 1.6 The structural similarities of chaperone and PPiase domains in SurA, PEB4 and Cj1289. A) The chaperone domain of SurA with helices shown as cylinders. The three stranded sheets containing extreme *N*- and *C*-termini are represented in yellow, as for all panels in this figure. **b)** As in A) but the PPIase domains I and II are coloured in dark and light grey respectively. **c)** The crystal structure of PEB4. Similar units to the chaperone domain of SurA are coloured. **d)** The chaperone domain of Cj1289 (helices and loops are in orange). **e)** As in d) but the PPIase domain is shown in grey. Image adapted from (Kale *et al.*, 2011).

1.7 Insertion and assembly of outer membrane proteins

1.7.1 Assembly of β-barrel proteins into the outer membrane of *E. coli*

OMPs require both periplasmic chaperones and OM assembly and folding machinery in order to be properly assembled into the OM. To explain how unfolded precursor proteins are transported across the hydrophilic environment of the periplasm, first, β-barrel proteins are translocated across the IM by the Sec machinery and transported through the periplasm by soluble molecular chaperones such as SurA in an unfolded status. They can be rescued by backup chaperones such as Skp if they fail to interact with the primary chaperone SurA (Bitto and McKay, 2002, Kleinschmidt, 2003, Sklar *et al.*, 2007, Walton and Sousa, 2004). Once they reach the OM, they are delivered to an oligomeric membrane-associated protein assembly complex known as β-barrel proteins Assembly Machinery (BAM) complex. This complex consists of a β-barrel integrated protein BamA (Misra, 2007) (previously known as YaeT) and outer membrane-associated lipoproteins, BamBCDE (previously known as YfgL, NlpB, YfiO, and SmpA respectively) (Hagan C., 2010, Sklar *et al.*, 2007) that physically interact with BamA. The Bam complex was found to have an essential role in the biogenesis of integral OMPs such as OmpA, LamB, OmpF/C, PhoE and TolC (Bos *et al.*, 2007, Ruiz *et al.*, 2006). Nevertheless, the mechanism required to fold β-barrel OMPs into their final location in the membrane has not been studied fully in most Gram-negative bacteria (Bos *et al*., 2007).

BamA is known as a key player in the assembly machinery of OMPs. It is a large multi-domain OMP containing an *N*-terminal periplasmic region consists of five domains, and a *C*-terminal integral OM β-barrel part (Gentle *et al.*, 2005, Knowles *et al.*, 2009, Sanchez-Pulido *et al.*, 2003, Voulhoux *et al.*, 2003). Kim *et al.* (2007) and Gatzeva-Topalova *et al*. (2008) resolved the crystal structure of POTRA domains 1 – 4 (residues 21 – 351), while domain 5 was characterised by Knowles *et al.* (2008). These periplasmic POTRA domains have been shown to bind OMP precursors prior to their folding into β-barrels and insertion into the OM (Knowles *et al.*, 2009, Robert *et al.*, 2006). Interestingly, diverse bacteria seem to be different in their requirements for periplasmic domain of BamA. For instance, in *E. coli* deletion of one POTRA domain is sufficient to impair the β-barrel assembly and reduce cell viability (Kim *et al.*, 2007). The cell can be viable with a single deletion of P1 domain or P2 domain, or double

deletion of P1 and P2. However, deletion of P3, P4 or P5 is lethal even in the presence of P1 and P2, suggesting that the minimum requirement to maintain cell viability is P3 – 5 together with the *C*-terminal membrane domain (Kim *et al.*, 2007). BamA seems to recognise a *C*-terminal motif in OMPs that includes a *C*-terminal Phe. It can interact with other Bam proteins, but in *E. coli* only BamA and BamD are essential for cell viability (Onufryk *et al.*, 2005). Although complete deletion of *bamA* and *bamD* are lethal in *E. coli*, insertions of the *C*-terminal part of the *bamD* gene produce viable mutants with OM assembly defects (Wu *et al.*, 2005). This indicates that only the *N*-terminal part of the protein is required for function. Furthermore, BamD is known to be an essential member in the BamCDE complex, as deletion of BamD is similar in consequences to deletion of BamA (Wu *et al.*, 2005). The sensible explanation is that both BamA and BamD are required to function differently but with an equal importance in the assembly of OMPs.

In *C. jejuni*, homology search lead to the identification of BamA and BamD homologue as Cj0129 and Cj1074c respectively (Kale *et al.*, 2011). However, extensive searches have revealed no homologues of BamB, BamC and BamE, suggesting either that BamAD is the minimal combination required for the assembly of OMPs, or that *C. jejuni* employs additional novel proteins involved in the assembly mechanism of OMPs.

1.7.2 Synthesis and interaction of the four outer membrane lipoproteins involved in the BAM complex

As other OMPs, lipoproteins involved in the BAM complex are synthesised in the cytoplasm as precursor proteins containing *N*-terminal signal sequences followed by a cysteine residue. After their translocation across the IM into the periplasm via the Sec-mediated translocation system, the signal peptide is cleaved by signal peptidase II (SP II), and modified by lipoprotein diacylglyceryl transferase (Narita *et al.*, 2004, Tokuda and Matsuyama, 2004). The mature protein is then transported across the periplasm and properly inserted into the OM. Studies on the BAM complex structure and function have demonstrated a direct interaction between BamA and both BamD and BamC, BamD and the *C*-terminus of BamC (Malinverni *et al.*, 2006). Also, direct interaction was reported between BamA and BamE, BamC and BamD (Sklar *et al.*, 2007).

Moreover, BamB and the Bam CDE complex interact with BamA in an independent manner, which means mutation in or deletion of BamB does not affect the physical interaction between BamA and BamCDE complex and vice versa (Kim *et al.*, 2007, Malinverni *et al.*, 2006, Sklar *et al.*, 2007). This suggests that BamB and BamCDE complex exhibits different roles and behave independently in the OMP assembly.

1.8 Lipid asymmetry in the outer membrane of Gram-negative bacteria

In the OM of Gram-negative bacteria, there is an asymmetric distribution of lipid with an outer leaflet contains lipid A conjugated to oligosaccharide (LPS/LOS) and an inner leaflet contains phospholipids (PLs) (Nikaido, 2003). Lipid A is acylated by six saturated fatty acids which allow Lipid A to anchor to the outer leaflet of the OM (Bishop, 2008). Both hydrophobicity of Lipid A and the strong lateral interaction between LPSs increase the efficacy of barrier nature of the OM (Nikaido, 2003). Under stress conditions, PLs from the IM are forced to move to the defective area of the OM and accumulate in the outer leaflet, therefore reducing their barrier role (Nikaido, 2005). To retain their functions, surface-exposed PLs have to be destroyed in the outer leaflet of the OM and therefore lipid asymmetry is maintained. Two systems have been known to do this function; phospholipase A (PldA) and PagP. PldA is an OM phospholipase that hydrolyses many PL substrates by removing the sn-1 and sn-2 fatty acid side chains from the glycerophosphodiester backbone of both PLs and lysophospholipids (lyso-PLs) (Dekker, 2000). PldA is normally found as an inactive monomer in the OM, but it can convert to a catalytically active PldA dimer when PLs and lyso-PLs present in the outer leaflet of the OM (Dekker, 2000). Therefore, its primary function is to maintain the asymmetric distribution of lipids in the OM under stress conditions. PagP is another OM β-barrel enzyme that acts by cleaving a palmitate moiety from PLs and transferring them to lipid A forming more hydrophobic compounds (Bishop, 2008). Similar to *pldA*, *pagP* is expressed at a low level in normal conditions (Jia *et al.*, 2004). However, expression is induced under stress conditions by the PhoP/Q stress response, which senses the restriction in divalent cations (Groisman, 2001).

1.8.1 Novel ABC transporters in *E. coli* and *C. jejuni* implicated in lipid transport

Recently, an entirely novel ABC (ATP-binding cassette) transport system has been identified in *E. coli* that maintains lipid asymmetry in the OM. Mla (**m**aintenance of **l**ipid **a**symmetry) system; a previously unidentified ABC-transporter and many associated proteins that remove PLs from the OM and drive them to the IM (Malinverni and Silhavy, 2009). Mutation in *mla* genes in *E. coli* results in a specific phenotype that has an increased sensitivity to SDS-EDTA mixture but does not show any growth defect or changes in OMP levels, indicating a damaged OM permeability barrier (Malinverni and Silhavy, 2009). The resulting defect in the OM can only be suppressed by increasing the level of OM phospholipase PldA. Therefore, the key function of Mla system is to maintain the asymmetric distribution of lipids in the OM by retrograde trafficking of PLs from the OM to the IM (Malinverni and Silhavy, 2009). It has been suggested that the Mla system is the most important pathway that maintains lipid asymmetry in the OM of *E. coli*. This suggestion was supported by two observations. The first shows that inactivation of *mla* genes results in increased OM permeability, whereas this permeability is not applicable with inactivation of *pldA* or *pagP*. The second observation shows that the Mla system prevents accumulation of PLs in the OM in the absence of PldA, while the opposite is not correct (Malinverni and Silhavy, 2009).

In *C. jejuni*, homology searches (see chapter 5) led to the identification of the ABC transporter genes (*cj1646-cj1648*) as one operon, and two other genes *cj1371* (*mlaA*) and *cj1372* (*mlaC*) form a different cluster. MlaA is proposed to function as a key protein that removes PLs from the OM and transfers them to MlaC in the periplasm before reaching the ABC system (Malinverni and Silhavy, 2009). Interestingly, *cj1371* mutant has been shown to be paraquat sensitive, suggesting an oxidative stress defect (Garenaux *et al.*, 2008). Nevertheless, this sensitivity could indicate a defect in OM structure. MlaA (Cj1371) is also a homologue of VacJ in *Shigella*, a crucial virulence factor known to facilitate intracellular spreading (Suzuki *et al.*, 1994).

1.9 Aims of the project

The overall aim of this project is to gain further insight into OM biogenesis in *C. jejuni*. After the structural and functional characterisation of the periplasmic chaperone and PPIase, PEB4, we wish to identify the structural characteristics and biological function of two periplasmic proteins; Cj1289 and Cj0694 that are homologous to PEB4/SurA proteins and are suggested to be additional periplasmic chaperones. Moreover, we suggest that mutation of one or both genes may cause growth retardation of the cell and may also affect the assembly of a number of OMPs, and consequently might lead to an OM permeability defect. Also, the phenotypic characterisation of single *cj1289, cj0694* mutants and a *cj1289/0694* double mutant on the assembly of OMPs and sensitivity to several antimicrobial agents and inhibitors will be investigated. Bioinformatics analysis suggests that Cj0694 is related to PpiD, a membrane anchored chaperone in *E. coli.* To investigate this, chaperone assays and PPIase assays will be performed as well as crystallography trials of this protein. We also aim to analyse the newly identified putative Mla ABC transport system by mutating the *mla* genes and compare their phenotypes to the wild-type strain, in terms of growth, motility and biofilm formation, and also sensitivity to a wide-range of OM damaging agents.

2 Materials and methods

2.1 Materials

All chemicals were supplied by Sigma-Aldrich, Oxoid, Melford and BDH. Antibiotics were purchased from Sigma-Aldrich, Ducheva, Applichem and Melford. DNA polymerase, restriction enzymes and DNA ligase were obtained from Promega, New England Biolab (NEB) and Bioline. Oligonucleotides were purchased from Sigma-Aldrich. Gases were supplied by BOC.

2.1.1 Antibiotics

All antibiotics used in this study were prepared in distilled water and filter sterilised, with the exception of chloramphenicol which was prepared in ethanol. Amphotericin B was prepared by dissolving in water with few drops of 10 M NaOH. They were stored at 4 °C and used as detailed in the table 2.1.

Table 2.1 Antibiotics used in this study.

Antibiotic	**Stock concentration**	**Final concentration**
Carbenicillin	50 mg mL^{-1}	50 µg mL^{-1}
Amphotericin B	10 mg mL^{-1}	10 µg mL^{-1}
Vancomycin	10 mg mL^{-1}	10 µg mL^{-1}
Kanamycin	50 mg mL^{-1}	50 µg mL^{-1}
Chloramphenicol	50 mg mL^{-1}	50 µg mL^{-1}

2.2 Bacterial strains and growth media

2.2.1 Bacterial strains

All bacterial strains used in this study are listed in table 2.2. *C. jejuni* strains were preserved at – 70 °C BHI (Brain Heart Infusion) broth (Oxoid) with 15 % *(v/v)* glycerol as a cryo-preservative. *E. coli* strains were preserved at – 70 °C in LB (Luria-Bertani) broth (Melford) with 20 % *(v/v)* glycerol.

Table 2.2 Bacterial strains used in this study.

Bacterial strain	**Genotype**	**Source**
Campylobacter jejuni NCTC 11168	Clinical isolate used for genome sequencing	Parkhill *et al*., 2000
Δ*cj1289*	11168 Δ*cj1289*	This study
Δ*cj1289/cj1289*$^{+}$	11168 Δ*cj1289* complemented with WT copy of *cj1289*	This study
Δ*cj0694*	11168 Δ*cj0694*	This study
Δ*cj1289*/Δ*cj0694*	11168 Δ*cj1289* Δ*cj0694* double mutant	This study
Δ*peb4*	11168 Δ*peb4*	This study
Δ*cj1371*	11168 Δ*cj1371*	This study
Δ*cj1371/cj1371*$^{+}$	11168 Δ*cj1371* complemented with WT copy of *cj1371*	This study
Δ*cj1372*	11168 Δ*cj1372*	This study
Δ*cj1372/cj1372*$^{+}$	11168 Δ*cj1372* complemented with WT copy of *cj1372*	This study
Δ*cj1373*	11168 Δ*cj1373*	This study
Eschericia coli DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Invitrogen™
Eschericia coli BL21(λDE3)	F– *omp*T *hsd*SB(rB–, mB–) *gal dcm* (DE3)	Invitrogen™
Eschericia coli TOP10	*hsd*R *mcrA lac*ZΔM15 *end*A1 *rec*A1	Invitrogen™

2.2.2 Media for growth of *C. jejuni*

Chocolate agar was routinely used for growth of *C. jejuni* as a solid medium. This consists of Columbia agar base (Oxoid), made according to manufacturer's instruction. Sterilisation was achieved by autoclaving (121 °C, 15 minutes, 15 psi). After cooling the sterile Columbia agar to approximately 70 °C, 5 % (*v/v*) sterile horse blood (Oxoid) was added. Vancomycin and amphotericin B (standard antibiotics added to *C. jejuni* to prevent bacterial and fungal contamination) were added to a final concentration of 10 µg mL^{-1} when chocolate agar was cooled to approximately 50 °C. Selective antibiotics (if required) were added to a final concentration of 50 µg mL^{-1}. Plates were stored at 4 °C for up to four weeks.

Muller-Hinton (MH) agar (Oxoid) was routinely used to grow *C. jejuni*. This was prepared according to manufacturer's instructions and sterilised by autoclaving (121 °C, 15 minutes, 15 psi).

Muller-Hinton (MH) broth (Oxoid) supplemented with 20 mM L-serine (MHS) or Brain-Heart infusion (BHI) broth (Oxoid) were used to grow *C. jejuni* in liquid cultures. Media were sterilised by autoclaving (121 °C, 15 minutes, 15 psi). Antibiotics were added to sterile media prior to inoculation to final concentrations as for solid media.

2.2.3 Media for growth of *E. coli*

Escherichia coli strains were routinely cultured in Luria-Bertani (LB) medium at 37 °C. For the preparation of solid LB medium, bacteriological agar (Oxoid) was added to a concentration of 1.5 % (*w/v).* Sterilisation was achieved by autoclaving (121 °C, 15 minutes, 15 psi) and, when required, selective antibiotics were added to appropriate concentrations prior to inoculation.

2.3 Growth of organisms

2.3.1 Growth of *C. jejuni*

Stock *C. jejuni* cultures were maintained on chocolate agar at 37 °C in microaerobic conditions (5 % *(v/v)* O_2, 10 % *(v/v)* CO_2, and 85 % *(v/v)* N_2) generated in a MACS-VA500 Microaerobic Workstation (Don Whitley Scientific Ltd). Cells were sub-cultured into fresh plates every 48 – 72 hours to maintain actively dividing organisms. Liquid cultures were routinely grown at 37 ^{o}C in MHS or BHI under standard microaerobic conditions (as described earlier) with culture volume equal to 1:5 conical flask capacity mixed by continuous shaking at 180 rpm. Liquid media were positioned in microaerobic conditions overnight for gas equilibration. Overnight starter cultures were allowed to grow from fresh 24 hours old cells grown on plates prior to inoculation of larger cultures.

2.3.2 Growth of *E. coli*

E. coli were cultured aerobically at 37 °C on LB agar or broth (Melford) which was sterilised by autoclaving. Selective *E. coli* growth was achieved with addition of appropriate antibiotics. Liquid cultures were grown with shaking (225-250 rpm).

2.4 Physiological experiments

2.4.1 Growth assays

One-day old cells harvested from chocolate agars were used to grow 25 mL MHS starter cultures containing appropriate selective antibiotics. After overnight incubation of starter cultures at 37 °C in standard microaerobic conditions, cells were pelleted by centrifugation (15,000 *xg*, 3 min) and resuspended in 1 mL plain MHS. Cell densities were adjusted to an OD_{600} of 0.1 in 50 mL sterile MHS cultures containing amphotericin B and vancomycin at 10 µg mL^{-1} each. Cultures were incubated for 24 hours and samples of 1 mL were collected every hour for the first 8 hours and a final

sample was collected at 24 hours. OD_{600} readings were plotted in a growth curve with an X axis represents the time (hr) and Y axis for OD_{600} for three biological replicates per each strain. Growth deficiency (if any) of mutants was compared to the one of the wild-type.

2.4.2 Disc diffusion assay

One-day-old cells from chocolate agar plates were used to prepare a 50-mL MH culture supplemented with 20 mM L-serine with addition of amphotericin B and vancomycin to a final concentration of 10 μg mL^{-1}. Cultures were grown under standard microaerobic conditions at 37 °C overnight. The OD_{600} of the stationary-phase cell culture was adjusted to 0.1, and 20 mL of this was used to inoculate 400 mL MH agar supplemented with amphotericin B and vancomycin to a final concentration of 10 μg mL^{-1} each. 20 mL of this was pipetted to each Petri dish and allowed to set at RT. After the plates have been dried for 10 minutes in a laminar flow cabinet, 5 μL stress agent (SDS) was applied to the centre of the 8-mm Whatman filter paper discs that had been pre-sterilised by autoclaving and positioned at the centre of the agar plates. Nine replicate plates were prepared per concentration of SDS per strain, and the solvent (water) was applied to the centre of the control plates. Plates inoculated with the relevant strains were incubated in a microaerobic conditions at 37 °C for 2 – 3 days, and the diameter of growth inhibition (including the filter paper disc) was measured. No growth inhibition was observed on the control plates.

2.4.3 Determination of cell viability and sensitivity to antimicrobials

Fresh one-day old *C. jejuni* cells grown on chocolate agar were harvested into 1 mL MHS broth by centrifugation (15,000 *xg*, 3 min) and resuspended in 100 μL MHS to inoculate 25 mL MHS starter culture that had been incubated overnight at 37 °C in standard microaerobic conditions. Samples from starter cultures (180 μL) were added to a 96-well microtiter plates containing antimicrobial agent (polymyxin B, polymyxin E or protamine sulfate) at final concentrations of 0, 50, 100 or 200 μg mL^{-1}, or human

cathelicidin (LL-37) at final concentrations range from 0 – 10 µM. Cultures were incubated microaerobically at 37 °C for 2 hours without shaking. Serial dilution was carried out by adding 20 µL of culture to sterile 180 µL MHS, mixing thoroughly using multichannel pipette. 10 µL from various dilutions were plated on square plates containing plain MHS agar, allowed to dry and incubated microaerobically at 37°C for 48 hours until defined single colonies were formed. Colony forming units (cfu) mL^{-1} were counted for each strain and compared to cfu mL^{-1} of wild type strain.

2.4.4 Motility assays

Motility assays were performed for Mla system mutants (Δ*cj1371-73)* and their complemented strains using 0.3 % MHS agar plates (motility plates). *C. jejuni* strains were allowed to grow in MHS broths containing appropriate selective antibiotics at 37 °C in microaerobic conditions overnight. 10 µL from cultures of each strain was pipetted and spotted on the centres of motility plates, incubated without inversion overnight at 37 °C in standard microaerobic conditions. The zone of motility was measured for each strain and compared to the motility zone of the wild type.

2.4.5 Biofilm assays

Biofilm formation assays were carried out in Sterile 96-well flat bottom plates (Fisher, UK). Each row of the plate contained one strain (12 replicates) plus a contamination control row with plain MHS. *C. jejuni* strains were pre-cultured overnight at 37 °C in standard microaerobic conditions in MHS containing appropriate selective antibiotics. Dilutions were applied to cultures with sterile plain MHS to an OD_{600} of 0.1 and 200 µL of the dilutions were added to each well. Plates were incubated in two different conditions; aerobic and microaerobic, without shaking, both at 37 °C for 24 hours. Cell densities of each 200 µL cultures were measured at OD_{600} nm in Victor^2 1420 Multilabel Counter plate scanner (Perkin Elmer, USA). Planktonic cells were carefully and fully poured off and 200 µL crystal violet (1 % *(v/v)* in dH_2O) stain was added to each well and allowed to stand for 5 min at RT. The crystal violet solution was then

poured off and plates were washed with dH_2O for three times until clean and allowed to dry for 10 min. The remaining dye in each well was resuspended in 200 µl of ethanol: acetone (4:1) and plates were incubated for further 20 min at RT with gentle agitation. The OD_{600} of the crystal violet was measured and the reading reflected the static biofilm formation. The MHS-only control was averaged and subtracted from the test wells to normalise data for both growth and biofilm formation. The OD_{600} reading of static Biofilm formation was divided by the OD_{600} reading of planktonic cells for each well to convert to arbitrary biofilm units, which take into account the amount of growth.

2.5 DNA preparation and manipulation

All reagents used for molecular biology techniques were purchased from Bioline, Boehringer Mannheim, Fermentas, New England Biolabs, Promega and Roche.

2.5.1 Plasmids used in this study

All plasmids used in this study are listed in table 2.3.

Table 2.3 Plasmids used in this study.

Plasmid	**Description**	**Resistance marker**	**Source**
pET21a(+)	An over-expression vector with *C*-terminal 6X Histidine residues used for protein over-production under the control of IPTG inducible T7 promoter	Amp^R	Novagen
pGEM®3Zf(-)	A cloning vector used in mutagenesis constructs. Multiple cloning site in frame with *lac* operon which allows blue/white colonies selection	Amp^R	Promega
pQE-70	A cloning vector used for over-production of *C*-terminally His tagged proteins	Amp^R	Qiagen
pJMK30	Cloning vector containing the *aph*AIII gene encoding kanamycin resistance	Amp^R Kan^R	Van Vliet *et al*., 1998
pAV35	Cloning vector containing the chloramphenicol acetyl transferase (*cat*) inserted at *BamH*I site	Amp^R Cm^R	Van Vliet *et al*., 1998

pBAD/His B	An over-expression vector with *N*-terminal polyhistidine adjacent to multiple cloning site used for protein over-production under the control of L-arabinose inducible *ara*BAD promoter	Amp^R	Invitrogen
pCOLD-TF	Cold shock expression plasmid used for increased production of soluble fraction of proteins under the regulation of *cspA* promoter	Amp^R	Takata
pGEM-T Easy	T7 and SP6 RNA polymerase promoters flanking a multiple cloning site within the α-peptide coding region of the enzyme β-galactosidase	Amp^R	Promega
pK46	Vector used for complementation of *C. jejuni* mutants by insertion of wild-type copy of DNA into the *cj0046* pseudogene	Kan^R	Gaskin *et al*., 2007
p*CfdxA*	Vector used for complementation of *C. jejuni* mutants by insertion of wild-type copy at the *cj0046* pseudogene, with *fdxA* promoter	Cm^R	Gaskin *et al*., 2007
p*1289cat*	pGEM3Zf(-) containing 400 bp upstream and 600 bp downstream flanking DNA of *cj1289* with the *cat* cassette inserted in place of the deleted sequence	Cm^R	This study
p*0694kan*	pGEM3Zf(-) containing 400 bp upstream and 600 bp downstream flanking DNA of *cj0694* with the *kan* cassette inserted in place of the deleted sequence	Kan^R	This study
p*peb4kan*	pGEM3Zf(-) containing 400 bp upstream and 600 bp downstream flanking DNA of *cj0596* with the *kan* cassette inserted in place of the deleted sequence	Kan^R	This study
pK*1289*	pK46 containing wild-type copy of *cj1289* inserted into the *cj0046* pseudogene	Kan^R	This study
p*CfdxA0694*	p*CfdxA* containing wild-type copy of *cj0694* inserted into the *cj0046* pseudogene	Cm^R	This study
p*CfdxApeb4*	p*CfdxA* containing wild-type copy of *peb4 gene* inserted into the *cj0046* pseudogene	Cm^R	This study
p*1371kan*	pGEM-T Easy with the *kan* cassette inserted in place of *cj1371*	Kan^R	Arnoud Van Vliet (IFR)
p*1372kan*	pGEM-T Easy with the *kan* cassette inserted in place of *cj1372*	Kan^R	Arnoud Van Vliet (IFR)

p*1373kan*	pGEM-T Easy with the *kan* cassette inserted in place of *cj1373*	Kan^R	Arnoud Van Vliet (IFR)
pC*fdxA1371*	pC*fdxA* containing wild-type copy of *cj1371* inserted into the *cj0046* pseudogene	Cm^R	Arnoud Van Vliet (IFR)
pC*fdxA1372*	pC*fdxA* containing wild-type copy of *cj1372* inserted into the *cj0046* pseudogene	Cm^R	Arnoud Van Vliet (IFR)
pC*fdxA1373*	pC*fdxA* containing wild-type copy of *cj1373* inserted into the *cj0046* pseudogene	Cm^R	Arnoud Van Vliet (IFR)
pET*0694H*	Plasmid construct with *cj0694* minus stop codon and *N*-terminal signal sequence, cloned into *Nde*I and *Xho*I sites of the pET21a(+) vector. Used for the production of recombinant Cj0694.	Amp^R	This study
pET*0694*	Plasmid construct with *cj0694* including stop codon cloned into *Nde*I and *Xho*I sites of the pET21a(+) vector. Used for the production of recombinant Cj0694 without the *C*-terminal polyhistidine	Amp^R	This study
pET*PEB4*	Plasmid construct with *cj0596* without stop codon cloned into *Nde*I and *Xho*I sites of the pET21a(+) vector. Used for the production of recombinant PEB4 protein.	Amp^R	This study
pBAD*0694*	Plasmid construct with *cj0694* including stop codon but minus *N*-terminal signal sequence, cloned into *Xho*I and *EcoR*I sites of the pBAD/His B vector. Used for the production of recombinant Cj0694 without the *N*-terminal signal sequence	Amp^R	This study
pCOLD*0694*	Plasmid construct with *cj0694* minus *N*-terminal signal sequence, cloned into *Nde*I and *EcoR*I sites of the pCOLD-TF vector. Used for the production of recombinant Cj0694.	Amp^R	This study
pNJ077	pQE-70 vector harbouring BamA cloned at *BamH*I site, used for over-production of BamA	Amp^R	Neil Oldfield (MBIG, Nottingham)

2.5.2 Primers used in this study

All primers used in this study were purchased from Sigma-Aldrich, UK.

Table 2.4 Primers used in this study**.** Restriction sites are illustrated in bold italics. Primer sequences which bind to DNA templates are underlined. Yellow sequences show isothermal assembly (ISA) cloning adapter regions.

Name	**Oligonucleotide 5' - 3' sequence**
1289-F1-F	GAGCTCGGTACCCGGGGATCCTCTAGAGTCGGCACGCAATTCTTCTAAT
1289-F1-R	CCCCGGGCTGCAGGAATTCCCAAGGAGAATTTAATAAAATTTTTTTCAT
1289-F2-F	GTACCCGGGATCCACTAGTCCAAGGTGTGCTATAGAATATTTAAGATAA
1289-F2-R	AGAATACTCAAGCTTGCATGCCTGCAGGTCATGAAACTGCTATTAAGG
0694-F1-F	GAGCTCGGTACCCGGGGATCCTCTAGAGTCTATCCTATTGCGAAAATC
0694-F1-R	AAGCTGTCAAACATGAGAACCAAGGAGAATTGTTGCATCCAAGTAAGC
0694-F2-F	GAATTGTTTTAGTACCTAGCCAAGGTGTGCTTTATTATAAAGGGAATT
0694-F2-R	AGAATACTCAAGCTTGCATGCCTGCAGGTCCTATCATCTAAACAAGCTAT
PEB4-F1-F	GAGCTCGGTACCCGGGGATCCTCTAGAGTCAAAAACATGAGTATCTACAG
PEB4-F1-R	AAGCTGTCAAACATGAGAACCAAGGAGAATACTAAAGAAAATTTTTTGAT
PEB4-F2-F	GAATTGTTTTAGTACCTAGCCAAGGTGTGCCTAAAGTGGAATATAAATAA
PEB4-F2-R	AGAATACTCAAGCTTGCATGCCTGCAGGTCATGCAAGAGCTACATCTTCT
1289+F	TAAT***CGTCTC***ACATGAAACGCGTTGTAAGTTTT
1289+R	TAAT***CGTCTC***ACATGAATTTTAATTGTAATTTTTA
0694+F	TAAT***CGTCTC***ACATGAGAACAAATATTTTT
0694+R	TAAT***CGTCTC***ACATGCTAATATATTCAAAATCA
PEB4+F	TAAT***CGTCTC***ACATGATTTTAGTTAATTAGCTT
PEB4+R	TAAT***CGTCTC***ACATGGTCTAAAACACCCA
1371-screen-F	AAATTTTTAGGAGTTTTTGC
1371-screen-R	ATATTTTTTTCATTAATTTCC
1372-screen-F	TTAGCAAATAAAGGAAATTA
1372-screen-R	TAAAAAACTTTAAAAGCTTT
1373-screen-F	CAAAAACTTGAAAGTATTGTCATAGAATA
1373-screen-R	AAATGAAAAATTTTTTAAAATATATAGCTA
1371+F	TAAT***CGTCTCACATG***TTGAGAATAAAATTT
1371+R	TAAT***CGTCTCACATG***TTATTTGCTAAGTTC
1372+F	TAAT***CGTCTCACATG***ATGAAAAAAATATTTTTA

1372+R	TAAT***CGTCTCACATG***TTATTCTATGACAATAC
kan-F-*sty*I	ATTCTCCTTGGTTCTCATGTTTGACAGCTTAT
kan-R-*sty*I	GCACACCTTGGCTAGGTACTAAAACAATTCAT
cat-F-*sty*I	ATTCTCCTTGGGAATTCCTGCAGCCC
cat-R-*sty*I	GCACACCTTGGACTAGTGGATCCCGG
0694-OEF-pCOLD	AATA***CTCGAG***GATTTTAATCTCAATAGA
0694-OER-pCOLD	AATA***GAATTC***TCAATTCCCTTTATAATAAAT
0694-OEF-pBAD	AATA***CTCGAGA***GATTTTAATCTCAATAGA
0694-OER-pBAD	AATA***GAATTC***TCAATTCCCTTTATAATAAAT
0694-OEF-pET	AATTATTA***CATATG***GATTTTAATCTCAATAG
0694-OER-pET(stop)	AATTATTA***CTCGAG***TCAATTCCCTTTATAAT
0694-OER-pET	AATTATTA***CTCGAG***ATTCCCTTTATAAT
PEB4-OEF	AATA***CATATG***ATGAAAAAATTTTCTTT
PEB4-OER	AATA***CTCGAG***ATTTATATTCCACTTTA
46F	GAGCCAATCCTATTTCATCAG
46R	CCAGCCCATAAAAGTAAAAGCGAGAC
M13F	GTAAAACGACGGCCAG
M13R	CAGGAAACAGCTATGAC
Omp85F	CGC**GGATCC**ATCGGATTAAATCATTTATCG
Omp85R	CGC**GGATCC**AAAGCGTGTTCCAAGATTAAATTC

2.5.3 Preparation of DNA

2.5.3.1 Isolation of genomic DNA

Genomic DNA of *C. jejuni* was extracted in large scale using Wizard® Genomic DNA Purification Kit (Promega). This DNA preparation kit produces quality DNA which can be used as PCR template by using various detergent and temperature changes for cell lysis and subsequently release of genomic DNA. Following removal of cell debis by centrifugation, genomic DNA was collected from supernatant and purified by ethanol precipitation. Manufacturer's instructions were followed.

2.5.3.2 Isolation of plasmid DNA

Plasmid DNA was routinely isolated using QIAprep® Spin Miniprep Kit (Qiagen) for small scale isolation, or the Wizard® Plus Midipreps DNA Purification System (Promega) for large scale isolation as instructed by manufacturers. The principle of spin column for DNA preparation is based on ion-exchange chromatography. DNA binds a DEAE sepharose gel filter in the spin column while debris and impurities pass through the filter during centrifugation. Plasmid DNA is then collected by treatment of high ionic strength elution buffer.

2.5.4 Manipulation of DNA

2.5.4.1 PCR amplification

Standard PCR reactions were implemented in reaction volume of 12.5 µl for either screening of *C. jejuni* strains or verifying DNA sequence of newly cloned plasmid constructs after transformation of *E. coli* strains using MyTaq™ Mix (Bioline). Alternatiely, 50 µl reaction volume was used when accurate DNA sequence was required using Accuzyme DNA polymerase (Bioline) in a Techne Techgene Thermal Cycler (Techne). Manufacturer's guidelines were followed for setting up reaction. At least 0.1 µg of template DNA was used and 10 µM primer stocks were added to a final concentration of 200 nM. The PCR cycle suggested by the manufacturers was followed with the annealing temperature of 5-10 ^{o}C lower than the primer melting temperature, and extension time dependent on size of DNA product for each reaction. PCR products were purified and concentrated using the QIAquick® PCR Purification Kit (Qiagen) and visualised by agarose gel electrophoresis.

2.5.4.2 Agarose gel electrophoresis

DNA product was visualized by agarose gel electrophoresis. Agarose gel was prepared by addition of 0.7% agarose to 1X TAE (40 mM Tris-acetate, 1mM EDTA, pH 8.0) buffer, and dissolved by heating. After addition of approximately 100 ng mL^{-1} ethidium

bromide, agarose solution was poured into a gel cast with a comb in place and allowed to set. The examined DNA samples were mixed with 5 x DNA loading buffer (Tri-Colour, Bioline) and loaded into the wells alongside Hyperladder I (Bioline). Electrophoresed was performed in 1 x TAE buffer at a constant 120 V until satisfactory migration had achieved, and visualised under an ultraviolet (UV) light source using a Gene Flash Gel Doc System (Syngene).

2.5.4.3 Isolation of DNA from agarose gel

When a DNA fragment from an agarose gel was required for further use, gel extraction was carried out using a QIAquick® Gel Extraction Kit (Qiagen) following manufacturer's instructions to obtain a pure DNA product from agarose gel.

2.5.4.4 Restriction digestion

Digestion of DNA with restriction enzymes was carried out in 20 µl reaction volume according to manufacturer's recommendations (NEB or Promega), in the presence of supplied buffer for optimum time and temperature recommended by manufacturers. For digest product which to be used for cloning, reaction mixture was heat inactivated and enzyme/buffer removed by the QIAquick® PCR Purification Kit (Qiagen).

2.5.4.5 Klenow polymerase treatment of DNA

DNA Polymerase I, Klenow Fragment (NEB) from *E. coli* was used to fill in 5′ overhangs on PCR products and vector termini for subsequent blunt-ended ligation. The reaction mixtures were incubated according to the manufacturer's guidelines at 37 °C for 30 minutes and reactions stopped by heat inactivation at 65 °C for 15 minutes.

2.5.4.6 Phosphatase treatment of DNA

To prevent vector DNA self-ligation during ligation reaction, Antarctic Phosphatase (New England Biolabs) was used to remove 5′-phosphoryl groups from the ends of digested plasmid DNA. Phosphatase treatment was carried out according to manufacturer's instructions. Typically, reaction mixtures were incubated at 37 °C for 30 minutes and heat inactivated at 65 °C for 10 minutes. Enzymes and buffers were removed using a QIAquick PCR purification kit (QIAGEN, UK) as instructed by manufacturer.

2.5.4.7 Ligation of DNA fragments

Ligation of DNA fragments was carried out at insert to vector molar ration of 1:1, 1:5, 3:1 and 5:1. Insert and vector concentrations were estimated from known volume loaded on agarose gel and compared to a standard molecular weight of known concentration (Hyperladder I [Bioline]).

The amount of DNA required to achieve different molar ratio was calculated according to the following equation:

$$\frac{concentration\ of\ vector\ (ng) \times size\ of\ insert\ (kb)}{size\ of\ vector\ (kb)} \times \frac{molar\ ratio\ of\ insert}{molar\ ratio\ of\ vector} = concentration\ of\ insert\ (ng)$$

Typically, 20 – 50 ng of vector DNA and calculated amount of insert DNA were used per ligation, plus 1 µl of 10 x T4 DNA ligase buffer (NEB) and 0.5 µl of T4 DNA ligase (NEB). The final volume of 20 µl was reached by dH_2O. An insert-free reaction control was included to observe the level of vector DNA self-ligation. Ligation reactions for cohesive-ended DNA fragments were carried out at 16 °C overnight or at room temperature (RT) for 1 hour. For blunt-ended DNA fragments, reactions were performed at 16 °C overnight or at RT for 2 hours. The ligation reaction was stopped by heat inactivation at 65 °C for 20 minutes prior to transformation of *E. coli* competent cells with the ligation mixture containing the plasmid construct.

2.5.5 Isothermal assembly (ISA) cloning

Isothermal assembly (ISA) cloning method was used as described by Gibson *et al* (2009), to generate plasmid construct for *C. jejuni* mutagenesis (Gibson *et al.*, 2009). The principle of the ISA cloning method is based on assembly of multiple overlapping DNA fragments in a single step reaction, in the presence of T5 exonuclease, Phusion polymerase and Taq ligase to generate an *in vitro* recombinant DNA fragment.

The DNA fragments to be assembled in the ISA reaction were prepared as follows. First, pGEM3Zf(-) was digested with *Hinc*II and phosphatase treated. The appropriate antibiotic resistance (Ab^R) cassette was PCR amplified using kan or cat primers. 50 bp primers (20 bp for gene of interest plus 30 bp for adapter) were designed to amplify two PCR products indicated here as F1; first 15 – 20 bases of gene of interest plus upstream flanking DNA, and F2; last 15 – 20 bases of gene of interest plus downstream flanking DNA). The adapter sequences used an overlapping region between the DNA fragments and Ab^R cassette or terminal sequence of the vector (Fig. 2.1). Digested vector and PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen) and DNA concentration was measured using a BioPhotometer (Eppendorf).

ISA mastermix was prepared by adding 40 µL ISA buffer (25 % polyethylene glycol [PEG-8000], 500 mM Tris-HCl pH 7.5, 50 mM $MgCl_2$, 50 mM dithiothreitol [DTT], 1 mM of each dNTP and 5 mM NAD) to 0.125 µL T5 exonuclease (Cambio), 2.5 µL Phusion polymerase (NEB) and 20 µL Taq ligase (NEB). The final volume of 150 µL was achieved by dH_2O, and divided into 15 µL aliquots and stored at – 20 °C. The ISA DNA fragments were combined in equimolar concentrations (10-100 ng of each) in a total volume that did not exceed 5 µL. This was added to 15 µL ISA mastermix and the volume made up to 20 µL with dH_2O. The reaction was allowed to progress overnight at 50 ^oC in a Techne Techgene Thermal Cycler (Techne Ltd). After overnight incubation, ISA reactions were purified using the QIAquick® PCR Purification Kit (Qiagen), eluting in 15 µL dH_2O. Only 3 – 5 µL of the resulting DNA was used to transform competent *E. coli* DH5α, plated on selective LB solid media containing the appropriate antibiotic. Positive colonies were screened by colony PCR using extreme fragment primers and correct assembly of plasmids was confirmed by automated DNA sequencing using M13 primers (Core Genomic Facility, University of Sheffield Medical School, UK).

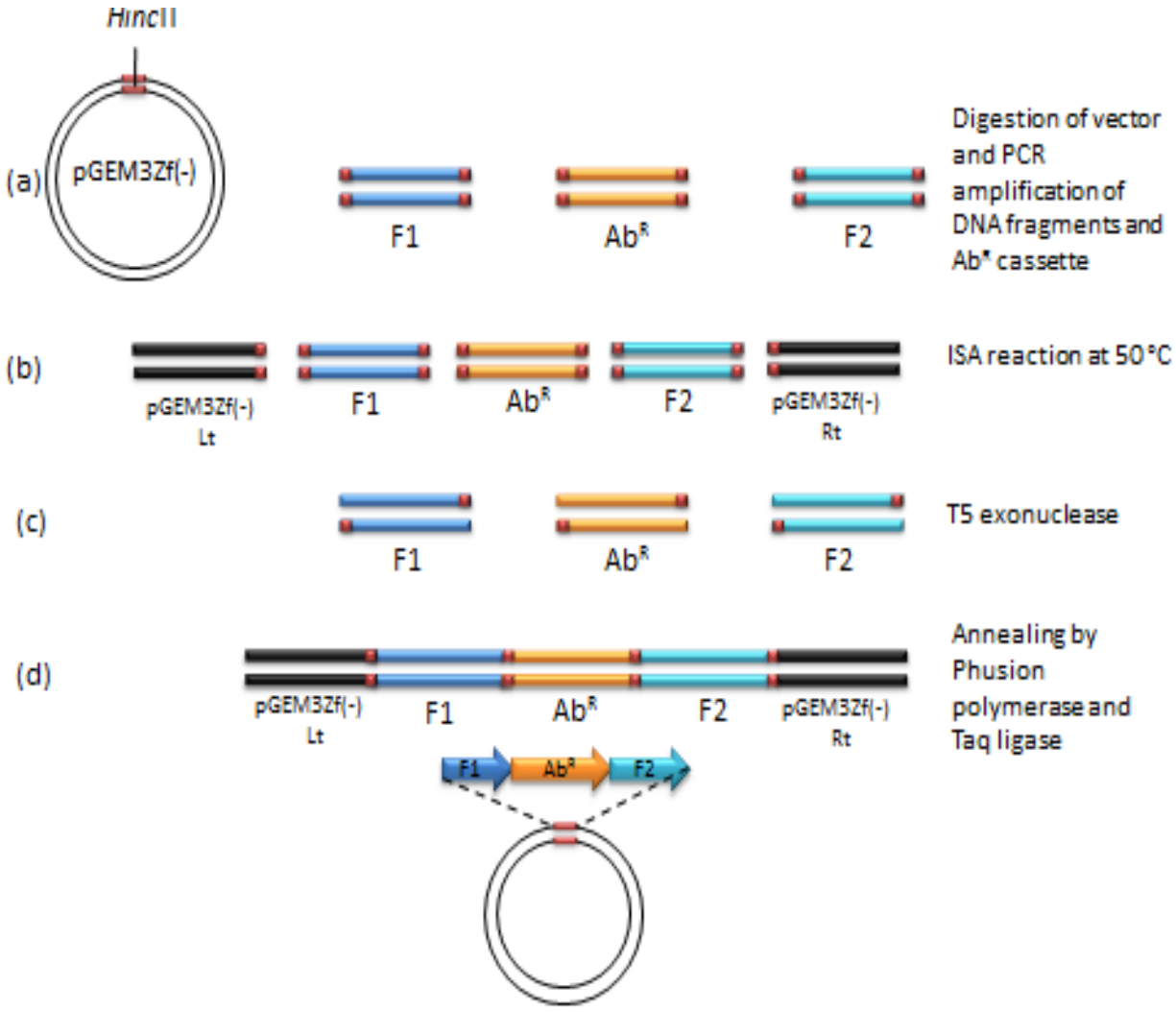


Figure 2.1 Isothermal assembly (ISA) cloning strategy. (a) The pGEM3Zf(-) vector was digested with *Hinc*II and treated with alkaline phosphatase to prevent self-ligation. DNA Fragments; F1, F2 and an antibiotic resistance cassette (Ab^R) were amplified by proof-reading DNA plymerase with adapter sequences at their termini. **(b)** The digested vector and amplified DNA fragments were incubated at 50 °C overnight. **(c)** T5 exonuclease removes nucleotides from the 5′ of double stranded DNA fragments. **(d)** Phusion DNA polymerase and *Taq* DNA ligase repair the double stranded DNA fragments. This generates a circular mutagenesis construct which possesses a replacement of 5′ end and upstream fragment, and 3′ end and downstream fragment of the gene of interest by Ab^R cassette. When *C. jejuni* strain is transformed by this construct, the gene of interest is deleted and replaced by Ab^R cassette. Figure and legend modified from (Gibson *et al.*, 2009).

2.6 Preparation and transformation of competent cells

2.6.1 Preparation and transformation of competent *C. jejuni*

Competent *C. jejuni* were prepared freshly when required for transformation of *C. jejuni*. A one-day old plate was harvested in 1 mL BHI and cells were collected by centrifugation (15,000 *xg*, 5 min, and 4 ^{o}C). Cells were resuspended in 1 mL wash buffer (10 % *(v/v)* glycerol, 9 % *(w/v)* sucrose) and pelleted as above. Washing was repeated three times and cells were resuspended in 100 µL wash buffer. Plasmid DNA (3 – 5 µL) was added to cell suspension and incubated in ice for 30 minutes. The transformation mixtures were transferred to pre-chilled electroporation cuvettes (Bio-Rad). Electroporation allowed to take place at pulse of 25 F. 2.5 kV and 200 Ω for 4 milliseconds in an *E. coli* pulser (Bio-Rad). Sterile BHI broth (100 µL) was added to the electroporated cells and 100 µL was plated in duplicate onto non-selective blood agar, incubated microaerobically at 37 ^{o}C for 24 hrs. Cells were harvested into 1 mL BHI broth, centrifuged (15,000 *xg*, 5 min, RT), resuspended in 200 µL BHI broth and plated (100 µL) in duplicate onto selective blood agar. Plates were incubated as above for 3 – 5 days to allow transformant single colonies resistant to selective antibiotics to appear.

2.6.2 Preparation of competent *E. coli*

Competent *E. coli* cells were prepared as described by (Hanahan, 1983). *E. coli* was grown in LB broth at 37 °C for 16 hours until mid-exponential phase was reached to give a cell density of 4 to 6 × 10^{7} viable cell mL^{-1} (O.D. $_{600}$ ~ 0.4 – 0.6). Cells were incubated on ice and centrifuged at 6000 *xg* for 20 minutes at 4 °C. The unwanted supernatants were discarded and pellets resuspended in 50 mL RF1 solution (100 mM KCl, 50 mM $MnCl_2 4H_2O$, 30 mM CH_3COOK, 10 mM $CaCl_2 \cdot 2H_2O$, 15 % *[w/v]* glycerol; adjusted to pH 5.8 with 0.2 M acetic acid). Cells were incubated on ice for 15 minutes, centrifuged as above and resuspended in 8 ml RF2 solution (10 mM MOPS, 10 mM KCl, 75 mM $CaCl_2 \cdot 2H_2O$, 15% *[w/v]* glycerol; adjusted to pH 6.8 with 5 M NaOH). Cells were incubated once more on ice for 20 minutes, aliquoted into pre-chilled eppendorfs and stored at – 70 ^{o}C. Viability of competent cell and transformation efficiency were tested by transformation with uncleaved vector.

2.6.3 Transformation of competent *E. coli*

Competent cells (200 µL) were freshly thawed on ice and 1 µL plasmid or 3 – 5 µL ligation reaction (10 – 20 ng) was added. Cells were incubated on ice for 30 minutes before heat shocking (42 ^{o}C for 1 minute, ice for 2 minutes). Sterile liquid LB (1 mL) was added to the mixture and incubater at 37 ^{o}C for 1 hour with shaking at 250 rpm. Following to incubation, cells were harvested by centrifugation at 1000 *xg* for 2 minutes, resuspended in 200 µL plain LB, and 100 µL was plated on LB agar containing appropriate selective antibiotic and incubated at 37 °C overnight. A negative control plate containing no insert was also routinely plated to determine the level of vector self-ligation.

2.7 Cell fractionation procedure

2.7.1 Preparation of *E. coli* cell-free extract

A 500 mL LB broth containing *E. coli* cells grown aerobically for 16 hours was pelleted by centrifugation (7,155 *xg*, 10 min and 4 °C) and cells resuspended in 10 mM Tris-HCl pH 8.0. the cell suspension was placed in ice and sonicated for 6 x 30 sec pulses at a frequency of 10 microns using a Soniprep 150 ultrasonic disintegrator (SANYO). The whole-cell lysate was kept in ice or – 20 °C for further analysis or otherwise, cell debris were removed by centrifugation (48,297 *xg*, 30 min and 4 °C), and the resulting supernatant was saved as the cell tree extract (CFE) in ice or – 20 °C.

2.7.2 Preparation of *C. jejuni* cell-free extract

Cells of *C. jejuni* strains were allowed to grow in 200 mL MHS broth under standard microaerobic conditions with shaking at 150 rpm for 16 hours after been harvested from on Columbia agar plates containing 5 % lysed horse blood. After achieving mid-exponential phase (~ 16 hours), cells were pelleted by centrifugation (7,155 *xg*, 10 min and 4 °C), and resuspended in 10 mM Tris-HCl pH 8.0 (unless otherwise stated). The resuspended solution was placed in ice and sonicated for 6 x 15 sec pulses at a

frequency of 10 microns using a Soniprep 150 ultrasonic disintegrator (SANYO) and cell debris were removed by centrifugation (48,297 *xg*, 30 min and 4 ^{o}C). The supernatant was kept as CFE and stored on ice or at -20 ^{o}C until further use.

2.7.3 Preparation of *C. jejuni* periplasm by osmotic shock procedure

A fresh culture of 0.5 – 1 L BHI broth containing *C. jejuni* grown until mid-log phase was achieved (~ 16 hrs) in standard microaerobic condition was harvested by centrifugation (7,155 *xg*, 15 min and 4 °C). Cells were gently resuspended in 20 mL STE (20 % *(w/v)* sucrose, 30 mM Tris-HCl pH 8.0 and 1 mM EDTA) buffer and incubated at RT with gentle shaking for 30 minutes. Then, cells were harvested by centrifugation (11,180 *xg*, 10 min and 4 °C) and supernatant was removed. Pink pellets were resuspended in ice-cold 10 mM Tris-HCl pH 8.0 buffer and incubated with gentle shaking at 4 °C and centrifuged (15,000 *xg*, 25 min and °C). The resulting supernatant containing periplasm was collected in 1.5 mL eppendorf tubes and stored at – 20 °C. The pellet was kept if required to be used in isolation of outer membrane.

2.7.4 Preparation of *C. jejuni* cell outer membrane

A culture of 1 L BHI broth containing *C. jejuni* grown overnight in standard microaerobic condition was harvested by centrifugation (7,155 *xg*, 15 min and 4 °C), and pellets were resuspended in 10 – 20 mL of 10 mM HEPES buffer pH 7.4. cell suspension was sonicated with ice cooling for 6 x 15 sec pulses at a frequency of 10 microns using a Soniprep 150 ultrasonic disintegrator (SANYO) and unbroken cells and cell debris were removed by centrifugation (27,167 *xg*, 30 min and 4 ^{o}C). The supernatant was kept in pre-chilled ultra-centrifuge tubes, and the inner and the outer membrane were pelleted by ultra-centrifugation (100,000 *xg*, 60 min and 4 °C). The red pellet containing both inner and outer membrane was resuspended in 2 mL of 10 mM HEPES buffer pH 7.4 and processed immediately or stored at – 20 °C. The inner membrane was dissolved by addition of 2 mL of 2 % sarkosyl (Sodium *N*-lauryl sarcosinate) dissolved in 10 mM HEPES buffer pH 7.4 and incubated at 37 °C for 30 minutes. A further centrifugation (48,297 *xg*, 30 min and 4 °C) was carried out to isolate

the OM. Supernatant containing the inner membrane was carefully and fully removed and the pellet contained the OM was washed three times prior to being homogenised by 0.5 – 1 mL of 10 mM HEPES buffer pH 7.4 and stored at – 20 °C.

2.7.5 Stable Isotope Labelling by Amino acids in Cell culture (SILAC)

The use of Stable Isotope Labelling by Amino acids in Cell culture (SILAC) developed by Ong *et al*., (2002) is highly applicable in quantitative proteomics in which the quantity and identity of relative differential protein change can be determined (Ong *et al.*, 2002). Basically, in this method, two groups of cells are grown on media that are identical in all contents except that one medium contains the light (^{12}C + ^{14}N) and the other medium contains the heavy (^{13}C + ^{15}N) forms of amino acids, typically Lys or Arg. The heavy and light amino acids are incorporated into all proteins during protein synthesis (these include periplasmic and OM protein). After isolation of the periplasms and OMs from both cell populations, proteins can be combined and analysed by mass spectroscopy for identification of any protein changes among the two cell groups.

2.7.5.1 Growth assays of wild-type and mutants in minimum Essential Media (MEM) for SILAC analysis

Phenol red-free MEM (Thermo) is a Minimum Essential Media that is deficient in L-lysine and L-arginine, and is specially used for SILAC analysis. Prior to performing a large-scale cultures for membrane isolation of the isotope-labelled wild-type and mutants, growth assays were conducted in MEM to ensure that all strains could grow with similar cell densities in the same volumes of cultures. One-day old cells were inoculated into 25 mL BHI pre-cultures contained 10 µg mL^{-1} amphotericin B and vancomycin, and 50 µg mL^{-1} kanamycin or chloramphenicol to select the growth of mutants. These were incubated overnight in microaerobic conditions at 37 °C. Cells were harvested and resuspended in 1 mL MEM that was pre-warmed to 25 °C. An additional harvesting and re-suspension step was applied to remove any remaining traces of BHI. Cells were adjusted to an $OD6_{00}$ of 0.1 in pre-warmed 50 mL MEM contained 10 µg mL^{-1} amphotericin B and vancomycin, 10 mM L-serine, 10 mM L-

aspartate, 50 µM ammonium ferric sulphate dissolved in 10 mM ascorbic acid, 0.4 mM L-arginine and 0.8 mM L-lysine. Cultures were incubated in microaerobic conditions at 37 °C for 24 hours and samples were collected from 0 – 8 hours and a final sample was collected at 24 hour. Absorbance was plotted in growth curves with an X axis represents the time (hr) and Y axis for OD_{600} using GraphPad Prism 6 (GraphPad Software Inc.).

2.7.5.2 Metabolic labelling with stable isotopes

Overnight cultures (25 mL) of the wild-type and mutants grown in BHI media containing 10 µg mL^{-1} amphotericin B and vancomycin, and 50 µg mL^{-1} of mutant selective antibiotics (kanamycin or chloramphenicol), were harvested and resuspended in1 mL pre-warmed MEM. The wild-type cells were added to 100 mL pre-warmed MEM contained in 500 mL conical flasks, also containing 50 µM ammonium ferric sulphate, 10 mM L-serine, 10 mM L-aspartate, 0.8 mM (U-$^{12}C_6$) L-lysine and 0.4 mM 'heavy' (U-$^{13}C_6$) L-arginine (Invitrogen). The mutant cells were added to identical media except for the L-arginine where 0.4 mM of 'light' (U-$^{12}C_6$) L-arginine was added. Cells were allowed to grow overnight in microaerobic conditions at 37 °C. After 24 hours of microaerobic growth, cultures of the wild-type and the mutant were pooled and periplasms and OMs were prepared as described in sections 2.7.3 and 2.7.4. Membrane fractions were sent to Dr Francis Mulholland (The Institute of Food Research, Norwich, UK) to be analysed by 1-D SDS-PAGE and mass spectroscopy. Figure 2.2 summarises the strategy for SILAC experiment approach.

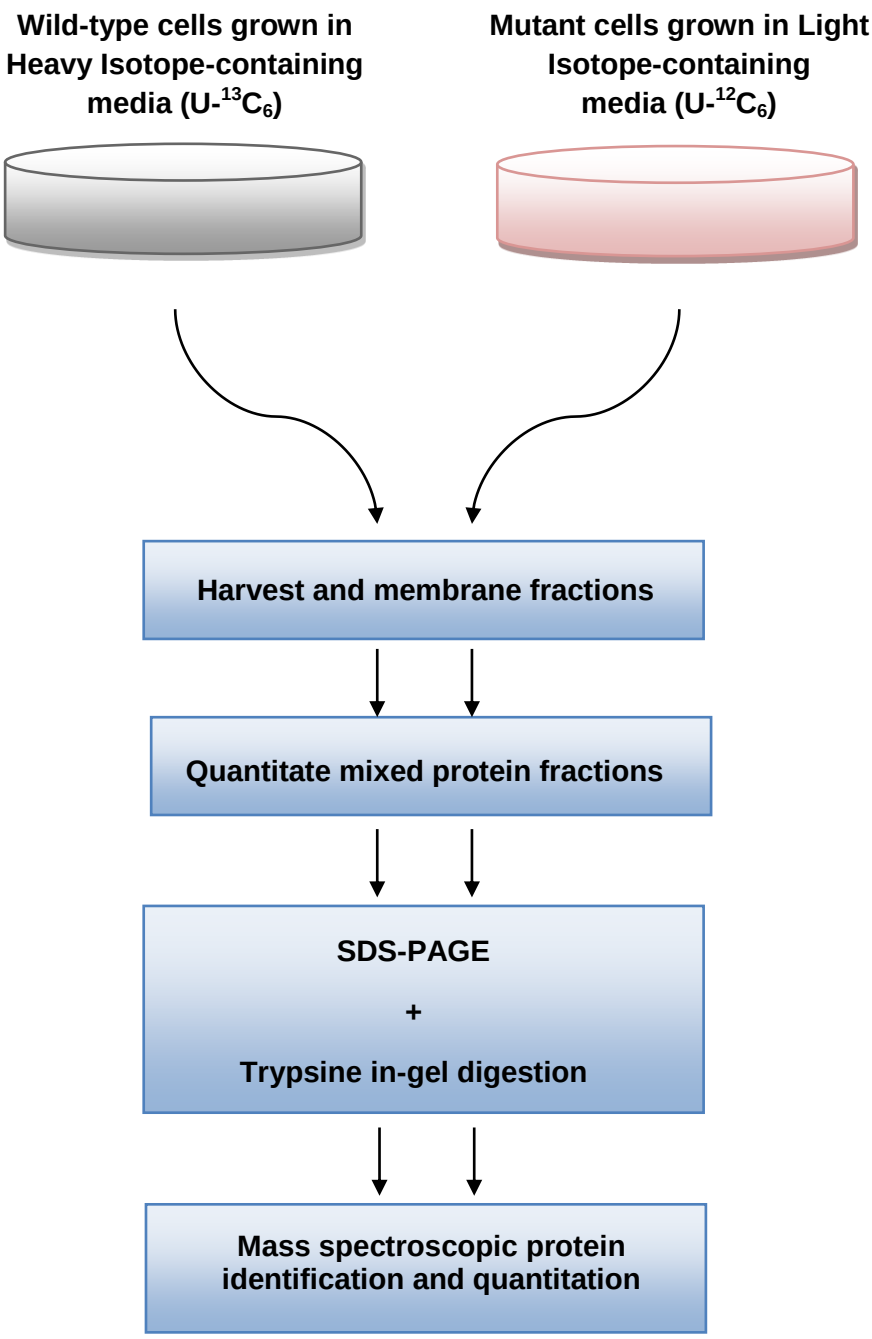


Figure 2.2 Strategy of SILAC experimental approach. The wild-type strain *C. jejuni* NCTC 11168 and the mutant cells were grown in MEM containing $^{12}C_6$-Lysine/$^{13}C_6$-Arginine and $^{12}C_6$-Lycine/$^{12}C_6$-Arginine, respectively. Following overnight growth, equal amounts of light and heavy stable isotope-containing cells were mixed and membrane fractions were prepared for separation by SDS-PAGE, trypsine digestion and identification by mass spectroscopy.

2.8 Protein manipulation

2.8.1 Determination of soluble protein concentration

Determination of protein concentration of purified proteins (>3 mg mL^{-1}) or cell-free extracts (CFE) was carried out using Bio-Rad Assay Kit as instructed by manufacturer. The principle of this assay is based on the Bradford Assay method, (Bradford, 1976), where binding of protein to a dye causes shift in the absorbance from 465 nm to 595 nm. Protein concentrations were calculated by comparison to a standard curve of a known concentration of Bovine serum albumin (BSA). Typically, 1 – 20 µL (1 – 10 µg) of protein were added to a 1.5 mL cuvette containing 0.8 mL milli-Q H_2O and 0.2 mL Bio-Rad reagent. Mixing was performed by inversion and absorbance measured at 595 nm. The protein concentration was determined according to the following formula:

$$\text{Protein concentration (mg mL}^{-1}) = \frac{Absorbance\ (595nm)}{Volume\ of\ protein\ (\mu L)} \times 15$$

For less concentrated proteins (<3 mg mL^{-1}), concentration was determined by measuring the absorbance at 280 nm (A_{280}) in a Biophotometer Spectrophotometer (Eppendorf, UK). Protein concentration (M) was measured according to Beer's Law ($A = \varepsilon \cdot C \cdot L$) using the following equation:

$$\text{Protein concentration (M)} = \frac{A280 - A320}{\varepsilon \cdot L} \times \text{Dilution factor}$$

A320 is subtracted from A280 to compensate for the effect of background absorbance and presence of any particles in the mixture. L represents the 1-cm optical path length of the cuvette, ε is the molar extinction coefficient of the protein, as computed by the ProtParam tool on the ExPASy website (http://expasy.org/tools/ protparam.html) based on a given amino acid sequence.

2.8.2 Determination of whole-cell protein concentration

Concentration of whole-cell proteins was measured by using a modified Lowry method (Markwell *et al.*, 1978). Protein samples were diluted at 1/20, 1/50 and 1/100 in dH_2O. Solution A (2 % *(w/v)* sodium carbonate, 0.4 % *(w/v)* sodium hydroxide, 0.16 % *(w/v)* sodium potassium tartrate, and 1% *(w/v)* SDS) and solution B (4 % *(w/v)* cupric sulphate) were mixed at a ratio of 100:1 to give solution C. 3 mL of solution C was added to the diluted protein samples, mixed thoroughly and incubated at RT for 60 minutes. After addition of 0.3 mL 1 N Folin-Ciocalteu's phenol reagent (Sigma-Aldrich) and thorough mixing, samples were incubated for a further 45 minutes at RT and absorbance was measured at 660 nm. BSA standard curve was used to compare with for calculation of protein concentration.

2.8.3 SDS-polyacrylamide gel electrophoresis

Bio-Rad Mini-PROTEAN® 3 or Mini-Protean® Tetra systems (both Bio-Rad) electrophoresis apparatus were routinely used for SDS-polyacrylamide electrophoresis (SDS-PAGE). Gel tanks were cleaned by ethanol, rined thoroughly with dH_2O and allowed to dry completely prior to use. Resolving gels made of 10 % or 12.5 % acrylamide (depending on experiments to be used for) were prepared. 30 % *(w/v)* acrylamide / 0.8 % *(w/v)* bisacrylamide was diluted in resolving gel buffer (1M Tris-HCl pH 8.8, 10 % *(w/v)* sodium dodecyl sulphate [SDS]) and distilled water creating a final concentration of 375 mM Tris-HCl pH 8.8, 0.1 % *(w/v)* SDS, 10 % or 12.5 % acrylamide mix. Polymerisation was originated with the addition of 0.1 % *(w/v)* ammonium persulphate (APS) and 0.01 % *(v/v)* N,N,N',N'-Tetramethylethylenediamine (TEMED). Acrylamide mixture was homogenised by gentle swirling, pipetted into pre-assembled gel cast and allowed to polymerise for 30 minutes. Stacking gels (6 %) were prepared by dilution of 30 % *(w/v)* acrylamide/0.8 % *(w/v)* bisacrylamide with stacking gel buffer (1 M Tris-HCl pH 6.8, 10 % *(w/v)* SDS) and distilled water to make a final concentration of a 125 mM Tris-HCl pH 6.8, 0.1 % *(w/v)* SDS, 6 % acrylamide mixture. Polymerisation was initiated as above. Components were mixed gently and the stacking gel was pipetted and added on top of the resolving gel with a comb inserted. The comb

was removed from the set gel, which was placed in a gel tank containing 1 x running buffer (25 mM Tris, 250 mM glycine, 0.1 % *(w/v)* SDS).

Protein samples to be analysed by SDS-PAGE were boiled for 5 minutes after addition of equal volume of sample buffer (60 mM Tris-HCl pH 6.8, 2 % *(w/v)* SDS, 0.005 % *(w/v)* bromophenol blue, 5 % *(w/v)* β-mercaptoethanol and 10 % *(w/v)* glycerol). Protein samples were loaded into the gel alongside molecular weight standard; PageRuler® (Plus) Prestained Protein Ladder (Fermentas). Electrophoresis was carried out at constant voltage of 180 V until sufficient migration of tracking dye had reached the bottom of the gel.

2.8.4 Coomassie blue staining of polyacrylamide gels

Gels were stained for at least 30 minutes with coomassie blue stain (50 % *(v/v)* methanol, 10 % *(v/v)* glacial acetic acid and 0.1 % *(w/v)* Coomassie Brilliant Blue R [Sigma-Aldrich]). Excess stain was poured off before gels were de-stained with de-stain solution (50 % *(v/v)* methanol, 10 % *(v/v)* glacial acetic acid) until individual protein bands were clearly visible.

2.8.5 Immunoblotting

Protein samples were prepared and loaded on 10 % SDS-PAGE as detailed previously. Proteins were transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences) using Mini Trans-Blot® Electrophoretic Cell (Bio-Rad). The transfer of protein was carried out at a constant current of 400 mA for 60 minutes at 4 °C in ice cold transfer buffer (25 mM Tris, 190 mM glycine, 20 % *(v/v)* methanol). All transfers were performed at RT with constant stirring. 1X TBS-T buffer (25 mM Tris-HCl pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1 % *(v/v)* Tween 20) was used as a base for washing the nitrocellulose membrane (20 min, 10 min and 5 min). 1X PBS-T (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 – to 1 L with dH_2O) was used as a base for blocking the membrane (5 % *(w/v)* dried skimmed milk incubated at least 1 hour with gentle shaking at RT or overnight at 4 °C). After blocking, membrane was washed with

1X TBS-T buffer with gentle shaking for 20 min and twice for 5 min. Primary polyclonal antibody raised in rat supplied at 1 mg mL^{-1} in phosphate buffer saline (PBS) pH 7.1-7.4 (BioServ UK Ltd, University of Sheffield, UK) was diluted in 1X TBS-T buffer (1:2000) and incubated with the membrane for 1 hour with gentle shaking or rolling. Membrane was washed with 1X TBS-T for 10 min and twice for 5 min. Secondary antibody (peroxidase-linked Anti-Rat IgG [whole molecule], Sigma-Aldrich) was diluted (1:2000) or (1:2500) in TBS-T and incubated with the membrane for another hour. Washing of the membrane by 1X TBS-T was for 10 min and three times for 5 min. After washing of the membrane, visualisation of antibody binding was performed according to the principle of enhanced chemi-luminescence (ECL Kit, GE Healthcare), following manufacturer's instructions. Exposure time was varied from 1 min to 5 min as required.

2.8.6 Over-production of recombinant proteins

The entire coding region of the protein of interest with or without the signal sequence and stop codon was PCR amplified using Accuzyme DNA polymerase (Bioline) and cloned into *Nde*I and *Xho*I sites (unless otherwise stated) of the pET21a(+) over-expression vector (Novagen). The correct insertion and orientation of the gene was confirmed by screening PCR using MyTaq^{TM} DNA polymerase (Bioline) or by automated DNA sequencing (Core Genomic Facility, University of Sheffield Medical School, UK), and the resulting recombinant plasmid was used to transform *E. coli* BL21(λDE3) competent cells (Invitrogen). The over-production of recombinant proteins was carried out under the control of IPTG inducible T7 promoter of the pET21a(+) vector which also has a *C*-terminal 6xHis tag. The addition of stop codon when cloning the coding region of the protein into pET21(+) stop the expression of the *C*-terminal 6xHis. The level of expression and solubility of recombinant proteins was monitored at different temperatures and times after the induction of IPTG. Cells were grown in small-scale cultures (50 mL LB containing 50 µg mL^{-1} carbenicillin) at 37 °C. When cells density reached OD_{600} ~ 0.6, 1 mM IPTG was added to different batches of cultures which had been then incubated at 37 °C or 25 °C. Samples were collected at 1, 3, 5 and 24 hours, pelleted by centrifugation (15,000 *xg*, 3 min). Equal volume of SDS

sample buffer was added to cells prior to be boiled and examined by SDS-PAGE in order to evaluate the level of over-production and solubility of proteins.

For over-production of proteins, variable volumes of cultures of over-expression *E. coli* cells harbouring the over-expression recombinant plasmid (depending on level of expression and solubility) were grown on LB broth containing 50 µg mL^{-1} carbenicillin at 37 °C until the OD_{600} ~ 0.6nm. Over-expression was induced by addition of 0.1 – 1 mM IPTG and cultures were incubated 37 °C or 25 °C with shaking at 250 rpm for 2 – 24 hours (pre-optimised growth conditions for best yield of protein). Cells were pelleted by centrifugation (7,155 *xg*, 20 min and 4 °C) and resuspended in binding buffer (see section 2.7.7) according to the volume of cultures. Cells were applied to sonication (6 X 30 sec pulses at a frequency of 10 amplitude microns in a Soniprep150 ultrasonic disintegrator, SANYO), and unbroken cells/debris were pelleted by centrifugation (48,297 *xg*, 30 min and 4 ^{o}C). The CFE was kept in ice used in further purification steps.

Over-production of recombinant proteins was also performed using pBAD/His B (Invitrogen) or pCOLD-TF (Takata) over-expression vectors. The former possesses 6xHis in the *N*-terminal adjacent to multiple cloning site and the over-expression was carried out under the control of L-arabinose inducible *ara*BAD promoter. The latter also has *N*-terminal 6xHis, and allows fusion of the 51.5 kDa trigger factor adjacent to the 6xHis residues, and has a Factor Xa protease cleavage site located before the multiple cloning site. It is used for increased recombinant protein yield, purity and solubility under the control of IPTG inducible *cspA* (cold shock protein) promoter, where the expression is performed at low temperature (~15 °C). Over-expression in pBAD/His B took place by transformation of *E. coli* TOP10 competent cells (Invitrogen) with recombinant plasmid construct. Cells were grown in LB broth containing 50 µg mL^{-1} carbenicillin at 37 °C until OD_{600} ~0.6. Over-expression was induced by addition of 0.02 % L-arabinose and cultures were incubated 37 °C with shaking at 250 rpm for 24 hours. Extraction of CFE and purification were as for pET21a(+). With regards to over-expression in pCOLD-TF vector, *E. coli* BL21(λDE3) competent cells (Invitrogen) were transformed by the recombinant plasmid, grown and induced as for pET21a(+). However, the expression was performed at 15 °C for 24 hours with shaking at 200 rpm. CFE was extracted and overexpressed protein was purified as for pET21a(+).

2.8.7 Purification of proteins

All protein purification steps were carried out usin Akta Prime plus purification system (GE Healthcare).

2.8.7.1 Ni-NTA affinity chromatography

Ice-cold cell free extracts were fractionated on a 5 ml HisTrap™ HP (GE Healthcare, UK), according to the manufacturers recommendation. Proteins were bound to the column in binding buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 20 mM imidazole) and eluted from the resin over 10 column volumes with a linear gradient of 20-500 mM imidazole in the same buffer. Protein-containing fractions were applied to SDS-PAGE analysis, stained with Coomassie blue in order to select the highly pure and concentrated fractions. These were then pooled for further purification or to be concentrated using Vivaspin 20 column (Sartorious Stedim Biotech) with an appropriate molecular weight cut off (MWCO) (at least 50 % smaller than size of protein of interest) until desired concentration was achieved.

2.8.7.1.1 Protein concentration, buffer exchange and protein dialysis

Vivaspin 20 column (Sartorious Stedim Biotech) with an appropriate molecular weight cut off (MWCO) (at least 50 % smaller than size of protein of interest) was used for concentration of proteins. Depending on the size of the protein, concentration was performed by centrifugation (6,000 or 8,000 *xg*, 30 min and 4°C). Buffer exchange was performed by applying the desired buffer (10 mL) to a concentrated protein sample in Vivaspin column and exchanged by centrifugation (6,000 *xg*, 30 min and 4°C). This was repeated at least twice until completed exchange had reached.

Protein dialysis was carried out overnight at 4 °C against 3 L of dialysis buffer (10 mM Tris-HCl, pH 8.0), to remove unrequired salts from the protein sample which could affect protein stability in structural or functional protein assays. The buffer was changed at 1, 4 and 24 hours. The 31 mm or 9 mm dialysis tubing cellulose membranes (both

from Sigma-Aldrich, UK) with a MWCO of 12 – 14 kDa or 2 kDa respectively, were prepared by incubating the membrane in 1 L dH_2O with 2 % *(w/v)* sodium bicarbonate and 1 mM EDTA at 80 °C for 30 minutes. The membrane was then rinsed thoroughly and stored in dH_2O at 4 °C for up to 2 days.

2.8.7.2 Ion-exchange chromatography

Cell extracts containing overexpressed recombinant protein, or fractions eluted from Ni-NTA affinity chromatography column were pooled, concentrated using Vivaspin 20 column to a final volume of 2-5 mL, and buffer exchanged into 50 mM Tris-HCl pH 8.0. These samples were loaded to a 5 ml HiTrap^{TM} DEAE FF column (GE Healthcare, UK) and proteins were eluted in the same buffer over 10 column volumes with a linear gradient of 0-1 M NaCl. Protein-containing fractions were loaded into SDS-PAGE to determine the most pure and concentrated fractions. The correct sequence of the protein was confirmed by *N*-terminal sequencing performed by Dr. Arthur Moir (Department of Molecular Biology and Biotechnology, University of Sheffield).

2.8.7.3 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) was used to purify Cj0694 protein which had been cloned into pET21a(+) without the signal sequence and with stop codon to stop the over-expression of *C*-terminal 6xHis. Cell extracts or protein fractions that had been adjusted to a volume of 2 ml in 50 mM Tris-HCl pH 8.5 with 1.5 M $(NH_4)_2SO_4$ were loaded into a 5 ml HiTrap^{TM} HIC column (GE Healthcare, UK). Protein was eluted over 10 column volumes in 1-0 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl pH 8.5. Fractions were analysed by SDS-PAGE to evaluate the most pure and concentrated fractions.

2.8.7.4 Gel filtration

Pooled protein fractions from ion-exchange chromatography and HIC were concentrated to a final volume of 2 mL using a Vivaspin 20 column (MWCO 15,000, Sartorious Stedim Biotech) and applied onto a Superdex 200 (10 × 300 mm) gel filtration column (GE Healthcare), using 10 mM Tris-HCl, pH 8.0 as the running buffer. Protein was eluted from the column in a single peak, fractions were pooled and purity of protein was examined by SDS-PAGE and stained by Coomassie stain.

2.8.7.5 Factor Xa protein digestion

Protein digestion using Factor Xa was used to cut the Cj0694 from the purified recombinant protein from pCOLD over-expression vector. Recombinant protein (~105.8 kDa) was diluted to a concentration of 1 mg mL^{-1}, buffer exchanged into Factor Xa buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM $CaCl_2$) using Vivaspin 20 column (Sartorious Stedim Biotech). Factor Xa (10 µg) was added to 10 mg mL^{-1} protein and incubated at RT for 2 hours. The reaction mixture was further purified by His-Trap purification column to trap the His-tagged fraction, and the flow-through fractions (a mL/tube) containing the Cj0694 were collected and stored on ice. Fractions were loaded into SDS-PAGE for visualisation and analysis before been pooled, concentrated and buffer exchanged into 10 mM Tris-HCl pH 7.2 by Vivaspin column and stored at 4 °C for further use.

2.9 Enzyme assays

2.9.1 PPIase enhanced refolding of reduced and carboxymethylated (RCM) RNase T_1

The wildtype ribonuclease T_1 from *Aspergillus oryzae* (Sigma-Aldrich) was used to evaluate the proline isomerisation-limited protein folding as described by Scholz *et al*. (1997). Disulphide reduced and S-carboxymethylated (RCM)-RNase T_1 was prepared following the method developed by Mücke and Schmid (1992). Firstly, 0.0362 µmol of RNase T_1 was incubated in 275 µL denaturation buffer (6 M guanidine-hydrochloride [GdnHCl] and 2 mM EDTA in 0.2 M Tris-HCl, pH 8.7) for 2 hours at 25 °C. Secondly, 30 µL of the reducing buffer (20 mM dithiothreitol [DTT], 6 M GdnHCl, and 2 mM EDTA in 0.2 M Tris-HCl, pH 8.7) was added, and protein reduction under argon was carried out at 25 °C for another 2 hours. Thirdly, 60 µL of the carboxymethylation buffer (0.6 M iodoacetate in 0.2 M Tris-HCl, pH 7.5) was added and the sample was incubated in the dark for 5 minutes at 25 °C. This step was essential to 'cap' the cysteine residues and prevent the formation of two disulphide bonds during the denaturation process. Finally, 100 µL of 0.5 M reduced glutathione in 0.2 M Tris-HCl, pH 7.5, was added to stop the reaction.

The RCM-RNase T_1 was immediately separated from the reagents by dialysis against 10 mM Tris-HCl pH 8.0 at 4 °C overnight. The refolding of a wildtype RCM-RNase T_1 was rate-limited by the prolyl *cis-trans* isomerisation of Pro39 and Pro55 (Mücke and Schmid, 1992), and was followed by monitoring the changes in the intrinsic tryptophan fluorescence. Refolding was initiated by a 50-fold dilution of the unfolded protein (stored in the absence of NaCl) to a final concentration of 1.2 µM in a buffer containing 0.1 M sodium acetate, pH 5.0, and 4 M NaCl. Changes in the steady-state Trp59 fluorescence were measured at 320 nm (10 nm bandwidth) with excitation at 268 nm (2.5 nm bandwidth) using a Varian Cary Eclipse spectrofluorometer for 15 minutes with the temperature maintained at 15 °C. PEB4 (0.5 µM) and Cj0694 (0.25 µM and 0.5 µM) were added to the RCM-RNase T_1 at a final concentration of 0.5 µM prior to the dilution. The relative fluorescence (%) was plotted against time (15 min) to illustrate the maximum saturation (100 %) of refolding of RCM-RNase T_1 in the absence or presence of PPIases.

2.9.2 Rhodanese refolding assay

Cj0694 was tested for its chaperone activity in the refolding of a chemically denatured bovine mitochondrial rhodanese (thiosulphate:cyanide sulphurtransferase; Sigma-Aldrich). In the presence of ferric nitrate, rhodanese activity is recovered by the formation of thiocyanate which formed an intensely red iron complex, $(FeSCN)^{2+}$ (Figure 2.3). This recovery was spectrophotometrically measured. As described by Horowitz (1995), denaturation of rhodanese (38 µM) was carried out for 2 hours at 25 °C in 50 mM Tris-HCl, pH 7.8, containing 6 M guanidine- HCl and 20 mM DTT. The denatured rhodanese (76-fold dilution) was added to refolding solution (50 mM Tris-HCl, pH 7.8, containing 50 mM $Na_2S_2O_3$, 10 mM KCN, and 10 mM DTT) to initiate renaturation and incubated at 37 °C. To stop all enzymatic reactions, 300 µL aliquots of the refolding mixture were added to 200 µL 38 % *(v/v)* formaldehyde at various time intervals (0 – 60 min). After the completion of the assay, samples were centrifuged for 5 minutes to pellet the precipitates. The supernatants were subsequently mixed with 500 µL ferric nitrate solution containing 165 mM $Fe(NO_3)_3 \cdot 9H_2O$ dissolved in 8.67 % *(v/v)* HNO3 and the absorbance at 460 nm was recorded. Separate measurements of native rhodanese activity (without denaturation) were used to determine the maximum (100 %) activity. Various concentration of Cj0694 (0.5 µM – 2.5 µM) or equimolar amount (0.5 µM) of BSA (negative control which lacks chaperone activity) were present in the refolding solution prior to the addition of the unfolded rhodanese.

Figure 2.3 Thiosulphate ($S_2O_3^{2-}$) is converted to thiocyanate SCN- in a reaction catalysed by rhodanese (Rho). When SCN- is bound to ferric nitrate $Fe(NO_3)_3$ in the presence of nitric acid, it produce an intense red compound $(Fe(SCN)^{2+}$, for which absorbance is measured at 460 nm.

2.9.3 Aggregation assay

To investigate the effect of Cj0694 on protein aggregation during renaturation, rhodanese and lysozyme were used as model proteins. The light scattering resulted from the formation of protein aggregates was measured by increase in the absorbance at 320 nm in a Shimadzu UV-2401PC spectrophotometer. Unfolding and refolding of rhrodanese and lysozyme was carried out as previously described (Indeno *et. al*., 2000). Typically, 30 µM rhodanese or lysozyme was first denatured for 2 hours at 25 °C in 50 mM Tris-HCl, pH 7.8, containing 6 M guanidine-HCl and 20 mM DTT. Renaturation was initiated by a 60- fold dilution in 50 mM Tris-HCl, pH 7.8, to reach a final concentration of 1.0 µM of rhodanese or lysozyme, and incubated at 25 °C in the absence or presence of Cj0694 (1.0 to 5.0 µM) or bovine serum albumin (1.0 µM) as a negative control).

2.10 Protein X-ray crystallography

All protein X-ray crystallography trials were conducted in collaboration with Dr. John Rafferty at the Department of Molecular Biology and Biotechnology, The University of Sheffield, UK. Purified proteins in 10 – 20 mM Tris-HCl pH 7.2-7.5 at a concentration of 5-10 mg mL^{-1} were used in crystal trials using vapour diffusion method. In this method, the protein and the crystallisation solution are mixed at one to one ratio, and the mixture is placed into a sealed environment next to a reservoir of a precipitant. Because the concentration of the reservoir is higher than that of the crystallisation mixture, water normally diffuses out from the sample mixture to the resirvoir. This results in an increase in the concentration of the sample mixture which leads the protein to come out of the solution. Crystallisation screening was performed using three common screens, PACT, $JCSG^{+}$ and Ammonium Sulphate screens (Qiagen) in 200 nL + 200 nL sitting drop vapour diffusion experiments (using a Matrix_Hydra II Plus One crystallisation robot). Small-scale trials were performed to minimise the variables, such as pH, temperature, protein concentration, type and purity, which may occur during crystallisation and may prevent macromolecules to form crystals. Moreover, vapour diffusion method was used as it is one of the most common and widely-used experiment in protein crystallisation, as it favours to use a small volume of purified protein.

2.11 Statistical data analysis

Three main statistical data analysis were applied to determine significance in results obtained by comparing the means of findings according to each experiment performed independently, and how groups of data sets are related (independent or dependent). Data obtained from same populations under the control of different variables were analysed by paired t-test. Otherwise, for grouped data analysis obtained from different group population under the control of one variable, Student's multiple t-test was applied. For more than two independent groups of populations in which one variable was tested, One-way Analysis of Variance (ANOVA) was applied.

3 The role of Cj1289 and Cj0694 in the translocation and assembly of outer membrane proteins in *Campylobacter jejuni*: A mutant phenotypic characterisation

3.1 Introduction

The well-characterised periplasmic protein SurA has been shown to play an essential role as a periplasmic chaperone and PPIase in most Gram-negative bacteria (Rahfeld *et al.*, 1994, Rudd *et al.*, 1995). In *C. jejuni*, the abundant periplasmic protein PEB4 has been identified to play a crucial role in the assembly of OMPs (Asakura *et al.*, 2007, Rathbun *et al.*, 2009, Rathbun and Thompson, 2009), and was found to be structurally similar to the SurA protein (Kale *et al.*, 2011). Moreover, a *peb4* null mutant made in strain NCTC 11168 was shown to be less able to adhere to INT407 cells than the wild-type, with lowered level and duration of colonisation to mouse intestinal epithelial cells and displayed a lower level of biofilm formation (Asakura *et al.*, 2007). In addition to PEB4, bioinformatics led to the identification of two other periplasmic proteins that possess SurA-like domains in *C. jejuni*. Cj1289 has been shown to be structurally more similar to SurA, and to have a SurA-like PPIase domain that was shown to be active in the refolding of a model synthetic oligopeptide although with less efficiency compared with PEB4, and also to have a SurA-like chaperone domain. However, it was shown to be unable to inhibit the refolding of a model protein substrate, suggesting that if it has a chaperone role, it might be more substrate specific (Kale *et al.*, 2011). The second periplasmic SurA-like protein is Cj0694, which has weak sequence similarity to the inner membrane anchored protein PpiD in *E. coli* which was first suggested to be a putative chaperone for assembly of OMPs (Dartigalongue and Raina, 1998), but later was shown to have no direct role in OMPs maturation (Matern *et al.*, 2010). Although the structure and *in vitro* biochemical function of Cj1289 has been characterised in a previous study (Kale *et al.*, 2011), the structure and biochemical function of Cj0694, the phenotypic characterisation of *cj1289* and *cj0694* null mutants and biological role of Cj1289 and Cj0694 has not been previously investigated.

Therefore, the main aim of this chapter was to identify the biological significance of these two periplasmic proteins and examine if they have a direct role in the maturation and assembly of OMPs in *C. jejuni*. The experimental approaches used in this chapter involved the physiological and phenotypic characterisation of these two proteins by generation of single and double null-*cj1289* and -*cj0694* mutants in NCTC 11168 strain and attempts to generate other mutant combinations such as *Δpeb4/Δcj0694* and *Δpeb4/Δcj1289* double mutants, and *Δpeb4/Δcj1289/Δcj0694* triple mutant in the same strain in order to try to define the contributions of each of these chaperones to OM assembly. These mutants were used for several physiological characterisations in comparison to the wild-type and the null-*peb4* mutant including the effect on microaerobic growth, membrane permeability, sensitivity to antimicrobial drugs/inhibitors and detergents, in order to indicate the severity of any membrane permeability defect. Moreover, these mutants were also used to assess the contribution of each of the proteins to OMP assembly and maturation by a comprehensive proteomic analysis of OM and periplasmic preparations using 2D-gel analysis, stable isotope labelling by amino acids in cell culture (SILAC) and mass spectrometry to indicate any proteomic alteration in OMP profiles in both periplasm and OM and to reveal the identity of their client proteins.

3.2 Results

3.2.1 Generation of *Δcj1289, Δcj0694, Δpeb4* mutants of *C. jejuni* NCTC 11168

The *Δcj1289, Δcj0694, Δpeb4* mutants were generated according to the isothermal assembly (ISA) cloning method described by Gibson *et al.*, 2009 (see section 2.4.5). This is based on producing a plasmid construct that contains the 5′ and 3′ flanking ends of the desired gene and complete replacement of this gene by an antibiotic resistance cassette. The upstream (F1) and downstream (F2) DNA fragments of the *cj1289* gene were amplified by PCR from genomic DNA of NCTC 11168 strain, using the following pairs of primers; 1289-F1-F, 1289-F1-R, and 1289-F2-F, 1289-F2-R. The resulting 419 bp and 619 bp products were then digested by *Hinc*II restriction enzyme together with the pGEM®3Zf(-) cloning vector. At the same time, an 850 bp antibiotic resistance cassette (cat^R) that encodes the chloramphenicol resistance protein was PCR amplified from the pAV35 vector using cat-F-*sty*I and cat-R-*sty*I primers. The resulting DNA was extracted from an agarose gel and treated with alkaline phosphatase to remove 5′-phosphoryl groups from the ends of the digested DNA fragment. The DNA fragments were adjusted to equimolar concentration in ISA master mix and incubated overnight at 50 °C to allow annealing and extension of overlapping regions under the control of T5 exonuclease and Phusion DNA polymerase in the ISA master mix (Figure 3.1A). The newly synthesised plasmid was named p*1289cat* (Figure 3.1B) and used for the transformation of *C. jejuni* NCTC 11168 by electroporation (Donahue *et al.*, 2000, Guerry *et al.*, 1994) to generate the *Δcj1289* mutant. The growth of the mutant was selected on chloramphenicol-containing media and the gene replacement was confirmed by colony PCR using cat-F-*sty*I and 1289-F2-R (Figure 3.1C). cat-F-*sty*I only binds to the cat^R cassette and a PCR product could only be produced as a result of correct insertion of the cassette into the genomic DNA of the mutant.

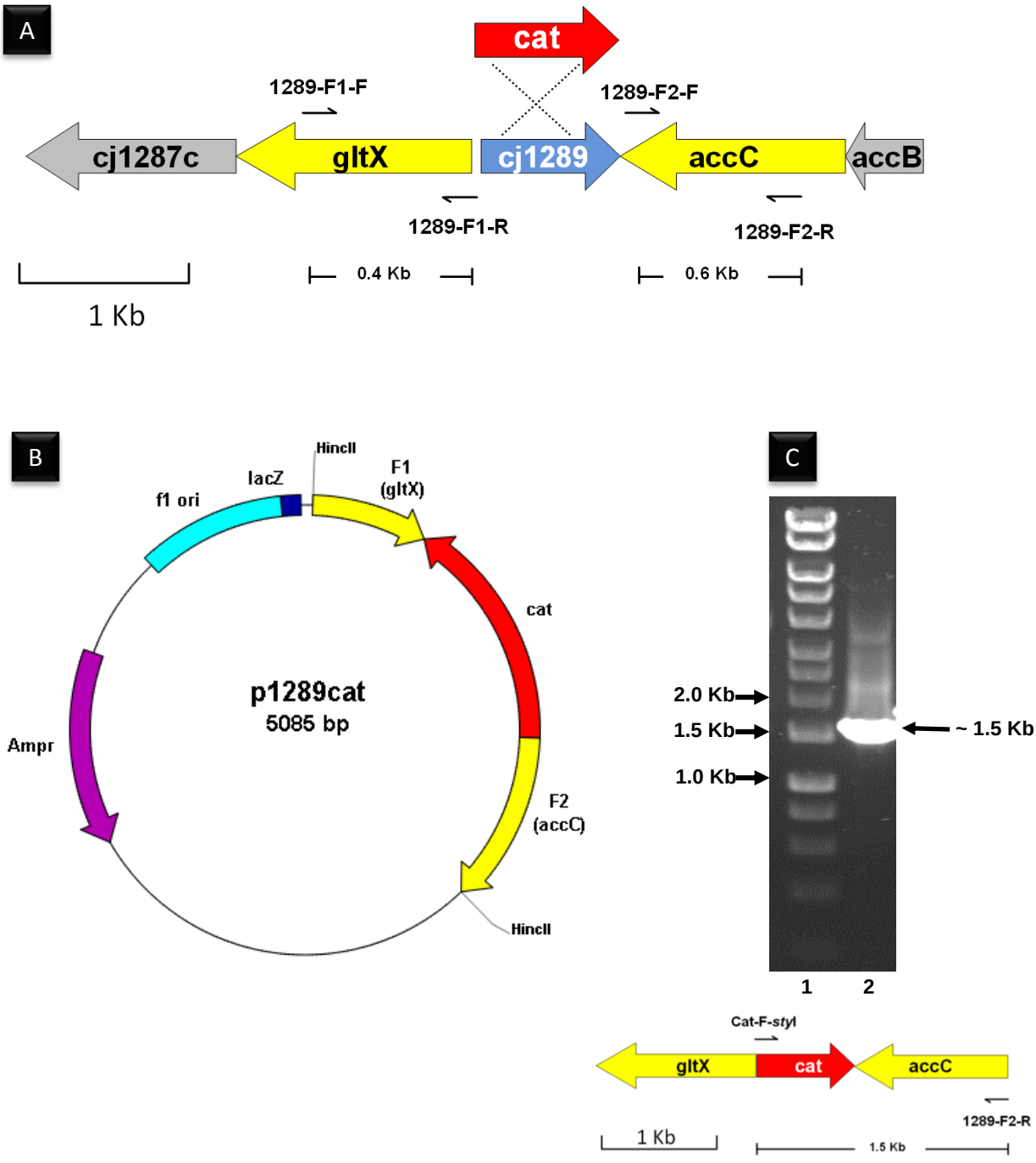


Figure 3.1 Generation of *Δcj1289* mutant in *C. jejuni* NCTC 11168. A) The upstream (F1) and downstream (F2) flanking DNA fragments of the *cj1289* gene were PCR amplified using the following primers; 1289-F1-F, 1289-F1-R, and 1289-F2-F, 1289-F2-R respectively. The chloramphenicol resistance cassette (cat^R) was also amplified using cat-*sty*I-F and cat-*sty*I-R. ISA cloning was conducted to generate p*1289cat* plasmid construct by fully replacing the *cj1289* with cat^R cassette. **B)** Plasmid map of p*1289cat* containing F1-cat^R-F2 DNA fragments cloned into *BsmB*I restriction site of pGEM3Zf(-) (Promega). **C)** Agarose gel electrophoresis confirms the deletion of *cj1289* and replacement with cat^R cassette. Lane 1: Hyperladder™ I molecular weight marker (Bioline, UK). Lane 2: PCR product (~1.5 Kb) of the mutant genomic DNA using cat-F-*sty*I and 1289-F2-R primers.

The *Δcj0694* mutant was generated by amplifying its 5′ and 3′ flanking DNA fragments using 0694-F1-F, 0694-F1-R and 0694-F2-F, 0694-F2-R pairs of primers (Figure 3.2A), whereas in *Δpeb4* mutant, PEB4-F1-F, PEB4-F1-R and PEB4-F2-F, PEB4-F2-R were used to amplify the 5′ and 3′ flanking DNA fragments (Figure 3.3A). The same strategy used to generate the *Δcj1289* mutant was followed to generate the *Δcj0694* and *Δpeb4* mutants. However, a 1.4 Kb antibiotic resistance cassette (kan^R) that encodes kanamycin resistance protein was obtained by PCR amplification from the pJMK30 vector using kan-F-*sty*I and kan-R-*sty*I primers. The resulting plasmids were named as p*0694kan* (Figure 3.2B) and p*peb4kan* (Figure 3.3B) and were used to transform *C. jejuni* NCTC11168 to generate the *Δcj0694* and *Δpeb4* mutants respectively. Both mutants were selected on kanamycin-containing media, and the mutations were confirmed by colony PCR for the *Δcj0694* mutant (Figure 3.2C) and for the *Δpeb4* mutant (Figure 3.3C). In the *Δcj0694* mutant, 0694-F1-F and kan-R-*sty*I primers were used to confirm correct insertion of the kan^R cassette into the genomic DNA of the *Δcj0694* mutant, while in *Δpeb4* mutant, peb4-F1-F and kan-R-*sty*I primers were used to confirm correct insertion of the kan^R cassette into the genomic DNA of the *Δpeb4* mutant.

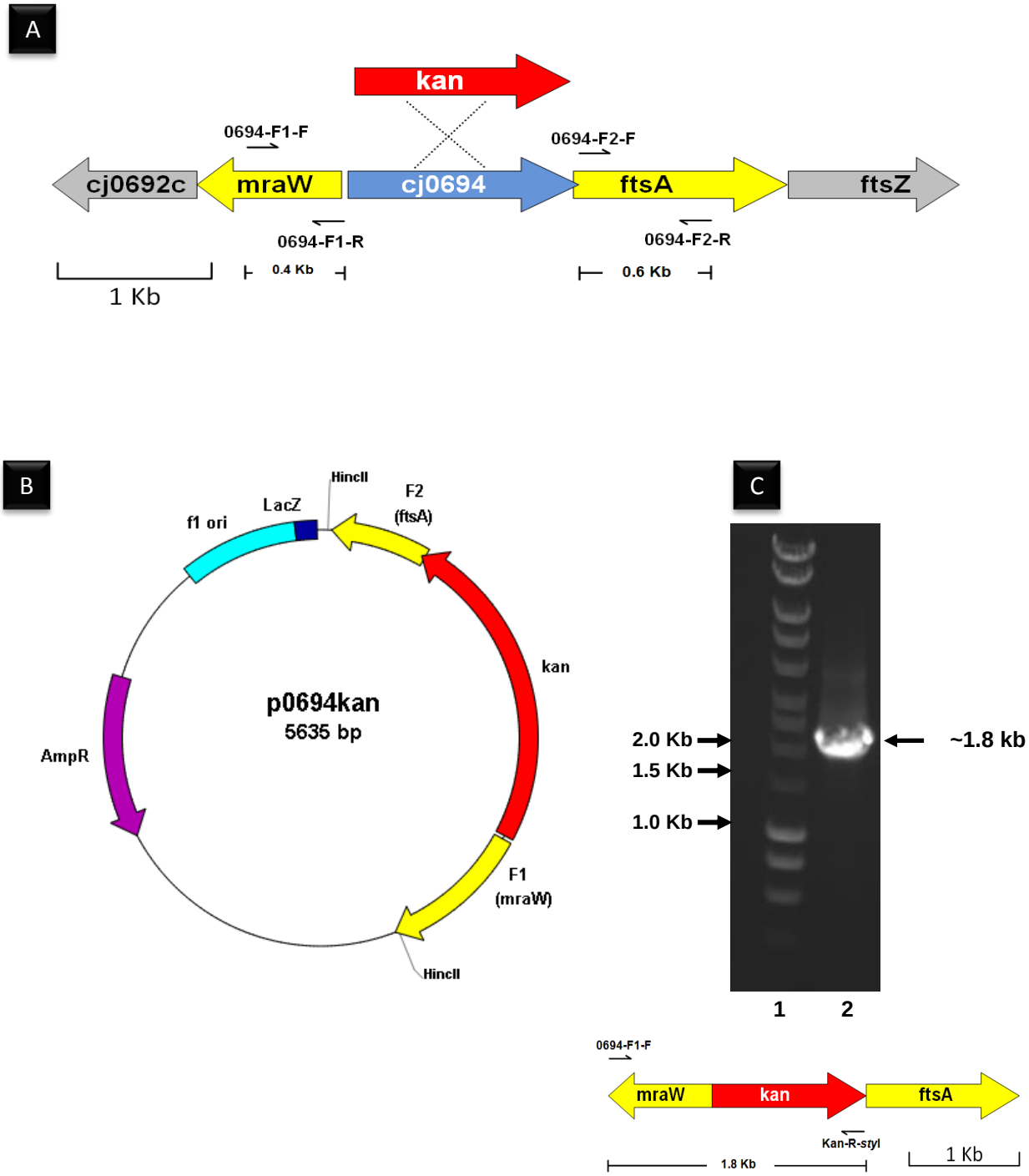


Figure 3.2 Generation of *Δcj0694* mutant in *C. jejuni* NCTC 11168. A) The upstream (F1) and downstream (F2) flanking DNA fragments of the *cj0694* gene were PCR amplified using the following primers; 0694-F1-F, 0694-F1-R, and 0694-F2-F, 0694-F2-R respectively. The kanamycin resistance cassette (kan^R) was also amplified using cat-*sty*I-F and cat-*sty*I-R. ISA cloning was conducted to generate p*0694kan* plasmid construct by fully replacing the *cj0694* gene with kan^R cassette. **B)** Plasmid map of p*0694kan* containing F1 and F2 DNA fragments of the *cj0694* gene and kan^R cassette cloned into *BsmB*I restriction site of pGEM3Zf(-) (Promega). **C)** Agarose gel electrophoresis showing deletion of *cj0694* and insertion of the kan^R cassette in the *cj0694* locus. Lane 1: Hyperladder™ I molecular weight marker (Bioline, UK). Lane 2: PCR product (~1.8 Kb) of the mutant genomic DNA using 0694-F1-F and kan-R-*sty*I primers.

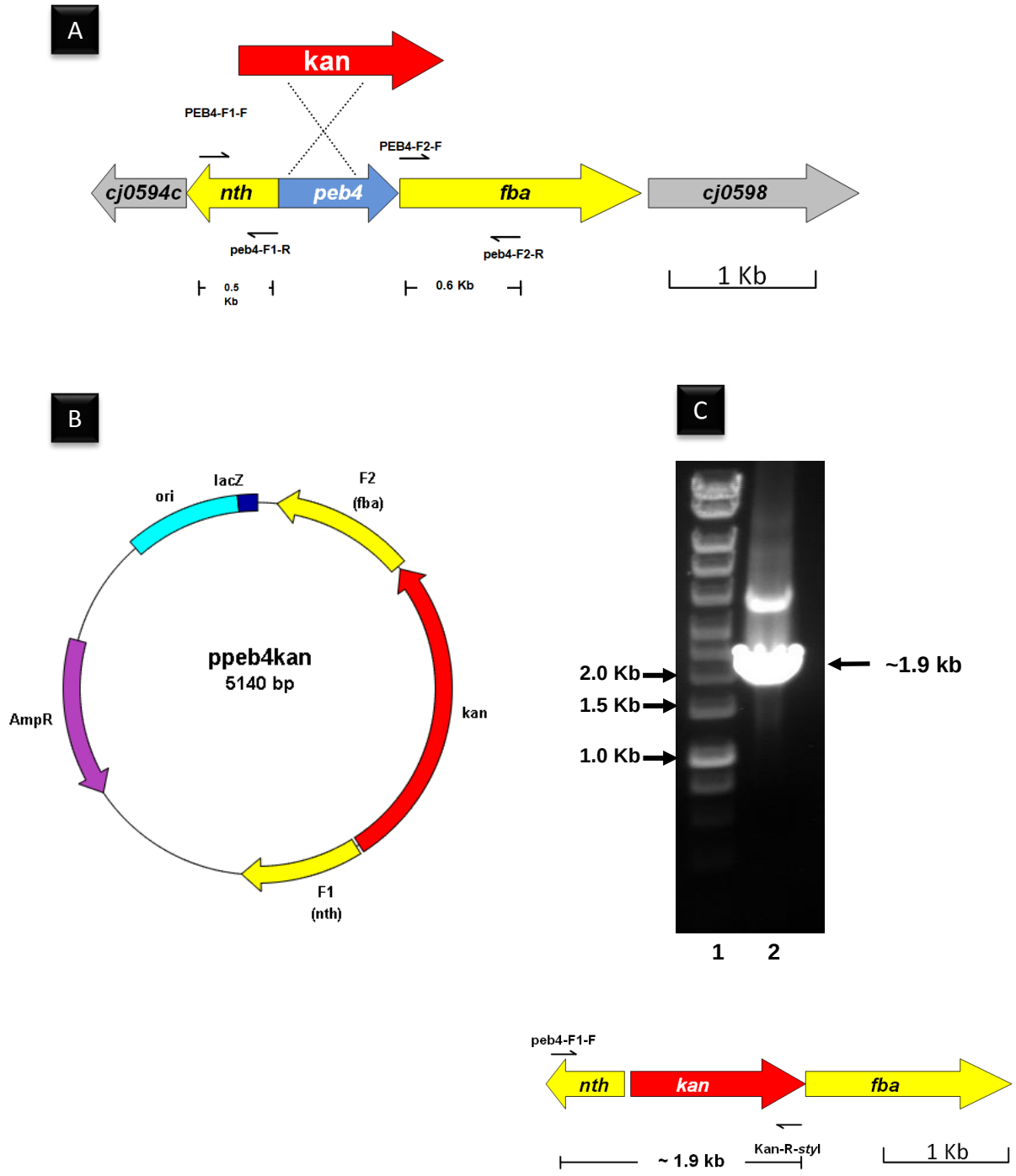


Figure 3.3 Generation of *Δpeb4* mutant in *C. jejuni* NCTC 11168. A) The upstream (F1) and downstream (F2) flanking DNA fragments of the *cj0596* (*peb4*) gene were PCR amplified using the following primers; peb4-F1-F, peb-F1-R, and peb-F2-F, peb-F2-R respectively. The kanamycin resistance cassette (kan^R) was also amplified using cat-*sty*I-F and cat-*sty*I-R. ISA cloning was conducted to generate p*peb4kan* plasmid construct by fully replacing the *peb4* gene with kan^R cassette. **B)** Plasmid map of p*peb4kan* containing F1 and F2 DNA fragments of the *peb4* gene and kan^R cassette cloned into *BsmB*I restriction site of pGEM3Zf(-) (Promega). **C)** Agarose gel electrophoresis confirming the deletion of *peb4* and replacement with kan^R cassette in the same gene location. Lane 1: Hyperladder™ I molecular weight marker (Bioline, UK). Lane 2: PCR product (~1.9 Kb) of the mutant genomic DNA using peb4-F1-F and kan-R-*sty*I primers.

3.2.2 Complementation of the *Δcj1289*, *Δcj0694* and *Δpeb4* mutants

Gene complementation for the mutants was performed to ensure that the phenotypic characteristics of the null mutants were solely credited to the deletion of these specific genes, and not due to polar effect of downstream genes resulting from insertion of the antibiotic resistance cassette. Copies of wild-type genes were integrated into different loci in the genomic DNA of the mutants. The pseudogene *cj0046* was used to integrate the wild-type copy of DNA. For complementation of the *Δcj1289* mutant, the entire coding region plus upstream promoter and ribosome binding site were PCR amplified using the primers 1289+F and 1289+R which both contain *Bsm*BI restriction sites (Figure 3.4A). The resulting 923 bp DNA fragment was digested by *Bsm*BI and cloned into pK46 (Gaskin *et al.*, 2007) to generate the plasmid pK*1289*, which was used to transform the *Δcj1289* mutant (Figure 3.4B). The complemented strain was selected on media containing both chloramphenicol and kanamycin. The presence of the wild-type gene integrated into the *cj0046* pseudogene was confirmed by colony PCR using 46F and 46R primers. An increase in the PCR product size from ~ 0.2 Kb to ~ 1.8 Kb confirms the correct integration of the WT *cj1289* gene into *cj0046* (Figure 3.4C).

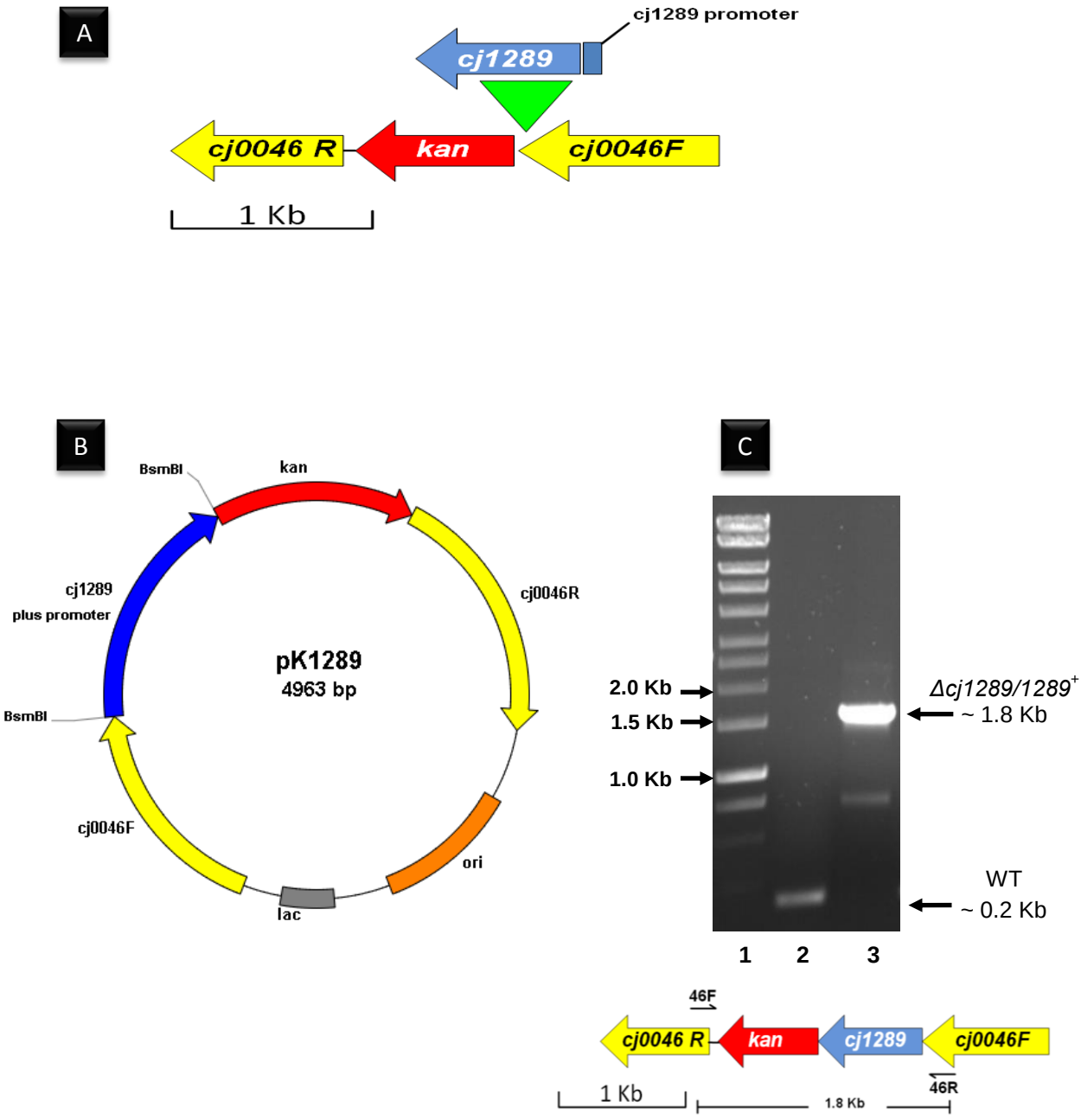


Figure 3.4 Complementation of the *Δcj1289* mutant. **A)** The entire coding sequence of the *cj1289* gene plus upstream promoter and ribosome binding site were PCR amplified using 1289+F and 1289+R primers, and cloned into *BsmB*I restriction sites of pK46 complementation vector in frame with *cj0046* and *kan*^{R} cassette. **B)** The plasmid map of the resulted pK1289 plasmid construct. **C)** Agarose gel electrophoresis confirms successful insertion of *cj1289* into the pK46. Lane 1: Hyperladder™ I molecular weight marker (Bioline, UK). Lane 2: PCR product (~ 0.2 Kb) of the *cj0046* from the wild-type genomic DNA using 46F and 46R primers. Lane 3: PCR product (~ 1.8 Kb) of the *cj0046* from the genomic DNA of the *Δcj1289/1289*$^{+}$ complemented strain using same set of primers.

For complementation of the *Δcj0694* mutant, the entire coding region of the gene was PCR amplified using the primers 0694+F and 0694+R which both contain *Bsm*BI restriction sites (Figure 3.5A). The resulting 1,491 bp DNA fragment was digested and cloned into the *Bsm*BI site of pC*fdxA* complementation vector (Gaskin *et al.*, 2007) to generate the plasmid pC*fdxA0694* (Figure 3.5B). The upstream promoter and ribosome binding site were not included in the DNA fragment as pC*fdxA* contains the strong *fdxA* promoter. Otherwise, the entire coding region of *cj0694* plus its upstream promoter and ribosome binding site was cloned into pC46 (which contains no additional promoter) and pC*metK* (contains a weak constitutive promoter), which was used to transform the *Δcj0694* mutant, with selection on media containing both kanamycin and chloramphenicol. Correct cloning of the wild-type copy of *cj0694* into the *cj0046* pseudogene in the pC*fdxA0694*, pC46 and pC*metK* plasmids was confirmed by PCR using 46F and 46R primers as seen by increasing in the PCR product size from ~ 0.2 Kb to ~ 2.5 Kb (Figure 3.5C). Despite numerous attempts of transformation of the *Δcj0694* mutant with pC*fdxA0694*, pC46 or pC*metK*, it was not possible to obtain a complemented *Δcj0694/cj0694*$^{+}$ strain.

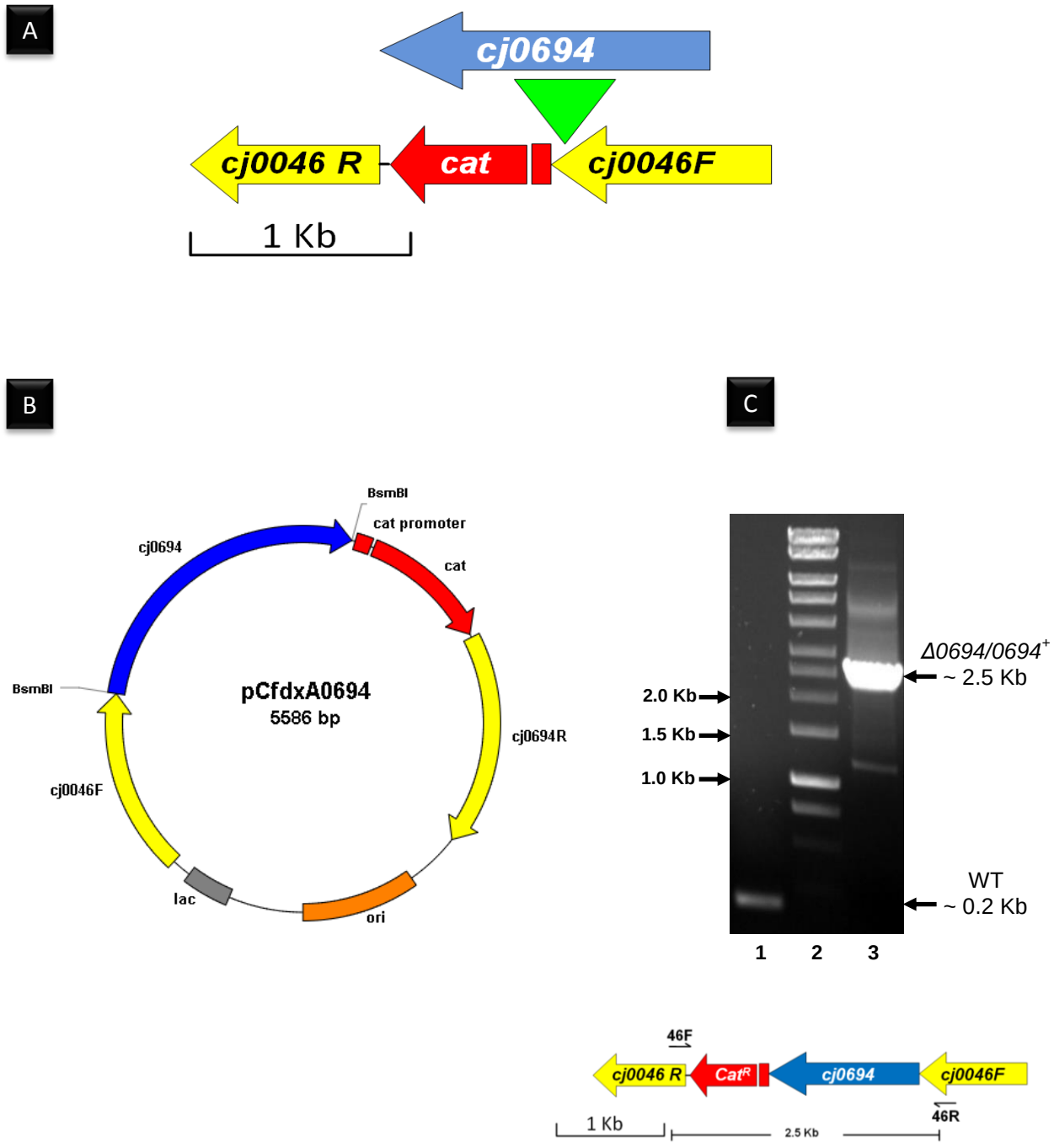


Figure 3.5 Construction of the pC*fdxA0694* complementation plasmid. **A)** The entire coding sequence of was PCR amplified using 0694+F and 0694+R primers, and cloned into *BsmB*I restriction sites of pC*fdxA* complementation vector in frame with *cj0046* and *cat*^{R} cassette. **B)** The plasmid map of the resulted pC*fdxA0694* plasmid construct. **C)** Agarose gel electrophoresis confirms successful insertion of *cj0694* into the pC*fdxA* plasmid. Lane 1: PCR product (~ 0.2 Kb) of the *cj0046* from the wild-type genomic DNA using 46F and 46R primers. Lane 2: Hyperladder™ I molecular weight marker (Bioline, UK). Lane 3: PCR product (~ 2.5 Kb) of the *cj0046* from the genomic DNA of the *Δcj0694/0694*$^{+}$ complemented strain using same set of primers.

For complementation of the *Δpeb4* mutat, the entire coding region of the *peb4* gene plus upstream promoter and ribosome binding site were PCR amplified using the primers PEB4+F and PEB4+R which both contain *Bsm*BI restriction sites (Figure 3.6A). The resulting 910 bp DNA fragment was digested by *Bsm*BI and cloned into pC46 to generate the plasmid pC*peb4*, which was used to transform the *Δpeb4* mutant (Figure 3.6B). The presence of the wild-type gene cloned into *cj0046* was confirmed by PCR using 46F and 46R primers as indicated by increasing in a product size from ~ 0.2 Kb to ~ 1.9 Kb (data not shown). Unfortunately, a complemented *Δpeb4/peb4*$^{+}$ strain could not be generated despite several attempts of electro-transformation of the *Δpeb4* mutant. Although single colonies of both putative *Δcj0694/cj0694*$^{+}$ and *Δpeb4/peb4*$^{+}$ strains could be obtained on selective plates, colony PCR screening using 46F and 46R primers resulted in only a single ~0.2 Kb DNA fragment representing the wild-type *cj0046* without insertion of complemented genes.

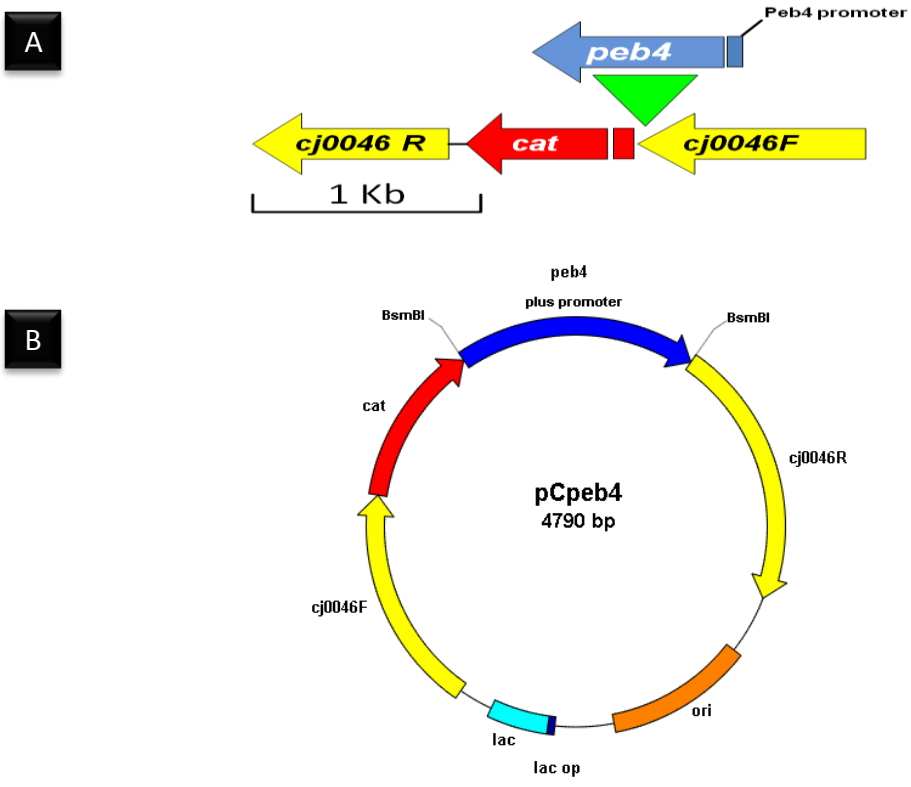


Figure 3.6 Strategy for complementation of the *Δpeb4* mutant. **A)** The entire coding sequence of *peb4* plus upstream promoter and ribosome binding site were PCR amplified using PEB4+F and PEB4+R primers, and cloned into *BsmB*I restriction sites of pC46 complementation vector in frame with *cj0046* and *cat*^{R} cassette. **B)** The plasmid map of the resulted pK1289 plasmid construct.

3.2.3 Generation of the *Δcj1289/Δcj0694* double mutant

To investigate whether Cj1289 and Cj0694 have a collaborative chaperone role in the periplasm, and also to monitor if deletion of one or both genes results in a different phenotype with more or less activity, a double *Δcj1289/Δcj0694* mutant was generated by introducing the p*0694kan* plasmid (which contains 400 bp upstream and 600 bp downstream flanking DNA of *cj0694* with the kan^{R} cassette inserted in place of the deleted sequence) into the *Δcj1289* mutant background. This resulted in a complete replacement of the coding sequences of the *cj1289* and *cj0694* with chloramphenicol and kanamycin resistance cassettes, respectively. In all cases the antibiotic resistance cassette had its own promoter and was inserted in the same transcriptional orientation as the gene of interest in order to minimise the chance of polar effects on downstream genes. The resulting double mutant was grown on selective plates containing chloramphenicol and kanamycin (50 µg mL^{-1}). Deletion of *cj1289* and *cj0694* were confirmed by PCR using two primers combination; 0694-F1-F, kan-R-*sty*I and cat-F-styI, 1289-F2-R (Figure 3.7) (data from agarose gel not shown).

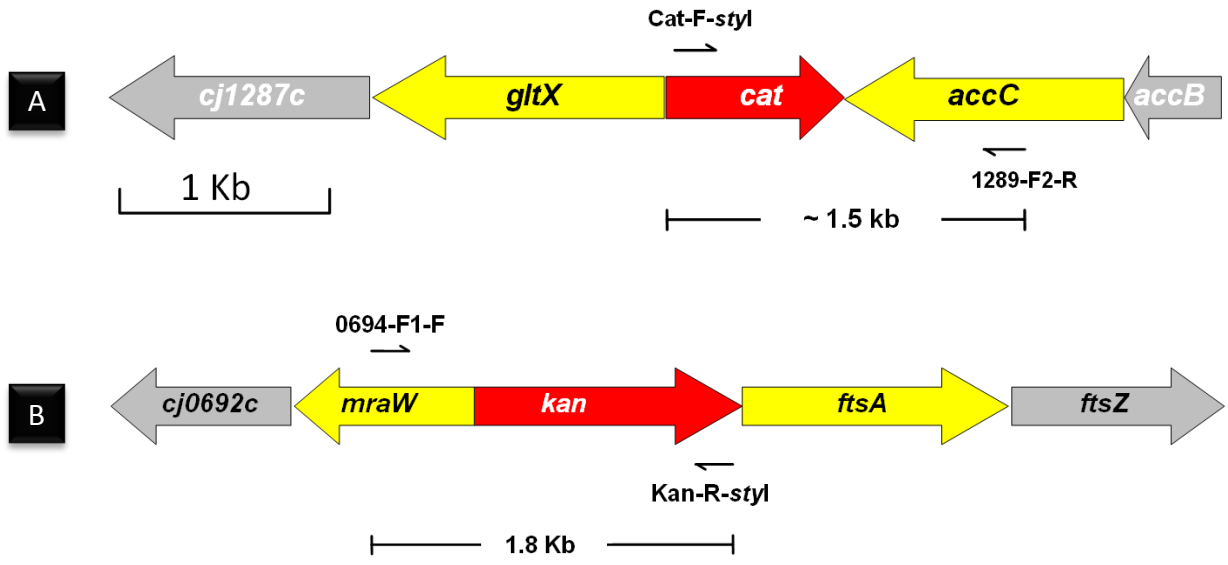


Figure 3.7 Schematic representation describes PCR screening of the *Δcj1289/Δcj0694* double mutant. A) Confirmation of correct replacement of *cj1289* by cat^{R} cassette in the genomic DNA of the *Δcj1289/Δcj0694* double mutant using cat-F-*sty*I and 1289-F2-R primers. **B)** Confirmation of correct replacement of *cj0694* by kan^{R} cassette in the genomic DNA of the *Δcj1289/Δcj0694* double mutant using 0694-F1-F and kan-R-*sty*I primers.

3.2.4 Attempts to generate double mutant containing a *peb4* gene deletion

PEB4 is known to have a major chaperone role in the periplasm for the assembly of OMPs (Kale *et al.*, 2011, Rathbun *et al.*, 2009). It has been suggested that combined deletion of *peb4* and other proposed periplasmic chaperone may result in severe defects in the OM. In order to investigate if PEB4 has an independent chaperone role which cannot be compensated by other chaperones, generation of double *Δpeb4/Δcj1289* mutants were attempted by introducing p*1289cat* (which contains 400 bp upstream and 600 bp downstream flanking DNA of *cj1289* with the cat^R cassette inserted in place of the deleted sequence) into *Δpeb4* mutant cells. This was supposed to result in a complete replacement of the coding sequences of the *peb4* and *cj1289* with kanamycin and chloramphenicol resistance cassettes, respectively. After several attempts of electro-transformation performed by introducing p*1289cat* into the *Δpeb4* mutant cells or by introducing p*peb4kan* into the *Δcj1289* mutant cells, double mutants could not be obtained, suggesting that deletion of *peb4* and *cj1289* might be synthetically lethal.

3.2.5 The *Δcj1289* and *Δcj0694* single mutations cause variable growth retardation, but this is more severe in a *Δcj1289/Δcj0694* double mutant

Previous work in our lab performed by Dr. Chatchawal Phansopa (Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield, UK) has reported that mutation in *peb4* resulted in a severe growth defect when compared to the wild-type strain 11168 grown under microaerobic conditions in MH broth supplemented with 20 mM L-serine. Here, the growth of the *Δcj1289* mutant in MH broth supplemented with 20 mM L-serine was assessed in comparison to the wild-type 11168, and it is clear that the *Δcj1289* mutant showed a slightly slower growth rate (Figure 3.8A). The *Δcj1289/cj1289*$^{+}$ complemented strain demonstrated a restoration in growth which was similar to that of the wild-type. The wild-type and the *Δcj1289/cj1289*$^{+}$ complemented strain have a doubling time of 2 hours, whereas the *Δcj1289* mutant has a doubling time of 2.5 hours. After an overnight incubation, cell densities of the wild-type, the *Δcj1289* mutant and the *Δcj1289/cj1289*$^{+}$ complemented strain were similar.

Also the microaerobic growth of the *Δcj0694* mutant in MH broth supplemented with 20 mM L-serine was monitored in comparison to the wild-type 11168 strain. The *Δcj0694* mutant showed a higher degree of growth defect than the *Δcj1289* mutant. The number of the *Δcj0694* mutant cells doubled every 3 hours (~2 hours in the wild-type), and reached similar density to that of the wild-type after overnight incubation (Figure 3.8B).

Moreover, the growth of the *Δcj1289/Δcj0694* double mutant was assessed in comparison to the wild-type, *Δcj1289* and *Δcj0694* mutants under microaerobic conditions. Interestingly, the *Δcj1289/Δcj0694* double mutant showed a more severe growth defect than the *Δcj1289* and *Δcj0694* single mutants. The *Δcj1289/Δcj0694* double mutant cells had a doubling time of 3.5 hours, which represents the slowest doubling time among all tested strains (Figure 3.8C). After overnight incubation, the cell density was similar to those of the wild-type and the single mutants.

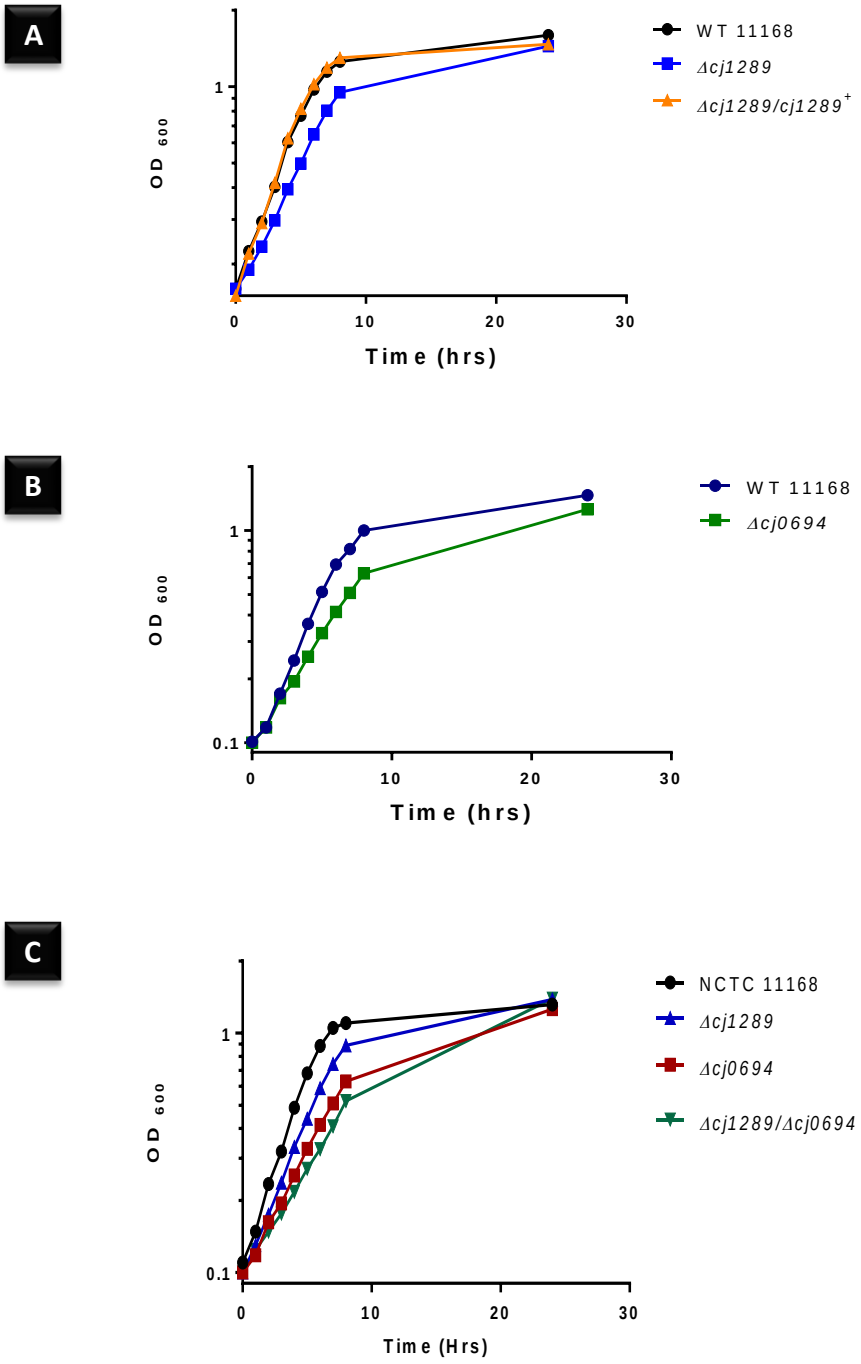


Figure 3.8 Growth of *C. jejuni Δcj1289, Δcj0694* single mutants and *Δcj1289/Δcj0694* double mutant. A) Microaerobic growth of the wild-type vs. the *Δcj1289* mutant. **B)** Microaerobic growth of the wild-type vs. the *Δcj0694* mutant. **C)** Microaerobic growth of the wild-type vs. the *Δcj1289/Δcj0694* double mutant. For microaerobic (oxygen sufficient) growth, 50 mL MHS media was contained in 250 ml conical flasks shaken at 180 rpm in a gas atmosphere of 10 % *(v/v)* oxygen, 5 % *(v/v)* carbon dioxide and 85 % *(v/v)* nitrogen. Samples were collected at time 0 – 8 hours and a final sample at 24 hours. Cell densities were measured spectrophotometrically at OD_{600}. Figures are representative experiments of several biological replicates with similar results.

3.2.6 Deletion of *cj1289* or *cj0694* genes increases sensitivity to SDS, but deletion of both genes shows a hypersensitive phenotype

To examine whether the Cj1289 or Cj0694 are involved in the assembly of the OMPs, a disc diffusion assay was carried out. It was hypothesised that deletions of one or both genes that encode for Cj1289 and Cj0694 may result in an increased sensitivity to antimicrobial agents that require entry through the OM to cause damage to the cell, due to an increased permeability of the OM with a defective integrity. If this is correct, then large-sized antimicrobial agents or detergents [such as sodium dodecyl sulphate (SDS)] can more easily gain entry into the bacterial cell through the defective OM and cause cell damage. The experiment involved addition of SDS to discs in the centre of MH plates containing wild-type or mutant strain. Sensitivity to SDS was represented by occurrence of a clear zone of growth inhibition around the antimicrobial agent-containing disc after 2 – 3 days of microaerobic growth. Results were obtained from three independent technical and biological replicates. The *Δcj1289* mutant was shown to be significantly more sensitive to 40 % SDS than the wild-type ($p < 0.01$). To confirm that this effect was due to the deletion of *cj1289* only, the same experiment was applied to the complemented *Δcj1289/Δcj1289*$^{+}$ strain that showed a restoration of the wild-type phenotype (Figure 3.9A).

The significance of sensitivity of the *Δcj0694* mutant to 40 % SDS was similar to that of the *Δcj1289* mutant ($p < 0.01$). Interestingly, the sensitivity of the *Δcj1289/Δcj0694* double mutant was twice more than each single mutant as represented by a significant increase in the size of the inhibition zone on sensitivity plates ($p < 0.0001$) (Figure 3.9B). This suggests that single mutant cells were less able to prevent entry of SDS molecules to the cell through the OM than the wild-type cells, and the case was more severe in the double mutant, which indicates that *cj1289* and *cj0694* are directly or indirectly involved in maintaining the integrity of the OM.

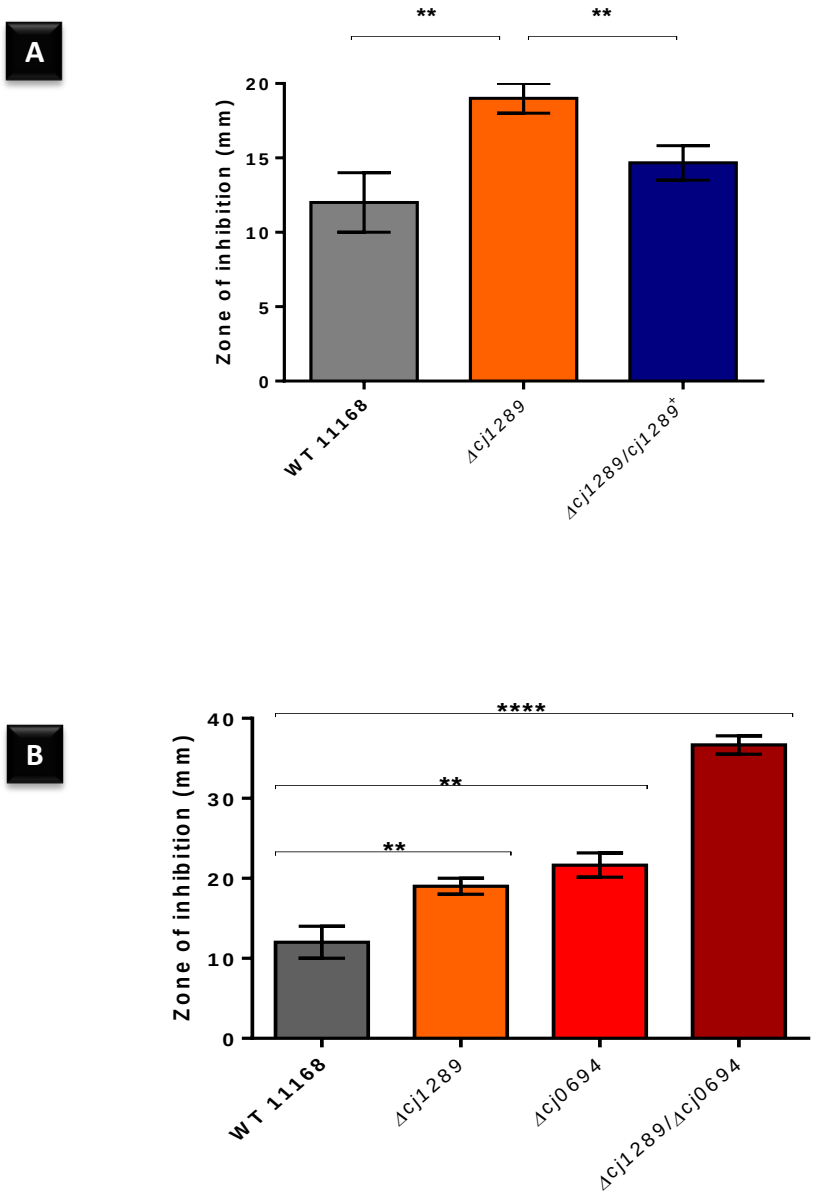
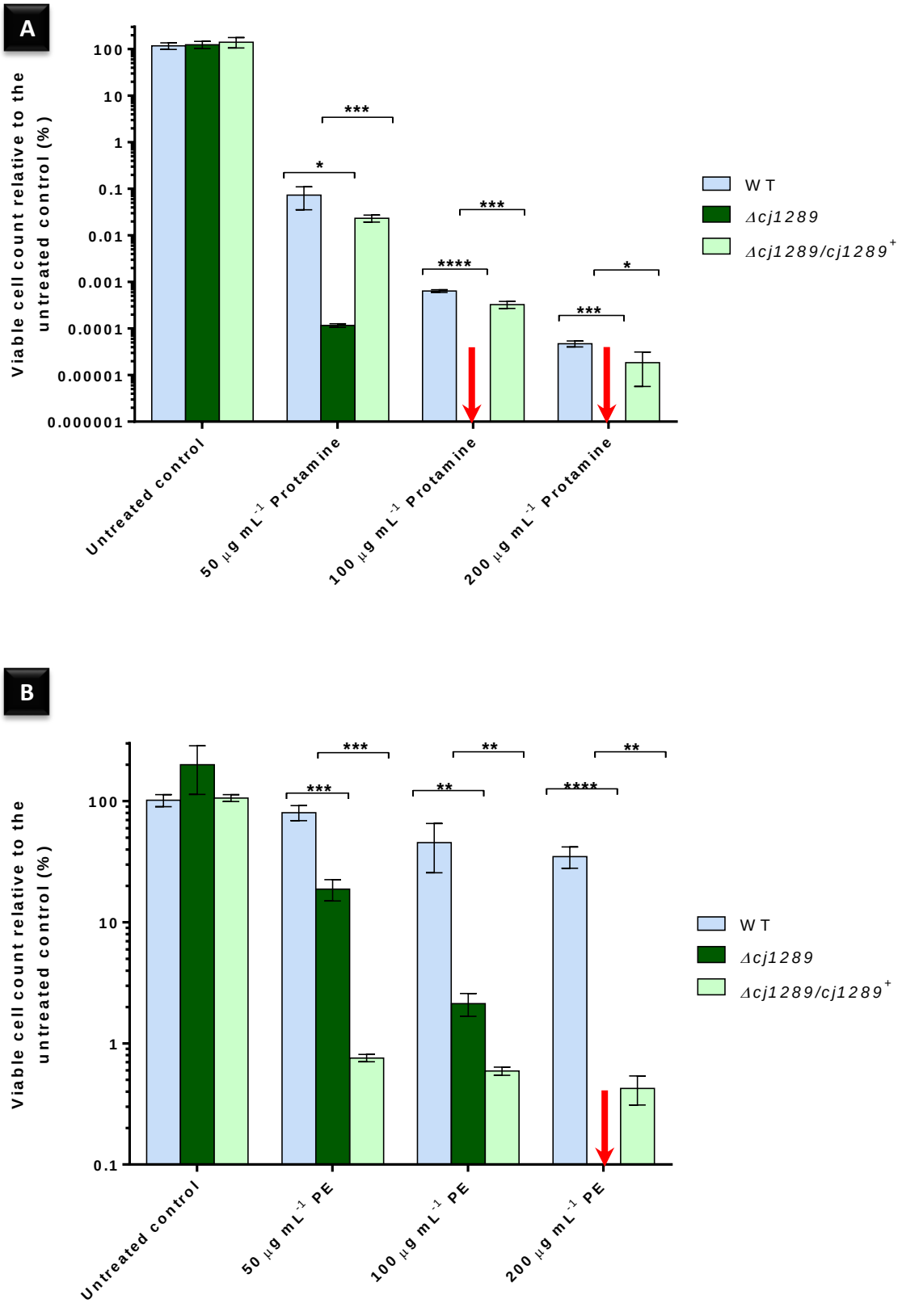


Figure 3.9 SDS disc diffusion assay for the *Δcj1289* and *Δcj0694* single mutants and *Δcj1289*/*Δcj0694* double mutant. A) Sensitivity of the *Δcj1289* mutant against the wild-type and the *Δcj1289*/*cj1289*$^{+}$ complemented strain. The zone of inhibition (mm) in the mutant was double that of the wild-type. The phenotype was restored in the complemented strain. **B)** Comparison between sensitivity of the *Δcj1289* and *Δcj0694* single mutants and *Δcj1289*/*Δcj0694* double mutant. The sensitivity of double mutant to SDS was approximately double these of both single mutants. Wild-type and mutant cells were allowed to grow on MHS agars and exposed to 40 % *(w/v)* SDS. Measurements were taken after 2 – 3 days of microaerobic growth from at least three replicate plates per strain. Error bars represents the standard deviation of these measurements. Statistical analysis was carried out using Student's multiple t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2.7 *cj1289*-deletion mutation results in an increased sensitivity to antimicrobial peptides

Purified Cj1289 has been shown to exhibit *in vitro* chaperone characteristics that may influence the assembly of OMPs, represented by inhibition of rhodanese refolding and aggregation (Kale *et al.*, 2011). To investigate if it has an *in vivo* role on the assembly of the OM, it was suggested that deletion of its cognate gene may result in an increased permeability of the OM and therefore, allow external agents to gain entry into cell through the OM. This can be represented by increased sensitivity of mutant cells to antimicrobial agents that require entry through the OM to cause cell damage. Here, three different antimicrobial compounds have been used to examine if the *Δcj1289* mutant has a defective OM, which can be represented by an elevated sensitivity level of the mutant compared to the wild-type. The first antimicrobial compound used was polymyxin B; a basic cationic polypeptide antibiotic derived from *Bacillus polymyxa*, which acts on Gram-negative ionic cell envelope components such as lipopolysaccharides (LPS) and phospholipids (PLs) by electrostatic and hydrophobic interaction inserts to the bacterial cell membrane, making it more permeable (Schindler and Teuber, 1975). The second compound was polymyxin E (also known as colistin); another peptide antibiotic produced by *Bacillus polymyxa*. The third antimicrobial peptide was protamine sulphate, a small 5.1 kDa cationic protein derived from Salmon which binds and precipitates DNA, but also has a strong antimicrobial effect on Gram-positive and Gram-negative bacteria (Johansen *et al.*, 1995). Overnight cultures of *Δcj1289* were exposed to various concentrations of antimicrobial agents for two hours and after making serial dilutions, they were allowed to grow on MH plates for 2 – 3 days until defined single colonies were detected. Despite that the *Δcj1289* mutant cells were viable in the absence of antimicrobial peptides on plain MH agar, it showed a dramatic increase in sensitivity to protamine sulphate (Figure 3.10A). The *Δcj1289* mutant was significantly more sensitive to 50 µg mL^{-1} protamine sulphate than the wild-type ($p < 0.05$) as represented by 3-logs reduction in the number of the *Δcj1289* mutant viable colony forming units (cfu) per mL relative to the wild-type under the same treatment. Although the wild-type viable cells reduced when exposed to 50 µg mL^{-1} protamine sulphate, the inhibition in the number of viable cells in the mutant was more than the wild-type by 3-logs. Complementation of The *Δcj1289* mutant showed a restoration of the wild-type activity as the number of the viable cells was recovered in

the complemented strain ($p < 0.001$). In each treatment, the complemented strain showed an increase in the viability compared to the mutant, thus providing an evidence for complementation. A concentration of 100 µg mL^{-1} of protamine sulphate was sufficient to kill all *Δcj1289* mutant cells. However, the wild-type phenotype was restored in the complemented strain ($p < 0.001$ and $p < 0.05$; at 100 and 200 µg mL^{-1} protamine sulphate, respectively). The *Δcj1289* mutant also showed a significant increase in the sensitivity to polymyxin E (Figure 3.10B). Approximately, a 1-log decrease in the number of the *Δcj1289* mutant viable cells relative to the wild-type at the same concentration of polymyxin E was observed when exposed to 50 µg mL^{-1} polymyxin E ($p < 0.001$), and 1.5 log decrease when exposed to 100 µg mL^{-1} polymyxin E ($p < 0.01$) while all the mutant cells were killed at a concentration of 200 µg mL^{-1} polymyxin E. Surprisingly, at concentrations of 50 and 100 µg mL^{-1} polymyxin E, the complementation was not able to restore the wild-type phenotype and showed lower number of viable cells than the mutant. However, at a higher concentration of polymyxin E (200 µg mL^{-1}) the phenotype was restored ($p < 0.01$). The *Δcj1289* mutant was also shown to be significantly more sensitive to polymyxin B than the wild-type at 50 and 100 µg mL^{-1} polymyxin B ($p <0.001$), and the sensitivity was more severe at 200 µg mL^{-1} ($p < 0.0001$) as represented by more than 1-log inhibition in the number of viable cells compared to the wild-type at the same concentration. The *Δcj1289/cj1289*$^{+}$ complemented strain had shown to be unable to restore the phenotype when treated with polymyxin B (Figure 3.10C). Unexpectedly, the complemented strain was killed more than the wild-type and the mutant. It would be expected to observe a restoration or partial restoration of the wild-type activity in the complemented strain, but in this case the complementation was not able to show such a restoration. Therefore, the results with complemented strain when exposed to polymyxin B are unexplainable, and provided no evidence for complementation. These findings together suggest that the Cj1289 may have a role in the permeability functions of the OM, as evidenced by the alteration in the sensitivity to protamine sulphate, polymyxin E and polymyxin B. Thus, the lack of Cj1289 results in more sensitive phenotype with an increased OM permeability.


A
Viable cell count relative to the untreated control (%)
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10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
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Untreated control
50 μg mL-1 Protamine
100 μg mL-1 Protamine
200 μg mL-1 Protamine
WT
Δcj1289
Δcj1289/cj1289+
B
Viable cell count relative to the untreated control (%)
100
10
1
0.1
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Untreated control
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Δcj1289
Δcj1289/cj1289+

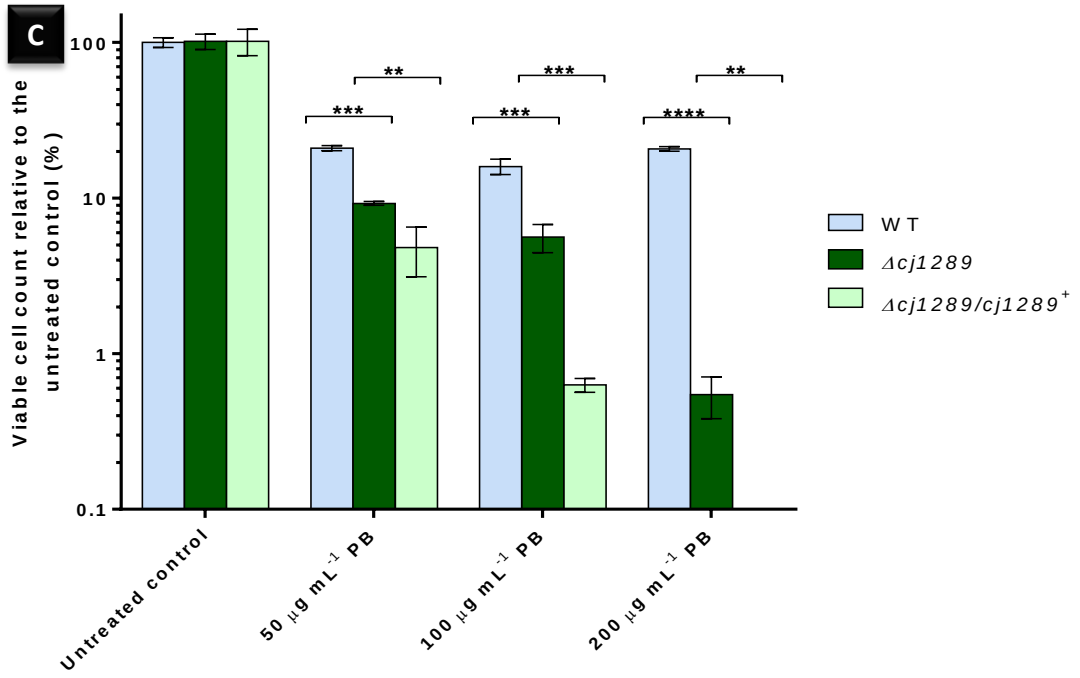
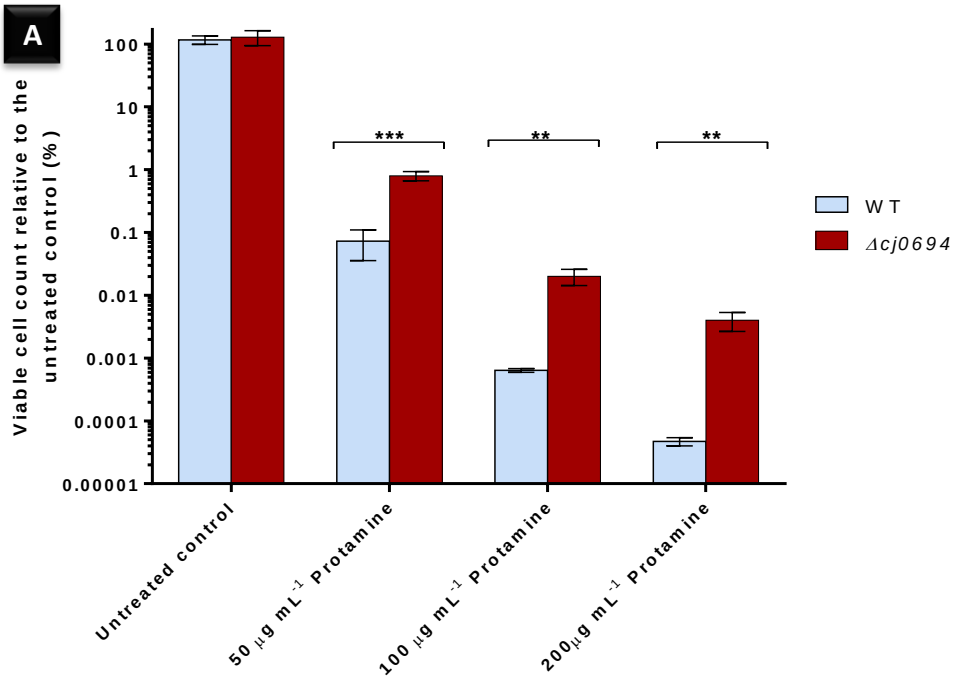


Figure 3.10 Sensitivity of the *Δcj1289* mutant to antimicrobial peptides. Overnight cultures of the *Δcj1289* mutant were exposed to various concentrations of antimicrobial peptides for two hours and were serially diluted to 1 X 10^{-8}. Ten microliters of each dilution were plated on MHS and incubated microaerobically for 2 – 3 days at 37 °C. **A)** Sensitivity of the *Δcj1289* mutant to protamine sulphate. The number of viable cell count was 3-logs reduced when exposed to 50 µg mL^{-1} protamine sulphate compared to the wild-type at the same concentration, while cells were killed when exposed to 100 µg mL^{-1} protamine sulphate. **B)** Sensitivity of the *Δcj1289* mutant to polymyxin E. A 1-log reduction in the number of the *Δcj1289* mutant viable cell count relative to the wild-type when exposed to 100 µg mL^{-1} polymyxin E, while 200 µg mL^{-1} was sufficient to kill the mutant cells. **C)** Sensitivity of the *Δcj1289* mutant to polymyxin B. A 1-log decrease in the number of the *Δcj1289* mutant viable cells compared to the wild-type when exposed to 200 µg mL^{-1} polymyxin B. Data obtained from three independent biological replicates. The cell viability values of the untreated controls were adjusted to 100% to reflect the maximum viability without treatment. Error bars represents the standard deviation of these measurements. Statistical analysis was carried out using Student's multiple t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2.8 *cj0694*-deletion mutation has a similar sensitivity profile to that of the wild-type

The amino acid sequence of Cj0694 protein is similar to that of the *E. coli* membrane-anchored protein PpiD, suggesting possible structural and functional similarities. Therefore, it was proposed that Cj0694 is an important periplasmic chaperone for maintaining the integrity of the OM. To investigate this, it was hypothesised that deletion of *cj0694* gene may result in a defective and more permeable OM. Hence, a sensitivity assay was carried out by using the same antimicrobial agents that were used with the *Δcj1289* mutant. It was found that the *Δcj0694* mutant cells were viable in the absence of antimicrobial peptides on plain MH agar as shown by 100 % viability (Figure 3.11). Interestingly, the degree of the inhibition in the number of viable cells was similar to that of the wild-type when exposed to the same concentration of antimicrobial peptides. Unexpectedly, the wild-type was killed more than the *Δcj0694* mutant when exposed to different concentrations of protamine sulphate for unknown reason. The sensitivity of the *Δcj0694* mutant to polymyxin E was similar to that of the wild-type, although the graph shows slight inhibition in the number of viable cells in the mutant. However, these inhibitions were statistically non-significant except for 200 µg mL^{-1} polymyxin E where the *Δcj0694* mutant shows a significant inhibition in the number of viable cells compared to the wild-type ($p < 0.01$) (Figure 3.11B). The sensitivity of the *Δcj0694* mutant to polymyxin B was not affected at 50 µg mL^{-1}. In contrast, the number of the wild-type viable cells was significantly reduced at this concentration. At higher concentrations of polymyxin B, the levels of killing were similar in the wild-type and the *Δcj0694* mutant. These data indicate that unlike the Cj1289, with the Cj0694 it does not seem that polymyxin or other antimicrobial peptides are having a great effect on the permeability functions of the OM in the *Δcj0694* mutant, suggesting a more important role for Cj1289 than Cj0694 in controlling OM permeability.


A
Viable cell count relative to the untreated control (%)
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10
1
0.1
0.01
0.001
0.0001
0.00001
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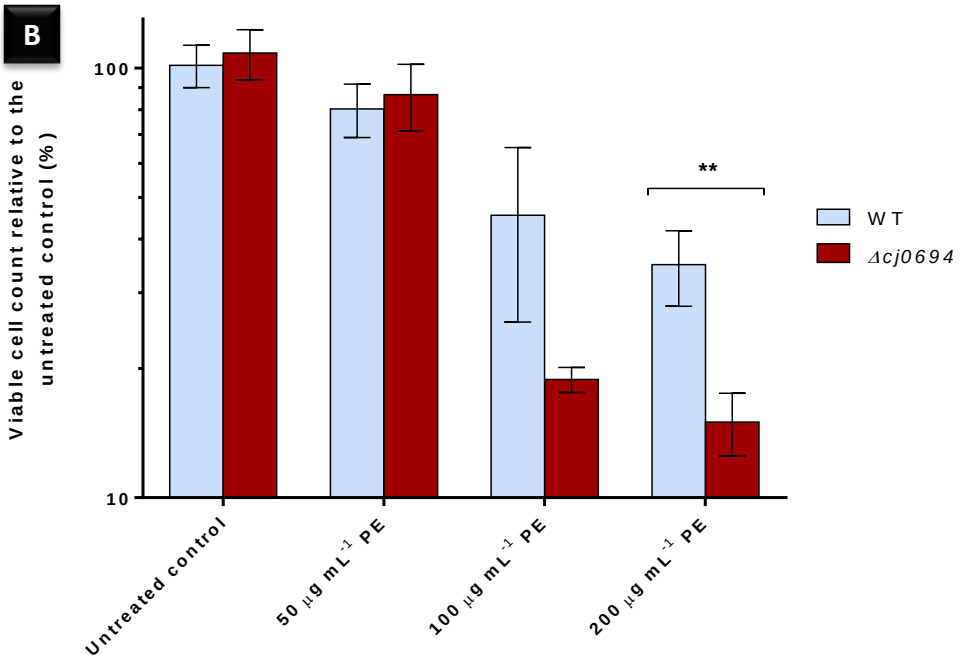
WT
Δcj0694
Untreated control
50 μg mL-1 Protamine
100 μg mL-1 Protamine
200μg mL-1 Protamine


B
Viable cell count relative to the untreated control (%)
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10
**
WT
Δcj0694
Untreated control
50 μg mL-1 PE
100 μg mL-1 PE
200 μg mL-1 PE

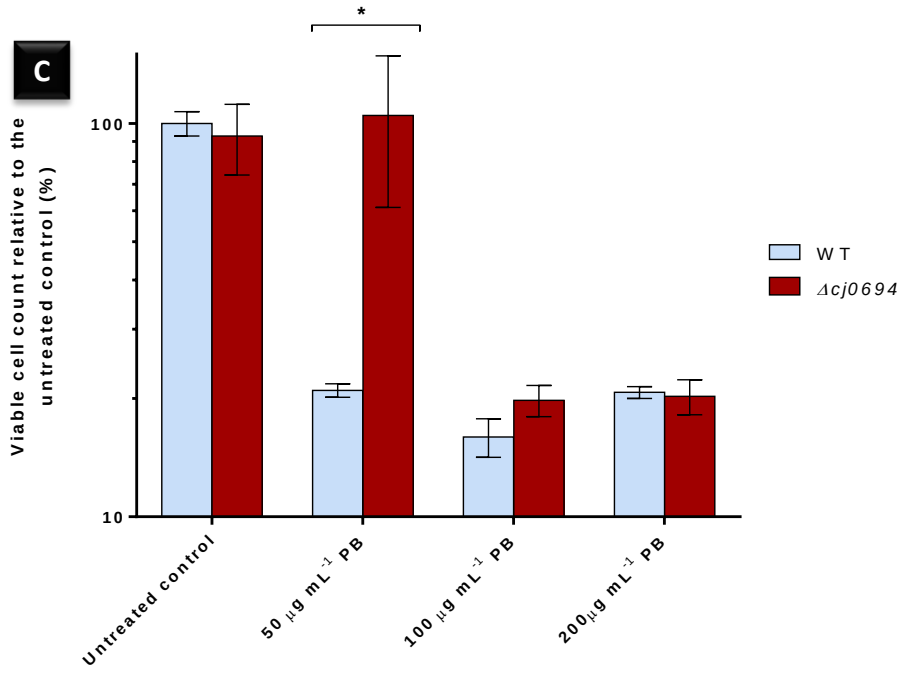
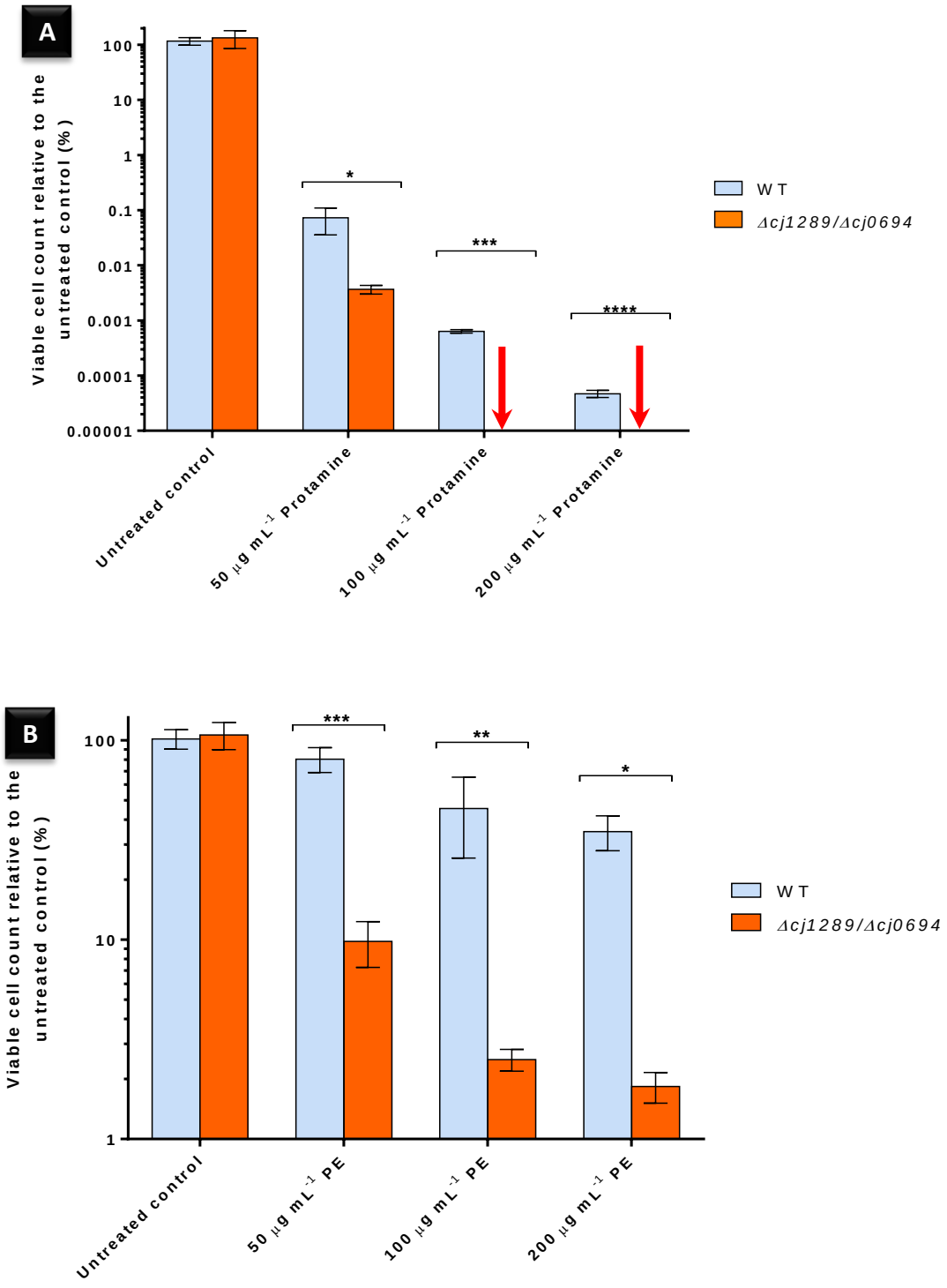


Figure 3.11 Sensitivity of the *Δcj0694* mutant to various antimicrobial peptides. The *Δcj0694* mutant shows viability in the absence of antimicrobial peptides represented by 100 % viability in the first column. **A)** The inhibitory effect of protamine sulphate on the *Δcj0694* mutant viability was less than that on the wild-type. The wild-type viability was significantly reduced by protamine sulphate compared to the *Δcj0694* mutant. **B)** The inhibitory effect of polymyxin E on the *Δcj0694* mutant viability. The sensitivity of the *Δcj0694* mutant was significantly more than that of the wild-type only when exposed to 200 µg mL^{-1} polymyxin E ($p < 0.01$). **C)** Sensitivity of the *Δcj0694* mutant to Polymyxin B. At 50 µg mL^{-1} polymyxin B, the sensitivity of the *Δcj0694* mutant was less than that of the wild-type. The number of the wild-type viable cells was significantly less than that of the mutant at this concentration ($p < 0.05$). At higher concentrations of polymyxin B, the levels of killing were similar in the wild-type and the *Δcj0694* mutant. Data are representative of three biological replicates. The cell viability values of the untreated controls were adjusted to 100% to reflect the maximum viability without treatment. Error bars represents the standard deviation of these measurements. Statistical analysis was carried out using Student's multiple t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. All replicate experiments were performed to all mutants at the same times. The wild-type data is obtained from the same experiment shown in Figure 3.10.

3.2.9 Deletion of both *cj1289* and *cj0694* showed more similar sensitivity profile to that of the *Δcj1289*

The *Δcj1289* mutant was shown to be severely sensitive to protamine sulphate and polymyxin E, whereas the *Δcj0694* mutant has not been shown to be severely affected. To examine if this effect was only due to the deletion of *cj1289* gene, and to examine if deletion of the *cj1289* and *Δcj0694* genes can result in more severe phenotype that can reveal a role of Cj0694 in OM permeability, the sensitivity of a double *Δcj1289Δcj0694* mutant was examined using the same antimicrobial peptides used with both single mutants. When the *Δcj1289/Δcj0694* double mutant was exposed to 50 μg mL^{-1} protamine sulphate, the number of viable cells was significantly reduced more than the wild-type that was treated with the same concentration of protamine ($p < 0.05$) as represented by 1-log inhibition in the number of viable cells in the double mutant. However, the double mutant cells were all killed at a concentration of 100 μg mL^{-1} (Figure 3.12A). This phenotype is more similar to that of the *Δcj1289* mutant. However, the degree of killing in double mutant was less severe than the *Δcj1289* single mutant at 50 μg mL^{-1} (Figure 3.10A). The inhibitory effect of polymyxin E on the *Δcj1289/Δcj0694* double mutant cell viability was shown to be more significant than that on the wild-type cells as represented by 1-log decrease in the number of viable cells relative to the wild-type at 50 μg mL^{-1} and the inhibition was more at higher concentrations. However, comparing to the *Δcj1289* single mutant, polymyxin E had similar effect on the double mutant at 50 and 100 μg mL^{-1} (Figure 3.12B and 3.14B), whereas it is more in the *Δcj1289* mutant at 200 μg mL^{-1}, suggesting that the extra mutation in *cj0694* in the double mutant does not increase the sensitivity to polymyxin E more than what is shown with the *Δcj1289* single mutant (Figure 3.12B). The effect of polymyxin B on the *Δcj1289/Δcj0694* double mutant cell viability was similar to that of the *Δcj0694* single mutant ($p < 0.01$ at 200 μg mL^{-1}). At 50 μg mL^{-1} polymyxin B, the wild-type cells were killed more than the double mutant for unknown reason, whereas the killing level of the wild-type cells remains constant at higher concentration, and the effect of polymyxin B on the double mutant started to be effective at 200 μg mL^{-1}, where the viability of the double mutant cells was significantly less than that of the wild-type ($p < 0.01$) (Figure 3.12C). These data indicate a more important role for Cj1289 than Cj0694 in controlling OM integrity.


A
Viable cell count relative to the untreated control (%)
100
10
1
0.1
0.01
0.001
0.0001
0.00001
*
***
****
WT
Δcj1289/Δcj0694
Untreated control
50 μg mL-1 Protamine
100 μg mL-1 Protamine
200 μg mL-1 Protamine
B
Viable cell count relative to the untreated control (%)
100
10
1
***
**
*
WT
Δcj1289/Δcj0694
Untreated control
50 μg mL-1 PE
100 μg mL-1 PE
200 μg mL-1 PE

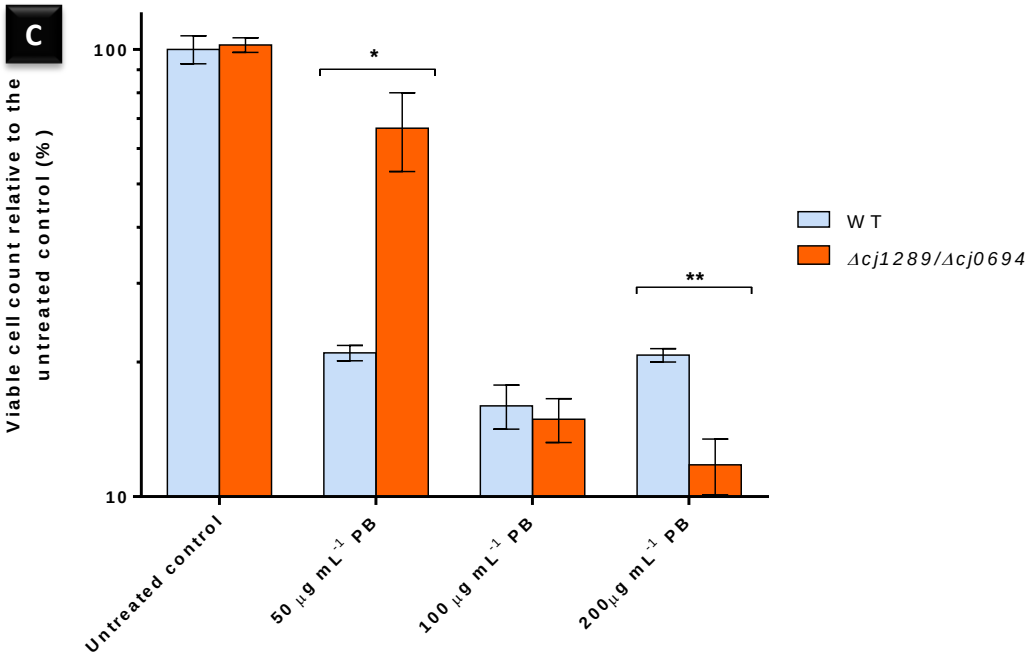
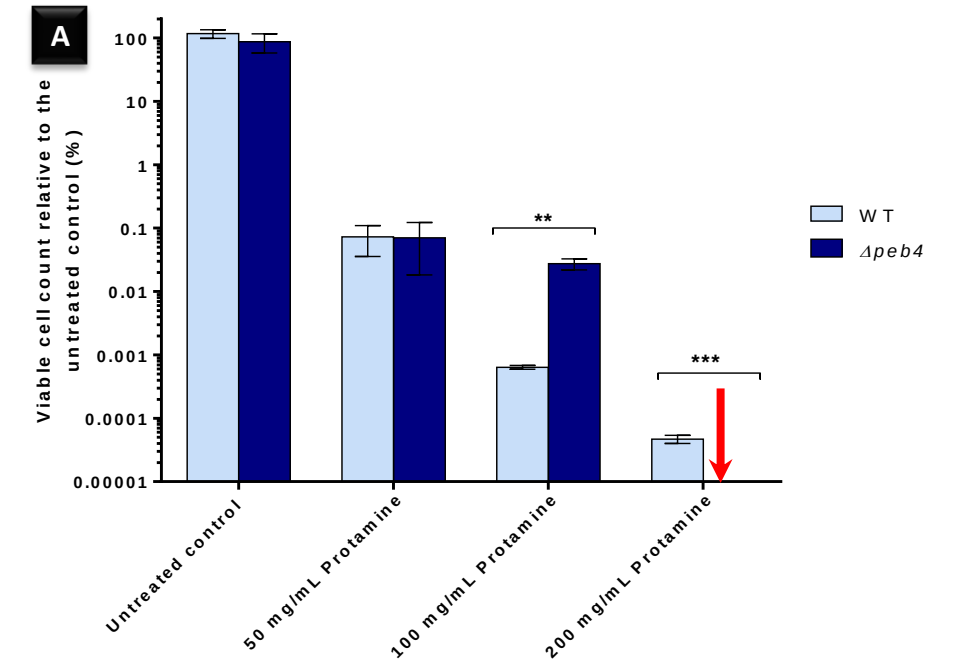
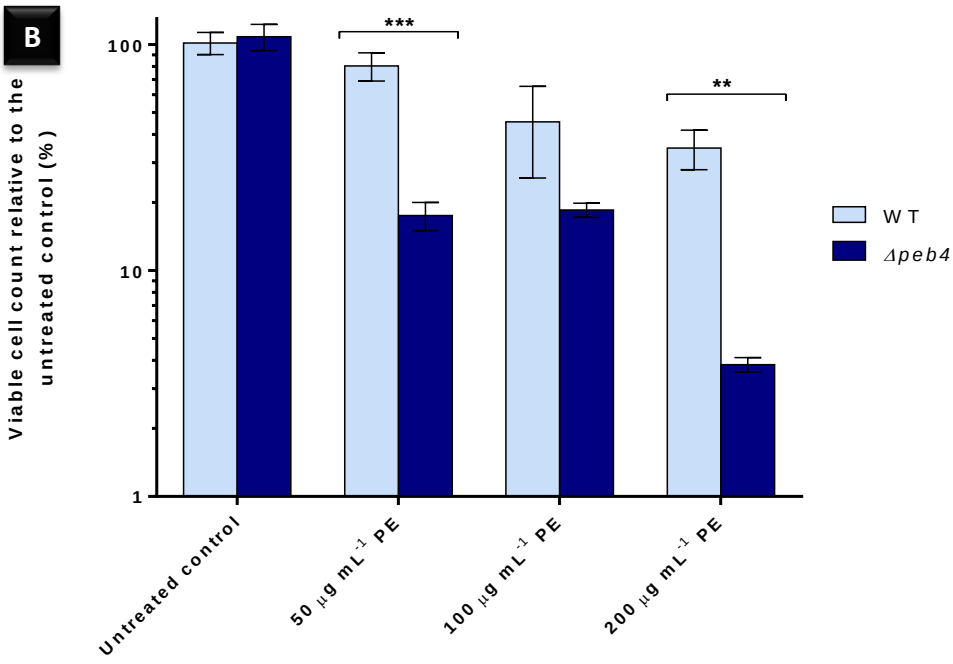


Figure 3.12 Sensitivity of the *Δcj1289/Δcj0694* double mutant to antimicrobial peptides. A) The effect of protamine sulphate on the *Δcj1289/Δcj0694* double mutant cell viability. Similar effect of protamine sulphate was observed in the *Δcj1289/Δcj0694* double mutant to that of the *Δcj1289* single mutant. The number of the *Δcj1289/Δcj0694* double mutant viable cells was 1-log less than that of the wild-type when exposed to 50 µg mL^{-1} protamine sulphate, while 100 µg mL^{-1} of protamine sulphate was sufficient to kill double mutant cells. **B)** The effect of polymyxin E on the *Δcj1289/Δcj0694* double mutant cell viability. Cell viability of the double mutant was significantly less than that of the wild-type as represented by 1-log decrease in the number of viable cells relative to the wild-type at 50 µg mL^{-1} and the inhibition was more at higher concentrations. Comparing to the *Δcj1289* single mutant, polymyxin E had similar effect on the double mutant. **C)** The effect of polymyxin B on the *Δcj1289/Δcj0694* double mutant cell viability. A similar effect was shown on the double mutant to that of the *Δcj0694* single mutant ($p < 0.01$ at 200 µg mL^{-1}). At 50 µg mL^{-1} polymyxin B, the wild-type cells were killed more than the double mutant. Data are representative of three biological replicates. All replicate experiments were performed to all mutants at the same times. The wild-type data is obtained from the same experiment shown in Figure 3.10. The cell viability values of the untreated controls were adjusted to 100% to reflect the maximum viability without treatment. Error bars represents the standard deviation of these measurements. Statistical analysis was carried out using Student's multiple t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.2.10 *peb4*-deletion mutation results in a defective strain with an increased sensitivity to antimicrobial peptides

The role of the periplasmic chaperone PEB4 in the assembly of the OM in *C. jejuni* was intensively investigated previously (Kale *et al.*, 2011). The mutant strain used in that study was generated by insertional mutagenesis in which an antibiotic resistance cassette is inserted into the gene sequence causing inactivation of the gene. Here, a *Δpeb4* mutant had been generated by deletion of *peb4* gene and fully replacing it with kan^R cassette. The viability of the *Δpeb4* mutant was monitored in comparison to the wild-type and both shows reduction in the number of viable cells with 3-logs decrease in the number of viable cells when treated with 50 µg mL^{-1} protamine sulphate comparing to the untreated controls. Surprisingly, at 100 µg mL^{-1} protamine sulphate the number of the *Δpeb4* mutant viable cells remained at the same level whereas the wild-type was significantly less than the mutant (p <0.01). However, the *Δpeb4* mutant cells were all killed at a concentration of 200 µg mL^{-1} protamine sulphate (Figure 3.13A). Therefore, the *Δpeb4* mutant only shows significant decrease in viability at higher concentrations, whereas at lower concentrations its viability is similar to that of the wild-type. The *Δpeb4* mutant had also shown to be sensitive to polymyxin E. The viability of the *Δpeb4* mutant cells was significantly less than that of the wild-type cells ($p < 0.001$) when exposed to 50 and 100 µg mL^{-1} polymyxin E as represented by 1-log decrease in the inhibition of the viability compared to the wild-type, and the inhibition increased at higher concentrations (Figure 3.13B). A similar effect was seen with polymyxin B as 50 and 100 µg mL^{-1} reduced the number of the *Δpeb4* viable cells (1-log decrease) comparing to the wild-type ($p < 0.0001$), whereas *Δpeb4* viable cells were reduced by 2-logs when exposed to 200 µg mL^{-1}, and its viability was significantly less than the wild-type ($p < 0.0001$) (Figure 3.13C).


A
Viable cell count relative to the untreated control (%)
100
10
1
0.1
0.01
0.001
0.0001
0.00001
**
***
W T
Δpeb4
Untreated control
50 mg/mL Protamine
100 mg/mL Protamine
200 mg/mL Protamine


B
Viable cell count relative to the untreated control (%)
100
10
1
***
**
W T
Δpeb4
Untreated control
50 μg mL-1 PE
100 μg mL-1 PE
200 μg mL-1 PE

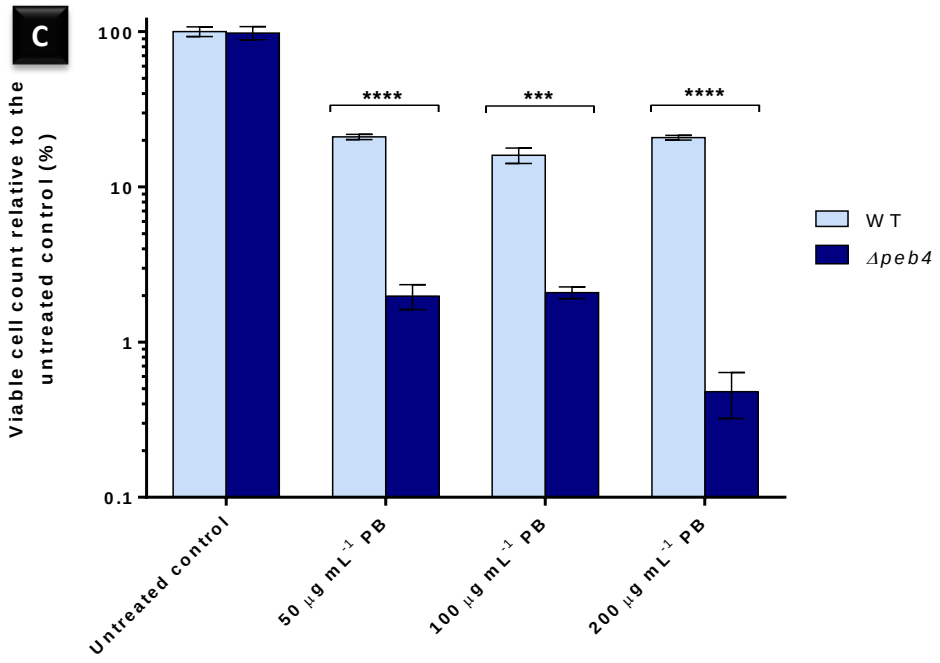


Figure 3.13 Sensitivity of the *Δpeb4* mutant to antimicrobial peptides. A) The effect of protamine sulphate on the viability of the *Δpeb4* mutant. A similar reduction in the number of viable cell count relative to the wild-type at the same treatment is seen when the mutant cells exposed to 50 and 100 µg mL^{-1} protamine sulphate, while mutant cells were killed when exposed to 200 µg mL^{-1} protamine sulphate. **B)** The effect of polymyxin E on the viability of the *Δpeb4* mutant. At 50 and 100 µg mL^{-1} polymyxin E, the viability of the *Δpeb4* mutant cells was significantly less than the wild-type ($p < 0.001$) represented by 1-log decrease in the inhibition of the viability compared to the wild-type, and the inhibition increased at higher concentrations. **C)** The effect of polymyxin E on the viability of the *Δpeb4* mutant. At 50 and 100 µg mL^{-1} causes 1-log decrease in the number of the *Δpeb4* viable cells comparing to the wild-type ($p < 0.0001$), whereas *Δpeb4* viable cells were reduced by 2-logs when exposed to 200 µg mL^{-1}, and its viability was significantly less than the wild-type ($p < 0.0001$). Data are representative of three biological replicates. All replicate experiments were performed to all mutants at the same times. The wild-type data is obtained from the same experiment shown in Figure 3.10. The cell viability values of the untreated controls were adjusted to 100% to reflect the maximum viability without treatment. Error bars represents the standard deviation of these measurements. Statistical analysis was carried out using Student's multiple t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

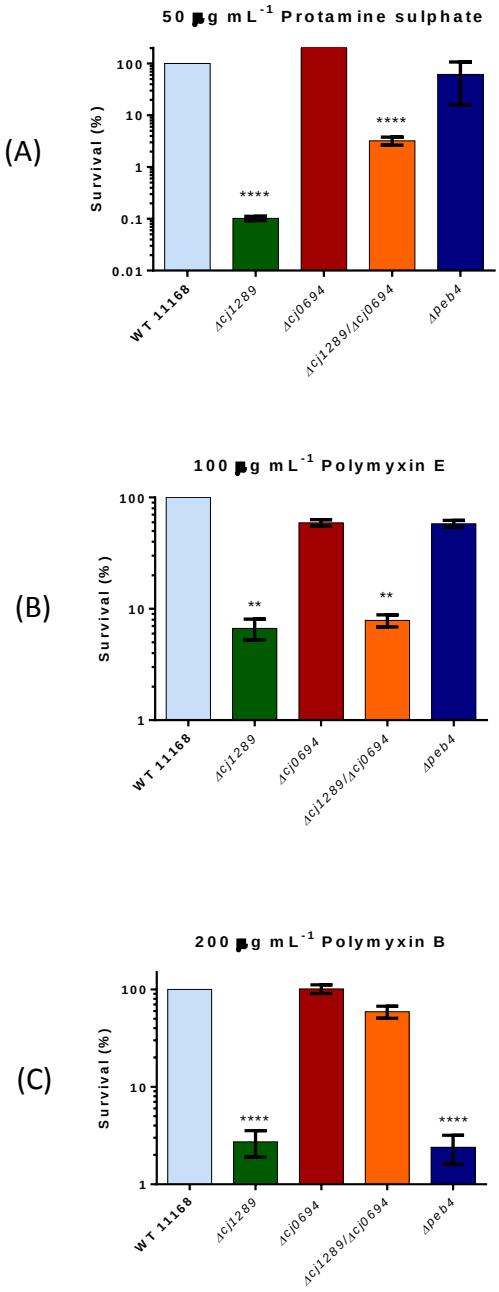


Figure 3.14 Summary of the increased susceptibility to killing by CAMPs in *C. jejuni* chaperone mutants. A) Sensitivity of chaperone mutants to 50 µg mL^{-1} protamine sulphate. **B)** Sensitivity of chaperone mutants to 100 µg mL^{-1} polymyxin E. **C)** Sensitivity of chaperone mutants to 200 µg mL^{-1} polymyxin B. The Δ*cj1289* mutant displays the highest sensitivity to all CAMPs ($p < 0.0001$), while the Δ*cj0694* mutant shows no significant change compared to the parent wild-type. The Δ*peb4* mutant shows significant increase in susceptibility to only polymyxin B (wild-type *C. jejuni* is usually resistant to polymyxin B). The cell viability values of the wild-type were adjusted to 100% to reflect the effect of the mutations on the cell viability under the same concentration of CAMPs. ** $p < 0.01$, **** $p < 0.0001$. Error bars represents the standard deviation of these measurements. Statistical analysis was carried out using One-way ANOVA.

3.2.11 Proteomic analysis of the OM and periplasmic profiles in the *Δcj1289* and *Δcj0694* mutants

To investigate the chaperone role of the Cj1289 and Cj0694 in the maturation and assembly of periplasmic and OMPs, it was suggested that mutation in their cognate genes may affect the pathway of either periplasmic or OMP folding and may, for example, result in an accumulation of un-inserted OMPs in the periplasm. Therefore, absence of Cj1289 or Cj0694 may lead to an alteration in the OM and periplasmic profiles of the mutants compared to those of the parent wild-type. Our ultimate goal in this section was to report the changes that may occur due to the alteration in the OM composition in the *Δcj1289* and *Δcj0694* mutants by identifying the proteins that are missing or down regulated in the OMs of the mutants and comparing them to the OM of the wild-type.

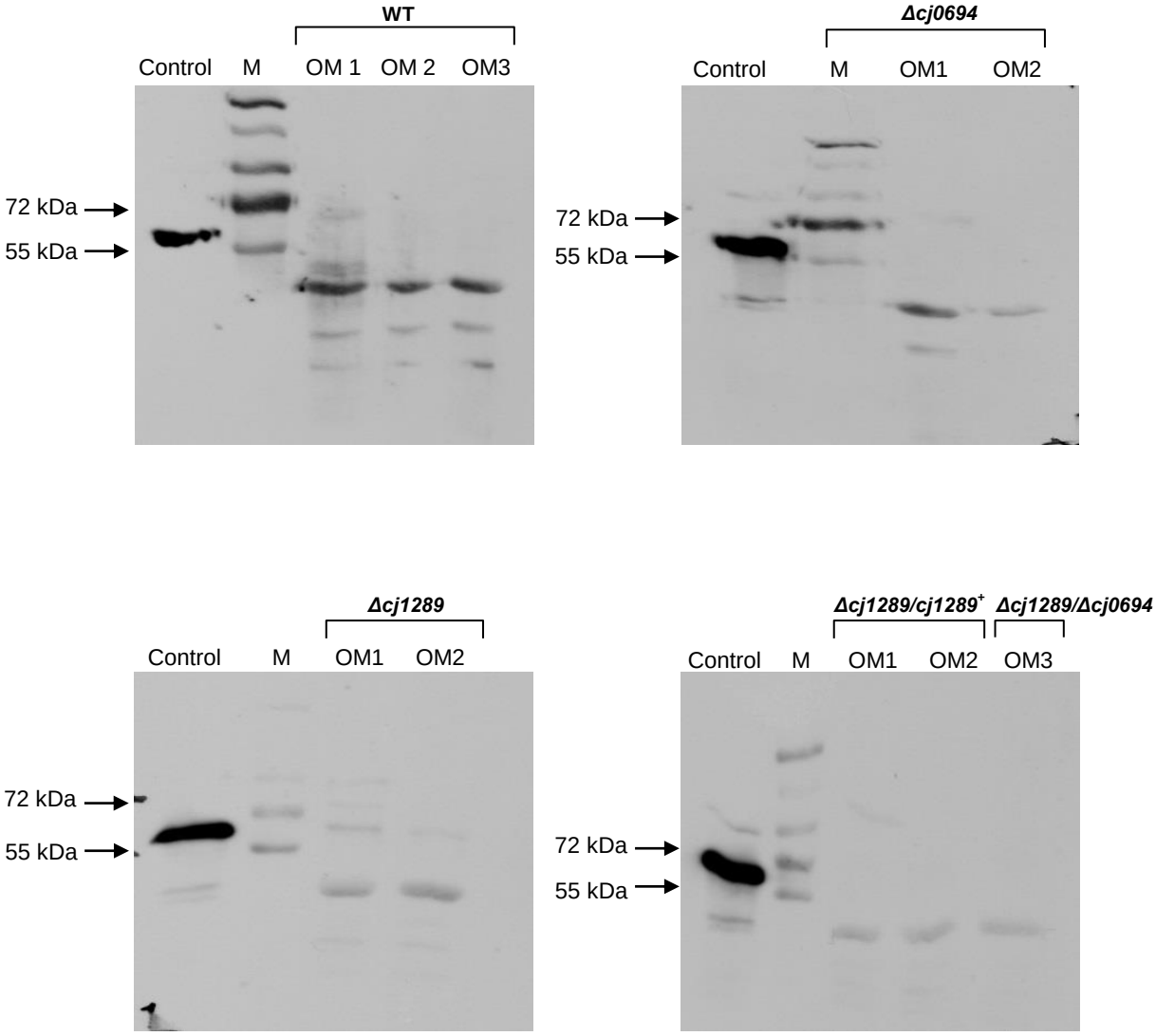
3.2.11.1 Isolation of outer membranes and periplasms of wild-type *C. jejuni*, *Δcj1289*, *Δcj1289/Δcj1289*$^{+}$, *Δcj0694* and *Δcj1289/Δcj0694* mutants

Cell fractionation was performed to isolate the OM and periplasm of the *Δcj1289* mutant, the *Δcj1289/Δcj1289*$^{+}$ complemented strain, the *Δcj0694* single mutant and the *Δcj1289/Δcj0694* double mutant, in order to identify the client OMPs that are dependent on Cj1289 or Cj0694 for their maturation and assembly in the OM. 2D-PAGE analysis was conducted in a collaborative work with Dr Francis Mulholland at The Institution of Food Research (IFR), Norwich, UK, to assess the alteration in OM and periplasmic profiles and compare these changes to those of the wild-type. The total membrane fractions of the wild-type *C. jejuni*, *Δcj1289* mutant, *Δcj1289/Δcj1289*$^{+}$ complemented strain, *Δcj0694* mutant and *Δcj1289/Δcj0694* double mutant were successfully isolated in the form of gel-like, sticky pellets. As described in 2.7.3 and 2.7.4, the pellets were washed twice and resuspended in 10 mM HEPES, pH 7.4, and incubated in 2 % (*w/v*) *N*-laurylsarcosinate at 37 °C for 30 minutes. Additional centrifugation was applied to remove the supernatants containing inner membrane proteins, and the remaining outer membrane pellets were washed and carefully resuspended in 10 mM HEPES, pH 7.4.

3.2.11.2 Western blot confirms the purity of cell fractions

Purity of both OMs and periplasms was confirmed by western blot using anti-MfrA, raised against the periplasmic fumarate reductase (MfrA) in *C. jejuni* (Guccione *et al.*, 2010), and anti-GroEL, raised against the cytoplasmic chaperone GroEL in *E. coli* (Lui and Kelly, Unpublished data). To confirm that OMs of all strains were pure from periplasmic contaminating proteins that may occur during OM preparation, the blots using anti-MfrA were performed against the OMs of mutants, using the wild-type periplasm as a control. These blots show that the ~ 65 kDa MfrA is localised in the periplasms of the wild-type but was absent in the OMs of all strains, confirming that the OMs of all strains were free from contaminating periplasmic proteins (Figure 3.15A). Also, Anti-GroEL was performed against the periplasms of all strains, using the wild-type cell-free extract (CFE) as a control, to confirm that the periplasms of all strains were free from contaminating cytoplasmic proteins that may occur in the periplasms during preparation. The blots show that the ~ 62 kDa GroEL is located in the CFE of the wild-type but not in the periplasms of all strains (Figure 3.15B). These blots confirmed that the prepared cell fractions were pure to be analyzed by 2D-gel electrophoresis.

(A) Western blot for outer membranes using anti-MfrA



(B) Western blot for periplasms using anti-GroEL

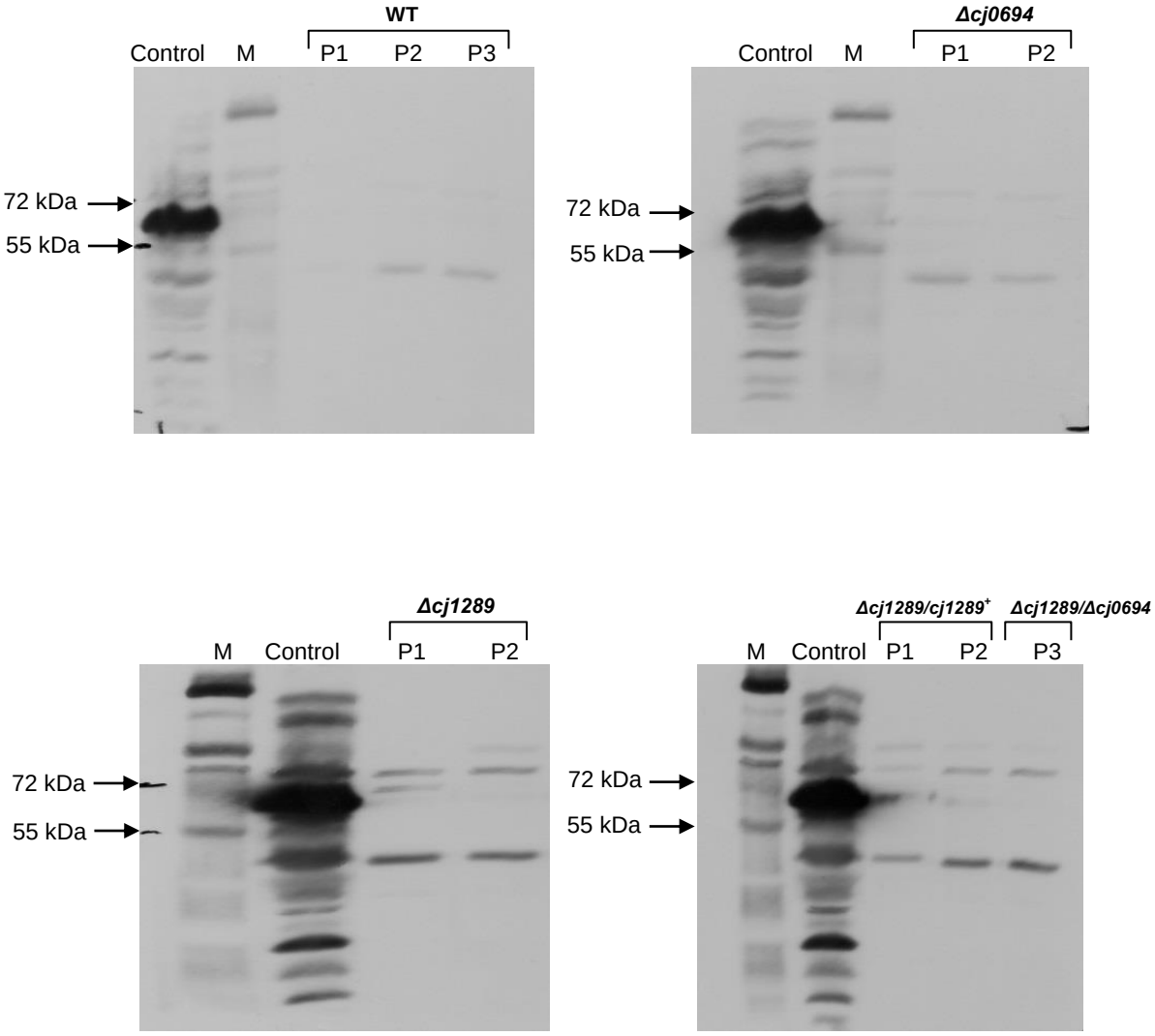


Figure 3.15 Confirmation of purity of OMs and periplasms by western blot. A) Western blot for outer membrane of wild-type and mutant strains using anti-MfrA. The localisation of ~ 65 kDa MfrA was confirmed in the periplasm of the control sample (wild-type periplasm) and was absent in the OMs of all strains. Lane (M) shows the PageRuler^{TM} pre-stained protein marker. Lanes in numbers show independently prepared OMs of each strain. **B)** Western blot for periplasms of wild-type and mutant strains using anti-GroEL. The localisation of the ~ 62 kDa cytoplasmic protein, GroEL was confirmed in the control sample (wild-type CFE) and was absent in the periplasms of all strains. Lane (M) shows the PageRuler^{TM} pre-stained protein marker. Lanes in numbers show independently prepared periplasms of each strain. At least two samples were used for each preparation except for the double mutant where only one sample was used.

3.2.11.3 Evaluation of the use of the 2D-PAGE and quantitative proteomics to determine the clients of periplasmic chaperones

The quantitative proteomic approaches such as mass spectrometric identification of proteins from 1-D and 2D-PAGE analysis are widely used in proteomics for the identification of client proteins and to determine the expression levels of OMPs that are handled by distinct chaperones (Asakura *et al.*, 2007, Rathbun *et al.*, 2009, Rathbun and Thompson, 2009). Here, OM and periplasmic fractions are isolated from the two cell populations that will be examined (e.g. wild-type and mutant) and run on 2D-gels (pI range 3 – 11). Protein spots observed to be changing between the two samples are picked, trypsin digested and identified by mass spectroscopy.

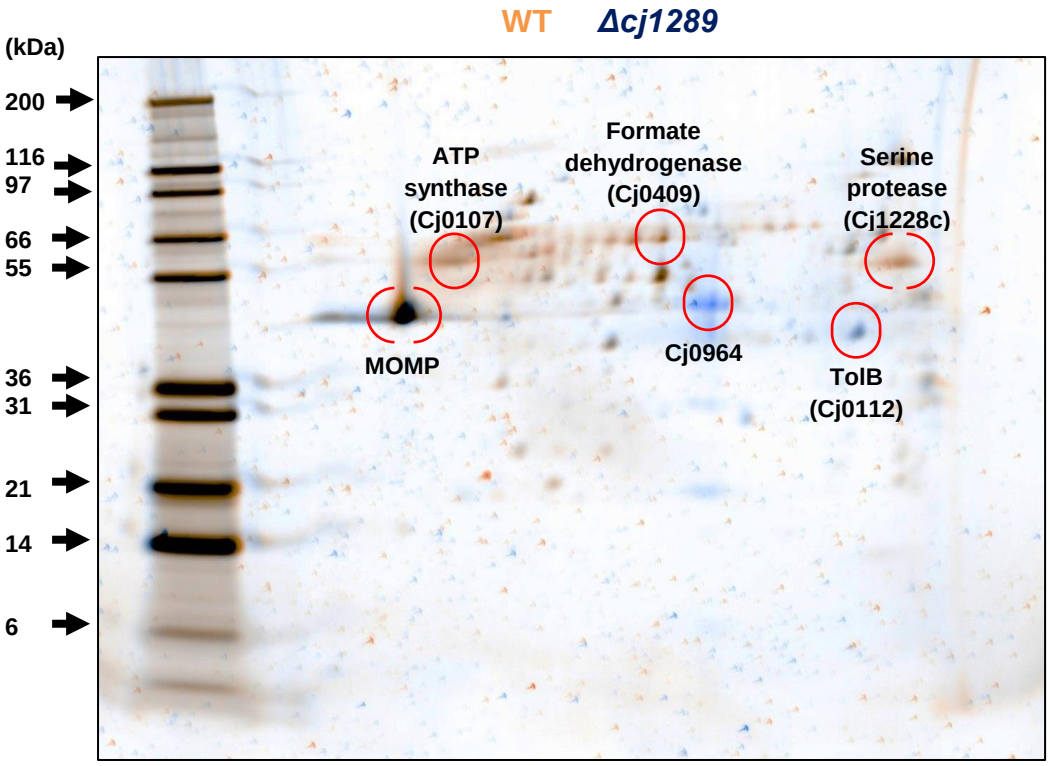
The OM and periplasmic fractions were analysed by 2D-PAGE in a collaborative work with Dr Francis Mulholland at IFR (Norwich, UK). OM and periplasm samples were processed in initial preparation steps prior to being analysed by proteomic 2D-PAGE analysis. These steps include an initial centrifugation of pellets that had been solubilised in rehydration lysis buffer (RHB; 7 M urea, 2 M thiourea, 2 % CHAPS). The resulted pellets were centrifuged for a second time and the supernatant was discarded, while the pellets were solubilised in 50 mM Tris-HCl, 0.3 % SDS, 0.2 M DTT. A third centrifugation of the re-dissolved pellets was applied and the final resulted pellets were resuspended in MilliQ water to be used for 1D and 2D-PAGE analysis. After overlying the 2D-gels for the OMs and periplasms of wild-type and each mutant, mass spectrometry analysis (Table 3.1) was conducted to identify each protein spot that is present in the OM of the wild-type and absent in that of the mutant, or present in the periplasm of the mutant and absent in that of the wild-type.

Overall, the 2D-gels of all mutants OMs provide clear evidence about an overall reduction in the OMPs in the mutants. However, the abundance of these proteins in the OMs is low. The PorA/MOMP is the strongest spot which can be used to adjust the imaging parameters. Therefore, if this spot is directly affected by mutation in any of these mutants, then the OM fraction must be enriched to exclude this factor. Overlaying the 2D-gels of the OMs of the wild-type (stained in orange) and the *Δcj1289* mutant (stained in blue) revealed that three outer membrane associated proteins are expressed only in the wild-type OM and are absent in the OM of the *Δcj1289* mutant (Figure 3.16A). These were the ATP synthase (Cj0107), formate dehydrogenase (Cj0409) and

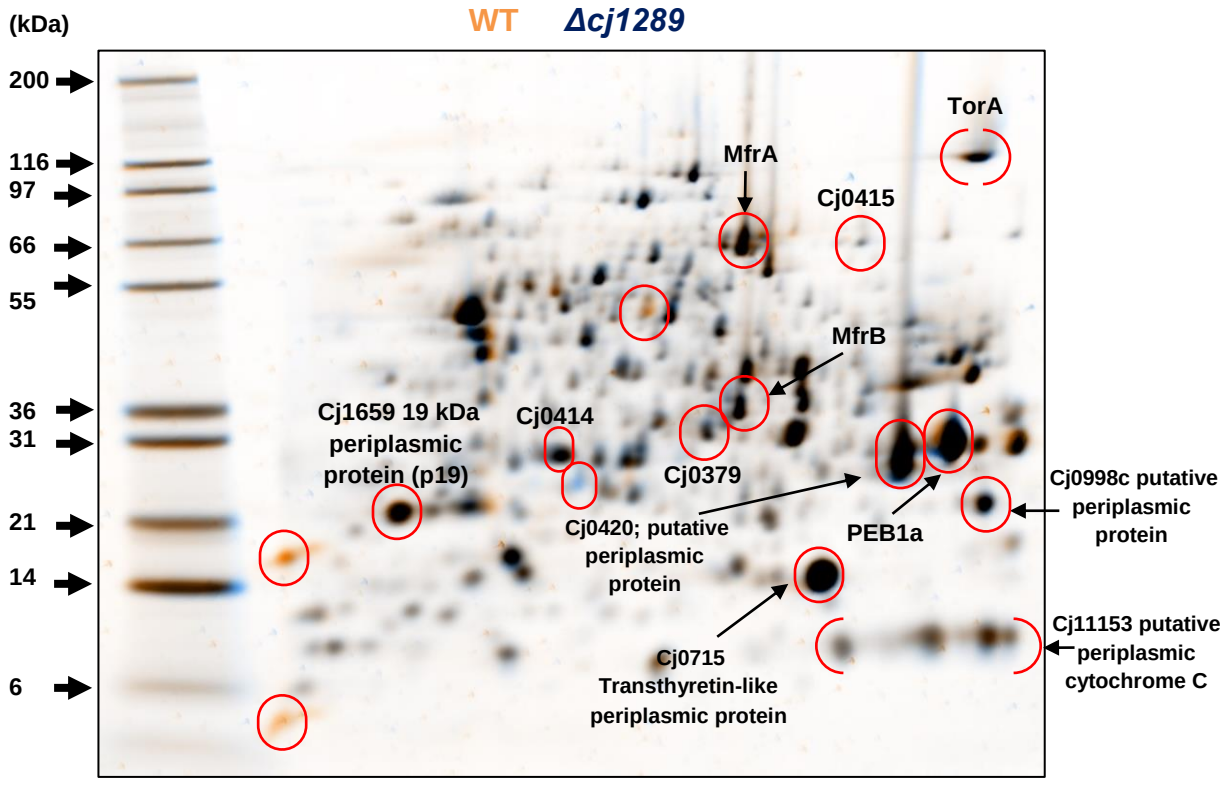
serine protease (Cj1228c) shown in orange. However, two periplasmic proteins were present in the OM of the *Δcj1289* mutant (shown in blue), and are considered to be contaminating proteins in the OM fraction. These were TolB (Cj0112) and a putative periplasmic protein (Cj0964). On the other hand, overlaying the 2D-gels of the periplasms of the wild-type (stained in orange) and the *Δcj1289* mutant (stained in blue) showed less alteration in the periplasmic profiles (Figure 3.16B). The PEB1a and the 19 kDa periplasmic protein (p19) appear to be good markers for the periplasms. These proteins were identified in a previous study (Hitchcock *et al.*, 2010), and were used here as markers that indicate a proper preparation of the periplasms. Overlaying the 2D-gels of the OMs of the wild-type (stained in orange) and the *Δcj0694* mutant (stained in blue) revealed that two outer membrane associated proteins are expressed only in the wild-type OM and are absent in the *Δcj0694* mutant (Figure 3.16C). These spots were stained in orange and are correspond to Ni-Fe hydrogenase (Cj1267c) and serine protease (Cj1228c). In contrast, overlaying the 2D-gels of the periplasms of the wild-type and the *Δcj0694* mutant shows more accumulation of proteins in the periplasm of the *Δcj0694* mutant (Figure 3.16D). Finally, in the 2D-gels of the OMs of the wild-type and the *Δcj1289*/*Δcj0694* double mutant we could identify two OMPs, ATP synthase (Cj0107) and serine protease (Cj1228c) which were found in the wild-type OM (orange) and were absent in the OM of the double mutant (Figure 3.16E). These proteins are persistent in both the *Δcj1289* single mutant and the *Δcj1289/Δcj0694* double mutant. The periplasmic picture of the double mutant revealed that only one protein stained in blue, indicating that it is related to the periplasm of the double mutant and is persistent in both the *Δcj1289* single mutant and the *Δcj1289/Δcj0694* double mutant (Figure 3.16F).

This can provide an initial indication of the accumulation of the OMPs in the periplasm of the mutants and failure to delivering them to the OM. However, the contaminating background in the 2D-gels is one of the main concerns that may make it difficult to confirm these findings, although the fractions were prepared according to standard methods and western blot data shows that these fractions were pure.

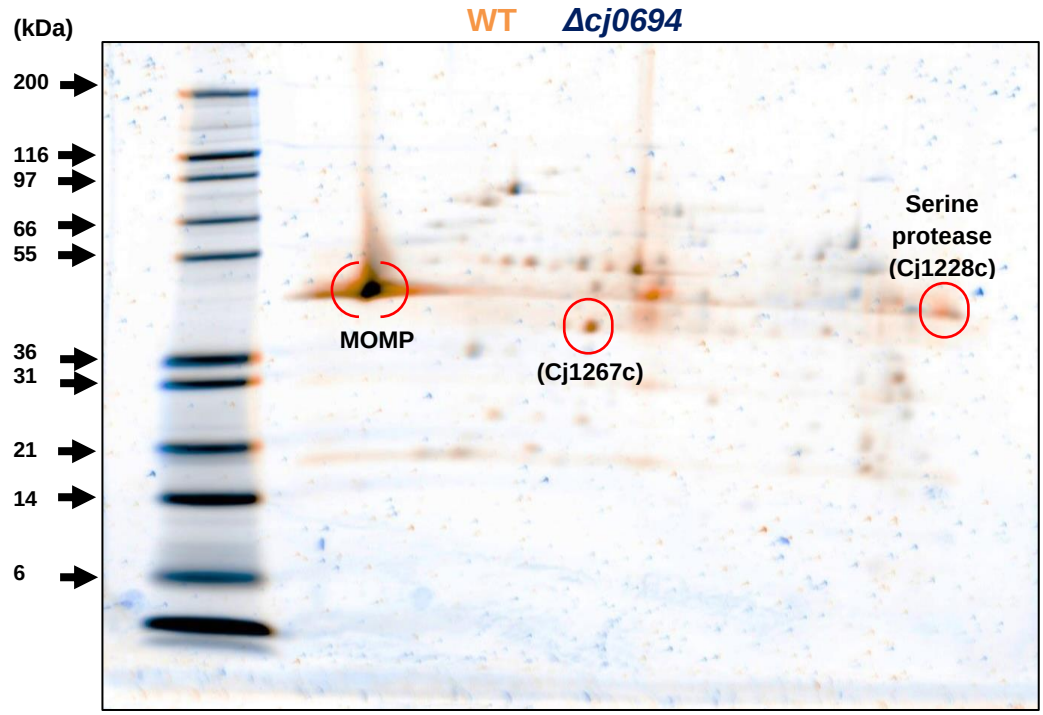
(A) The wild-type Vs. the *Δcj1289* mutant OMs



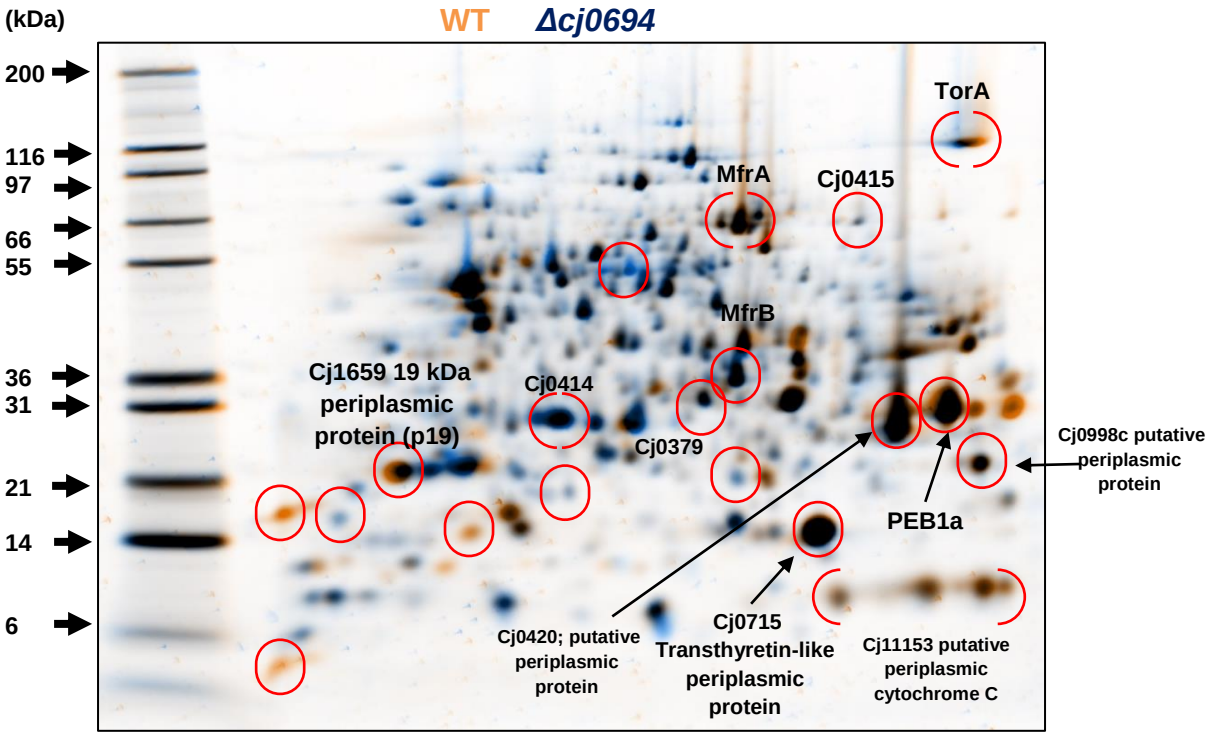
(B) The wild-type Vs. the *Δcj1289* mutant periplasms



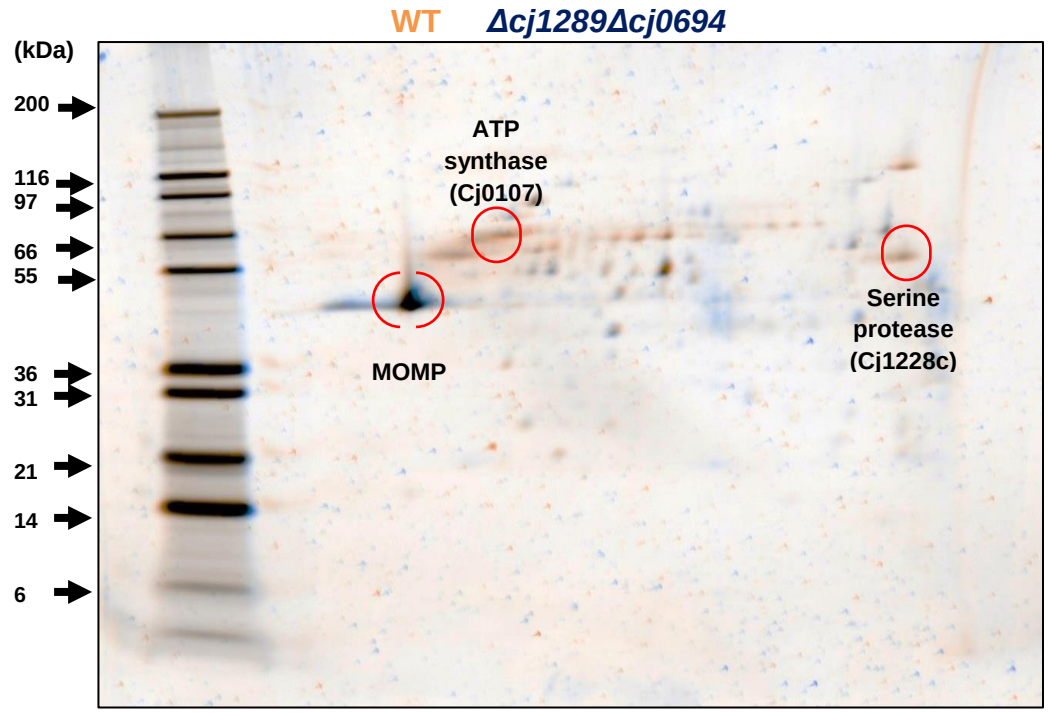
(C) The wild-type Vs. the *Δcj0694* mutant OMs



(D) The wild-type Vs. the *Δcj0694* mutant periplasms



(E) The wild-type Vs. the *Δcj1289Δcj0694* double mutant OMs



(F) The wild-type Vs. the *Δcj1289Δcj0694* double mutant periplasms

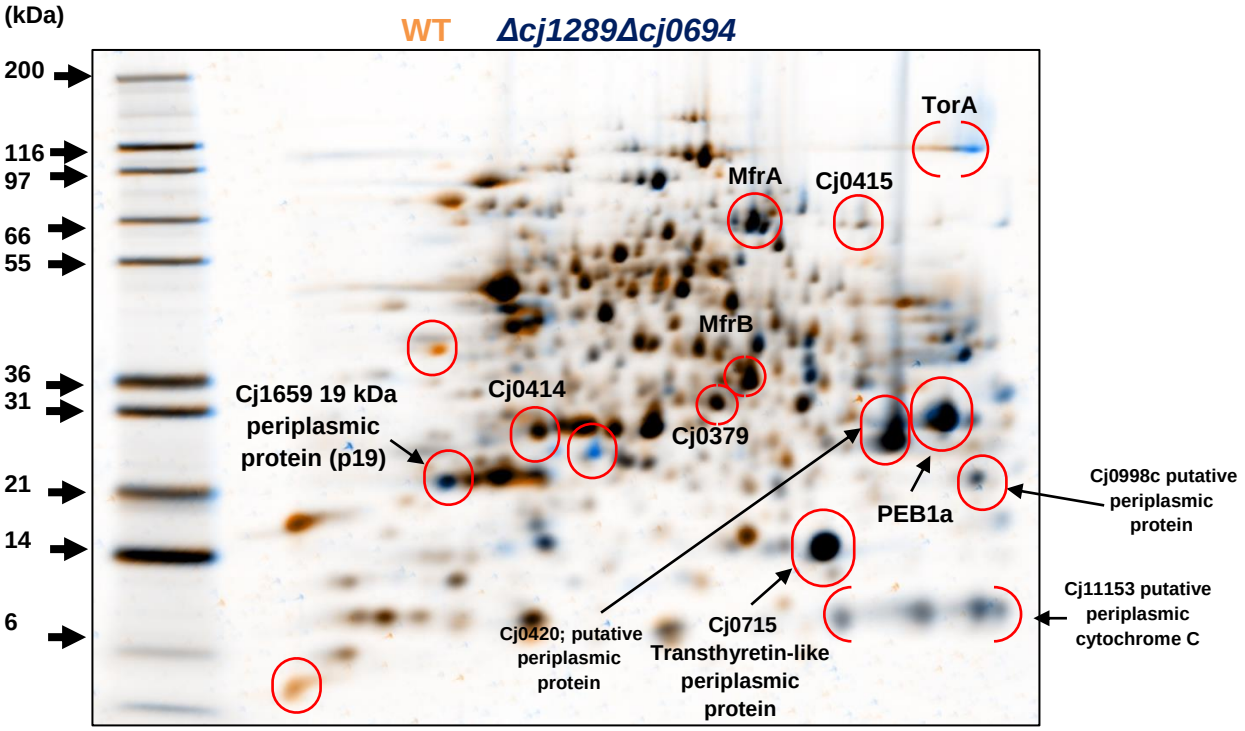


Figure 3.16 2D-PAGE analysis of the periplasmic and outer membrane proteins of the wild-type (orange) and mutants (blue). Protein samples were prepared as described in 2.7.3 and 2.7.4 and separately resolved by 2D PAGE. The overlays descriptions are indicated on the top of each gel picture. Orange spots represent proteins found in the wild-type fractions only, and absent in the mutant fractions. Blue spots represent proteins found in mutants fractions only, and absent in the wild-type fractions. Black spots represent proteins found in both the wild-type and mutant fractions. Labelled protein spots in 2D-gels correlate to the OMs were identified by mass spectrometry analysis as indicated in Table 3.2, whereas proteins spots in gels correlate to the periplasms were previously published (Hitchcock *et al*., 2010). **A)** Comparative analysis of the OMs of the wild-type and the *Δcj1289* mutant. **B)** Comparative analysis of the periplasms of the wild-type and the *Δcj1289* mutant. **C)** Comparative analysis of the OMs of the wild-type and the *Δcj0694* mutant. **D)** Comparative analysis of the periplasms of the wild-type and the *Δcj0694* mutant. **(E)** Comparative analysis of the OMs of the wild-type and the *Δcj1289*/*Δcj0694* double mutant. **F)** Comparative analysis of the periplasms of the wild-type and the *Δcj1289*/*Δcj0694* double mutant. At least two samples were analysed for each strain and all indicate similar results, except for the double mutant where only one sample was analysed. Gels were stained by SYPRO® Ruby (Invitrogen, UK). This work was performed by Dr. Francis Mulholland at the Institute of Food Research (Norwich, UK).

Table 3.1 Mass spectrometric data obtained from 2D-gel analysis performed by Dr Francis Mulholland at the Institute of Food Research (IFR), Norwich, UK.

Sample Name	Well No	Orbitrap Data file name	Top Hit	Mascot Score	Secondary Hits	Mascot Score
Cj0694-om 13233 pick 1_1	A01	**FM120816_24**	ATP synthase subunit beta; EC=3.6.3.14 (Cj0107)	1854		
Cj0694-om 13233 pick 1_2	A02	**FM120816_02**	Fumarate reductase iron-sulfur protein; EC=1.3.99.1 (Cj0410)	369	Putative lipoprotein (Cj0396c)	261
Cj0694-om 13233 pick 2_1	A03	**FM120816_03**	Putative NADH dehydrogenase I chain G; EC=1.6.5.3 (Cj1573c)	1071	Protein translocase subunit SecA (Cj0942c)	449
Cj0694-om 13233 pick 2_2	A04	**FM120816_04**	Putative formate dehydrogenase large subunit (Selenocysteine containing); EC=1.2.1.2 (Cj1511c)	601		
Cj0694-om 13233 pick 2_3	A05	**FM120816_25**	ATP synthase subunit alpha; EC=3.6.3.14 (Cj0105)	475		
Cj0694-om 13233 pick 3_1	A06	**FM120816_29**	Fumarate reductase flavoprotein subunit; EC=1.3.99.1 (Cj0409)	1490		
Cj0694-om 13233 pick 3_2	A07	**FM120816_05**	Ni/Fe-hydrogenase large subunit; EC=1.12.5.1 (Cj1266c)	444	Succinate dehydrogenase flavoprotein subunit; EC=1.3.99.1 (Cj0437)	141
Cj0694-om 13233 pick 3_3	A08	**FM120816_06**	Ni/Fe-hydrogenase small chain; EC=1.12.5.1 (Cj1267c)	280	Outer membrane protein (Cj0129c)	118
Cj0694-om 13233 pick 4_1	A09	**FM120816_07**	Putative periplasmic protein (Cj0151c)	368		
Cj0694-om 13233 pick 4_2	A10	**FM120816_26**	Putative peptidyl-prolyl cis-trans isomerase Cbf2 (Cj0596)	1357	Possible periplasmic protein (Cj1289)	494
Cj0694-om 13233 pick 4_3	A11	**FM120816_08**	Protein TolB (Cj0112)	340	Putative cytochrome C551 peroxidase; EC=1.11.1.5 (Cj0358)	328
Cj0694-om 13233 pick 4_4	A12	**FM120816_27**	Serine protease (Protease DO); EC=3.4.21 (Cj1228c)	2056	Putative periplasmic protein (Cj0114)	272
Cj0694-om 13233 pick 5_1	B01	**FM120816_09**	Protein TolB (Cj0112)	366	Putative cytochrome C551 peroxidase; EC=1.11.1.5 (Cj0358)	308
Cj0694-om 13233 pick 5_2	B02	**FM120816_10**	Major cell-binding factor; AltName: Full=CBF1; AltName: Full=PEB1 (Cj0921c)	561	Putative periplasmic protein (Cj1380)	500
Cj0694-om 13233 pick 5_3	B03	**FM120816_11**	Ubiquinol-cytochrome c reductase iron-sulfur subunit (Cj1186c)	278	Putative periplasmic protein (Cj1380)	103
Cj0694-om 13233 pick 5_4	B04	**FM120816_12**	5-hydroxyisourate hydrolase (Cj0715)	228	Putative lipoprotein (Cj0950c)	156
Cj0694-om 13233 pick 6_1	B05	**FM120816_13**	Serine protease (Protease DO); EC=3.4.21 (Cj1228c)	831	Glutamate-1-semialdehyde 2,1-aminomutase (Cj0853)	318

Cj0694-om 13233 pick 6_2	B06	**FM120816_14**	50S ribosomal protein L5 (Cj1695c)	470	50S ribosomal protein L25 (Cj0311)	438
Cj0694-om 13233 pick 6_3	B07	**FM120816_15**	Ni/Fe-hydrogenase small chain; EC=1.12.5.1 (Cj1267c)	174		
Cj0694-om 13233 pick 6_4	B08	**FM120816_16**	ATP synthase subunit alpha; EC=3.6.3.14 (Cj0105)	922	50 kDa outer membrane protein (Cj1170c)	333
Cj0694-om 13233 pick 7_1	B09	**FM120816_17**	50S ribosomal protein L6 (Cj1692c)	639	50S ribosomal protein L25 (Cj0311)	285
Cj0694-om 13233 pick 7_2	B10	**FM120816_18**	Putative peptidase M23 family protein (Cj1215)	671	Branched-chain amino-acid ABC transport system,periplasmic binding protein (Cj1018c)	276
Cj0694-om 13233 pick 7_4	B12	**FM120816_28**	Protein TolB (Cj0112)	2115		
Plate sample upper piece (Probably B11)	C01	**FM120816_19**	Putative formate dehydrogenase large subunit (Selenocysteine containing); EC=1.2.1.2 (Cj1511c)	548		
Plate sample lower piece (Probably A11)	C02	**FM120816_20**	Putative periplasmic protein (Cj0964)	708	Putative cytochrome C551 peroxidase; EC=1.11.1.5 (Cj0358)	396
Cj wt om 13235 pick 1_1	C03	**FM120816_21**	50 kDa outer membrane protein (Cj1170c)	529	Putative periplasmic protein (Cj0964)	273
Cj wt om 13235 pick 1_2	C04	**FM120816_22**	Putative succinate dehydrogenase iron-sulfur protein; EC=1.3.5.1 (Cj0438)	592	Putative cytochrome C551 peroxidase; EC=1.11.1.5 (Cj0358)	363
Cj wt om 13235 pick 1_3	C05	**FM120816_23**	Putative periplasmic protein (Cj0964)	88		

3.2.11.4 Stable Isotope Labelling by Amino acids in Cell culture (SILAC)

An alternative approach used in protein determination is the use of Stable Isotope Labelling by Amino acids in Cell culture (SILAC) developed by (Ong *et al.*, 2002). This approach requires the growth of two groups of cell populations in media that have identical contents, except that one contains the light (^{12}C + ^{14}N) and the other contains the heavy (^{13}C + ^{15}N) form of amino acids Lysine and Arginine. Both forms of amino acids are incorporated into all cellular proteins during cell division. Then, proteins from both cell populations can be combined and applied to 1-D PAGE for visualisation. The resulting bands can be then digested from the gel and analysed by mass spectrometry. The ratio of peak intensities in the mass spectrum exactly indicates the abundance ratio for the two proteins. This approach can be used for analysis of membrane proteins of low abundance.

3.2.11.4.1 Growth assays in Minimum Essential Media (MEM) for SILAC method

Because SILAC is a quantitative comparative proteomic analysis, the cell densities and protein quantities are required to be similar in both cell groups to exclude any variations occurring due to variable cell densities. It was therefore important to assess the growth of the wild-type and mutants in the MEM prior to incorporating the stable isotopes into the normal protein synthesis of these strains in large-scale cultures for the isolation of the isotope-labelled membrane proteins in the wild-type and mutants. This is to ensure that all strains grow with similar cell densities in the same volumes of cultures. The growth of mutants has already been assessed in complex media such as MHS and BHI (section 3.2.5), and the mutants have shown to have slight growth defects compared to the wild-type. However, their overnight growths were similar to that of the wild-type. Here, the MEM does not contain carbon sources that can be utilised by *Campylobacter*. Although the MEM contains glucose, *Campylobacter jejuni* has been known to be unable to utilise external sugars because it lacks the key glycolytic enzyme phosphofructokinase (Kelly, 2001, Parkhill *et al.*, 2000). Therefore, the growth of the wild-type and mutants in MEM was supplemented by 10 mM L-serine and 10 mM L-aspartate as carbon sources. Also, the media was supplemented with ordinary 'light' L-arginine (0.4 mM) and L-lysine (0.8 mM). Cultures were incubated in microaerobic

conditions at 37 °C for 24 hours and samples were collected from 0 – 8 hours and a final sample was collected at 24 hour. Interestingly, the *Δcj1289* and the *Δcj0694* mutants showed similar growth to that of the wild-type (Figure 3.17), although with slight defects which can be credited to the MEM, which can also explain that the growth of all strains was less than that reported in complex media (OD_{600} ~ 0.5).

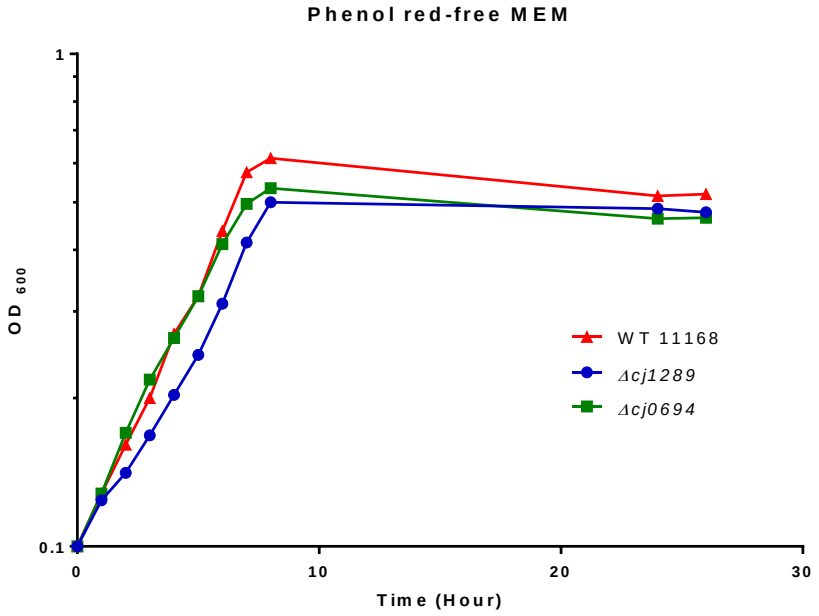


Figure 3.17 Growth of the wild-type *C. jejuni* 11168 strain, the *Δcj1289* and the *Δcj0694* mutants in Minimum Essential Media. Microaerobic growth was performed in 50 mL Minimum Essential Media supplemented with 10 mM L-serine, 10 mM L-aspartic acid, 50 µM ammonium ferric sulphate, 0.4 mM L-arginine and 0.8 mM L-lysine. The media was contained in 250 ml conical flasks shaken at 100 rpm in a gas atmosphere of 10 % *(v/v)* oxygen, 5 % *(v/v)* carbon dioxide and 85 % *(v/v)* nitrogen. Cells were inoculated at an OD_{600} of 0.1. Samples were collected at time 0 – 8 hours and a final sample at 24 hours. Cell densities were measured spectrophotometrically at OD_{600}.

3.2.11.4.2 Metabolic labelling with stable isotopes

Overnight cultures of the wild-type and mutants cells grown in MHS media were adjusted to an OD_{600} of 0.1 and inoculated into MEM supplemented with exactly same components except that the wild-type culture contained the 'heavy' (^{13}C + ^{15}N) L-arginine (0.4 mM) and the mutants contained the 'light' (^{12}C + ^{14}N) L-arginine (0.4 mM). It was found from the growth experiment in the MEM that the optimum time for cells to achieve highest density (late log phase) was 8 – 12 hours. Therefore, cultures were allowed to grow in MEM for 12 hours prior to membrane fractionation. At time 12 hours, cell densities of the wild-type and mutants were measured and confirmed to be equal, and cells were mixed, harvested and membrane fractions were prepared as described in section 2.7.3 and 2.7.4. In collaboration with Dr Francis Mulholland (IFR), membrane fractions were run on SDS-PAGE, trypsin digested and analysed by mass spectroscopy in order to identify client proteins that may be handled by Cj1289 or Cj0694 in the periplasm. Interestingly, MS data revealed that some OMPs were found to be less abundant in the OMs of the *Δcj1289* and *Δcj0694* mutants compared to the wild-type. The data are shown in the Appendix, in which the abundance ratio of the heavy (H) to light (L) labelled proteins is indicated in the column named as (Ratio H/L normalised). The higher the number, the more present in the wild-type, as that was used as the heavy label. Conversely the lower the number the more present in the mutant. It can be clearly observed that several OMPs are present in higher abundance in the wild-type than mutants. The proteins that have a heavy to light (H/L) ratio greater than 1 are less abundant in the mutants compared to the wild-type. These proteins could be chaperone clients.

Table 3.2 shows the key proteins which are differentially expressed in the *Δcj1289* and *Δcj0694* mutants. The CBF1 (PEB1) was found to be less abundant in the OMs of the *Δcj1289* and *Δcj0694* mutant (H/L ratio = 2-2.5). In addition, Cj0371, a membrane lipid-anchor lipoprotein was found in less abundance in the *Δcj1289* mutant (H/L ratio = 1.35), whereas the CBF1 (PEB1), Cj0755 (ferric enterobactin uptake receptor) and Cj0129c (BamA) were found to be less in the OM of the *Δcj0694* mutant (H/L ratios = 2.0554, 1.75 and 1.34, respectively). These findings strongly suggest a possible role of the Cj1289 and Cj0694 as periplasmic chaperones for the assembly of the OMPs in *C. jejuni*. Nevertheless, MS data also showed a presence of other contaminating proteins in

the OMs, which could be due to an insufficient incorporation of isotope-labelled proteins in mutants cells. Also it could be due to the instability of the OMs of the mutants, which may have prevented the proper cell fractionation.

Table 3.2 The key proteins that are differentially expressed in the outer membrane of the chaperone mutants.

Mutant	Gene number	Protein name	Ratio H/L normalised
Δcj1289	*cj0921c*	Major cell-binding factor (CBF1) (PEB1)	2.6865
	cj0371	UPF0323 lipoprotein Cj0371	1.3491
	cj0113	Peptidoglycan associated protein (Omp18)	1.1806
Δcj0694	*cj0921c*	Major cell-binding factor (CBF1) (PEB1)	2.0554
	Cj0755	Ferric enterobactin uptake receptor	1.7470
	Cj0129c	Outer membrane protein assembly factor (BamA)	1.3362

3.3 Discussion

3.3.1 Single mutation in *cj1289* or *cj0694* results in a defective growth of *C. jejuni* NCTC11168 strain and increases sensitivity to SDS, but is more severe in double mutant

The outer membrane proteins are one of the most important virulence factors known in *C. jejuni* that act in adhesion and colonisation of *C. jejuni* to host epithelial cells. One of the key features of *C. jejuni* that enable us to understand the mechanism by which OMPs are translocated through the periplasm and correctly inserted into the OM is the role of periplasmic chaperones in *C. jejuni*. Two periplasmic proteins have been suggested to exhibit chaperone activity that may handle and transport OMPs in the periplasm in *C. jejuni* known as Cj1289 and Cj0694. Previous work has revealed structural similarity of Cj1289 to the periplasmic chaperone SurA, and weak sequence similarity between Cj0694 and the inner membrane anchored protein PpiD in *E. coli* (Kale *et al.*, 2011). Single mutants of *cj1289* and *cj0694* were successfully generated by deletion of the respected gene and replacement by an antibiotic resistance cassette. It was suggested that deletion of *cj0694* may be lethal in *C. jejuni* (Kale *et al.*, 2011). This hypothesis relied on (*i*) the fact that *cj0694* shares the same promoter region with the downstream cell division gene *ftsA,* and are co-transcribed as a single mRNA, and (*ii*) the overlapping between the 3′ of *cj0694* at position 652,533 and the 5′ of *ftsA* at position 652,530. This hypothesis was disputed by Stahl and Stintzi, (2011), who confirmed that *ftsA* and *ftsZ* are among non-essential genes for cell survival, even though they are required as essential genes for cell division in other bacteria. Moreover, a viable *ppiD* deletion mutant was obtained in *E. coli*, suggesting a possible success in deleting *cj0694* as they have sequence similarities. Therefore, based on the fact that deletion of *ppiD* resulted in a viable mutant and that the co-transcription of *cj0694* with *ftsA* and overlapping region do not preclude the deletion of *cj0694*, a *cj0694* deletion mutant was successfully generated. Single mutants were constructed by allelic replacement of *cj1289* and *cj0694* by (cat^R) and (kan^R), respectively. The use of different antibiotic resistance cassettes allowed the generation of a double mutant strain that contains both antibiotic resistance cassettes replacing both genes. Complementation of deleted *cj1289* was successfully performed by inserting the wild-type copy of the gene into the pseudogene locus *cj0046.* This insertion was generated to restore the

phenotype of the mutant, and to ensure that the resulting effect is due to the deletion of the respected gene. In the complementation of the *Δcj1289* mutant, there is no possibility of polar effect because the *cj1289* gene is encoded in one DNA strand and the downstream gene is encoded in the other strand. This means that the *cj1289* gene has its own promoter that drives the expression of the gene. Therefore, mutation in the *cj1289* gene is an independent transcriptional unit and does not affect the transcription of the downstream gene. With regards to the complementation of the *cj0694* gene it is important to note that the downstream gene is co-transcribed with the *cj0694*. Therefore, there is a possibility of polar effect from the downstream gene, and we cannot rule out that the resulting mutant phenotype is not solely due to the deletion of the *cj0694* gene, but it might be due to mutation in the downstream gene *ftsA*. It would be highly valuable to obtain a complemented *Δcj0694/Δcj0694*$^{+}$ strain and examine whether the integration of the wild-type gene into the chromosome will restore the phenotype, and the phenotype is due to mutation in *cj0694* and not a secondary mutation. However, if this is not possible, an alternative approach can be used to ensure that the resulting phenotype is not due to polar effect of the downstream gene. RT-PCR can be used to examine whether mRNA can be made from the downstream gene in the mutant. If mRNA cannot be made, then the expression of the *cj0694* may be affected by the expression of the downstream gene. Other experiment that can be performed is enzyme activity assay, in which the activity of the protein produced from the downstream gene is measured in the mutant. If the activity of this protein does not change between the wild-type and the mutant, then the mutation of the *cj0694* gene does not affect the expression of its downstream gene. It would be highly beneficial to generate different combinations of double mutants such as *Δcj1289/Δpeb4* and *Δcj0694/Δpeb4* to analyse the effect of deletion of PEB4 with other candidate periplasmic chaperones on the cell growth and sensitivity to antimicrobial agents. However, several attempts to generate double mutants containing *peb4* deletion were unsuccessful. A possible explanation of this finding is that these combinations which include deletion of *peb4* with other periplasmic chaperone cognate genes may be lethal, which means that Cj1289 functions as a rescuer chaperone in the absence of PEB4. Rizzitello *et al* (2001) found that a *surA/skp* double mutant in *E. coli* is synthetically lethal, and that they may be functionally redundant. Also, deletion of *surA* and *ppiD* was found to be lethal (Dartigalongue and Raina, 1998). However, the lethality of *surA/ppiD* had been later disputed (Justice *et al.*, 2005). In this study, unsuccessful generation of the

Δcj1289/Δpeb4 double mutants suggests that the PEB4 and Cj1289 are functionally related in the same mechanism. Therefore when one of these genes is deleted, the other gene may compensate this deletion, but deletion of both genes can be lethal.

The growth of *Δcj1289* and *Δcj0694* single mutants in microaerobic conditions was found to be poorer than the wild-type (2.5 hours for the *Δcj1289* mutant and 3 hours for the *Δcj0694* mutant) in Muller-Hinton media. Interestingly, the growth retardation was more severe in the *Δcj1289/Δcj0694* double mutant. The possible explanation of this severity might be that both chaperones function in the same pathway, and one chaperone can compensate for the deletion of the other chaperone. However, the defective growth of the *Δcj0694* mutant might be also affected by the mutation of its downstream gene *ftsA* which share the same promoter. This cannot be confirmed unless we obtain data from its complemented strain. Similar phenomenon was seen in *E. coli* where elevated levels of PpiD production was found to serve as a rescuer for *surA skp* cells from lethality by increasing the folding stress in the cell envelope as a result of loss of periplasmic chaperone activity (Matern *et al.*, 2010). Although PpiD exhibits a rescue role in the network of periplasmic chaperones, it has no major role in the maturation of OMPs and cannot compensate for the lack of SurA in the periplasm (Matern *et al.*, 2010). Others favoured the possibility that both Skp and SurA function in the same pathway, where Skp may work as a holding chaperone to prevent aggregation of OMPs in the periplasm and SurA works as a folding chaperone (Bos *et al.*, 2007). Healthy growth of *C. jejuni* requires the ability of the cell to divide in a maintained manner, and because many proteins required for cell division are located in the membrane surface of microorganisms, it was inferred that the Cj1289 and Cj0694 can be directly or indirectly involved in the maturation of surface proteins, and therefore, deletion of one of their cognate genes results in growth defect of the microorganism.

3.3.2 Increased susceptibility to killing by detergents and cationic antimicrobial peptides displayed by chaperone mutants

The maintenance of membrane integrity has a major impact on the bacterial defence mechanism against external factors, and serves in increasing the resistance mechanism to detergents (such as SDS) and antimicrobial agents such as antimicrobial peptides

(AMPs). Disc diffusion assay has been widely used to determine the sensitivity of microorganisms to different antimicrobial agents (Douglas *et al.*, 2006, Gabhainn *et al.*, 2004). Here, we determined the sensitivity of the *Δcj1289* and *Δcj0694* mutant cells to SDS which was applied to a disc in the centre of MH agar containing mutant cells. It was found that deletion of the *cj1289* or *cj0694* gene results in a more sensitive strain to SDS as demonstrated by disc diffusion assay. These phenotypic alterations may be a result of increased outer membrane permeability allowing an uncontrolled entry of SDS which became lethal at higher concentrations. The more severe effect of *cj1289* and *cj0694* deletion in double mutant in sensitivity to SDS was similar to that seen in the growth assay indicating a more related role of both chaperones in the OM assembly.

Because all bacterial cell membranes are negatively charged, cationic antimicrobial peptides (CAMPs) can kill most Gram-positive and Gram-negative strains by the formation of carpet-like coating over the bacterial membrane causing disintegration of the membrane and consequent bacterial death (Tjabringa *et al.*, 2005). *C. jejuni* is known to be highly resistant to polymyxin B, an antimicrobial peptide usually added to growth media to select for *C. jejuni* (Tjabringa *et al.*, 2005). Lin *et al*., (2009) have identified seven genes responsible for this resistance. These are involved in synthesis of cell-surface carbohydrates, modification of intracellular targets, signal transduction and modulation of transmembrane potential (Lin *et al.*, 2009). Among these genes is the *galU* gene which is responsible for the production of uridine diphosphate glucose (UDP-glucose), an essential product for carbohydrate synthesis. Other well-known CAMPs are cathelicidins, small cationic peptides that possess broad-spectrum antimicrobial activities, also known as 'natural antibiotics' (Nizet and Gallo, 2003). The 18 kDa human cathelicidin, also known as human cationic antimicrobial protein (hCAP-18), is expressed by neutrophils and epithelial cells. The major cleavage product of hCAP-18 is the *C*-terminal region, LL-37 (Nizet and Gallo, 2003), which is required at low concentrations to kill *Campylobacter* species (0.6 – 2.5 µM). Sochacki *et al*., (2011) imaged the attack of LL-37 on a single *E. coli* cell in real time to demonstrate the distribution of LL-37 on the bacterial cell surface membrane, and to measure the time required by LL-37 to enter the periplasmic space of the bacterial cell to cause halting in growth (Sochacki *et al.*, 2011) (Reviewed in (Cederlund *et al.*, 2011)). Testing the efficacy of bacterial cell membrane and its components to stand against antimicrobial agents has been widely used in antimicrobial research, and has been known to provide a

better understanding about how microorganisms can evolve biological mechanisms to resist the surrounding environments. A study carried out by van Mourik *et al*., (2010) tested the effect of polymyxin B, colistin and LL-37 on the modification of lipid A of bacterial lipo-poly saccharide (LPS). They found two genes; namely *gnnA* and *gnnB* that enable addition of an *N*-linked acyl chain instead of an *O*-linked acyl chain to lipid A backbone. *gnnA* and *gnnB* mutants were more susceptible to killing by these AMPs than the isogenic parent wild-type (van Mourik *et al.*, 2010). However, LPS is not the sole direct binding target for CAMPs as primary studies on action mechanisms of CAMPs on bacterial membrane revealed. Chang *et al*., (2011) identified an outer membrane lipoprotein Lpp, which works as a cell-surface receptor for cationic AMPs in *Enterobacteriacae* and *V. cholerae*, and was identified to be responsible for susceptibility to cationic α-helical AMPs. Previously, they identified another outer membrane protein known as OprI, responsible for susceptibility of *Pseudomonas aeruginosa* to hRNase 7, a lysine-enriched cationic protein derived from human skin (Lin *et al.*, 2010).

Our major goal was to investigate the effect of the mutagenesis of the periplasmic chaperone genes on the biological properties of the outer membrane that maintain its role as a protective barrier for the cell. We could demonstrate that deletion of one or more genes encoding for the production of the periplasmic proteins Cj1289, Cj0694 or PEB4 increases the susceptibility to killing by AMPs. Three AMPs were used to assess the integrity of OMs of all mutants. These include polymyxin B, Polymyxin E (also known as colistin) and protamine sulphate. Our findings indicate that the chaperone mutants were more sensitive to all CAMPs than the wild-type *C. jejuni*. Figure 3.14 summarises the most significant changes in sensitivity of chaperone mutants to CAMPs compared to the parent wild-type. The Δ*cj1289* mutant was found to be the most susceptible strain to killing by all tested CAMPs. This strongly suggests a direct role of Cj1289 in OM assembly and maintenance of its barrier function against external antimicrobial agents. Although Kale *et* al., (2011) revealed a strong structural similarity of Cj1289 to the *E. coli* periplasmic chaperone SurA, and found that it is structurally more similar than PEB4, they found that Cj1289 could not demonstrate chaperone refolding activity by assessing the refolding rate of a model substrate, rhodanese. Thus, suggesting that Cj1289 possibly has a strong substrate specificity associated to its physiological role in the periplasm of *C. jejuni*. Given the fact that the Cj1289 is similar

to SurA in *E. coli*, it would be highly interesting to compare the antimicrobial peptides sensitivity of the Cj1289 to that of the SurA. However, it is hard to do such a comparison as it seems that no previous works have been published with regards to SurA sensitivity to CAMPs. The substrate specificity of Cj1289 can be more investigated by intensive proteomic analysis of the OM and periplasm of the mutant to identify the exact client OMPs of Cj1289. In contrast to the Δ*cj1289* mutant, our findings indicate that the CAMPs do not increase the susceptibility of the Δ*cj0694* mutant to killing, and its viability was not affected by increasing concentrations of peptides. From the findings of growth assays and SDS sensitivity (sections 3.2.5 and 3.2.6, respectively) that show growth inhibition and increased sensitivity to SDS by the Δ*cj0694* mutant, we suggested that Cj0694 may have an indirect role in OM assembly.

This study is the first study to examine the sensitivity of the Δ*peb4* mutant to CAMPs. Interestingly, the Δ*peb4* mutant was only significantly sensitive to polymyxin B ($p < 0.0001$), whereas the number of its viable cells was similar to the wild-type in the presence of 50 µg mL^{-1} protamine sulphate and 100 µg mL^{-1} polymyxin E. *C. jejuni* is normally resistant to Polymyxin B. Therefore, mutation in *peb4* gene might indicate a severe alteration in the composition of the OM that changes its properties to be severely sensitive to an antimicrobial peptide that *C. jejuni* is normally resistant to. The downstream gene of *peb4* is the *fba* gene, which encodes for fructose biphosphate aldolase (Fba); an important metabolic enzyme that catalyses the reversible aldole condensation of glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) to fructose-1,6-bisphosphate (FBP). It was shown previously that mutation of *peb4* gene does not negatively alter or abolish the expression of *fba* (Phansopa C, PhD thesis). Therefore, the resuting phenotype is credited to the deletion of the *peb4* gene and is not due to polar effect of its downstream gene *fba*. Our findings are consistent with previous studies that confirmed that the lack of PEB4 results in a defective growth, impaired motility, and reduced levels of invasion and colonisation as well as increased susceptibility to antimicrobial agents (Kale *et al.*, 2011, Rathbun *et al.*, 2009, Rathbun and Thompson, 2009).

Overall, we suggest that the increased membrane permeability in mutants due to uncompleted process of correct assembly of OMPs in the OM might be the main reason for the increased susceptibility to killing by SDS and CAMPs. This is because one or more periplasmic chaperones are non-functional in mutants.

3.3.3 The use of comparative proteomic analysis to determine the expression levels of the periplasmic chaperones client OMPs

Previous studies have demonstrated a direct correlation between the changes in OM protein expression levels and virulence. Asakura *et al*. (2007) have shown that a *peb4* mutant in *C. jejuni* strain NCTC 11168 shows less ability to adhere to INT407 cells than the wild-type, and lower level and duration of intestinal colonisation of mouse model, and was also unable to form biofilm. Moreover, the mutant cellular protein profile showed changes in the expression levels of several proteins compared to the wild-type strain. They found decreases in abundance of several outer membrane and periplasmic proteins in *peb4* mutant, including the major outer membrane protein (MOMP), porins (OmpA, Omp50), the haemin outer membrane receptor (CirA), the Cysteine binding protein (Cj0982) and the iron receptor (FepA) (Asakura *et al.*, 2007). In addition to this study, Rathbun *et al*. (2009) found that a *peb4* mutant generated in a highly pathogenic strain 81-176 has a lower ability to colonise mice. Mass spectroscopic analysis revealed a decrease in the level of three OMPs, the MOMP, the fibronectin binding protein (CadF) and the Omp50 porin (Rathbun *et al.*, 2009, Rathbun and Thompson, 2009).

Our ultimate goals in the use of comparative proteomic analysis were to examine if the Cj1289 and Cj0694 periplasmic proteins are involved in OM assembly in *C. jejuni*, and to identify client proteins that may be handled by Cj1289 and Cj0694 in the periplasm. OM and periplasmic preparations from the wild-type and chaperone mutant strains were prepared and resolved by SDS-PAGE. Then, western blot was performed to check the purity levels of OM and periplasmic fractions before they had been resolved by 2D-PAGE for comparative analysis and mass spectroscopy identification. By using anti-MfrA antibody, we could be able to demonstrate that OM fractions were free from contaminating periplasmic proteins that may occur during the preparation procedure. Periplasmic sample of the wild-type was used as a positive control to show that the MfrA is localised in the periplasm. The western blot images confirmed that the OM fractions were free from the periplasmic protein MfrA. On the other hand, antibody raised against the cytoplasmic chaperone, GroEL was used to demonstrate that the periplasmic fractions were free from contaminating cytoplasmic proteins. A cell-free extract from the wild-type was used as a positive control to show that GroEL is localised in the cytoplasm, while periplasmic fractions were free from contaminating

cytoplasmic proteins. After assuring that the OMs and periplasms were pure and could be used for comparative proteomics, they were applied to 2D-PAGE analysis developed by Dr. Francis Mulholland at The Institute of Food Research (IFR), Norwich, UK. As demonstrated in section 3.2.11.3 an overall reduction in the expression level of the OMPs was observed in the OMs of all mutants. Mass spectrometric analysis was applied to these preparations in order to identify each protein spot that has been changed among the wild-type and the mutants fractions. These protein spots may represent the un-inserted proteins to the OMs due to the mutational effect. Three OMPs were found to be expressed in the OM of the wild-type and were absent in the OM of the Δ*cj1289* mutant. These proteins were identified by mass spectrometry as ATP synthase, formate dehydrogenase and serine protease (Cj1228c). The ATP synthase has been shown to be localised in the membrane surface of *E. coli* (Wilkens *et al.*, 2000), while the formate dehydrogenase is an outer membrane-bound protein but is facing the periplasm (Smart *et al.*, 2009). Mass spectrometry identification of these proteins suggests that when the Cj1289 is not expressed, it results in an inability of these OMPs to be translocated and inserted into the OM, suggesting an important chaperone role of the Cj1289 in the periplasm. Unexpectedly, other periplasmic proteins were found in the OM of the Δ*cj1289* mutant. These were present as low level contaminating proteins from the periplasm during cell fractionation. These proteins were identified by MS as TolB and a putative periplasmic protein Cj0964. TolB, which was first suggested to be found in two forms, a large 47.5 kDa membrane associated protein and a small 43 kDa periplasmic protein (Levengood and Webster, 1989). However, it was later found that the mature form of the protein (the small protein) is localised in the periplasm and that the large TolB was its precursor which was observed only when the protein was over-expressed (Isnard *et al.*, 1994). On the other hand, overlaying the 2D-gels of the wild-type and the Δ*cj0694* mutant revealed that two OMPs were not expressed in the OM of the mutant. These were Ni-Fe hydrogenase and serine protease. Interestingly, in the OM of the Δ*cj1289/*Δ*cj0694* double mutant, two OMPs were not expressed in the OM, and were similar to those in the Δ*cj1289* single mutant. These were ATP synthase and serine protease. From these findings it can be noticed that serine protease is the only protein which is consistently not expressed in all mutants, whereas in the Δ*cj1289* mutant and the Δ*cj1289*/Δ*cj10694* double mutant ATP synthase is also not expressed. This can suggest that deletion of the *cj0694* gene does not alter the membrane profile. These findings are consistent with the data obtained from the sensitivity assays of the mutants

to detergents and antimicrobial peptides. Thus, it can be concluded that the Cj1289 plays a major periplasmic role in the OM assembly and the maintenance of the permeability functions of the OM, whereas the Cj0694 role may be more general in the periplasm and may be indirectly involved in the OM assembly.

Data obtained from mass spectrometry revealed low levels of contamination by periplasmic proteins in the OM samples, although the purity of the OMs was checked by western blot. In addition to the contaminating background occurred in the 2D-gels, the difficulty of normalising the loading of OM and periplasmic fractions from wild-type and mutants on gel affects the quality of the data obtained from the proteomic analysis using the 2D-PAGE technique. Therefore, it was not possible to conclude with high confidence that the relative abundance of proteins in the outer membranes or periplasms, as shown by size and intensity of the protein spots was entirely due to *cj1289* or *cj0694* deletion rather than the effect of an other factor such as unequal loading of protein samples across multiple gels. Thus, it has been decided to use a quantitative proteomic analysis such as Stable Isotope Labelling by Amino acids in Cell culture (SILAC) developed by Ong *et al*., (2002).

Interestingly, from the MS data it can be clearly shown that the Cj1289 and Cj0694 are implicated in some way in the OM biogenesis as described by the lower abundance level of some OMPs in their mutants compared to the wild-type. These possible client proteins might be handled by one of the periplasmic chaperones and mutation in one of their cognate genes resulted in an incomplete delivery to the OM. As far as possible client proteins are identified, a further verification step can be performed to ensure the dependence of these OMPs on periplasmic chaperones. Marker antibodies can be raised against one or more of these OMPs and western blot can be applied to the OM of each mutant. Therefore, if there is an actual dependence of these OMPs on periplasmic chaperones, then the OMs of the *Δcj1289* and *Δcj0694* should be deficient in these OMPs.

The presence of cytoplasmic and periplasmic proteins in the OM fractions could be due to the increased instability of the OM during cell fractionation, which in turn could be a consequence of the deletion of periplasmic chaperones due to mutation in their cognate genes. Another possible explanation for this contamination can be the low incorporation level of the labelled L-arginine during protein synthesis. Moreover, the high sensitivity

of the mass spectrometry could detect proteins in very low abundance which cannot be detected by visualization of proteins in 2D-gels or by using marker antibodies. Therefore, this high sensitivity could be another reason in the presence of contaminating proteins in the MS data.

3.3.4 The nature of BAM complex in *C. jejuni*

A few studies have focused on the assembly machinery required for the folding of β-barrel OMPs into their final location in the outer membrane of Gram-negative bacteria, mainly in *E. coli* and *Neisseria meningitidis* (Bos *et al.*, 2007c). BamA is a key player in the BAM complex (as discussed in section 1.7.1) and is a large multi-domain OMP that possesses five repeat domains which interact with client proteins by recognising the *C*-terminal motif in OMPs that contains a *C*-terminal Phe (Bos *et al.*, 2007c). The fact that the *E. coli* BamA and BamD are essential for cell viability (Onufryk *et al.*, 2005) and the lack of homologues of other Bam protein in *C. jejuni* suggests that BamAD is the minimal complex required for the assembly of OM, and also suggests that the BAM complex may contain novel proteins in *C. jejuni*. Therefore, we aimed to investigate the role of the outer membrane-integrated BamA (Cj0129) and BamD (Cj1047c) in the assembly and insertion of OMPs in the OM of *C. jejuni,* in order to obtain a better understanding of the mechanism by which OMPs are correctly assembled and localised in the OM. The original experimental objectives were to detect proteins that interact with BamA and BamD. This would involve cloning, over-expression of BamA and BamD, and polyclonal antibody production for immune-precipitation of *C. jejuni* OM extracts, in order to determine the biochemical properties of BamA and BamD in catalysing the correct assembly and insertion of OMPs in the OM of *C. jejuni*. The vector pNJ077 (Figure 3.18) was obtained as a generous gift from Dr Neil Oldfield (Molecular Bacteriology and Immunology Group, The University of Nottingham, Nottingham, UK). This contains the entire coding sequence of BamA protein excluding the *N*-terminal signal sequence inserted into the *BamH*I restriction site of the pQE-70 vector (Qiagen). Despite several attempts to optimise the expression conditions, such as monitoring the expression at different temperatures and times, all attempts resulted in only insoluble form of the protein. BamA protein is known to have a *C*-terminal integral OM β-barrel part (Gentle *et al.*, 2005, Knowles *et al.*, 2009, Robert *et al.*, 2006,

Sanchez-Pulido *et al.*, 2003). The membrane integrated state of this protein might be the most likely reason for not being easily produced as a soluble protein. Although it can be possible to solubilise the insoluble form of BamA by generic procedures by denaturing and refolding the protein, the resulting purified protein may be inactive, and therefore, is not appropriate to utilise in biochemical characterisation. In view of this, no further work on BamA was performed.

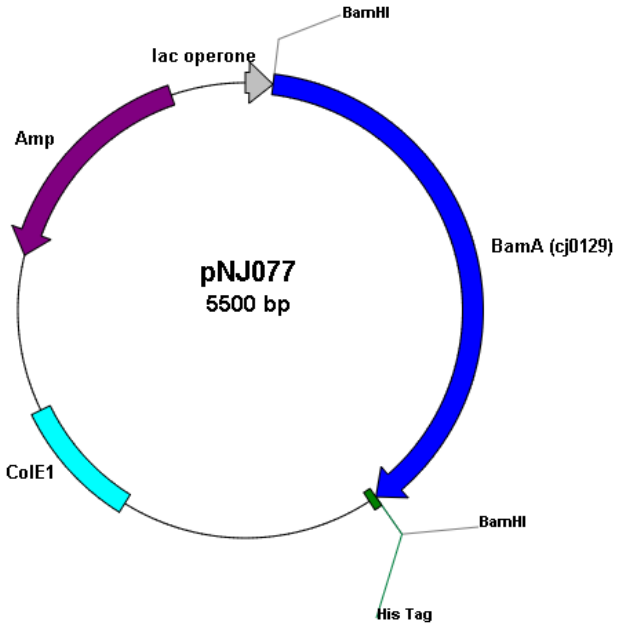


Figure 3.18 Construction of pNJ077 plasmid for over-expression. The pQE-70 over-expression vector was utilised for IPTG inducible production of protein under the control of the T7 promoter. **A)** The plasmid map of pNJ077. The entire coding region of BamA protein minus the *N*-terminal signal sequence was cloned into the *BamH*I restriction site of the pQE-70 vector (Qiagen). The *C*-terminal 6X histidine tag in the vector was utilised to aid in the purification.

4 Biochemical characterisation of the putative chaperone Cj0694 in *Campylobacter jejuni*

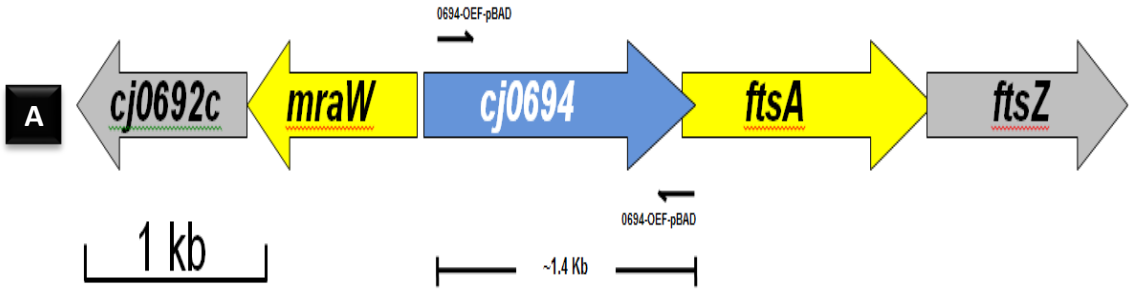
4.1 Introduction

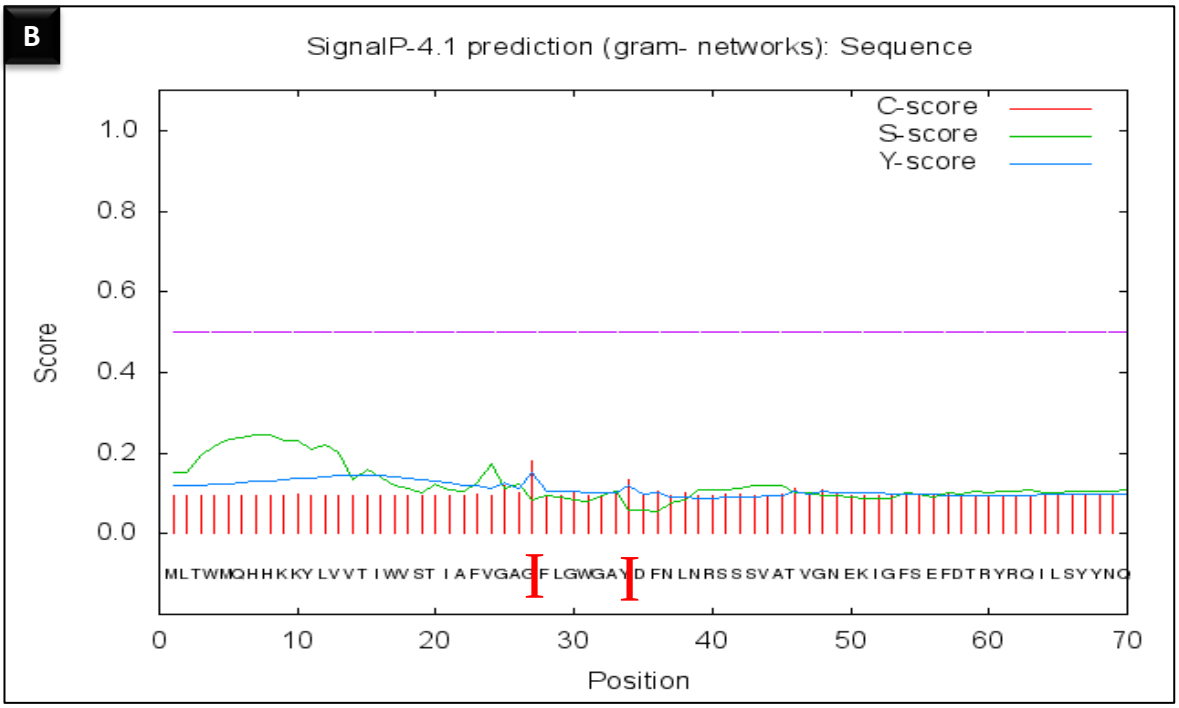
The structure and biochemical characteristics of the periplasmic facing PPIase in *E. coli*, PpiD have been studied since more than a decade (Dartigalongue and Raina, 1998). It has been shown to have a membrane-anchored domain and three periplasmic domains. The first and third domain (residues 35 – 263 and 358 – 623 respectively) are proposed to be chaperone domains, while the middle domain (residues 264 – 357) is a parvulin-like domain (Stymest and Klappa, 2008). Despite the fact that PpiD is structurally similar to the first parvulin-like domain in SurA (Weininger *et al.*, 2010), it has been shown to be catalytically inactive (Weininger *et al.*, 2010). This might be because the first PPIase domain of the SurA is known to be inactive (Behrens *et al.*, 2001). In *C. jejuni*, homology searches lead to the identification of Cj0694, a SurA-like protein that shows weak sequence similarity to the PpiD of *E. coli* (Kale *et al.*, 2011). It was interesting to know whether Cj0694 is structurally similar to the PpiD, however, Kale *et al.* (2011) could not obtain a soluble form of recombinant Cj0694. Here, it was aimed to study the structural and biochemical function of Cj0694 by cloning and over-production of Cj0694 using modified methods and expression vectors to obtain a soluble form of the protein for structural analysis. It was also aimed to study the catalytic activity of Cj0694 by examining its chaperone and PPIase activities, and comparing them to those of the PpiD in *E. coli*. Bringing these findings together with those of the phenotypic characteristics of the *Δcj0694* mutant (discussed in chapter 3) would be beneficial for identification of the biological role of Cj0694 in the translocation and maturation of OMPs and periplasmic proteins in *C. jejuni*.

4.2 Results

4.2.1 Generation of pBAD*0694* plasmid for over-expression

The coding sequence of *cj0694* excluding the proposed signal sequence (residues 1 – 34) (Figure 4.1A and B) was PCR amplified from *C. jejuni* NCTC11168 genomic DNA using the primers 0694-OEF-pBAD and 0694-OER-pBAD. The gene fragment was digested with *Xho*I and *EcoR*I restriction enzymes prior to cloning into the *Xho*I and *EcoR*I restriction sites of the pBAD/His B expression vector which was digested by the same set of enzymes, in frame with the *N*-terminal hexahistidine tag (Figure 4.1C). The resulting pBAD*0694* expression vector was verified by automated DNA sequencing (Core Genomic Facility, University of Sheffield Medical School, UK) which showed the insert sequences were correct and in-frame with the His-tag (data not shown). In addition, colony PCR was performed to confirm the correct insertion of the *cj0694* DNA sequence into pBAD/His B vector using the primers 0694-OEF-pBAD and 0694-OER-pBAD (Figure 4.1D). pBAD*0694* was used to transform competent TOP10 cells. Small-scale over-expression trials were performed to determine the optimum over-expression duration after L-arabinose induction. This was achieved by growing TOP10 cells harbouring the over-expression plasmid pBAD*0694* in separate 50 mL LB cultures at 37 °C, in the presence of 50 µg mL^{-1} carbenicillin. After inducing the over-production of Cj0694 by 0.02 % L-arabinose, samples were collected at 1, 3, 5 and 24 hours, and applied to SDS-PAGE for visualisation and analysis (Figure 4.1E). The best over-production level of the ~ 58 kDa recombinant Cj0694 was achieved when cells were incubated at 37 °C, 5 hours after induction with 0.02 % L-arabinose.

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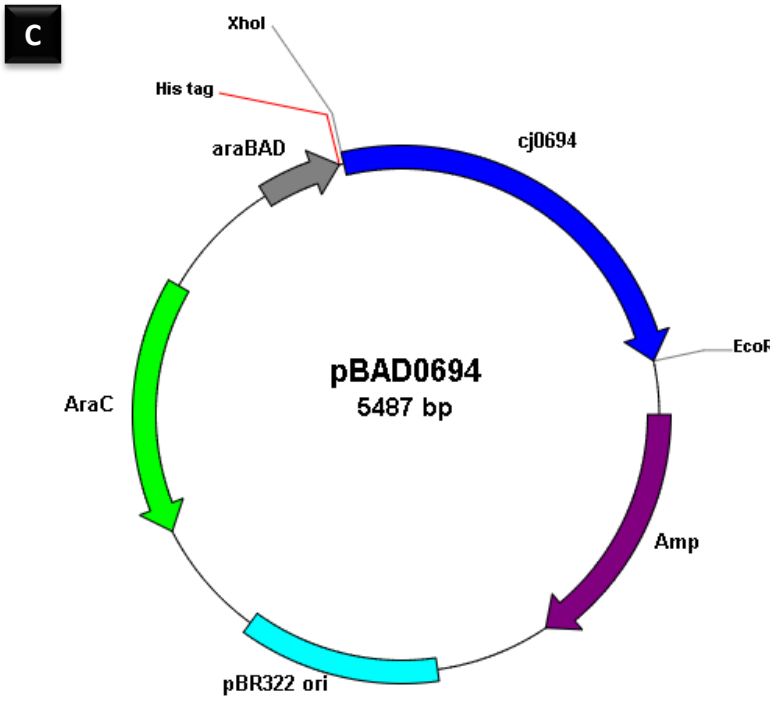
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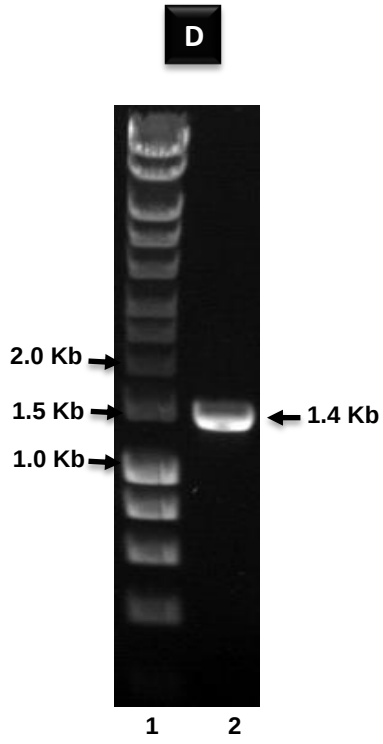
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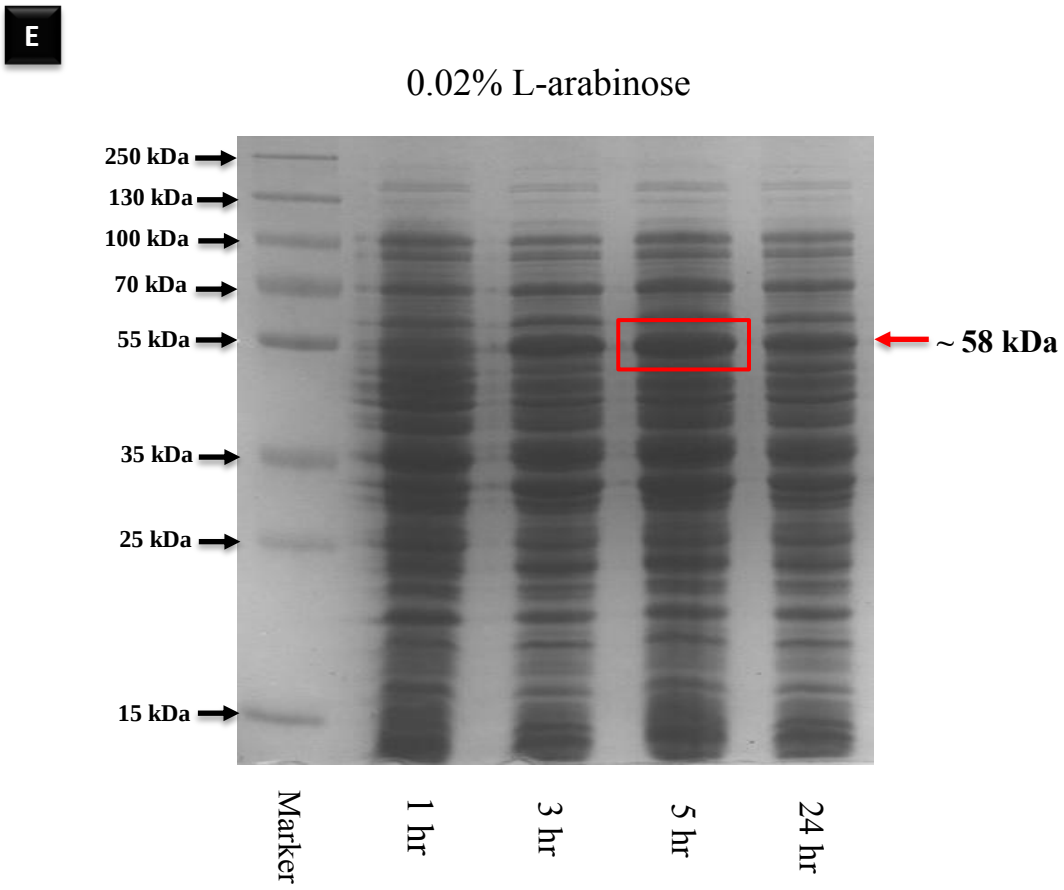


Figure 4.1 Generation of pBAD*0694* plasmid construct for over-production of Cj0694. A) The entire coding sequence of *cj0694* minus the possible signal peptide cleavage site was amplified using the primers 0694-OEF-pBAD and 0694-OER-pBAD from the genomic DNA of *C. jejuni* NCTC 11168. **B)** Primary amino acid sequence analysis shows the most likely signal peptidase I cleavage site of Cj0694 was not well-defined, but positions 27 and 34 in the amino acid sequence indicate the highest scores for raw cleavage site (C-score). Data obtained from SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). **C)** The plasmid map of pBAD*0694*. The full length *cj0694* minus the *N*-terminal signal sequence was cloned into the *Xho*I and *EcoR*I restriction sites of the pBAD/His B expression vector (Invitrogen). The *N*-terminal 6X histidine tag in the vector was utilised to aid in the purification. **D)** A 0.7 % agarose gel electrophoresis confirms successful insertion of *cj0694* into the pBAD/ His B over-expression vector to generate pBAD*0694* over-expression plasmid using the primers 0694-OEF-pBAD and 0694-OER-pBAD. Lane 1: Hyperladder™ I molecular weight marker (Bioline, UK). Lane 2: PCR product (~ 1.4 Kb) of the *cj0694* DNA sequence minus the *N*-terminal signal sequence amplified from pBAD*0694* plasmid DNA. **E)** 12.5 % SDS-PAGE shows over-expression trials for Cj0694. The *E.* coli TOP10 cells harbouring the pBAD*0694* were grown in (50 mL) LB media containing 50 µg mL^{-1} carbenicillin. After inducing the over-expression by 0.02 % L-arabinose, samples were collected at times 1, 3 5 and 24 hours and visualised by SDS-PAGE against PageRuler™ prestained protein ladder (Fermentas). The optimum over-expression level is shown in red box.

4.2.2 Over-production and purification of Cj0694

Cultures of TOP10 cells (4 L) transformed with pBAD*0694* were grown aerobically at 37 ºC in LB medium containing carbenicillin (50 µg mL^{-1}), and the production of Cj0694 was induced by 0.02 % L-arabinose after 5 hours of aerobic incubation with shaking at 250 rpm. The Cj0694 recombinant protein containing an *N*-terminal hexahistidine tag was purified by affinity chromatography using a Ni-NTA His-Trap™ column (GE Healthcare, UK). However, although this resulted in a considerable enrichment of Cj0694, the protein was clearly not pure (Figure 4.2A). Therefore, protein-containing fractions were pooled, dialysed overnight and buffer exchanged, and further purified by anion-exchange chromatography using a HiTrap™ DEAE FF column (GE Healthcare, UK). Samples taken from different purification fractions after gradient elution were then analysed by SDS-PAGE (Figure 4.2B). The production level of Cj0694 was constantly high, and the purified protein was stable at 4 °C. The purity of the recombinant protein was verified by Coomassie blue stain of polyacrylamide gel as shown in Figure 4.2A and B. The identity of Cj0694 was confirmed by *N*-terminal sequencing in collaboration with Dr. Arthur Moir at the Department of Molecular Biology and Biotechnology, The University of Sheffield. However, we could not demonstrate the actual *N*-terminal sequence obtained from the purified Cj0694 because it was lost. The purified Cj0694 was used in several biochemical experiments.

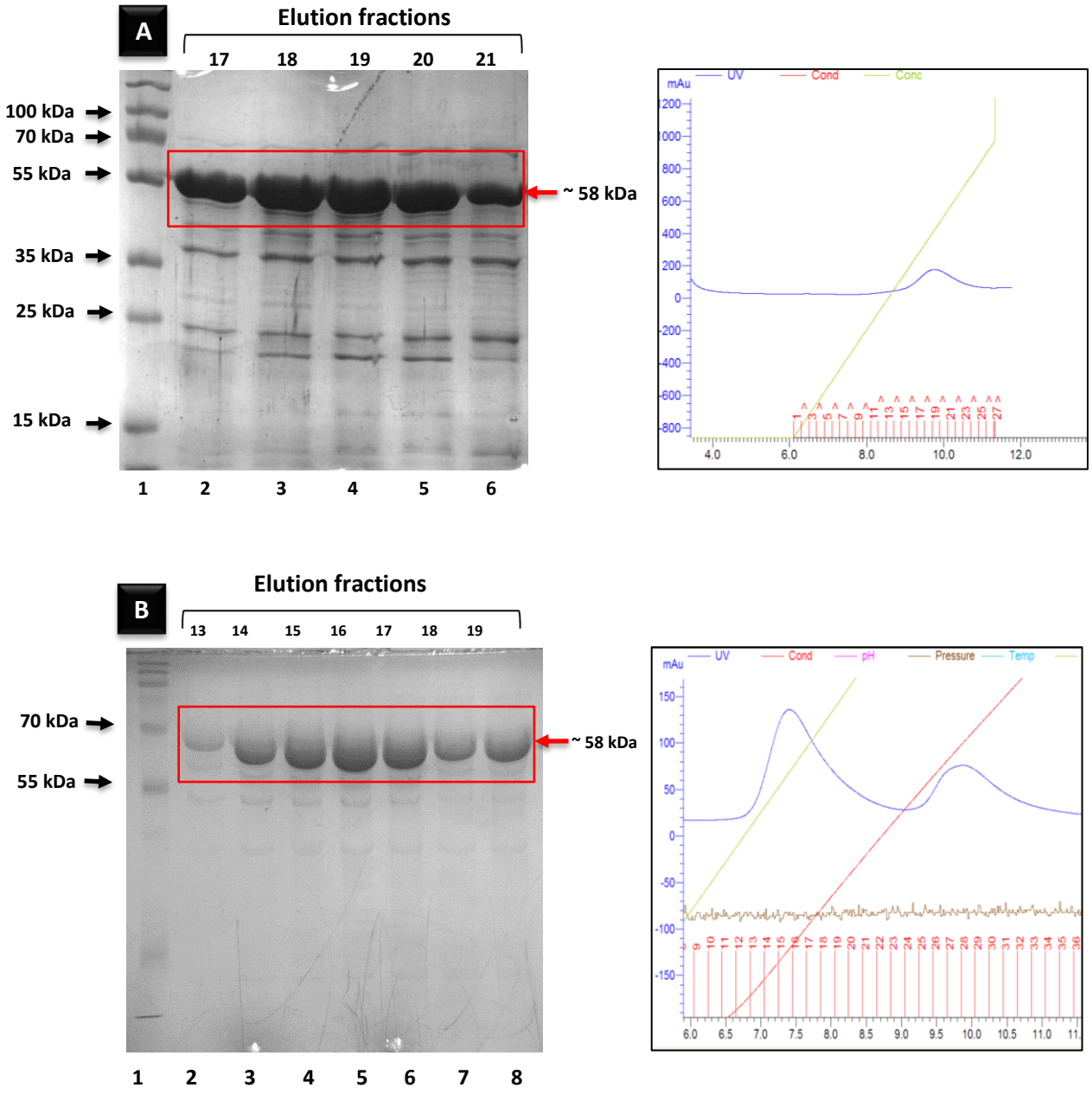


Figure 4.2 Purification of Cj0694 from pBAD*0694* over-expression plasmid. A) (Left) Overloaded 12.5 % SDS-PAGE showing Cj0694 fractions (lane 2 – 6) eluted following affinity chromatography using a Ni-NTA His-Trap™ column (GE Healthcare, UK). The ~ 58 kDa Cj0694 band is showed in the red box. The fainter bands are predicted to be contaminating proteins eluted in the same fractions. Lane 1 shows the PageRuler™ prestained protein ladder (Thermo). **(Right)** UV protein traces showing eluted protein fractions using gradient elution (20 – 500 mM imidazole) in 20 mM Tris-HCl pH 8.0, 500 mM NaCl elution buffer. **B) (Left)** additional SDS-PAGE showing Cj0694 fractions (lane 2 – 8) after been applied to anion-exchange chromatography using DEAE FF column (GE Healthcare, UK). The same sized Cj0694 is shown in red box with increased (~ 95 %) purity. Lane 1 shows the PageRuler™ prestained protein ladder (Thermo). **(Right)** UV protein traces showing eluted protein fractions using gradient elution (0 – 1 M NaCl) in 50 mM Tris-HCl pH 8.0, elution buffer.

4.2.3 Generation of pET*PEB4* plasmid for over-expression

PEB4 was shown to have a potential dual PPIase and chaperone function in the *C. jejuni* periplasm, as demonstrated by significant PEB4-dependent acceleration of refolding rate of ribonuclease T_1 in a PPIase assay, and that PEB reduced the yield of active protein in the refolding assay using a model protein substrate rhodanese (Kale *et al.*, 2011). To examine if the Cj0694 protein has a possible periplasmic PPIase role, PEB4 was cloned, over-expressed and purified in order to be used as a positive control in the PPIase assay. The entire coding sequence of *peb4* (*cj0596*) gene including the *N*-terminal signal sequence (Figure 4.3A and B) was PCR amplified from *C. jejuni* NCTC11168 genomic DNA using the primers PEB4-OEF and PEB4-OER. The gene fragment was digested with *Nde*I and *Xho*I restriction enzymes prior to cloning into the *Nde*I and *Xho*I restriction sites of the pET21a(+) expression vector which was digested by the same set of enzymes, in frame with the *C*-terminal hexahistidine tag to generate pET*PEB4* over-expression plasmid (Figure 4.3C). The resulting pET*PEB4* expression plasmid was verified by colony PCR to confirm the correct insertion of the *peb4* DNA sequence into pET21a(+) vector using the primers PEB4-OEF and PEB4-OER (data not shown). pET*PEB4* was used to transform competent BL21 (λDE3) cells, which were grown aerobically at 25 °C with shaking at 250 rpm under the induction of 1 mM IPTG, and was used for the production of PEB4.

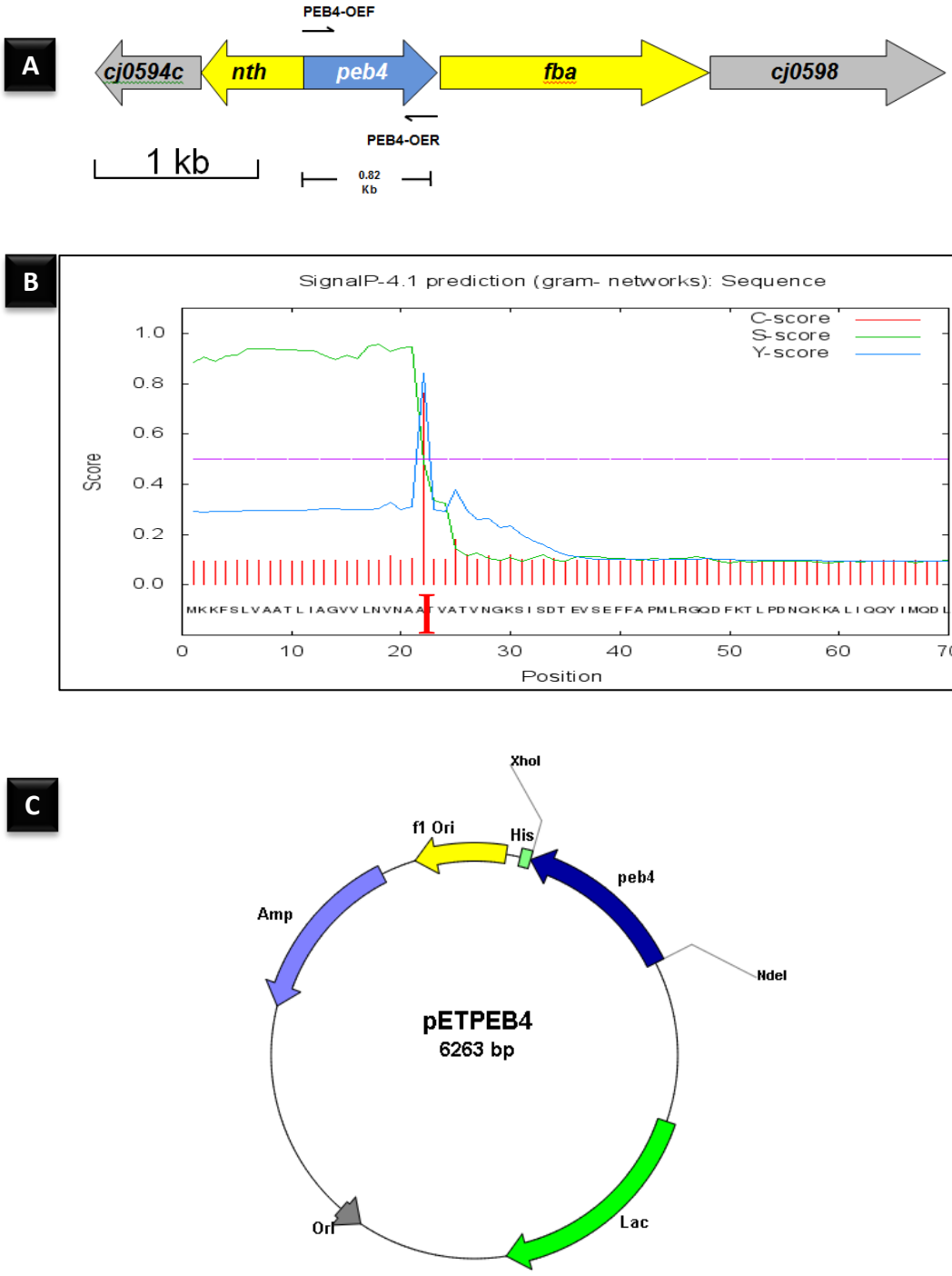


Figure 4.3 Generation of pET*PEB4* plasmid construct for over-production of PEB4. A) The entire coding sequence of *peb4* was amplified using the primers PEB-OEF and PEB-OER from the genomic DNA of *C. jejuni* NCTC 11168. **B)** Primary amino acid sequence analysis shows the signal peptidase I cleavage site of PEB4 between the amino acids at position 21 and 22 as indicated by the highest scores for raw cleavage site (C-score). Data obtained from SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). **C)** The plasmid map of pET*PEB4*. The full length *cj0596* including the *C*-terminal signal sequence was cloned into the *Nde*I and *Xho*I restriction sites of the pET21a(+) expression vector (Novagen). The *C*-terminal 6X histidine tag in the vector was utilised to aid in the purification.

4.2.4 Over-production and purification of PEB4

Cultures of BL21 (λDE3) cells (1 L) transformed with pET*PEB4* were grown aerobically at 37 ºC in LB medium containing carbenicillin (50 µg mL^{-1}), and the production of PEB4 was induced by 1.0 mM IPTG after 24 hours of aerobic incubation with shaking at 250 rpm at 25 °C. The PEB4 recombinant protein containing a *C*-terminal hexahistidine tag was purified by affinity chromatography using a Ni-NTA His-Trap™ column (GE Healthcare, UK). The PEB4 was judged to be pure by Coomassie blue staining of SDS-PAGE (Figure 4.4). The production level of PEB4 was regularly high, and the purified protein was stable at 4 °C.

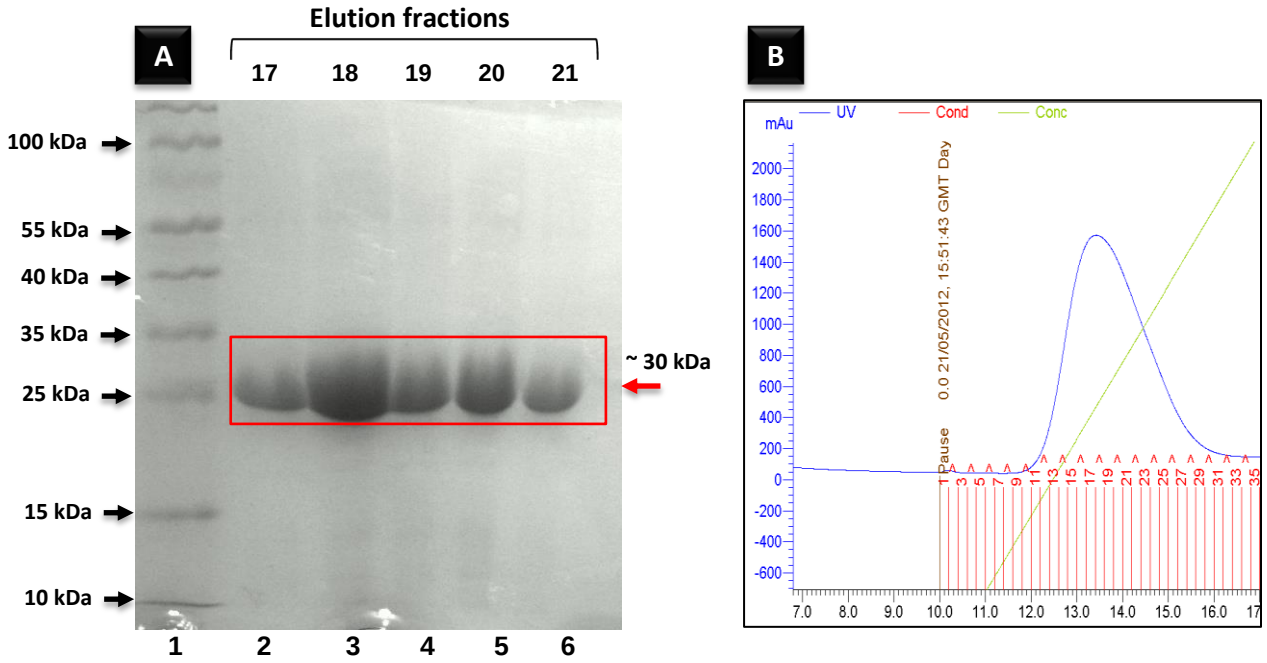


Figure 4.4 Purification of PEB4 from pET*PEB4* over-expression plasmid. A) Overloaded 12.5 % SDS-PAGE showing PEB4 fractions (lanes 2 – 6) eluted following affinity chromatography using a Ni-NTA His-Trap™ column (GE Healthcare, UK). The ~ 30.5 kDa PEB4 band is showed in the red box. Lane 1 shows PageRuler™ pre-stained protein marker (Thermo). **B)** UV protein traces showing eluted protein fractions using gradient elution (20 – 500 mM imidazole) in 20 mM Tris-HCl pH 8.0, 500 mM NaCl elution buffer.

4.2.5 Cj0694 accelerates the refolding kinetics of reduced and carboxymethylated ribonuclease T_1

Bioinformatics analysis revealed that Cj0694 is a homologue of PpiD in *E. coli* (Kale *et al.*, 2011). Since PpiD in *E. coli* has a parvulin domain [residues 227 to 357; (Dartigalongue and Raina, 1998)], this means that Cj0694 may have a parvulin domain similar to that of PpiD. In order to gain evidence for potential PPIase activity for Cj0694, the activity of the Cj0694 PPIase domain in accelerating the rate of proline isomerisation limited refolding of reduced and carboxymethylated ribonuclease T_1 (RCM-RNase T_1) was examined using a classical PPIase assay. The isomerisation of the two structural proline conformations; *cis* and *trans* may limit the refolding of proteins. This isomerisation can be catalysed by ubiquitous enzymes known as peptydyl-prolyl *cis*/*trans* isomerases (PPIases) which can accelerate slow steps in protein refolding that are limited by *cis*/*trans* isomerisations (Rudd *et al.*, 1995). Reducing and carboxymethylating the RNase T_1 enhance protein stability and make it suitable to examine the PPIase activity in protein unfolding (Mucke and Schmid, 1992). The two disulphide bonds in RNase T_1 (Cys2-CyslO and Cys6-CyslO3) are essential in maintaining its conformational stability (Oobatake *et al.*, 1979, Pace, 1990, Pace *et al.*, 1988). Therefore, breaking these bonds results in unfolding the protein under native conditions such as pH 8.0 and 15 °C. The RCM-RNaseT_1, like the native RNaseT_1, can be stabilised and becomes catalytically active in the presence of 2 M NaCl (Pace *et al.*, 1988). Thus, folding of this protein can be enhanced simply by increasing the concentration of NaCl. RNase T_1 has a single tryptophan side chain Trp^{59} which is located in a hydrophopic environment and is hidden from solvents (Moors *et al.*, 2009). Refolding of the RNase T_1 results in an accumulation of Trp residues which in turn can yield fluorescence that can be reported kinetically.

The PPIase activity of Cj0694 was demonstrated by monitoring the acceleration of the proline isomerisation limited refolding of reduced and carboxymethylated ribonuclease T_1, as reported by tryptophan fluorescence in the presence of 4 M NaCl. RNase T_1 was denatured in a solution contained 6 M guanidine-hydrochloride [GdnHCl] and 2 mM EDTA in 0.2 M Tris-HCl, pH 8.7 for 2 hours at 25 °C. The reduction was carried out in 20 mM dithiothreitol [DTT], 6 M GdnHCl, and 2 mM EDTA in 0.2 M Tris-HCl, pH 8.7 under argon at 25 °C. The carboxymethylation was carried out in 0.6 M iodoacetate in

0.2 M Tris-HCl, pH 7.5 in the dark for 5 minutes at 25 °C. This step was essential to 'cap' the cysteine residues and prevent the formation of two disulphide bonds during the denaturation process. Refolding was initiated by a 50-fold dilution of the unfolded protein (stored in the absence of NaCl) to a final concentration of 1.2 µM in a buffer containing 0.1 M sodium acetate, pH 5.0, and 4 M NaCl. Changes in the steady-state Trp59 fluorescence were measured at 320 nm with excitation at 268 nm for 15 minutes with the temperature maintained at 15 °C. The periplasmic protein PEB4 was used as a positive control for PPIase activity, which previously showed a marked acceleration of the refolding rate (Kale *et al.*, 2011). Cj0694 (0.25 µM and 0.5 µM) or PEB4 (0.5 µM) were added to the RCM-RNase T_1 prior to the dilution. The relative fluorescence (%) was plotted against time (15 min) to illustrate the maximum saturation (100 %) of refolding of RCM-RNase T_1 in the absence or presence of PPIases. Interestingly, a strong Cj0694-dependent acceleration of the RCM-RNase T_1 refolding rate was found as reported by tryptophan fluorescence (Figure 4.5).

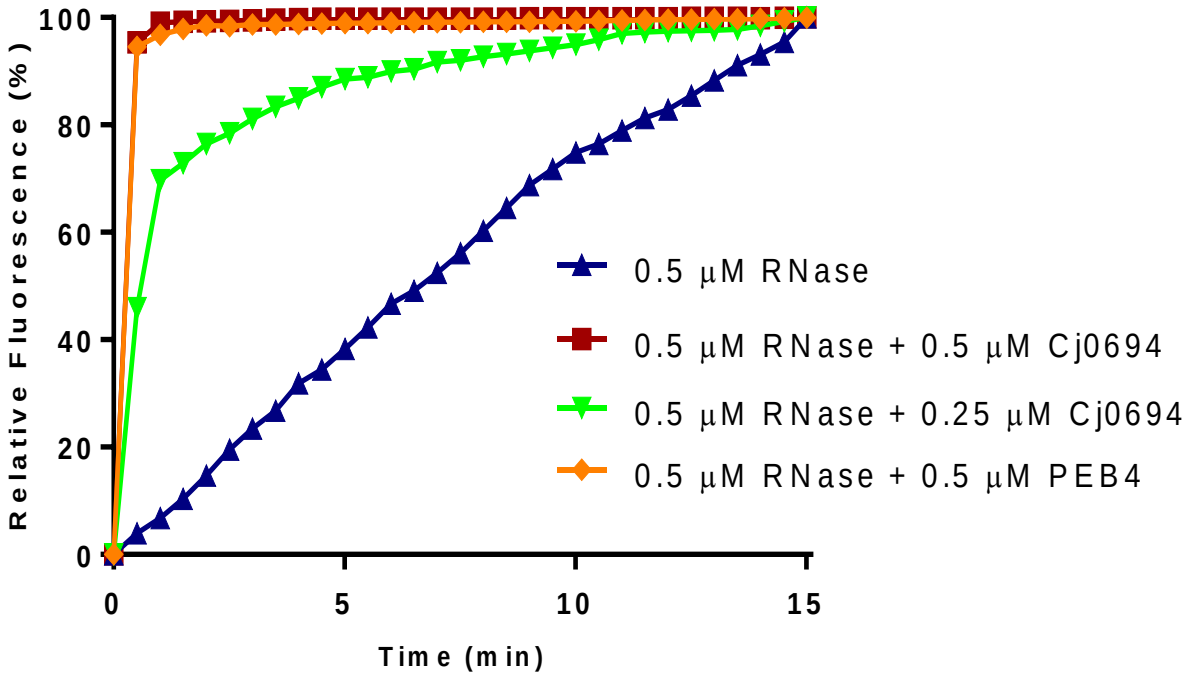
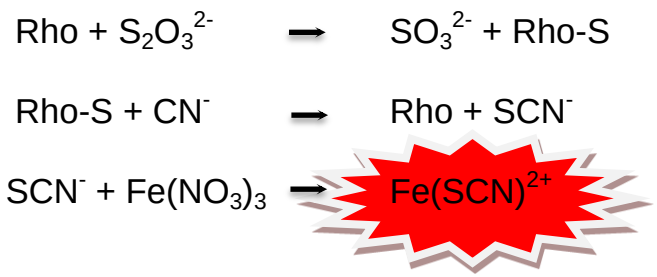


Figure 4.5 PPIase activity of Cj0694 as measured by the refolding rate of reduced and carboxymethylated ribonuclease T_1. Refolding kinetics of 0.5 µM RCM-RNase T_1 in the presence of 4 M NaCl was measured by change in the fluorescence at 320 nm after excitation at 268 nm. Denaturation, reduction and carboxymethylation of RNase T_1 was performed as described in 2.9.1. At time zero, refolding was initiated by a 50-fold dilution in the presence of 4 M NaCl. The change in the fluorescence was measured at 25 °C, and the data shown are representative of three independent experiments. Fluorescence changes show that increasing the concentration of Cj0694 accelerates the refolding of RNase T_1. PEB4 was used as a positive control for the acceleration of the refolding kinetics. Data were fitted by using Prism 6 (GraphPad Software Inc.).

4.2.6 Cj0694 does not inhibit the refolding of the chemically denatured rhodanese, but is capable of preventing aggregation

It has been shown by Matern *et al*, (2010) that PpiD possesses *in vitro* chaperone activity by having the capability to prevent the aggregation of thermally denatured citrate synthase. To examine if Cj0694 possesses *in vitro* chaperone activity, a model protein, rhodanese (a thiosulphate:cyanide sulphurtransferase) was used, in which the refolding is not limited by proline isomerisation. Thiosulphate ($S_2O_3^{2-}$) is converted to thiocyanate SCN^- in a reaction catalysed by rhodanese. In the presence of ferric nitrate ($Fe(NO_3)_3$), rhodanese activity is recovered by the formation of thiocyanate which formed an intensely red iron complex, $(FeSCN)^{2+}$. The restoration of the enzyme activity can be measured spectrophotometrically at 460 nm.



Denaturation of rhodanese was carried out in the presence of 6 M guanidine- HCl and 20 mM DTT. Renaturation of rhodanese was initiated by 76-fold dilution of the denatured rhodanese into refolding solution (50 mM Tris-HCl, pH 7.8, containing 50 mM $Na_2S_2O_3$, 10 mM KCN, and 10 mM DTT) and incubated at 37 °C. The reaction was finally mixed with ferric nitrate solution containing 165 mM $Fe(NO_3)_3 \cdot 9H_2O$ dissolved in 8.67 % *(v/v)* HNO_3 and the absorbance at 460 nm was recorded. It was found that Cj0694 has no effect of the inhibition of rhodanese refolding when compared to the negative control protein, BSA. Various concentrations of Cj0694 (0.5 – 2.5 µM) were used to assess whether the inhibition of rhodanese is concentration-dependent (Figure 4.6). However, all these concentrations showed no effect of the inhibition of the rhodanese refolding.

Nevertheless, an alternative assay was conducted to examine the *in vitro* chaperone activity of Cj0694. Protein aggregation is a classic assay used to examine the capability of candidate chaperones to inhibit aggregation of substrate proteins, where the correct refolding of substrate proteins is not required, and the formation of protein aggregates results in light scattering that can be measured spectrophotometrically by increase in the absorbance. Two candidate substrate proteins were used; rhodanese and lysozyme.

Unfolding and refolding of rhrodanese and lysozyme was carried out as previously described (Ideno *et al.*, 2000). Denaturation of both substrates was carried out in a solution contained 6 M guanidine-HCl and 20 mM DTT for two hours at 25 °C. Whereas renaturation was initiated by a 60- fold dilution in 50 mM Tris-HCl, pH 7.8, to reach a final concentration of 1.0 µM of rhodanese or lysozyme, and incubated at 25 °C in the absence or presence of Cj0694 (1.0 to 5.0 µM) or BSA (1.0 µM) as a negative control). During the renaturation of both proteins, increasing concentration of Cj0694 progressively inhibited the aggregation of both denatured proteins as measured by light scattering kinetics at 320 nm (Figure 4.7). BSA was used as a negative control with no effect on the inhibition of protein aggregation. The aggregation rate of rhodanese and lysozyme was highly affected by Cj0694 concentration, which may reflect that the chaperone domain of Cj0694 is active in preventing substrate proteins to aggregate, and therefore, suggest that Cj0694 is a chaperone that prevents protein aggregation, a role consistent with binding client proteins maintained in only a partially folded state before transfer to the BAM complex for insertion in the OM.

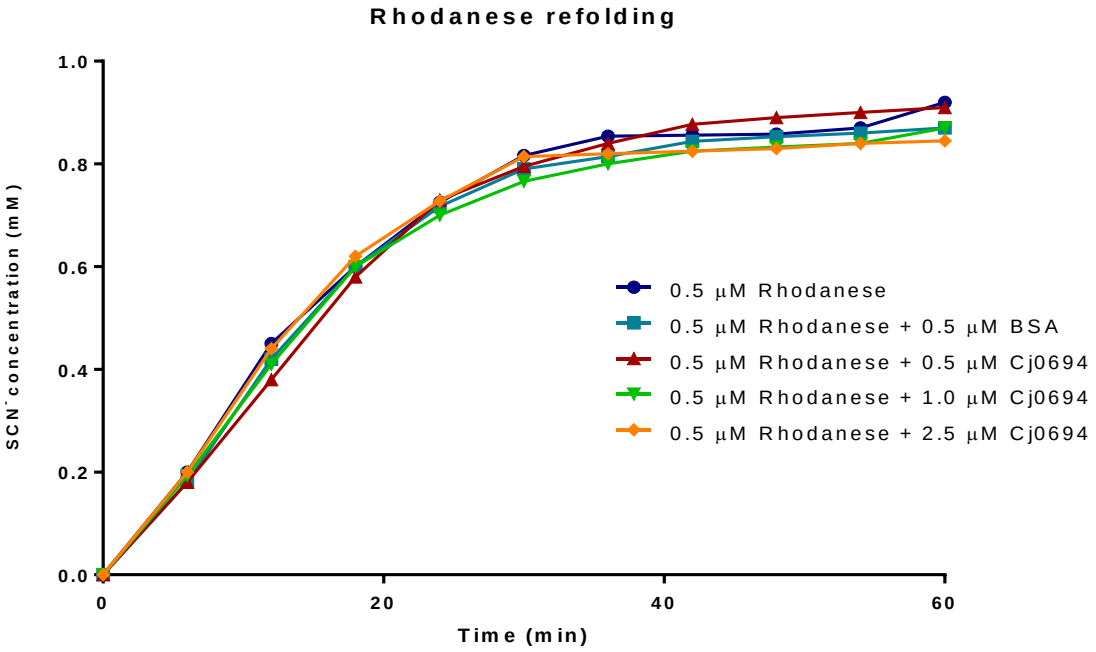


Figure 4.6 Chaperone activity of Cj0694 as represented by inhibition of rhodanese refolding. Cj0694 shows no effect on inhibition of the refolding of chemically denatured rhodanese. At time zero, refolding of chemically denatured rhodanese was initiated by 76-fold dilution into 50 mM Tris-HCl pH 7.8 containing 50 mM $Na_2S_2O_3$, 10 mM KCN and 10 mM DTT and incubated at 37 °C. Enzymatic reactions were stopped by adding 300 µL aliquots of the refolding mixture to 200 µL 38 % *(v/v)* formaldehyde at various time intervals (0 – 60 min). After the completion of the assay, samples were centrifuged to pellet the precipitates, and the supernatants were mixed with 500 µL ferric nitrate solution containing 165 mM $Fe(NO_3)_3 \cdot 9H_2O$ dissolved in 8.67 % *(v/v)* HNO_3 and the absorbance at 460 nm was recorded. Separate measurements of native rhodanese activity (without denaturation) were used to determine the maximum (100 %) activity. Various concentrations of Cj0694 (0.5 µM – 2.5 µM) or equimolar amount (0.5 µM) of BSA (negative control which lacks chaperone activity) were present in the refolding solution prior to the addition of the unfolded rhodanese.

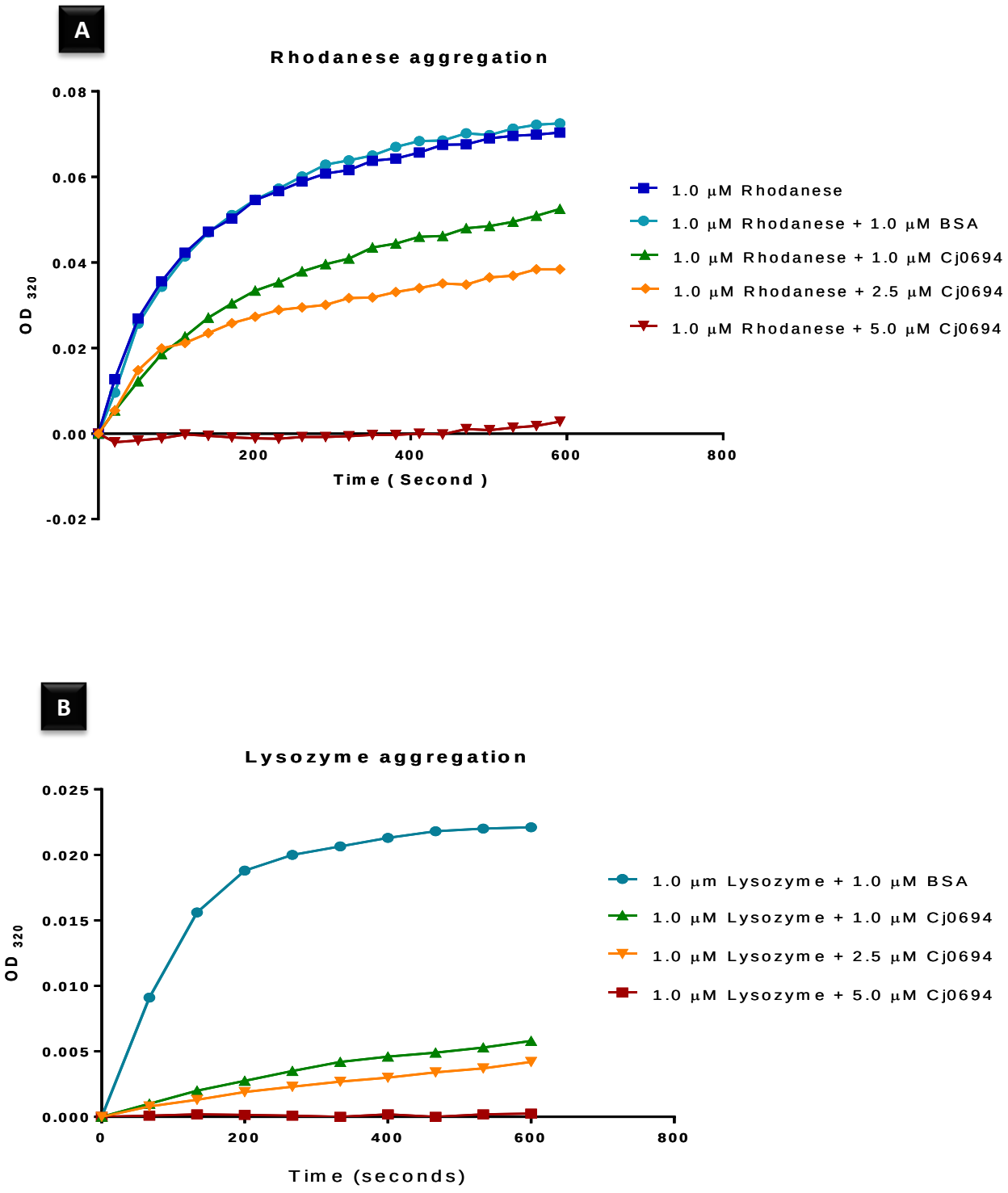


Figure 4.7 Chaperone activity of Cj0694 as represented by inhibition of rhodanese and lysozyme aggregation. A) Effect of Cj0694 on rhodanese aggregation. Aggregation of 1.0 µM rhodanese was initiated by 60 fold dilution and measured by monitoring the increase in optical density at 320 $_{nm}$. Cj0694 significantly slowed the formation of rhodanese aggregates, whereas addition of equimolar concentration of BSA had no effect. **B)** Effect of Cj0694 on lysozyme aggregation. Cj0694 showed a similar effect to that of rhodanese as it significantly inhibited the aggregation of lysozyme. BSA was used as a negative control as for rhodanese aggregation assay.

4.2.7 Crystallography trials

Although purified Cj0694 from pBAD*0694* over-expression vector was effectively used to examine the biochemical characteristics of Cj0694, all attempts to use the same protein for structural studies were unsuccessful. Crystallography trials were conducted in collaboration with Dr. John Rafferty at the Department of Molecular Biology and Biotechnology, The University of Sheffield, UK. We used sitting drop technique which is based on vapour diffusion method, a mixture contained 1:1 ration of the protein and the crystallisation solution is placed into a sealed environment next to a reservoir of a precipitant. Due to the higher concentration of the reservoir, water normally diffuses out from the sample mixture to the reservoir. This results in an increase in the concentration of the sample mixture which leads the protein to come out of the solution. We performed small-scale screening using three common screens, PACT, JCSG^{+} and Ammonium Sulphate screens (Qiagen) in 200 nL + 200 nL sitting drop vapour diffusion experiments (using a Matrix_Hydra II Plus One crystallisation robot). Small-scale trials were performed to minimise the variables, such as pH, temperature, protein concentration, type and purity, which may occur during crystallisation and may prevent macromolecules to form crystals. Moreover, vapour diffusion method was used as it is one of the most common and widely-used experiment in protein crystallisation, as it favours to use a small volume of purified protein.

One of the main reasons for failure to obtain crystals might be due to the long (34 residues) flanking *N*-terminal amino acid sequence between the *N*-terminal hexahistidine tag and the Cj0694 sequence in the pBAD*0694* vector. To overcome this problem, other over-expression systems were used to produce a higher solubility form of the protein with fewer additional amino acids. Here, two over-expression vectors were used; the cold shock expression vector pCOLD TF (Takata) and the pET21a(+) expression vector (Novagen).

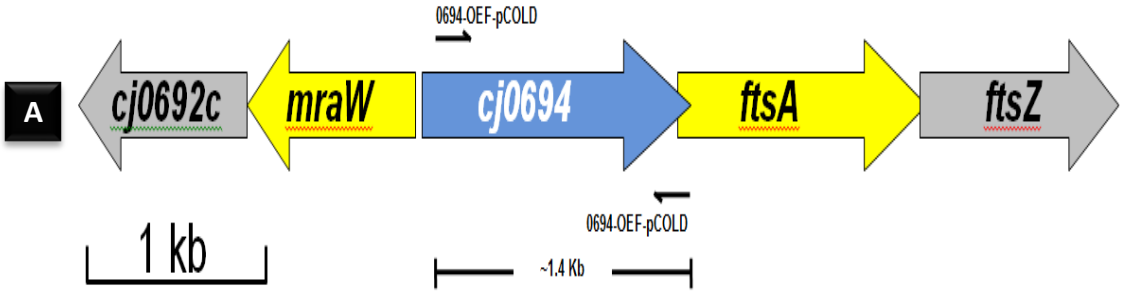
4.2.7.1 Generation of pCOLD*0694* plasmid for over-expression

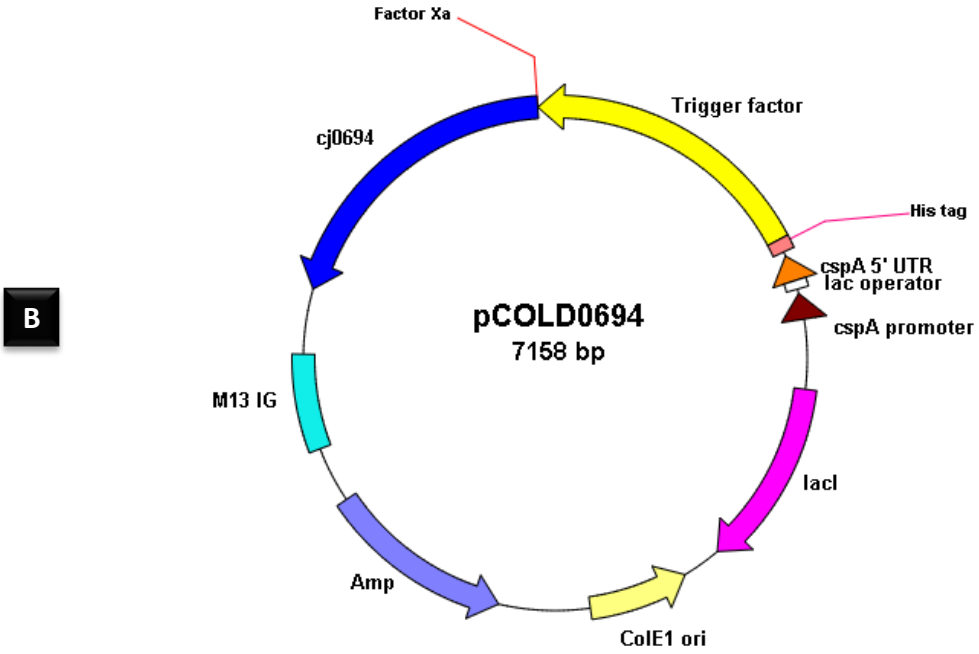
The pCOLD vector offers high *in vivo* protein yield, purity, and solubility for recombinant proteins using "cold shock" system (Hayashi and Kojima, 2008). In more detail, the *cspA* (cold shock protein A) promoter and related elements are inserted into these vectors to upregulate production of target protein at low incubation temperatures (37 °C – 15 °C). This decrease in temperature also suppresses production of other cellular proteins and temporarily stops overall cell growth. This process allows production of target proteins at high yield. Moreover, pCOLD vector is a fusion cold shock expression vector that produces Trigger Factor (TF) chaperone as a soluble tag. Trigger Factor is a prokaryotic ribosome-associated chaperone protein which facilitates co-translational folding of newly expressed polypeptides. Because TF is derived from *E. coli*, it is highly produced in *E. coli* expression systems. Furthermore, the vector contains Factor Xa protease cleavage site immediately adjacent to the multiple cloning site, so that it provides a protein with fewer additional undesirable amino acids (Figure 4.8B).

The full-length *cj0694* excluding the signal sequence was PCR amplified from *C. jejuni* NCTC11168 chromosomal DNA using the primers 0694-OEF-pCOLD and 0694-OER-pCOLD (Figure 4.8A). The gene fragment was cloned into the *Xho*I and *EcoR*I restriction sites of the pCOLD TF expression vector, in frame with the *N*-terminal hexahistidine tag and trigger factor, and immediately after the Factor Xa cleavage site (Figure 4.8B). The resulting pCOLD*0694* expression plasmid was verified by colony PCR which was used to confirm the correct insertion of the *cj0694* DNA sequence into pCOLD vector using the primers 0694-OEF-pCOLD and 0694-OER-pCOLD (Figure 4.8C). pCOLD*0694* was used to transform *E. coli* competent BL21(λDE3) cells and small scale growths were conducted to determine the amount of soluble fraction of the protein under the control of isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible *csp* promoter. Over-produced Cj0694 was found to be highly soluble (Figure 4.8D) when both CFE and cell pellets were examined by SDS-PAGE.

4.2.7.2 Over-production of Cj0694 in pCOLD*0694*

Cultures of *E. coli* BL21(λDE3) cells transformed with pCOLD*0694* were grown aerobically at 37 °C in LB medium containing carbenicillin (50 µg mL^{-1}) until OD_{600} ~ 0.1. Over-expression was induced by the addition of 1 mM IPTG to the cultures at an OD_{600} ~ 0.1. The over-expression took place for 24 hours at 15 °C to allow the activation of the *csp* (cold shock protein) promoter, and suppression of the expression of other cellular proteins. The ~ 105.8 kDa recombinant protein containing the Cj0694 (~ 54.3 kDa) and the *N*-terminal hexahistidine tag plus the trigger factor and protease recognition sites (~ 51.5 kDa) was purified by affinity chromatography using a Ni-NTA column (GE Healthcare, UK), and the resulted recombinant pure protein was used to perform small-scale cleavage trials by utilising the cleavage characteristics of Factor Xa protease. Small-scale cleavage trials were performed to determine the best cleavage condition required for optimum cleavage. After addition of an appropriate concentration of Factor Xa protease (1 µg Factor Xa cleaves 50 µg fusion protein), samples (50 µL) were collected at times; 30 minutes, 1 hour, 2 hours, 4 hours and 24 hours, and applied to SDS-PAGE for visualisation of cleavage (Figure 4.9A). After one hour of cleavage, the recombinant protein was partially cleaved and complete cleavage was achieved after two hours. Therefore, the pure protein was applied to a "large-scale" protease cleavage (5 mg mL^{-1} of protein) to obtain a highly pure Cj0694. However, after several attempts of Factor Xa cleavage in a large-scale concentration, only degraded forms of Cj0694 were obtained as observed by SDS-PAGE (Figure 4.9B). This may be due to the large concentration of the recombinant protein used against Factor Xa or may be due to the non-specific cleavage of Factor Xa that resulted in multiple digestions of the protein.


A
0694-OEF-pCOLD
cj0692c
mraW
cj0694
ftsA
ftsZ
1 kb
0694-OEF-pCOLD
~1.4 Kb


B
Factor Xa
Trigger factor
cj0694
His tag
cspA 5' UTR
lac operator
cspA promoter
pCOLD0694
7158 bp
M13 IG
lacI
Amp
ColE1 ori

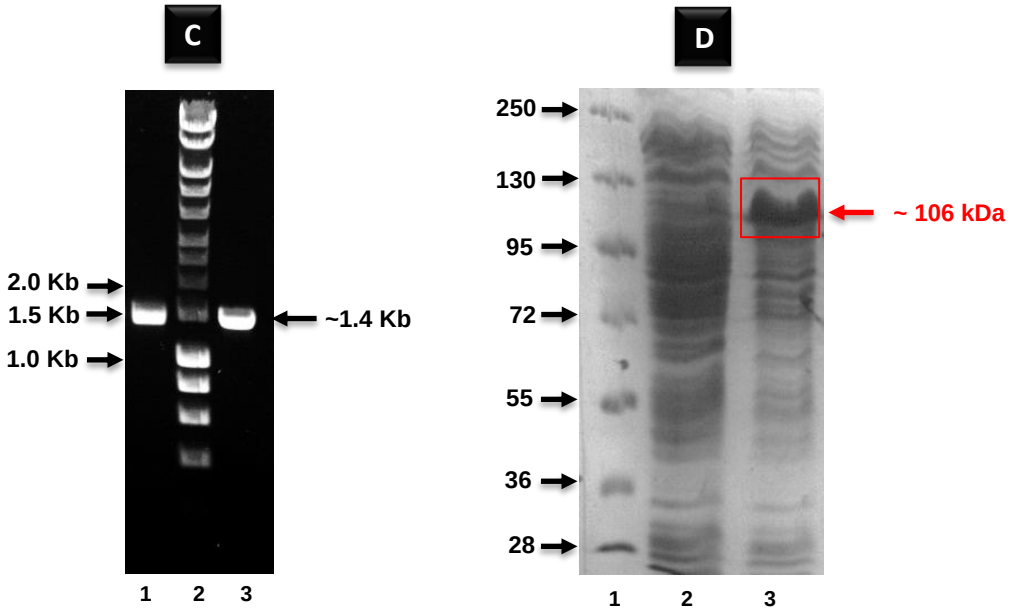

C
2.0 Kb
1.5 Kb
1.0 Kb
~1.4 Kb
1
2
3
D
250
130
95
72
55
36
28
~ 106 kDa
1
2
3

Figure 4.8 Generation of pCOLD*0694* over-expression plasmid construct. A) The entire coding sequence of *cj0694* minus the possible signal peptide cleavage site was amplified using the primers 0694-OEF-pCOLD and 0694-OER-pCOLD from the genomic DNA of *C. jejuni* NCTC 11168. **B)** The plasmid map of pCOLD*0694*. Full-length *cj0694* without the *N*-terminal signal sequence was cloned into the *Xho*I and *EcoR*I restriction sites of the pCOLD TF expression vector immediately after the Factor Xa cleavage site. The *N*-terminal hexahistidine was utilised for purification. **C)** An overloaded 0.7 % agarose gel electrophoresis representing colony PCR used to confirm the correct insertion of *cj0694* sequence into pCOLD TF expression vector. Lane 1: the *cj0694* amplified from the wild-type genomic DNA. Lane 2: Hyperladder™ I molecular weight marker (Bioline, UK). Lane 3: the inserted *cj0694* from the pCOLD*0694* plasmid construct using the primers 0694-OEF-pCOLD and 0694-OER-pCOLD. **D)** An overloaded 12.5 % SDS-PAGE showing that the Cj0694 is highly produced in soluble fractions. The ~ 106 kDa recombinant protein containing the Cj0694 (~ 54.3 kDa) and the trigger factor (~ 51.5 kDa) was highly produced in a soluble form in the *E. coli* BL21(λDE3) cells harbouring pCOLD*0694* grown aerobically at 15 °C in LB medium containing 50 µg mL^{-1} carbenicillin after induction with 1 mM IPTG. Lane 1: PageRuler^{TM} prestained protein ladder (Thermo). Lane 2 and 3: insoluble and soluble fractions of BL21(λDE3) cells harbouring pCOLD*0694*, respectively.

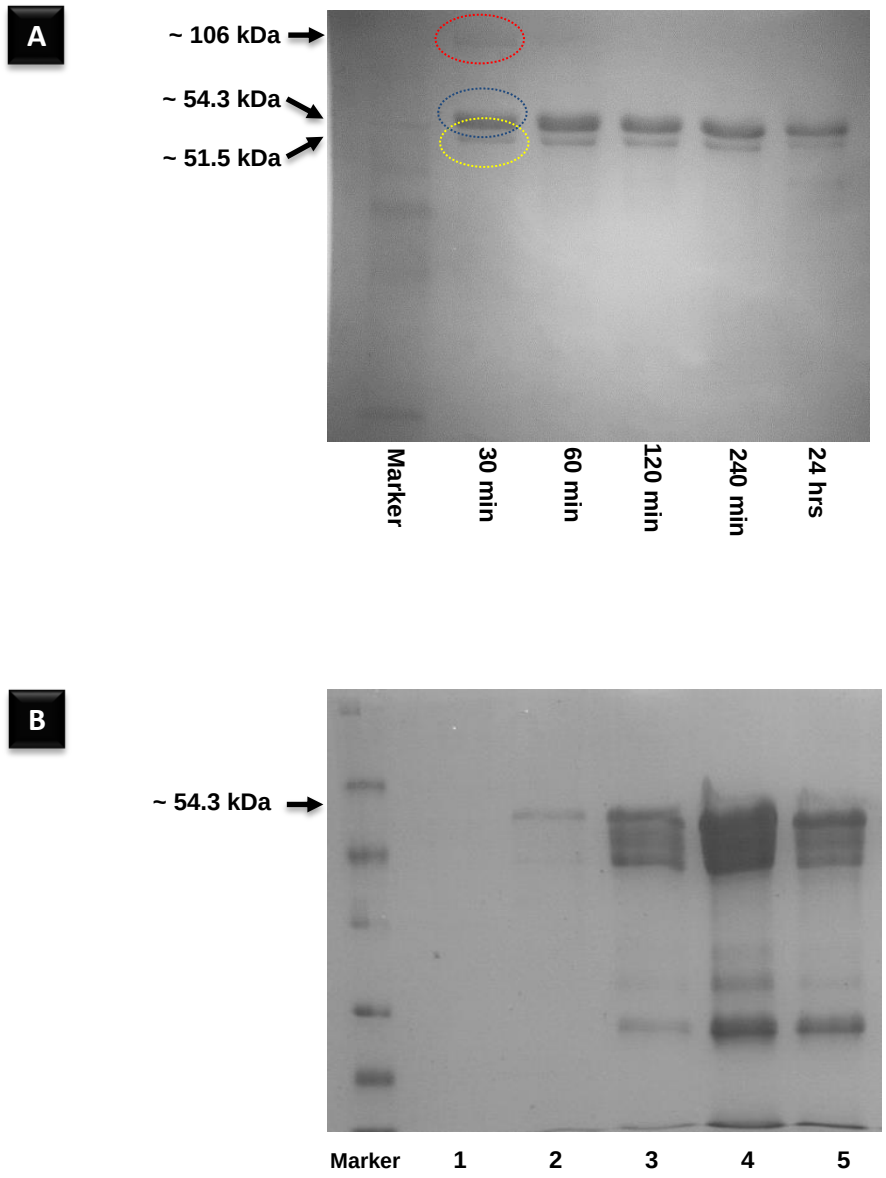


Figure 4.9 Factor Xa cleavage and over-production of Cj0694 in pCOLD*0694* over-expression vector. A) Overloaded 12.5 % SDS-PAGE representing cleavage trials of Cj0694 from pCOLD*0694* vector using Factor Xa protease. The purified recombinant protein (~ 106 kDa) was incubated with appropriate concentration of Factor Xa, and 50 µL samples were collected at times 30 minutes, 1, 2, 4 and 24 hours. Undigested protein (~ 106 kDa) is shown in red circle, while Cj0694 (~ 54 kDa) and trigger factor (~ 51 kDa) are shown in dark blue and yellow circles respectively. **B)** Overloaded 12.5 % SDS-PAGE showing Cj0694 fractions (lane 1 – 5; ~ 54.3 kDa) digested with Factor Xa and applied to a further affinity chromatography using a Ni-NTA His-Trap™ column (GE Healthcare, UK). The (~ 54.3 kDa) Cj0694 fractions were collected from the flow-through of the purification column as the *N*-terminal His tag and the trigger factor were trapped in the column. Lower bands are predicted to be degraded products of Cj0694.

4.2.7.3 Generation of pET*0694* over-expression plasmid

The entire coding region of *cj0694* including the stop codon minus the *N*-terminal signal peptide sequence was PCR amplified from the chromosomal DNA of *C. jejuni* using the primers 0694-EOF-pET and 0694-OER-pET(stop) (Figure 4.10A). The stop codon was included in the reverse primer to stop expression immediately before the *C*-terminal hexahistidine tag. Previous attempts to over-produce the Cj0694 with the *C*-terminal hexahistidine tag were unsuccessful and resulted in an insoluble form of the protein (C. Phansopa Ph.D thesis). This might be because the His-tag can reduce the solubility of some proteins (Woestenenk *et al.*, 2004). The resulting amplicon was digested with *Nde*I and *Xho*I to generate sticky ends that were cloned into *Nde*I and *Xho*I sites of pET21a(+) which was digested by the same enzymes (Figure 4.10B). The insertion of *cj0694* into pET21a(+) was verified by colony PCR and diagnostic restriction digest using *Nde*I and *Xho*I restriction enzymes (data not shown) and the resulting pET*0694* was used to transform *E. coli* BL21(λDE3) over-expression cells. To determine the amount of solubility, small-scale over-expression in LB cultures (50 mL) containing 50 μg mL^{-1} carbenicillin was performed under the control of isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible T7 promoter. Approximately, similar amount of protein was found in both soluble and insoluble fractions (Figure 4.10C).

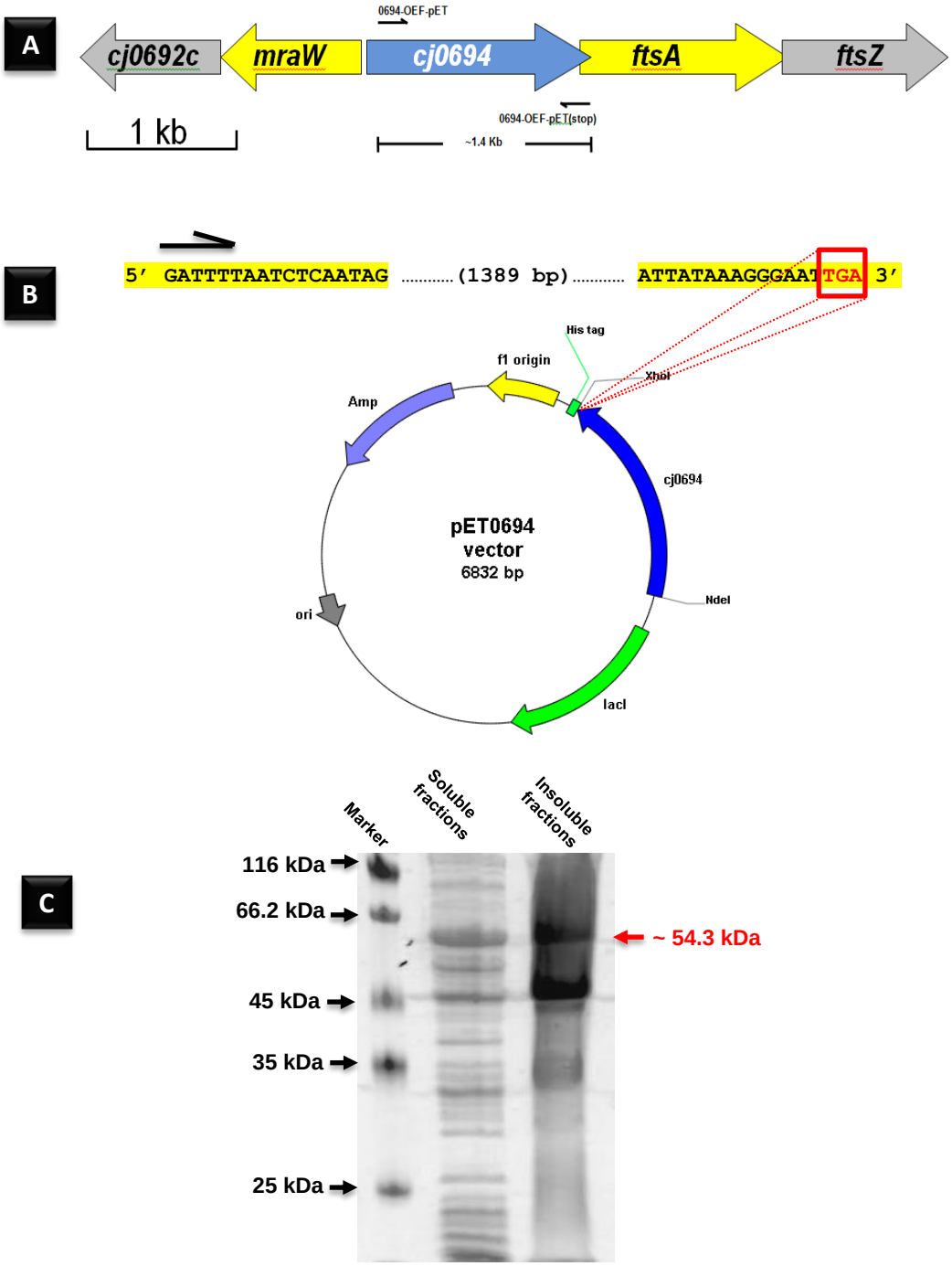


Figure 4.10 Generation of pET*0694* over-expression plasmid. A) The entire coding sequence of *cj0694* minus the possible signal peptide cleavage site was amplified using the primers 0694-OEF-pET and 0694-OER-pET from the genomic DNA of *C. jejuni* NCTC 11168. **B)** Plasmid map of pET*0694*. The complete coding sequence of *cj0694* excluding the most likely *N*-terminal signal sequence but including the stop codon (**TGA**) was cloned into the *Xho*I and *Nde*I sites of the pET21a(+) expression vector. The *C*-terminal His tag is not expressed due to the insertion of the stop codon in the 3' of the *cj0694* (shown in red box). **C)** Overloaded 12.5 % SDS-PAGE showing that the Cj0694 is highly produced in soluble fractions. The ~ 54 kDa Cj0694 is highly produced as a soluble protein in the *E. coli* BL21(λDE3) cells harbouring pET*0694* grown aerobically at 37 °C in LB medium containing 50 µg mL^{-1} carbenicillin after induction with 1 mM IPTG. Lane 1 represents unstained MW protein marker (Thermo).

4.2.7.4 Over-production and purification of Cj0694 in pET21a(+) system

Cultures (2L) of *E. coli* BL21(λDE3) cells harbouring the pET*0694* expression vector were grown aerobically at 37 °C in LB medium containing carbenicillin (50 µg mL^{-1}). Over-production of Cj0694 was performed under the control of isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible T7 promoter (Figure 4.11). Purification of Cj0694 was performed by hydrophobic interaction chromatography (HIC), ion-exchange chromatography and gel filtration which was aided by S.E. Sedelnikova (The University of Sheffield, UK). The purity of Cj0694 was judged by Coomassie blue staining on overloaded SDS-PAGE gels (Figure 4.12). Nevertheless, all attempts to obtain high purity yield of Cj0694 were unsuccessful. In view of this, no further work to obtain pure Cj0694 for structural studies was performed.

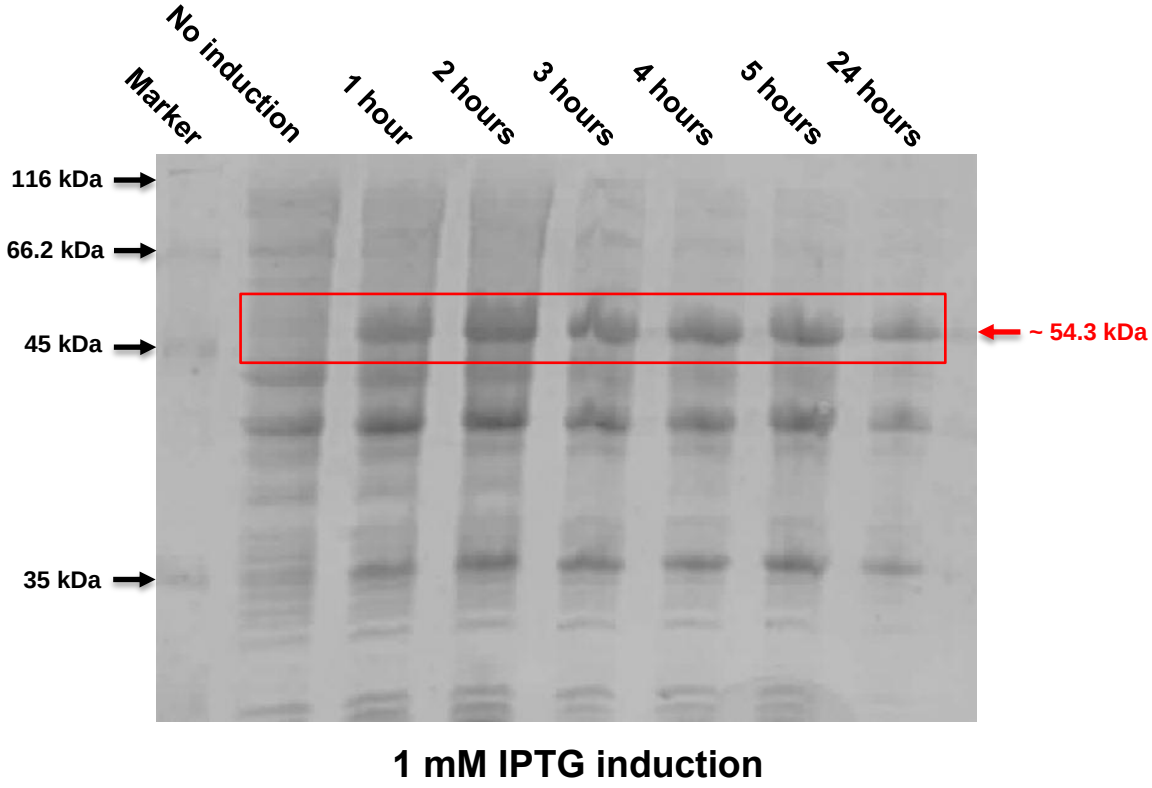
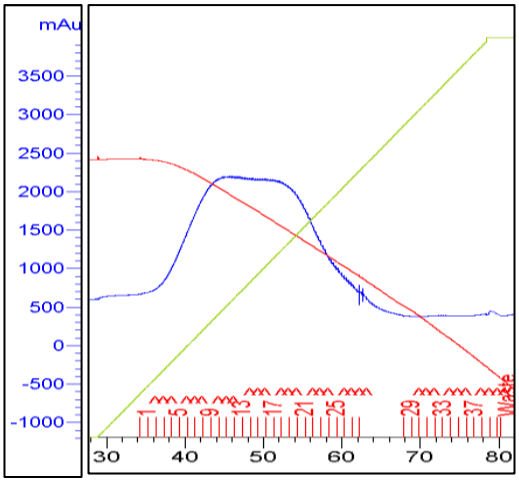
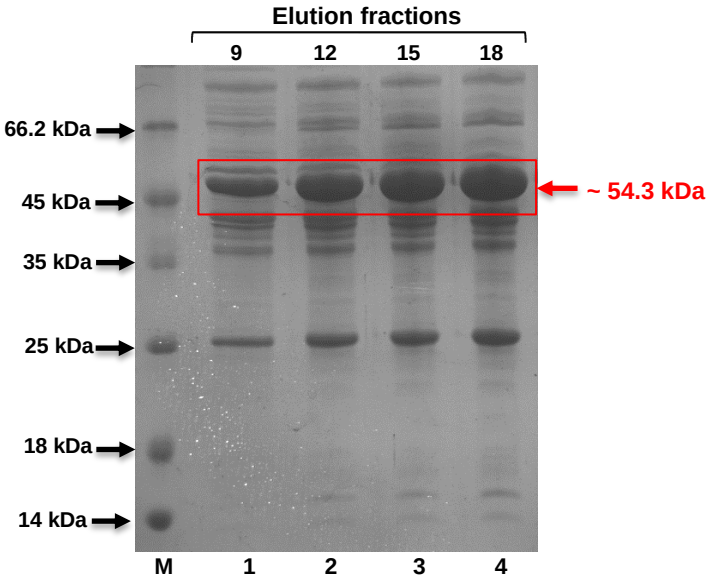


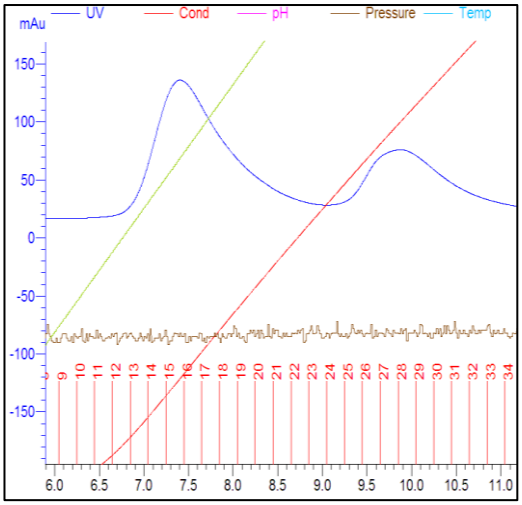
Figure 4.11 Over-expression trials for Cj0694 in pET21a(+) vector. The *E.* coli BL21(λDE3) cells harbouring the pET*0694* expression vector were grown aerobically at 37 °C in LB medium containing 50 µg mL^{-1} carbenicillin. After inducing the over-expression by 1 mM IPTG, samples were collected after 1 and 2 hours and visualised by SDS-PAGE. A sample of non-induced expression was also loaded alongside the induced-expression samples. Lane 1 represents unstained MW protein marker (Thermo).

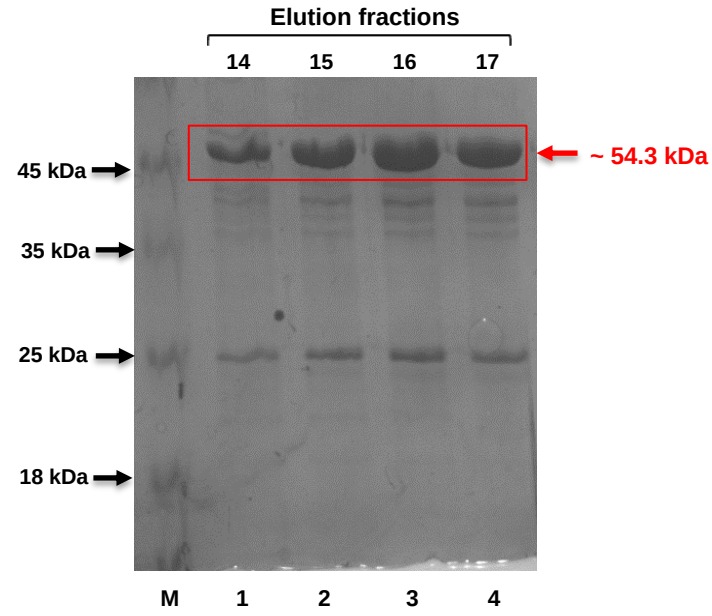
(1) HIC



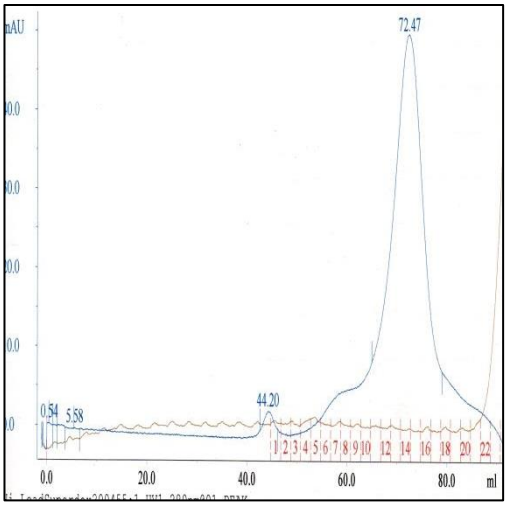


(2) Anion-exchange





(3) Gel filtration



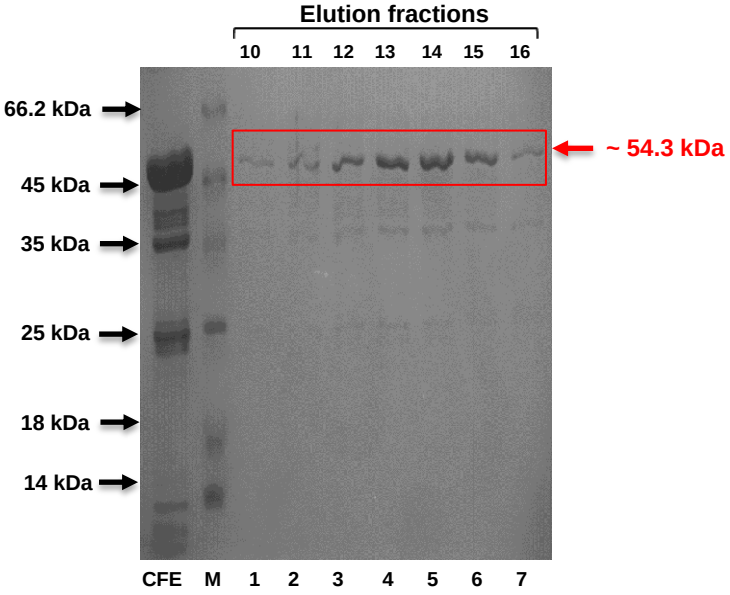


Figure 4.12 Purification of Cj0694 from the pET*0694* plasmid. (1) Cell-free extracts containing the over-produced Cj0694 were adjusted to a volume of 2 ml in 50 mM Tris-HCl pH 8.5 with 1.5 M $(NH_4)_2SO_4$ and were loaded into a 5 mL HiTrap^{TM} HIC column (GE Healthcare, UK) for hydrophobic interaction chromatography (HIC). Protein was eluted over 10 column volumes in 1-0 M $(NH_4)_2SO_4$ in 50 mM Tris-HCl pH 8.5 (2) Protein-containing fractions were subsequently pooled, buffer exchanged into 50 mM Tris-HCl pH 8.0 and purified by anion-exchange chromatography. Protein was loaded to a 5 ml HiTrap^{TM} DEAE FF column (GE Healthcare, UK) and was eluted in the same buffer over 10 column volumes with a linear gradient of 0-1 M NaCl. (3) Protein-containing fractions eluted from anion-exchange chromatography were pooled and applied to a final purification step using Superdex 200 gel filtration column (GE Healthcare). Lane M contains unstained MW protein marker (Thermo). Lanes labelled in numbers represent purification-elution fractions. Lane CFE represents concentrated cell-free extracts obtained from antion-exchanged chromatography. Fainter bands are predicted to be contaminating proteins.

4.3 Discussion

The aims of this chapter were to study the biochemical characteristics and catalytic activities of the periplasmic protein Cj0694, in order to determine its chaperone role and PPIase activity in the periplasm, and to compare them to those of the previously obtained data from the Cj1289 and PEB4 in *C. jejuni* (Kale *et al.*, 2011), and the inner membrane-anchored periplasmic protein PpiD in *E. coli*. Also it was aimed to obtain the crystal structure of Cj0694 by over-producing it in an over-expression vector as a soluble protein. Despite successful cloning, over-production and purification of Cj0694 for biochemical characterisation, gaining a pure Cj0694 for structural determination remains an obstacle. However, this set of different over-expression vectors used for over-production and purification of Cj0694 and the preliminary findings obtained in this study and discussed below provide a beneficial background for future work.

4.3.1 Cj0694 is a periplasmic SurA-like protein that may be localised in the periplasmic face of the of the inner membrane of *C. jejuni*

Bioinformatics had revealed Cj0694 as a periplasmic protein belonging to the SurA superfamily (Kale *et al.*, 2011). However, unlike Cj1289 which has a well-defined 17 amino acids signal sequence, suggesting that Cj1289 is located in the periplasm, Cj0694 has no positively charged *N*-terminal sequence and Ala-X-Ala signal peptidase I cleavage site as indicated by primary amino acid sequence analyser (SignalP 4.1) (http://www.cbs.dtu.dk/services/SignalP/). This indicates that Cj0694 is not likely to be a Sec-dependent secretory protein, and provides more evidence that it is more related to its homologues; the inner-membrane anchored protein PpiD in *E. coli*. The role of PpiD in immediate interaction with several proteins exiting the Sec-system was suggested by the retardation of the release of OmpA in the periplasm via the Sec system in *ppiD* mutant (Antonoaea *et al.*, 2008), suggesting that it has a similar role in the periplasm as the Trigger factor (Matern *et al.*, 2010). Since bioinformatics indicated that the *C. jejuni* Cj0694 is a PpiD orthologue, the lack of Sec-dependent secretion signal sequence can indicate that Cj0694 is an inner membrane-anchored protein. However, more direct structural evidence is still required to prove such functional and localisation similarities. Indeed, its exact membrane localisation can be experimentally determined by western

blotting, in which membrane fractions of the wild-type *C. jejuni* can be prepared to obtain the inner membrane and the periplasm. Monoclonal antibodies can be raised against the *C. jejuni* Cj0694, and western blot can be then carried out to each fraction using anti-Cj0694.

Despite previous unsuccessful over-production of Cj0694 as a soluble protein using the pET21/BL21 (λDE3) system, here, the Cj0694 was successfully over-produced and purified using the pBAD/His B over-expression vector. Cloning, over-expression and purification of Cj0694 was successfully achieved in order to examine its biochemical function and catalytic activities as a periplasmic chaperone and PPIase.

4.3.2 The PPIase domain of Cj0694 is catalytically active as demonstrated by RCM-RNase T_1 refolding

The catalytic activity of Cj0694 in acceleration of the refolding rate of the reduced and carboxymethylated ribonuclease T_1 was tested by PPIase assay. This assay showed a remarkable Cj0694-dependent acceleration of the refolding rate of RCM-RNase T_1. In the same assay, PEB4 was used as a positive control as it previously showed significant activity in the acceleration of the refolding rate of the same substrate (Kale *et al.*, 2011). Interestingly, this is the first evidence that confirmed that the PPIase domain of Cj0694 has a high catalytic activity as a PPIase. Compared to PpiD, it has been shown that there is weak sequence similarity between the two proteins (Kale *et al.*, 2011). However, PpiD was shown to be a periplasmic PPIase with no catalytic activity in the acceleration of the refolding rate of protein isomerisation as examined in *E. coli* (Matern *et al.*, 2010, Weininger *et al.*, 2010) and in *Yersinia psudotuberculosis* (Obi *et al.*, 2011). As reviewed in Chapter 1, PpiD consists of α-helical transmembrane domain and three periplasmic domains. The first and third domains are proposed to be chaperone domains and the second domain (residues 227 – 357) was identified as a parvulin-like domain (Dartigalongue and Raina, 1998), which was structurally confirmed by NMR spectroscopy using a shorter length of protein (residues 264 – 357) (Weininger *et al.*, 2010). Moreover, PpiD was shown to have high structural similarities to the first parvulin domain of SurA (Weininger *et al.*, 2010). However, when its catalytic activity

in acceleration of prolyl isomerisation was examined by PPIase assay in the same study, it was not active. This might be because the PpiD is structurally similar to the first parvulin-like domain of SurA and this domain is known to be inactive (Behrens *et al.*, 2001). Therefore, despite the fact that Cj0694 in *C. jejuni* and PpiD in *E. coli* have a weak sequence similarity, Cj0694 has shown to be a periplasmic PPIase that has a high catalytic activity in the acceleration of the refolding rate of RNase T_1. The biological role of the single PPIase domain of Cj0694 is still unidentified, and cannot certainly be investigated until structural data of Cj0694 becomes available to be assessed together with the biochemical and phenotypic characteristics. The well-identified periplasmic protein SurA is known to bind proteins having the *C*-terminal Ar-Ar or Ar-X-Ar motif, which most OMPs possess (Bitto and McKay, 2002, Hennecke *et al.*, 2005, McKay and Bitto, 2004). The inactive parvulin-like domain of SurA is substrate specific, which interacts with the aromatic residues of the tested peptide (Xu *et al.*, 2007). The *C*-terminal aromatic residues are also found in *C. jejuni* OMPs, which can indicate a direct specific role of Cj0694 as a periplasmic PPIase that may play a role in accelerating the folding of OMPs.

4.3.3 Cj0694 exhibits a general periplasmic chaperone function that may assist in early stages of protein folding in the periplasm

The nature of Cj0694 as a periplasmic chaperone was tested by refolding assays using a classical model protein rhodanese (a sulfurtransferase). Although chaperone activity of Cj0694 could not be demonstrated in inhibiting the rhodanese refolding, even when increasing concentration of Cj0694 (0.5 – 2.5 µM), it was found that Cj0694 was active in preventing aggregation of rhodanese, and the reaction was found to be concentration-dependent. Also, lysozyme was used as an alternative substrate for rhodanese to confirm this activity, and was shown to give a similar effect. It is thus possible that Cj0694 has a more general role in the periplasm as a chaperone for both periplasmic and outer membrane proteins, which is consistent with work which indicate that PpiD has less substrate specificity than SurA (Stymest and Klappa, 2008). Matern *et al.*, (2010) tested the chaperone activity of the full-length PpiD and also a PpiD variant lacking the PPIase domain (PpiDΔParv) to evaluate the hypothesis that the chaperone activity can be independent of the PPIase domain. Their findings were consistent with the chaperone

role of PpiD as it prevented the aggregation of the model protein, citrate synthase. However, PpiDΔParv variant was shown to be more effective than the full-length protein in preventing aggregation of the substrate protein. The chaperone role of PpiD was suggested to be carried in the *N*-terminal region of the protein, which shows sequence similarity to the chaperone domain in SurA (Matern *et al.*, 2010). Later, Kale *et al.*, (2011) tested the chaperone activity of the periplasmic proteins, PEB4 and Cj1289, using rhodanese as a model protein. PEB4 was found to reduce the yield of active protein in the refolding assay by inhibiting its correct folding during renaturation, and also prevented the unfolded rhodanese to aggregate. However, Cj1289 was not able to inhibit rhodanese to fold or aggregate, suggesting that if it has a chaperone role in the periplasm, it might be substrate specific (Kale *et al.*, 2011).

The classical role of a model chaperone is to aid in protein folding. This is often driven by an ATP-dependent process as seen in the cytoplasmic chaperonin GroEL and its co-chaperone GroES. This is not the case with periplasmic chaperones, where ATP is absent. Therefore, periplasmic chaperones must have evolved a catalytic mechanism that is ATP-independent and is appropriate for this function in the periplasm. It can be useful to show the effect of over-production of Cj0694 in *peb4* mutant, which may demonstrate a rescue role of Cj0694 that compensates for the lack of PEB4. Additionally, a Cj0694 variant lacking the PPIase domain can be constructed to examine if the PPIase domain of Cj0694 contributes in the overall chaperone activity. Based on these findings we can conclude with high confidence that Cj0694 is a periplasmic PPIase in *C. jejuni* that plays a role in the network of periplasmic chaperones. It is not yet clear if it is directly involved in the maturation of OMPs, or if it can be a more general periplasmic chaperone for periplasmic and outer membrane proteins, that assists in early stages of periplasmic folding of newly Sec-translocated proteins. Although Cj0694 might possesses a distinct chaperone role in the periplasm, together with other previously identified periplasmic chaperones in *C. jejuni* they can form an essential network in the periplasm that aids in OMPs maturation or a rescue role in the absence of other essential chaperones.

4.3.4 Cloning, over-expression and purification of Cj0694 in other expression systems

To obtain a comprehensive study about the role of Cj0694 in the periplasm, which provides better understanding its biological function, phenotypic and biochemical analysis and structural determination have to be available. Cj0694 was successfully over-expressed in pBAD/His B expression vector, and purified for biochemical analysis. However, the pure Cj0694 obtained from pBAD/His B system could not be utilised for structural studies, despite several crystallography trials by trying several screens using variety of conditions. The possible reason for these unsuccessful trials using the pBAD/His B vector might be due to the long flanking *N*-terminal sequence (34 residues) in the over-expression plasmid pBAD*0694* which is located between the *N*-terminal His-tag and the Cj0694 sequence. This long sequence may have interfered with the crystals formation which is usually resulted from the collision between amino acid molecules. In addition, several variables contribute to generate successful crystallisation. These include the protein purity, the amount of macromolecules, the protein type, concentration, pH and temperature. We performed initial (small-scale) screening trials to be able to screen a variety of conditions that favours crystal growth. These trials were based on vapour diffusion experiment, in which small volumes of protein is required. Basically, vapour diffusion enables a protein-precipitant mixture to equilibrate against a large reservoir solution containing the same precipitant, by allowing 1:1 ratio of protein and crystallisation solution. Then, vapour diffusion occurs between the drop and the reservoir. The resulting water transfer from the protein solution to the reservoir causes an increase in the concentration of protein and precipitant, which is accompanied by formation of crystals of variable sizes, or precipitates, or nothing.

This led us to clone the full-length *cj0694* excluding the signal sequence into "cold shock" vector, pCOLD TF which provides high *in vivo* protein yield, purity, and solubility for recombinant proteins using "cold shock" system. The vector harbours the cold shock protein A (*cspA*) with its promoter that upregulates the production of target protein at low incubation temperatures (37 °C – 15 °C), in which the production of other cellular proteins is supressed and the cell growth is temporarily stops. Therefore, the target protein is produced at high yield and purity. Moreover, pCOLD vector is a fusion

cold shock expression vector that produces Trigger Factor (TF) chaperone as a soluble tag. Trigger Factor is a prokaryotic ribosome-associated chaperone protein which facilitates co-translational folding of newly expressed polypeptides (Hesterkamp and Bukau, 1996, Hesterkamp *et al.*, 1996, Stoller *et al.*, 1995). Because TF is originated from *E. coli*, it is highly produced in *E. coli* expression systems. Furthermore, it consists of Factor Xa protease cleavage site immediately adjacent to the multiple cloning site, so that it provides more pure proteins with least additional undesirable amino acids.

The major reason for cloning the *cj0694* into this expression vector were (*i*) to exclude the long flanking *N*-terminal residues which may interfere with crystal formation due to the presence of Factor Xa cleavage site immediately adjacent to the fusion protein, and (*ii*) the low temperature used for protein expression that stops the expression of other cellular proteins and provides higher purity of target protein yield. The main principle of using the pCOLD TF vector is the production of a full-length recombinant protein that consists of the target protein and the fusion trigger-factor (TF). The entire recombinant protein is then cleaved by Factor Xa protease immediately before the sequence of the target protein to obtain two fragments of the recombinant protein, of which one is for the target protein and the other is for the TF that contains an *N*-terminal His.tag that can be easily trapped during the purification process. Despite that Cj0694 was successfully produced as a soluble protein with high purity, and that the small-scale Factor Xa cleavage trials yielded maximum cleavage after 2 hours, obtaining a pure Cj0694 after "large-scale" protease cleavage (5 mg mL^{-1} of protein) was not successful and resulted in degradation of Cj0694 although several attempt in changing the cleavage incubation temperature and duration were performed. This may be due to the large concentration of the recombinant protein used against Factor Xa or may be due to the non-specific cleavage of Factor Xa that resulted in multiple digestions of the protein.

With all previous attempts to obtain a purified Cj0694 with high solubility for structural determination, it was finally decided to clone its coding sequence into the pET21a(+) over-expression system. The pET21a(+) vector utelises the T7 polymerase promoter to activate the expression of the T7 polymerase in the bacterial phage λDE3 on the chromosome of the BL21 cells. This promoter is induced by IPTG which is not found naturaly as the L-arabinose in the pBAD/Top10 system. Although previous attempts in our lab to over-express the Cj0694 in the vector yielded an entirely insoluble protein,

we managed to obtain the Cj0694 in a soluble form by cloning its cognate gene including the 3′ stop codon to stop the expression prior to the *C*-terminal His-tag, so that the protein is produced without the His-tag which can negatively affect its solubility (Woestenenk *et al.*, 2004). Moreover, using the pET/BL21(λDE3) system instead of the pBAD/Top10 system allows the Cj0694 recombinant protein to be purified without additional amino acid residues. This is because the nucleotide sequence can be cloned between the *Nde*I and *Xho*I restriction sites, leaving no additional residues that can interfere with proein crystallisation. Because His-tag was not expressed with the recombinant Cj0694, protein purification was carried out by alternative methods of chromatography. These were hydrophobic interaction chromatography (HIC), anion-exchange chromatography and gel filtration. In contrast to the His-Trap purification, in which only the histidine-tagged protein can bind to the purification column, HIC is based on the interaction between the hydrophobic groups on the surface of the molecule and the hydrophobic ligands which are attached to the gel matrix. Based on this principle, many proteins in the cell-free extracts might have closely similar hydrophobicity, which results in eluting more than one pure protein in the same fraction during sample fractionation. Despite that Cj0694 was highly expressed as a soluble protein, and purified by three different purification strategies, the presence of other contaminating proteins seen on the SDS gels of the three purification steps was an obstacle to carry out further crystallographic studies. However, the protein was used in crystal trials due to its high concentration anticipating that small chance of crystal formation could be useful, even in the presence of other contaminating proteins. However, all attempts to obtain crystal formation were unsuccessful.

Overall, our findings support the suggestion that Cj0694 acts as a periplasmic PPIase that may have a general chaperone role for periplasmic and outer membrane proteins. Similar to the inner-membrane anchored PpiD in other bacteria, it shows ability in preventing *in vitro* aggregation of unfolded substrate proteins. However, it shows a distinct activity in catalysing the *cis/trans* isomerisation of synthetically denatured RNase T_1, whereas all previous studies on PpiD in other organisms demonstrated that the PPIase domain is inactive. It would be beneficial to study the structural characteristics of each domain in the Cj0694. However, our attempt to obtain a pure protein that can be used for structural analysis may be useful as a start point for future work.

5 The role of the Mla pathway in the maintenance of the barrier function in the outer membrane of *Campylobacter jejuni*

5.1 Introduction

The asymmetric distribution of lipid contents in the OM of Gram-negative bacteria has a crucial role in increasing the barrier function of the OM, and is known to be maintained by three maintenance systems; phospholipase A (PldA), PagP (Dekker, 2000), and a newly identified Mla (Maintenance of Lipid Asymmetry) system described in *E. coli* (Malinverni and Silhavy, 2009). PldA is an OM phospholipase that hydrolyses many phospholipid (PL) substrates by removing the sn-1 and sn-2 fatty acid side chains from the glycerophosphodiester backbone of both PLs and lysophospholipids (lyso-PLs) (Dekker, 2000). It is normally found as an inactive monomer in the OM, but it can convert to a catalytically active PldA dimer when PLs and lyso-PLs present in the outer leaflet of the OM (Dekker, 2000). Therefore, its primary function is to maintain the asymmetric distribution of lipids in the OM under stress conditions. PagP is another OM β-barrel enzyme that acts by cleaving a palmitate moiety from PLs and transferring them to lipid A forming more hydrophobic compounds (Bishop, 2008). Similar to *pldA*, *pagP* is expressed at a low level in normal conditions (Jia *et al.*, 2004). However, expression is induced under stress conditions by the PhoP/Q stress response, which senses the restriction in divalent cations (Groisman, 2001). The newly identified Mla system consists of a previously unidentified ATP-binding cassette (ABC) transporter and other associated proteins that stops PLs to accumulate in the outer leaflet of the OM in the absence of extracellular stress and remove PLs from the OM when the cell is stressed, and drive them to the IM. *E. coli mla* mutants have a very specific phenotype that indicates a damaged OM permeability barrier, an increase in sensitivity to SDS-EDTA mixture, but do not show any growth defect or change in OMPs expression level, due to partial suppression of the phenotype by PldA. However, *mla pldA* double mutants are more compromised. The encoding genes of this pathway were named *mlaA–F* according to their roles in maintenance of lipid asymmetry in the OM (Malinverni and Silhavy, 2009). Accumulation of PLs in the outer leaflet of the OM increases membrane permeability to hydrophobic molecules such as bile salts. Therefore, most Gram-negative organisms tend to remove or destroy surface exposed PLs from the OM to retain membrane integrity and barrier role. Inactivation of *mla*

genes increases membrane permeability while inactivation of *pldA* or *pagP* does not show similar increased permeability (Malinverni and Silhavy, 2009). Moreover, the Mla system prevents PLs from accumulating in the absence of PldA. Considering these facts together, it is suggested that the Mla system is the most important pathway that maintains lipid asymmetry in Gram-negative bacteria (Malinverni and Silhavy, 2009). Interestingly, *mlaA* had been previously identified as a homologue of *vacJ*, a virulence gene required for intracellular spreading of *Shigella flexneri* (Suzuki *et al.*, 1994). However, mutation in *mlaA* orthologs does have a direct effect on the early steps of pathogenesis, and MlaA is required together with other factors to evade host response (Malinverni and Silhavy, 2009, Suzuki *et al.*, 1994). Therefore, the Mla pathway is considered as an important virulence factor, in enteric bacteria.

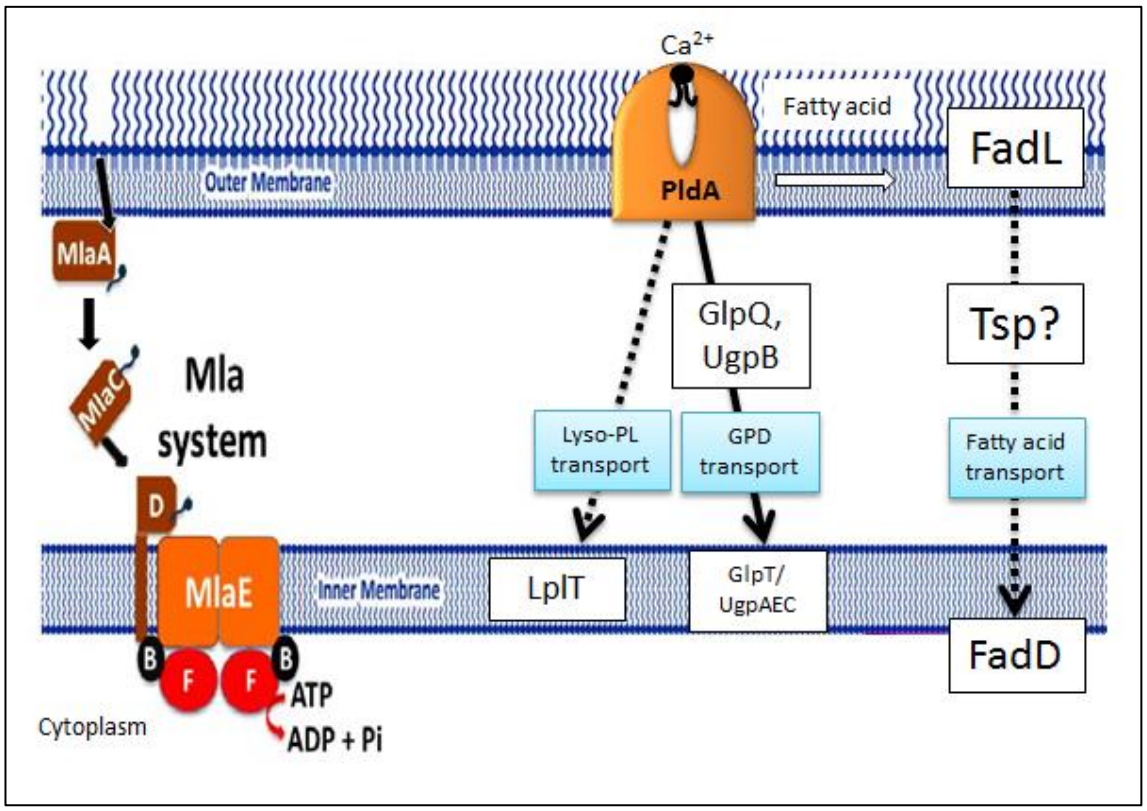


Figure 5.1 Schematic representation of the Mla and PldA pathways. In the Mla pathway, PLs are removed from the inner leaflet of the OM or after migration to the outer leaflet of the OM by MlaA, and delivered by the periplasmic substrate binding protein MlaC to the MlaFEDB complex at the IM. Other retrograde PL trafficking systems may exist but still unknown. High levels of PldA destroy surface-exposed PL, and result in producing free fatty acids, lyso-PLs and glycerophosphodiesters (GPDs). Proteins shown in white boxes have not been directly proven to be involved in the PldA pathway. Dashed arrows represent unknown modes of transport across the periplasm. Image adapted from (Malinverni and Silhavy, 2009).

In this study, all of the components of a putative Mla system in *C. jejuni* 11168 have been identified by homology search. The ABC transporter genes seem to form an operon (*cj1646–cj1648*), while two other genes *mlaA* (*cj1371*) and *mlaC* (*cj1372*) form a separate cluster. It was also found that the *mlaC* downstream gene (*cj1373*) is located in the same operon and shares the ribosomal binding site of *mlaC*, suggesting that it might have a possible role in the Mla pathway. The function of the MlaA homologue in *C. jejuni* has not been previously fully investigated, and it remains unclear whether it is possibly involved in the intracellular spreading of *C. jejuni* into host intestinal cells. This chapter will provide an insight into the biological function of the Mla system in *C. jejuni* and its role in maintaining lipid asymmetry and membrane integrity based on phenotypic evidence. The effect of mutagenesis of Mla candidate genes; *mlaA* (*cj1371*), *mlaC* (*cj1372)* and (*cj1373*) on the growth of *C. jejuni* under stress conditions will be presented here. Also, the mutagenesis effect of these genes on the sensitivity to SDS and antimicrobial peptides will be demonstrated in a comparative analysis between the wild-type and the *mla* mutants cells. Finally, the effect of mutagenesis of *mla* genes on the *in vitro* motility and biofilm formation will be discussed.

5.2 Results

5.2.1 Analysis of the complete genome sequence of *C. jejuni* NCTC 11168 reveals the presence of Mla homologues

BLAST searches of the genome sequence of *C. jejuni* NCTC 11168 using the MlaA and MlaC of the *E. coli* as query proteins revealed two proteins encoded in this genome homologous to MlaA and MlaC, namely Cj1371 and Cj1372. The Cj1371 shows 33 % identity to MlaA in 196 amino acid overlap (residue 37 – 228 and 29 – 222); score 203 E (10000): 4e-21 (Figure 5.2A). The Cj1372 shows 24 % identity to MlaC in 133 aa overlap (residue 59 – 186 and 68 – 200); score: 171 E (10000): 1.8e-10 (Figure 5.2B). In *C. jejun* NCTC 11168, the *cj1371* gene is located upstream of *cj1372* and *cj1373*, and has an upstream promoter and ribosome binding site (RBS) whereas *cj1372* and *cj1373* share the same RBS (Figure 5.2F), suggesting that the two genes (*cj1372* and *cj1373*) are possibly co-transcribed and translationally coupled. We also could identify the ABC transporter genes in *C. jejuni* NCTC 11168 that appears to form one operon (*cj1646 – cj1648*) by BLAST search using MlaD, MlaE and MlaF of *E. coli* as query sequences. The Cj1646 shows 27.6 % identity to MlaE in 214 aa overlap (61-274:152-364); score: 343 E (10000): 2.5e-27 (Figure 5.2C). The best candidate for MlaD (the periplasmic lipid binding protein in *E. coli*) is Cj1648. It shows 24.6 % identity to MlaD in 118 aa overlap (1-117:1-116); score: 118 E (10000): 5.7e-05 (Figure 5.2D). The Cj1647 is equivalent to MlaF as BLAST search shows 31.8 % identity in 239 aa overlap (8-245:2-240); score: 447 E (10000): 4.9e-38 (Figure 5.2E).

The protein sequence of Cj1373 indicates that it is a huge transmembrane protein which is conserved in most *Campylobacter* species. BLAST search using the *E. coli* MlaD as a query sequence indicated that Cj1373 is clearly not a homologue of MlaD in *E. coli*, therefore indicating that it is an unrelated protein in *E. coli* but is present in *C. jejuni*. However, it is clearly co-transcribed with *mlaC* and could be related to the function of Mla system. It has conserved domains related to the RND transporter family, in which members share the machinery for the biogenesis of hopanoid lipids. Moreover, genes in this family are usually located proximal to other components of this biological process. Therefore, it is possibly an exporter for PLs which is not present in the *E. coli* Mla system.

A

```
       30        40        50        60        70        80
MlaA   SDPLEGFNRTMYNFNFNVLDPYIVRPVAVAWRDYVPQPARNGLSNFTGNLEEPAVMVNYF
       .::: :.:..: .::  . : : .:::  ..   .:.  : :  ::  ::  :  .:.
Cj1371 NDPLSGYNKAMTSFNVALYD-YGLRPVLKGYNAITPEFIRLGARNFFDNLLAPLRFVSNV
         40        50         60        70        80        90

       90       100       110       120       130       140
MlaA   LQGDPYQGMVHFTRFFLNTILGMGGFIDVAGMANPKLQRTEPHRFGSTLGHYGVGYGPYV
       ::    ..  .: ::  :::.:.::..:::.  . :    .:  .:..:.:.::: : ..
Cj1371 LQFKFEEAGEEFKRFTANTIMGFGGLMDVASKMGLK---KHPADLGTVLAHWGVGSGFHI
         100       110       120       130          140       150

      150       160       170         180       190       200
MlaA   QLPFYGSFTLRDDGGDMADGLYPVLSWL--TWPMSVGKWTLEGIETRAQLLDSDGLLRQS
        ::. :  .:::     :       ...  ::   . .    : :   .: . : . ...
Cj1371 VLPILGPSNLRDTLTLPATWYASFTAYIDPTWASIAISAYGFGNELSFRLDEIDEIYHNT
            160       170       180       190       200       210

        210       220
MlaA   SDPYIMVREAYFQRHD
        . : ..:.:: ::..
Cj1371 PNLYPFLRDAYEQRRN
            220
```

B

```
        70        80        90       100       110       120
MlaC   VQVKYAGALVLGQYYKSATPAQREAYFAAFREYLKQAYGQALAMYHGQTYQIAPEQPLGD
       .. :  . : :.. :.. :: ..: . .::.  ::... . :..:. :. ..   .  ..
Cj1372 IDYKLMAKLSLSKNYSKLTPKEQEKFTTAFETSLKKSFTDKLSLYKDQVLKVKNGELKNE
       60        70        80        90       100       110

       130       140       150       160       170       180
MlaC   KTIVPIRVTIIDPNGRPPVRLDFQWRKNSQTGNWQAYDMIAEGVSMITTKQNEWGTLLRT
       :        ..: . .     .. ..  ....::  ::. . :::.. : ....: .: .
Cj1372 KRYFLTTSMVVDGEEK-----NIIFKFYNDNNNWLIYDVDVLGVSIVQTYRSQFGDILAN
      120       130            140       150       160       170

       190       200
MlaC   KGIDGLTAQLKSI
       .:.:.:  .:.::
Cj1372 QGFDALLQKLESI
           180
```

C

```
              70        80        90       100       110       120
MlaE   LVRQLYNVGVLSMLIIVVSGVFIGMVLGLQGYLVLTTYSAETSLGMLVALSLLRELGPVV
       .. .. : .  .. :.......:.::. :.   :. ..:.  .  :...:  :::.:..
Cj1646 FLYHIENSAFKALPIVILTALLVGVVLAYQAAYQLAQFGANIFIVDLMGISATRELAPLI
             160       170       180       190       200       210

              130       140       150       160       170       180
MlaE   AALLFAGRAGSALTAEIGLMRATEQLSSMEMMAVDPLRRVISPRFWAGVISLPLLTVIFV
       ::...:::..:. ::.::.:. :.....:. :.   .. .. ::  : :...::...:
Cj1646 AAIVIAGRSASSYTAQIGVMKITDEIAAMNTMGFRSFEFIVIPRVMALVVAMPLIVAISD
             220       230       240       250       260       270

              190       200       210       220       230       240
MlaE   AVGIWGGSLVGVSWKGIDSGFFWSAMQNAVDWRMDLVNCLIKSVVFAITVTWISLFNGYD
       :..: :: .:.     :. . :   ..:::: .  ... : :. .:.. .  :. : :..
Cj1646 AISIIGGMMVAKLNLDISFAEFLRRFREAVDIKHIFIG-LAKAPIFGFLIGLIACFRGFE
             280       290       300       310       320       330

              250       260       270
MlaE   AIPTSAGISRATTRTVVHSSLAVLGLDFVLTALM
       .  :. .:.  ::..::.. . :...: ......
Cj1646 VKNTTQSIGIYTTKSVVNAIFWVIAFDALFSVIL
             340       350       360
```

D

```
         10        20        30        40        50        60
MlaD   MQTKKNEIWVGIFLLAALLAALFVCLKAANVTSIRTEPTYTLYATFDNIGGLKARSPVSI
       :... : ..::.:.....:.:   :  .. .. ..   : .. : ....::  ..:: .
Cj1648 MENRANYFFVGLFVFGVFFASLGFILWLGGYSKEESFKYYEIH-TQESVAGLGIKAPVRL
              10        20        30        40         50

              70        80        90        100       110
MlaD   GGVVVGRVADITLDPKTYLPRVTLEIEQRYNH-IPDTSSLSIRTSGLLGEQYLALNVG
        :: :: : .:..  .  :  :...:. . :  : . .  ... .:. : ... :. :
Cj1648 LGVEVGSVEEISIYNQDELG-VNIRIKVKNNTPIKEDTFATLQLQGITGLKFIQLQGG
      60        70         80        90       100       110
```

E

```
        10        20        30        40        50        60
MlaF   LVDMRDVSFTRGNRCIFDNISLTVPRGKITAIMGPSGIGKTTLLRLIGGQIAPDHGEILF
       ..  ...    :.. . :..:. . ...: .:.: :: ::..::. .      : ::  .
Cj1647 VIKAQNIITKFGEKIVHDGVSFEIKKNEIFGILGGSGSGKSVLLKQMLMLEHFDDGEYEI
             10        20        30        40        50        60

        70        80        90       100       110       120
MlaF   DGENIPAMSRSRLYTVRKRMSMLFQSGALFTDMNVFDNVAYPLREHTQLPAPLLHSTVMM
        : ..  ...    ..::. ...:: .:::. .::..:.: ::.:.:.:    ..  :.:
Cj1647 LGYKLKNINEEDALALRKKWGVVFQFAALFSFFNVYENIAIPLKEYTHLDENSIQELVLM
             70        80        90       100       110       120

       130       140       150       160       170       180
MlaF   KLEAVGL-RGAAKLMPSELSGGMARRAALARAIALEPDLIMFDEPFVGQDPITMGVLVKL
       ::. ::: ... : .::::::: .:.:.:::.::.  :...:::  : :: .   .  :
Cj1647 KLKMVGLNESVLKQFPSELSGGMQKRVAIARALALDSKLLFLDEPTSGLDPHSSREFDDL
             130       140       150       160       170       180

        190       200       210       220       230       240
MlaF   ISELNSALGVTCVVVSHDVPEVLSIADHAWILADKKIVAHGSAQALQANPDPRVRQFLD
       . ::.... .. ..:.::   . .. :.  :: .::.   :. . :. . .   ..:..
Cj1647 VLELKKSFDLNIILVTHDKESMKNLLDRFIILENKKVGFCGTYEELRLQNERLFKRFME
             190       200       210       220       230       240
```

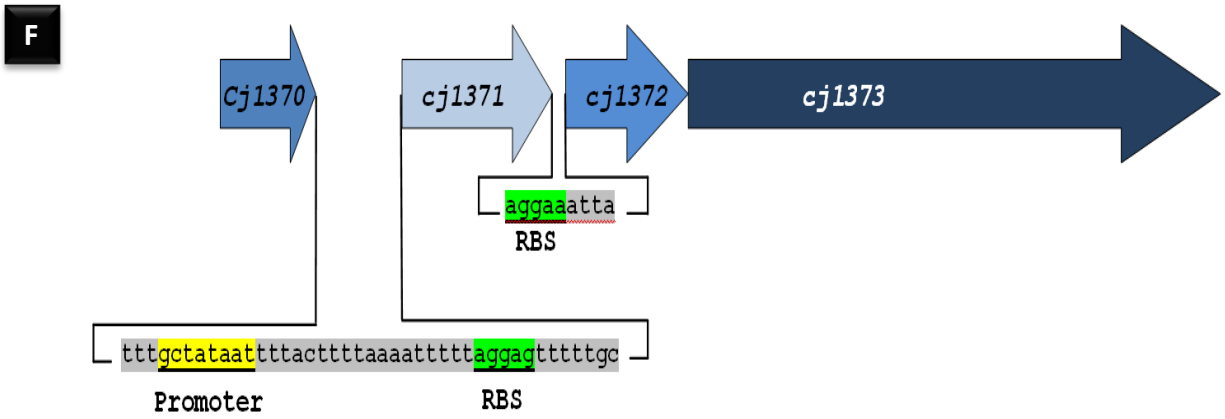
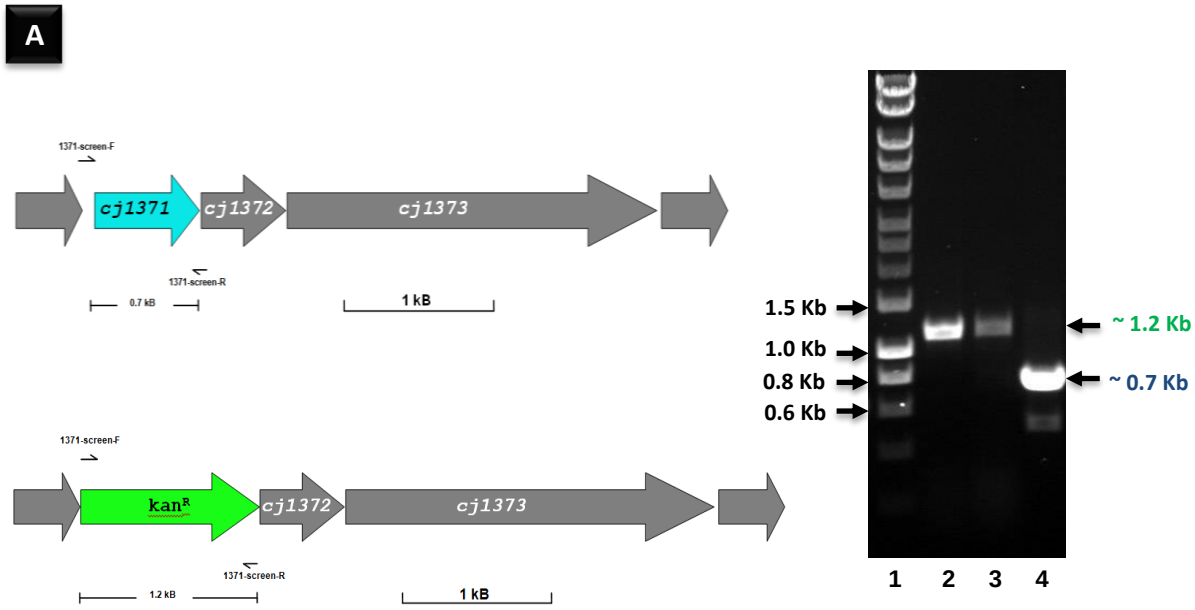


Figure 5.2 The *E. coli* Mla homologues in *C. jejuni* NCTC 11168. A) Sequence alignment analysis of the 251 aa MlaA and the 232 aa Cj1371shows a 33 % identity in the 196 amino acids overlap (residue 55 – 246 and 29 – 222, respectively; score 282 E (10000): 2e-21) as reported by LALIGN alignment tool of the Swiss EMBnet node server (http://www.ch.embnet.org/software/LALIGN_form.html). **B)** Sequence alignment analysis of the 211 aa MlaC and the 189 aa Cj1372 shows 24 % identity in 133 aa overlap (residues 68 – 200 and 59 – 186, respectively); score: 171 E (10000): 1.8e-10. **C)** Sequence alignment analysis of the 260 aa MlaE and the 369 aa Cj1646 shows 27.6 % identity in 214 aa overlap (residues 61 – 274 and 152 – 364, respectively); score: 343 E (10000): 2.5e-27. **D)** Sequence alignment analysis of the 183 aa MlaD and the 296 aa Cj1648 shows 24.6 % identity in 118 aa overlap (residues 1 – 117); score: 118 E (10000): 5.7e-05. **E)** Sequence alignment analysis of the 269 aa MlaF and the 240 aa Cj1647 shows 31.8 % identity in 239 aa overlap (restudies 8 – 245 and 2 – 240, respectively); score: 447 E (10000): 4.9e-38. **F)** Diagrammatic representation of the genes *cj1371-73* region in the genome of *C. jejuni* NCTC 11168. Upstream of the *cj1371* is promoter sequence (underlined and highlighted in yellow) and ribosome binding site (RBS) (underlined and highlighted in green). The *cj1372* and *cj1373* share the same RBS which is located upstream of the *cj1372* (underlined and highlighted in green).

5.2.2 Generation of *mla* mutants

Plasmids containing deletions of the putative *mla* genes (*Δcj1371*, *Δcj1372* and *Δcj1373*) were generated by Dr Arnoud van Vliet (Institute of Food Research, Norwich, UK), using *C. jejuni* NCTC 81116 genomic DNA, and were kindly gifted to be used in transformation of *C. jejuni* NCTC 11168 strain. The knock-out constructs were generated by SOE (Splicing by Overlapping Extension) PCR, in which the wild-type genes were replaced by a kanamycin resistance cassette (kan^R) in the same orientation as the genes. DNA fragments were cloned into pGEM-T Easy vector to generate p*1371*, p*1372* and p*1373*. These plasmid constructs were utilised to electro-transform the *C. jejuni* NCTC 11168 strain to generate *Δcj1371*, *Δcj1372* and *Δcj1373* single mutants. The growth of the mutants was selected on kanamycin-containing media and the gene replacement was confirmed by colony PCR. For screening of the *Δcj1371* mutant, the primers 1371-screen-F and 1371-screen-R were used (Figure 5.3A) which shows a replacement of the wild-type *cj1371* genes with the kan^R cassette, while for screening of the *Δcj1372* mutant the primers 1372-screen-F and 1372-screen-R were used (Figure 5.3B) which shows a replacement of the wild-type *cj1372* genes with the kan^R cassette. For screening of the *Δcj1373* mutant the primers 1373-screen-F and 1373-screen-R were used (Figure 5.3C). The resulting ~1.2 Kb PCR product is representative for replacement of the wild-type *cj1373* genes with the kan^R cassette, which confirms successful mutation.



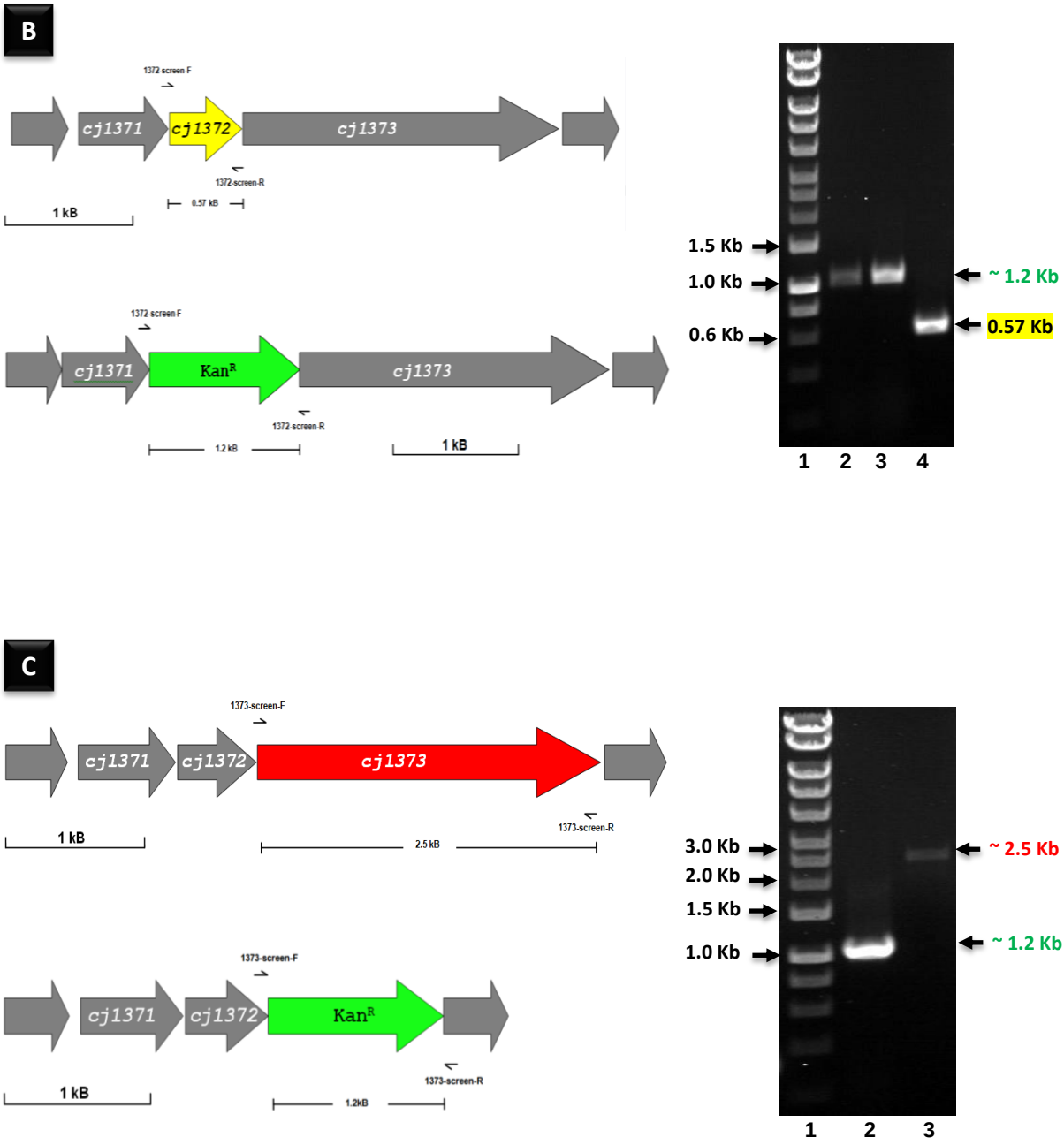


Figure 5.3 Overloaded 0.7 % agarose gel electrophoresis confirm the deletion of mla genes and replacement with kan^R cassette. A) Screening of the *Δcj1371* mutant. Lane 1: Hyperladder™ I molecular weight marker (Bioline, UK). Lane 2 and 3: kan^R cassette from the *Δcj1371* mutant cells. Lane 4: the wild-type *cj1371* from the *C. jejuni* 11168 chromosomal DNA. **B)** Screening of the *Δcj1372* mutant. Lane 1: Hyperladder™ I molecular weight marker (Bioline, UK). Lane 2 and 3: kan^R cassette from the *Δcj1372* mutant cells. Lane 4: the wild-type *cj1372* from the *C. jejuni* 11168 chromosomal DNA. **C)** Screening of the *Δcj1373* mutant. Lane 1: Hyperladder™ I molecular weight marker (Bioline, UK). Lane 2: kan^R cassette from the *Δcj1373* mutant cells. Lane 3: the wild-type *cj1373* from the *C. jejuni* 11168 chromosomal DNA.

5.2.3 Complementation

As for *mla* mutants, complementation plasmids were obtained as a generous gift from Dr Arnoud van Vliet (Institute of Food Research, Norwich, UK). Copies of DNA sequences of the mutated genes were integrated into the *C. jejuni* NCTC 11168 *cj0046* pseudogene or equivalent in NCTC 81116 strain, under the control of the *fdxA* promoter with chloramphenicol resistance cassette (cat^R) in the pCfdxA complementation vector. The resulting complementation plasmids p*CfdxA1371*, p*CfdxA1372* and p*CfdxA1373* were used to transform the *Δcj1371*, *Δcj1372* and *Δcj1373* mutants respectively to generate *Δcj1371/cj1371*$^+$, *Δcj1372/cj1372*$^+$ and *Δcj1373/cj1373*$^+$ complemented strains. Successful complementation was achieved to generate *Δcj1371/cj1371*$^+$ and *Δcj1372/cj1372*$^+$ complemented strains, but not *Δcj1373/cj1373*$^+$ although several attempts were performed. The growth of complemented strains was selected on chloramphenicol- and kanamycin-containing media. Colony PCR using 46F and 46R primers was performed to confirm the correct insertion of wild-type copies of mutated genes into the *cj0046* pseudogene (Figure 5.4).

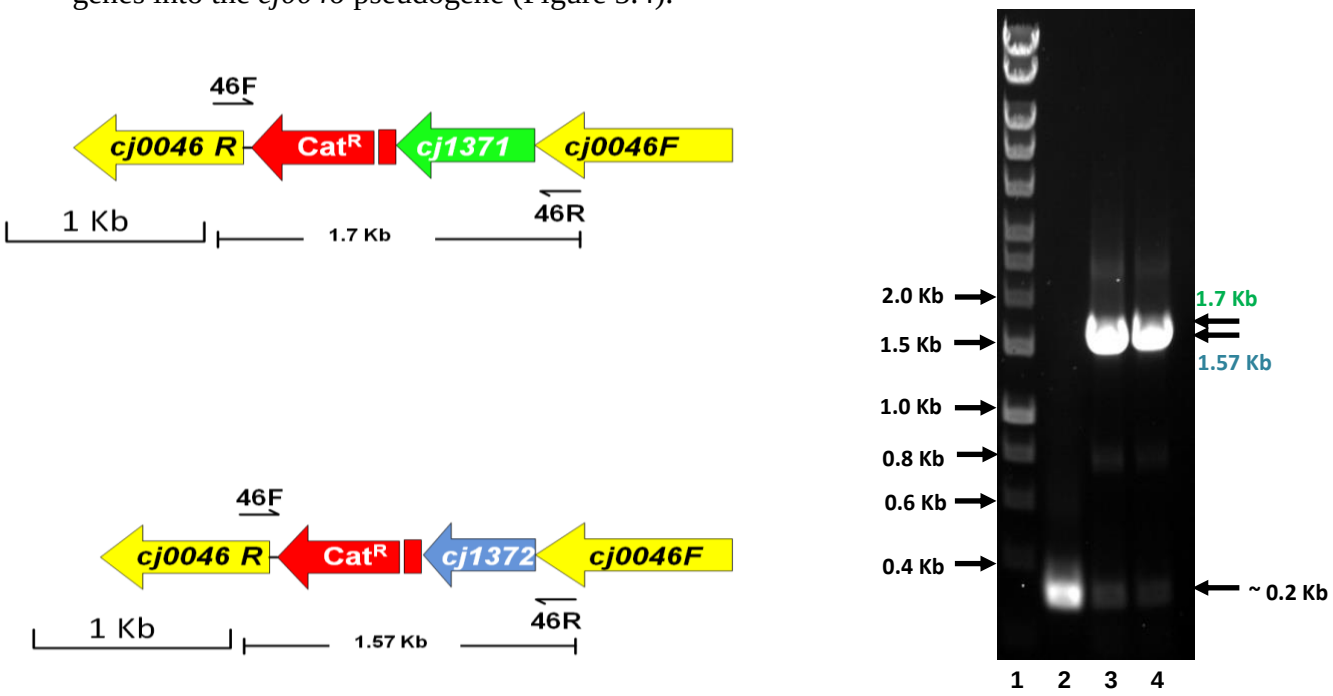


Figure 5.4 Agarose gel electrophoresis showing gene complementation of the *Δcj1371* and *Δcj1372* mutants. Lane 1: Hyperladder™ I molecular weight marker (Bioline, UK). Lane 2: PCR product (~ 0.2 Kb) of the *cj0046* from the wild-type genomic DNA using 46F and 46R primers. Lane 3: PCR product (~ 1.6 Kb) of the *cj0046* from the genomic DNA of the *Δcj1372/1372*$^+$ complemented strain using same set of primers. . Lane 4: PCR product (~ 1.7 Kb) of the *cj0046* from the genomic DNA of the *Δcj1371/1371*$^+$ complemented strain using same set of primers.

5.2.4 Growth assays of *mla* mutants in *C. jejuni*

5.2.4.1 Hyperosmotic stress causes a defective growth of the *Δcj1371* mutant

In stress conditions, phospholipids from the IM are moved to the outer leaflet of the OM and accumulate in the defective area causing reduced membrane barrier role (Nikaido, 2005). Previous work on the newly identified Mla pathway in *E. coli* showed that Mla proteins inhibit phospholipids from accumulating in the outer leaflet of the OM (Malinverni and Silhavy, 2009), causing re-maintenance of the lipid asymmetry in the OM. Here, the growth phenotype of the *mlaA* homologue mutant (*Δcj1371*) was examined to determine the effect of *cj1371* gene deletion on the growth of *C. jejuni* under stress conditions. The microaerobic growth of *Δcj1371* in cultures containing MHS media in the presence or absence of hyperosmotic stress agent (1.0 % NaCl) was monitored and compared to that of the wild-type and complemented strains. In the presence of stress agent (1.0 % NaCl) the microaerobic growth of the *Δcj1371* mutant was significantly retarded comparing to those of the wild-type and the *Δcj1371/cj1371*$^{+}$ complemented strain (Figure 5.4). Also, the retardation was more severe than that of the *Δcj1371* mutant grown in the absence of 1.0 % NaCl (Figure 5.5). The *Δcj1371* mutant had a doubling time of 6.5 hours when grown under hyperosmotic stress or 3.5 hours in absence of stress agent. The wild-type and the *Δcj1371/cj1371*$^{+}$ complemented strain were not affected significantly by hyperosmotic stress where they had a doubling time of 3 hours in the presence or absence of hyperosmotic stress agent.

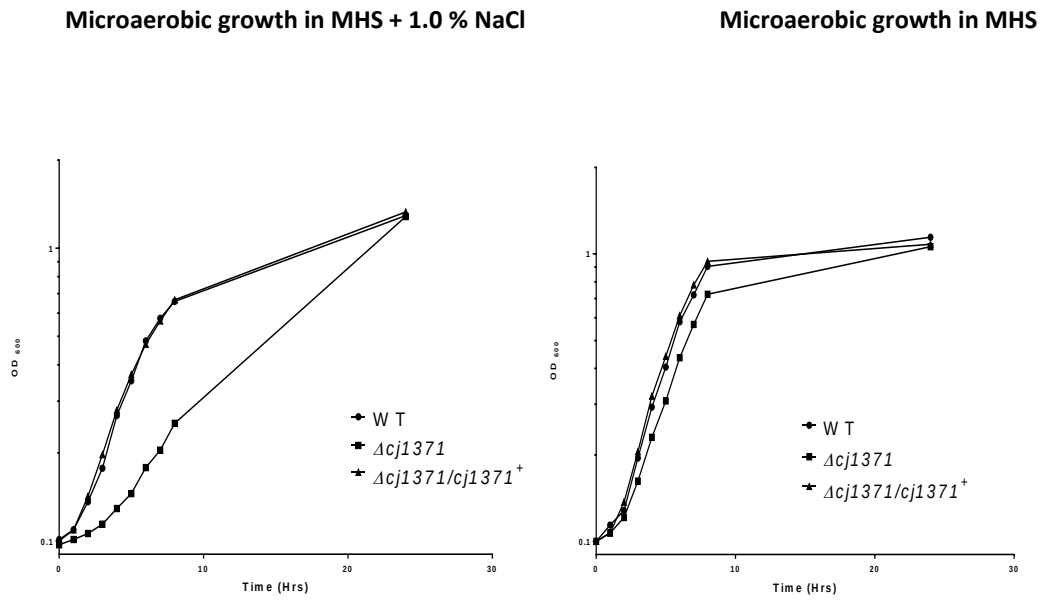


Figure 5.5 Hyperosmotic stress effect on the growth of the *Δcj1371* mutant. The wild-type, *Δcj1371* mutant and *Δcj1371/cj1371*$^{+}$ complemented strain cells were grown microaerobically in 50 mL cultures containing MHS (left) or MHS + 1.0 % NaCl as a hyperosmotic stress agent (right). Cultures were contained in 250 mL conical flasks shaken at 180 rpm in a gas atmosphere of 10 % *(v/v)* oxygen, 5 % *(v/v)* carbon dioxide and 85 % *(v/v)* nitrogen. Samples were collected at time 0 – 8 hours and a final sample at 24 hours. Cell densities were measured spectrophotometrically at OD_{600} and data were plotted on graphs using GraphPad Prism 6 (GraphPad Software Inc.). Figures are representative experiments of at least two biological replicates with similar results. The doubling time for the *Δcj1371* mutant was 6.5 hours in the presence of 1.0 % NaCl, and 3.5 hours in absence of 1.0 % NaCl. The doubling time for the wild-type and the *Δcj1371/cj1371*$^{+}$ complemented strain was 3 hours in the presence or absence of 1.0 % NaCl, unless for the late-log phase where the growth rate reduced.

5.2.4.2 The growth rate of the *Δcj1372* mutant is slightly lowered by hyperosmotic stress

The microaerobic growth of the *mlaC* homologue mutant (*cj1372* encoding the periplasmic binding protein) in *C. jejuni* (*Δcj1372*) was also examined to investigate the effect of stress conditions on *C. jejuni* lacking the *mlaC* gene. The *Δcj1372* mutant was grown in cultures containing MHS in the presence or absence of hyperosmotic stress agent (1.0 % NaCl). Under stress conditions, the microaerobic growth of the *Δcj1372* mutant was slightly retarded in comparison to the wild-type and *Δcj1372/cj1372*$^{+}$. It had a doubling time of 3 hours and 45 minutes in the presence of 1.0 % NaCl, or 3 hours and 15 minutes in the absence of 1.0 % NaCl (Figure 5.6) which was similar to the growth of the wild-type and the *Δcj1372/cj1372*$^{+}$ complemented strain when grown in MHS lacking stress agent. This suggests that if Cj1372 has a role in the maintenance of lipid asymmetry in the OM of *C. jejuni*, it may not have a major direct role in controlling PLs in the OM.

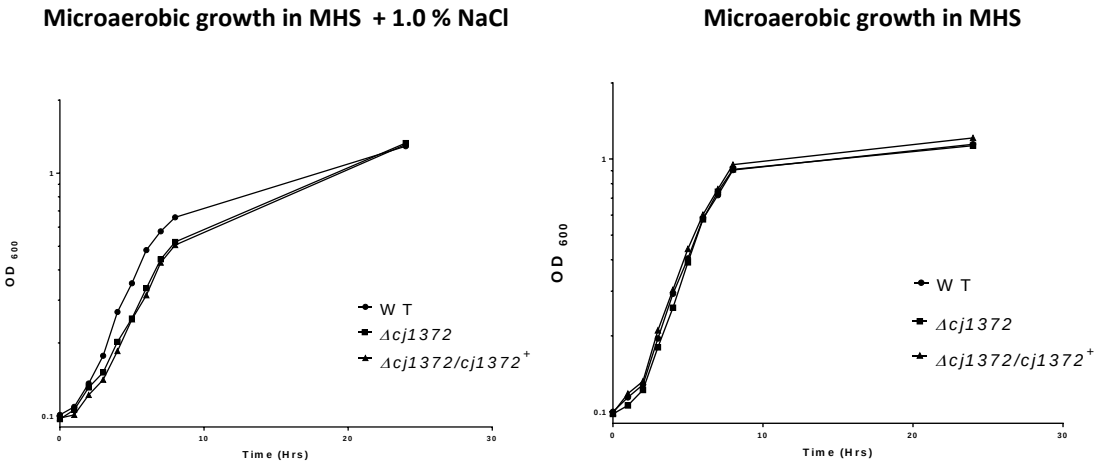


Figure 5.6 Hyperosmotic stress effect on the growth of the *Δcj1372* mutant. The wild-type, *Δcj1372* mutant and *Δcj1372/cj1372*$^{+}$ complemented strain cells were grown microaerobically in 50 mL MHS cultures contained in 250 mL conical flasks shaken at 180 rpm in the gas atmosphere of 10 % *(v/v)* oxygen, 5 % *(v/v)* carbon dioxide and 85 % *(v/v)* nitrogen. Growth was tested in MHS media in the presence (left) or absence (right) of 1.0 % NaCl, and data are representative of at least two independent experiments with similar results. The doubling time of the *Δcj1372* mutant was 3 hours and 45 minutes in the presence of 1.0 % NaCl, and had a similar growth to the wild-type and the *Δcj1372/cj1372*$^{+}$ complemented strain (3 hours and 15 minutes) in the absence of 1.0 % NaCl.

5.2.4.3 Deletion of *cj1373* does not alter the growth of *C. jejuni* under hyperosmotic stress conditions.

The third protein which might be involved in the Mla pathway is the inner membrane anchored substrate-binding protein Cj1373. The microaerobic growth of the *Δcj1373* mutant was monitored in cultures of MHS in the presence or absence of hyperosmotic stress factor (1.0 % NaCl). Under hyperosmotic stress conditions, the growth of the *Δcj1373* mutant was similar to that of the wild-type strain with no significant growth defect in comparison to the wild-type. The doubling time of both strains was 3 hours under hyperosmotic stress, or 2 hours and 45 minutes in media lacking stress agent (Figure 5.7). Thus, the growth rate of both strains in the late log-phase was slightly reduced in the presence of stress agent.

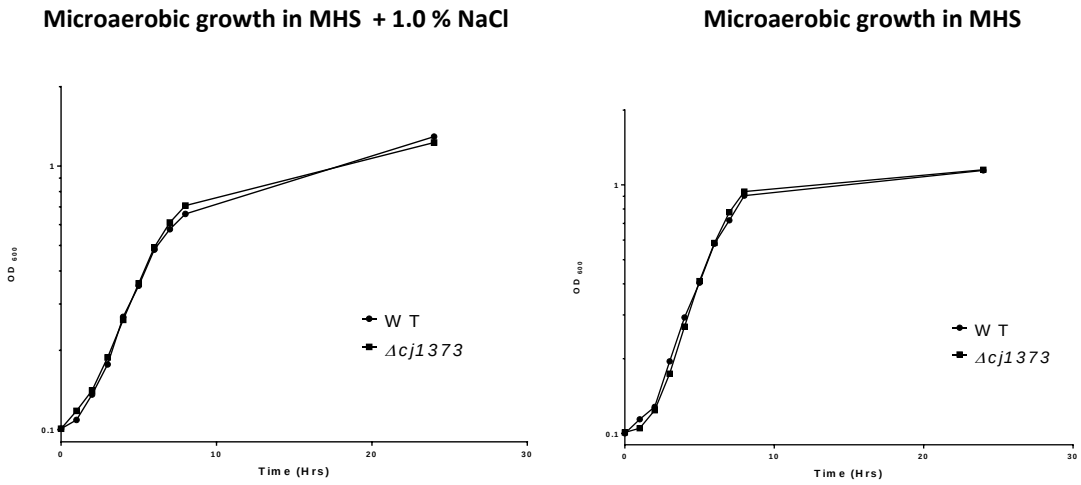


Figure 5.7 Hyperosmotic stress effect on the growth of the *Δcj1373* mutant. The wild-type and the *Δcj1373* mutant cells were grown microaerobically in 50 mL MHS cultures contained in 250 mL conical flasks shaken at 180 rpm in the gas atmosphere of 10 % *(v/v)* oxygen, 5 % *(v/v)* carbon dioxide and 85 % *(v/v)* nitrogen. Growth was tested in MHS media in the presence (left) or absence (right) of 1.0 % NaCl. The doubling time of the *Δcj137*3 mutant and the wild-type was similar (3 hours) in the presence of 1.0 % NaCl, and 2 hours and 45 minutes in the absence of 1.0 % NaCl. Data are representative of at least two independent experiments with similar results.

5.2.5 Deletion of *cj1371* or *cj1372* results in an increased sensitivity of *C. jejuni* to antibiotics and SDS

To investigate whether the Mla proteins in *C. jejuni* are involved in maintaining the integrity of the OM, it was suggested that mutation in one or more of their cognate genes may result in phenotypes that indicate a damaged or permeable OM, and therefore, more sensitive phenotypes to various antimicrobial agents that require entry to the bacterial cell through the OM. This was tested by addition of antimicrobial agent to paper disc in the centre of MHS plates containing the wild-type or mutant strain. The sensitivity is represented by formation of a clear zone of inhibition around the antimicrobial agent-containing disc after 2 – 3 day of microaerobic incubation. Four antimicrobial agents were used including ampicillin (200 µg mL^{-1}), polymyxin B (200 µg mL^{-1}), vancomycin (400 µg mL^{-1}) and SDS (40 %). Results were obtained from three independent biological replicates. The *Δcj1371* mutant was found to be significantly more sensitive to all tested agents than the wild-type ($p < 0.01 – 0.0001$) whereas the *Δcj1372* mutant showed no significant difference to the wild-type with ampicillin and SDS but was significantly more sensitive to polymyxin B and vancomycin ($p < 0.01$) (Figure 5.8). These findings suggest that the mutants cells have lowered ability to prevent the entry of antimicrobial agents into the cell, and that the MlaA and MlaC proteins may have a direct or indirect role in maintaining the OM integrity as deletion of their cognate gene results in more sensitive phenotypes.

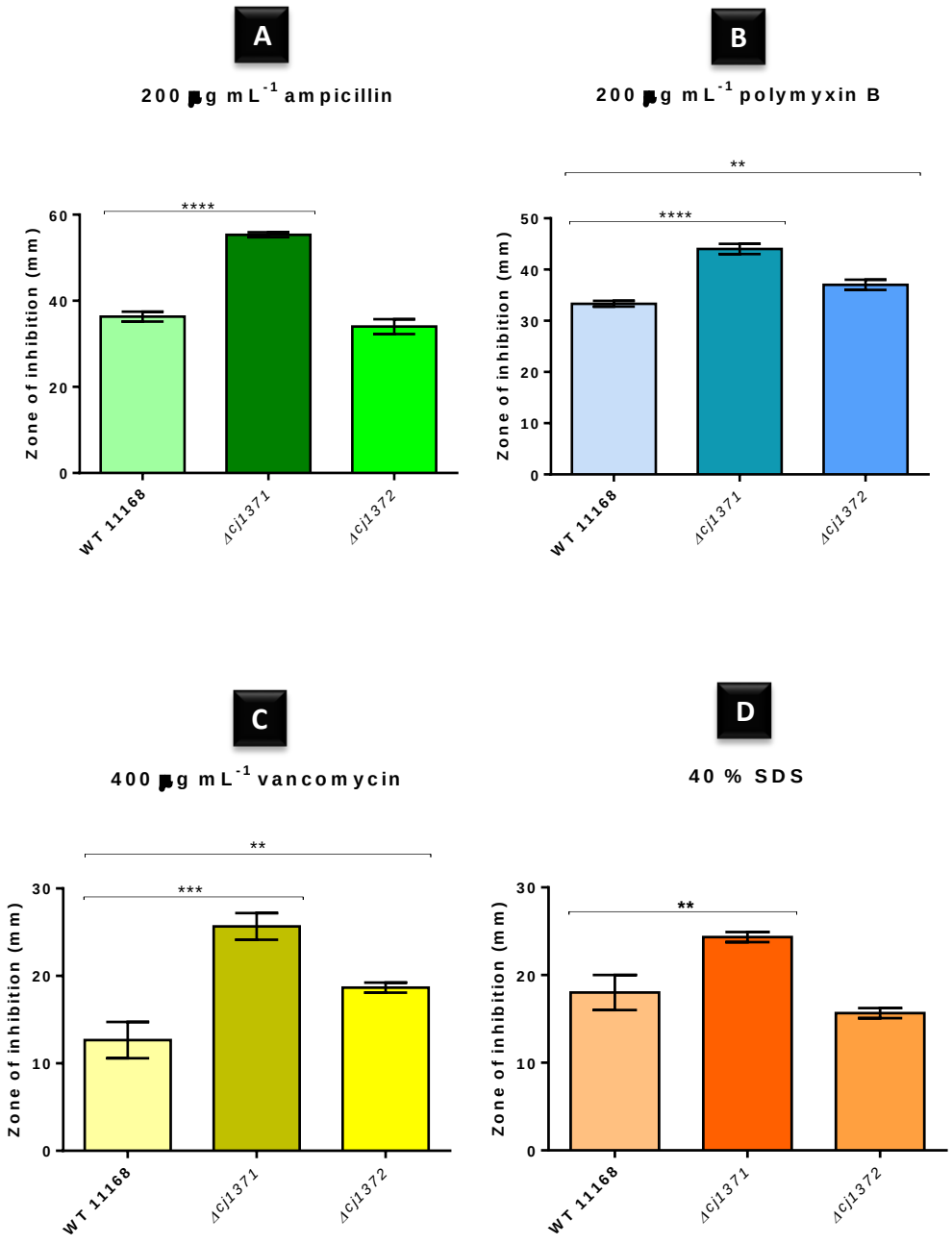


Figure 5.8 Disc diffusion assay for the *Δcj1371* and *Δcj1372* mutants. **A)** Sensitivity of the *Δcj1371* and *Δcj1372* mutants to 200 µg mL^{-1} ampicillin. **B)** Sensitivity of the *Δcj1371* and *Δcj1372* mutants to 200 µg mL^{-1} polymyxin B. **C)** Sensitivity of the *Δcj1371* and *Δcj1372* mutants to 400 µg mL^{-1} vancomycin. **D)** Sensitivity of the *Δcj1371* and *Δcj1372* mutants to 40 % SDS. The wild-type and mutant cells were allowed to grow on MHS agars and exposed to various concentrations of antimicrobial agents. Measurements were taken after 2 – 3 days of microaerobic growth from at least three biological replicate plates per strain. Error bars represents the standard deviation of these measurements. Statistical analysis was carried out using Student's multiple t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.2.6 Sensitivity of *mla* mutants to antimicrobial peptides

5.2.6.1 A *cj1371*-deletion mutation increases sensitivity of *C. jejuni* to various antimicrobial peptides

Lipopolysaccharides (LPS) have been known to act as the first barrier in the OM of Gram-negative bacterial cells against antibiotics. Their role can be defined by obstructing the diffusion of chemicals through the OM into the cell (Delcour, 2009). Therefore, if Cj1371 is involved in Mla pathway in *C. jejuni*, then it may have a direct role in maintaining the integrity of the OM under stress conditions. It would be expected that deletion of its cognate gene may lead to an increase in membrane permeability when mutant cells are stressed, allowing large-molecules like antimicrobial peptides (AMPs) to gain entry through the cell membrane causing damage to the cell. AMPs are essential factors of innate immunity which act as a primary line in defence against bacterial infection. They have been known as endogenous antibiotics that act in eliminating micro-organisms by disrupting their cell membrane (Cederlund *et al.*, 2011). They have a high affinity to cell wall molecules such as LPS, and can interact with LPS by breaking the divalent cations and causing loss of LPS compactness, which allows peptides to transport across the OM (Hancock, 1997). Because all bacterial cell membranes are negatively charged, cationic antimicrobial peptides (CAMPs) can kill most Gram-positive and Gram-negative strains by the formation of carpet-like coating over the bacterial membrane causing disintegration of the membrane and consequent bacterial death (Tjabringa *et al.,* 2005). Among these CAMPs is the *C*-terminal region of human cathelicidin (LL-37). It is composed of 37 amino acids with highly hydrophobic *N*-terminal and *C*-terminal regions that form α-helical conformation in the presence of negatively charged lipids (Pasupuleti *et al.,* 2012). This hydrophobicity allows the LL-37 to bind to bacterial membrane and LPS and displays broad spectrum antimicrobial properties (Larrick *et al.*, 1995a, Larrick *et al.*, 1995b, Pasupuleti *et al.*, 2012).

To investigate this, sensitivity of mutant cells to several antimicrobial peptides was examined. These peptides included human cathelicidin (LL-37), polymyxin B, polymyxin E (colistin) and protamine sulphate. Overnight cultures of the wild-type 11168 strain, the *Δcj1371*mutant and the *Δcj1371/cj1371*$^{+}$ complemented strain grown in MHS media were exposed to various concentrations of the above peptides for two

hours and after being serially diluted, they were plated on MHS agar. Sensitivity was measured after 2 days incubation in microaerobic conditions. The *Δcj1371* mutant was shown to be slightly more sensitive to LL-37 than the wild-type. However, the mutant showed a significant increase in sensitivity ($p < 0.05$) compared to the wild-type at 2.0 µM LL-37 as represented by 1-log inhibition in the viability of the mutant compared to the wild-type under the same treatment (Figure 5.9A). On the other hand, the *Δcj1371* mutant was found to be significantly more sensitive than the wild-type to polymyxin B ($p < 0.01 – 0.001$) as represented by 2-logs decrease in the viable cell count relative to the wild-type at 50, 100 and 200 µg mL^{-1} polymyxin B (Figure 5.9B). Complementation of the *Δcj1371* mutant showed a restoration of the wild-type activity as the number of the viable cells was recovered in the complemented strain ($p < 0.05 – 0.01$). In each treatment, the complemented strain showed an increase in the viability compared to the mutant, thus providing an evidence for complementation. In contrast, the sensitivity of the *Δcj1371* mutant was shown to be similar to that of the wild-type strain to polymyxin E with no significant changes (Figure 5.9C). Surprisingly, at concentrations of 50, 100 and 200 µg mL^{-1} polymyxin E, the complementation was not able to restore the wild-type phenotype and showed a significantly lower number of viable cells than the mutant ($p < 0.01$). It would be expected to observe a restoration or partial restoration of the wild-type activity in the complemented strain, but in this case the complementation was not able to show such a restoration. Therefore, the results with complemented strain when exposed to polymyxin E are unexplainable, and provided no evidence for complementation. However, the *Δcj1371* mutant was shown to be significantly more sensitive to protamine sulphate than the wild-type strain ($p < 0.05$) as represented by 2-logs, 3-logs and 4-logs decrease in the viable cell count relative to the wild-type at 50, 100 and 200 µg mL^{-1} protamine sulphate, respectively (Figure 5.9D). Complementation of the *Δcj1371* mutant showed a restoration of the wild-type activity as shown by a significant increase in the number of viable cells compared to the mutant ($p < 0.01 – 0.0001$).

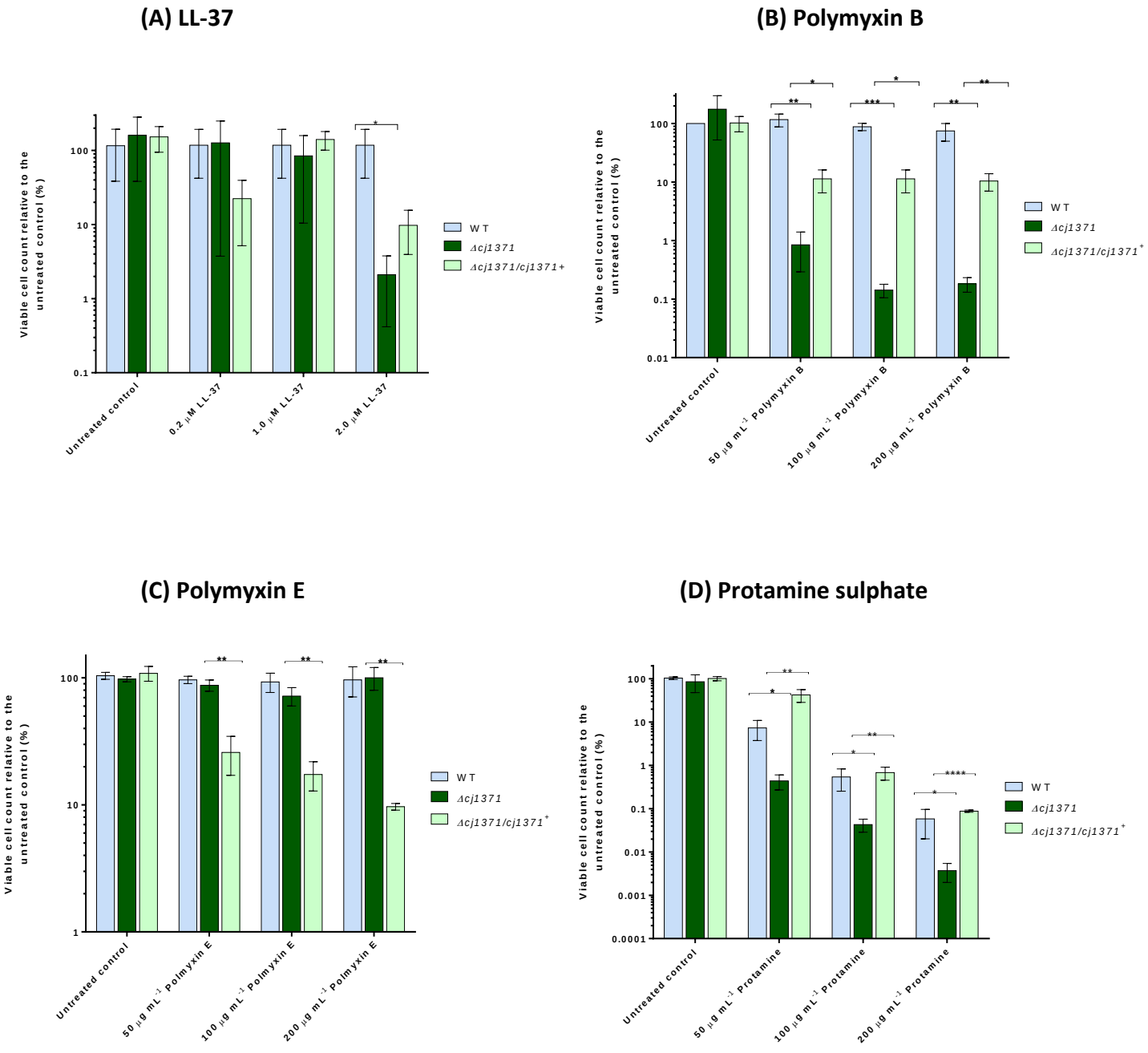


Figure 5.9 Sensitivity of the *Δcj1371* mutant to antimicrobial peptides. Overnight cultures of the *Δcj1371* mutant were exposed to various concentrations of antimicrobial peptides for two hours and were serially diluted to 1 X 10^{-8}. Ten microliters of each dilution were plated on plain MHS, and incubated microaerobically for 2 – 3 days at 37 °C. **A)** Sensitivity of the *Δcj1371* mutant to human cathelicidin (LL-37). **B)** Sensitivity of the *Δcj1371* mutant to polymyxin B. **C)** Sensitivity of the *Δcj1371* mutant to polymyxin E. **D)** Sensitivity of the *Δcj1371* mutant to protamine sulphate. Data are representative of three biological replicates. The cell viability values of the untreated controls were adjusted to 100% to reflect the maximum viability without treatment. Error bars represents the standard deviation of these measurements. Statistical analysis was carried out using Student's multiple t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.2.6.2 The *Δcj1372* mutant is more sensitive to antimicrobial peptides

The sensitivity of the *Δcj1372* mutant to antimicrobial peptides was examined to assess the role of the Cj1372 periplasmic protein in maintaining the integrity of the OM. The same antimicrobial peptides used in the sensitivity of the *Δcj1371* mutant were used here, and the same protocol described in section 5.2.6.1 was followed. The sensitivity of the *Δcj1372* mutant was shown to be significantly increased to LL-37 compared to the wild-type ($p < 0.05$) as represented by 1-log, 2-logs and 3-logs decrease in the viable cell count relative to the wild-type at 50, 100 and 200 µg mL^{-1} LL-37, respectively (Figure 5.10A). Complementation of the *Δcj1372* mutant was unable to show a restoration of the wild-type activity for unknown reason, and the sensitivity of the complemented strain was similar to that of the mutant. The sensitivity of the *Δcj1372* mutant to polymyxin B and polymyxin E was slightly lower than that of the LL-37. With polymyxin B, the *Δcj1372* mutant showed a significant increase in the sensitivity to polymyxin B compared to the wild-type ($p < 0.01$ at 100 µg mL^{-1} and $p < 0.001$ at 200 µg mL^{-1}) as shown by 3-logs decrease in the viable cell count relative to the wild-type (Figure 5.10B). With polymyxin E, the *Δcj1372* mutant showed a less significant increase in the sensitivity to polymyxin E than polymyxin B compared to the wild-type ($p < 0.05$) as shown by 2-logs decrease in the viable cell count relative to the wild-type at 100 µg mL^{-1} polymyxin E, and 3-logs decrease in viability compared to the wild-type at 200 µg mL^{-1} polymyxin E (Figure 5.10C). The wild-type activity was restored in the complemented strain when treated with polymyxin B and E, as represented by significant increases in the cell viability of the complemented strain compared to the mutant ($p < 0.05$), thus, providing an evidence of complementation. Nevertheless, the protamine sulphate showed a similar sensitivity effect to the *Δcj1372* mutant and the wild-type. Similar to LL-37, the complemented strain could not show a restoration of the wild-type activity, and its viability was significantly lower than the mutant at 100 and 200 µg mL^{-1} protamine sulphate ($p < 0.05$). These findings suggest an important role of the Cj1372 periplasmic protein in maintaining the OM integrity, as deletion of the *cj1372* gene showed a phenotype with high sensitivity to various antimicrobial peptides.

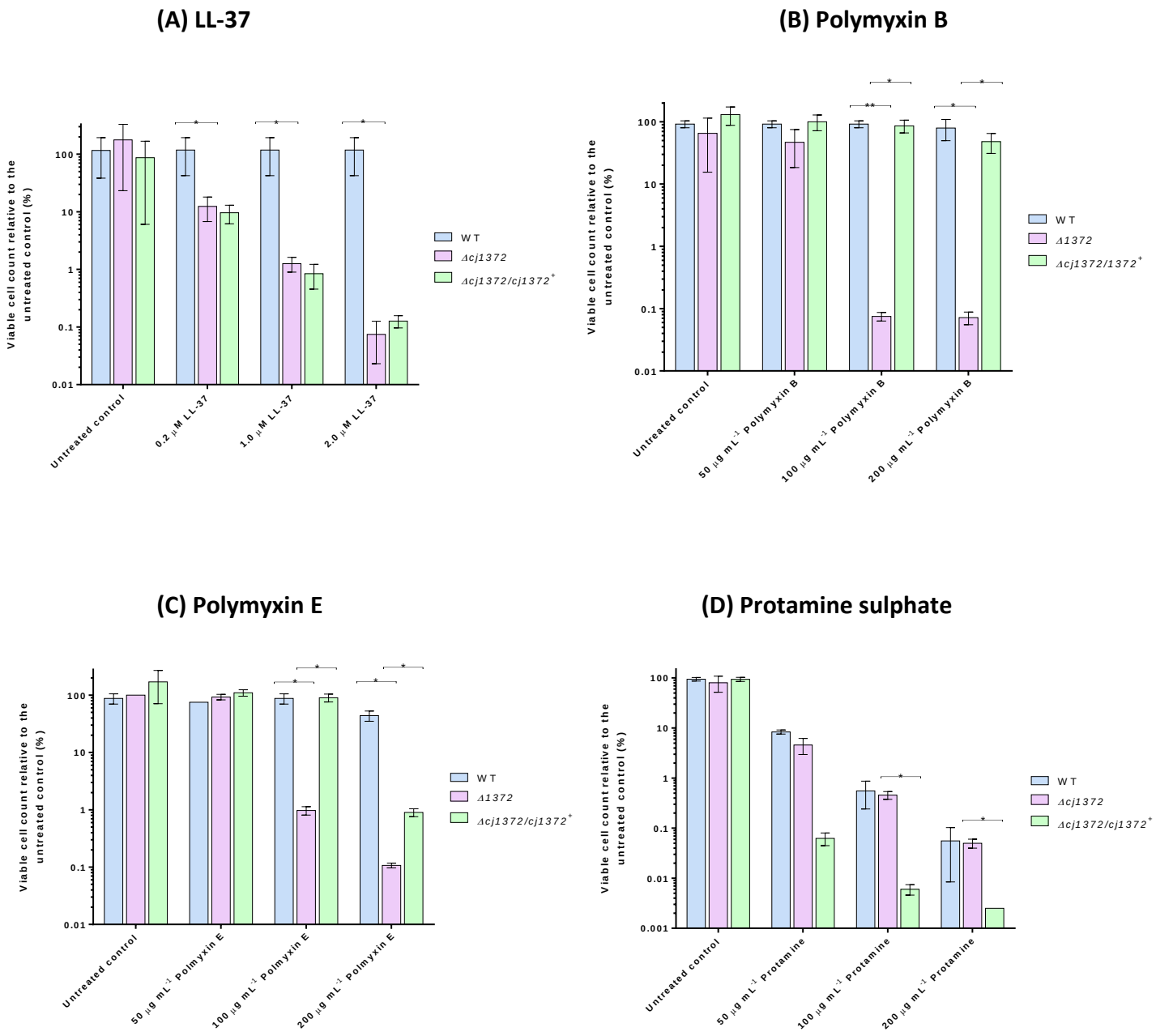


Figure 5.10 Sensitivity of the *Δcj1372* mutant to various antimicrobial peptides. A) Sensitivity of the *Δcj1372* mutant to human cathelicidin (LL-37). **B)** Sensitivity of the *Δcj1372* mutant to polymyxin B. **C)** Sensitivity of the *Δcj1372* mutant to polymyxin E. **D)** Sensitivity of the *Δcj1372* mutant to protamine sulphate. Data are representative of three biological replicates. The cell viability values of the untreated controls were adjusted to 100% to reflect the maximum viability without treatment. Error bars represents the standard deviation of these measurements. Statistical analysis was carried out using Student's multiple t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.2.6.3 A *cj1373*-deletion mutation does not increase the sensitivity of *C. jejuni* to antimicrobial peptides.

The third substrate-binding protein that might be involved in the Mla system pathway is the Cj1373. To test if this protein has a role in the Mla pathway in the maintenance of the OM integrity, a mutant lacking the *cj1373* gene was generated and the sensitivity of the *Δcj1373* mutant to the same antimicrobial peptides used with the two former mutants was examined. As expected, the *Δcj1373* mutant showed a similar sensitivity profile to that of the wild-type (Figure 5.11A, B and D) except for polymyxin E (Figure 5.11C), where the *Δcj1373* showed a significant increase in the sensitivity to polymyxin E compared to the wild-type ($p < 0.05$), as represented by 1-log decrease in the viable cell count relative to the wild-type at 200 µg mL^{-1} polymyxin E. These data suggest that the absence of Cj1373 may not directly affect the OM integrity.

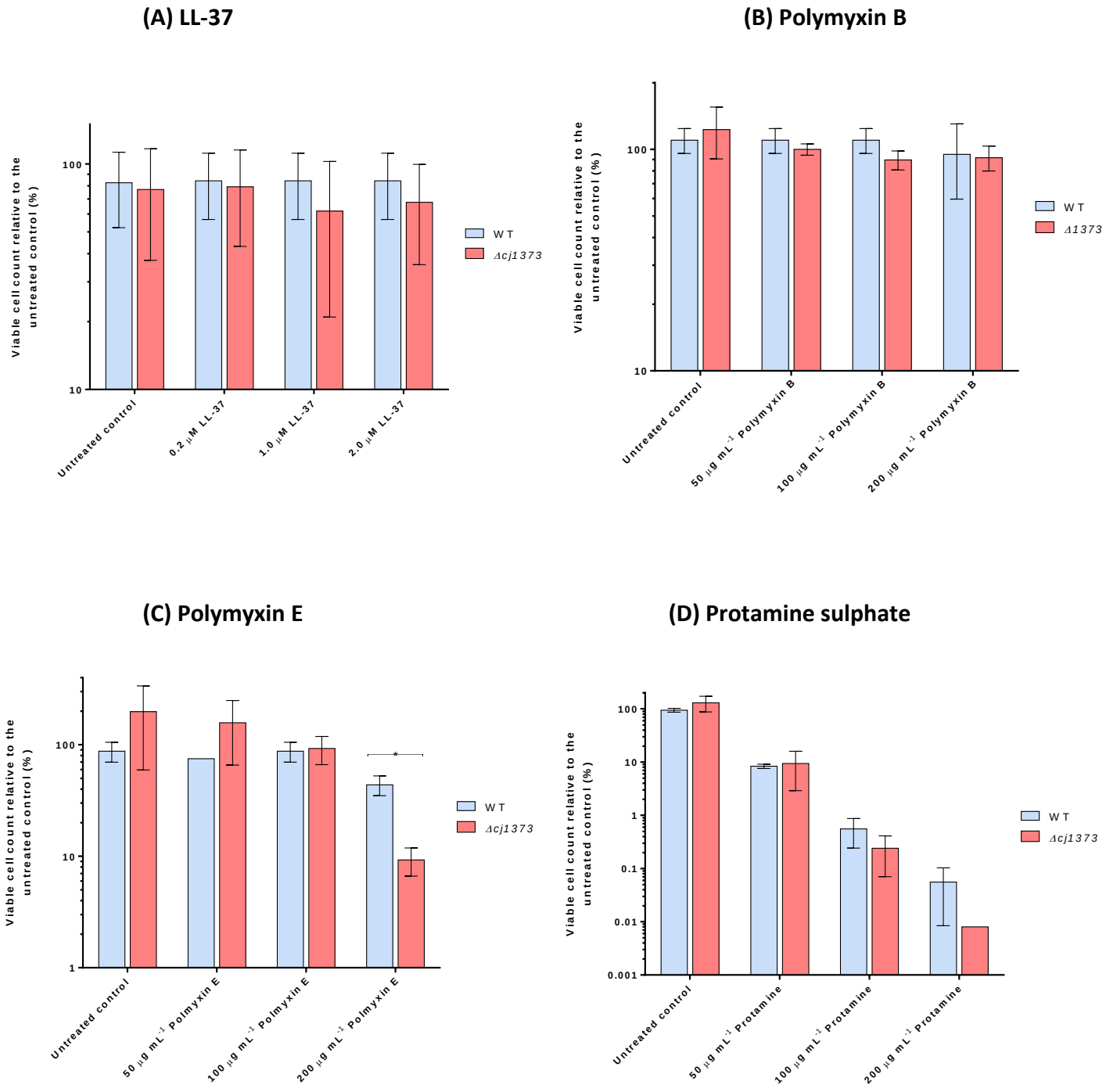


Figure 5.11 Sensitivity of the *Δcj1373* mutant to antimicrobial peptides. A) Sensitivity of the *Δcj1373* mutant to human cathelicidin (LL-37). **B)** Sensitivity of the *Δcj1373* mutant to polymyxin B. **C)** Sensitivity of the *Δcj1373* mutant to polymyxin E. **D)** Sensitivity of the *Δcj1373* mutant to protamine sulphate. Data are representative of three biological replicates. The cell viability values of the untreated controls were adjusted to 100% to reflect the maximum viability without treatment. Error bars represents the standard deviation of these measurements. Statistical analysis was carried out using Student's multiple t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.2.7 Single mutations in the *cj1371-73* genes increases *in vitro* motility of *C. jejuni* under hyperosmotic stress conditions

To investigate whether deletion of one of the *mla* genes results in an increased motility of the *C. jejuni* 11168 strain, motility assays were carried out using soft (0.3 %) agar. Exactly 10 µL of overnight cultures of mutants cells grown in MHS containing 10 µg mL^{-1} of both amphotericin and vancomycin, and 50 µg mL^{-1} of kanamycin were applied to centres of motility plates (0.3 % MHS agar) in the presence or absence of 1.0 % NaCl, and incubated overnight in microaerobic conditions. The wild-type 11168 strain was used as a negative control for motility test. In contrast to the wild-type, all *mla* mutants showed increased spreading "swarming" motility on salt-containing media while in salt-deficient media, they represented similar motility to that of the wild-type (Figure 5.12). This indicates that the Cj1371, Cj1372 and Cj1373 proteins all can be involved in the *in vitro* spreading phenotype under stress conditions, suggesting a possible role of these lipoproteins in the *in vivo* colonisation of host epithelial cells. However, further investigations still need to be done to confirm this suggestion.

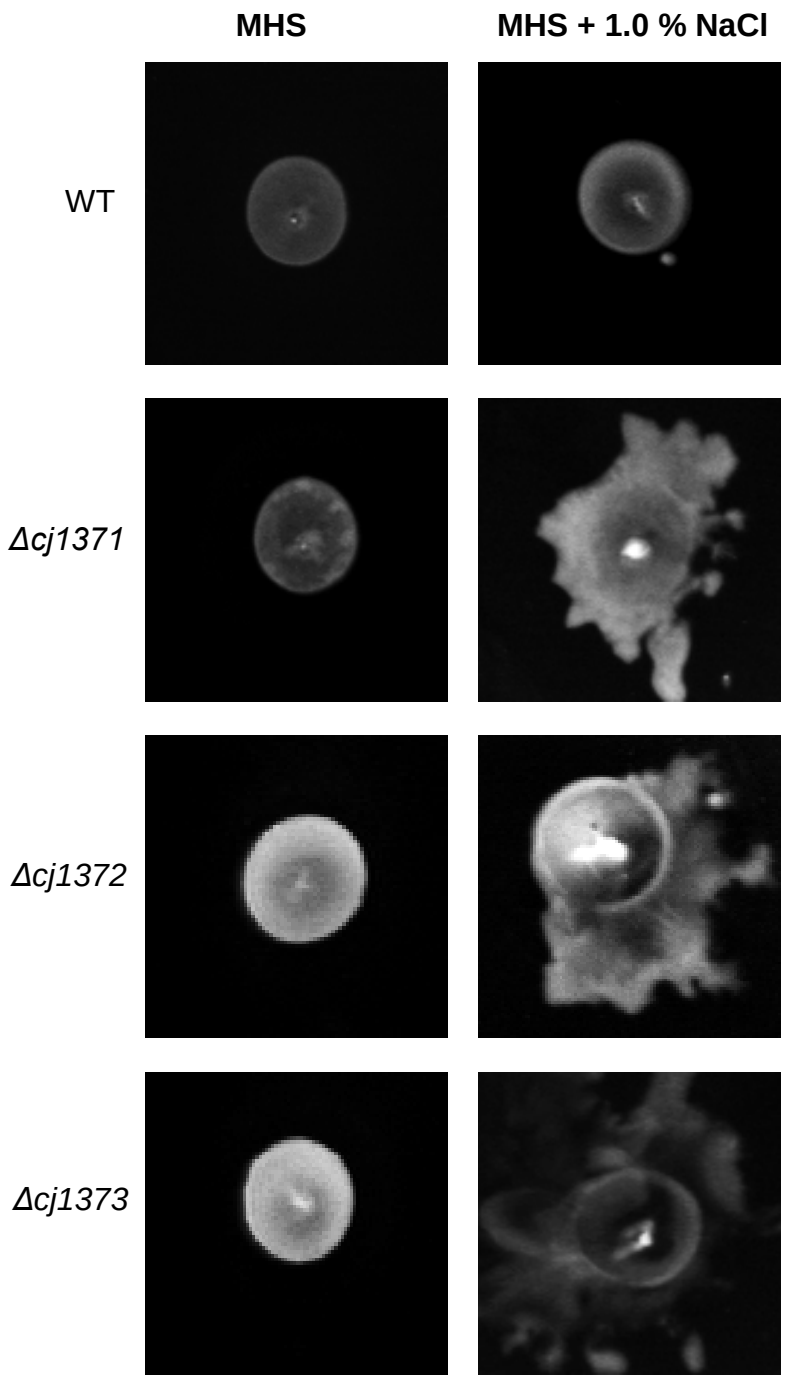


Figure 5.12 Motility assays of the *Δcj1371, Δcj1372* and *Δcj1373* mutants. Overnight cultures (10 µL) of the *Δcj1371, Δcj1372* and *Δcj1373* mutants were spotted into centres of motility plates (0.3 % MHS) with or without hyperosmotic stress agent (1.0 % NaCl), and incubated microaerobically overnight. All mutants showed spreading "swarming" phenotypes under stress conditions whereas in normal conditions, where the salt is absent, they were similar to the wild-type.

5.2.8 Deletion of one of the *mla* genes in *C. jejuni* reduces the ability for biofilm formation under aerobic conditions

Biofilm formation is a well-considered bacterial mechanism that is utilised by *C. jejuni* in its growth and survival by which the cells can be protected and shed from stressful surrounding environments. Indeed, biofilm formation can increase bacterial resistance to disinfectants, and antimicrobial agents by more than 1000-fold than the planktonic cells (Fux *et al.*, 2005). Studies have demonstrated that biofilm can increase the survival of *Campylobacter* as twice in atmospheric conditions and in water environment (Asakura *et al.*, 2007, Joshua *et al.*, 2006). Flagella are shown to play a crucial role in biofilm formation in *C. jejuni*, as mutation in *flabAB* genes resulted in decreased biofilm formation (Reeser *et al.*, 2007). Reuter *et al*., (2010) examined the effect of loss of flagellar motility on biofilm formation of *C. jejuni* under microaerobic conditions. They found that the motile *C. jejuni* 11168 strain was able to form > 50 % more biofilm than the non-motile strain. The importance of biofilm formation in *C. jejuni* can be more appreciable under stress conditions (e.g. atmospheric oxygen level), in which the organism makes its transfer between hosts. Hence, the effect of atmospheric growth of *C. jejuni* on biofilm formation was tested by Reuter *et al*., (2010). Interestingly, in the motile wild-type strain, the biofilm formation was double that recorded in microaerobic conditions. Also, the non-motile wild-type and the *flabAB* mutant showed increased levels of biofilm formation, although overall levels were less than that of the motile wild-type (Reuter *et al.*, 2010).

Having said that, biofilm formation was investigated using biofilm assay for the *mla* mutants in comparison to the wild-type to further examine if the *mla* genes products are possibly involved in biofilm formation when the cells are oxygen stressed. Cells were incubated microaerobically or aerobically under static conditions for 24 hours and stained with crystal violet to determine the level of biofilm formation. Growth of the *Δcj1371* mutant was similar to the wild-type under both aerobic and microaerobic conditions (Figure 5.13A). Biofilm formation of the *Δcj1371* mutant in aerobic conditions was significantly less than that of the wild-type ($p < 0.0001$). Because the growth of both strains was similar under both conditions, biofilm units followed the biofilm formation and were significantly less in the *Δcj1371* mutant than the wild-type under aerobic conditions.

The growth of the *Δcj1372* mutant was similar to the wild-type under both conditions (Figure 5.13B). However, biofilm formation and biofilm units of the mutant were higher than the wild-type under microaerobic conditions ($p < 0.05$). This was exactly opposite under aerobic conditions. Biofilm formation and biofilm units significantly decreased in the mutant under aerobic conditions compared to the wild-type ($p < 0.0001$). The *Δcj1373* mutant was found to have a higher growth rate than the wild-type under microaerobic and aerobic conditions ($p < 0.0001$ and $p < 0.01$ respectively) but its biofilm formation significantly dropped under aerobic conditions compared to the wild-type ($p < 0.0001$) (Figure 5.13C). These data represent significant reduction in the biofilm units in all *mla* mutants under aerobic conditions and therefore, can explain that the Mla proteins which are not produced in these mutants can be essential for the *C. jejuni* in maintaining the integrity of the cell, as the lack of these proteins result in a significantly reduced biofilm formation under stress conditions which is directly dependent on cell-cell interaction.

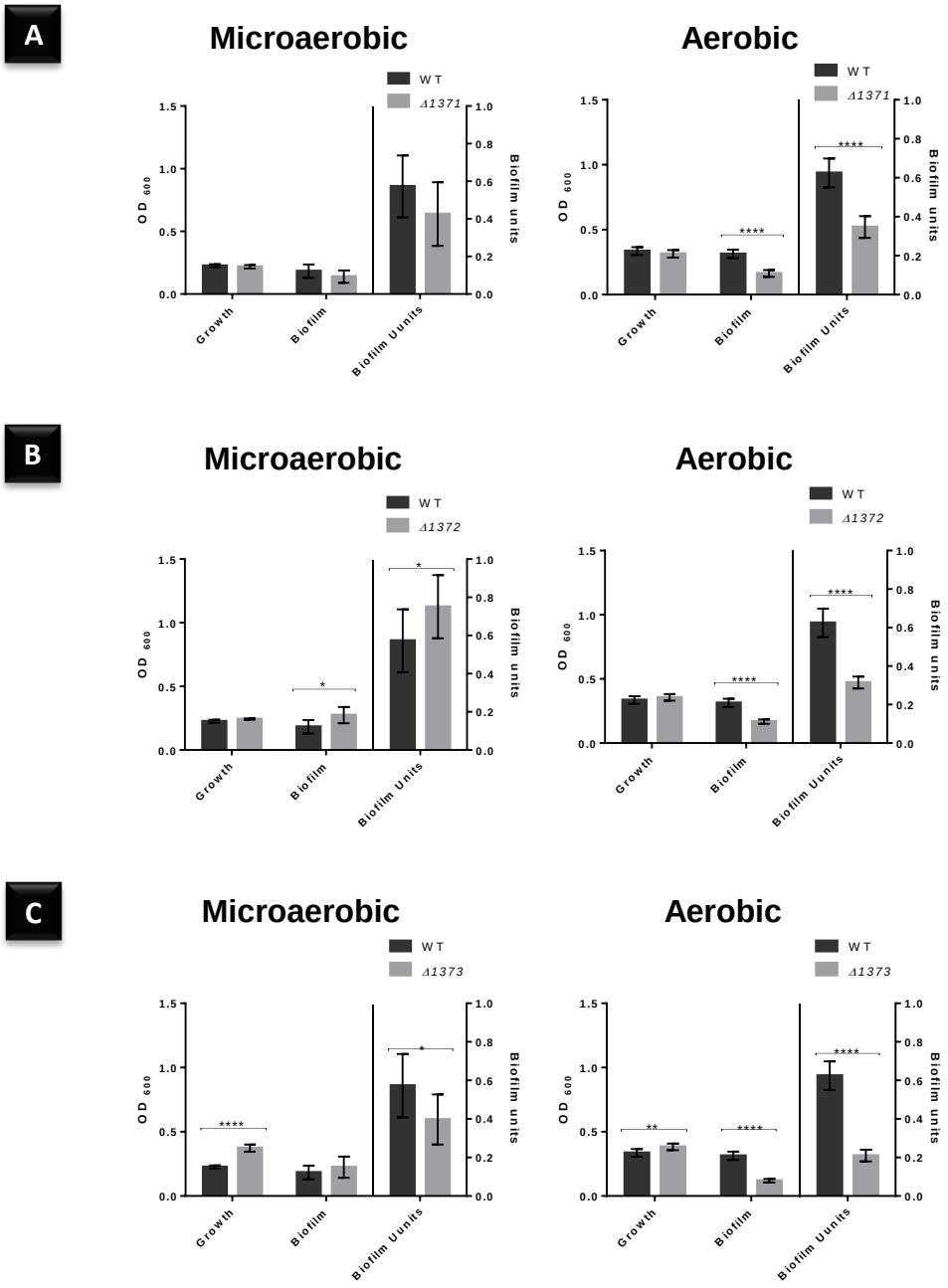


Figure 5.13 Biofilm formation of the wild-type and the *mla* mutants under microaerobic and aerobic conditions. The wild-type and the *mla* mutants were grown microaerobically until the early stationary phase was reached. Cultures were adjusted to an OD_{600} of 0.1. 200 µL were added to 8 wells of 96-well microtiter plate and incubated statically for 24 hours under microaerobic (left) or aerobic (right) conditions. OD_{600} of planktonic and biofilm growths was measured. Biofilm units were calculated by dividing the OD_{600} of the biofilm growth by the OD_{600} of the planktonic growth. Error bars represent the standard deviations from means of 8 technical replicates. Statistical analysis was carried out using Student's multiple t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.3 Discussion

Gram-negative bacteria, including *Campylobacter jejuni*, are normally more resistant to antibiotics, detergents and other chemicals than Gram-positive bacteria. This is credited to the complex composition of the OM. The asymmetric distribution of the lipid contents in the OM, including lipopolysaccharides (LPSs) in the outer leaflet and phospholipids (PLs) in the inner leaflet plays an important role in increasing the barrier function of the OM (Nikaido, 2003). PLs accumulate in the outer leaflet of the OM when the cell is stressed, causing an increase in cell permeability and loss of barrier function of the OM (Nikaido, 2005). The newly identified Mla system (Malinverni and Silhavy, 2009) stops PLs from accumulation in the outer leaflet of the OM in unstressed cells, and remove PLs from the OM when the cell is stressed. Interestingly, the Mla system is suggested to be the most important pathway in Gram-negative bacteria among the previously identified systems, PldA and PagP, that maintain lipid asymmetry in the OM (Malinverni and Silhavy, 2009). Hence, the Mla pathway is considered as an important virulence factor, in enteric bacteria.

5.3.1 The essential elements of the Mla pathway is highly conserved in Gram-negative bacteria.

One of the main aims in this chapter was to identify the candidate genes that are involved in the Mla pathway in *C. jejuni*. We already know from previous studies that the *mla* genes are highly conserved in Gram-negative bacteria (Malinverni and Silhavy, 2009). However, identification and localisation of these genes in *C. jejuni* have not been previously studied. The *mlaA* gene was first discovered in *Shigella flexneri* and named as a *vacJ* gene. Its amino acid sequence indicates that it encodes a 28 kDa surface-exposed lipoprotein with an *N*-terminal signal sequence (Suzuki *et al.*, 1994). Moreover, the VacJ protein was found to be essential for intracellular spreading of *Shigella flexneri* and enteroinvasive *E. coli* (Suzuki *et al.*, 1994). The availability of the complete genome sequence of *C. jejuni* (Parkhill *et al.*, 2000) eases the mission of searching for homologues of thousands of genes including *vacJ* homologue. In *C. jejuni* NCTC 11168 strain, BLAST searches of the genome sequence using the *mlaA* and *mlaC* of the *E. coli* as query genes revealed two genes in *C. jejuni* genome homologues to *mlaA* and *mlaC*,

namely *cj1371* and *cj1372*. We found that the *cj1371* is located in a locus containing two other genes, *cj1372* and *cj1373*. Interestingly, the *cj1371* has an upstream promoter and ribosome binding site, and a downstream ribosome binding site between *cj1371* and *cj1372*. In contrast, there is no ribosome binding site between the *cj1372* and *cj1373*, suggesting that the two gens are transcriptionally coupled. In addition, we identified the ABC transporter genes in *C. jejuni* which form an operon (*cj1646 – cj1648*), which are equivalent to *mlaE, mlaD* and *mlaF*, respectively. That only leaves MlaB, which is a small protein that seems to interact with MlaE and may be involved in its assemble.

We also aimed to gain phenotypic evidence about their *in vivo* roles in maintaining the integrity of the OM by generating mutations in these genes, and comparing the growth, motility and biofilm formation, as well as the antimicrobial sensitivity of the mutants to the wild-type strain. Here, we used *cj1371*, *cj1372* and *cj1373* knockout mutants to gain experimental evidence for the Mla dependence of the OM integrity and barrier functions in *C. jejuni*. The knockout plasmid constructs were generated by Dr Arnoud van Vliet at the Institute of Food Research (IFR), Norwich, UK, based on allelic replacement of *cj1371*, *cj1372* and *cj1373* by kan^R cassette. These constructs were obtained as a generous gift to transform *C. jejuni* 11168 strain to generate knockout mutants. Complementation plasmid constructs were also obtained from Dr van Vliet to transform the knockout mutants to generate complemented strains with wild-type copies of the deleted genes. We successfully generated three mutants which are *Δcj1371, Δcj1372* and *Δcj1373* mutants and two complemented strains which are *Δcj1371/Δcj1371*$^+$ and *Δcj1372/Δcj1372*$^+$ complemented strains. The *Δcj1373/Δcj1373*$^+$ complemented strain could not be obtained, although several attempts were made. All the three genes involved in the Mla pathway are encoded in one DNA strand, i.e, their promoters drive the expression of all three genes in one direction. Therefore, mutation in one gene is not an independent transcriptional unit and may affect the transcription of the downstream gene. Hence, the main reasons of complementation were to observe the restoration of the wild-type phenotype, and to ensure that the resulting effect is due to the deletion of the respected gene and is not due polar effect of downstream gene. Alternative approaches can be used to ensure that the resulting phenotype is not due to polar effect of the downstream gene. RT-PCR can be used to examine whether mRNA can be made from the downstream gene in the mutant. If mRNA cannot be made, then the expression of the deleted gene may be affected by the expression of the downstream gene. Other

experiment that can be performed is enzyme activity assay, in which the activity of the protein produced from the downstream gene is measured in the mutant. If the activity of this protein does not change between the wild-type and the mutant, then the gene deletion does not affect the expression of its downstream gene.

5.3.2 The *C. jejuni* MlaA homologue is crucial in OM stability during cell division under stress conditions.

The nature of Gram-negative cells allows PLs to be moved from the IM and accumulate in the defective area of the OM when the cell is stressed (Nikaido, 2005). Previous work showed that the Mla proteins inhibits PLs from accumulating in the outer leaflet of the OM (Malinverni and Silhavy, 2009), causing re-maintenance of the lipid asymmetry in the OM. Here, we assessed the growth phenotype of the *mla* homologues mutants (*Δcj1371, Δcj1372* and *Δcj1373* mutants) to determine the effect of the deletion of these genes on the growth of *C. jejuni* under stress conditions. The *Δcj1371* mutant was shown to have severe growth retardation under hyperosmotic stress conditions (1.0 % NaCl) compared to the wild-type and the complemented strain. MlaA was previously reported as an OM lipoprotein with an unknown function (Juncker *et al.*, 2003, Suzuki *et al.*, 1994). Because of the OM localisation of the Cj1371, and its crucial role in moving the accumulated PLs in the outer leaflet of the OM in stressed cells, thus, a possible explanation of the severe growth defect observed in the *mlaA* homologue mutant (*Δcj1371*) under stress conditions is that the lack of Cj1371 may result in an accumulation of PLs in the OM causing instability of the OM during cell division, suggesting that the product of the *C. jejuni mlaA* homologue (*cj1371*) may play an essential role in the maintenance of the OM integrity by maintaining the lipid asymmetry in the OM when *C. jejuni* cells are subjected to stress conditions. Therefore, the lack of *mlaA* gene results in a severe growth defect which may be due to the severe interruption of the OM during cell division. On the other hand, the growth retardation shown by the *Δcj1372* mutant was less severe than that of the *Δcj1371* mutant under hyperosmotic stress. MlaC is located in the periplasm and is predicted to be a substrate-binding protein (Linton and Higgins, 1998, Lopez-Campistrous *et al.*, 2005). Therefore, the lack of the MlaC in the *Δcj1372* mutant does not have a severe effect on the growth of *C. jejuni*. This can possibly be explained by the fact that the Cj1372 is localised in

the periplasm, and if the Cj1372 has a role in the maintenance of lipid asymmetry in the OM of *C. jejuni*, it may not have a major direct role in controlling PLs in the OM. Alternatively, its role may be in a later phase in the pathway, specifically in the periplasm, after MlaA removes the PLs from the outer leaflet of the OM toward the IM. The growth of the *Δcj1373* mutant was not affected by the presence of hyperosmotic stress factor in the media. The protein sequence of Cj1373 indicates that it is a huge transmembrane protein which is conserved in most *Campylobacter* species. Therefore, it can presumably be located in the periplasmic face of the inner membrane. Although *cj1373* is co-transcribed with *cj1372*, homology search clearly indicated that it is not a homologue to the *E. coli* MlaD, and therefore indicating that it is an unrelated protein in *E. coli* but is present in *C. jejuni.* It has conserved domains related to the RND transporter family, so one possibility is that it is somehow involved in lipid transport relating to the Mla system, but this requires further investigation.

Because the Cj1373 sequence suggests that it is a transmembrane protein, it can be suggested that it can be functional at a late phase in the MlaFEDB complex in the IM (Figure 5.1), and therefore, the absence of the *cj1373* gene may be compensated by other genes involved in the MlaFEDB complex or it is not essential to the system to function. Therefore, the absence of Cj1373 may not directly affect the growth *C. jejuni* under stress conditions, although there was a clear biofilm deficient phenotype in the mutant.

5.3.3 Mutation in *mla* genes increases susceptibility to killing by antibiotics, peptides and detergents.

The asymmetric distribution of lipids in the OM has a major role in maintaining the integrity of the OM to provide the right barrier function, which increases the bacterial defence mechanism against external factors, and serves in increasing the resistance mechanism to detergents (such as SDS) and antimicrobial agents such as antimicrobial peptides (AMPs). Here, we determined the sensitivity of the *mla* mutant cells to various antimicrobial agents to assess whether the Mla proteins have a direct role in maintaining the integrity of the OM. We suggested that mutation in the *mla* genes may result in phenotypes with a damaged or permeable OM, and therefore, more sensitive phenotypes

to various antimicrobial agents that require entry to the bacterial cell through the OM. We used ampicillin, polymyxin B, vancomycin and SDS in disc diffusion method to examine the ability of the *mla* mutants to grow in the presence of these agents. It was found that deletion of the *cj1371* gene results in a more sensitive strain to these antimicrobial agents, whereas deletion of the *cj1372* results in a strain which displays a lower effect of these agents on the membrane integrity, as demonstrated by clear zone of growth inhibition around the discs that contain these agents. These phenotypic alterations could be resulted from increased outer membrane permeability allowing an uncontrolled entry of various antimicrobial agents which became lethal at higher concentrations. These findings suggest that the mutants cells have lowered ability to prevent the entry of antimicrobial agents into the cell, and that the MlaA and MlaC proteins may have a direct or indirect role in maintaining the OM integrity as deletion of their cognate gene results in more sensitive phenotypes.

Because all bacterial cell membranes are negatively charged, cationic antimicrobial peptides (CAMPs) can kill most Gram-positive and Gram-negative strains by the formation of carpet-like coating over the bacterial membrane causing disintegration of the membrane and consequent bacterial death (Tjabringa *et al.*, 2005). *C. jejuni* is known to be highly resistant to polymyxin B, an antimicrobial peptide usually added to growth media to select for *C. jejuni* (Skirrow, 1977). Other well-known CAMPs are cathelicidins, small cationic peptides that possess broad-spectrum antimicrobial activities, also known as 'natural antibiotics' (Nizet and Gallo, 2003). The 18 kDa human cathelicidin, also known as human cationic antimicrobial protein (hCAP-18), is expressed by neutrophils and epithelial cells. The major cleavage product of hCAP-18 is the *C*-termilal region, LL-37 (Nizet and Gallo, 2003), which is required at low concentrations to kill *Campylobacter* species (0.6 – 2.5 µM). Testing the efficacy of bacterial cell membrane and its components to resist antimicrobial agents has been widely used in antimicrobial research, and has been known to provide a better understanding about how microorganisms can evolve biological mechanisms to resist the surrounding environments. Lipid A in bacterial OM is known to have a crucial role to evade innate immunity by host, and most Gram-negative bacteria have evolved various physiological mechanisms which modify the structure of lipid A (van Mourik *et al.*, 2010). The modification of lipid A of bacterial lipo-poly saccharide (LPS) has been previously tested by using polymyxin B, colistin and LL-37 (van Mourik *et al.*, 2010).

Two genes were found to be responsible for the addition of an *N*-linked acyl chain instead of an *O*-linked acyl chain to lipid A backbone, namely *gnnA* and *gnnB*. Mutation in these genes resulted in a more susceptible phenotype to AMPs than the isogenic parent wild-type (van Mourik *et al.*, 2010).

Our major goal was to investigate the effect of the mutagenesis of the *mla* genes on the OM integrity. We could demonstrate that deletion of *mlaA* or *mlaC*, which encode for MlaA and MlaC, essential component in the Mla pathway, increases the susceptibility to killing by AMPs. We tested the viability of the mutants cells in the presence of four AMPs to examine the integrity of OMs in all mutants. These include the *C*-terminal region of the hCAP-18, LL-37, polymyxin B, Polymyxin E (also known as colistin) and protamine sulphate. As expected, our findings indicate that the Δ*mlaA* and Δ*mlaC* mutants were more sensitive to all CAMPs than the wild-type *C. jejuni*, whereas the sensitivity of the Δ*cj1373* mutant was significantly more in the presence of polymyxin E and protamine sulphate. Figure 5.14 summarises the most significant changes in sensitivity of chaperone mutants to CAMPs compared to the parent wild-type, the Δ*cj1371* and Δ*cj1372* mutants were found to be the most susceptible strain to killing by most tested CAMPs. This strongly suggests that the Cj1371 surface-exposed lipoprotein and the Cj1372 periplasmic binding protein have direct roles in maintaining the OM integrity and barrier function against external antimicrobial agents. In contrast, our findings indicate that the deletion of *cj1373* does not increase the sensitivity to most of the CAMPs tested even when increasing the peptides concentrations. Thus, it was suggested from the findings of growth and sensitivity assays that the *C. jejuni* MlaA and MlaC are the key players in the Mla pathway that maintains the OM integrity, while the absence of Cj1373 may not directly affect the OM integrity and its loss may be compensated by another member of the MlaFEDB complex in the IM.

Overall, we suggest that the increased membrane permeability and loss of its integrity in *mla* mutants is due to unsuccessful removal of migrated PLs from the IM under stress, or due to inability of the Mla system to prevent PLs from accumulation in the outer leaflet of the OM in unstressed cells. This is because one or more proteins involved in the Mla system are absent or non-functional in mutants. According to the primary function of the Mla system described by (Malinverni and Silhavy, 2009), it prevents PLs from accumulation in the outer leaflet of the OM in unstressed cells, and remove PLs from the OM when the cell is stressed. We demonstrated the role of the Mla system

in preventing the accumulation of PLs in the OM in unstressed cells by testing the mutants sensitivity to CAMPs. It would be highly valuable to prove its role in removing PLs from the outer leaflet of the OM and transporting them to the IM under stress conditions. This could be shown by testing the viability of mutants cells under the effect of CAMPs in the presence of stress factor, such as 1.0 % sodium chloride.

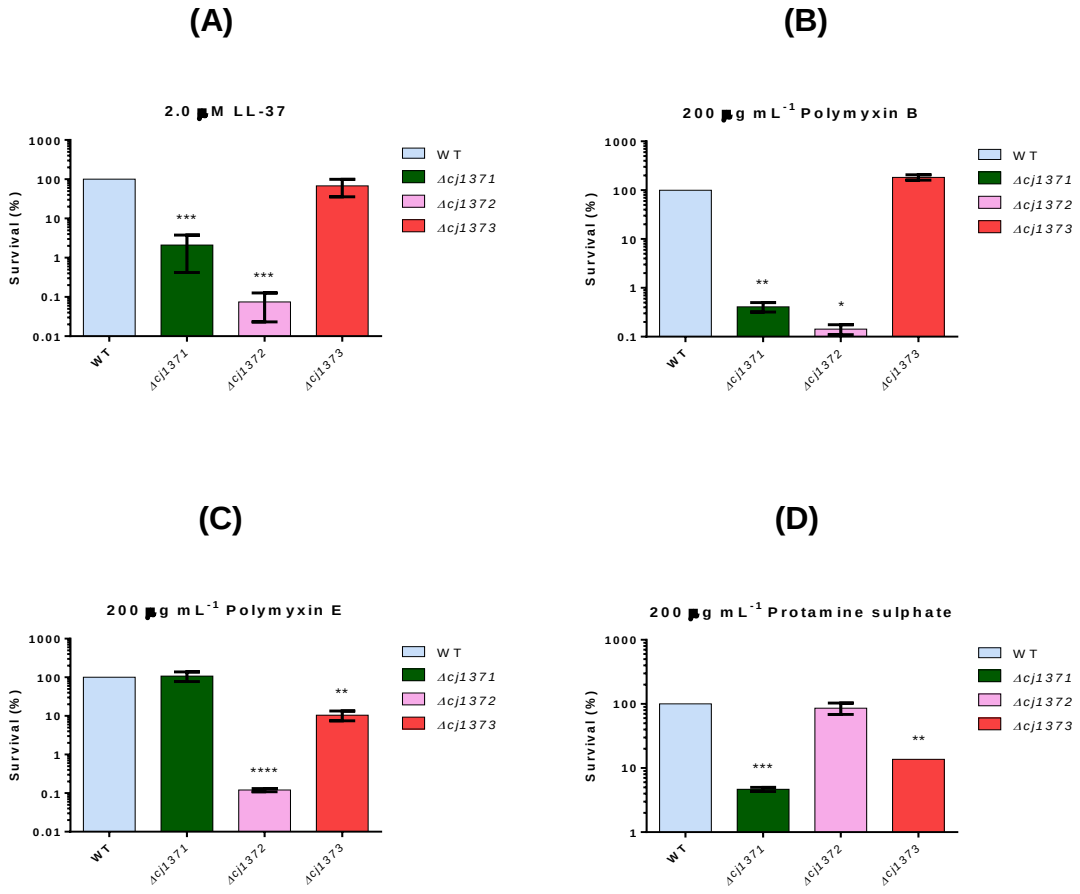


Figure 5.14 Summary of the increased susceptibility to killing by CAMPs in *C. jejuni mla* mutants. A) Sensitivity of *mla* mutants to 2.0 µM LL-37. **B)** Sensitivity of *mla* mutants to 200 µg mL^{-1} polymyxin B. **C)** Sensitivity of *mla* mutants to 200 µg mL^{-1} polymyxin E. **D)** Sensitivity of *mla* mutants to 200 µg mL^{-1} protamine sulphate. The Δ*cj1371* mutant displays significant increases in sensitivity to LL-37, polymyxin B and protamine sulphate ($p < 0.001$), while its sensitivity to polymyxin E was similar to the wild-type at the same concentration. The Δ*cj1372* mutant shows a significant increase in the sensitivity to LL-37 ($p < 0.001$), polymyxin B and polymyxin E ($p < 0.0001$), while no significant change occurred when treated with protamine sulphate compared to the parent wild-type. The sensitivity of the Δ*cj1373* mutant to LL-37 and polymyxin B was similar to the wild-type treated with the same peptides. However, it shows significant increase in susceptibility to polymyxin E and protamine sulphate ($p < 0.01$). The cell viability values of the wild-type were adjusted to 100% to reflect the effect of the mutations on the cell viability under the same concentration of CAMPs. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Error bars represents the standard deviation of these measurements. Statistical analysis was carried out using One-way ANOVA.

5.3.4 The Mla lipoproteins may be involved in intracellular spreading and early steps of host cells colonisation of *C. jejuni* as shown by *in vitro* motility.

The VacJ protein was found to be essential for intracellular spreading of *Shigella flexneri* and enteroinvasive *E. coli* (Suzuki *et al.,* 1994). However, the function of the *vacJ* homologue product (Cj1371) in *C. jejuni* is currently unknown. Because bacterial motility is an important factor in invasion of host epithelial cells, and is important for animal colonisation, we wanted to investigate by motility assays if the *mlaA* (*cj1371*) gene in *C. jejuni* and the other two genes involved in this pathway (*mlaC* and possibly *cj1373*) are responsible for increased motility of *C. jejuni* under stress conditions. An increased motility may be an indicative of an alteration in the surface composition of the OM of the *mla*-deficient strains. This was demonstrated by testing the swarming motility of the *mla* mutants over soft agar plate (0.3 % MHS), using the wild-type 11168 strain as a negative control. All mutants showed swarming phenotypes that were demonstrated by spreading of cells over the entire plates when incubated under stress conditions, while they formed solid thick spots, similar to that of the wild-type when incubated in the absence of stress agent. This observation can provide evidence for an uncontrolled motility of the mutant cells when they were exposed to a stress factor such as hyperosmotic environment. This phenotype can therefore explain that the Mla proteins can be involved in the maintenance of the OM integrity, as the lack of one of the *mla* genes results in an uncontrolled *in vitro* motility. It is important to note that the plasmids of the *mla* mutants were generated from the genomic DNA of *C. jejuni* 81116 strain, and were used to transform the 11168 strain in our study. Although the gene sequences in both strains are exactly typical, the 11168 strain is known to exhibit a less ability of chicken colonisation, invasion and motility compared to other strains (Gaynor *et al.*, 2004). Therefore, the increased swarming motility observed in the *mla* mutants is an indicative of an impaired function of the OM compositions.

In a previous study, Szymanski *et al.,* (2003) suggested that selective pressure can lead the bacterium to alter surface antigens in order to escape the host immune response (Szymanski *et al.,* 2003). We suggest from our results of motility assays that the Mla proteins may play a role in evading host immune defence. Because our findings indicated that elimination of Mla proteins results in an increased motility, we suggest that the *mla* mutants might be more invasive than the parent wild-type. Nevertheless,

more experiments need to be done to examine the ability of the *mla* mutants in adherence, invasion and intracellular survival as well as host cells colonisation. A gentamicin protection assay, a model adherence, invasion and intracellular survival assay, can be carried out using INT407 intestinal epithelial cells to test the ability of *mla* mutants in adherence and invasion (Pajaniappan *et al.*, 2008, Rathbun *et al.*, 2009, Wassenaar *et al.*, 1991). In brief, mutant cells are grown in an enriched media under standard microaerobic conditions for 20 hours. Then, cells are harvested and resuspended in PBS buffer and added to semi-confluent INT407 cells monolayers. Quantification of viable cells can be determined by CFU mL^{-1}. The cells are then incubated microaerobically for 3 hours, washed and lysed with triton X-100. The number of adherent cells can be quantified by viable count. For invasion determination, INT407 cells are incubated for 3 hours with bacterial cells, and gentamicin is then added to kill extracellular bacteria. Quantification of intracellular bacteria can be carried out by viable count.

5.3.5 The Mla system is important in biofilm formation under aerobic conditions

Accumulation of PLs in the outer leaflet of the OM does not only reduce its barrier role (Nikaido, 2005), but also reduces the ability of *C. jejuni* to form biofilms when the cells are stressed due to a non-stable membrane. Biofilm formation is considered as a defence mechanism that bacterial cells use to resist external damaging agents such as detergents and antimicrobial agents (Fux *et al.*, 2005). Because *C. jejuni* lives in two hosts, i.e., poultry and human, it has to face the atmospheric environment during its transfer between hosts. Therefore, it requires evolving a mechanism to protect itself from the high oxygen level. We previously know that the Mla system is important in the removal of accumulated PLs in the OM under stress conditions, and thus maintaining the barrier function of the OM (Malinverni and Silhavy, 2009). Hence, we became interested to know whether mutation in the *mla* genes can alter the ability of *C. jejuni* to form biofilm under stress conditions. The biofilm formation in the wild-type is known to be higher in aerobic conditions (Reuter *et al.*, 2010). However, our findings indicated that mutation in *mla* genes resulted in an opposite behaviour. The decreased ability of all *mla* mutants to form biofilms in atmospheric environments compared to the parent wild-type can be an indicative of loss of membrane integrity, and this could be due to the accumulation

of PLs in the OM because the cell is stressed. Thus, it is speculated that the Mla proteins are not produced to remove the accumulated PLs from the OM, causing decreased OM integrity. Although mutants were able to form biofilms under microaerobic conditions, in which the proposed function of the Mla system is not required, their ability to form biofilms under stress conditions was impaired. Therefore, it can be stated that the Mla proteins are essential players when cells are stressed, i.e., in host's intestine where the oxygen is limited. These data can explain that the Mla proteins which are not produced in these mutants can be essential for the *C. jejuni* in maintaining the integrity of the cell, as the lack of these proteins result in a significantly reduced biofilm formation under stress conditions which is directly dependent on cell-cell interaction.

6 General conclusion and future directions

This study has provided a clearer insight about the role of periplasmic chaperones in the translocation and assembly of the periplasmic and surface-exposed OMPs that can be highly immunogenic or considered as antimicrobial targets. In Chapter 3, mutation in *cj1289* and *cj0694* demonstrated phenotypes which are poorer in their growth compared to the wild-type, and have decreased membrane integrity as shown by antimicrobial sensitivity assays. In addition, alteration in the OM profiles in each mutant suggests possible periplasmic chaperone roles of the Cj1289 and Cj0694 proteins consistent with previous studies. Mass spectrometry data indicated that some OMPs are less abundant in the chaperone mutants compared to the wild-type. Therefore, providing evidence that these proteins could be chaperone clients. In Chapter 4, Cj0694 could be successfully over-expressed and purified in order to investigate its role in the periplasm based on biochemical and enzymatic evidence. Bioinformatics evidence that revealed sequence similarities to the inner membrane-anchored PpiD in *E. coli* suggest a possibly similar localisation and function of Cj0694. This was supported by the fact that Cj0694 does not have a defined *N*-terminal signal peptidase cleavage site, therefore suggesting that it is not a Sec-dependent secretory protein. Moreover, Cj0694 was demonstrated to be an active periplasmic PPIase as shown by a remarkable Cj0694-dependent acceleration of the refolding rate of RCM-RNase T_1. Also, Cj0694 was found to exhibit a general periplasmic chaperone function that may help in early protein folding in the periplasm. Although chaperone activity of Cj0694 could not be demonstrated in inhibiting the refolding of the tested substrate, rhodanese, it was found that Cj0694 was active in preventing aggregation of rhodanese, and the reaction was found to be concentration-dependent. Thus, providing evidence that Cj0694 has a more general role in the periplasm as a chaperone for both periplasmic and outer membrane proteins, consistent with previous findings of PpiD. Attempts to obtain a defined crystal structure of Cj0694 were not successful. However, cloning, over-production and purification using different expression systems can be useful background for further studies. In Chapter 5, further evidence was provided about the involvement of the Mla transport system in the maintenance of the OM function in *C. jejuni*. The lack of MlaA results in a severe salt-stress growth defect which may be due to a compromised OM function affecting cell growth under these conditions. The Δ*mlaA* and Δ*mlaC* mutants were more sensitive to AMPs than the wild-type *C. jejuni*, whereas the sensitivity of the Δ*cj1373* mutant was

significantly more in the presence of polymyxin E and protamine sulphate, suggesting that the MlaA and MlaC have direct roles in maintaining the OM integrity and barrier function against external antimicrobial agents, whereas Cj1373 may have a distinct role in the Mla pathway. Moreover, the decreased ability of the *mla* mutants to form biofilm suggests loss of membrane integrity, and this could be due to the accumulation of PLs in the OM because the cell is stressed. The highly motile phenotype shown in *mla* null mutants under stress conditions may be indicative for an alteration in the surface composition of the OM.

In the past few years, our understanding of the mechanism by which *C. jejuni* uses for the biogenesis of OMPs was not completely clear. However, taking these new findings in consideration, we would like to introduce an initial model that describes the pathway of newly translocated proteins from their entry to maturation in the periplasm. As shown in Figure 6.1, PEB4 and Cj1289 are soluble proteins indicating that they are mobile chaperones in the periplasm in this model, whereas Cj0694 is an insoluble PPIase anchored to the periplasmic face of the inner membrane, similar to PpiD. In the *E. coli* model of OMPs biogenesis, the majority of the OMPs are SurA-dependent proteins that are delivered to the BAM insertion and assembly complex in a partially folded state. The *E. coli* periplasmic chaperone, Skp plays a rescue role for misfolded proteins or carries them to DegP for degradation. In our proposed model, PEB4 may be the major periplasmic chaperone by which OMPs are carried across the periplasm. This can be supported by the fact that double deletion of *peb4* gene and other proposed periplasmic chaperone genes is lethal, suggesting that they can be functionally redundant. Cj1289 is suggested to be a more substrate-specific chaperone, whereas Cj0694 may play a role as a general chaperone for periplasmic and OMPs. The PEB4 PPIase domain can offer a local prolyl isomerisation for a bound client protein, whereas the PPIase domain of Cj0694 may serve as a calling point where precursor proteins bind to it rapidly for prolyl isomerisation and then passed on to other chaperones, such as the DegP homologue Cj1228c, for further maturation.

We also proposed the Mla system pathway in the periplasm that maintains the OM function in C. *jejuni*. It is supposed to do this function by retrograde trafficking of PLs from the OM to their original site in the IM. MlaA is proposed to be the primary binding protein that binds PLs in the inner leaflet of the OM, whereas MlaC binds free

PLs in the periplasm. Cj1373 is suggested to have a distinct role in the system where it may act at later stages in the IM.

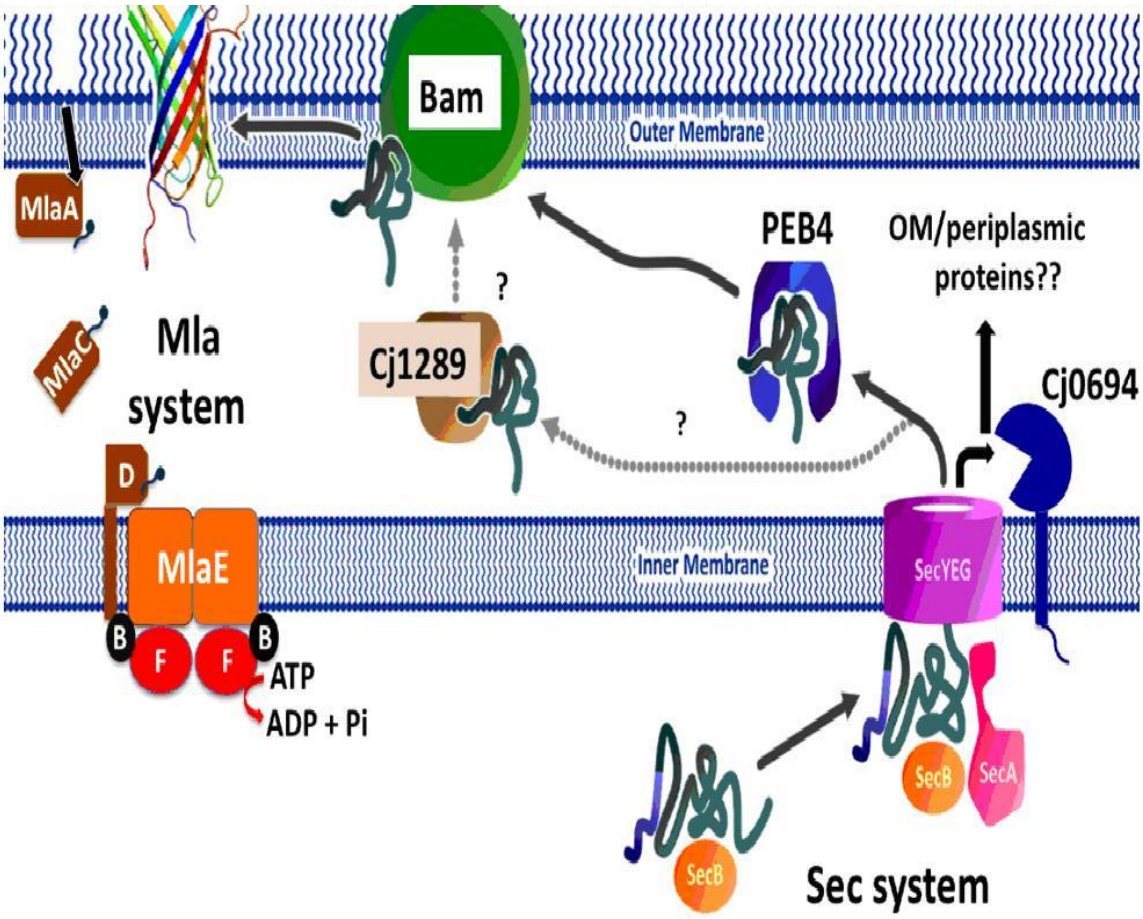


Figure 6.1 Our current understanding of OM biogenesis in *C. jejuni*. OMPs are synthesised in the cytoplasm as precursers and recognised by SecA and SecB chaperones prior to their entry to the IM via the SecYEG translocon. Once entered the periplasm, the substrate proteins are assisted by periplasmic chaperones across the periplasm. Two periplasmic proteins have been known to exhibit similar chaperone function of SurA in *E. coli*; PEB4 and Cj1289. PEB4 is a holdase-type chaperone that prevents aggregation of substrate proteins. Cj1289 has shown a less chaperone role than PEB4, suggesting that its role might be substrate specific. Cj0694 is a third chaperone-like periplasmic protein that is membrane anchored to the IM by its *N*-terminal domain. Cj0694 possesses sequence similarity to the inner membrane-anchored chaperone PpiD in *E. coli*. The folding of a secreted protein can be catalysed by PEB4 or earlier upon entry to the periplasm by Cj0694. The substrate proteins are then delivered to the BAM insertion and assembly complex to catalyse insertion into the OM. The Mla system removes the PLs from the OM and delivers them to the IM, firstly by binding of MlaA at the inner leaflet of the OM and then the periplasmic substrate-binding protein MlaC binds free PLs in the periplasm. Cj1373 may have a distinct role in the Mla pathway. The destiny of PLs is unclear but they might be re-introduced at the IM.

Our current proposed model of outer membrane protein assembly pathway, in which periplasmic chaperones bind to OMPs entering from the Sec system in a partially folded state and handle them to the BAM complex in the OM, has come mainly from studies on only *E. coli* and *Neisseria meningitidis* (Bos *et al*., 2007; Hagen *et al*., 2010). In these bacteria, five proteins (Bam A–E) form the Bam complex, of which the key player is BamA (YaeT / Omp85). In *C. jejuni*, homology search led to the identification of Cj0129c as the BamA homologue. In addition, a probable BamD homologue is encoded by cj1074c. However, extensive searches have revealed no homologues of BamB (YfgL), BamC (NlpB) or BamE (SmpA), suggesting either that BamAD is the minimal complex required for OMP assembly, or that *C. jejuni* employs additional novel proteins in its Bam complex. In order to identify the complete Bam complex in *C. jejuni*, two experimental approaches can be established; the first involves a complete or partial loss of function mutations, and the second is by identifying the interaction partners of BamA and BamD.

Despite the fact that complete deletions of *bamA* and *bamD* are lethal in *E. coli*, insertions of the *C*-terminal part of the *bamD* gene result in viable mutants with OM assembly defects (Wu *et al*., 2005). In *C. jejuni*, insertional mutagenesis of *cj0129c* and *cj1074c* in the 5' and 3' regions can be attempted to determine the importance of these proteins. By assessing the nature and severity of the OMP assembly defects in these mutants through phenotypic and proteomic analyses, it would be possible to determine the identities of the OMPs that are handled by the Bam complex. In order to identify proteins that interact with Cj0129c (BamA) and Cj1074c (BamD), polyclonal antibody raised against these proteins can be used for immuno-precipitation of *C. jejuni* OM extracts. Alternatively, antibody produced against *E. coli* BamA (Robert *et al*., 2006) can be employed, as the nature of this protein in all Gram-negative bacteria is highly conserved. Potential interaction partners are then identified by mass spectrometry, following the SDS-PAGE of immune complexes.

The Mla system pathway, which is proposed in this study has been shown to be involved in the maintenance of the OM function in *C. jejuni*. It will be particularly interesting to investigate the potential relation of the Mla system to other systems that are involved in the maintenance of lipid asymmetry in the OM, particularly, the PldA system. This can be achieved by construction of double *mla/pldA* mutants and testing their growth, viability in the presence of AMPs, sensitivity to antimicrobial agents and

detergents, motility and biofilm formation in comparison to their parent wild-type. Moreover, localisation experiments of the Mla proteins can provide clearer evidence about their roles in the Mla pathway. This can be achieved by raising polyclonal antibodies against these proteins for immuno-precipitation of *C. jejuni* membrane fractions.

As far as the Mla proteins are considered to maintain the asymmetric distribution of the lipids in the OM, it would be highly recommended to perform lipid analysis by Thin Layer Chromatography (TLC) for the lipid compositions of the OMs of the *mla* mutants, and comparing them to that of the wild-type. This can provide an overall picture of the alteration in the lipid composition in the OM of the *mla* mutants, and can initially indicate lipids that are up-regulated or down-regulated in the OM. Then, the membrane profile can be analysed by mass spectrometry to identify each component that has been altered. One-dimensional TLC can be sufficient to obtain a fair separation quality of the lipids. This approach can be good indication for the changes occurring in the OM profiles of the *mla* mutants, as a result of alteration in the lipid composition due to the lack of the production of Mla components.

Providing clearer evidence about the biogenesis of the OM in *C. jejuni* may be valuable for the identification of novel OM targets for vaccines and chemotherapeutic intervention, and can provide a better understanding of assembly mechanisms of many OM virulence factors and vaccine candidates. Also, it can deliver novel strategies for engineering OM permeability properties to increase *C. jejuni* sensitivity to current antibiotics, or to allow the use of novel ones that are normally impermeable.

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Appendix

SILAC Mass spectrometry data

Table 1. Mass spectrometry data obtained from SILAC analysis of wild-type (heavy labelled) versus *Δcj1289* (light labelled).

(Outer membrane).

Gene No	Protein name	Ratio H/L	Ratio H/L normalized	Ratio H/L variability [%]
Cj0437	Succinate dehydrogenase flavoprotein subunit (EC 1.3.99.1)	2.622	2.8408	17.31
Cj0921c	Major cell-binding factor (CBF1) (PEB1)	2.523	2.6865	10.517
Cj1357c	Putative periplasmic cytochrome C (EC 1.7.2.2)	1.884	2.0636	19.355
Cj0358	Putative cytochrome C551 peroxidase (EC 1.11.1.5)	1.534	1.8021	16.427
Cj1339c;Cj133	Flagellin A;Flagellin B	1.708	1.7692	21.494
Cj1112c	Putative SelR domain containing protein	1.493	1.7392	2.3463
Cj0689	Acetate kinase (EC 2.7.2.1) (Acetokinase)	1.675	1.7247	49.988
Cj0920c	Putative ABC-type amino-acid transporter permease protein	1.655	1.6971	14.709
Cj0762c	Aspartate aminotransferase (EC 2.6.1.1)	1.500	1.5515	13.244
Cj0706	Putative uncharacterized protein	1.309	1.4645	10.969
Cj1059c	Glutamyl-tRNA(Gln) amidotransferase subunit A (Glu-ADT subunit A)	1.431	1.4616	13.209
Cj1214c	Putative exporting protein	1.409	1.4412	16.304
Cj1624c	L-serine dehydratase (EC 4.3.1.17)	1.291	1.4044	11.842
Cj0057	Putative periplasmic protein	1.236	1.3824	10.973
Cj0442	3-oxoacyl-[acyl-carrier-protein] synthase 2 (EC 2.3.1.179)	1.301	1.3707	13.482
Cj0371	UPF0323 lipoprotein Cj0371	1.269	1.3491	23.198

Table 2. Mass spectrometry data obtained from SILAC analysis of wild-type (heavy labelled) versus *Δcj1289* (light labelled).

(Periplasm).

Gene No	Protein name long	Ratio H/L	Ratio H/L normalized	Ratio H/L variability [%]
Cj1157	Putative DNA polymerase III subunit gamma (EC 2.7.7.7)	0.81031	0.69968	9.3368
Cj0914c	CiaB protein	0.81284	0.69901	38.511
Cj0100	ParA family protein	0.78596	0.69537	9.0816
Cj1476c	Pyruvate-flavodoxin oxidoreductase (EC 1.2.7.-)	0.69805	0.68747	17.001
Cj0008	Putative uncharacterized protein	0.79091	0.68302	9.784
Cj1266c	Ni/Fe-hydrogenase large subunit (EC 1.12.5.1)	0.78156	0.67667	37.309
Cj1117c	Ribosomal protein L11 methyltransferase (L11 Mtase) (EC 2.1.1.-)	0.79332	0.67399	13.637
Cj0410	Fumarate reductase iron-sulfur protein (EC 1.3.99.1)	0.75591	0.67084	58.567
Cj0066c	3-dehydroquinate dehydratase (3-dehydroquinase) (EC 4.2.1.10)	0.68761	0.67032	27.589
Cj1070	30S ribosomal protein S6	0.65521	0.66406	4.5316
Cj1259	Major outer membrane protein (Porin)	0.6647	0.65733	14.81
Cj1514c	Putative uncharacterized protein	0.68102	0.64632	7.0323
Cj1595	DNA-directed RNA polymerase subunit alpha (RNAP subunit alpha)	0.73527	0.64184	8.5615
Cj0127c	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	0.73734	0.63974	6.6

Gene No	Protein name long	Ratio H/L	Ratio H/L normalized	Ratio H/L variability [%]
Cj1518	Putative molybdopterin converting factor,subunit 2	0.66998	0.63963	20.918
Cj1041c	Putative periplasmic ATP/GTP-binding protein	0.73576	0.63537	5.1569
Cj1169c	Putative periplasmic protein	0.67509	0.63427	20.664
Cj0298c	3-methyl-2-oxobutanoate hydroxymethyltransferase	0.74302	0.632	10.898
Cj0543	Proline--tRNA ligase (EC 6.1.1.15) (Prolyl-tRNA synthetase) (ProRS)	0.71887	0.63003	7.7582
Cj0843c	Putative secreted transglycosylase	0.70097	0.62976	12.137
Cj0443	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	0.69115	0.61788	15.386
Cj0994c	Ornithine carbamoyltransferase (OTCase) (EC 2.1.3.3)	0.7123	0.6158	8.025
Cj0640c	Aspartate--tRNA ligase (EC 6.1.1.12)	0.64808	0.60853	8.0969
Cj1516	Putative periplasmic oxidoreductase	0.70141	0.60806	12.62
Cj0283c	Chemotaxis protein	0.64295	0.60798	6.0989
Cj0279	Carbamoyl-phosphate synthase large chain (EC 6.3.5.5)	0.61691	0.6065	12.044
Cj1548c	Putative NADP-dependent alcohol dehydrogenase (EC 1.1.1.2)	0.67339	0.60558	7.7717
Cj0912c	Cysteine synthase B (CSase B) (EC 2.5.1.47)	0.65406	0.59389	7.0056
Cj0775c	Valine--tRNA ligase (EC 6.1.1.9) (Valyl-tRNA synthetase) (ValRS)	0.67709	0.59256	82.575
Cj0771c	Putative NLPA family lipoprotein	0.66256	0.59079	16.237
Cj0772c	Putative NLPA family lipoprotein	0.65155	0.58034	5.8041
Cj1534c	DNA protection during starvation protein (EC 1.16.-.-)	0.57876	0.57695	12.183
Cj0419	Putative histidine triad (HIT) family protein	0.534	0.57611	2.7316
Cj0409	Fumarate reductase flavoprotein subunit (EC 1.3.99.1)	0.64809	0.57423	26.87
Cj1112c	Putative SelR domain containing protein	0.52933	0.56755	5.9368
Cj0132	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase (EC	0.65992	0.56205	14.191
Cj0334	Alkyl hydroperoxide reductase (EC 3.4.-.-)	0.59975	0.56195	14.501
Cj0613	Putative periplasmic phosphate binding protein	0.62275	0.56084	9.1763
Cj0896	Phenylalanine--tRNA ligase beta subunit (EC 6.1.1.20)	0.60964	0.55445	36.426
Cj1586	Single domain haemoglobin	0.55139	0.55383	5.7821
Cj1426c	Putative methyltransferase family protein	0.62491	0.54969	23.642
Cj0509c	Chaperone protein ClpB	0.61337	0.54138	16.143
Cj0239c	Nitrogen fixation protein NifU	0.62776	0.53832	35.201
Cj0139	Putative endonuclease	0.54768	0.52809	24.039
Cj0284c	Chemotaxis histidine kinase (EC 2.7.3.-)	0.59698	0.51386	40.22
Cj0169	Superoxide dismutase [Fe] (EC 1.15.1.1)	0.57138	0.50864	14.309
Cj1200	Putative NLPA family lipoprotein	0.56389	0.50851	76.638
Cj1072	30S ribosomal protein S18	0.47604	0.50428	12.771
Cj1725	Putative periplasmic protein	0.52092	0.49227	3.221
Cj1366c	Glutamine--fructose-6-phosphate aminotransferase [isomerizing]	0.5475	0.4786	9.7645
Cj0897	Phenylalanine--tRNA ligase alpha subunit (EC 6.1.1.20)	0.53868	0.473	21.416
Cj1708c	30S ribosomal protein S10	0.4771	0.46764	40.862
Cj0240c	Cysteine desulfurase (NifS protein homolog) (EC 2.8.1.7)	0.48468	0.46577	11.849
Cj0575	Acetolactate synthase small subunit (EC 2.2.1.6)	0.45219	0.45335	0.77265
Cj0779	Probable thiol peroxidase (EC 1.11.1.-)	0.48601	0.4528	27.057
Cj0947c	Putative carbon-nitrogen hydrolase	0.4437	0.45018	130.99
Cj1605c	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase	0.48834	0.44349	112.08
Cj0474	50S ribosomal protein L11	0.4298	0.43443	6.354
Cj1698c	30S ribosomal protein S17	0.40393	0.43092	11.264
Cj1253	Polyribonucleotide nucleotidyltransferase (EC 2.7.7.8)	0.46638	0.42712	14.117
Cj1690c	30S ribosomal protein S5	0.43043	0.42698	2.1529
Cj0037c	Putative cytochrome C	0.45835	0.42055	35.557
Cj1592	30S ribosomal protein S13	0.41372	0.41494	33.406
Cj0450c	50S ribosomal protein L28	0.37964	0.40929	0.5788
Cj0370	30S ribosomal protein S21	0.37993	0.40857	8.4814
Cj1507c	Putative regulatory protein	0.44083	0.4008	189.76
Cj1058c	Inosine-5'-monophosphate dehydrogenase (IMP dehydrogenase)	0.46432	0.40018	95.4
Cj1584c	Putative peptide ABC-transport system periplasmic peptide-binding	0.45471	0.39853	17.427
Cj1377c	Putative ferredoxin	0.45309	0.39682	12.424
Cj0942c	Protein translocase subunit SecA	0.42367	0.38929	25.497
Cj1027c	DNA gyrase subunit A (EC 5.99.1.3)	0.43422	0.38659	29.919
Cj0479	DNA-directed RNA polymerase subunit beta' (RNAP subunit beta')	0.39001	0.38529	47.104

Gene No	Protein name long	Ratio H/L	Ratio H/L normalized	Ratio H/L variability [%]
Cj0478	DNA-directed RNA polymerase subunit beta (RNAP subunit beta)	0.37697	0.37428	72.203
Cj1691c	50S ribosomal protein L18	0.36053	0.37415	17.486
Cj1718c	3-isopropylmalate dehydrogenase (EC 1.1.1.85)	0.43884	0.37326	9.703
Cj0884	30S ribosomal protein S15	0.34506	0.36939	16.288
Cj1693c	30S ribosomal protein S8	0.36799	0.3684	0.98843
Cj1697c	50S ribosomal protein L14	0.36116	0.36483	29.863
Cj0710	30S ribosomal protein S16	0.33948	0.36321	2.7341
Cj1083c	Putative nuclease (EC 4.2.99.18)	0.38401	0.36249	182.3
Cj0289c	Major antigenic peptide PEB3	0.40271	0.35378	28.466
Cj0714	50S ribosomal protein L19	0.34674	0.35138	60.706
Cj1707c	50S ribosomal protein L3	0.37602	0.34957	10.846
Cj1479c	30S ribosomal protein S9	0.33864	0.34245	4.0467
Cj1201	5-methyltetrahydropteroyltriglutamate--homocysteine	0.38562	0.33127	34.641
Cj0492	30S ribosomal protein S7	0.31185	0.32642	30.264
Cj1689c	50S ribosomal protein L15	0.33102	0.32446	9.9369
Cj1480c	50S ribosomal protein L13	0.3377	0.32304	16.493
Cj1338c	Flagellin B	0.34837	0.32098	116.32
Cj1594	30S ribosomal protein S4	0.33654	0.31713	4.9
Cj0699c	Glutamine synthetase (EC 6.3.1.2)	0.36604	0.31429	230
Cj0106	ATP synthase gamma chain (ATP synthase F1 sector gamma subunit)	0.35999	0.30728	17.024
Cj0105	ATP synthase subunit alpha (EC 3.6.3.14)	0.3275	0.28194	0.48087
Cj0007	Glutamate synthase (NADPH) large subunit (EC 1.4.1.13)	0.27861	0.26951	20.566
Cj1659	Periplasmic protein p19	0.24812	0.23716	2.742
Cj1727c	Putative O-acetylhomoserine (Thiol)-lyase (EC 2.5.1.49)	0.22342	0.21195	56.924
Cj1221	60 kDa chaperonin (GroEL protein) (Protein Cpn60)	0.20896	0.17976	8.8305
Cj0939c	Putative uncharacterized protein	0.1713	0.17653	81.068
Cj0415	Putative GMC oxidoreductase subunit	0.15593	0.14	179.73
Cj0777	Putative ATP-dependent DNA helicase	0.13731	0.13069	78.986
Cj0237	Carbonic anhydrase (EC 4.2.1.1)	0.1291	0.1214	5.8383
Cj1325	Putative methyltransferase	0.10834	0.10302	3.7419
Cj0414	Putative oxidoreductase subunit	0.08352	0.078985	301.74
Cj1393	Putative cystathionine beta-lyase (EC 4.4.1.8)	0.07409	0.066526	14.299

Table 3. Mass spectrometry data obtained from SILAC analysis of wild-type (heavy labelled) versus *Δcj0694* (light labelled).

(Outer membrane).

Gene No	Protein name	Ratio H/L	Ratio H/L normalized	Ratio H/L variability [%]
Cj1674	Putative uncharacterized protein	4.1104	6.2538	257.47
Cj0066c	3-dehydroquinate dehydratase (3-dehydroquinase) (EC 4.2.1.10)	2.4689	4.2574	184.11
Cj0112	Protein TolB	1.3841	2.1904	33.554
Cj0921c	Major cell-binding factor (CBF1) (PEB1)	1.6282	2.0554	23.051
Cj0706	Putative uncharacterized protein	1.3522	2.0028	25.711
Cj1337	PseE protein	1.1844	1.938	134.87
Cj0078c	Cytolethal distending toxin B	1.0996	1.8629	6.6786
Cj1266c	Ni/Fe-hydrogenase large subunit (EC 1.12.5.1)	1.1494	1.7905	101.5
Cj1516	Putative periplasmic oxidoreductase	1.0935	1.7539	24.596
Cj0755	Ferric enterobactin uptake receptor	1.0842	1.747	16.083
Cj1339c;Cj1338c	Flagellin A;Flagellin B	1.1924	1.7027	19.886
Cj1357c	Putative periplasmic cytochrome C (EC 1.7.2.2)	1.0696	1.6828	14.042

Gene No	Protein name	Ratio H/L	Ratio H/L normalized	Ratio H/L variability [%]
Cj1269c	N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)	1.0288	1.6479	21.952
Cj0077c	Cytolethal distending toxin C	1.2179	1.6239	17.352
Cj0475	50S ribosomal protein L1	0.94126	1.6164	4.446
Cj1289	Possible periplasmic protein	1.0609	1.5766	26.498
Cj0092	Putative periplasmic protein	0.94999	1.5624	35.865
Cj0114	Putative periplasmic protein	0.90665	1.5538	7.0377
Cj1479c	30S ribosomal protein S9	1.0616	1.5424	17.559
Cj0561c	Putative periplasmic protein	1.0257	1.5202	10.998
Cj1704c	50S ribosomal protein L2	0.96805	1.4932	15.825
Cj1700c	50S ribosomal protein L16	1.0837	1.4897	4.2495
Cj1651c	Methionine aminopeptidase (EC 3.4.11.18)	0.9003	1.4697	1.0983
Cj0511	Putative secreted protease	0.77606	1.4352	11.801
Cj0886c	DNA translocase FtsK	0.91653	1.4281	29.249
Cj1701c	30S ribosomal protein S3	0.82458	1.4133	19.208
Cj0093	Putative periplasmic protein	0.85458	1.3879	29.134
Cj1702c	50S ribosomal protein L22	0.94819	1.3736	2.5786
Cj1689c	50S ribosomal protein L15	0.89892	1.3731	11.308
Cj1500	Putative integral membrane protein	0.82261	1.3553	15.368
Cj1181c	Elongation factor Ts (EF-Ts)	1.166	1.3509	15.938
Cj1592	30S ribosomal protein S13	1.0711	1.3503	15.836
Cj0089	Putative lipoprotein	0.85715	1.3404	13.731
Cj0129c	Outer membrane protein assembly factor BamA	0.88825	1.3362	22.697
Cj1214c	Putative exporting protein	1.0621	1.3335	35.202
Cj0245	50S ribosomal protein L20	0.9243	1.3306	18.288
Cj0492	30S ribosomal protein S7	0.89393	1.3216	16.746
Cj0884	30S ribosomal protein S15	1.0663	1.3125	13.561
Cj0812	Threonine synthase. Functional classification-Amino acid biosynthesis-	1.1412	1.3009	30.589

Table 4. Mass spectrometry data obtained from SILAC analysis of wild-type (heavy labelled) versus *Δcj0694* (light labelled).

(Periplasm).

Gene No	Protein name	Ratio H/L	Ratio H/L normalized	Ratio H/L variability [%]
Cj1691c	50S ribosomal protein L18	0.6141	0.69689	11.919
Cj0069	Putative uncharacterized protein	0.60351	0.69517	9.4542
Cj1070	30S ribosomal protein S6	0.62221	0.69347	0.81139
Cj0994c	Ornithine carbamoyltransferase (OTCase) (EC 2.1.3.3)	0.60154	0.69115	4.4986
Cj0194	GTP cyclohydrolase 1 (EC 3.5.4.16) (GTP cyclohydrolase I) (GTP-CH-I)	0.60191	0.68682	11.121
Cj1624c	L-serine dehydratase (EC 4.3.1.17)	0.59727	0.68429	17.909
Cj1724c	NADPH-dependent 7-cyano-7-deazaguanine reductase (EC 1.7.1.13)	0.61483	0.68272	3.708
Cj0643	Two-component response regulator	0.59978	0.68271	8.9054
Cj1543	Putative allophanate hydrolase subunit 2	0.56354	0.68252	10.09
Cj0833c	Putative oxidoreductase	0.55182	0.68074	16.185
Cj0688	Putative phosphate acetyltransferase (EC 2.3.1.8)	0.57145	0.67748	16.108
Cj1221	60 kDa chaperonin (GroEL protein) (Protein Cpn60)	0.56504	0.67641	11.415
Cj0518	Chaperone protein HtpG (Heat shock protein HtpG) (High	0.5759	0.67514	12.604
Cj1607	Bifunctional enzyme IspD/IspF [Includes: 2-C-methyl-D-erythritol 4-	0.55369	0.67458	29.388
Cj1534c	DNA protection during starvation protein (EC 1.16.-.-)	0.44008	0.6727	22.017
Cj1594	30S ribosomal protein S4	0.58986	0.67088	15.079
Cj1595	DNA-directed RNA polymerase subunit alpha (RNAP subunit alpha)	0.54794	0.6661	7.3095
Cj0759	Chaperone protein DnaK (HSP70) (Heat shock 70 kDa protein) (Heat	0.55187	0.66609	27.904
Cj1478c	Outer membrane fibronectin-binding protein	0.57565	0.66426	42.814

Gene No	Protein name	Ratio H/L	Ratio H/L normalized	Ratio H/L variability [%]
Cj0016	7-cyano-7-deazaguanine synthase (EC 6.3.4.20)	0.52467	0.65914	3.5848
Cj0533	Succinyl-CoA ligase [ADP-forming] subunit beta (EC 6.2.1.5) (Succinyl-	0.56376	0.65724	15.472
Cj1542	Putative allophanate hydrolase subunit 1	0.55781	0.65483	6.8005
Cj0779	Probable thiol peroxidase (EC 1.11.1.-)	0.57386	0.65283	87.524
Cj0534	Succinyl-CoA ligase [ADP-forming] subunit alpha (EC 6.2.1.5)	0.55543	0.65122	17.459
Cj0427	Putative uncharacterized protein	0.5764	0.64955	9.2226
Cj0113	Peptidoglycan associated lipoprotein (Omp18)	0.57161	0.64769	29.124
Cj1288c	Glutamate--tRNA ligase 2 (EC 6.1.1.17) (Glutamyl-tRNA synthetase 2)	0.57527	0.64532	12.14
Cj1201	5-methyltetrahydropteroyltriglutamate--homocysteine	0.34777	0.64247	35.039
Cj1537c	Acetyl-coenzyme A synthetase (AcCoA synthetase) (Acs) (EC 6.2.1.1)	0.53704	0.63748	32.245
Cj0953c	Bifunctional purine biosynthesis protein PurH (EC 2.1.2.3)	0.52515	0.63254	9.2057
Cj0583	Putative uncharacterized protein	0.54845	0.6322	64.389
Cj0896	Phenylalanine--tRNA ligase beta subunit (EC 6.1.1.20) (Phenylalanyl-	0.33417	0.62833	134.81
Cj1436c	Aminotransferase	0.54927	0.62433	9.5865
Cj0701	Putative protease	0.56151	0.62378	11.689
Cj0642	DNA repair protein RecN (Recombination protein N)	0.53988	0.62285	6.1036
Cj1426c	Putative methyltransferase family protein	0.52715	0.62177	15.822
Cj0196c	Amidophosphoribosyltransferase (ATase) (EC 2.4.2.14) (Glutamine	0.52335	0.61982	12.308
Cj0206	Threonine--tRNA ligase (EC 6.1.1.3) (Threonyl-tRNA synthetase)	0.52983	0.61779	7.0202
Cj1548c	Putative NADP-dependent alcohol dehydrogenase (EC 1.1.1.2)	0.54156	0.61704	9.003
Cj1498c	Adenylosuccinate synthetase (AMPSase) (AdSS) (EC 6.3.4.4) (IMP--	0.5616	0.61642	5.2484
Cj1399c	Putative Ni/Fe-hydrogenase small subunit	0.5406	0.61146	6.7497
Cj0775c	Valine--tRNA ligase (EC 6.1.1.9) (Valyl-tRNA synthetase) (ValRS)	0.33106	0.61065	35.547
Cj1085c	Transcription-repair coupling factor	0.38493	0.61064	7.713
Cj0384c	2-dehydro-3-deoxyphosphooctonate aldolase (EC 2.5.1.55)	0.51779	0.60924	5.9383
Cj0681	Putative uncharacterized protein	0.58104	0.60857	13.561
Cj0342c	Excinuclease ABC subunit A	0.43072	0.60329	14.473
Cj0845c	Glutamate--tRNA ligase 1 (EC 6.1.1.17) (Glutamyl-tRNA synthetase 1)	0.54183	0.60074	43.258
Cj0269c	Branched-chain amino acid aminotransferase (EC 2.6.1.42)	0.49013	0.5978	18.79
Cj0478	DNA-directed RNA polymerase subunit beta (RNAP subunit beta) (EC	0.37756	0.59387	27.081
Cj1190c;Cj0448	Bipartate energy taxis response protein cetA;Putative MCP-type	0.51458	0.59158	15.524
Cj0389	Serine--tRNA ligase (EC 6.1.1.11) (Seryl-tRNA synthetase) (SerRS)	0.54269	0.58922	104.42
Cj0891c	D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95)	0.48873	0.58469	17.964
Cj0169	Superoxide dismutase [Fe] (EC 1.15.1.1)	0.47426	0.57648	12.43
Cj1686c	DNA topoisomerase 1 (EC 5.99.1.2) (DNA topoisomerase I)	0.31523	0.57387	68.089
Cj0783	Periplasmic nitrate reductase, electron transfer subunit (Diheme	0.51109	0.57094	9.9869
Cj1418c	Putative transferase	0.30861	0.56951	57.885
Cj0884	30S ribosomal protein S15	0.4498	0.56011	14.854
Cj1690c	30S ribosomal protein S5	0.50059	0.55815	5.3156
Cj0372	Putative glutathionylspermidine synthase (EC 6.3.1.8)	0.46119	0.54808	12.009
Cj1164c	Putative uncharacterized protein	0.43835	0.54409	53.292
Cj1707c	50S ribosomal protein L3	0.47116	0.54369	41.696
Cj1481c	Putative helicase (EC 3.6.1.-)	0.36169	0.5413	1.5088
Cj0139	Putative endonuclease	0.29319	0.53652	15.928
Cj1112c	Putative SelR domain containing protein	0.47871	0.53467	12.592
Cj0274	Acyl-[acyl-carrier-protein]--UDP-N-acetylglucosamine O-	0.45943	0.53371	16.818
Cj0572	3,4-dihydroxy-2-butanone 4-phosphate synthase (DHBP synthase)	0.46501	0.52715	7.3751
Cj0074c	Putative iron-sulfur protein	0.44322	0.51687	6.1836
Cj0279	Carbamoyl-phosphate synthase large chain (EC 6.3.5.5) (Carbamoyl-	0.32739	0.51686	24.728
Cj0240c	Cysteine desulfurase (NifS protein homolog) (EC 2.8.1.7)	0.42449	0.51616	15.181
Cj1364c	Fumarate hydratase class II (Fumarase C) (EC 4.2.1.2)	0.44334	0.50568	19.302
Cj0914c	CiaB protein	0.42904	0.50211	69.463
Cj0485	Putative oxidoreductase	0.43459	0.50034	7.8948
Cj0700	Putative uncharacterized protein	0.41255	0.49841	71.515
Cj1274c	Uridylate kinase (UK) (EC 2.7.4.22) (Uridine monophosphate kinase)	0.40219	0.4942	4.3899
Cj1271c	Tyrosine--tRNA ligase (EC 6.1.1.1) (Tyrosyl-tRNA synthetase) (TyrRS)	0.45491	0.49368	4.701
Cj1476c	Pyruvate-flavodoxin oxidoreductase (EC 1.2.7.-)	0.31839	0.49057	14.99
Cj0073c	Putative uncharacterized protein	0.42736	0.48966	7.2603

Gene No	Protein name	Ratio H/L	Ratio H/L normalized	Ratio H/L variability [%]
Cj0008	Putative uncharacterized protein	0.41602	0.48822	127.05
Cj1377c	Putative ferredoxin	0.41913	0.48623	12.829
Cj1325	Putative methyltransferase	0.39602	0.47487	182.95
Cj0838c	Methionine--tRNA ligase (EC 6.1.1.10) (Methionyl-tRNA synthetase)	0.39109	0.47319	17.7
Cj0942c	Protein translocase subunit SecA	0.29968	0.47211	35.84
Cj0371	UPF0323 lipoprotein Cj0371	0.40904	0.47196	1.0471
Cj1236	Putative uncharacterized protein	0.39789	0.47182	111.15
Cj1480c	50S ribosomal protein L13	0.41595	0.46935	7.7842
Cj0575	Acetolactate synthase small subunit (EC 2.2.1.6)	0.42021	0.46536	11.146
Cj0409	Fumarate reductase flavoprotein subunit (EC 1.3.99.1)	0.35349	0.45882	32.18
Cj0197c	4-hydroxy-tetrahydrodipicolinate reductase (HTPA reductase) (EC	0.39904	0.45735	7.0055
Cj1157	Putative DNA polymerase III subunit gamma (EC 2.7.7.7)	0.37627	0.45257	7.9662
Cj0026c	Thymidylate synthase ThyX (TS) (TSase) (EC 2.1.1.148)	0.39634	0.44293	7.937
Cj1672c	Enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (2-	0.39577	0.44082	29.432
Cj1400c	Enoyl-[acyl-carrier-protein] reductase [NADH] (EC 1.3.1.9)	0.37849	0.43992	10.925
Cj0640c	Aspartate--tRNA ligase (EC 6.1.1.12) (Aspartyl-tRNA synthetase)	0.36587	0.43988	15.556
Cj1259	Major outer membrane protein (Porin)	0.37043	0.43979	19.914
Cj0474	50S ribosomal protein L11	0.3891	0.43597	9.7937
Cj0284c	Chemotaxis histidine kinase (EC 2.7.3.-)	0.23928	0.43581	43.09
Cj1479c	30S ribosomal protein S9	0.37662	0.43469	129.87
Cj0192c	ATP-dependent Clp protease proteolytic subunit (EC 3.4.21.92)	0.3742	0.42699	5.0272
Cj0843c	Putative secreted transglycosylase	0.3564	0.42484	18.037
Cj0298c	3-methyl-2-oxobutanoate hydroxymethyltransferase (EC 2.1.2.11)	0.3499	0.42224	3.2833
Cj0401	Lysine--tRNA ligase (EC 6.1.1.6) (Lysyl-tRNA synthetase) (LysRS)	0.35582	0.4154	37.552
Cj1359	Polyphosphate kinase (EC 2.7.4.1) (ATP-polyphosphate	0.2586	0.41428	45.936
Cj0105	ATP synthase subunit alpha (EC 3.6.3.14) (ATP synthase F1 sector	0.35539	0.41323	2.5921
Cj0023	Adenylosuccinate lyase (EC 4.3.2.2)	0.36813	0.41313	11.93
Cj0136	Translation initiation factor IF-2	0.27645	0.41238	11.958
Cj1519	Putative molybdopterin biosynthesis protein	0.36709	0.41046	0.24199
Cj1287c	Malate oxidoreductase (EC 1.1.1.38)	0.38009	0.41038	56.913
Cj1234	Glycine--tRNA ligase beta subunit (EC 6.1.1.14) (Glycyl-tRNA	0.34223	0.40703	30.261
Cj1523c	Putative CRISPR-associated protein	0.28577	0.40474	19.429
Cj0087	Fumarate hydratase class II (Fumarase C) (EC 4.2.1.2)	0.36247	0.39948	35.98
Cj0537	OORB subunit of 2-oxoglutarate:acceptor oxidoreductase	0.33841	0.39619	10.823
Cj1110c	Putative MCP-type signal transduction protein	0.35939	0.39583	8.0077
Cj0536	OORA subunit of 2-oxoglutarate:acceptor oxidoreductase	0.34647	0.39113	22.037
Cj0778	Major antigenic peptide PEB2	0.34313	0.38855	12.172
Cj0665c	Argininosuccinate synthase (EC 6.3.4.5) (Citrulline--aspartate ligase)	0.35297	0.37691	14.422
Cj1027c	DNA gyrase subunit A (EC 5.99.1.3)	0.21474	0.36636	47.986
Cj0710	30S ribosomal protein S16	0.29884	0.3486	5.5912
Cj1592	30S ribosomal protein S13	0.30883	0.34843	6.0148
Cj0641	Probable inorganic polyphosphate/ATP-NAD kinase (Poly(P)/ATP	0.28711	0.33985	25.692
Cj0807	Putative oxidoreductase	0.31117	0.33944	11.413
Cj0622	Carbamoyltransferase (EC 2.1.3.-)	0.19344	0.33684	40.342
Cj0699c	Glutamine synthetase (EC 6.3.1.2)	0.27858	0.33653	53.199
Cj0392c	Pyruvate kinase (EC 2.7.1.40)	0.28794	0.3323	39.941
Cj0283c	Chemotaxis protein	0.26776	0.30723	64.789
Cj1718c	3-isopropylmalate dehydrogenase (EC 1.1.1.85) (3-IPM-DH) (Beta-	0.23037	0.29014	0.15542
Cj0913c	DNA-binding protein HU (HCj)	0.24466	0.28784	6.1195
Cj0237	Carbonic anhydrase (EC 4.2.1.1)	0.24961	0.28589	5.5503
Cj0543	Proline--tRNA ligase (EC 6.1.1.15) (Prolyl-tRNA synthetase) (ProRS)	0.2183	0.26281	13.5
Cj1689c	50S ribosomal protein L15	0.22964	0.253	6.2074
Cj1266c	Ni/Fe-hydrogenase large subunit (EC 1.12.5.1)	0.18314	0.21662	21.846
Cj1727c	Putative O-acetylhomoserine (Thiol)-lyase (EC 2.5.1.49)	0.13855	0.21498	94.995
Cj0499	Putative histidine triad (HIT) family protein	0.18117	0.20412	190.61
Cj1393	Putative cystathionine beta-lyase (EC 4.4.1.8)	0.09115	0.10547	199.39