Chapter 1 – Literature Review

1.1 *Shigella*

Shigella are Gram-negative intracellular bacterial pathogens that inhabit the gastrointestinal tract of humans and are the causative agent of shigellosis. Shigellosis is a current health burden which is endemic and estimated to affect 80-165 million individuals annually. Ninety-nine percent of infections caused by *Shigella* occur in developing countries, and the majority of cases, and cases of deaths, occur among children less than 5 years of age (Kotloff *et al.*, 1999; WHO, 2005).

1.1.1 Disease and symptoms

Shigellosis is exclusively a human disease and is usually acquired through contaminated food and water sources (DuPont *et al.*, 1989). *Shigella* infect via the oral-faecal route and infection is transmissible with as few as 100 micro-organisms (DuPont *et al.*, 1989), partly due to the bacterium's ability to survive the high acidity of the host's stomach (Small *et al.*, 1994). The high incidence of *Shigella* in developing countries is considered to be attributed to the lack of clean water, poor sanitation, malnutrition, and the cost of antibiotic treatment (Jennison & Verma, 2004). Infection is known to produce a range of symptoms, and range from watery diarrhoea to severe dysentery. Severe dysentery is characterised by fever, abdominal pains, and acute permanent bloody and mucoid diarrhoea (Phalipon & Sansonetti, 2007). In the absence of effective treatments, patients with shigellosis can develop secondary complications such as septicaemia and pneumonia (Bennish, 1991; Jennison & Verma, 2004).

1.2 Classification

Shigella belongs to the family of *Enterobacteriaceae*. They share common characteristics and genetic relatedness with members of the genus *Escherichia* (in particular to enteroinvasive *E. coli* [EIEC] which is also responsible for shigellosis in humans), and are now moving towards being classified as a subtype of *E. coli* (Lan & Reeves, 2002; Parsot, 2005). Analyses on the evolution of *Shigella* (as well as EIEC) have suggested that *Shigella* originated from non-invasive *E. coli* but unlike most commensal and other pathogenic *E. coli* strains, have acquired the ability to invade cells through the gain of a ~220 kb virulence plasmid and other virulence genes (pathogenicity islands) as enhancers, and loss of virulence-suppressor genes (such as *cadA*) and genes which are no longer required for living in the intracellular niche (e.g. lactose utilisation and motility). However for the purpose of this study, *Shigella* will be italicised to keep to the old nomenclature.

1.2.1 Species and serotypes

Shigella strains can be divided into four species/groups (or clones of *E. coli* as denoted by Lan & Reeves (2002)), which can be further divided into serotypes based on biochemical differences and variations in their O antigen (Oag) chains. They include: *Shigella dysenteriae* (Group A), which is made up of 15 serotypes; *Shigella flexneri* (Group B), which consists of the 14 classical serotypes and subserotypes; *Shigella boydii* (Group C), which has 20 serotypes; and *Shigella sonnei* (Group D), which has a single serotype (Levine *et al.*, 2007). For *S. flexneri*, 13 of the serotypes (with the exception of serotype 6 (Dmitriev *et al.*, 1979)), share an identical linear backbone which consists of a tetrasaccharide repeat unit composed of the following sugars: →2)-α-L-rhamnose-(1→2)-α-L-rhamnose-(1→3)-α-L-rhamnose-(1→3)-β-D-*N*-acetylglucosamine-(1→ (Morona *et al.*, 1995b; Simmons & Romanowska, 1987). The basic Oag structure is known as serotype Y (having group antigen 3,4) and the

addition of glucosyl and/or O-acetyl groups to different sugars in the tetrasaccharide unit (summarised in Fig. 1.1) forms the basis of serotype conversion (Allison & Verma, 2000). *S. flexneri* serotype 2a is the most predominant in developing countries (Kotloff *et al.*, 1999) and causes greater mortality than any other *Shigella* strain (Bennish & Wojtyniak, 1991).

1.3 *Shigella* pathogenesis

Shigella invade the human colonic and rectal mucosa of the gut epithelium and cause severe inflammation and eventual mucosal destruction by intracellular and intercellular spreading of *Shigella* inside epithelial cells (Bernardini *et al.*, 1989; LaBrec *et al.*, 1964). This model of infection is summarised in Fig. 1.2.

1.3.1 Invasion of the gut epithelium

During *Shigella* infection, ingested organisms that survive the gastric acidity of the stomach pass through to the small intestine and eventually reach the colon where they translocate through the follicular associated epithelium (FAE) via the Membranous epithelial cells (M cells). Bacteria then gain access to the underlying lymphoid nodules and infect resident macrophages, evade the killing mechanisms of macrophages by IpaB-mediated lysis of the phagocytic vacuole (High *et al.*, 1992), and induce macrophage cell death by apoptosis or oncosis (Nonaka *et al.*, 1999). Infected macrophages release increasing amounts of interleukin-1β (IL-1β) and interleukin-8 (IL-8) cytokines. IL-1β gives rise to the strong inflammatory response (Suzuki & Sasakawa, 2001; Zychlinsky *et al.*, 1994), and together with IL-8 helps to recruit increased amounts of polymorphonuclear cells (such as neutrophils) to the site of infection and disrupt the integrity of the epithelium (Beatty & Sansonetti, 1997; Perdomo *et al.*, 1994).

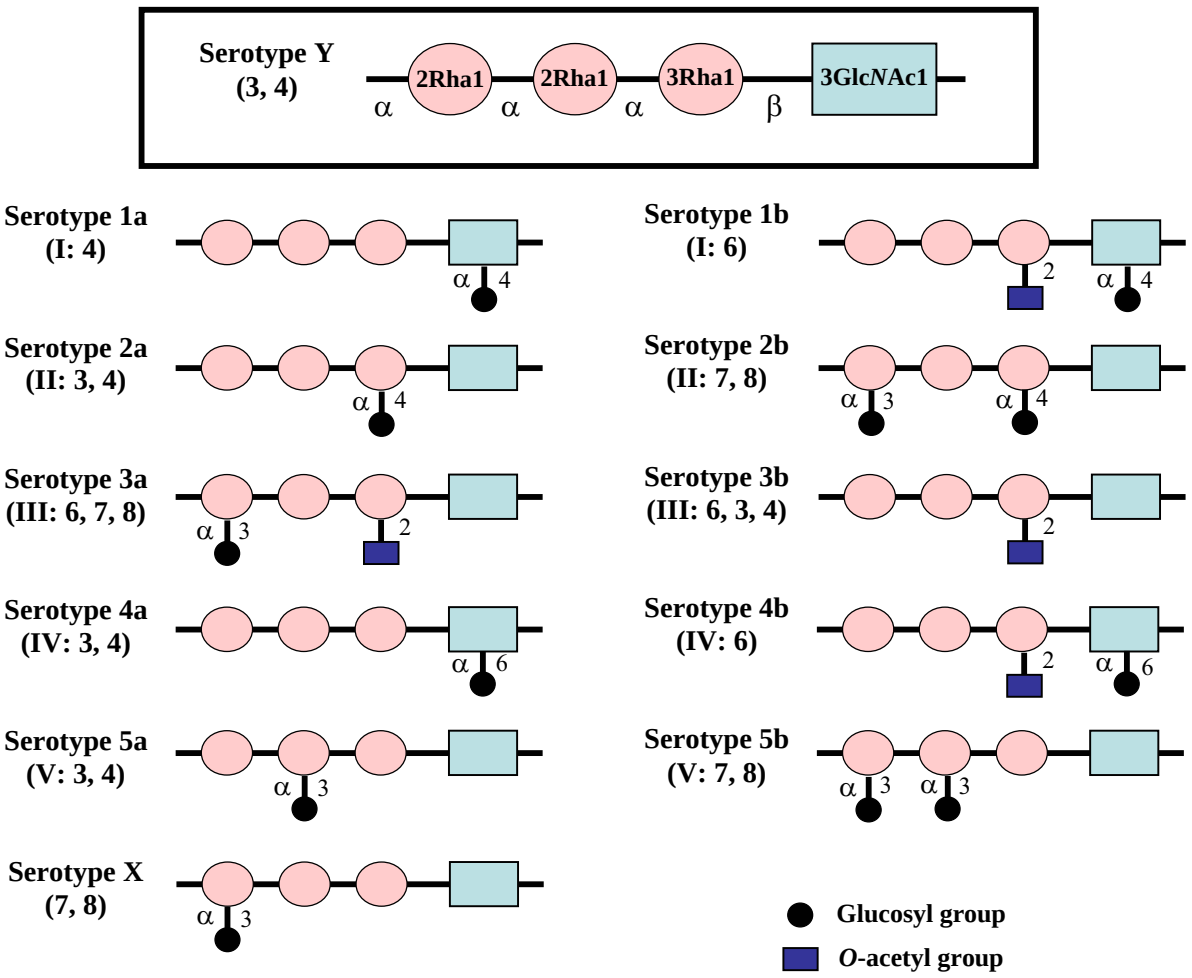


Fig. 1.1 *S. flexneri* 2a serotypes

The basic Oag structure (serotype Y) consists of the tetrasaccharide repeat unit [→2)-α-L-rhamnose-(1→2)-α-L-rhamnose-(1→3)-α-L-rhamnose-(1→3)-β-D-*N*-acetylglucosamine-(1→]. The additions of glucosyl and/or *O*-acetyl groups to different sugars in the tetrasaccharide unit are indicated for each serotype. Each serotype has one type-specific (Roman numeral) and one or more group-specific (Arabic numeral) antigen determinants. Abbreviations: Rha, rhamnose; Glc*N*Ac, *N*-acetylglucosamine (diagram adapted from Allison and Verma, 2000).

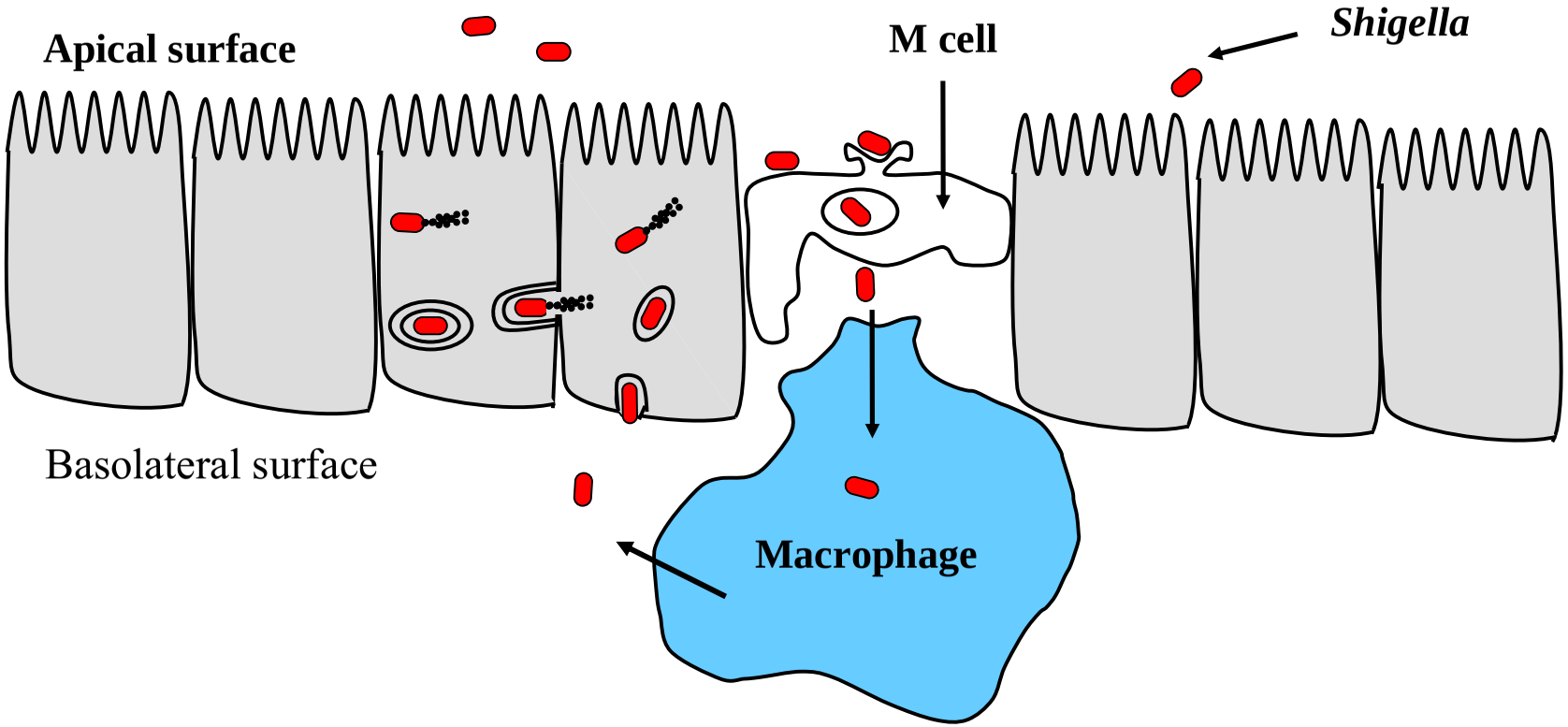


Fig. 1.2 ***Shigella*** **pathogenesis**

Shigella invade the gut epithelium via M cells and translocate into the underlying lymph nodules where they infect resident macrophages and induce cell death by apoptosis or oncosis. *Shigella* then enter epithelial cells by bacterial mediated endocytosis via the basolateral surface and, once inside cells, lyse the encapsulating endocytic vacuole and escape into the cytoplasm where they form F-actin comet tails at one pole of the bacterium. *Shigella* move to form protrusions at the epithelial cell surface and spread into adjacent cells where encapsulating endocytic vacuoles are again lysed and the spread process repeated.

1.3.2 Entry into epithelial cells

Shigella entry into epithelial cells is achieved by bacterial mediated endocytosis, usually through the basolateral surface of epithelial cells (Mounier *et al.*, 1992). *Shigella* invasion proteins required for entry into epithelial cells are delivered by a Type III secretion system (TTSS) which results in localised reorganisation of the cell's cytoskeleton at the point of entry. Bacteria become enveloped by the resulting localised membrane ruffling and are internalised (Menard *et al.*, 1996b). Once internalised, the encapsulating endocytic vacuole is then lysed and the bacterium escapes into the cytoplasm.

The genes required for entry and lysis of the phagocytic vacuole are located on a 30 kb region of the large 220 kb *Shigella* virulence plasmid (Baudry *et al.*, 1987; Maurelli *et al.*, 1985; Sasakawa *et al.*, 1988). *Shigella* strains cured of the virulence plasmid are avirulent and unable to penetrate cultured epithelial cells (Sansonetti *et al.*, 1982). The genes required for entry and lysis include *ipgC* (invasion plasmid gene), *ipaB*, *ipaC*, *ipaD* (invasion plasmid antigens), and the majority of the 20 *mxi-spa* (membrane expression of Ipas-surface presentation of antigens) genes (Menard *et al.*, 1996a). The three secreted Ipa proteins (i.e. IpaB, IpaC and IpaD), together with lipopolysaccharide (LPS), are the dominant antigens in the humoral response during shigellosis (DuPont *et al.*, 1972a; DuPont *et al.*, 1972b; Hale *et al.*, 1985; Jennison & Verma, 2004). These secretory proteins contact the eukaryotic cell surface to trigger the phagocytic process, and subsequently carry out lysis of the vacuole membrane to release the bacterium into the cytoplasm (High *et al.*, 1992; Menard *et al.*, 1993). The large set of *mxi* and *spa* genes encode the components of the TTSS necessary for the secretion of Ipa proteins from the bacterial cytoplasm to the cytoplasmic membrane of epithelial cells (Allaoui *et al.*, 1993; Andrews *et al.*, 1991; Hueck, 1998). The regulatory genes, *virF* and *virB*, are located on the *Shigella* virulence plasmid and activate expression of *ipa, mxi* and *spa* operons in bacteria growing at 37°C (Dorman & Porter, 1998).

1.3.3 Intracellular and Intercellular spread

Once *Shigella* escape into the cytoplasm of epithelial cells, the bacteria multiply and utilise host cell machinery to initiate actin polymerisation and form comet-like F-actin tails at one pole of the bacterium which can be seen by staining with FITC-phalloidin. During the multiplication step, *Shigella* escapes the autophagy system by secreting IcsB via the TTSS. IcsB then binds IcsA, an OM protein required to initiate actin polymerisation, to inhibit binding by the autophagy protein, Atg5 (Ogawa *et al.*, 2003; Ogawa *et al.*, 2005). Polymerisation of actin enables *Shigella* to propel through the cytoplasm and form protrusions at the epithelial cell surface into adjacent cells, hence initiating *Shigella* movement from one cell to another (Goldberg, 2001; Monack & Theriot, 2001). This type of movement is described as actin-based motility (ABM).

1.3.3.1 Actin based motility

Shigella motility was first observed by Ogawa *et al*. in 1968 by phase contrast cinemicrography (Ogawa *et al.*, 1968), and was observed to be independent of organelle movement and to exhibit polarity. Also identified at the time by Ogawa *et al*. (1968) was the presence of motile bacteria occasionally seen within protrusions extending the host cell surface (Ogawa *et al.*, 1968; Suzuki & Sasakawa, 2001). This has since led to the discovery of the IcsA gene (also known as *virG*). IcsA is also located on the large 220 kb virulence plasmid of *S. flexneri* (Shere *et al.*, 1997; Venkatesan *et al.*, 2001) and is essential for the assembly of actin at the pole of the bacterium for ABM (Bernardini *et al.*, 1989; Lett *et al.*, 1989; Makino *et al.*, 1986). In experimental models, mutants of *Shigella* that do not assemble actin have been shown to be greatly attenuated in human volunteers, monkeys and mice (Kotloff *et al.*, 1996; Kotloff *et al.*, 2002; Lett *et al.*, 1989; Makino *et al.*, 1986; Sansonetti *et al.*, 1991).

1.3.3.2 IcsA

IcsA is a 120 kDa cell surface exposed outer membrane (OM) protein essential for intracellular and cell-to-cell spread (Bernardini *et al.*, 1989; Lett *et al.*, 1989). Composed of a 1,120 amino acids (aa), IcsA contains 3 distinctive domains; the N-terminal signal sequence (residues 1 to 52), the 706 aa surface exposed α-domain (residues 53-758), and the 355 aa OM embedded pore-forming β-core (residues 759-1102) (Goldberg *et al.*, 1993b; Lett *et al.*, 1989; Suzuki *et al.*, 1995). IcsA is polarly localised and essential for the movement of *Shigella* in mammalian cells, as well as spread between epithelial cells needed to elicit *Shigella* pathogenesis (Bernardini *et al.*, 1989; Goldberg *et al.*, 1993b). Previous studies have demonstrated that a deletion in the gene encoding IcsA produces a mutant that does not polymerise actin on its surface, nor move within the host cell and form protrusions into adjacent cells (Bernardini *et al.*, 1989; Lett *et al.*, 1989; Makino *et al.*, 1986).

Newly synthesised IcsA appear to be directed to the old division pole where it is autotransported to the OM (Charles *et al.*, 2001; Jain *et al.*, 2006; Steinhauer *et al.*, 1999). To initiate actin polymerisation required for *Shigella* ABM, the surface exposed α-domain of IcsA at the bacterial pole interacts with the host Neural Wiskott-Aldrich syndrome protein (N-WASP) via glycine-rich repeat binding sites located in the α-domain of IcsA (Suzuki *et al.*, 1996; Suzuki *et al.*, 1998). IcsA activates N-WASP which in turn interacts with the actin related protein (Arp) 2/3 complex to mediate local actin nucleation and elongation (Egile *et al.*, 1999; Suzuki *et al.*, 1998; Suzuki *et al.*, 2000). Interaction of vinculin (an actin-binding cytoskeleton protein) with IcsA, vasodilator-stimulating phosphoprotein (VASP), and profilin has also been proposed to contribute to enhancing barbed-ends growth of actin filaments (Laine *et al.*, 1997; Suzuki & Sasakawa, 2001). This localised actin assembly at the site of IcsA localisation provides the force for *Shigella* movement. In wild-type *S. flexneri* 2457T

serotype 2a, IcsA is cleaved by a member of the Omptin family of proteases, known as IcsP, which is also localised to the OM.

1.4 The Gram-negative outer membrane

The cell envelope of Gram-negative bacteria such as *Shigella* consists of two cell membranes, the inner membrane (IM) and the outer membrane (OM) (reviewed by Bos *et al*., 2007). Both differ in composition and structure and are separated by the periplasm containing the peptidoglycan layer. The IM is a phospholipid bilayer and integral IM proteins tend to typically span the membrane in the form of hydrophobic α–helices (Bos *et al.*, 2007). The OM in contrast, is an asymmetrical bilayer consisting of phospholipids and LPS in the inner and outer leaflet, respectively. It acts as a selective barrier that protects the bacteria from harmful components in its environment, and allows nutrients to pass through by passive diffusion via OM porin proteins (Bos *et al.*, 2007; Nikaido, 2003). OM proteins generally consist of anti-parallel β–strands that fold into cyclindrical β–barrels with a hydrophilic interior, and hydrophobic resides pointing outwards to face the membrane lipids (Bos *et al.*, 2007; Koebnik *et al.*, 2000). There are generally two types of OM proteins, which include OM lipoproteins that are normally anchored to the OM by an N-terminal lipid tail, and OM integral proteins (for example IcsA) which contain membrane-spanning regions (Bos & Tommassen, 2004).

1.4.1 Surface distribution of OM proteins

In *Shigella*, the OM protein IcsA has been shown to be polarly localised on the cell surface. However, unlike IM proteins such as FtsZ (Lutkenhaus & Addinall, 1997) and MreB (Tamaki *et al.*, 1980) known to be involved in cell division and shape, and shown to be assembled in long filaments that spiral across the IM, the subcellular distribution of only a few OM proteins

have been determined. Lynne *et al*. (2007) recently detected the presence of surface Iss and Bor proteins all over the surface of *E. coli* with no distinct pattern (Lynne *et al.*, 2007), while Gibbs *et al*. (2004) recently characterised the *E. coli* OM porin LamB to exist as two populations on the cell surface: one that diffused in a helical pattern, and one that was relatively immobile (Gibbs *et al.*, 2004). In addition to this, recent data from Ghosh *et al*. (2005) found that a number of *E. coli* OM proteins (non-specific) were organised in stable helical swaths, and that LPS itself possessed a helical distribution across the cell surface of *E. coli* (Ghosh & Young, 2005). Their results suggest that the OM may have a defined non-random organisation in which OM proteins (as well as LPS) can be embedded in stable domains at the poles and along a number of helical ribbons which span the length of the Gram-negative bacterium (Ghosh & Young, 2005). No *Shigella* OM protein to date has been shown to display this helical distribution. IcsP belongs to the family of OM proteases which has no known distribution to date.

1.5 Outer membrane proteases

1.5.1 The Omptin family

The outer membrane protease (Omptin) family of enterobacterial proteases is composed of 6 members; OmpT and OmpP of *Escherichia coli*, Pla of *Yersinia pestis*, PgtE of *Salmonella enterica*, Pla endopeptidase A (PlaA) of *Erwinia pyrifoliae*, and IcsP of *Shigella flexneri*. A protein sequence alignment is shown in Fig. 1.3. Members of the Omptin proteases are highly related in structure and share at least 50% sequence identity (Kukkonen & Korhonen, 2004) (the % identity between IcsP and other Omptins is summarised in Table 1.1). Typically, Omptin sequences lack cysteine and possess a β–barrel fold, which in Gram-negative bacteria is restricted to OM proteins (Koebnik *et al.*, 2000; Schulz, 2000). Omptins cleave proteins or peptide substrates preferentially between two basic aa residues (Kukkonen & Korhonen,

Fig. 1.3 Omptin protein alignments

The protein sequences of the six members of the Omptin family of proteases (IcsP of *S. flexneri*, OmpT of *E. coli*, OmpP of *E. coli*, Pla of *Y. pestis*, PlaA of *E. pyrifoliae* and PgtE of *S. enterica*) were aligned using DNAMAN software. The black, pink and blue areas of shading represent areas of 100%, >75%, and >50% homology between all Omptin aa sequences respectively. The consensus sequence for the identical aa is shown below. Numbers on the right represent the aa position.

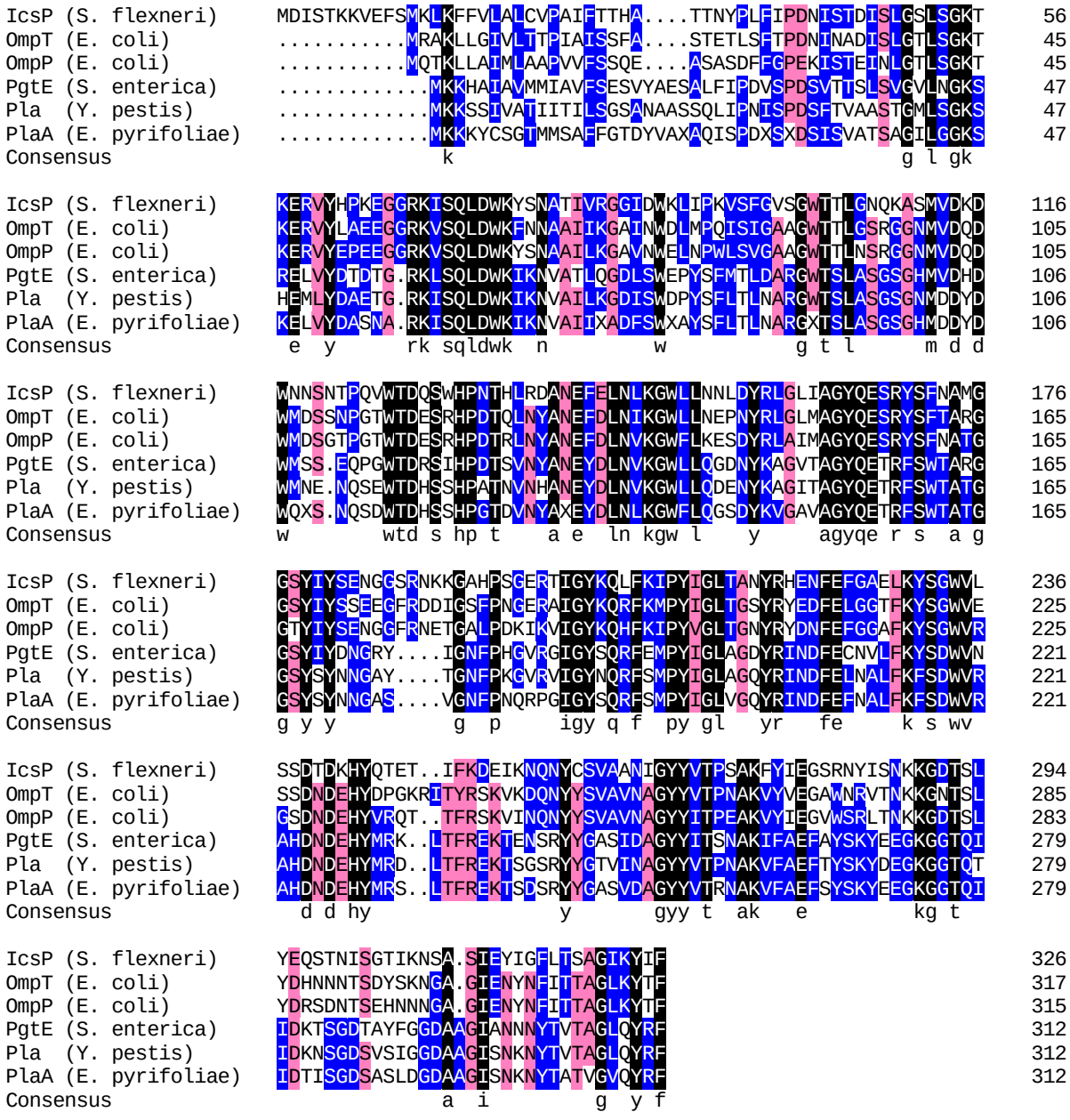

IcsP (S. flexneri) MDISTKKVEFSMKLKFFVLALCVPAIFTTHA....TTNYPLFIPDNISTDISLGSLSGKT 56
OmpT (E. coli) ..........MRAKLLGIVLTTPIAISSFA....STETLSFTPDNINADISLGTLSGKT 45
OmpP (E. coli) ..........MQTKLLAIMLAAPVVFSSQE....ASASDFFGPEKISTEINLGTLSGKT 45
PgtE (S. enterica) ............MKKHAIAVMMIAVFSESVYAESALFIPDVSPDSVTTSLSVGVLNGKS 47
Pla (Y. pestis) ............MKKSSIVATIITILSGSANAASSQLIPNISPDSFTVAASTGMLSGKS 47
PlaA (E. pyrifoliae) ............MKKKYCSGTMMSAFFGTDYVAXAQISPDXSXDSISVATSAGILGGKS 47
Consensus k g l gk
IcsP (S. flexneri) KERVYHPKEGGRKISQLDWKYSNATIVRGGIDWKLIPKVSFGVSGWTTLGNQKASMVDKD 116
OmpT (E. coli) KERVYLAEEGGRKVSQLDWKFNNAAIIKGAINWDLMPQISIGAAGWTTLGSRGGNMVDQD 105
OmpP (E. coli) KERVYEPEEGGRKVSQLDWKYSNAAILKGAVNWELNPWLSVGAAGWTTLNSRGGNMVDQD 105
PgtE (S. enterica) RELVYDTDTG.RKLSQLDWKIKNVATLQGDLSWEPYSFMTLDARGWTSLASGSGHMVDHD 106
Pla (Y. pestis) HEMLYDAETG.RKISQLDWKIKNVAILKGDISWDPYSFLTLNARGWTSLASGSGNMDDYD 106
PlaA (E. pyrifoliae) KELVYDASNA.RKISQLDWKIKNVAIIXADFSWXAYSFLTLNARGXTSLASGSGHMDDYD 106
Consensus e y rk sqldwk n w g t l m d d
IcsP (S. flexneri) WNNSNTPQVWTDQSWHPNTHLRDANEFELNLKGWLLNNLDYRLGLIAGYQESRYSFNAMG 176
OmpT (E. coli) WMDSSNPGTWTDESRHPDTQLNYANEFDLNIKGWLLNEPNYRLGLMAGYQESRYSFTARG 165
OmpP (E. coli) WMDSGTPGTWTDESRHPDTRLNYANEFDLNVKGWFLKESDYRLAIMAGYQESRYSFNATG 165
PgtE (S. enterica) WMSS.EQPGWTDRSIHPDTSVNYANEYDLNVKGWLLQGDNYKAGVTAGYQETRFSWTARG 165
Pla (Y. pestis) WMNE.NQSEWTDHSSHPATNVNHANEYDLNVKGWLLQDENYKAGITAGYQETRFSWTATG 165
PlaA (E. pyrifoliae) WQXS.NQSDWTDHSSHPGTDVNYAXEYDLNLKGWFLQGSDYKVGAVAGYQETRFSWTATG 165
Consensus w wtd s hp t a e ln kgw l y agyqe r s a g
IcsP (S. flexneri) GSYIYSENGGSRNKKGAHPSGERTIGYKQLFKIPYIGLTANYRHENFEFGAELKYSGWVL 236
OmpT (E. coli) GSYIYSSEEGFRDDIGSFPNGERAIGYKQRFKMPYIGLTGSYRYEDFELGGTFKYSGWVE 225
OmpP (E. coli) GTYIYSENGGFRNETGALPDKIKVIGYKQHFKIPYVGLTGNYRYDNFEFGGAFKYSGWVR 225
PgtE (S. enterica) GSYIYDNGRY....IGNFPHGVRGIGYSQRFEMPYIGLAGDYRINDFECNVLFKYSDWVN 221
Pla (Y. pestis) GSYSYNNGAY....TGNFPKGVRVIGYNQRFSMPYIGLAGQYRINDFELNALFKFSDWVR 221
PlaA (E. pyrifoliae) GSYSYNNGAS....VGNFPNQRPGIGYSQRFSMPYIGLVGQYRINDFEFNALFKFSDWVR 221
Consensus g y y g p igy q f py gl yr fe k s wv
IcsP (S. flexneri) SSDTDKHYQTET..IFKDEIKNQNYCSVAANIGYYVTPSAKFYIEGSRNYISNKKGDTSL 294
OmpT (E. coli) SSDNDEHYDPGKRITYRSKVKDQNYYSVAVNAGYYVTPNAKVYVEGAWNRVTNKKGNTSL 285
OmpP (E. coli) GSDNDEHYVRQT..TFRSKVINQNYYSVAVNAGYYITPEAKVYIEGVWSRLTNKKGDTSL 283
PgtE (S. enterica) AHDNDEHYMRK..LTFREKTENSRYYGASIDAGYYITSNAKIFAEFAYSKYEEGKGGTQI 279
Pla (Y. pestis) AHDNDEHYMRD..LTFREKTSGSRYYGTVINAGYYVTPNAKVFAEFTYSKYDEGKGGTQT 279
PlaA (E. pyrifoliae) AHDNDEHYMRS..LTFREKTSDSRYYGASVDAGYYVTRNAKVFAEFSYSKYEEGKGGTQI 279
Consensus d d hy y gyy t ak e kg t
IcsP (S. flexneri) YEQSTNISGTIKNSA.SIEYIGFLTSAGIKYIF 326
OmpT (E. coli) YDHNNNTSDYSKNGA.GIENYNFITTAGLKYTF 317
OmpP (E. coli) YDRSDNTSEHNNNGA.GIENYNFITTAGLKYTF 315
PgtE (S. enterica) IDKTSGDTAYFGGDAAGIANNNYTVTAGLQYRF 312
Pla (Y. pestis) IDKNSGDSVSIGGDAAGISNKNYTVTAGLQYRF 312
PlaA (E. pyrifoliae) IDTISGDSASLDGDAAGISNKNYTATVGVQYRF 312
Consensus a i g y f

Table 1.1 The Omptin family of OM proteases

Bacterium	Omptin	Identity to IcsP (%)	GenBank #	Reference
Shigella flexneri	IcsP	100	AF386526	(Jin *et al.*, 2002)
Escherichia coli	OmpT	56	CAA30008	(Grodberg *et al.*, 1988)
Escherichia coli	OmpP	56	P34210	(Kaufmann *et al.*, 1994)
Yersinia pestis	Pla	38	P17811	(Sodeinde & Goguen, 1989)
Salmonella enterica	PgtE	37	P06185	(Yu & Hong, 1986)
Erwinia pyrifoliae	PlaA	35	NP 758736	(McGhee *et al.*, 2002)

2004), and the substrate specificity of OmpT has been most studied, with the consensus sequence of the OmpT cleavage site determined as (Arg/Lys)↓(Arg/Lys)-Ala (Dekker *et al.*, 2001; Okuno *et al.*, 2002; Sugimura & Higashi, 1988; Sugimura & Nishihara, 1988). Functionally, the Omptins are atypical proteases which lack the signature sequences of classical protease families and are resistant against inhibitors typical for a protease class (Bond & Butler, 1987; Kukkonen & Korhonen, 2004; Sugimura & Higashi, 1988; Sugimura & Nishihara, 1988). Based on the structure of OmpT (shown in Fig. 1.4), Omptins are classified as aspartyl proteases (family A26) and have a highly conserved active site (Vandeputte-Rutten *et al.*, 2001).

1.5.2 Omptins and infectious diseases

The infectious diseases caused by Omptin-expressing bacterial species vary in severity, invasiveness as well as pathogenetic mechanisms. They range from the highly invasive and fatal zoonosis of plague caused by *Y. pestis* (Perry & Fetherston, 1997), to the beneficial commensalism in the intestines by most *E. coli* species (some infectious diseases are associated with specific subgroups of *E. coli*, such as enteropathogenic *E. coli* and enterohaemorrhagic *E. coli* (Nataro & Kaper, 1998)). Other infectious diseases caused by Omptin-expressing bacterial species include the severe mucous and bloody diarrhoeal disease of bacillary dysentery caused by *S. flexneri* (Kotloff *et al.*, 1999; Parsot & Sansonetti, 1996), gastroenteritis caused by *S. enterica* (Ohl & Miller, 2001) and the bacterial infections in fruit trees caused by *E. pyrifoliae* (Kim *et al.*, 1999).

1.5.3 OmpT of *E. coli*

E. coli OmpT (previously known as protease VII) is biochemically the best characterised member of the Omptin family, and to date is the only Omptin for which the crystal structure has been solved (Vandeputte-Rutten *et al.*, 2001). It is a ~33.5 kDa OM protease (Grodberg *et*

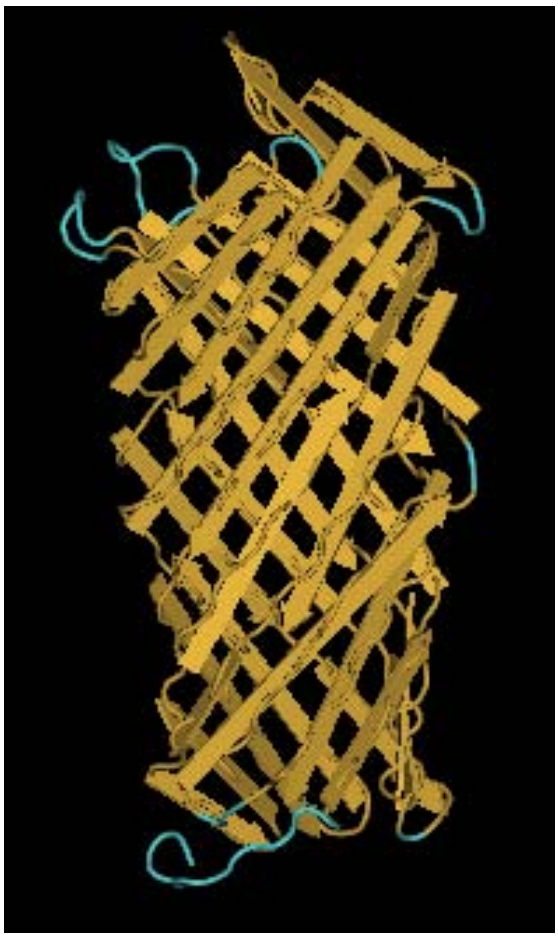


Fig. 1.4 OmpT structure

The β-barrel structure of OmpT modeled using Cn3D 4.1 software.

al., 1988), and along with OmpP, has the highest sequence identity to *Shigella* IcsP (refer to Table 1.1) and has been shown to efficiently cleave IcsA from the cell surface of an *E. coli* strain at the same cleavage site as IcsP (Nakata *et al.*, 1993). Characterisation of purified OmpT reveals a unique substrate specificity (Sugimura & Nishihara, 1988) and that it displays optimal activity at alkaline pH (Kramer *et al.*, 2000). Its activity appears to be dependant on the presence of LPS structure (Kramer *et al.*, 2000).

OmpT has been shown to be associated with complicated urinary tract diseases by cleaving protamine, a highly basic antimicrobial peptide that is secreted by epithelial cells of the urinary tract (Stumpe *et al.*, 1998). In addition to this, it has been known to activate human plasminogen (Leytus *et al.*, 1981), degrade a number of exogenous and endogenous recombinant proteins during their purification (for example keratinocyte growth factor-2 (Laird *et al.*, 2004), cyclin A (Yam *et al.*, 2001), H-NS (Goldberg *et al.*, 1997) and T7 RNA polymerase (Grodberg & Dunn, 1988) to name a few), and cleave colicins A, E1, E2 and E3 (Cavard & Lazdunski, 1990).

1.5.4 OmpP of *E. coli*

E. coli OmpP is an F plasmid-encoded OM protease that exhibits 71% aa sequence identity with OmpT (Hwang *et al.*, 2007). Similar to OmpT, OmpP is able to cleave a number of recombinant proteins during their purification, such as SecY (also cleavable by OmpT (Akiyama & Ito, 1990)) which forms part of the SecYEG complex and acts as a protein channel for the passage of precursor secretory proteins (Matsuo *et al.*, 1999), and T7 RNA polymerase (Grodberg & Dunn, 1988; Hwang *et al.*, 2007). Purified OmpP is also active only in the presence of LPS and has been shown to confer resistance to elevated concentrations of protamine (Hwang *et al.*, 2007). There appears to be very little difference between OmpT and

OmpP in the current literature but unlike OmpT, *ompP* has been shown to be subjected to catabolite repression, whereas *ompT* is not (Kaufmann *et al.*, 1994).

1.5.5 IcsP of *S. flexneri*

S. flexneri IcsP, also known as SopA (Egile *et al.*, 1997), is a 36 kDa OM protease which slowly cleaves IcsA at the Arg_{758} – Arg_{759} bond position (Fukuda *et al.*, 1995) to secrete a ~95 kDa α-domain IcsA fragment which can be detected in culture supernatants (Goldberg *et al.*, 1993b; Goldberg & Theriot, 1995). IcsP bears most sequence identity with OmpT and OmpP (Table 1.1), but does not appear to be closely related to the other members of the Omptin family as shown in the phylogram in Fig. 1.5.

1.5.5.1 Regulation of IcsP

IcsP is encoded by the *icsP* gene which has an open reading frame of 981 bp, and is located in an isolated region of the *Shigella* virulence plasmid with no genes present immediately upstream or downstream of the coding sequence (Shere *et al.*, 1997). Except for the additional 11 aa at the start of the *icsP* sequence (which is also present before the start of the *sopA* sequence), *icsP* is identical to the *sopA* gene described by Egile *et al*. (1997). The expression of IcsP is regulated by the transcriptional regulators VirF and VirB (Wing *et al.*, 2004), and VirB in particular has been shown to significantly enhance *icsP* transcription even in the absence of VirF (Wing *et al.*, 2004). Further data by Wing *et al*. (2005) have also shown that a *virK*::Tn*10* mutant displays increased IcsP expression by Western immunoblotting (refer to Section 1.7.2 below).

1.5.5.2 Role of IcsP in ABM

S. flexneri icsP and *sopA* mutants have reduced amounts of secreted α-domain IcsA fragment in culture supernatants compared to the wild-type strains, and display IcsA across the entire

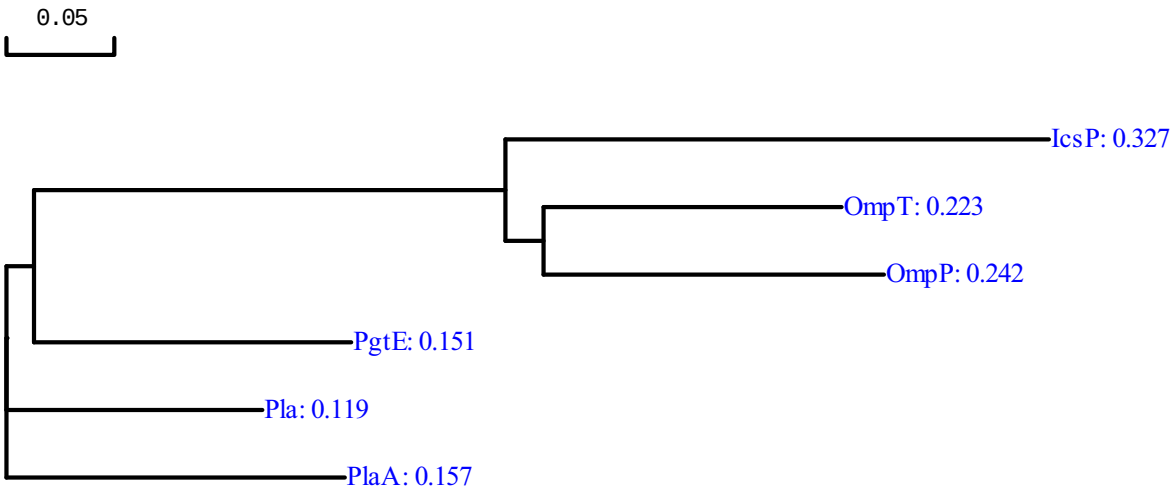


Fig. 1.5 Omptin phylogram

A phylogram showing the protein relationship between the six members of the Omptin family (IcsP of *S. flexneri*, OmpT of *E. coli*, OmpP of *E. coli*, Pla of *Y. pestis*, PlaA of *E. pyrifoliae* and PgtE of *S. enterica*) was constructed using DNAMAN version 4.22 software (bootstrapping value = 100). The sequence weight for each protease is shown. The scale bar represents 0.05 units branch distance.

cell surface (on lateral regions and at the cell poles), suggesting that IcsP is important for maintaining IcsA polar localisation (d'Hauteville *et al.*, 1996; Egile *et al.*, 1997; Shere *et al.*, 1997; Steinhauer *et al.*, 1999). However, studies involving IcsA production in *E. coli* K-12 *ompT*$^{-}$ showed that unipolar localisation of IcsA was not dependant on virulence plasmid determinants such as IcsP (Sandlin & Maurelli, 1999), and that IcsA possessed polarity targeting sequences (Charles *et al.*, 2001). In addition to this, a *sopA* mutant constructed by Egile *et al*. (1997) was shown to produce plaques half the size of wild-type M90T plaques (Egile *et al.*, 1997), suggesting that IcsP may be important in *Shigella* cell-to-cell spread, but an *icsP* mutant studied by Shere *et al*. (1997) was shown to display no significant difference in plaque size compared to the wild-type 2457T strain (Shere *et al.*, 1997). Whether IcsP plays a role in ABM is still incompletely understood. Differences in the findings by Egile *et al*. (1997) and Shere *et al*. (1997) may be explained by the difference in cell lines used to perform the plaque assays, and serotype strains of *S. flexneri* studied by each author.

1.5.5.3 Role of IcsA cleavage in ABM

Studies involving *icsA* mutants with aa changes at the cleavage site required to secrete IcsA have also been carried out (d'Hauteville *et al.*, 1996; Fukuda *et al.*, 1995; Van den Bosch & Morona, 2003). These mutants are commonly termed IcsA*. Studies described by d'Hauteville *et al*. (1996) suggested that IcsA* mutants expressed abnormal intracellular movement due to non-polar localisation of IcsA (d'Hauteville *et al.*, 1996). However, Fukuda *et al*. (1995) showed that IcsA* mutants were capable of F-actin aggregation at one pole of the bacterium and spread into adjacent cells with no apparent defect in ABM (Fukuda *et al.*, 1995), suggesting that the ability to cleave and secrete the N-terminal (α-) domain of IcsA in *Shigella* was not a requirement for intracellular spreading. The importance of IcsA cleavage in ABM is unclear.

1.5.5.4 Distribution of IcsP on the cell surface

Steinhauer *et al*. (1999) showed that IcsP, and a significant amount of the membrane anchored β-core domain of IcsA, are localised to the OM. They hence suggested that IcsP cleavage of IcsA may occur after translocation to the OM. Steinhauer *et al*. (1999) also showed that IcsA at the old pole was susceptible to cleavage by IcsP and proposed a model whereby IcsP cleaved IcsA over the entire bacterial surface in *S. flexneri*. They predicted that IcsP might localise uniformly across the cell surface, but unfortunately were unable to perform detection with their IcsP antibody or an antibody against a His-tagged IcsP (Steinhauer *et al.*, 1999). The OM distribution of IcsP and other Omptin proteases has never been shown.

1.5.6 Pla of *Y. pestis*

Pla of *Y. pestis* is required for the migration of *Y. pestis* from the subcutaneous infection site into the blood circulation (Sodeinde *et al.*, 1992) and contributes to the enhanced disease state of plague (Kukkonen *et al.*, 2001; Lahteenmaki *et al.*, 2001). Pla actives plasminogen (Plg) to plasmin (Beesley *et al.*, 1967), and this activity has been shown to require the absence of LPS O antigen (Oag) (Kukkonen *et al.*, 2004). Pla has also been shown to cleave the complement C3 component (Sodeinde *et al.*, 1992), modify *Y. pestis* OM proteins (Sodeinde *et al.*, 1988), proteolytically inactivate α_2-antiplasmin (α_2AP) (Kukkonen *et al.*, 2001), enhance bacterial adherence to human cells and is a laminin-specific adhesion (Lahteenmaki *et al.*, 1998), and potentially possesses intrinsic adhesive properties (Lobo, 2006). α_2AP is an inhibitor of plasmin and its cleavage is suggested to promote uncontrolled proteolysis and contribute to the invasive character of plague (Kukkonen *et al.*, 2004).

1.5.7 PgtE of *S. enterica*

PgtE of *S. enterica* is required for the systemic spread of *S. enterica* (Lahteenmaki *et al.*, 2005). Similar to Pla of *Y. pestis*, PgtE has also been shown to active Plg to plasmin (but only in the absence of LPS Oag) (Kukkonen *et al.*, 2004), cleave complement components (specifically C3b, C4b, and C5) (Ramu *et al.*, 2007), proteolytically inactivate α_2AP (Lahteenmaki *et al.*, 2005), and mediate bacterial adhesion to human cells (Kukkonen *et al.*, 2004). Other known activities of PgtE are similar to OmpT, and include the ability to degrade T7 RNA polymerase (Grodberg & Dunn, 1989) and cleave cationic microbial peptides (specifically α-helical cationic microbial peptides) (Guina *et al.*, 2000).

1.5.8 Pla endopeptidase A of *E. pyrifoliae*

Pla endopeptidase A (PlaA) of *E. pyrifoliae* possesses most sequence identity to the Pla protease of *Y. pestis* (78%) but little else is known about this protease in the current literature. *E. pyrifoliae* is a pathogen known to cause Asian pear blight (Kim *et al.*, 1999).

1.6 Lipopolysaccharide

The OM of Gram-negative bacteria contains LPS composed of three distinct regions; lipid A, core sugars and Oag polysaccharide chains (Fig. 1.6). These different LPS regions are synthesised at the cytoplasmic leaflet of the IM. *S. flexneri* strains containing LPS with all three regions are known as smooth LPS strains, while *Shigella* mutants (such as *rmlD* mutants) lacking the Oag component in their LPS structure are known as rough LPS strains. The lipid A region anchors the molecule to the OM, and Oag chains are linked to lipid A via the core sugar region and extend into the external milieu as shown in Fig. 1.6. Lipid A and the inner core tend to be highly conserved within *Enterobacteriaceae* (Bruneteau & Minka, 2003; Heinrichs *et al.*, 1998). The Oag is the most variable part of the LPS structure and is an

Fig. 1.6 Lipopolysaccharide

LPS consists of three distinct regions (lipid A, core oligosaccharide of repeating subunits (n), and O antigen) as indicated in (**A**), and is located in the outer leaflet of the OM of the Gram negative cell envelope as depicted in (**B**) (diagram was adapted from Gerald Murray's thesis (2004)).

important virulence factor of a range of enterobacterial pathogens, as it mediates resistance to complement-mediated and phagocytic killing of bacteria (Rautemaa & Meri, 1999). The LPS of *S. flexneri* 2a has Oag chains of two modal lengths which have been shown to be optimized for virulence: a) short-type modal length (11-17 Oag repeat units) which is determined by the chromosomally located wzz_{SF}, and b) very long-type model lengths (>90 Oag repeat units) which is determined by the pHS-2 plasmid located wzz_{pHS2} and confers resistance to serum (Hong & Payne, 1997; Morona *et al.*, 2003; Stevenson *et al.*, 1995).

1.6.1 LPS and IcsA

The maintenance of IcsA polarity, which has been suggested to be contributed by IcsP, also appears to be associated with the presence of smooth LPS. *S. flexneri* strains expressing a defect in Oag production, such as rough LPS strains, have been shown to display IcsA on polar and lateral regions of the cell surface, and have a defect in virulence (Charles *et al.*, 2001; Sandlin *et al.*, 1995; Sandlin *et al.*, 1996; Steinhauer *et al.*, 1999; Van den Bosch *et al.*, 1997). Notably, Van Den Bosch and Morona (2003) showed that the virulence defect in a *S. flexneri rmlD* mutant expressing rough LPS was not due to the loss of IcsA polarity, as IcsA* mutants (which also displayed IcsA on polar and later regions) did not express a defect in virulence (Van den Bosch & Morona, 2003), suggesting that the presence of Oag may contribute to IcsA mediated ABM. Interestingly, Morona and Van Den Bosch (2003) also showed that the presence of Oag chains masked the presence of IcsA present on polar and lateral regions of the cell surface in *S. flexneri* smooth LPS strains from IcsA antibody (Morona & Van Den Bosch, 2003). The masking effect of LPS has also been previously reported for other OM proteins, such as for *E. coli* PhoE (van der Ley *et al.*, 1986a; van der Ley *et al.*, 1986b) and *Y. pseudotuberculosis* Inv (Voorhis *et al.*, 1991).

1.6.2 LPS and Omptins

LPS regions are known to interact with members of the Omptin family. Kramer *et al*. (2000) observed that no OmpT enzymatic activity was present in purified protein (extracted from inclusion bodies) in the absence of LPS, despite re-folding into its native state. They concluded that active OmpT could only be obtained when LPS was added to the protein (Kramer *et al.*, 2000). Additionally, the single known three-dimensional structure of a protein in complex with LPS is that of FhuA (Ferguson *et al.*, 1998; Ferguson *et al.*, 2000), and three of the four residues of the consensus LPS binding motif of FhuA are present in the X-ray structure of OmpT (Vandeputte-Rutten *et al.*, 2001). This suggests that OmpT interacts with LPS. Since *Shigella* IcsP shares most identity to *E. coli* OmpT and possesses a similar structure based on computer modelling (Section 4.2.2), IcsP may also interact with LPS. The sequences of *Y. pestis* Pla and *S. enterica* PgtE also contain two arginines of the LPS motif predicted to interact with the lipid A component of LPS (Kukkonen & Korhonen, 2004).

PgtE of *S. enterica* and Pla of *Y. pestis* possess 33% and 34% identity with IcsP, respectively (Table 1.1). Both members of the Omptin family have been shown to activate human Plg, and are likely to be important in the ability of *S. enterica* and *Y. pestis* to create uncontrollable proteolysis during infection (Lahteenmaki *et al.*, 2005; Sodeinde *et al.*, 1992). It is unknown whether IcsP activates Plg. However, Kukkonen *et al*. (2004) noted that the presence of Oag repeats (i.e. smooth LPS) on wild-type or recombinant *S. enterica*, *Y. pseudotuberculosis* or *E. coli* prevented Plg activation by PgtE and Pla (Kukkonen *et al.*, 2004). They showed that loss of Oag allowed activation of Plg by *S. enterica* and facilitated Pla function and invasiveness of *Y. pestis* (Kukkonen *et al.*, 2004). Additionally, PgtE mediated inactivation of α_2AP (which inhibits plasmin) was also shown to be inhibited in the presence of Oag (Lahteenmaki *et al.*, 2005). There is no data on whether Plg activation (or α_2AP inactivation) plays a role in diarrhoeal diseases, or data on whether *Shigella* IcsP acts on Plg (or α_2AP).

However, LPS appears to influence many proteases with homology to IcsP, and it is likely that IcsP is no exception.

1.7 VirK

The *virK* gene was originally discovered to affect intracellular spreading of *Shigella flexneri* and was found to be highly conserved among the large virulence plasmids of *Shigella* and enteroinvasive *E. coli* (Nakata *et al.*, 1992). *virK* is located in an operon (summarised in Fig. 1.7) on the *Shigella* virulence plasmid with genes known to modify the lipid A of LPS (specifically *rfbU* and *msbB2* (D'Hauteville *et al.*, 2002; Fallarino *et al.*, 1997).

1.7.1 VirK and IcsA

Nakata *et al*. (1992) showed that a *S. flexneri* 2a *virK*::Tn*10* insertion mutant formed smaller plaques (i.e. foci) compared to wild-type by the Fp-test (focus plaque forming assay), and expressed low levels of IcsA by Western immunoblotting which was not due to an affect on the transcription levels of *icsA* (Nakata *et al.*, 1992). Complementation studies performed by Nakata *et al*. (1992) (by cloning a 1.4 kb DNA fragment containing *virK* into pBR322 and introducing the plasmid into the *virK*::Tn*10* mutant) resulted in restoration of the virulence phenotype of the *virK*::Tn*10* mutant (Nakata *et al.*, 1992). When compared to an *icsA* mutant however, a *virK*::Tn*10* mutant produced greater sized foci. Further comparison of polar deposition of F-actin in a *virK*::Tn*10* mutant showed some deposition of F-actin surrounding the bacteria with occasional polar deposition observed (Nakata *et al.*, 1992). These results suggest that VirK function is an essential virulence determinant for *Shigella*.

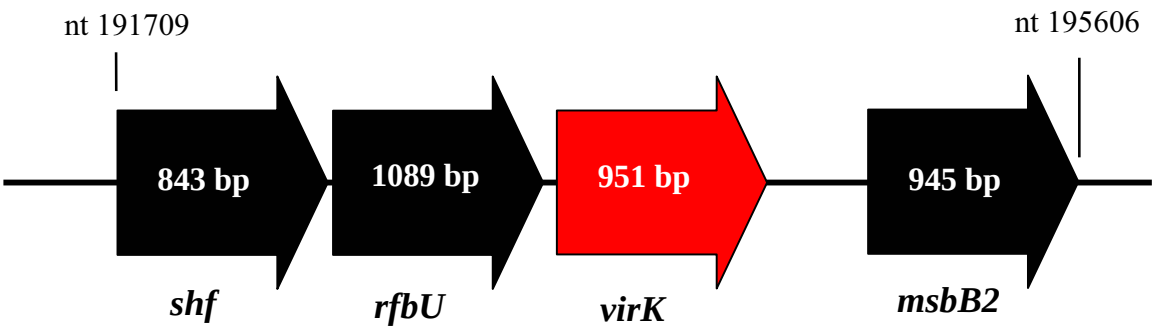


Fig. 1.7 *virK* operon

A diagram of the gene operon located on the *Shigella* virulence plasmid which contains the *virK* gene (981 bp). The other known genes of the operon are indicated, and their gene sizes (bp) specified. The nt position at the start of *shf* and the end of *msbB2* are indicated above (GenBank # AF386526).

1.7.2 VirK and IcsP

A recent study by Wing *et al*. (2005) showed that the defect in the intracellular spread of the *S. flexneri virK*::Tn*10* mutant by Nakata *et al*. (1992) was due in part to the increase in IcsP expression and IcsP-mediated cleavage of IcsA. This was supported by the artificial increase in IcsP expression levels (via a plasmid-bourne IcsP gene induced with IPTG) in a *Shigella* strain which resulted in a marked defect in actin assembly and intracellular spread (measured by the presence and size of plaques on cell monolayers). They concluded that IcsP expression was an essential *Shigella* virulence factor (Wing *et al.*, 2005).

1.7.3 VirK and LPS

The location of the *virK* gene in *S. flexneri* suggests that it may potentially affect LPS. Immediately upstream of *virK* is the *rfbU* gene which has been shown to be essential for Oag biosynthesis in *V. cholerae* (Fallarino *et al.*, 1997). Immediately downstream of *virK* is the *msbB2* gene which *S. flexneri* actually carries two gene copies of, one located on the chromosome (*msbB1*) and one on the virulence plasmid (*msbB2*) next to *virK* (Fig. 1.7) (D'Hauteville *et al.*, 2002). The *msbB* genes in *S. flexneri* have been shown to encode proteins required for maximal acylation of lipid A and a mutant with both *msbB* genes inactivated, was shown to be unable to cause rupture and destruction of the gut epithelium in a rabbit model of shigellosis (D'Hauteville *et al.*, 2002).

Two genes with homology to *Shigella virK* are found in *Salmonella* and include the *Salmonella virK* gene (which has 51% homology), and the *Salmonella somA* gene (which has 36% identity) (Detweiler *et al.*, 2003). *somA* has been shown to be functionally linked to LPS modifications (Murray *et al.*, 2001). An insertional mutation in *somA* suppresses insertional mutations in *Salmonella msbB* (which encodes the enzyme MsbB that also myristoylates lipid A of LPS) (Murray *et al.*, 2001). Data on *Salmonella virK* suggests that its product may

confer *Salmonella* resistance against specific microbiocidal mechanisms of the macrophage (Brodsky *et al.*, 2005; Detweiler *et al.*, 2003). In addition to this, *virK* and *somA* in *S. typhimurium* appear to require the response regulator *PhoP* for expression (Detweiler *et al.*, 2003) and it has been demonstrated that different PhoP-mediated modifications in lipid A are necessary for resistance to different antimicrobial peptides in *Salmonella* (Shi *et al.*, 2004). However to date there is no direct evidence for VirK function.

1.11 Aims of this study

The specific aims of this thesis were:

1. To construct an *icsP* mutant and characterise its phenotype, this includes:
    - comparing the virulence of an *icsP* mutant in a *S. flexneri* 2a and a *S. flexneri* 5a strain

2. To determine the distribution of IcsP on the bacterial cell surface in smooth and rough LPS strains.

3. To investigate the effect of the *virK* and *rmlD* mutations on IcsP and *Shigella* virulence.

4. To investigate alternative substrates for IcsP.

Chapter 2 – Materials and Methods

2.1 Reagents used in this study

2.1.1 Buffers and solutions

The buffers and solutions used in this study (unless otherwise indicated) are summarised in Table 2.1. All reagents were prepared with RO (Millipore) or MQ water (MilliQ water; Millipore, 18.2 $M\Omega cm^{-1}$).

2.1.2 Oligonucleotides

The oligonucleotides used in this study were purchased from GeneWorks (Thebarton, Adelaide, Australia) and are summarised in Table 2.2.

2.1.3 Antibodies

The antibodies used in this study, and their relevant concentrations and dilutions used for Western immunoblotting and IF microscopy, are outlined in Table 2.3. Anti-IcsP antiserum was produced in this study by immunisation of a rabbit with purified His_6-tagged IcsP expressed from a pQE60 construct (refer to Section 2.9.4.1 below). All antibodies were stored at -20°C and (except for anti-LPS purchased from Denka Seiken) made up in 50% (v/v) glycerol.

Table 2.1 Buffers and media

Procedure	Buffer/Media	Composition
Bacterial culture	-70°C glycerol medium	30% (w/v) glycerol (Invitrogen), 1% (w/v) Bacto peptone (Difco)
	LB broth	0.5% (w/v) NaCl, 0.5% (w/v) Yeast Extract (Difco), 1% (w/v) Bacto Tryptone Peptone (BD)
	LB agar	LB broth, 1.5% (w/v) Bacto agar (BD)
	Modified LB agar	LB broth (with no NaCl), 6% (w/v) sucrose, 1.5% (w/v) Bacto agar (BD)
	Congo red agar	30% (w/v) Bacto Tryptic Soy broth (BD), 0.02% (v/v) Congo red (Sigma) and 1.5% (w/v) Bacto agar (BD)
	SOC media	2% (w/v) Tryptone (Oxoid), 0.5% (w/v) Yeast Extract (Oxoid), 0.04 M NaCl, 0.01 M KCl, 0.01 M $MgCl_2$, 0.01 M $MgSO_4$
Sequencing	Ethanol/NaAc solution	3 µl 3 M NaAc, 62.5 µl non-denatured 95% (v/v) ethanol, made up to 80 µl with MQ
Agarose gel electrophoresis	Loading dye	0.1% (w/v) bromophenol blue (Sigma), 20% (v/v) glycerol, 0.1 mg/ml RNase (Qiagen)
	10x TBE buffer	0.5 M Tris, 5 M Boric acid, 0.001 M EDTA
	Agarose gel	1% (w/v) DNA grade agarose (Quantum Scientific) in 1x TBE buffer
SDS-PAGE	2x sample buffer	4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol (Sigma), 0.04% (w/v) bromophenol blue (Sigma), 0.125 M Tri-HCl, pH 6.8
	5x SDS- PAGE running buffer	0.5% (w/v) SDS, 1 M glycine, 0.125 M Tris-HCl
	Coommassie blue stain	0.09% (w/v) Coommassie Blue G250 (Sigma) in 5% (v/v) perchloric acid
	Destaining solution	5% glacial acetic acid
Protein purification	1x PBS	16% (w/v) NaCl, 0.4% (w/v) KCl, 0.4% (w/v) KH_2PO_4, 2.3% Na_2HPO_4, pH 7.6
	HEPES buffer	0.01 M HEPES (Sigma), 1 mM $MgCl_2$
	Triton/$MgCl_2$ buffer	2% (v/v) Triton X-100 (Sigma), 0.001 M $MgCl_2$, 0.25 M Tris-HCl, pH 8.0

	Triton/Urea buffer	1% (v/v) Triton X-100 (Sigma), 10% (v/v) glycerol, 0.1 M NaH_2PO_4, 0.5 M NaCl, 8 M urea, 0.02 M Tris-HCl, pH 8.0
	NaH_2PO_4 equilibration buffer	0.05 M NaH_2PO_4, 0.3 M NaCl, pH 8.0
	Wash buffer C	Same composition as Triton/Urea buffer, except pH 6.3
	Wash buffer D	Same composition as Triton/Urea buffer, except pH 5.9
	Wash buffer E	Same composition as Triton/Urea buffer, except pH 4.5
	Elution buffer	Same composition as Triton/Urea buffer, including 0.1 M EDTA
	Dialysis buffer	1% (v/v) Triton X-100 (Sigma), 10% (v/v) glycerol, 0.1 M NaH_2PO_4, 0.15 M NaCl, 0.02 M Tris-HCl, pH 8.0
Western immunoblotting	1x TTBS buffer	0.016 M Tris, 0.05% (v/v) Tween 20 (Sigma), 0.12 M NaCl
	1x TBS buffer	0.016 M Tris, 0.12 M NaCl
	Transfer buffer	5% (v/v) methanol, 0.025 M Tris, 0.2 M glycine
	Ponceau S stain	0.1% (w/v) Ponceau S (Sigma), 5% (v/v) glacial acetic acid
LPS analysis	Lysing buffer	2% (w/v) SDS, 4% (v/v) β-mercaptoethanol (Sigma), 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue (Sigma), 0.66 M Tris-HCl, pH 7.6
	Proteinase K solution	2.5 mg/ml in lysing buffer
	Fixing solution	5% (v/v) glacial acetic acid, 40% (v/v) ethanol
	Oxidising solution	5% (v/v) glacial acetic acid, 40% (v/v) ethanol, 0.7% (w/v) periodic acid
	Staining solution	2 ml NH_3OH, 0.12 g NaOH, 5 ml 20% (w/v) $AgNO_3$, made up to 150 ml with MQ
	Developing solution	50 mg citric acid, 500 µL formaldehyde in 1 L MQ (warmed to 56ºC)
	Stopping solution	4% (v/v) glacial acetic acid
IF microscopy	GTE buffer	0.05 M glucose, 0.01 m EDTA, 0.02 M Tris-HCl, pH 7.5
Antimicrobial assays	MOPS buffer	0.05 M MOPS, 0.02 M MgSO4, pH 7.7

Table 2.2 Oligonucleotides

Primer name	Oligonucleotide sequence (5' → 3') [a, b, c]	Target	GenBank # /Source	nt [d] position
HA-encoding primers				
ET18	tacccgtacgacgtcccggactacgccagtaccaatatatctggcac	*icsP* gene	AF386526	221158
ET19	ggcgtagtccgggacgtcgtacgggtattgctcataaagagatgtatc	*icsP* gene	AF386526	221157
icsP-specific primers				
ET1	gcgcatatggacatttcaaccaaaaaag	*icsP* gene	AF386526	220267
ET2	cgcgcggccgcaaaaatatactttatacctgcgg	*icsP* gene	AF386526	221244
ET3	gcggatccgtattgcttctgccatttcc	484 bp upstream *icsP*	AF386526	219783
ET4	gcgagctcgtccctgatagcactgttc	371 bp downstream *icsP*	AF386526	221618
ET9	ggccatggacatttcaaccaaaaaag	*icsP* gene	AF386526	220267
ET10	gcggatccaaaaatatactttatacctgcg	*icsP* gene	AF386526	221244
ET22	gcggtaccataaagtaagaagatcatggac	16 bp upstream *icsP*	AF386526	220251
ET25	gggaagctttcaaaaaatatactttatacctg	*icsP* gene	AF386526	221247
pKD4 specific primers				
ET28	ccgggctagctgtgtaggctggagctgcttcg	FRT flanked *kan*^{R} priming site 1	AY048743	31
ET29	gcccgctagccatatgaatatcctcctta	FRT flanked *kan*^{R} priming site 2	AY048743	1488
ET33	[b]*ttgaaaccaaaaaacgcccgaaatacatcatcaagagagtcaaaaaatga*gtgtaggctggagctgcttc	FRT flanked *kan*^{R} priming site 1	AY048743	31
ET34	[b]*taggttggtattatagcttgtgcgcgccatgattggcgcgcaatttaaac*catatgaatatcctccttag	FRT flanked *kan*^{R} priming site 2	AY048743	1488
ET40	[c]*taataaaactgcgttaaaaatactcacattagcgaagcacaagtaacaat*tgtgtaggctggagctgcttcg	FRT flanked *kan*^{R} priming site 1	AY048743	31
ET41	[c]*gcccctaccataatcatcattaatattttattcagagaatacatctaacc*catatgaatatcctccttа	FRT flanked *kan*^{R} priming site 2	AY048743	1488

pGEMT specific primer				
M13R	tcacacaggaaacagctatgac	pGEMT vector	Promega	197
pQE60 specific primers				
Promoter region	cccgaaaagtgccacctg	pQE vector	Qiagen	3340
Reverse sequencing	gttctgaggtcattactgg	pQE vector	Qiagen	190
Type III/IV	cggataacaatttcacacag	pQE vector	Qiagen	69
virK-specific primers				
ET38	gcgggatcctactgcatggagattac	898 bp upstream *virK*	AF386526	192749
ET39	cgcggatccgcgctggcaatgcat	879 bp downstream *virK*	AF386526	195476
yfdI-specific primers				
ET35	cgcccgaaatacatcatca	32 bp upstream *yfdI*	U00096	2467121
ET36	tgtgcgcgccatgattgg	32 bp downstream *yfdI*	U00096	2468516
ET37	gtataagcaaaggtcgaacg	*yfdI* gene	U00096	2468415

[a]Underlined sequences indicate the nucleotides that encode the HA epitope
[b]Italisied sequences indicate overhang nucleotides homologous to *yfdI* gene
[c]Italisied sequences indicate overhang nucleotides homologous to *virK* gene
[d]nt = nucelotide

Table 2.3 Antibodies

Antibody	Concentration	Dilutions		Source/Reference
		Western	IF	
Primary antibodies				
Rabbit polyclonal				
Anti-IcsP [#]	-	1:250	-	Morona Lab; Section 2.9.6.1
Anti-IcsA	-	1:1000	1:100	Morona Lab; Van Den Bosch *et al*. (1997)
Anti-Plg	10 mg/ml	1:1000	-	Accurate Chemical & Scientific Corporation
Type II anti-LPS *	-	-	-	Denka Seiken, Oxoid
Type IV anti-LPS *	-	-	-	Denka Seiken, Oxoid
Type V anti-LPS *	-	-	-	Denka Seiken, Oxoid
Group 3,4 anti-LPS *	-	-	1:100	Denka Seiken, Oxoid
Mouse monoclonal				
Anti-α_2AP	0.5 mg/ml	1:1000	-	R&D systems
Anti-His_6	0.2 mg/ml	1:1000	-	Novogen
Anti-HA	1 mg/ml	1:500	1:50	Sigma
MASF-B	-	-	1:50	Carlin and Lindberg (1987)
MASF-V	-	-	1:50	Carlin and Lindberg (1987)
MASF Y-5	-	-	1:20	Carlin and Lindberg (1987)
Secondary antibodies				
HRP-conjugated goat anti-mouse IgG	1 mg/ml	1:30,000	-	KPL
HRP-conjugated goat anti-rabbit IgG	1 mg/ml	1:30,000	-	KPL
Alexa Fluor 488 donkey anti-mouse IgG	2 mg/ml	-	1:100	Molecular Probes; Invitrogen
Alexa Fluor 488 donkey anti-rabbit IgG	2 mg/ml	-	1:100	Molecular Probes; Invitrogen
Alexa Fluor 594 donkey anti-rabbit IgG	2 mg/ml	-	1:100	Molecular Probes; Invitrogen

[#] Detection of IcsP by Western immunoblotting was performed with ECL Advance substrate (Amersham); the secondary antibody was diluted further by 1/10.
***** Antibodies used for Oag typing.

2.2 Bacterial strains and growth conditions

2.2.1 Strains and plasmids

The bacterial strains and plasmids used in this study are described in Table 2.4 and Table 2.5, respectively.

2.2.2 Growth media and conditions

The media used in this study are included in Table 2.1. Bacteria were stored at -70°C in glycerol medium and routinely cultured at 37ºC for 16 h in either Luria-Bertani (LB) broth with aeration, or on LB agar for *E. coli*, and Congo red agar for *S. flexneri*. Red colonies were selected for *S. flexneri* strains maintaining the large virulence plasmid, and white colonies for virulence plasmid negative (VP^{-ve}) strains. To cure the virulence plasmid in *S. flexneri* strains, bacteria were grown on LB agar supplemented with rifampicin and white colonies selected. All VP^{-ve} strains were PCR checked twice with *icsP* or *virK* primers for the absence of the virulence plasmid. Where appropriate, antibiotics were added to the following concentrations: ampicillin (Amp), 100 μg/ml; chloramphenicol (Cml), 25 μg/ml; kanamycin (Kan), 50 μg/ml; rifampicin (Rif), 8 μg/ml; streptomycin (Strep), 100 μg/ml and tetracycline (Tet), 10 μg/ml.

2.3 DNA preparation

2.3.1 Preparation of chromosomal DNA

Broth cultures (10 ml) grown for 16 h were centrifuged (4,500 rpm, 10 min, Sigma 3K15) and the pellets resuspended in 3 ml 0.85% (w/v) saline by vortexing prior to the addition of 3 ml Tris-equilibrated phenol (pH 7.5). The mixture was then vortexed intermittently over 2 min, re-centrifuged (as above), and the aqueous layer added to 3 ml of cold 100% isopropanol.

Table 2.4 Bacterial Strains

Strain	Description	LPS[a]	Source/Reference
E. coli K-12			
DH5α	*endA hsdR supE44 thi-1 recA1 gyrA relA* Δ(*lacZYA-argF*) U169 [ϕ80d*lac*Δ(*lac*Z) M15)	rough	Gibco-BRL
M15	NaI^s Str^s Rif^s *thi*$^-$ *lac*$^-$ *ara*$^+$ *gal*$^+$ *mtl*$^-$ *F*$^-$ $RecA^+$ Uvr^+ Lon^+	rough	Qiagen
UT5600	*F*$^-$ *ara14 leuB6 secA6 lacY1 proC14 tsx-67 Δ(ompT-fepC)266 entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi-1*; *ompT* mutant; $Strep^R$	rough	Lab collection
S7-1	*hsdR pro* RP4-2-Tc::Mu-Km::Tn7	rough	(Morona *et al.*, 1994)
TOP10F'	F'*lac*I^q::Tn*10* *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G; Tet^R, $Strep^R$	rough	Invitrogen
ETRM16	DH5α (pSL1180-*icsP*::*kan*^{R})	rough	This study; Ch. 3
ETRM70	M15 (pREP4)(pQE60::*icsP*-His_6)	rough	This study; Ch. 4
ETRM71	M15 (pREP4)(pQE60)	rough	This study; Ch. 4
ETRM156	UT5600 (pJRD215) (pBAD30::*icsP*^{HA})	rough	This study; Ch. 4
ETRM158	UT5600 (pJRD215) (pBAD30)	rough	This study; Ch. 4
ETRM168	UT5600 (pRMA154) (pBAD30::*icsP*^{HA})	smooth	This study; Ch. 4
ETRM170	UT5600 (pRMA154) (pBAD30)	smooth	This study; Ch. 4
ETRM174	UT5600 (pRMA154) (pBAD30::*icsP*)	smooth	This study; Ch. 4
ETRM205	UT5600 Δ*yfdI*::scar^{FRT}	rough	This study; Ch. 4
ETRM207	ETRM205 (pJRD215)	rough	This study; Ch. 4
ETRM209	ETRM205 (pRMA154)	smooth	This study; Ch. 4
ETRM211	ETRM207 (pBAD30::*icsP*^{HA})	rough	This study; Ch. 4
ETRM213	ETRM207 (pBAD30)	rough	This study; Ch. 4
ETRM215	ETRM209 (pBAD30::*icsP*^{HA})	smooth	This study; Ch. 4
ETRM217	ETRM209 (pBAD30)	smooth	This study; Ch. 4
ETRM237	DH5α (pCACTUS::*virK*)	rough	This study; Ch. 5
ETRM287	DH5α (pCACTUS::*virK*)(pKD46)	rough	This study; Ch. 5
ETRM290	DH5α (pCACTUS-Δ*virK*::*kan*^{R})	rough	This study; Ch. 5
RMA156	S17-1 (JRD215)	rough	Lab collection; (Macpherson *et al.*, 1991)
RMA160	S17-1 (pRMA154)	rough	Lab collection; (Morona *et al.*, 1994)
E. coli K53	Colicin E1-producing strain	rough	(Burman & Nordstrom, 1971)
S. flexneri 2a			
2457T	wild type strain	smooth	(Van den Bosch *et al.*, 1997)
ETRM22	2457T *icsP*::*kan*^{R}; Kan^R	smooth	This study; Ch. 3

ETRM29	ETRM22 (pWSK29)	smooth	This study; Ch. 3
ETRM31	ETRM22 (pWSK29::*icsP*)	smooth	This study; Ch. 3
ETRM81	ETRM22 (F'*lac*I^{q}::Tn*10*)	smooth	This study; Ch. 4
ETRM84	ETRM81 (pQE60::*icsP*-His$_6$)	smooth	This study; Ch. 4
ETRM85	ETRM81 (pQE60)	smooth	This study; Ch. 4
ETRM117	ETRM22 (pBAD30::*icsP*^{HA})	smooth	This study; Ch. 4
ETRM118	ETRM22 (pBAD30)	smooth	This study; Ch. 4
ETRM124	RMA2161 (F'*lac*I^{q}::Tn*10*)	rough	This study; Ch. 4
ETRM125	ETRM124 (pQE60::icsP-His$_6$)	rough	This study; Ch. 4
ETRM131	ETRM124 (pQE60)	rough	This study; Ch. 4
ETRM138	RMA2161 (pBAD30::*icsP*^{HA})	rough	This study; Ch. 4
ETRM139	RMA2161 (pBAD30)	rough	This study; Ch. 4
ETRM143	ETRM22 (pBAD30::*icsP*)	smooth	This study; Ch. 4
ETRM187	RMA2519 (pBAD30::*icsP*^{HA})	smooth	This study; Ch. 4
ETRM189	RMA2519 (pBAD30)	smooth	This study; Ch. 4
ETRM230	2457T *rmlD*::*kan*^{R}; Kan^{R}	rough	This study; Ch. 5
ETRM233	2457T *rmlD*::scar^{FRT}	rough	This study; Ch. 5
ETRM240	ETRM233 *icsP*::*kan*^{R}; Kan^{R}	rough	This study; Ch. 5
ETRM243	ETRM240 (pBAD30::*icsP*^{HA})	rough	This study; Ch. 6
ETRM245	ETRM240 (pBAD30)	rough	This study; Ch. 6
ETRM292	2457T Δ*virK*::*kan*^{R}; Kan^{R}	smooth	This study; Ch. 5
ETRM306	2457T Δ*virK*::scar^{FRT}	smooth	This study; Ch. 5
ETRM309	ETRM320 *icsP*::*kan*^{R}; Kan^{R}	rough	This study; Ch. 5
ETRM318	ETRM306 *icsP*::*kan*^{R}; Kan^{R}	smooth	This study; Ch. 5
ETRM320	ETRM233 Δ*virK*::scar^{FRT}	rough	This study; Ch. 5
RMA2041	2457T Δ*icsA*::*tet*^{R}; Tet^{R}	smooth	(Van den Bosch & Morona, 2003)
RMA2161	2457T VP$^{-ve\ (1)}$ *rmlD*::*kan*^{R}; Kan^{R}	rough	(Morona & Van Den Bosch, 2003)
RMA2519	2457T VP$^{-ve\ (1)}$	smooth	Lab collection
S. flexneri 5a			
M90T	wild type strain	smooth	Lab collection (PE856)
PE647	wild type strain	smooth	(Chua *et al.*, 1999)
PE780	M90T wild type strain	smooth	Lab collection (S. Formal)
ETRM108	M90T *icsP*::*kan*^{R}; Kan^{R}	smooth	This study; Ch. 3
ETRM112	M90T *icsP*::*kan*^{R} (pWSK29::*icsP*)	smooth	This study; Ch. 3
ETRM114	M90T *icsP*::*kan*^{R} (pWSK29)	smooth	This study; Ch. 3
ETRM294	M90T VP^{-ve} (pBAD30)	smooth	This study; Ch. 4
ETRM296	M90T VP^{-ve} (pBAD30::*icsP*^{HA})	smooth	This study; Ch. 4
S. sonnei P9	Colicin E2-producing strain	smooth	(Burman & Nordstrom, 1971)

[a] lipopolysaccharide status
(1) virulence plasmid negative

Table 2.5 Plasmids

Plasmid	Description	Source/Reference
pACYC184	Cloning vector; Cml^R, Tet^R	(Rose, 1988)
pBAD30	Arabinose-inducible pBAD promoter vector; Amp^R	(Guzman *et al.*, 1995)
pBAD30::*icsP*	pBAD30 with *icsP* gene; Amp^R	This study; Ch. 4
pBAD30::$icsP^{HA}$	pBAD30 with *icsP* gene and inserted HA epitope; Amp^R	This study; Ch. 4
pCACTUS	Suicide vector; Cml^R; 30ºC	(Morona *et al.*, 1995)
pCACTUS-*icsP*::kan^R	pCACTUS with *icsP* gene disrupted by a kan^R gene; Cml^R, Kan^R; 30ºC	This study; Ch. 3
pCACTUS-*rmlD*::kan^R	pCACTUS with *rmlD* gene disrupted by a kan^R gene; Cml^R,, Kan^R; 30°C	This study; Ch. 5
pCACTUS::*virK*	pCACTUS with *virK* gene; Cml^R, 30ºC	This study; Ch. 5
pCACTUS-Δ*virK*::kan^R	pCACTUS with *virK* gene deleted and substituted for kan^R gene; Cml^R, Kan^R; 30°C	This study; Ch. 5
pCP20	FLP recombinase; Amp^R, Cml^R; 30ºC	(Datsenko & Wanner, 2000)
pGEMT-Easy	Cloning vector; Amp^R	Promega
pGEMT-Easy::*icsP*	pGEMT-Easy with *icsP* gene; Amp^R	This study; Ch. 3
pGEMT-Easy::$icsP^{HA}$	pGEMT-Easy with *icsP* gene and inserted HA epitope; Amp^R	This study; Ch. 4
pGEMT-Easy::kan^R	pGEMT-Easy with kan^R gene; Amp^R, Kan^R	This study; Ch. 5
pGEMT-Easy::*virK*	pGEMT-Easy with *virK* gene; Amp^R	This study; Ch. 5
pJRD215	Cosmid vector; Kan^R	(Davison *et al.*, 1987)
pKD4	vector containing FRT-flanked kan^R gene; Kan^R	(Datsenko & Wanner, 2000)
pKD46	Red λ plasmid; Amp^R; 30ºC	(Datsenko & Wanner, 2000)
pKTUWE	pACYC184 with kan^R gene; Kan^R	(Murray *et al.*, 2003)
pMN4	pHS-2 with cml^R gene inserted downstream of wzz_{pHS-2}; Cml^R	(Hong & Payne, 1997)
pQE60	Expression vector with a C-terminal His_6 tag; Amp^R	Qiagen
pQE60::*icsP*-His_6	pQE60 with *icsP* tagged with His_6; Amp^R	This study; Ch. 4
pREP4	*lac* repressor vector; Kan^R	Qiagen
pRMA154	pJRD215 containing *S. flexneri rfb* region; Kan^R	(Morona *et al.*, 1994)
pRMA718	pUC1318 containing *S. flexneri rfb* region; Amp^R	(Van den Bosch *et al.*, 1997)
pRMA718-*rmlD*::kan^R	pRMA718 containing *rmlD* disrupted by a kan^R gene; Kan^R	This study; Ch. 5
pSL1180	Cloning vector; Amp^R	(Brosius, 1989)
pSL1180-*icsP*	pSL1180 with *icsP* gene; Amp^R	This study; Ch. 3
pSL1180-*icsP*::kan^R	pSL1180 with *icsP* gene disrupted by a kan^R gene; Amp^R, Kan^R	This study; Ch. 3
pWSK29	Low copy cloning vector; Amp^R	This study; Ch. 3

pWSK29::*icsP*	pWSK29 with *icsP* gene; Amp^R	This study; Ch. 3
F'*lac*I^q::Tn*10*	Large plasmid which contains the *lac*I^q mutation; *lac* repressor vector; Tet^R	Invitrogen

Chromosomal DNA was precipitated by vortexing and incubation at -20°C for 20 min, and collected by spooling using a heat sealed Pasteur pipette. DNA was then washed in 70% (v/v) ethanol, resuspended in 1 ml MQ water, and stored at 4°C. One microlitre of a 1/100 dilution was used for PCR.

2.3.2 Preparation of boiled lysates for PCR amplification

Boiled bacterial lysates for PCR amplification were made by resuspending colonies of bacteria in 100 µl of sterile MQ water using a sterile pipette tip, and incubating at 100°C for 5 min. Samples were then centrifuged (13,000 rpm, 1 min, Eppendorf centrifuge 5415-R), and 1 µl of the supernatant was used for PCR.

2.3.3 Preparation of DNA using a kit

DNA purified from agarose gels were purified using the QIAEX II Gel extraction kit (Qiagen). PCR products and restriction enzyme digests were purified for further use with the QIAquick PCR purification kit (Qiagen). Plasmid extraction was performed with the QIAprep Spin Miniprep kit (Qiagen) and 1 µl diluted 1/100 used for PCR.

2.4 Polymerase Chain Reaction (PCR)

2.4.1 General PCR

PCR oligonucleotides used in this study are summarised in Table 2.2. PCR amplification was performed in a 50 µl reaction volume consisting of 200 µM dNTPs (Sigma), 1x PCR buffer (NEB), 100 µM oligonucleotide primers and 0.25 U *Taq* polymerase (NEB). Reactions described here and elsewhere were amplified using an Eppendorf Mastercycler Gradient PCR

machine. Standard PCR conditions involved 25 cycles of denaturation (95°C, 30 sec), annealing (56°C, 30 sec) and extension (72°C, 1 min/kb product).

2.4.2 Amplification of products for cloning PCR

Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) or Phusion High-Fidelity DNA Polymerase (Finnzymes) was used to amplify PCR products for cloning. PCR was carried out as described by the manufacturer.

2.4.3 Splicing by overlap extension PCR

Splicing by Overlap Extension (SOE) PCR was performed based on the method by Horton *et al*. (1990). In the first part of this two-step PCR technique, upstream and downstream amplicons were amplified from *S. flexneri* 2457T chromosomal DNA using HA encoding primers (ET18 and ET19) and *icsP*-specific primers (ET3 and ET10) (Table 2.2). 2457T chromosomal DNA was PCR amplified in two 50 µl reaction volumes consisting of 200 µM dNTPs (Sigma), 1x High Fidelity PCR buffer (Invitrogen), 100 µM oligonucleotide primers, 2 mM $MgSO_4$ buffer (Invitrogen), and 1 U Platinum High Fidelity *Taq* polymerase (Invitrogen). PCR conditions involved 25 cycles of denaturation (94°C, 30 sec), annealing (60°C, 30 sec) and extension (68°C, 1 min/kb product). The secondary PCR involved mixing 5 µl of each of the two amplicons from the primary PCR, and using this mix as a template for PCR with *icsP*-specific primers ET3 and ET10, under the same PCR conditions above. The HA encoding regions here overlap and prime one another in this second reaction to give the resultant *icsP* fragment tagged with a HA epitope.

2.4.4 DNA sequencing

DNA sequencing was conducted using the ABI Prism Big Dye Terminator version 3.1. Each 20 µl reaction contained 1 µl double stranded DNA template, 4 µl Big Dye terminator mix

and 1 µl of a single primer. Sequencing reactions involved 30 cycles of heat denaturation at 95°C for 30 sec, primer annealing at 50°C for 15 sec and extension at 60°C for 4 min.

After amplification, Big Dye Terminator labelled-DNA was precipitated via ethanol/sodium acetate (NaAc) precipitation. Briefly, 80 µl of ethanol/NaAc solution was added to each 20 µl sequencing sample and vortexed. Samples were left at room temperature (RT) (~25°C) for 2 h and the DNA pelleted by centrifugation (13,000 rpm, 20 min, 4°C, Eppendorf centrifuge 5415-R), then washed in 250 µl of 70% (v/v) ethanol, and re-centrifuged (13,000 rpm, 5 min, 4°C, Eppendorf centrifuge 5415-R). After removal of the supernatant, pellets were dried in a centrifuge vacuum for 5 min to remove residual alcohol. Sequencing reactions were sent to and read by the Australian Genomic Research Facility, Level 5, Gehrmann Laboratories, Research Road, The University of Queensland.

2.5 Sequence analysis

DNA sequence data were analysed using DNAMAN version 4.22 software. Alignments of deduced amino acid (aa) sequences were also performed using the DNAMAN software. BLAST searches (Altschul *et al*., 1990) were conducted using resources available at GenBank (NCBI).

2.6 Analysis of DNA

2.6.1 Agarose gel electrophoresis

Restricted DNA and PCR product were mixed with 0.4 volumes of loading dye and separated on 1% (w/v) agarose gels with 1x Tris-borate-EDTA (TBE) buffer at 120 V for 1 h. *Bacillus*

subtilis SPP-1 bacteriophage DNA restricted with *Eco*RI was used as the DNA marker (made in-house by Morona laboratory), and is described in Table 2.6. Gels were stained by incubation in an ethidium bromide solution (and destained in RO water) or in a GelRed (Biotium) solution, and visualised under UV light. DNA bands were visualised using a 312 nm UV transilluminator (model TC-312A, Spectroline) and photographed using a Tracktel GDS-2 gel documentation system (Vision System). Images were printed on Mitsubishi K65HM-CE high-density thermal paper.

2.7 *In vitro* cloning of DNA

2.7.1 General techniques

Restriction endonuclease digestion of DNA and ligation were performed as described by the manufacturer (NEB). Ligation with the pGEMT-Easy vector kit was performed as described by the manufacturer (Promega). For blue-white colony selection of transformants, X-gal in dimethylformamide (DMF) was used at a concentration of 32 μg/ml LB agar.

2.7.2 Preparation of competent cells

2.7.2.1 Chemically competent cells

Chemically competent cells were made from 16 h cultures diluted 1/20 in 10 ml LB broth and incubated with aeration at 37°C to an OD_{600} of ~0.8. Cultures were then centrifuged (4,500 rpm, 10 min, Sigma 3K15) and the pellet resuspended in 5 ml 0.1 M (w/v) $MgCl_2$ prior to re-centrifugation (as above). Cells were then resuspended in 1 ml 0.1 M $CaCl_2$, left on ice for 1 h, centrifuged in an 1.5 ml reaction tube (13,000 rpm, 1 min, 4°C, Eppendorf centrifuge 5415-R) and finally resuspended in 0.5 ml 0.1 M $CaCl_2$ solution containing 15% (v/v) glycerol. Aliquots of 200 μl were dispensed to cold 1.5 ml tubes and cells were placed at -70°C for storage.

Table 2.6 SPP-1 restricted with *Eco*RI

Fragment	Size (kb)	Molecular weight
1	8.51 kb	5.62×10^6
2	7.35 kb	4.85×10^6
3	6.11 kb	4.03×10^6
4	4.84 kb	3.19×10^6
5	3.59 kb	2.37×10^6
6	2.81 kb	1.85×10^6
7	1.95 kb	1.28×10^6
8	1.86 kb	1.23×10^6
9	1.51 kb	0.99×10^6
10	1.39 kb	0.92×10^6
11	1.16 kb	0.76×10^6
12	0.98 kb	0.64×10^6
13	0.72 kb	0.47×10^6
14	0.48 kb	0.32×10^6
15	0.36 kb	0.24×10^6

2.7.2.2 Electrocompetent cells

Electrocompetent cells were made from 16 h cultures diluted 1/20 in 10 ml LB broth and incubated with aeration at 30°C or 37°C (depending on the strain) to an OD_{600} of ~0.8. Cultures were then centrifuged (4,500 rpm, 10 min, Sigma 3K15) and the pellet resuspended in 1 ml cold 10% (v/v) glycerol. Cells were then transferred to a 1.5 ml reaction tube, washed twice in 10% (v/v) glycerol by centrifugation (13,000 rpm, 1 min, 4°C, Eppendorf centrifuge 5415-R), with final resuspension in 1 ml 10% (v/v) glycerol. Aliquots of 200 µl were dispensed to cold 1.5 ml reaction tubes and cells were placed at -70°C for storage.

2.7.3 Bacterial cell transformation

2.7.3.1 Heat shock transformation

Chemically competent cells thawed on ice for 5-10 min were mixed with ~10 µl DNA and left on ice for 20 min. Cells were then heat shocked by placing the cells at 37°C for 3 min and returning to ice for 5 min. Approximately 1 ml SOC media (containing 0.2% [w/v] glucose) was added and cells were expressed at 37°C for 15-30 min for transformants required to be selected on Amp, and 60-90 min for transformants selected on other antibiotics, before plating. Plates were incubated at 37°C for 16 h to obtain transformants.

2.7.3.2 Transformation by electroporation

Electrocompetent cells thawed on ice for 5-10 min were mixed with ~5 µL DNA and the mixture transferred to a sterile electroporation cuvette (0.1 cm or 0.2 cm gap, Bio-Rad). Cells were then electroporated (Bio-Rad Gene Pulser, 2 kV (for 1 cm gap) or 2.5 kV (for 0.2 cm gap), 25 µF, Capacitance extender 960 µF, Pulse Controller 200 Ω) and diluted in 0.5 ml LB or SOC media (containing 0.2% [w/v] glucose). Transformants selected on ampicillin were

plated directly, while transformants selected on other antibiotics were incubated at either 30°C or 37°C for 60-90 min prior to plating. Transformant colonies were obtained after 16 h at either 30°C or 37°C.

2.7.3.3 Conjugation

Conjugation was performed from 16 h cultures of donor and recipient strains centrifuged (4,500 rpm, 10 min, Sigma 3K15) and washed twice in 10 ml LB, with final resuspension in 10 ml LB. Donor and recipient cultures were then mixed in a 1:10 ratio and centrifuged as above. The pellet was resuspended in 100 µl LB and spread onto a sterile cellulose acetate membrane filter (0.45 µm, type HA, Millipore) placed centrally on a pre-warmed (37°C) LB plate and incubated at 37°C for 4 h. Bacteria were then collected off the filter by transferring the membrane to 10 ml LB broth and vortexing. The cell suspension was then diluted, spread on selective agar, and incubated at 37°C for 16 h for ex-conjugates.

2.8 Creation of chromosomal and virulence plasmid mutations

2.8.1 Mutagenesis using pCACTUS

Selected chromosomal and virulence plasmid mutations were constructed with the suicide vector pCACTUS, possessing a temperature sensitive origin of replication (ori_{ts}), chloramphenicol resistance marker (Cml^R) and *sacB*. Strains containing mutagenesis constructs were grown for 16 h in LB broth at 30ºC with aeration and antibiotic selection. Broths were then diluted and plated onto pre-warmed (42ºC) LB plates containing Kan and incubated at 42ºC. After 16 h, colonies were inoculated to 10 ml LB broths and grown for 16 h at 37ºC with no antibiotic selection. Cultures were diluted and plated onto modified LB agar with Kan, and incubated at 30ºC for 16 h to resolve cointegrates. The resultant isolates were

patched onto plates containing Cml to confirm loss of the plasmid construct. Mutations were confirmed by PCR with appropriate primers.

2.8.2 Mutagenesis using the λ red phage mutagenesis system

2.8.2.1 Mutagenesis of *E. coli*

Mutagenesis using the λ Red mutagenesis system in *E. coli* was performed as described by Datsenko and Wanner (2000). Primers were designed to PCR amplify the FRT-Kan-FRT cassette from pKD4 with homologous tag sequences to the gene to be mutated. PCR products (from 8 x 50 µl reaction tubes) were purified and concentrated in a 100 µl volume (Section 2.3.3), digested with *Dpn*I, and re-purified and concentrated in a final volume of 50 µl. *E. coli* UT5600 carrying pKD46 was then grown at 30°C in the presence of 0.2% (v/v) arabinose and made electrocompetent (Section 2.7.2.2), before 50 µl of cells was mixed with 50 µl of PCR product and electroporated in a 1 mm gap cuvette (Section 2.7.3.2). The resultant transformants were patched onto plates containing Amp to confirm loss of pKD46 and chromosomal/virulence plasmid mutations were confirmed by PCR. Where indicated, elimination of the FRT-Kan-FRT cassette was performed by electroporating pCP20 into the strain, incubating at 30ºC for 60-90 min prior to selection on LB plates containing Amp, and incubating plates at 30°C for 16 h. Colonies were then inoculated to 10 ml LB and incubated at 42°C for 16 h, diluted, and then plated onto LB agar and incubated at 37°C for another 16 h. The absence of the temperature sensitive plasmids pCP20 and loss of Kan resistance was confirmed by plating on LB containing appropriate antibiotics. Mutations were confirmed by PCR with appropriate primers.

2.8.2.2 Mutagenesis of *S. flexneri*

Mutagenesis of *Shigella* using the λ Red mutagenesis system was performed based on the method described by Nicholas West, via personal communication. This method is a slight

modification of the Datsenko & Wanner (2000) method described above and involves two parts. In the first part of this method, primers were designed to PCR amplify the FRT-Kan-FRT cassette from pKD4 with homologous tag sequences to the gene to be mutated in *Shigella*. The resultant PCR product was purified by precipitation as described in Section 2.8.2.1. A second set of primers were then designed to anneal ~900 bp upstream and downstream of the gene to be mutated in *Shigella* (with specific restriction enzyme sites), and the resultant PCR amplified product digested and cloned into the 30°C temperature sensitive pCACTUS (via pGEMT-Easy) prior to electroporation into *E. coli* DH5α carrying pKD46 (also at 30°C). This resultant strain was made electrocompetent (again as described in Section 2.8.2.1), and electroporated with 50 µl of purified FRT-Kan-FRT PCR product (amplified from the initial PCR). Transformants were selected on Kan to give an *E. coli* DH5α strain carrying the mutated gene disrupted with a FRT-Kan-FRT cassette.

In the second part of the method, the primers used to anneal ~900 bp upstream and downstream of the gene to be mutated above were used to PCR amplify the mutated gene from the above Kan^R transformant. The resultant PCR product was then purified, *Dpn*I treated, and concentrated as previously described (Section 2.8.2.1), and introduced into electrocompetent *S. flexneri* carrying pKD46 via electroporation (cells were made electrocompetent by growth at 30°C in the presence of 0.2% (w/v) arabinose) (Section 2.8.2.1). Kan^R transformants were patched onto plates containing Amp to confirm loss of pKD46. Chromosomal/virulence plasmid mutations were confirmed by PCR with appropriate primers. Where indicated, elimination of the FRT-Kan-FRT cassette was performed as described in Section 2.8.2.1.

2.9 Protein techniques

2.9.1 General preparation of whole cell lysates

Whole cell lysates were prepared by centrifuging (13,000 rpm, 1 min, Eppendorf centrifuge 5415-R) and 1 ml cultures standardised to an OD_{600} of 1 (~$5x10^8$ cells/ml) in 2x sample buffer.

2.9.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were detected after samples were solubilised by heating at 100ºC for 5 min, then separated on SDS 15% polyacrylamide gels in PAGE running buffer at 200 V (Bio-Rad MiniProtean) or 220 V (14.5 cm Vertical Gel Electrophoresis Unit; Sigma) and stained with Coomassie Blue for visualisation. Coomassie Blue staining was performed by incubating the gel at RT in Coomassie staining solution with agitation for 16 h, then incubating in destaining solution until removal of background staining. Low molecular weight standards (Amersham) or BenchMark Prestained molecular weight markers (Invitrogen) were used as molecular size markers (Table 2.7).

2.9.3 Western transfer and detection

Proteins separated as above were transferred to nitrocellulose membranes (NitroBind, Pure nitrocellulose, 0.45 μm, GE Water & Process Technologies) for either 1 h at 200 mA (Bio-Rad MiniProtean) or 2 h at 200 mA (14.5 cm Vertical Gel Electrophoresis Unit; Sigma) in transfer buffer. The membrane was then blocked for 30 min in TTBS containing 5% (w/v) skim milk pre-warmed at 37°C, and subsequently incubated with the primary antibody in TTBS containing 2.5% (w/v) skim milk for 16 h at RT with rocking. The relevant concentration and dilutions of antibodies used is described in Table 2.3. After 3x 10 min

Table 2.7 Protein markers

Low molecular weight standards (kDa) (Amersham)			BenchMark Pre-Stained markers (Invitrogen)	
Band #	Molecular weight (kDa)		Band #	Molecular weight (kDa)
1	97	(phosphorylase B)	1	190
2	66	(bovine serum albumin)	2	120
3	45	(ovalbumin)	3	85
4	30	(carbonic anhydrase)	4	60
5	20.1	(soybean trypsin inhibitor)	5	50
6	14.4	(α-lactalbumin)	6	40
			7	25
			8	20
			9	15
			10	10

washes in TTBS the membrane was incubated with secondary antibody (also in TTBS containing 2.5% (w/v) skim milk) for 2 h at RT with rocking, washed 3x in TTBS (5 min each), and then 3x in TBS (5 min each). For IcsP protein detection, detection was performed using the ECL Advance Western Blotting Detection kit as described by the manufacturer (Amersham). For the detection of all other proteins, either BM chemiluminescence ELISA substrate (POD) reagents (Roche) or Chemiluminescent Peroxidase substrate-3 (Sigma) was used as described by the manufacturer. All detection was carried out on X-ray (AGFA) film. Film was developed using a CP100 medical X-ray film processing machine (AGFA).

2.9.3.1 Stripping and re-probing nitrocellulose membranes

Membranes were stripped free of antibodies and re-probed by washing 3x in TTBS (5 min each) and incubating in 0.2 M glycine (adjusted to a pH of 2.2 with HCl) for 15 min at RT with shaking. The membrane was re-blocked in TTBS containing 5% (w/v) skim milk, incubated with new primary and secondary antibody (as described above), and protein detected (as described above).

2.9.4 Wild-type IcsP and IcsA expression

Wild-type IcsP and IcsA expression were detected from 16 h cultures of *S. flexneri* strains diluted 1/20 in 10 ml LB broth and incubated with aeration at 37°C to an OD_{600} of ~0.2-0.4.

2.9.5 His-tagged protein over-expression and purification from pQE60

2.9.5.1 IPTG induced over-expression

Expression of C-terminal 6xHis-tagged IcsP was achieved from the pQE60 vector (Qiagen). The expression construct pQE60::*icsP*-His_6 was electroporated into an *E. coli* M15 expression strain containing pREP4 to repress leaky expression of the IcsP protein. Cultures grown for 16 h at 37°C in LB were diluted 1/20 in 10 ml LB (containing Kan and Amp) and grown at

37°C with aeration to an OD_{600} of 0.8. A 1 ml sample was obtained, and the cultures induced with 0.05 mM IPTG (BioVectra). Cultures were then left at RT for 16 h for expression of IcsP protein. His-tagged IcsP protein was checked by Western immunoblotting (Section 2.9.3) and a purified 6xHis-tagged *S. pneumococcus* PsaA protein (His_6-PsaA) of ~40 kDa (kindly donated by Dr. David Ogunniyi) was used as a control.

2.9.5.2 Purification of His-tagged protein

Four flasks of 50 ml cultures grown at 37°C with aeration for 16 h were centrifuged (8,000 rpm, 15 min, JA14 rotor, Beckman centrifuge J2-21M) and diluted in two 5 L conical flasks containing 2 L of LB broth with Amp in each. Diluted cultures were grown at 37°C with aeration to an OD_{600} of 0.8 prior to induction with 0.05 mM IPTG and left at RT for 16 h. Induced cultures were centrifuged (8,000 rpm, 15 min, 4°C, JA10 rotor, Beckman centrifuge J2-21M) and pellets washed briefly in 12 ml 50 mM Tris (pH 8.0) on ice, resuspended in 20 ml HEPES buffer, and run once through a French press chamber (FRENCH pressure cell press, SLM Aminco Instruments) pre-cooled to 4°C. Broken cellular debris was removed by centrifugation (4,500 rpm, 15 min, Sigma 3K15) and the supernatant ultracentrifuged (35,000 rpm, 1 h, 4°C, 80Ti rotor, Beckman Coulter Optima L-100 Ultracentrifuge) to isolate membrane factions. Membrane pellets were stored at –20°C overnight.

To remove inner membrane (IM) proteins, pellets corresponding to 1 L of initial culture were resuspended in 2.5 ml Triton/$MgCl_2$ buffer, incubated at RT for 30 min with constant gentle rocking, and ultracentrifuged (35,000 rpm, 1 h, 4°C, 80Ti rotor, Beckman Coulter Optima L-100 Ultracentrifuge). The pellet was then treated with 1 ml Triton/Urea buffer and incubated at RT for 30 min with constant gentle rocking. The soluble OM proteins fraction was then isolated by ultracentrifugation (35,000 rpm, 1 h, 4°C, 80Ti rotor, Beckman Coulter Optima L-

100 Ultracentrifuge). Supernatants were collected and stored at 4°C in 1.5 ml reaction tubes (1 ml corresponds to ~1 L initial culture) overnight.

For every 1 ml supernatant, 0.5 ml Profinity IMAC Ni-charged resin (BioRad) was placed in a 10 ml reaction tube and equilibrated by washing the resin beads three times with NaH_2PO_4 equilibration buffer and centrifugation (2,100 rpm, 5 min, #8179 swing-out rotor, Heraeus Labofuge 400R centrifuge). After the final addition of equilibration buffer, protein supernatant was added to the equilibrated Ni-charged resin and the mixture incubated at RT for 1 h with gentle agitation. This was followed by centrifugation (as above), and removal of the supernatant. Resin was then washed three times with ~3 ml wash buffer C for 5 min, three times with buffer D for 5 min, twice with buffer E for 5 min, and centrifuged (as above). Protein was eluted from the resin by incubating the beads with 0.5 ml elution buffer at RT for 30 min with gentle agitation, centrifugation (as above), and collection of the supernatant to obtain elution fraction Ef1. The elution process was repeated for elution fractions Ef2 (0.4 ml elution buffer) and Ef3 (0.2 ml elution buffer). All protein fractions were placed individually in dialysis tubing and dialysed in 4 L of dialysis buffer with gentle stirring at 4°C over 1-2 nights. Samples were then removed from the dialysis tubing and protein was stored at 4°C.

2.9.6 Antisera techniques

2.9.6.1 Production of polyclonal anti-IcsP antiserum

Polyclonal anti-IcsP antiserum was produced by injecting a rabbit subcutaneously six times over a period of 2-3 months with purified IcsP protein mixed with an adjuvant. Specifically, the first inoculum for rabbit injection was prepared by mixing 200 µl of elution Ef1, 200 µl phosphate buffered saline (PBS) and 200 µl of Freund's complete adjuvant (FCA). After emulsification (by forcing the mixture between two 3 ml luer-connected syringes for 15 min), 600 µl of the inoculum was injected at up to 4 sites on the rabbit subcutaneously. At 3, 6, 8,

10 and 12 weeks after the first immunisation, booster immunisations were prepared by mixing 700 µl of a mixture of elution Ef1 and Ef2, with 700 µl of Freund's incomplete adjuvant (substituted for FCA) and administered as above. At 5 and 10 weeks, a test bleed was obtained and tested by Western immunoblotting to confirm the presence of IcsP antibodies in antiserum (Section 2.9.3). After 14 weeks, the rabbit was bled. The collected blood was clotted by incubation at 37°C and the supernatant centrifuged twice in a 50 ml reaction tube (2,000 rpm, 5 min, #8179 swing-out rotor, Heraeus Labofuge 400R centrifuge) and stored at -20°C in 10 ml aliquots. Working aliquots of serum were stored at 4°C.

2.9.6.2 Purification of antiserum by absorption with live bacteria

Broth cultures (10 ml, grown for 16 h) of ETRM71 (*E. coli* M15 [pREP4][pQE60]) (control expression strain) and ETRM22 (*S. flexneri* 2457T *icsP*::*kan*^{R}) were centrifuged (4,500 rpm, 10 min, Sigma 3K15) and the pellets resuspended in ~10 ml antiserum. The mixture was gently inverted for 3-4 h at RT before bacteria were removed by centrifugation (as above). Following this, another set of 16 h cultures (of the strains above) diluted 1/20 in 10 ml LB broth and incubated with aeration at 37°C to an OD_{600} of ~0.8, were then centrifuged and the pellets resuspended in the antiserum. Mixtures were then gently inverted for 3-4 h at RT transferred to 4°C, and gently inverted for another 16 h before bacteria were again removed by centrifugation (as above). After ~9 days of repeated absorption, bacteria were removed by repeated centrifugation (as above) until no pellet remained. The absorbed antiserum (~5 ml) was then filter sterilised using a 10 ml syringe fitted with a 0.2 µm filter, and stored at 4°C.

2.9.6.3 Affinity purification of antisera

Purified His-tagged IcsP (~70 µg) was electrophoresed on a SDS 15% polyacrylamide gel and transferred to a nitrocellulose membrane by Western transfer (Section 2.9.3). Protein transferred to the membrane was visualised by staining the membrane with Ponceau S stain

(refer to Table 2.1) for 5 min, and the protein band removed using a sharp blade. After destaining (5 min in RO water) the membrane strip was incubated in 10 ml PBS containing 5% (w/v) skim milk and 0.1% Tween 20 for 1 h at RT (~25°C) with shaking to block non-specific membrane absorption. The blocking solution was then removed and the membrane incubated in 5 ml of antiserum (absorbed against live bacteria, see above) for 4 h at RT with shaking, washed 3x in PBS containing 0.1% Tween 20 (15 min each, with shaking), once in PBS (15 min, with shaking), and then antibodies eluted by mixing at RT for 30 min in 0.7 ml of 0.2 M glycine (pH adjusted to 2.2 using HCl). The pH of the eluate was neutralised by the addition of ~0.2 ml of 1 M K_2HPO_4 and the antiserum dialysed against 4 L PBS for 16 h at 4°C. Aliquots were stored at -20°C in 50% (v/v) glycerol. The resultant purified antiserum was used at 1/100 dilution for Western immunoblotting.

2.9.7 HA-tagged protein over-expression from pBAD30

2.9.7.1 Arabinose induced over-expression

Broth cultures (10 ml) of strains harbouring pBAD30::*icsP* or pBAD30::*icsP*^{HA} (grown for 16 h at 37°C with aeration) were diluted 1/20 in 10 ml LB containing Amp and 0.3% (w/v) glucose, and grown at 37°C with aeration to an OD_{600} of 0.2-0.4. Cultures were then pelleted by centrifugation (4,500 rpm, 10 min, Sigma centrifuge 3K15), washed twice in LB and resuspended in a final volume of 10 ml LB. Unless otherwise stated, arabinose was added at 0.2% (w/v) to induce expression and the culture grown at 37ºC with aeration for 1 h.

2.9.8 Cell fractionation

2.9.8.1 Triton/$MgCl_2$ solubilisation

Broth cultures (200 ml) were grown and induced with arabinose as described above (Section 2.9.7.1), harvested by centrifugation (8,000 rpm, 15 min, 4ºC, JA14 rotor, Beckman centrifuge J2-21M), washed briefly in 50 mM Tris-HCl (pH 8.0), and resuspended in 5 ml 10

mM HEPES in 1 mM $MgCl_2$. The suspension was passed through a pre-cooled French Pressure cell (SLM Aminco) once, centrifuged to remove unbroken cells (4,500 rpm, 10 min, 4ºC, Sigma centrifuge 3K15), and membranes collected by ultracentrifugation (35,000 rpm, 1 h, 4ºC, 80Ti rotor, Beckman Coulter Optima L-100 XP ultracentrifuge). The resulting whole membrane (WM) pellet was resuspended in 2 ml Triton/$MgCl_2$ buffer, incubated at RT for 30 min with rocking, and the outer membrane (OM) pellet collected by ultracentrifugation (as above), followed by pellet resuspension in 1 ml Triton/Urea buffer and incubation at RT for 30 min with rocking. The soluble fraction containing IcsP protein was collected by ultracentrifugation (as above). A small sample (~1 µl) of the insoluble pellet, and 10 µl of the supernatant fraction, after each ultracentrifugation step was resuspended in 100 µl 2x sample buffer, and 10 µl of each sample analysed on SDS 15% polyacrylamide gels.

2.9.8.2 Sucrose density gradient fractionation

Sucrose gradient centrifugation was performed according to the method of Osborn and Munson (1974). In brief, 200 ml cultures were grown and induced with arabinose as described previously (Section 2.9.7.1), and the WM pellet obtained as described above (Section 2.9.8.1) but resuspended in 0.8 ml 25% (w/w) sucrose in 5 mM EDTA and applied to a 10 ml sucrose gradient of 30-55% (w/w) sucrose in 5 mM EDTA, prepared by first layering 1.2 ml 55% (w/w) sucrose solution followed successively by 2 ml each of 45%, 40%, 35% and 30% solutions. Centrifugation to equilibrium was performed with a Beckman SW40Ti swing out rotor (35,000 rpm, 20 h, 4ºC, Beckman Coulter Optima L-100 XP ultracentrifuge) and 0.5 ml fractions collected through the pierced bottom of the tube. Samples (10 µl) of each fraction were resuspended in 2x sample buffer and IcsP protein detected as described in Section 2.9.3.

2.9.8.3 Refractive Index readings of sucrose fractions

The refractive index value of sucrose fractions was read using a standard refractometer at 26°C. A sucrose density standard curve was drawn using the refractive index (RI) readings obtained from the 25%, 30%, 35%, 40%, 45% and 55% (w/v) sucrose buffers, and the "Density and refractive indexes of sucrose" table found at http://homepages.gac.edu/~cellab/chpts/chpt3/table3-2.html to determine sucrose buffer density. Data were analysed and graphs generated using were Microsoft Excel 2002.

2.9.9 Preparation of cell associated and soluble IcsA

Broth cultures of *S. flexneri* strains expressing IcsA (grown for 16 h) were diluted 1/20 in 50 ml LB broth and incubated with aeration at 37°C to an OD_{600} of ~0.8. Whole cell protein extracts were obtained from the pellet of 1 ml culture resuspended in 2x sample buffer. Cultures were then centrifuged (8,000 rpm, 10 min, 4ºC, JA14 rotor, Beckman centrifuge J2-21M) and the supernatant treated with cold 5% (v/v) trichloroacetic acid (TCA) for 1 h at 4ºC. Precipitated supernatant protein extracts were collected by centrifugation (12,000 rpm, 1 h, 4ºC, JA14 rotor, Beckman centrifuge J2-21M) and the pellet washed in 0.5 ml cold acetone, re-centrifuged (13,000 rpm, 5 min, 4ºC, Eppendorf centrifuge 5415-R), air dried, and finally resuspended in 1x sample buffer. Ten microlitre samples of whole cell protein extracts and 20 µl samples of supernatant protein extracts were electrophoresed on SDS 15% polyacrylamide gels.

2.10 Lipopolysaccharide techniques

2.10.1 O antigen typing

Agglutination of bacteria by antisera was assessed by emulsifying bacterial growth from fresh grown cultures on LB plates (2-3 colonies) in 20 µl saline, and adding an equal volume of

antisera. Agglutination was determined by eye and light microscopy. Agglutination was scored as positive if it occurred within 15 sec.

2.10.2 Preparation of LPS samples

LPS was prepared by the method of Hitchcock and Brown (1983). In brief, 1 ml of 16 h cultures were standardised to an OD_{600} of 2 (1 x 10^9 cells/ml) was pelleted by centrifugation (13,000 rpm, 10 min, Eppendorf centrifuge 5415-R) and resuspended in 50 µl of lysing buffer. After 10 min incubation at 100ºC, 10 µl of 25 mg/ml proteinase K solution diluted 1/10 in lysing buffer was added and samples incubated for 16 h at 56ºC. LPS samples were stored at -20ºC.

2.10.3 Analysis of LPS samples by silver-stained SDS-PAGE

LPS samples were incubated at 100ºC for 5 min prior to loading 10 µl on SDS 15% (w/v) polyacrylamide gels as described by Macpherson *et al*. (1991). Samples were electrophoresed at 12 mA for 16 h or until the dye front was eluted from the gel. Silver-staining was performed using the method described by Tsai and Frasch (1982). In brief, gels were fixed for 2.5 h in fixing solution with agitation, oxidised for 5 min in oxidising solution, washed 4x in MQ water for 15 min each time, then stained for 10 min in staining solution and washed 5x in MQ water for 10 min each. The gel was then developed with developing solution pre-warmed to 56ºC, and stopped by the addition of stopping solution.

2.11 Immunofluorescence (IF) microscopy techniques

2.11.1 Formalin-fixation of cells

For IF microscopy, 1 ml of bacterial culture induced with arabinose as described above (Section 2.9.7.1) was washed once in PBS, centrifuged (13,000 rpm, 1 min, Eppendorf

centrifuge 5415-R), resuspended in 1 ml 3.7% (w/v) formaldehyde (Sigma) in saline (0.85% [w/v] NaCl), and incubated at RT for 20 min. Bacteria were then washed in PBS, with final resuspension in 100 µl PBS (~$5x10^8$ cells). For bacteria to be treated with Sf6 tailspike protein (TSP), bacteria ($1x10^9$ cells) were formalin-fixed as above in 1% (w/v) formaldehyde in saline and resuspended in a final volume of 100 µl saline.

2.11.2 Sf6 TSP treatment of cells

Formalin-fixed bacteria in saline (0.1 ml, ~$1x10^9$) was mixed with (and without) 100 µl of 1.8 mg/ml purified Sf6 phage TSP diluted 1/20 in MQ, incubated at 37ºC for 1 h, then washed 3x in either MQ water (for LPS analysis by SDS-PAGE (Section 2.9.2)), or PBS (for IF microscopy (Section 2.11.4)), and resuspended in a final volume of 100 µl of MQ or PBS.

2.11.3 Lysozyme treatment of cells

Formalin-fixed bacteria in PBS (0.1 ml, ~$5x10^8$ cells) was centrifuged (13,000 rpm, 1 min, Eppendorf centrifuge 5415-R) and resuspended in 100 µl GTE buffer. Lysozyme was added to a final concentration of 1 µg/ml and incubated at RT for 1.5 min. Bacteria was then centrifuged (13,000 rpm, 30 sec, Eppendorf centrifuge 5415-R), washed once in PBS, and resuspended in a final volume of 50 µl of PBS.

2.11.4 Indirect epi-fluorescence microscopy and deconvolution

Formalin-fixed bacteria on coverslips (~4 µl) were labelled with primary antibody (1:100 in PBS with 10% foetal calf serum (FCS)) for 1 h at RT, washed 3x with PBS, and incubated with secondary antibody for 30 min at 37ºC. Antibody concentrations used are described in Table 2.3. Coverslips were again washed 3x, mounted on glass microscope slides with Mowiol 4-88 (Calbiochem) containing 20 µg/ml *p*–phenylenylenediamine (Sigma), and sealed with nail polish. Bacteria were observed by epi-fluorescent microscopy using 100x

Phase contrast, oil immersion objective lens with an Olympus 1X70 microscope (connected to a Hamamatsu ORCA-ER camera controller, ASI MFC-2000 automatic focus fine-tuning instrument, lamda 10-C shutter instrument, and a Uniblitz2 VMM-D3 three channel shutter drive). Images and optical sectioning to obtain a series of 16-18 Z stack images taken in successive planes with a Z distance of 0.2 μm were taken with Metamorph v6.3r7 software (Molecular Devices).

2.11.5 Microscopy image analysis

Viewing and manipulation of images was performed with Adobe Photoshop CS v8.0 software (Adobe). Sharpening of Z stack images by 3-dimensional deconvolution was performed with AutoDeBlur (X1.14) software (AutoQuant). ImageJ v1.37c software was used to determine the percentage of labelling overlap in samples labelled with two primary antibodies.

2.12 Tissue culture techniques

2.12.1 Growth, maintenance, and incubation of cell monolayers

Human cervical cancer HeLa and monkey kidney CV-1 cells were grown and maintained in tissue culture media consisting of Modified Eagle's Media (MEM) (Gibco) containing 0.225% (w/v) $NaHCO_3$, 10% (v/v) foetal calf serum (FCS), 100 U/ml penicillin/streptomycin in 0.85% (v/v) saline, and supplemented with 2 mM L-glutamine every 7 days. Growth, maintenance and incubation of cell monolayers (for tissue culture assays) were always performed at 37°C in a humidified atmosphere with 5% CO_2 unless otherwise stated.

2.12.2 Splitting cells and seeding trays/flasks

For plaque assays, cells grown to confluency in a 75 cm^2 tissue culture medium flask (BD Falcon) were split (the day before conducting the plaque assay) and seeded into 35 mm diameter 6-well Falcon trays (Becton Dickinson) at a seeding density of ~$1x10^6$ cells to obtain confluency in wells on the day of the plaque assay. Splitting/seeding cells was performed by washing the cells in PBS for 4 min, incubating in 2 ml 0.1% (w/v) trypsin containing 0.02% (w/v) EDTA in PBS (to dislodge cells from the flask bottom), then resuspending cells in ~14 ml tissue culture media and dispensing ~1.5 ml of diluted cells into each well. Wells were topped with ~1.5 ml tissue culture media extra for added cell nutrients. For invasion assays, cells were seeded into 15 mm diameter 24-well Falcon trays (Becton Dickinson) containing sterile round glass coverslips at a seeding density of ~$0.2x10^6$ cells to obtain semi-confluency in wells on the day of invasion assay. In brief, cells were split (as above), resuspended in ~18 ml tissue culture media, diluted 1 in 4, and ~1.5 ml of diluted cells dispensed into each well. Remaining cells were seeded into a new medium flask as required for subsequent assays.

2.12.3 Preparation of bacteria for infection of cells

Broth cultures of bacterial strains (grown for 16 h) were diluted 1/50 in 10 ml LB broth and grown at 37°C with aeration for 1.5 h (to an OD_{600} of ~0.2-0.4). Strains requiring arabinose induction were centrifuged, washed and induced as described in Section 2.9.7.1. Cultures (1 ml) were then centrifuged (13,000 rpm, 1 min, Eppendorf centrifuge 5415-R), and resuspended in 1 ml Dulbecco's MEM (DMEM) (Gibco) containing 0.37% (w/v) $NaCO_3$ for the plaque assay, or 1 ml Dulbecco's PBS (D-PBS; PBS containing 0.1% [w/v] $CaCl_2$ and 0.1% [w/v] $MgCl_2$) for the invasion assay.

2.12.4 Plaque assay

Plaque assay for *Shigella* species was performed based on a modified method of Oaks *et al*. (1985). In brief, cells grown to confluency in 60 mm wells were washed and aspirated twice with D-PBS, then infected with 200 µl of bacterial suspension diluted 1/1000 or 1/10,000 in FCS-free and antibiotic-free MEM. Monolayers were incubated for 90 min with rocking every 15 min to ensure uniform distribution of the bacteria. The bacterial inoculum was then aspirated and an agarose overlay (4 ml) consisting of DMEM (with 0.37% [w/v] $NaCO_3$), 5% (v/v) FCS, 20 µg/ml gentamycin, and 0.5% (w/v) agarose (SeaKem ME; Cambrex) was applied. For plaque assays involving CV-1 cells, infected monolayers were incubated for ~20 h before a second agarose overlay supplemented with 0.01% (w/v) neutral red (Sigma) in 1% (v/v) glacial acetic acid) was applied and after ~7-8 h of incubation, digital photo images of plaques were taken. For plaque assays involving HeLa cells, infected monolayers were incubated for ~28 h before applying the second agarose layer and digital photo images taken after another 16 h of incubation.

2.12.5 Invasion assay

Invasion assay was performed based on a modified method of Oaks *et al*. (1985). In brief, cells grown to semi-confluency in 15 mm wells were washed and aspirated 3x with D-PBS, once with antibiotic-free MEM containing 10% (v/v) FCS (leaving some media behind so that cells have some nutrients), and infected with 80 µl of bacterial suspension diluted in D-PBS. Infected monolayers were then centrifuged (2,000 rpm, 7 min, RT, Heraeus Labofuge 400R centrifuge) to allow bacteria to contact the cell monolayer, and incubated for 1 h. The bacterial inoculum was then aspirated and washed 3x with D-PBS before incubating the infected monolayer in MEM containing 10% (v/v) FCS and 40 µg/ml gentamycin (to kill extracellular bacteria) for 90 min. At RT, infected monolayers were then washed 3x with D-PBS, fixed by incubating with 3.7% formaldehyde in (0.85%) saline for 20 min, washed once

with D-PBS, incubated with 50 mM NH_4Cl in D-PBS for 10 min, followed by incubation with 0.1% (v/v) Triton X-100 (Sigma) in PBS for 5 min, and then blocked with 10% (v/v) FSC in PBS for 15 min. Incubation of coverslips with primary antibody and/or FITC conjugated phalloidin (Sigma) (1 mg/ml stock diluted 1/200 for F-actin tail staining) in 10% (v/v) FCS was performed for 30 min (at 37°C in a humidified atmosphere with 5% CO_2), followed by 3x washes in PBS, incubation with secondary antibody (also in 10% (v/v) FCS) for 1 h, and then another 3x washes with PBS. For further DAPI staining of bacteria and HeLa cell nuclei, coverslips were washed once with MQ, incubated with 40 µl of 2 mg/ml DAPI (Sigma) diluted 1/1000 in MQ for 1 min at RT, followed by 2x washes with MQ, and 2x wash with PBS. Coverslips then were mounted onto slides and imaged by microscopy as described in Section 2.11.4.

2.13 Plasminogen/α_2AP cleavage assay

Detection of cleaved plasminogen (Plg) and alpha$_2$-antiplasmin (α_2AP) products in culture supernatants was performed based on a modified method of Kukkonen *et al*. (2001). In brief, 16 h cultures were diluted 1/20 in 10 ml LB and grown at 37°C with aeration to an OD_{600} of 0.8. $5x10^8$ cells was then collected and resuspended in 100 µl PBS. For the detection of cleaved α_2AP, cells were incubated with 0.4 µg/ml human plasma α_2AP protein (Calbiochem) in a 37°C waterbath and 40 µl samples taken at 3 h were centrifuged (13,000 rpm, 1 min, Eppendorf centrifuge 5415-R), and the supernatant resuspended in 20 µl 2x sample buffer. For detection of cleaved Plg, cells were incubated with 0.2 µg/ml human glu-plasminogen protein (American Diagnostics Inc.) with (and without) 0.4 µg/ml human plasma α_2AP protein. Cells were incubated at 37°C and samples taken as described above. Samples (15 µl) were loaded on SDS 15% polyacrylamide gels and the presence of cleaved protein detected by Western immunoblotting (Section 2.9.3) with anti-α_2AP or anti-Plg, respectively.

2.14 Antimicrobial assays

2.14.1 Growth of bacteria for bactericidal assay

Broth cultures of bacterial strains (grown for 16 h) were diluted 1/20 in 10 ml LB and grown at 37°C with aeration to an OD_{600} of 0.8. Cultures (1 ml) were then pelleted by centrifugation (13,000 rpm, 1 min, Eppendorf centrifuge 5415-R) and resuspended in either PBS (for complement assay) or 3-(N-Morpholino)-propanesulfonic acid (MOPS) (Sigma) buffer (for protamine assay) and serially diluted to $1x10^6$ cells/ml in the same buffer.

2.14.1.1 Complement bactericidal assay

For complement bactericidal assay, $1x10^6$ cells/ml bacteria were centrifuged (13,000 rpm, 1 min, Eppendorf centrifuge 5415-R) and resuspended in PBS containing 25 µg/ml Cml to prevent continual growth during the assay. A 360 µl aliquot of diluted bacterial suspension was added to 40 µl guinea pig serum or human serum (obtained from Gerald Murray, the preparation of which is described in his PhD thesis (2004)) and vortexed. A 50 µl sample (time = 0 min) was initially obtained, and the assay mix incubated at 37°C. Samples (50 µl) were taken every 30 min for 2 h and serially diluted three times in 450 µl PBS prior to plating 50 µl for viable counts on LB agar. Plates were incubated at 37°C for 16 h. Viable counts were expressed as a percentage of initial concentration (% survival). Heat inactivated complement (HIC) was used in place of complement as a control.

2.14.1.2 Protamine assay

Protamine assays were performed by incubating $1x10^6$ cells in 1ml MOPS buffer with 10 µl of fresh 1 mg/ml protamine (CAT# 539122.5, Calbiochem) in 50 mM MOPS (pH 7.0) diluted

1/100 in the same buffer. A 10 µl sample was taken (time = 0 min) and the assay mix was incubated at 37°C with shaking. Samples (10 µl) were taken every 30 min for 2 h and serially diluted twice in 90 µl MOPS buffer prior to plating 25 µl for viable counts on LB agar. Plates were incubated and viable counts expressed as described above (Section 2.13.1.1).

2.15 Colicin sensitivity assay

The double layer test method of colicin sensitivity assay was performed as described by Renato Morona's PhD thesis (1982) and Masi *et al*. (2007). In brief, 16 h cultures of strains producing colicin (Table 2.4) (grown without aeration at 37°C) were used to soak sterile cotton swabs to streak parallel lines of growth across 20 ml LB agar plates. Plates were incubated at 37°C for 16 h and colicinogenic strains killed with 400 µl of chloroform (added to a piece of Whatman paper placed inside the lid of the upturned plate) by incubation at RT for 30 min. Plates were then overlaid with 20 ml LB agar, allowed to set, and the strains to be tested streaked across and perpendicular to the original colicinogenic streak line. Plates were incubated at 37°C for 16 h and clear zones of inhibitory growth by colicin-sensitive bacteria recorded.

2.16 Data Analysis

The statistical significance of tissue culture plaque assay sizes and the results obtained from antimicrobial assays were calculated by performing a two-tailed unpaired *t*-test with GraphPad Prism version 5.00 (Graphpad Software 2007). Graphs were also generated using GraphPad Prism version 5.00.

Chapter 3 – Characterisation of IcsP

3.1 Introduction

IcsP cleaves IcsA to secrete a ~95 kDa α-domain IcsA fragment which can be detected in culture supernatants (d'Hauteville *et al.*, 1996; Goldberg *et al.*, 1993a; Goldberg & Theriot, 1995). IcsA is a polarly localised OM protein. *Shigella icsP* mutants have been shown to display IcsA on both polar and lateral regions of the cell surface (d'Hauteville *et al.*, 1996; Egile *et al.*, 1997; Shere *et al.*, 1997; Steinhauer *et al.*, 1999). The role of IcsP in *Shigella* ABM however remains controversial. The *sopA* mutant of Egile *et al*. (1997) had a defect in F-actin comet tail formation and displayed reduced plaque size compared to wild-type, while the *icsP* mutant of Shere *et al*. (1997) had no defect in either property. Shere *et al*. (1997) suggested that the different *S. flexneri* strains used in each study may play a role in these observed differences. To resolve the discrepancy in the literature regarding the importance of *icsP*, this chapter describes the cloning and mutagenesis of *icsP* in *S. flexneri* 2457T serotype 2a and *S. flexneri* M90T serotype 5a. Constructed *icsP* mutants from both strains were characterised and compared to their respective wild-types with emphasis on the ability to mediate IcsA cleavage, display polar IcsA distribution, form plaques and F-actin comet tails.

3.2 Mutagenesis of *icsP* in *S. flexneri* 2457T and M90T

To investigate the role of IcsP in *Shigella* ABM and intercellular spread, the virulence plasmid located *icsP* gene in *S. flexneri* 2457T and M90T (Table 2.4) were inactivated by insertion of a kanamycin resistance gene (kan^R) using allelic exchange mutagenesis. Shere *et al*. (1997) noted that an extra 11 aa was found at the start of the 2457T *icsP* gene compared to

the M90T *sopA* gene described by Egile *et al*. (1997). Comparisons of both genes in this study found that the *sopA* gene described by Egile *et al*. (1997) actually starts at a downstream ATG site in the *icsP* sequence, but is otherwise identical to *icsP*. Briefly, the *icsP* gene was PCR amplified from *S. flexneri* 2457T genomic DNA with upstream and downstream primers ET3 and ET4 (containing *Bam*HI and *Sac*I restriction sites) (Fig. 3.1 A, lane 2). PCR amplification was performed as described in the Section 2.4.2. The resultant 1.8 kb PCR fragment was cloned into pGEMT-Easy to give pGEMT-Easy::*icsP* (Table 2.5), which was then digested with *Bam*HI and *Sac*I, and the 1.8 kb *Bam*HI-*Sac*I fragment sub-cloned into likewise digested pSL1180 (pSL1180::*icsP*). Further digestion with *Cla*I allowed insertion of the 1.25 kb *Acc*I-*Acc*I digested kan^R gene from pKTUWE to give pSL1180-*icsP*::kan^R (Table 2.5) and electroporated into DH5α to give ETRM16. Disruption of the *icsP* gene was confirmed by PCR with primers ET3 and ET4 for the presence of a 3 kb fragment corresponding to *icsP*::kan^R (Fig. 3.1 A, lane 3). Subsequently, pSL1180-*icsP*::kan^R was digested with *Bam*HI and *Sac*I, and the 3 kb fragment sub-cloned into pCACTUS to give pCACTUS-*icsP*::kan^R (Table 2.5). The resulting suicide plasmid construct was transformed into *S. flexneri* 2457T and M90T via electroporation and allelic exchange mutagenesis undertaken as described in Section 2.8.1.

The presence of the 2.23 kb *icsP*::kan^R disrupted gene in the virulence plasmid was confirmed by PCR with primers ET1 and ET2 in 2457T *icsP* mutant ETRM22 and M90T *icsP* mutant ETRM108 (Table 2.2) in Figure 3.1B (lanes 3 and 5, respectively). The wild-type 0.981 kb *icsP* gene was detected in *S. flexneri* wild-type strains 2457T and M90T as expected (Fig. 3.1 B, lanes 2 and 4). Subsequent Western immunoblotting analysis on whole cell samples of ETRM22 (Section 5.6) and ETRM108 (data not shown) using anti-IcsP antibody confirmed that the *icsP* mutants were unable to synthesize the IcsP protein.

Fig. 3.1 *icsP* PCR analysis

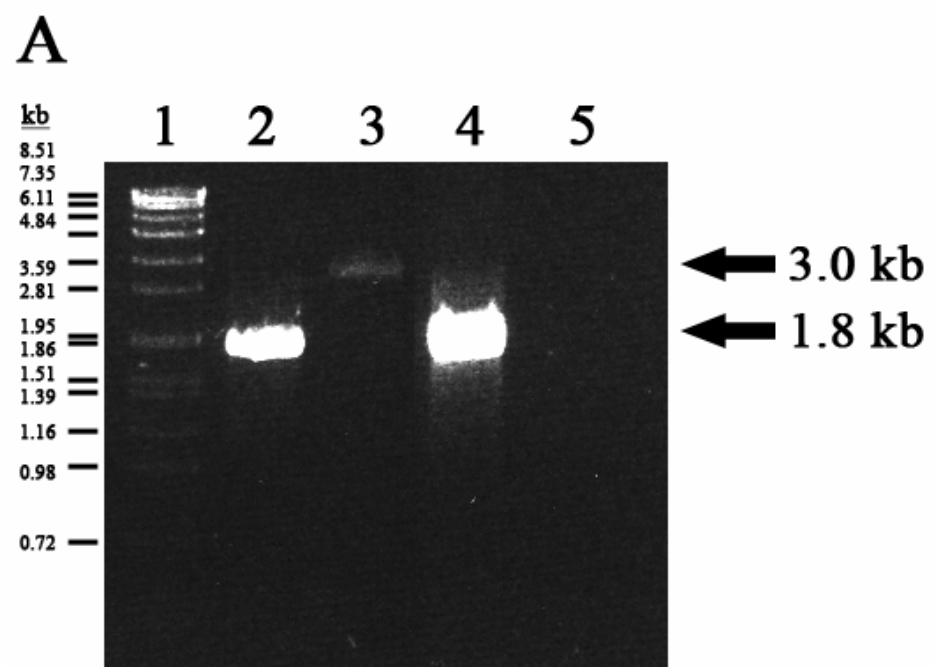
PCR was performed on strains with *icsP*-specific primers ET3 and ET4 for (**A**), and ET1 and ET2 for (**B**) (Table 2.2). The size of the wild-type *icsP* gene (1.8 kb for (**A**) and 0.98 kb for (**B**)), and disrupted *icsP* gene (3.0 kb for (**A**) or 2.23 kb for (**B**)), is indicated on the right. The migration positions of the SPP1 DNA marker is indicated on the left in kb. *S. flexneri* 2a 2457T genomic DNA (diluted 1/100) was used as a positive control and MQ water was used as a template for the negative control. The lane order is as follows:

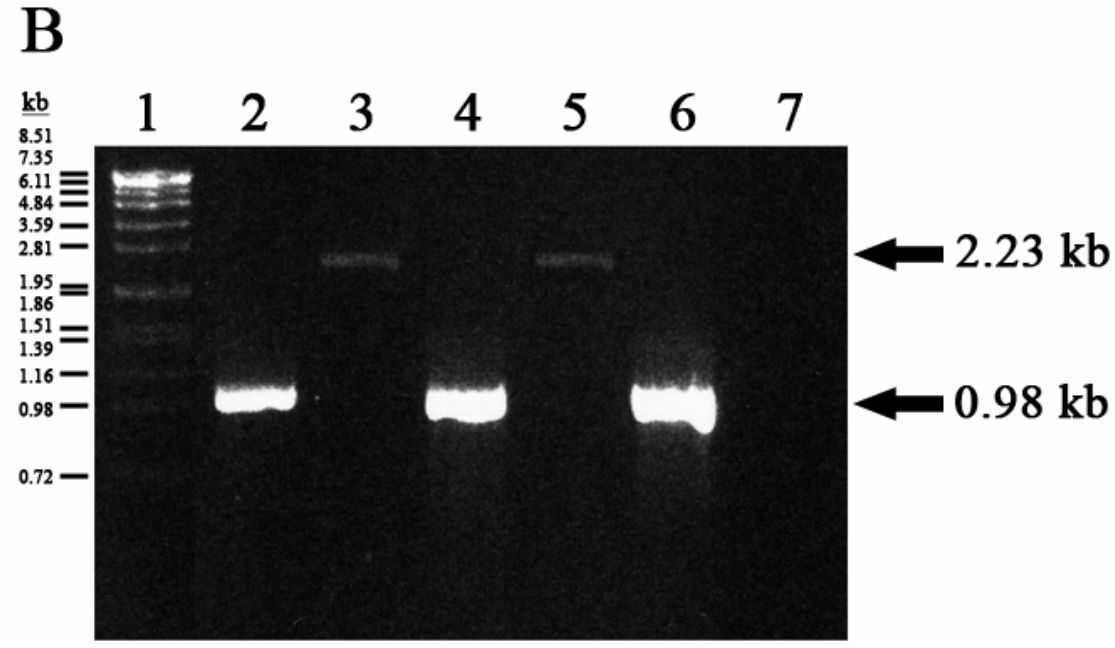
A

1. SPP1 DNA marker
2. *S. flexneri* 2a 2457T
3. ETRM16 (DH5α [pSL1180-*icsP*::*kan*^{R}])
4. *S. flexneri* genomic DNA control
5. MQ control

B

1. SPP1 DNA marker
2. *S. flexneri* 2a 2457T
3. ETRM22 (2457T *icsP*::*kan*^{R})
4. *S. flexneri* 5a M90T
5. ETRM108 (M90T *icsP*::*kan*^{R})
6. *S. flexneri* genomic DNA control
7. MQ control


A
kb
1
2
3
4
5
8.51
7.35
6.11
4.84
3.59
2.81
1.95
1.86
1.51
1.39
1.16
0.98
0.72
3.0 kb
1.8 kb


B
kb
1
2
3
4
5
6
7
8.51
7.35
6.11
4.84
3.59
2.81
1.95
1.86
1.51
1.39
1.16
0.98
0.72
2.23 kb
0.98 kb

To confirm that the *icsP* mutations were non-polar, the following plasmid and strains were constructed. The 1.8 kb undisrupted *icsP* fragment was digested from pGEMT-Easy::*icsP* (using *Bam*HI and *Sac*I) and sub-cloned into likewise digested pWSK29 to produce pWSK29::*icsP* (Table 2.5). This low-copy number plasmid construct was electroporated into ETRM22 (*S. flexneri* 2a 2457T *icsP*::*kan*^{R}) and ETRM108 (*S. flexneri* 5a M90T *icsP*::*kan*^{R}) to give ETRM31 (*S. flexneri* 2a 2457T *icsP*::*kan*^{R} [pWSK29::*icsP*]) and ETRM112 (*S. flexneri* 5a M90T *icsP*::*kan*^{R} [pWSK29::*icsP*]), respectively (Table 2.4). Control strains were also made by electroporating pWSK29 into ETRM22 and ETRM108 to give ETRM29 (*S. flexneri* 2a 2457T *icsP*::*kan*^{R} [pWSK29]) and ETRM114 (*S. flexneri* 5a M90T *icsP*::*kan*^{R} [pWSK29]).

3.3 Characterisation of *S. flexneri icsP* mutants

3.3.1 Analysis of IcsA cleavage by *S. flexneri icsP* mutants

Loss of IcsP function by ETRM22 (2457T *icsP*::*kan*^{R}) and ETRM108 (M90T *icsP*::*kan*^{R}) was determined by preparation of cell associated and soluble IcsA samples (Section 2.9.9). Samples were subjected to SDS 15% PAGE and Western immunoblotting with an anti-IcsA antibody. Figure 3.2 shows the presence of the full length 120 kDa IcsA protein in the whole cell samples of 2457T, ETRM22, ETRM31 (2457T *icsP*::*kan*^{R} [pWSK29::*icsP*]) and ETRM29 (2457T *icsP*::*kan*^{R} [pWSK29]) (Fig. 3.2, lanes 1, 3, 5 and 7) as expected. Similarly, Figure 3.3 shows the presence of the full length 120 kDa IcsA protein in the whole cell samples of M90T, ETRM108, ETRM112 (M90T *icsP*::*kan*^{R} [pWSK29::*icsP*]) and ETRM114 (M90T *icsP*::*kan*^{R} [pWSK29]) (Fig. 3.3, lanes 1, 3, 5 and 7) as expected. The presence of the ~95 kDa cleaved form of IcsA was observed only in strains carrying a functional *icsP* gene, i.e. wild-type strains 2457T and M90T (Fig. 3.2 and 3.3, lane 2) and complemented strains ETRM31 and ETRM112 (Fig. 3.2 and 3.3, lane 6). IcsA fragments less than 95 kDa in size are degraded IcsA products. The absence of the cleaved ~95 kDa IcsA fragment in the culture

Fig. 3.2 IcsA cleavage by 2457T *icsP* mutants

S. flexneri strains (as indicated below) were grown in LB at 37°C to an OD_{600} of ~0.2-0.4. For the detection of cleaved IcsA in culture supernatants, whole cell protein samples were obtained from the pellet of 1 ml culture and supernatant protein samples were obtained from 50 ml culture supernatants treated with 5% (v/v) TCA (Section 2.9.9). Samples were electrophoresed on SDS 15% polyacrylamide gels prior to Western immunoblotting with anti-IcsA. The size of the mature IcsA protein (120 kDa) and cleaved IcsA fragment (~95 kDa) is indicated on the right. Bands smaller than 95 kDa are degraded IcsA fragments and are not labelled. Lanes containing whole cell samples represent $5x10^7$ bacteria. Supernatant protein samples were prepared from 50 ml volumes of culture. Lanes are as follows:

1. 2457T whole cell
2. 2457T supernatant
3. ETRM22 (2457T *icsP*::*kan*^{R}) whole cell
4. ETRM22 (2457T *icsP*::*kan*^{R}) supernatant
5. ETRM31 (2457T *icsP*::*kan*^{R} [pWSK29::*icsP*]) whole cell
6. ETRM31 (2457T *icsP*::*kan*^{R} [pWSK29::*icsP*]) supernatant
7. ETRM29 (2457T *icsP*::*kan*^{R} [pWSK29]) whole cell
8. ETRM29 (2457T *icsP*::*kan*^{R} [pWSK29]) supernatant

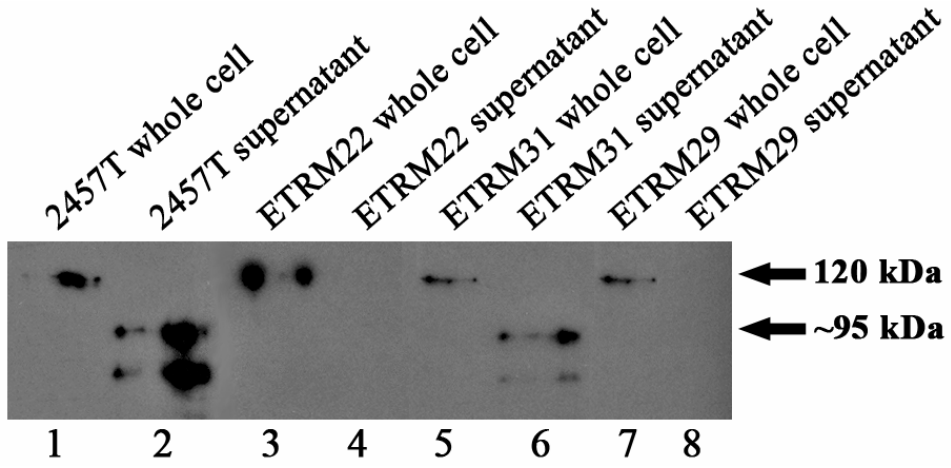
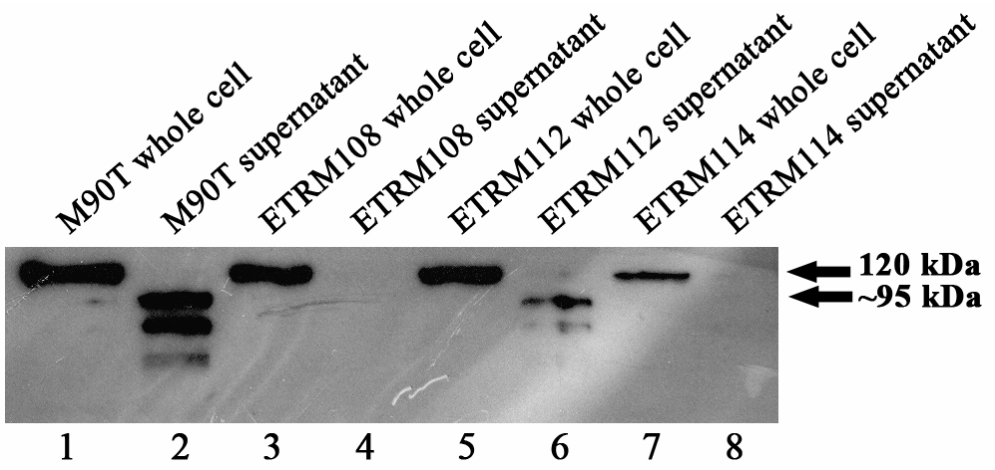

2457T whole cell
2457T supernatant
ETRM22 whole cell
ETRM22 supernatant
ETRM31 whole cell
ETRM31 supernatant
ETRM29 whole cell
ETRM29 supernatant
120 kDa
~95 kDa
1
2
3
4
5
6
7
8

Fig. 3.3 IcsA cleavage by M90T *icsP* mutants

S. flexneri strains (as indicated below) were grown in LB at 37°C to an OD_{600} of ~0.2-0.4. For the detection of cleaved IcsA in culture supernatants, whole cell protein samples were obtained from the pellet of 1 ml culture and supernatant protein samples were obtained from 50 ml culture supernatants treated with 5% (v/v) TCA (Section 2.9.9). Samples were electrophoresed on SDS 15% polyacrylamide gels prior to Western immunoblotting with anti-IcsA. The size of the mature IcsA protein (120 kDa) and cleaved IcsA fragment (~95 kDa) is indicated on the right. Bands smaller than 95 kDa are degraded IcsA fragments and are not labelled. Lanes containing whole cell samples represent $5x10^7$ bacteria. Supernatant protein samples were prepared from 50 ml volumes of culture. Lanes are as follows:

1. M90T whole cell
2. M90T supernatant
3. ETRM108 (M90T *icsP*::*kan*^{R}) whole cell
4. ETRM108 (M90T *icsP*::*kan*^{R}) supernatant
5. ETRM112 (M90T *icsP*::*kan*^{R} [pWSK29::*icsP*]) whole cell
6. ETRM112 (M90T *icsP*::*kan*^{R} [pWSK29::*icsP*]) supernatant
7. ETRM114 (M90T *icsP*::*kan*^{R} [pWSK29]) whole cell
8. ETRM114 (M90T *icsP*::*kan*^{R} [pWSK29]) supernatant


M90T whole cell
M90T supernatant
ETRM108 whole cell
ETRM108 supernatant
ETRM112 whole cell
ETRM112 supernatant
ETRM114 whole cell
ETRM114 supernatant
120 kDa
~95 kDa
1
2
3
4
5
6
7
8

supernatant samples of ETRM22 and ETRM108 (Fig. 3.2 and 3.3, lane 4), and the vector control strains ETRM29 and ETRM114 (Fig. 3.2 and 3.3, lanes 8), indicate loss of IcsP activity on IcsA in the constructed *S. flexneri icsP* mutants.

3.3.2 Analysis of surface distribution of IcsA in *S. flexneri icsP* mutants

To further characterise the constructed 2457T and M90T *icsP* mutants, the distribution of IcsA on the cell surface of ETRM22 (2457T *icsP*::*kan*^{R}) and ETRM108 (M90T *icsP*::*kan*^{R}) was investigated by IF with an anti-IcsA antibody and compared to control strains. The results obtained showed that ETRM22 and ETRM108 (Fig. 3.4 B and F), and control strains ETRM29 (2457T *icsP*::*kan*^{R} [pWSK29]) and ETRM114 (M90T *icsP*::*kan*^{R} [pWSK29]) (Fig. 3.4 D and H), displayed IcsA on polar and lateral regions of the cell surface. Polar localisation of IcsA on the cell surface was observed in the wild-type strains 2457T and M90T (Fig. 3.4 A and E), and complemented strains ETRM31 (2457T *icsP*::*kan*^{R} [pWSK29::*icsP*]) and ETRM112 (M90T *icsP*::*kan*^{R} [pWSK29::*icsP*]) (Fig. 3.4 C and G). A *S. flexneri icsA* mutant (RMA2041) was used as a negative control and showed no antibody labelling of IcsA, as expected (Fig. 3.4 I). The results show that the constructed *S. flexneri icsP* mutants have a similar phenotype as those described by other authors (Egile *et al.*, 1997; Shere *et al.*, 1997).

3.4 An analysis of *S. flexneri icsP* mutants with respect to virulence related properties

3.4.1 Analysis of intercellular spread by *S. flexneri icsP* mutants

To determine whether IcsP played a role in *S. flexneri* intercellular spreading, the ability of *icsP* mutants to spread from cell-to-cell was investigated by quantitation of plaque formation in HeLa and CV-1 cell monolayers (Fig. 3.5 and Fig. 3.7 respectively). Plaque assays were preformed as described in Section 2.12.4. Plaque assays with HeLa cells showed that

Fig. 3.4 IF detection of IcsA localisation in *icsP* mutants

Strains were grown in LB at 37°C to an OD_{600} of ~0.2-0.4 and formalin fixed. Cell surface IcsA was detected by indirect IF staining with a rabbit anti-IcsA antibody and a donkey anti-mouse Alexa Fluor 488 IgG secondary antibody. Bacteria were observed by epi-fluorescence microscopy. Within each IF image an enlargement of a typical bacterium is shown. Scale bars represent 1 μm in size. The images are as follows:

A. *S. flexneri* 2a 2457T

B. ETRM22 (2457T *icsP*::*kan*^{R})

C. ETRM31 (2457T *icsP*::*kan*^{R} [pWSK29::*icsP*])

D. ETRM29 (2457T *icsP*::*kan*^{R} [pWSK29])

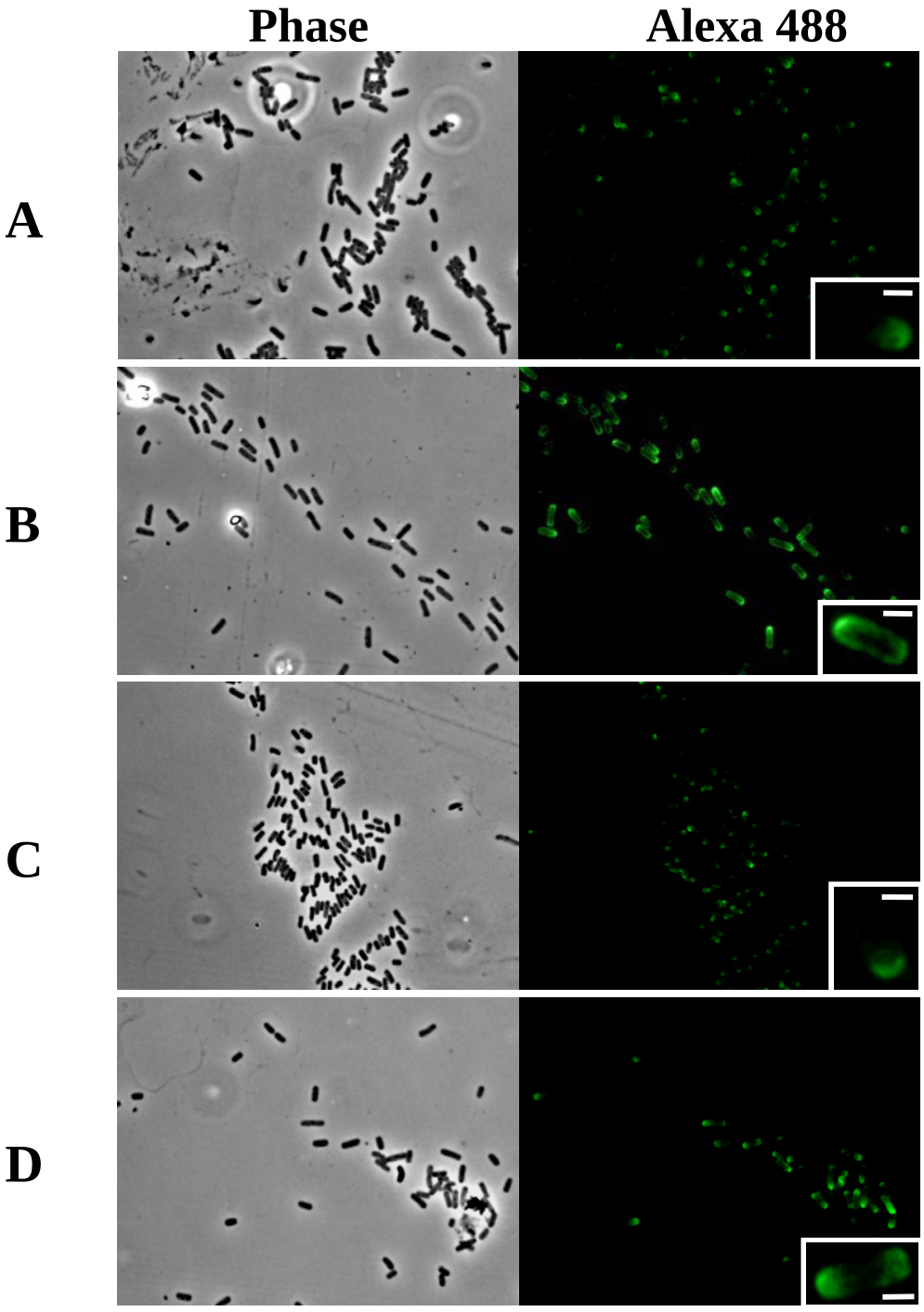
E. *S. flexneri* 5a M90T

F. ETRM108 (M90T *icsP*::*kan*^{R})

G. ETRM112 (M90T *icsP*::*kan*^{R} [pWSK29::*icsP*])

H. ETRM114 (M90T *icsP*::*kan*^{R} [pWSK29])

I. RMA2041 (2457T *icsA*::*tet*^{R})


Phase
Alexa 488
A
B
C
D

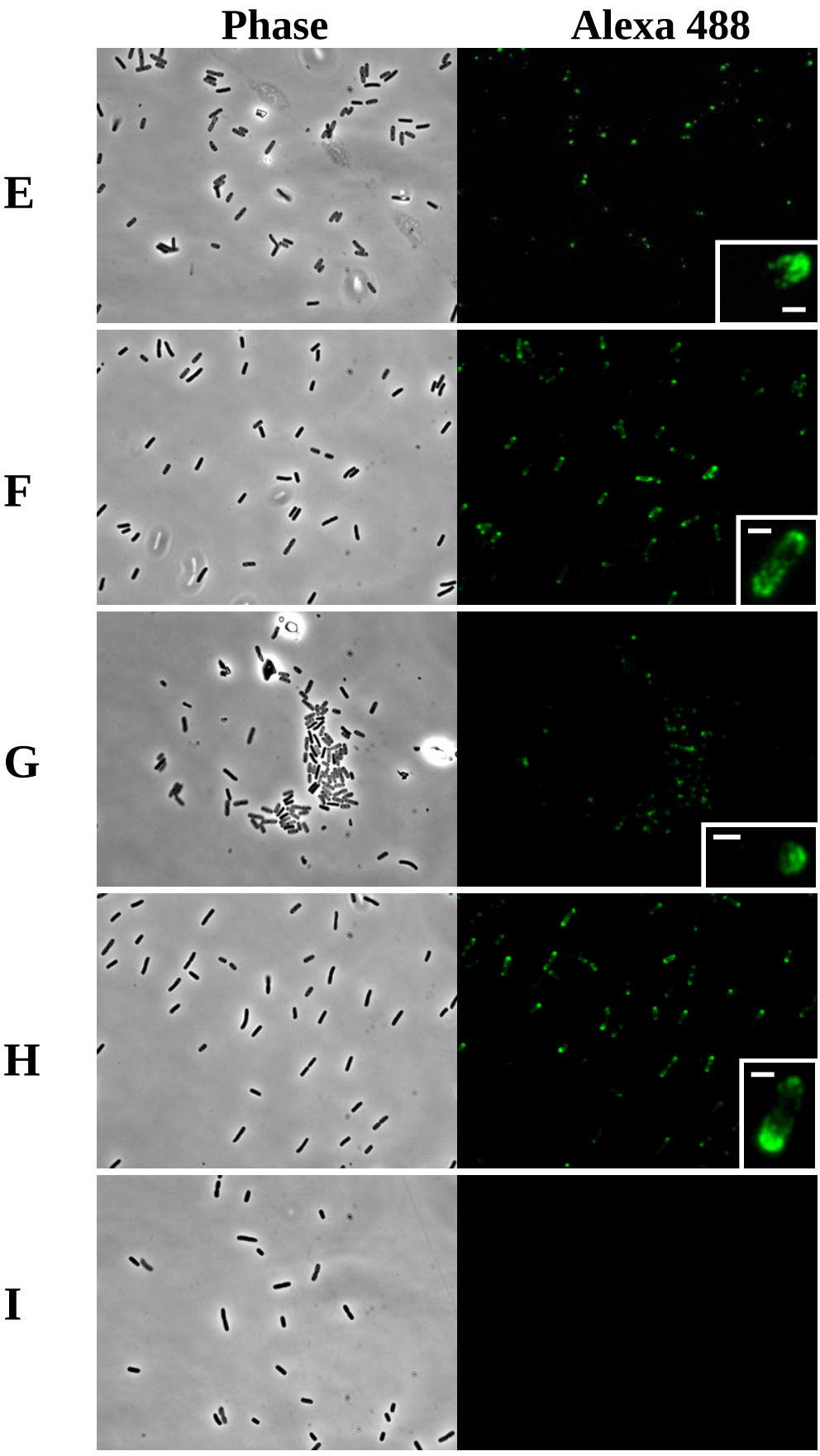

Phase
Alexa 488
E
F
G
H
I

Fig. 3.5 HeLa cell plaque assays with *icsP* mutants

Confluent HeLa cell monolayers were infected with LB grown *Shigella* strains (as indicated below) for 2 h, overlaid with agarose containing gentamycin and incubated at 37°C with 5% CO_2 for 28 h, then overlaid with a second agaroseose layer containing Neutral Red and incubated for a further 16 h prior to taking pictures (refer to Section 2.12.4). The experiment was repeated four times with consistent results. The average size of 20 plaques ± the standard error mean for each strain is shown in the top corner of each image. The images are as follows:

A. HeLa infected with 2457T

B. HeLa infected with ETRM22 (2457T *icsP*::*kan*^{R})

C. HeLa infected with ETRM31 (2457T *icsP*::*kan*^{R} [pWSK29::*icsP*])

D. HeLa infected with ETRM29 (2457T *icsP*::*kan*^{R} [pWSK29])

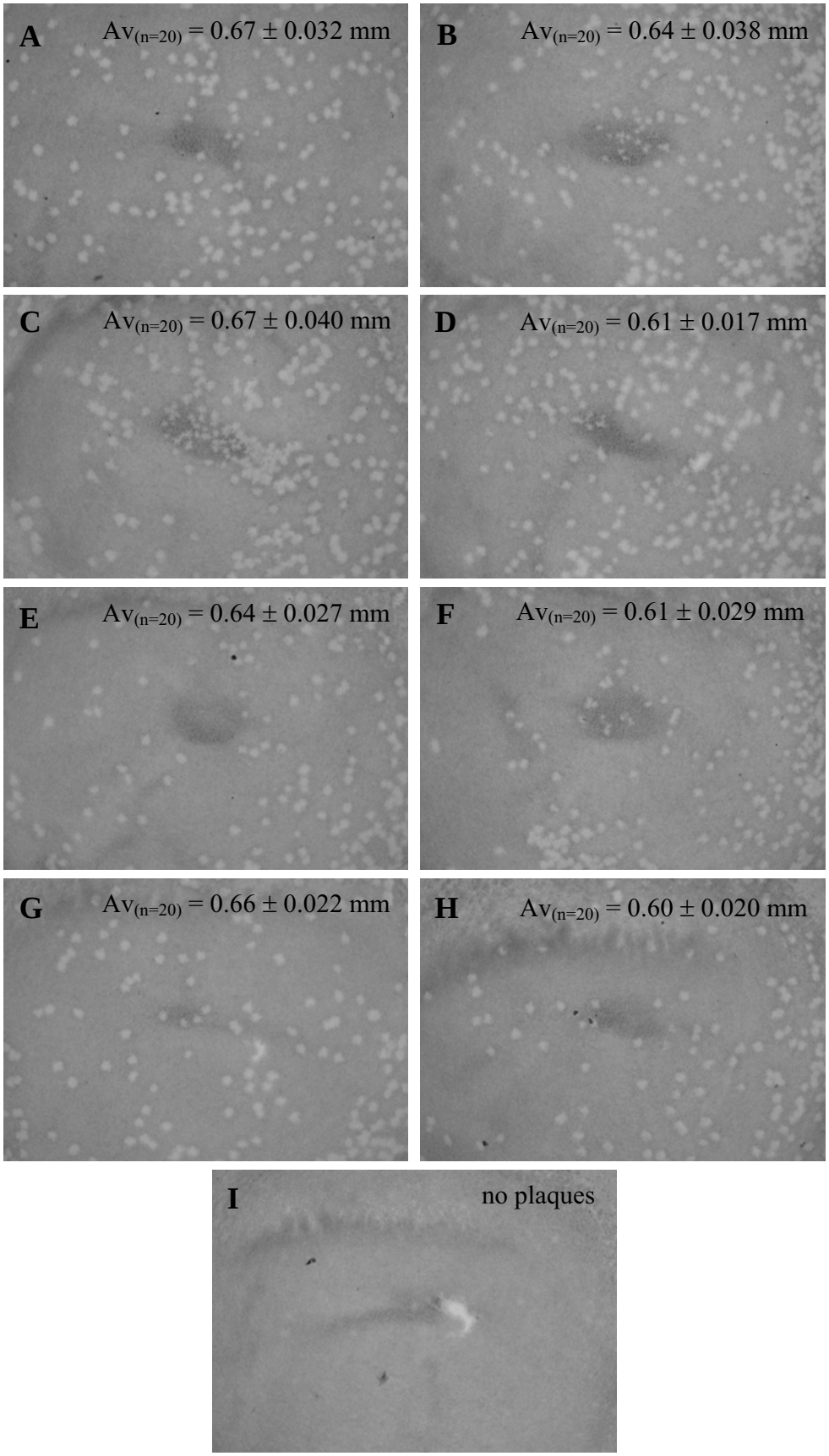
E. HeLa infected with M90T

F. HeLa infected with ETRM108 (M90T *icsP*::*kan*^{R})

G. HeLa infected with ETRM112 (M90T *icsP*::*kan*^{R} [pWSK29::*icsP*])

H. HeLa infected with ETRM114 (M90T *icsP*::*kan*^{R} [pWSK29])

I. HeLa infected with RMA2041 (2457T Δ*icsA*::*tet*^{R})


A
Av(n=20) = 0.67 ± 0.032 mm
B
Av(n=20) = 0.64 ± 0.038 mm
C
Av(n=20) = 0.67 ± 0.040 mm
D
Av(n=20) = 0.61 ± 0.017 mm
E
Av(n=20) = 0.64 ± 0.027 mm
F
Av(n=20) = 0.61 ± 0.029 mm
G
Av(n=20) = 0.66 ± 0.022 mm
H
Av(n=20) = 0.60 ± 0.020 mm
I
no plaques

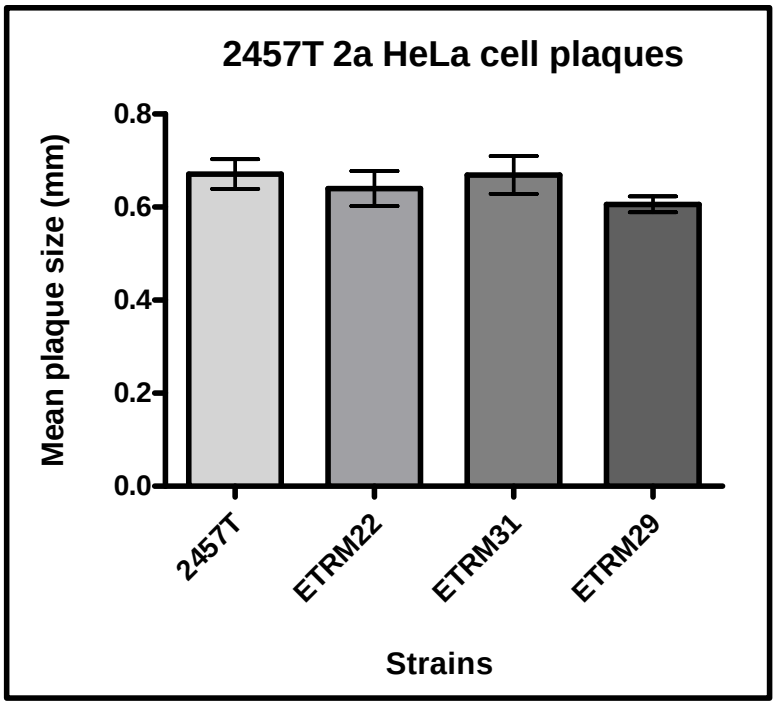
ETRM22 (2457T *icsP*::*kan*^{R}) and ETRM108 (M90T *icsP*::*kan*^{R}) (Fig. 3.5 B and F) formed plaques of similar sizes to their wild-type parents 2457T and M90T (Fig. 3.5 A and E), suggesting that both *icsP* mutants were able to invade and spread intercellularly with no apparent defect in the ability to move from cell-to-cell. The plaque sizes measured for the complemented and vector control strains ETRM31, ETRM29, ETRM112, and ETRM114 (Table 2.4) (Fig. 3.5 C and D, G and H) were also similar to their respective wild-type strains. The experiment was repeated four times with consistent results. A two-tailed unpaired *t*-test was performed on the average plaque sizes for each strain and the graphs shown in Fig. 3.6 show that the plaque size differences were statistically insignificant from wild-type.

In plaque assays with CV-1 cells however, ETRM22 (2457T *icsP*::*kan*^{R}) and ETRM108 (M90T *icsP*::*kan*^{R}) (Fig. 3.7 B and F) formed plaques that were consistently larger in size than those of their corresponding wild-type strains 2457T and M90T (Fig. 3.7 A and E). The experiment was repeated four times with consistent results. Statistical analysis using a two-tailed unpaired *t*-test (as described above) showed that the observed plaque sizes obtained for *icsP* mutants ETRM22 and ETRM108 were significantly larger than the plaques formed by 2457T and M90T (Fig. 3.8 A and B). The strains complemented with a functional *icsP* gene, i.e. ETRM31 (2457T *icsP*::*kan*^{R} [pWSK29::*icsP*]) and ETRM112 (M90T *icsP*::*kan*^{R} [pWSK29::*icsP*]), showed restoration of plaque sizes to that of their wild-type parents (Fig. 3.7 C and G, compared to A and E). Vector control strains ETRM29 (2457T *icsP*::*kan*^{R} [pWSK29]) and ETRM114 (M90T *icsP*::*kan*^{R} [pWSK29]) (Fig. 3.7 D and H) showed plaque sizes similar to their *icsP* mutant equivalents, as expected. A *S. flexneri icsA* mutant (RMA2041) was used as a negative control for all assays and showed no formation of plaques (Fig. 3.5 I and 3.7 I).

Fig. 3.6 Statistical analysis of HeLa cell plaque sizes

Confluent HeLa cell monolayers were infected with the following LB grown *Shigella* strains: (**A**) 2457T, ETRM22 (2457T *icsP*::*kan*^{R}), ETRM31 (2457T *icsP*::*kan*^{R} [pWSK29::*icsP*]) and ETRM29 (2457T *icsP*::*kan*^{R} [pWSK29]), and (**B**) M90T, ETRM108 (M90T *icsP*::*kan*^{R}), ETRM112 (M90T *icsP*::*kan*^{R} [pWSK29::*icsP*]) and ETRM114 (M90T *icsP*::*kan*^{R} [pWSK29]). After 2 h, infected monolayers were overlaid with agarose containing gentamycin and incubated at 37°C with 5% CO_2 for 28 h, then overlaid with a second agaroseose layer containing Neutral Red and incubated for a further 16 h prior to calculating plaque sizes (refer to Section 2.12.4). The graph represents mean plaque sizes relative to 2457T $\pm$ standard error (**A**) or M90T $\pm$ standard error (**B**); error bars are shown for each column. Strains were not statistically significant from 2457T and M90T as determined by a two-tailed unpaired *t*-test.

A


2457T 2a HeLa cell plaques
Mean plaque size (mm)
0.8
0.6
0.4
0.2
0.0
2457T
ETRM22
ETRM31
ETRM29
Strains

B

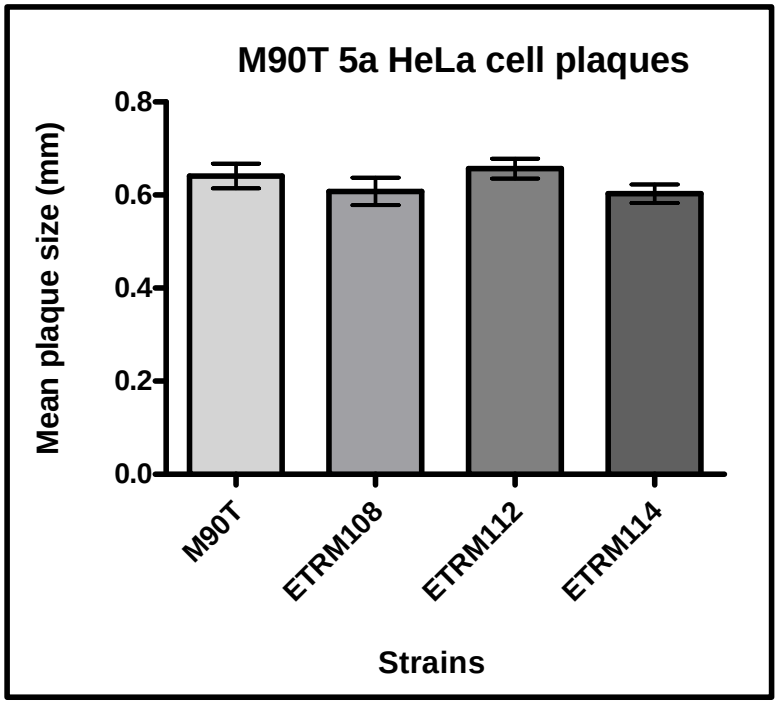

M90T 5a HeLa cell plaques
Mean plaque size (mm)
0.8
0.6
0.4
0.2
0.0
M90T
ETRM108
ETRM112
ETRM114
Strains

Fig. 3.7 CV-1 cell plaque assays with *icsP* mutants

Confluent CV-1 cell monolayers were infected with LB grown *Shigella* strains (as indicated below) for 2 h, overlaid with gentamycin-containing agarose and incubated at 37°C with 5% CO_2 for 20 h, then overlaid with a second agarose layer containing Neutral Red and incubated for a further 8 h prior to taking pictures (refer to Section 2.12.4). The experiment was repeated four times with consistent results. The average size of 20 plaques ± the standard error mean for each strain is shown in the top corner of each image. The images are as follows:

A. CV-1 infected with 2457T

B. CV-1 infected with ETRM22 (2457T *icsP*::*kan*^{R})

C. CV-1 infected with ETRM31 (2457T *icsP*::*kan*^{R} [pWSK29::*icsP*])

D. CV-1 infected with ETRM29 (2457T *icsP*::*kan*^{R} [pWSK29])

E. CV-1 infected with M90T

F. CV-1 infected with ETRM108 (M90T *icsP*::*kan*^{R})

G. CV-1 infected with ETRM112 (M90T *icsP*::*kan*^{R} [pWSK29::*icsP*])

H. CV-1 infected with ETRM114 (M90T *icsP*::*kan*^{R} [pWSK29])

I. CV-1 infected with RMA2041 (2457T Δ*icsA*::*tet*^{R})

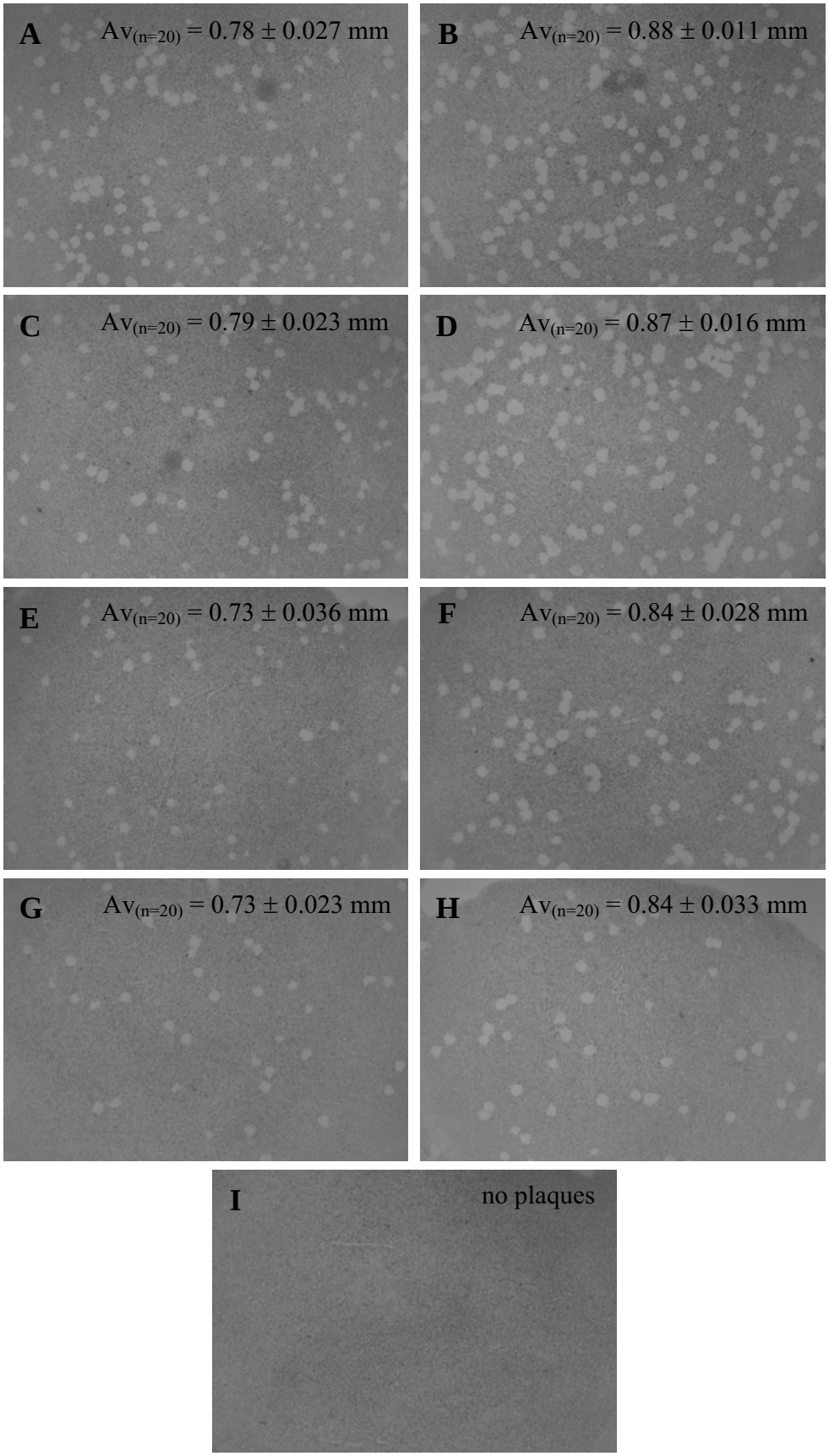
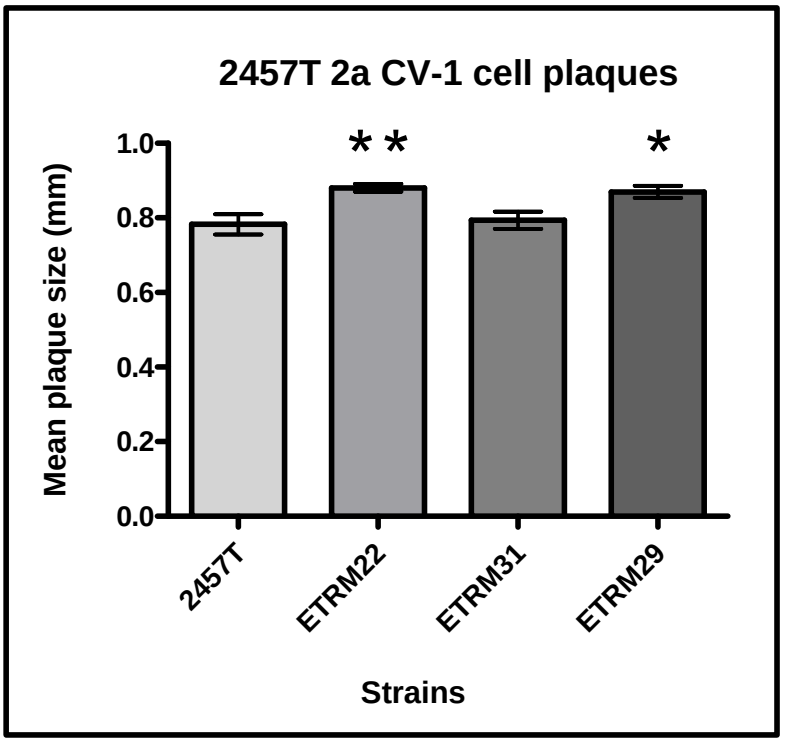

A
$Av_{(n=20)}$ = 0.78 ± 0.027 mm
B
$Av_{(n=20)}$ = 0.88 ± 0.011 mm
C
$Av_{(n=20)}$ = 0.79 ± 0.023 mm
D
$Av_{(n=20)}$ = 0.87 ± 0.016 mm
E
$Av_{(n=20)}$ = 0.73 ± 0.036 mm
F
$Av_{(n=20)}$ = 0.84 ± 0.028 mm
G
$Av_{(n=20)}$ = 0.73 ± 0.023 mm
H
$Av_{(n=20)}$ = 0.84 ± 0.033 mm
I
no plaques

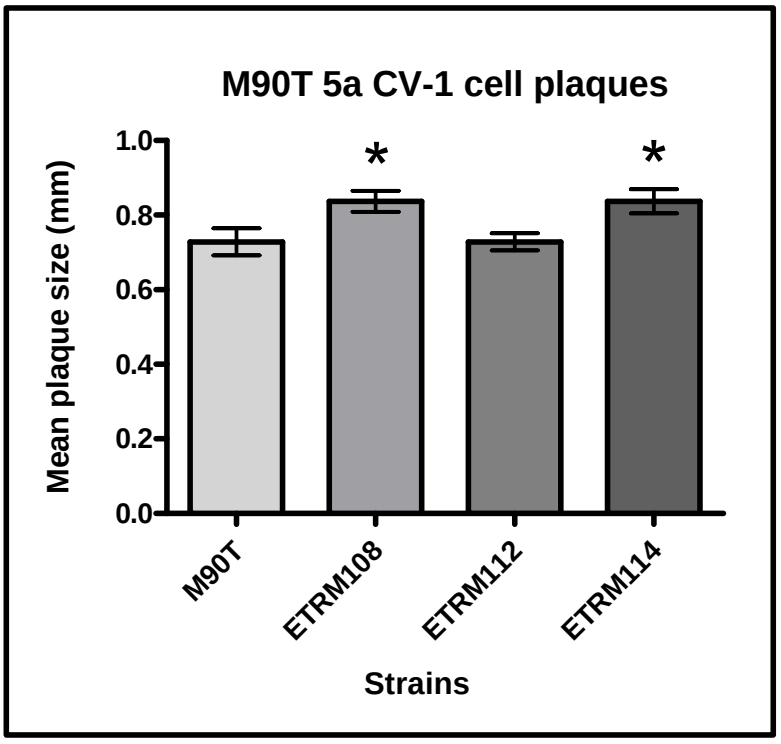
Fig. 3.8 CV-1 cell plaque sizes

Confluent CV-1 cell monolayers were infected with the following LB grown *Shigella* strains: (**A**) 2457T, ETRM22 (2457T *icsP*::*kan*^{R}), ETRM31 (2457T *icsP*::*kan*^{R} [pWSK29::*icsP*]) and ETRM29 (2457T *icsP*::*kan*^{R} [pWSK29]), and (**B**) M90T, ETRM108 (M90T *icsP*::*kan*^{R}), ETRM112 (M90T *icsP*::*kan*^{R} [pWSK29::*icsP*]) and ETRM114 (M90T *icsP*::*kan*^{R} [pWSK29]). After 2 h, infected monolayers were overlaid with gentamycin-containing agarose and incubated at 37°C with 5% CO_2 for 20 h, then overlaid with a second agarose layer containing Neutral Red and incubated for a further 8 h prior to calculating plaque sizes (refer to Section 2.12.4). The graph represents mean plaque sizes relative to 2457T $\pm$ standard error (**A**) or M90T $\pm$ standard error (**B**); error bars are shown for each column. Strains which are statistically significant from 2457T or M90T are summarised above each column (whereby ** represents $P<0.01$, * represents $P<0.05$) as determined by a two-tailed unpaired *t*-test.

A


2457T 2a CV-1 cell plaques
Mean plaque size (mm)
1.0
0.8
0.6
0.4
0.2
0.0
* *
*
2457T
ETRM22
ETRM31
ETRM29
Strains

B


M90T 5a CV-1 cell plaques
Mean plaque size (mm)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
M90T
ETRM108
ETRM112
ETRM114
Strains

3.4.2 Analysis of F-actin comet tail formation by *S. flexneri icsP* mutants

Since a difference in plaques sizes was observed by the *icsP* mutants in CV-1 cell monolayers, F-actin comet tail formation by 2457T, M90T and the *icsP* mutant derivatives were compared by staining CV-1 infected cells with FITC-phalloidin. The results obtained showed that ETRM22 and ETRM108 (Fig. 3.9 B and F) formed normal F-actin comet tails with no apparent abnormalities compared to wild-type strains 2457T and M90T (Fig. 3.9 A and E). Similar results were obtained for complemented and vector control strains ETRM31, ETRM29, ETRM112, and ETRM114 (Table 2.4) (Fig. 3.9 C and D, G and H).

3.5 Summary

This chapter describes the inactivation of the *icsP* gene in *S. flexneri* 2a 2457T and *S. flexneri* 5a M90T. Mutagenesis of *icsP* was performed using allelic exchange mutagenesis and the resultant *icsP* mutant strains ETRM22 (*S. flexneri* 2a 2457T *icsP*::*kan*^{R}) and ETRM108 (*S. flexneri* 5a M90T *icsP*::*kan*^{R}) were shown to have an inactivated *icsP* gene and were unable to release IcsA into culture supernatants. Furthermore staining of IcsA on the cell surface of these mutants showed that IcsA was present on both polar and lateral regions of the cell surface, as previously described by other authors (d'Hauteville *et al.*, 1996; Egile *et al.*, 1997; Shere *et al.*, 1997; Steinhauer *et al.*, 1999).

Subsequent investigation of the *icsP* mutants using tissue culture based plaque assays showed that *icsP* in both serotype backgrounds did not appear to be essential for cell-to-cell spread in human cervical epithelial HeLa cells, but the absence of *icsP* enhanced cell-to-cell spread in monkey kidney fibroblast CV-1 cells, suggesting that IcsP may play a role in limiting the spread of *S. flexneri* inside host cells. Additionally, F-actin comet tail staining of bacteria

Fig. 3.9 F-actin tail formation inside CV-1 cells

Semi-confluent CV-1 cell monolayers were infected with LB grown *S. flexneri* strains (as indicated below) for 1 h, followed by centrifugation and 90 min incubation in medium containing 40 μg/ml gentamycin. Monolayers were then washed, fixed in 3.7% formaldehyde solution, incubated in DPBS + 50mM NH_4Cl, and then in PBS + 0.1% Triton X-100 prior to blocking in 10% FCS (refer to Section 2.12.5). Coverslips containing *S. flexneri* infected cells were then labelled with a rabbit group 3,4 anti-LPS antibody and a donkey anti-rabbit Alexa Fluor 594 IgG secondary antibody to stain the bacteria red; FITC-phalloidin was used to stain F-actin green. A typical F-actin comet tail is indicated by a white arrow for each image. Scale bars represent 4 μm in size. Images are as follows:

A. CV-1 infected with 2457T

B. CV-1 infected with ETRM22 (2457T *icsP*::kan^R)

C. CV-1 infected with ETRM31 (2457T *icsP*::kan^R [pWSK29::*icsP*])

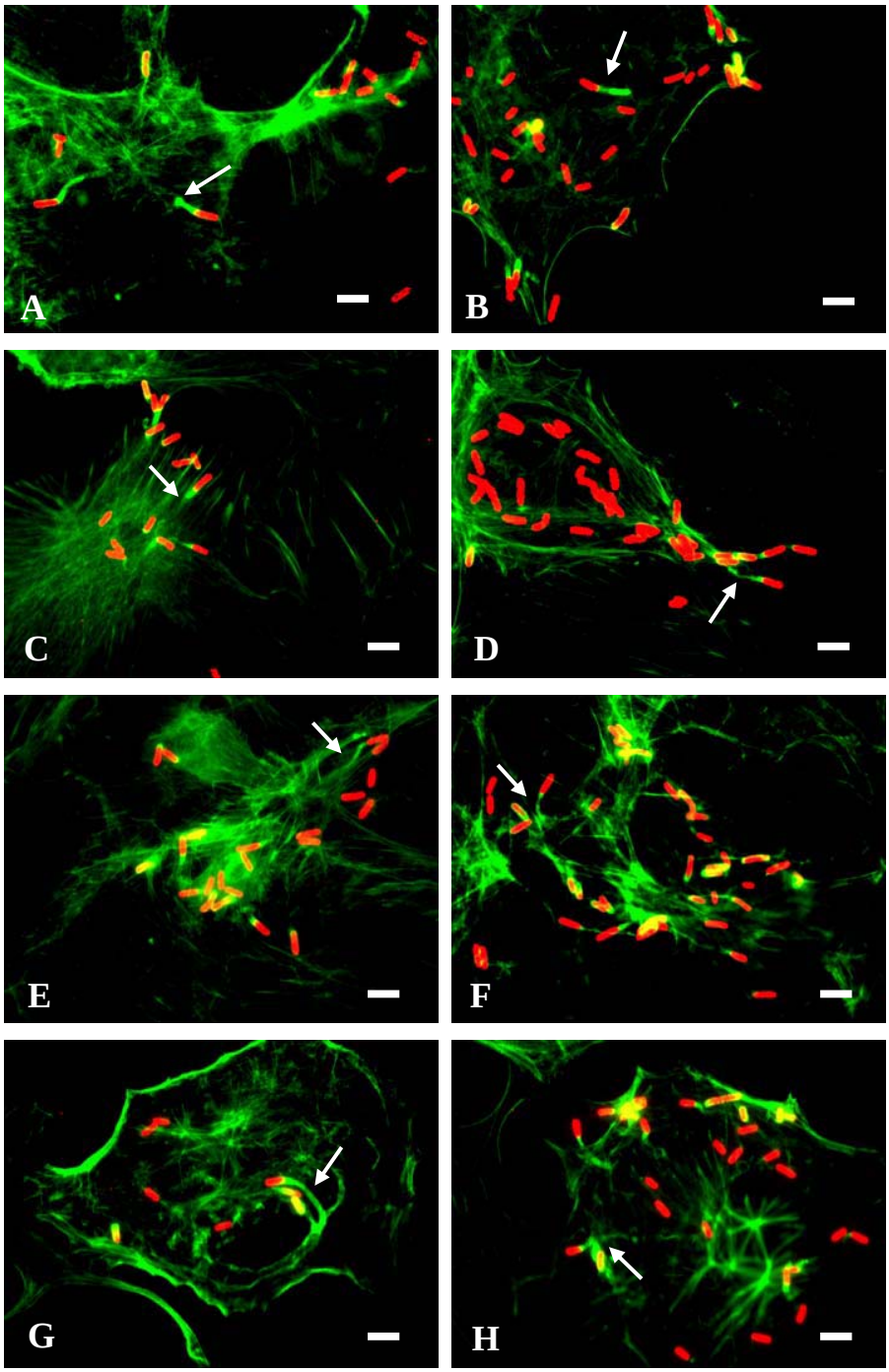
D. CV-1 infected with ETRM29 (2457T *icsP*::kan^R [pWSK29])

E. CV-1 infected with M90T

F. CV-1 infected with ETRM108 (M90T *icsP*::kan^R)

G. CV-1 infected with ETRM112 (M90T *icsP*::kan^R [pWSK29::*icsP*])

H. CV-1 infected with ETRM114 (M90T *icsP*::kan^R [pWSK29])


A
B
C
D
E
F
G
H

inside CV-1 cells showed that F-actin comet tail formation by both *icsP* mutants were similar to that of the wild-type parent strains.

In conclusion, this chapter has shown that *S. flexneri* IcsP may be required to limit *Shigella* intercellular spread, as shown in CV-1 cells, and hence potentially limit disease. Importantly, this role does not appear to be dependant on the strain serotype. The construction of *S. flexneri* 2a 2457T and *S. flexneri* 5a M90T *icsP* mutants allowed further investigation of the role of IcsP in *Shigella* virulence in association with other virulence factors such as LPS and VirK in subsequent chapters.