Immunoproteome analysis, vaccine development and acid resistance pathways of *Shigella flexneri* 2457T

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Corrections

1. pg ix, last line, *In vivo*.
2. pg xi, line 16, possibly reduced.
3. pg xi, line 26, *in vitro*.
4. pg xi, line 28, *mgl*.
5. pg xi. line 29, *S. flexneri*.
6. pg 8, line 12, An interaction.
7. pg 38, line 10, SLA3000 rotor.
8. pg 38, last line, Falcon tube.
9. pg 40, line 11, samples were.
10. pg 41, line 26, 8 mL SuperSignal.
11. pg 41, section 2.13.5, the lines" Sera from the five patients was stored at – 70 °C. There was no detailed patient information available for these samples." should be added after the second sentence of the paragraph.
12. pg 44, line 20, solution in Eppendorf tubes.
13. pg 50, line 20, to a solution in Eppendorf tube on ice.
14. pg, 53, section 2.22.2, "vaccine strains" should read "liposome preparations".
15. pg 57, section 3.2.1, the sixth sentence of the paragraph should read "The membranes were probed with pooled sera from five *S. flexneri* patients and a map of immunoreactive proteins was visualised by chemiluminescence."
16. pg 65, line 10, antigen perspective, but as they.
17. pg 73, section 4.2.9, the last sentence should only state that cells are attenuated in growth during conditions of low carbon – not in low nitrogen availability.
18. pg 75, section 4.3.1, the fifth sentence of the 2nd paragraph should have the first comma replaced with a hyphen.
19. pg 76, line 22, co-linear.
20. pg 88, section 5.2.7, the final sentence of the 2nd paragraph should read "It is most likely that MglB is present in this protein extaction…"
21. pg 100, line 29, used as a baseline.
22. pg 109, line 15, of IpaD reduced.
23. pg 121, line 10, truncated and possibly reduced.
24. pg 130, line 22, as an attenuation target in.
25. Figure legend 3.5, the final line of the legend should read " Identical amounts of protein were loaded in each lane".
26. Figure legend 4.7, the formula should read intracellular bacteria divided by total bacteria.

Conference Presentations arising from thesis:

- April 2006: Seminar presented at the 158th Meeting for Society for General Microbiology, United Kingdom, Microbial Infection/Clinical Microbiology Joint Symposium. "Visualising the antigenome of *Shigella flexneri* 2457T; identification of novel vaccine candidate antigens"
- September 2005: Seminar presented at the Annual Joint Scientific Meeting and Exhibition of the Australian Society for Microbiology. "Visualising the antigenome of *Shigella flexneri* 2457T; identification of novel vaccine candidate antigens"
- May 2005: Seminar presented at Australian Society for Microbiology BacPath VIII Conference. "Visualising the antigenome of *Shigella flexneri* 2457T; identification of novel vaccine candidate antigens"
- September 2004: BD Student Winner Seminar Presentation at the Annual Joint Scientific Meeting and Exhibition of the Australian Society for Microbiology.
- June 2004: Seminar presented at Australian Society for Microbiology Students Speaker Night. "Acid Resistance in *Shigella flexneri* 2457T".

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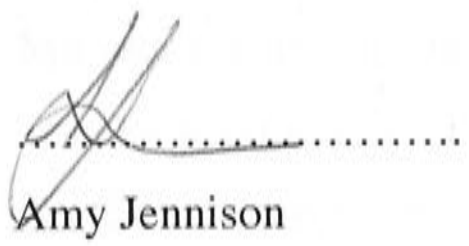
Declaration

I declare that this thesis is an original work and is an account of research carried out by myself while enrolled as a PhD candidate at The Australian National University between 2003-2006. To the best of my knowledge, this thesis does not contain material that has been accepted for the award of any other degree or diploma in any University.



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Finally, I would like to dedicate this work to my two grandfathers, who passed away during this work. They were both proud, strong men who lived amazing lives. Their love and pride in me was always inspiring and I miss them very much.

Abstract

Shigella flexneri causes shigellosis, the most infectious form of bacterial diarrhoea. Shigellosis is endemic in developing countries and kills over 1.1 million people per year. Despite over sixty years of research into developing a vaccine against *S. flexneri* and designation as a high priority of the World Health Organisation, there is no commercially available vaccine. Further research is required into *S. flexneri* virulence and immunogenicity in order to identify new, promising candidates for vaccine development. This study describes the identification of immunogenic proteins reactive to sera from shigellosis patients, and the assessment of a number of these proteins in both *S. flexneri* 2457T pathogenesis and vaccine development.

Proteomics was used to visualise the immunoreactive proteins of *S. flexneri* 2457T. Eight proteins from soluble and membrane protein extractions were identified across three repeats. Six of these proteins have never been reported as immunogenic in *S. flexneri* natural infection. A further four proteins were identified as immunogenic in only one repeat each. Two of the immunogenic proteins were assessed for their role in *S. flexneri* virulence by constructing disruptions in the *udp* and *S3195* genes and assessing any changes in phenotype. S3195 does not appear to be important for *S. flexneri* invasion or intra- and intercellular spread in epithelial cells according to *in vitro* cell culture plaque assays. Udp, a uridine phosphorylase, was discovered to be important for the *in vitro* replication of *S. flexneri* within the epithelial cell cytoplasm. This growth defect was not sufficient to prevent the *udp S. flexneri* mutant from causing disease symptoms in the *in vivo* virulence assay, the Sereny test. Therefore, Udp may contribute to the intracellular survival and replication of *S. flexneri*, but is not essential, and is therefore not a significant candidate for construction of single mutation attenuated vaccine strains.

Virulence studies on a *mgl* operon disruption mutant determined not only that the parent strain was avirulent in the Sereny test, but that the deletion of the *mgl* operon restores virulence to the strain. Cell culture studies indicated that the deletion of the *mgl* operon improves the intra- and intercellular spread of the attenuated *S. flexneri* strain, and that complementation with a functional *mgl* operon represses spread. Western blot and microscopy analysis revealed that removal of the *mgl* operon restores outer membrane

levels of IcsA, an essential protein for *S. flexneri* intra- and intercellular spread. These results suggest a novel role for the proteins of the Mgl galactose transport system in the regulation of IcsA transport to the outer membrane.

One immunogenic protein, IpaD, was investigated as a vaccine candidate in the development of subunit vaccines for *S. flexneri*. It was determined by antibody inactivation plaque assays that antibodies raised against IpaD may play a protective role against *S. flexneri* infection. IpaD, delivered in liposomes to mice was no more immunogenic than the protein alone, even with the inclusion of dendritic and antigen presenting cell targeting molecules. However, very promising results were produced with the administration of IpaD to mice with the adjuvants gamma inulin, cochleates and aluminium hydroxide. Mice immunised with these subunit vaccine preparations were able to clear a *S. flexneri* infection more rapidly than mice not immunised with IpaD in a challenge experiment. These preliminary vaccine studies suggest that IpaD delivered with some adjuvants is a promising vaccine strategy for *S. flexneri,* which may be able to produce multiple serotype protection simultaneously.

The stationary acid resistance pathways of *S. flexneri* 2457T were characterised for the first time in this study. Acid resistance is an important phenotype for enteric pathogens as it contributes to the survival of the strain as it passes through the stomach during infection. Two pathways previously identified in other *S. flexneri* strains were characterised in *S. flexneri* 2457T; however, a novel acid resistance phenotype was also detected. Disruption mutants constructed in three known acid resistance genes revealed that they are not contributing to this resistance, suggesting it may be a novel pathway or the oxidative pathway operating in the presence of glucose. Furthermore, it was determined that *S. flexneri* 2457T expresses a truncated RpoS protein, which does not appear to be important for any of the acid resistance pathways of *S. flexneri* 2457T. This is the first report of a *S. flexneri* strain displaying RpoS-independent regulatory mechanisms for its oxidative acid resistance pathway.

Abbreviations

2-DE	two-dimensional electrophoresis
aa	amino acid
ANOVA	analysis of variance
ANU	Australian National University
APAF	Australian Proteome Analysis Facility
BHK	baby hamster kidney cells
BRF	Biomolecular Resource Facility
BSA	bovine serum albumin
cfu	colony forming units
CRP	cyclic AMP receptor protein
DNA	deoxyribonucleic acid
EG	minimal salts E glucose
ELISA	enzyme linked immunosorbent assay
FAE	follicular associated epithelium
GABA	gamma aminobutyric acid
GALT	gut associated lymphoid tissue
GDAR	glutamate dependant acid resistance
HLPC	high pressure liquid chromatography
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
Ipa	invasion plasmid antigen
IPTG	isopropylthiogalactoside
kb	kilobase
kDa	kilodalton
LB	Luria Bertarni broth
LBG	Luria-Bertarni broth glucose
LPS	lipopolysaccharide
M	molar
mA	milliamps
MALDI-TOF	matrix assisted laser desorption ionisation time of flight

Mb	megabase
MCS	multiple cloning site
MEM	minimal essential medium
MES	2-(N-morpholino)ethanesulphonic acid
MHC	major histocompatibility complex
mM	millimolar
MOPS	3-Morpholinopropanesulfonic acid
Mw	molecular weight
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMN cell	polymorphonuclear cell
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
rpm	revolutions per minute
ScFv Ab	single chain full length variable antibody fragment
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sIgA	secretory IgA
TCA cycle	tricarboxylic acid cycle
TGF-β	transforming growth factor beta
Th1	T lymphocyte helper 1
Th2	T lymphocyte helper 2
TLR	Toll-like receptors
TNF-α	tumour necrosis factor alpha
TNF-β	tumour necrosis factor beta
TSB	tryptic soy broth
RBS	ribosomal binding site
V	volts
WHO	World Health Organisation
WRAIR	Walter Reed Army Institute of Research

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Introduction and literature review

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Introduction and literature review

1.1 Introduction to Shigella flexneri

1.1.1 Taxonomy of *Shigella*

Members of the genus *Shigella* are Gram-negative facultative anaerobes that belong to the family, Enterobactericae. They share many common characteristics with the *Escherichia* genus and appear to be clones of *E. coli* (181). There are four species in the *Shigella* genus *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei* and *Shigella dysenteriae*. The species are further divided into serotypes based on modifications to their O-antigen structure and biochemical differences. According to this classification scheme, *Shigella flexneri* is traditionally divided into 13 serotypes (9). Two *S. flexneri* serotype 2a strains and one serotype 5b strain have been sequenced, revealing that the *S. flexneri* genome consists of roughly 4 600 000 bp and a 221 618 bp virulence plasmid (150, 249, 403).

1.1.2 Clinical symptoms of shigellosis

Shigellosis is the most infectious form of bacterial diarrhoea. *Shigella* species invade the colon of humans and higher primates, destroying the intestinal epithelium and generating the acute mucosal inflammation characteristic of shigellosis. Infection is generally restricted to the superficial layer of the colonic mucosa, where the inflammation and severe tissue destruction results in abscesses and ulceration (251). The damage to the epithelial layer generates the clinical symptoms of watery diarrhoea, severe abdominal pain and cramping, eventuating in the bloody mucoid stool characteristic of bacillary dysentery (149). Severe symptoms may eventuate, including shedding of the colonic epithelium, loss of protein and dehydration (251). The average duration of illness is 5-7 days (251). Without effective treatment, susceptible shigellosis patients particularly the elderly, malnourished children and immunocompromised patients, may develop severe complications such as septicaemia, pneumonia and haemolytic uremic syndrome (23).

1.1.3 Epidemiology of *S. flexneri*

Over 164.7 million people suffer from shigellosis per year, with 1.1 million annual fatalities. The majority of cases, 163.2 million, occur in developing countries and 69% of infections are in children under the age of five (178). *S. flexneri* is responsible for more fatalities than any other *Shigella* species and is endemic in most developing countries (24). Most *S. flexneri* isolates in the developed world are serotype 2a, whilst the predominant serotypes in developing countries vary but include serotypes 1b, 2a, 3a, 4a and 6 (178). The occurrence of shigellosis in Australia is low, with an average of 544 notifications per year (389). Outbreaks of shigellosis in developed countries usually occur in child-care centres and custodial institutions (251). The high incidence of shigellosis in the developing world is generally attributed to the lack of clean water, poor sanitation, overcrowding, malnutrition and the high cost of antibiotic treatment. Transmission occurs commonly via the faecal-oral route, which is augmented by poor hygiene and close personal contact (405). *S. flexneri* is highly infectious, requiring as little as 100 cells to cause disease in healthy adult volunteers (80).

1.1.4 Treatment of *S. flexneri*

Shigellosis is self-limiting in healthy adults and the decision to prescribe antibiotic therapy is based on the severity of the disease, the age of the patient and the likelihood of further transmission (251). An effective antimicrobial can reduce the duration of symptoms from 5-7 days to 3 days and shorten the excretion of *Shigella* in the stool, diminishing the release of *Shigella* into the environment (77, 251). Rehydration therapy is an essential first step in treatment of any diarrhoea and can greatly reduce morbidity. According to the World Health Organisation (WHO) guidelines, when a presumptive diagnosis of shigellosis is made the patient should be treated with antibiotics, and if symptoms have not improved within 48 hours an alternative antibiotic should be given. Adhering to these guidelines is proving more difficult, as there is increasing antibiotic resistance emerging in *Shigella* strains (319). Continual monitoring by a surveillance system is becoming essential to ensure that effective therapy and control measures are used against shigellosis (252). However, such measures and the need to constantly substitute antibiotics further strain the limited health services of developing countries. Consequently, the WHO has prioritised the development of a safe and effective vaccine against shigellosis (178).

1.2 Virulence genes of S. flexneri

A number of virulence genes have been identified for *S. flexneri* that are crucial for the invasion and colonisation of the intestinal epithelium (Table 1.1). At least three pathogenicity islands have been identified on the *S. flexneri* chromosome, encoding important virulence factors such as the lipopolysaccharide and genes for the temperature-dependant regulation of the virulence plasmid genes (241, 301, 376). However, the majority of virulence genes are located on a 220 kb plasmid, known as the virulence plasmid, which is essential for pathogenicity (222). The genes required for epithelial cell invasion and induction of macrophage death are located in a 30 kb region of the virulence plasmid. This region encodes the components of a type III secretion apparatus, known as the *mxi/spa* and the substrates of this secretion apparatus including IpaB, IpaC and IpaD. Other important virulence genes are scattered throughout the plasmid, including *virA*, *ipaH* and *icsA* (183). Expression of the genes on the virulence plasmid is regulated by the *virF* and *virB* genes in a temperature-dependant manner (368). *virB* transcription is repressed by the binding of the histone-like protein, H-NS, to the *virB* promoter region, blocking the VirF binding site, at 30 °C (368). Growth at 37 °C alters the DNA conformation leading to expression of VirB and thus, expression of the virulence plasmid based genes (73). Gene expression from the virulence plasmid destabilises the plasmid, resulting in a high rate of plasmid loss from the strain (337). Therefore, it is essential to monitor *S. flexneri* strains for maintenance of the virulence plasmid, which is most often done by plating on Congo red agar plates, where Congo red binding by components of the virulence plasmid is detected as red pigmentation in the colonies (63). It is also possible to detect the virulence plasmid using PCR with primers directed against particular regions of the plasmid (366).

Table 1.1. Main plasmid and chromosomal virulence genes of *S. flexneri*

Gene location	Proteins	Virulence role
Virulence plasmid	IpaA	Vinculin recruitment in host cell cytosol (114).
	IpaB	Macrophage apoptosis, epithelial cell entry and vacuole escape (109).
	IpaC	Assists in epithelial cell entry and vacuole escape (219).
	IpaD	Regulates Ipa secretion and assists in insertion of IpaB and IpaC into cell membranes (293).
	IpaH	Assists in escape from macrophage vacuole (91).
	IcsA	Intra- and intercellular spread (25).
	IcsB	Secreted by type III secretion system, involved in post-invasion step (269).
	Mxi/Spa	Encodes the type III secretion system required for cell entry (328).
	VirB	Regulation of virulence plasmid proteins (21).
	VirF	Regulates VirB (368).
	VirK	Regulates IcsP (409).
	IpgD	Assists in bacterial cell entry (281).
Chromosome	ShET-1, ShET-2	Enterotoxins (253).
	LPS biosynthesis genes	Invasion, inflammation and host resistance (132).
	VacB	Regulation of Ipa proteins and IcsA (367).
	VacC	Regulation of Ipa proteins (81).
	VacJ	Required for intercellular spread (358).
	KcpA	Regulation of IcsA (412).
	Fur	Regulates acid resistance and iron acquisition genes (270).
	VirR	Regulates VirF (134).

1.3 Pathogenesis of S. flexneri

The pathogenesis of *S. flexneri* is complex, requiring penetration of the colonic epithelium, cytokine-mediated inflammation and necrosis of the mucosa. The bacteria are ingested and must pass through the high acidity of the host's stomach in order to reach the site of invasion, the large intestine. It is believed that the ability of *S. flexneri* to survive the acid shock of the gastric fluid, contributed by at least two acid resistance pathways, is essential for the low infectious dose of *S. flexneri,* which is several log lower than *Salmonella* and *Vibrio cholerae* (105, 202). The process of colonic invasion and destruction is outlined in Figure 1.1 and is discussed stepwise in the following sections.

Figure 1.1 Pathogenesis of *S. flexneri* (Figure adapted from Jennison and Verma, 2004).

1. Lumenal bacteria invade the colonic epithelial layer by three known mechanisms. *S. flexneri* can manipulate the tight junction proteins expressed by epithelial cells, allowing paracellular movement of bacteria into the sub-mucosa.

2. PMN cells recruited by IL-8 and IL-1β produced in response to *S. flexneri* invasion create gaps between epithelial cells, through which *S. flexneri* can transmigrate into the sub-mucosa.

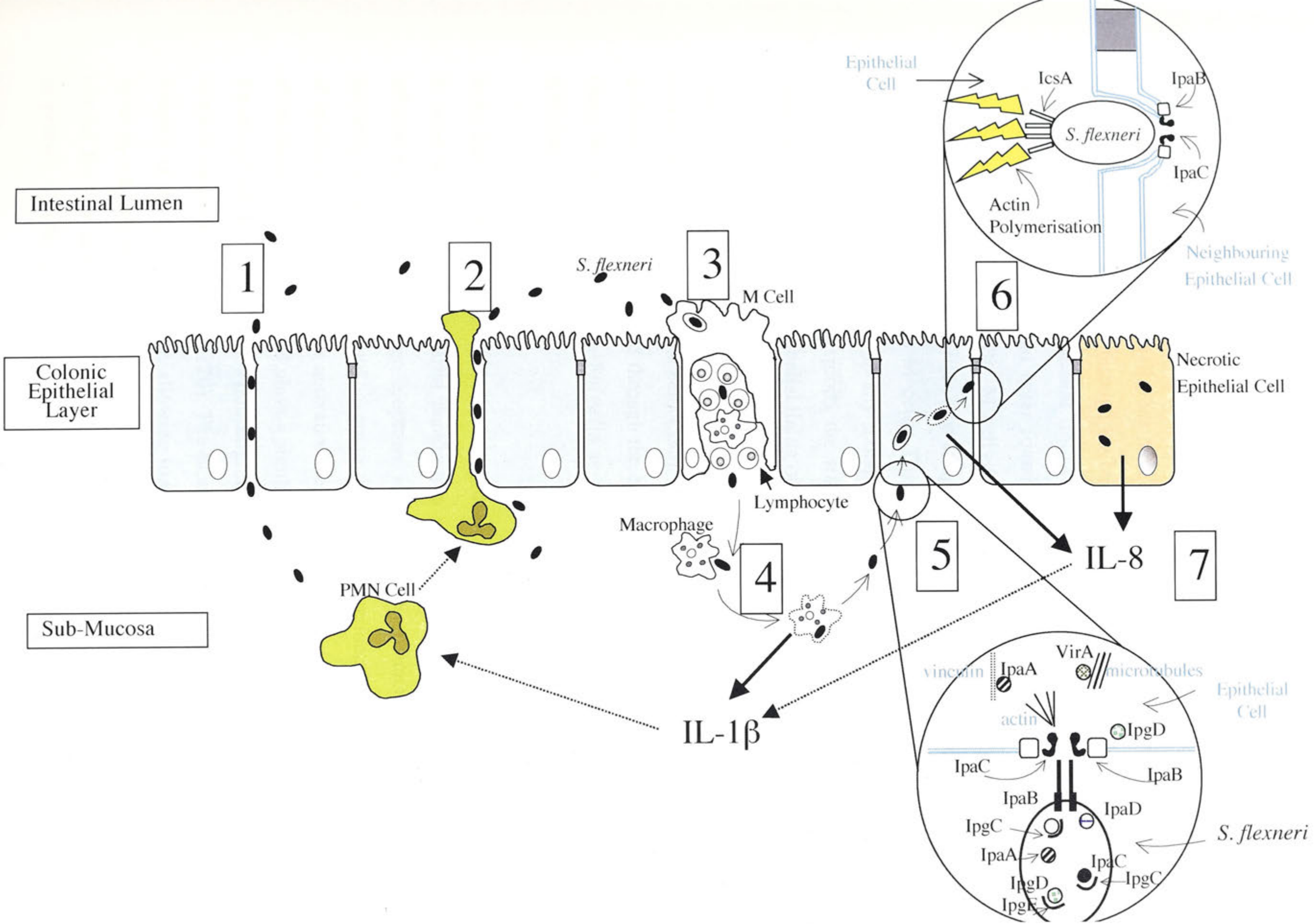
3. Endocytic M cells transcytose bacteria, releasing them into an intraepithelial pocket filled with B and T lymphocytes and macrophages.

4. Macrophages phagocytose the bacteria. *S. flexneri* escapes the phagosome and induces the macrophage to undergo apoptosis. The apoptotic macrophage releases IL-1β.

5. Sub-mucosal *S. flexneri* contact the basolateral membrane of epithelial cells, activating secretion of proteins through their type-III secretion system. Proteins chaperoned in the cytosol of *S. flexneri* are secreted into the epithelial cell's cytoplasm through a pore formed by IpaB and IpaC. IpaC polymerises actin, IpgD dissociates the plasma membrane from the actin cytoskeleton, VirA destabilises microtubules and IpaA forms a complex with vinculin, depolymerising actin. This results in an entry foci forming around the bacterium, driving the epithelial cell to take up *S. flexneri* into a vacuole.

6. IpaB and IpaC lyse the vacuole, releasing *S. flexneri* into the epithelial cell's cytoplasm. The *S. flexneri* protein, IcsA is displayed on only one pole of the bacterium, creating a polymerised actin tail behind the bacterium. This propels *S. flexneri* through the cytoplasm until it contacts the plasma membrane, the force of the contact creates a protrusion into the neighbouring epithelial cell. Both membranes are lysed by IpaB and IpaC, releasing S. *flexneri* into the neighbouring epithelial cell.

7. Intracellular *S. flexneri* induces the epithelial cell to release IL-8. IL-8 and the IL- 1β released from apopotic macrophages are chemotactic to PMN cells (represented by dotted arrows), attracting and inducing them to migrate through the epithelial layer to the lumen. This epithelial disruption amplifies *S. flexneri* invasion of the epithelial layer.


Intestinal Lumen
Colonic Epithelial Layer
Sub-Mucosa
1
2
3
4
5
6
7
S. flexneri
M Cell
Lymphocyte
Macrophage
PMN Cell
IL-1β
IL-8
Necrotic Epithelial Cell
Epithelial Cell
IcsA
IpaB
IpaC
S. flexneri
Actin Polymerisation
Neighbouring Epithelial Cell
VirA
vinculin
IpaA
microtubules
Epithelial Cell
actin
IpgD
IpaC
IpaB
IpaB
IpaD
IpgC
S. flexneri
IpaA
IpaC
IpgD
IpgC
IpgE

1.3.1 Crossing the colonic epithelial layer

The epithelial layer acts as a barrier to pathogens found in the gut lumen (212). Experiments using polarised cell lines have demonstrated that the majority of *S. flexneri* epithelial cell invasion occurs through the basolateral pole of colonic epithelial cells (242). *S. flexneri* penetrates the epithelial lining through the follicular associated epithelium (FAE), which is the epithelial layer found above the mucosa-associated lymph nodes containing the highly endocytic M cells (330). M cells sample and transport lumenal antigen across the epithelial barrier, liberating it into an intraepithelial pocket formed by the basolateral membrane of the M cells. This pocket is occupied with lymphocytes and macrophages waiting to take up any transported antigen and initiate a mucosal immune response (248). *S. flexneri* exploits the transcytotic properties of M cells in order to penetrate the impermeable epithelial lining of the mucosa (393).

Shigella can induce their own uptake by M cells, inducing the same membrane ruffling seen in the invasion of epithelial cells (330). Once internalised in the endocytic vacuole, the bacteria are rapidly transported through the cell and released into the intraepithelial pocket. Once the FAE is penetrated, *Shigella* is able to access the basolateral membrane of epithelial cells.

In the later stages of *Shigella* infection, shigellae exploit the host's inflammatory response in order to amplify the bacterial penetration of the colonic epithelium. Macrophages infected with *S. flexneri* undergo apoptosis, releasing large amounts of IL-1, which recruits polymorphonuclear (PMN) leuokocytes to the site of infection. Furthermore, the invasion of epithelial cells by *Shigella* activates the transcription and secretion of IL-8. IL-8 is chemotactic for PMN cells and plays a significant role in the recruitment of PMN cells to the subepithelial area, where they transmigrate through the epithelial lining to the gut lumen to reach bacteria (Figure 1.1) (20). The passage of PMN cells to the lumen disrupts the integrity of the epithelial layer, allowing lumenal bacteria to access the subepithelial area by an M-cell independent mechanism (284). This inflammatory recruitment of PMN cells is crucial for the development of the inflammation and tissue destruction typical of shigellosis. The blocking of IL-1 and IL-8 in *Shigella* infection of rabbit ligated-intestinal-

loops almost abolishes inflammation, cellular destruction and notably decreases bacterial invasion (325, 327). Additionally, the blocking of CD18, an adhesion molecule used by PMN cells during migration, in the same animal model, also diminished tissue damage and bacterial invasion (284). However, although the PMN-mediated interruption of the barrier function of the epithelial layer promotes the local spread of *Shigella*, the same PMN cells appear to be responsible for restricting infection to the submucosa and preventing systemic dissemination (327). Recent research has also revealed that *S. flexneri* may also be able to penetrate the colonic epithelium independently of both M cells and PMN cells by manipulating the tight-junction associated proteins of human intestinal epithelial cells, allowing bacterial paracellular movement through a model intestinal barrier (Figure 1.1) (320).

1.3.2 Macrophage apoptosis

Once released into the intraepithelial pocket of the M cell, *Shigella* are engulfed by resident macrophages, possibly by a bacterial-driven macropinocytic event similar to *Shigella* entry of epithelial cells (180). *S. flexneri* evades the killing mechanisms of the macrophage by IpaB-mediated lysis of the phagocytic vacuole, where the membrane lysing properties of the IpaB invasin allows the bacteria to gain free access to the cytoplasm (127). Once in the macrophage cytosol, secreted IpaB binds and activates caspase-1, a member of the pro-apoptotic cysteine proteases (52). Caspase-1 dependant apoptosis is not an immunologically silent death, as activated caspase-1 cleaves and activates the pro-inflammatory cytokines IL-1β and IL-18 (71). There is a level of discrepancy in *Shigella* macrophage studies with a number of recent reports suggesting that the macrophages are not undergoing apoptosis but are actually being destroyed by necrosis (110, 172, 254). However, it has been suggested that necrosis and apoptosis are not independent mechanisms but may be a common pathway, which may explain the confusion over the exact mechanism of *Shigella* induced death of macrophages (164). Macrophage cell death occurs within four hours of *in vivo Shigella* infections, releasing bacteria into the sub-mucosa, proximal to the basolateral membrane of epithelial cells (427).

1.3.3 Adhesion to the basolateral membrane of colonic epithelial cells

S. flexneri preferentially invades epithelial cells through their basolateral membrane (168). The apical membrane of colonic epithelial cells is covered with glycolipids, which form a mucin layer (212). This layer may act as a physical barrier, preventing *S. flexneri* from gaining access to the apical membrane, interfering with the type-III secretion system delivery of the invasion plasmid antigens (Ipa), crucial for *Shigella* invasion of epithelial cells (260). Furthermore, the basolateral membrane of epithelial cells appear to display proteins utilised by *S. flexneri* as cell adhesion receptors; the Ipa proteins interact with the α5β1 integrin, a basolateral receptor, which binds the extracellular matrix located beneath the epithelium and IpaB is also capable of binding the CD44 receptor, a major cell surface receptor for hyaluronic acid found on the basolateral membrane of epithelial cells (6, 345, 394). As both α5β1 integrin and CD44 are cytoskeleton linkers it is possible that on the binding of *S. flexneri*, they may contribute to the cytoskeletal reorganisation seen during epithelial cell invasion (372). More recently, bacterial adherence to epithelial cells was shown to be dependant on the length and the presence of the O-antigen (168). A number of cell receptors capable of binding lipopolysaccharide (LPS) have been characterised including CD14 and the Toll family of receptors (TLRs), all of which are located on the basolateral membrane of epithelial cells (2, 139).

1.3.4 *S. flexneri* uptake by the epithelial cell

Shigella cells use a special means to invade epithelial cells, called the "trigger mechanism of entry", which requires a macropinocytic process where *S. flexneri* induces rearrangements of the host cell cytoskeleton to engulf bacteria in vacuoles (268). The type III secretion system is activated by contact of the bacterium with the epithelial cell, releasing IpaA, IpaB, IpaC and IpaD, which are the effectors of bacterial entry into the host cell. The *mxi-spa* operon, which encodes the type III secretion system and IpaB, IpaC and IpaD are essential for *in vitro* epithelial cell invasion (229, 333).

The exact mechanism by which the Ipa proteins generate *Shigella* entry into epithelial cells is not entirely understood. The Ipa proteins are synthesised and stored within the bacterium, where they are associated with chaperone proteins until secretion of the type III system is

activated (231, 277). An IpaB-IpaD complex may regulate this secretion by acting as a "plug" in the type III needle (230). Upon activation, large pools of IpaB and IpaC are rapidly released through the needle and the N-terminus of IpaC binds to IpaB forming a complex (84, 119). As both proteins are hydrophobic, the complex can insert into the epithelial cell membrane and form a pore. It is assumed that the other effector molecules, delivered by the type III secretion secreton, access the host cytoplasm through this pore (32). The C-terminal domain of IpaC activates the host cell Rho GTPases, triggering actin polymerisation and filopodial extensions of the epithelial cell membrane in the vicinity of the bacteria (371). IpaA-vinculin complexes depolymerise the actin filaments, forming an entry foci around the bacterium (33, 370). IpgD is injected into the cell, where it acts as a phosphoinositide phosphatase, uncoupling the plasma membrane from the actin cytoskeleton, creating membrane extensions (250). A interaction of VirA with host cell tubulin destabilises microtubules around the bacterial site of entry, which may stimulate Rac1, a Rho family GTPase, creating lamellipodial extensions in the host cell membrane (417). The cytoskeletal rearrangements induced by the *Shigella* effector proteins results in bacterial cells being internalised by the epithelial cell within a micropinocytic vacuole (Figure 1.1).

1.3.5 Replication within the epithelial cell

The macropinocytic vacuole containing an internalised *Shigella* cell is rapidly lysed by the IpaB invasin, which acts as a membranolytic toxin inserting into the phagosome membrane, releasing the bacterium into the host cell cytoplasm (127). The lysis of the phagosome may also require IpaC, which contains hydrophobic regions capable of inserting into and disrupting phospholipid membranes (65). *S. flexneri* can replicate inside the cytosol of cultured epithelial cells with a doubling time of forty minutes. Epithelial cells undergo necrosis during *S. flexneri* infection, and although it was originally thought that this was caused by *Shigella* multiplication within the cytosol, it now seems more likely that the epithelial cells are being destroyed by the host's inflammatory response (285). The nutrient and growth requirements of *Shigella* during multiplication within epithelial cells are not well understood. However, it is known that aromatic mutants of bacteria are unable to replicate within eukaryotic cells indicating that synthesis pathways are important and that a

number of metabolites must not be readily available to bacteria intracellularly; likewise iron transport systems are essential for the intracellular survival of *Shigella* (157, 280).

1.3.6 Intra- and intercellular spread of *S. flexneri* within epithelial cells

Shigella is able to exploit the host cell's actin assembly machinery to move through the cytoplasm and into neighbouring epithelial cells (Figure 1.1). This intra- and intercellular spread is a crucial step in the virulence of *S. flexneri* and is driven by the outer membrane protein, IcsA (25, 186). IcsA is expressed in an unipolar fashion on the bacterial outer membrane, with the greatest concentration localised to the old pole of the bacterium (103). Newly synthesised IcsA appears to be targeted to the old pole by protein targeting interactions with two internal regions of IcsA and unknown protein partners (49, 350). These targeting partners may help direct IcsA to cross the cytoplasmic membrane through the Sec transport system at the old pole (35). IcsA appears to autotransport itself across the outer membrane by inserting its C-terminal β-domain into the membrane to form a barrel-shaped pore (35).

The maintenance of the unipolar localisation of IcsA is essential for intracellular movement, and appears to be mediated by both the protease IcsP and the structure of the LPS. IcsP cleaves and inactivates outer membrane IcsA, releasing a 95 kDa N-terminal fragment into the surrounding environment. IcsA appears to diffuse from the old pole once it has inserted into the outer membrane but IcsP cleavage appears to occur more slowly than the display of IcsA on the old pole, therefore, IcsP cleavage acts to "sharpen" the unipolar gradient by inactivating diffusing IcsA (350) Furthermore, mutants of *S. flexneri* missing the O-antigen, expressing a partial O-antigen structure or lacking a modal distribution of O-antigen chain length display non-polar localisation of IcsA and are consequently reduced in their intra- and intercellular spread (322, 323, 382). It is possible that the LPS maintains IcsA polarity by forming interlocking microdomains with the O-antigen side chains on the outer membrane, preventing IcsA from diffusing away from the old pole (313). Another study has suggested that *S. flexneri* must display two O-antigen chain lengths, with each contributing differently to the virulence of the strain; short chains that do not shield IcsA and long chains that confer resistance to serum (239).

Polar IcsA interacts with the host protein, neural Wiskott-Aldrich syndrome protein (N-WASP) and possibly with vinculin (356, 359). IcsA specifically binds N-WASP, stimulating the actin-related protein (Arp) 2/3 complex-mediated actin polymerisation. This ligand specificity for N-WASP, and not other WASP family proteins, may be responsible for determining which cells allow *Shigella* to use actin-based motility (357). The actin polymerisation at the old pole of the bacterium creates propulsive force, driving the bacterium through the cell until it contacts the host cell membrane, forming a protrusion into the neighbouring epithelial cell (237). The protrusion is actively endocytosed by the neighbouring cell in a myosin light chain kinase and cadherin dependant manner (307, 329). The bacteria can lyse the two cellular membranes with type III-secreted IpaB, IpaC and VacJ, releasing it into the cytoplasm of the neighbouring cell (276, 358). Thus, in this manner, *S. flexneri* is able to replicate and spread within the intestinal epithelial layer whilst avoiding exposure to the extracellular environment and its circulating immune cells.

1.4 O-antigen modification

Lipolysaccharides (LPS) are found on the outer membrane of gram-negative bacteria (309). *S. flexneri* LPS is an important virulence factor required for the resistance to hosts defences, intercellular spread, the induction of inflammation and possibly in the bacterial entry of epithelial cells (20, 168, 309). The O-antigen is a polysaccharide chain of repeating units that extends from the core of the LPS and is used to serologically classify *S. flexneri* into thirteen serotypes. More recently, an additional serotype, 1c, has been detected in a number of countries (83, 362). The basic structure of the *S. flexneri* O-antigen is known as serotype Y and the addition of glucosyl residues and/or O-acetyl groups creates the serotype-specific structure of each serotype's O-antigen (9). The genes encoding the enzymes responsible for the biosynthesis of the basic O-antigen are found in the *rfb*/*rfc* loci in the *S. flexneri* chromosome (408). However, the factors involved in the serotype-specific modification of the O-antigen are located within serotype-converting bacteriophages capable of lysogenising *S. flexneri*. The modification is carried out by a three-gene glucosyltransferase locus, *gtrA*, *gtrB* and *gtr* (*type*) (9). As the O-antigen is highly immunogenic and a major target of the host's immune response, altering the O-antigen structure can assist in the avoidance of host defences by *S. flexneri* (391). In natural infection the majority of the host's immune response appears to be directed against the O-antigen as immunity is

serotype-specific. Furthermore, the different structures of the O-antigen may also play a role in the virulence of the strain, with some serotype-specific structures possibly optimising the virulence of *S. flexneri* (406).

1.5 Virulence assays for S. flexneri

Shigellosis is a disease of humans and higher primates. Smaller animals are resistant to shigellosis, possibly because their intestinal microflora prevent *Shigella* colonisation (213, 361). Consequently, there is no simple intestinal animal model available, particularly as the use of monkeys is expensive and ethically questionable. A number of alternative animal models have been developed, which use mucosal surfaces other than the colon, including the Sereny test in guinea pigs and the rabbit-ligated ileal loop model (285, 339). The Sereny test consists of inoculating a suspension of bacteria into the kerato-conjunctival sac of a guinea pig. Virulent *Shigella* are able to invade the conjunctival epithelium causing conjunctivitis (291). The rabbit-ligated ileal loop model requires sections of rabbit ileal to be externalised, so it can be inoculated with large amounts of bacteria and the degree of subsequent inflammation measured (393). Mice can be used for *Shigella* vaccine research by utilising the mucosal surface of the lung in the pulmonary model. Although again the organ specificity is not accurate, intranasally delivered *Shigella* is able to invade the tracheo-bronchial tract resulting in an inflammatory broncho-tracheo-alveolitis, activating both systemic and local immune responses (215, 387). Furthermore, immunised mice can be challenged in this model and the protective efficacy can be scored by measuring the percentage of survival (381).

Cell culture assays have also proved extremely useful in the study of the host-cell invasion steps of *Shigella*. Studies have traditionally used the non-polarised epithelial cell line, HeLa. However, it has been determined that the kidney epithelial cell line, BHK, is ideal for plaque assays, as the plaques produced in this cell line are both clearer and larger than those produced in HeLa cells (233). The plaque assay measures the initial cellular invasion of epithelial cells, and also the intra- and intercellular spread (266). The invasion assay and intracellular survival assay are used to study the degree of bacterial entry and bacterial intracellular survival and replication, respectively (111, 203). All these cell culture assays are dependant on the use of gentamycin, which is membrane impermeable, allowing

intracellular bacteria to survive antibiotic treatment of the infected monolayer. Thus, the levels of intracellular bacteria can be determined without extracellular bacteria interfering with bacterial counts (291). It is through the use of these and other virulence techniques that it has been possible to piece together the pathophysiology of shigellosis and assess the initial suitability of *Shigella* vaccines. Furthermore, the application and further development of these methods will continue to contribute to the knowledge of *S. flexneri* infection and immunity.

1.6 Acid resistance pathways of S. flexneri *2457T*

The pathogenesis of shigellosis is dependant upon the bacterium's ability to invade and colonise the gastrointestinal tract. However, in order to do so, *S. flexneri* must pass through the acidity of the human stomach, which can be at a pH < 3 (99). Additionally, enteric bacteria must survive low pH stress external to the host such as that encountered during passage through the environment, in acidic foods and from fermenting acidified faecal material (26, 202). The ability of *S. flexneri* to survive these low pH levels appears to be an important pathogenic characteristic and may be a contributing factor to the low infectious dose of 10-100 organisms required for shigellosis to occur (105).

The *in vitro* study of bacterial acid resistance measures stationary phase bacterial survival after 2 hours of acid exposure at pH 2.5, where an inoculum survival ≥10% is considered resistant (105). It is believed that these conditions mimic the pH and gastric emptying time of a normal fasting stomach (105). Bacteria able to survive this length of acid exposure are theoretically capable of breaching the gastric acid barrier, and able to reach the more favourable environment of the intestine.

Acid resistance studies have revealed that *S. flexneri* has at least two acid resistance pathways detectable in *in vitro* studies (202). These include the little understood oxidative pathway, which requires oxygenated growth and is repressed in the presence of glucose, and the well-characterised glutamate dependant acid resistance pathway (GDAR). The components of the oxidative pathway are unknown, although it has an absolute requirement for the sigma factor, RpoS, and recently, the iron regulator, Fur, has been discovered to be important (270, 397).

The GDAR pathway consists of two interchangeable glutamate decarboxylase enzymes, GadA and GadB, and an antiporter, GadC (126, 396). The system acts to reverse the membrane potential of the bacterial cell when in an acidic environment, creating an internal positive charge and alkalising the extracellular medium by converting glutamate to gamma-aminobutyric acid (GABA). During this conversion, a proton is incorporated into GABA. The GABA is antiported from the cell by GadC in exchange for more glutamate, so that *Shigella* is effectively removing protons from the cytoplasm, preventing acidification of the cell. The complex regulation of the GDAR pathway has been described in *E. coli* (211, 220).

1.7 The host's immune response to S. flexneri

1.7.1 Innate immunity

The severe inflammation generated by shigellosis can persist in the gut for over a month, with a general up-regulation in a number of cytokines, including IL-1, TNF-α, IL-6, IFN-γ, TNF-β, IL-4, IL-10, TGF-β and IL-8 (304). Although some of the clinical symptoms of shigellosis may actually be a direct consequence of the cytokines, they are also important for the control and containment of the infection.

Resident macrophages and infiltrating monocytes are not able to efficiently kill *S. flexneri* inside their phagosomes, and instead succumb to an apoptotic-like cell death (123, 426). The IL-18 released by the apoptotic macrophages activates natural killer (NK) cells and T cells to produce IFN-γ (28). IFN-γ plays an essential role in the early protection against *S. flexneri*, as it activates macrophages and fibroblast cells. IFN-γ-treated activated macrophages actually have increased bactericidal activity against *S. flexneri*, promote bacterial clearance and possibly inhibit bacterial replication within epithelial cells (184, 399). IFN-γ deficient mice are five times more susceptible to *S. flexneri* infection (399).

The *S. flexneri* protein, OspG, is secreted by the type III secretion system and may interfere with the activation of NK-κB in epithelial cells (163). The NK-κB pathway activates the production of chemostimulatory molecules such as IFN-γ and IL-8 (15). Thus, *Shigella*

may be attempting to suppress the early inflammatory response in order to safely invade the intestinal epithelium and suppress the activation of macrophages (163).

Paradoxically, it is the massive inflammation induced by the IL-8 released from infected epithelial cells and the IL-18 from apoptotic macrophages that assists in the *Shigella*-mediated epithelial destruction and acts to amplify the number of *Shigella* cells able to penetrate the intestinal mucosa, by the recruitment of PMN cells to the site of infection. The PMN cells transmigrate through the tight junctions of the epithelial cells, destroying the impermeability of the epithelial lining by generating intercellular gaps (Figure 1.1). Lumenal *S. flexneri* can cross the epithelial layer via these gaps, resulting in a massive influx of bacteria into the submucosa (284, 285). However, *Shigella* are unable to escape the phagocytic vacuole of the PMN cells and are killed within the phagosome by human neutrophil elastase (NE) (217, 404). Thus, PMN cells ultimately play a crucial role in the control of *Shigella* infection, confining extracellular bacteria to the mucosa preventing deeper tissue invasion and systemic spread (420).

Other innate defence mechanisms that can protect against *Shigella* include the glycoprotein lactoferrin, which is present in mucosal secretions and impairs the ability of *S. flexneri* to invade epithelial cells by exposing IpaB-IpaC complexes to protease degradation, through the disruption of the bacterial surface (104).

1.7.2 Cellular immunity

Very little is known about the host's cellular immune response to *S. flexneri*, especially in comparison to other intracellular bacteria. Studies have shown increased T cell activation in shigellosis patients, and T cells have been isolated that proliferate *in vitro* in response to *S. flexneri* antigen (141-143). The cytokines detected in vaccines studies are suggestive of both Th1 and Th2 lymphocyte responses (176, 381). A recent study has found that the induction and maintenance of inflammatory cytokine production actually depends on the presence of lymphocytes, including T cells (184). The ShiA protein of *S. flexneri* has been identified as capable of suppressing the T-cell response to *S. flexneri* in mice, suggesting that it may be used by the bacterium to reduce the T cell response during *Shigella* infection (140). Furthermore, the increased susceptibility of AIDS patients, deficient in $CD4^+$ T cells,

to shigellosis suggests that cell-mediated immunity may play a role in protective immunity against *Shigella* (247).

However, in contradictory evidence, a study attempting to determine the contribution of T cells to the host's protective immunity against *Shigella*, vaccinated mice deficient in T cells against *S. flexneri*. These mice were suitably protected against challenge with wild type *S. flexneri*, despite their deficiency in T cells, suggesting that even though T cell responses develop against *Shigella*, they are not essential for protection (401).

1.7.3 Humoral immunity

Numerous serological studies of infected humans and animal model experiments have generated information on the host's humoral response against *S. flexneri* (197, 198, 262, 264). The data suggests that the humoral arm of the immune response is the major constituent of the protective immunity against shigellosis, with both the systemic and mucosal components activated against the LPS and some proteins, particularly those encoded by the virulence plasmid (57, 142, 267).

The serotype-specific structure of the LPS is assumed to be a major target of the host's antibody response, as natural and experimental infections with *S. flexneri* confer serotype-specific immunity, where previous infection or vaccination offers little to no protection against heterologous serotypes (79, 96, 227). However, there is some cross-reactivity between antibodies directed against epitopes shared between O-antigen structures, indicating that it may be possible to induce a protective immune response against multiple serotypes simultaneously (379). The overall significance of the antibody response to *S. flexneri* infection has been demonstrated in a study that showed that the reduced and delayed humoral response in children, in comparison to adult patients, is the likely cause of the increased susceptibility of children to shigellosis (305).

Secretory IgA (sIgA), especially anti-LPS IgA has been implicated in the generation of immunity against *S. flexneri* and has been detected in humans suffering natural shigellosis in a number of studies (142, 267, 306). sIgA is made up of two IgA units and two polypeptides, the J chain and the secretory component (SC). sIgA transcytoses into the

lumenal cavity of the intestine, where the secretory component binds the mucosal coating of the epithelial cells and forms an antibody shield over the cells (289). The outer membrane of lumenal bacteria can also become coated with IgA, blocking invasion by preventing their attachment to mucosal surfaces. Furthermore, IgA can mediate antibody-dependant cellular cytotoxicity and interfere with the bacterial utilisation of growth factors (138). The anti-LPS sIgA produced in the breast milk of *S. flexneri* infected mothers may be responsible for the decreased severity of shigellosis in *Shigella*-infected infants. Additionally, the implantation of an anti-LPS sIgA hybridoma on the back of mice protected them against intranasal challenge with a lethal dose of *S. flexneri* (290).

Serum antibodies IgG and IgM raised against the LPS and virulence plasmid antigens are detected in natural human infections, despite shigellosis generally being a localised mucosal infection (57, 262, 267, 380). Anti-LPS IgG and possible IgM appears to play a protective role in *Shigella* immunity produced in mice studies. The challenge of IgA deficient vaccinated mice demonstrated that in the absence of an IgA response, the mice were still fully protected against *S. flexneri* infection (400). Furthermore, mice deficient in T cells were protected from wild-type *S. flexneri* challenge by a predominately anti-LPS IgM response (401). It is still unclear what role serum antibodies are playing in the generation of protective immunity but it seems that they may be stimulating complement killing of the bacteria or mediating antibody-dependant cellular cytotoxicity in the mucosal area (58, 209, 308). However, it must be noted that studies on the protective role of serum antibodies in *S. flexneri* infection has predominately focused on mice and warrants further investigation, particularly as some human vaccination data suggests that the parenteral stimulation of serum Ig may not correlate with protection (16, 22, 59).

1.8 Vaccine development for S. flexneri

Despite over sixty years of research into the production of a safe *Shigella* vaccine, there is to date, no vaccine available commercially. The cost of treating shigellosis in the developing world with antibiotics is unrealistic and the emerging antibiotic resistances in *S. flexneri* are adding increased strain on the health services of these areas. An ideal vaccine for shigellosis should activate the immune response in a long lasting manner, must be cheap to manufacture, simple to administer and ideally would simultaneously offer protection

against multiple serotypes (204). A variety of vaccine strategies have been undertaken for *S. flexneri* vaccine development and are reviewed in the following sections.

1.8.1 Subunit vaccines

It is possible that subunit vaccines may avoid the safety issues associated with live vaccines. Subunit vaccines are advantageous as they allow selective display of just a few antigens to the immune system, essentially directing the immune response to the most protective targets of a pathogen. Furthermore, subunit vaccines utilise adjuvants, which can be used to enhance the strength and duration of the immune response and can manipulate the types of cell-mediated and antibody responses produced (218).

S. flexneri LPS complexed to proteosomes, delivered intranasally to humans, can induce a serotype-specific immune response (97). *S. flexneri* LPS has also been linked to carrier proteins and delivered parentally to volunteers (11, 56). This vaccine was safe in humans and induced a strong serum antibody response and is currently in Phase 3 clinical trials (189). Other subunit vaccines are yet to be evaluated in humans. A parenteral ribosomal vaccine was capable of generating protective immunity in guinea pigs and monkeys (187). However, the immune response is directed against the O-antigen polysaccharides purified with the ribosomal preparations, and the O-antigen content varies between ribosomal preparations, making a consistent vaccine difficult to manufacture (188). A purified complex of *S. flexneri* antigens, named Invaplex, which contains IpaB, IpaC, IpaD and LPS has shown promise in animal models, where intranasal immunisation induced significant protection in challenged mice and guinea pigs (265). It is possible that by developing a subunit vaccine with common *S. flexneri* proteins, the vaccine may be protective against multiple serotypes simultaneously.

1.8.2 Killed oral vaccines

Early challenge experiments revealed that orally administered acetone-killed and dried *Shigella* was unable to protect monkeys from infection (95). More recently, an oral heat-killed *S. flexneri* vaccine evaluated in a rabbit model was found to be 100% protective (48). In a further study, the same vaccine was able to confer significant protection to immunised

guinea pigs (244). However, it is yet to be established if orally delivered killed vaccines are of any use in protecting humans from *S. flexneri* infection.

1.8.3 Non-invasive live vaccines

Non-invasive live vaccine strains have been constructed for *S. flexneri* by disrupting genes in either the chromosome or virulence plasmid. Most of these strains are safe in humans and can produce some degree of protective immunity in volunteers (Table 1.2). Probably the most successful of these vaccines is the *S. flexneri* 2a Istrati T_{32} strain, which is 100% safe in humans and provides up to an 85% protective efficacy. However, it must be administered in large multiple doses, which is labour intensive, expensive and impractical for developing countries (226).

Table 1.2. Live non-invasive *S. flexneri* vaccines that have been assessed in monkeys or humans. Adapted from Jennison and Verma, 2004.

Vaccine	Description	Safety	Efficacy	Comment	Ref.
S. flexneri 2a 2457O	Spontaneous avirulent mutant, *virF* inactivated by insertion.	Reverts to virulence in humans.	Monkeys are protected. Caused dysentery in 34% of human volunteers.	Reactogenic in humans.	(78, 95, 234)
S. flexneri 2a Istrati T_{32}	Spontaneous deletion of *ipaBCDA*, *invA* and *icsA* from the virulence plasmid.	Safe in humans, mild adverse effects in very few volunteers at 2×10^{11} cfu	Around 80% protection in humans when administered in 5 doses.	Protection lasts 6 months. Reduces attack rate of heterologous *Shigella* serotypes.	(226, 384)
S. flexneri streptomycin dependant strains	Spontaneous mutants incapable of growing in the absence of streptomycin.	Reversion to streptomycin independence in volunteers. Diarrhoea and vomiting in 15 -35% of volunteers (5×10^{10} cfu).	Up to 90% protection in field trials with multiple doses. US trials found marginal protection.	Unstable phenotype and inconsistent protective efficacy.	(79, 190, 228)

1.8.4 Invasive live vaccines

Vaccine strategies using invasive live *Shigella* vaccine strains are increasingly being investigated, as an invasive strain is capable of delivering antigen to the mucosal immune system, provoking a strong immune response. As the genetic understanding of *S. flexneri* virulence has improved, so have the strategies for constructing safe invasive vaccine

strains. Invasive vaccines are generally attenuated by mutations in either virulence genes necessary for pathogenesis after cell entry, or in metabolic genes that prevent the bacteria from replicating and spreading in the host after invasion.

Mutations in either IcsA and/or in a variety of metabolic genes have produced attenuated vaccine strains, which are safe and capable of 100% protection when delivered in multiple doses in monkeys (Table 1.3). A number of auxotrophic strains, some also carrying mutations in virulence genes, have been assessed for their safety and immunogenicity in human volunteers in clinical trials (158, 175, 176, 196). These strains vary in their levels of attenuation and immunogenicity (Table 1.3).

The CVD attenuated vaccines have demonstrated promising immunogenicity and minimal reactogenicity. The latest CVD vaccine, CVD 1208, contains auxotrophic mutations as well as deletions in the *icsA* virulence gene and the two enterotoxin genes, and is presently undergoing clinical evaluation (100, 177). Furthermore, the *S. flexneri* 2a vaccine strain, SC602, has proceeded to Phase 2 clinical trials (62). This strain has deletions in *icsA* and the aerobactin *iuc* locus, which is involved in iron transport. SC602 is capable of generating protection to immunised humans challenged with wild-type *S. flexneri*; however, there is severe reactogenicity observed for volunteers administered a high dose, whilst lower doses do not appear to be sufficiently immunogenic (62, 100). Thus, despite promising results, attenuated live vaccines are proving difficult to design for *S. flexneri* as there is only a slight margin between the level of attenuation required for immunogenicity and that needed to prevent reactogenicity.

1.8.5 Hybrid vaccines

E. coli vaccine candidates have also been used to develop hybrid vaccines expressing *Shigella* antigens. Early attempts with *Shigella-E. coli* hybrid vaccines developed invasive vaccines that either caused symptoms in human volunteers or were not protective (87, 192). Strains of *E. coli* K12 engineered to carry the group and type antigens of *S. flexneri* 2a and the *S. flexneri* 5a virulence plasmid were unable to induce significant protection in immunised volunteers (173, 174). Alternatively, *S. flexneri* candidate vaccine strains can be used to express the O-antigen structures of other *Shigella* species. The *S. flexneri* 2a

Vaccine	Description	Safety	Efficacy	Comment	Ref.
S. flexneri 5a SC560	Deletion in *icsA*.	Mucoid diarrhoea and mild clinical symptoms in some monkeys with 5×10^{10} cfu.	100% protective in monkeys after 3 doses.	Slightly reactogenic in monkeys.	(326)
S. flexneri 5a SC433	Deletion in *envZ* and *ompR*.	Mucoid diarrhoea and mild clinical symptoms in some monkeys with 5×10^{10} cfu.	100% protective in monkeys after 3 doses.	Slightly reactogenic in monkeys.	(326)
S. flexneri 5a SC445	Deletion in *envZ*, *icsA* and *ompR*.	100% safe in monkeys at 5×10^{10} cfu.	One out of five monkeys challenged became sick.	Level of attenuation may be too severe for consistent protection.	(326)
S. flexneri 2a vc77	Auxotrophic for purine (Pur^-) and rifampicin resistant.	No symptoms in adults or children up to 3×10^9 cfu.	Dysentery in 2 out of 4 adults challenged.	Poor protective capabilities.	(70, 205, 206)
S. flexneri Y TSF-21	Thymine requiring (Thy^-) and temperature sensitive (Ts^-).	No symptoms in monkeys at 1×10^{11} cfu.	100% protection in monkeys after 2 doses.	Temperature sensitivity mutation has a high rate of reversion.	(5, 12)
S. flexneri Y SFL114	Insertional inactivation of *aroD*.	No symptoms in monkeys at $2\text{-}3 \times 10^{10}$ cfu.	100% protection in monkeys after four doses.	Some reversion of phenotype observed in laboratory conditions.	(160)
S. flexneri Y SFL124	Deletion of *aroD*.	Very mild symptoms in some human volunteers at 2×10^9 cfu and children at up to 1×10^9 cfu.	100% protection in monkeys after three doses of 1×10^{11} cfu.	Strain is advantageous as it can be converted to new serotypes.	(159, 194-196)
S. flexneri 2a SFL1070	Deletion of *aroD*.	Mild symptoms in volunteers at $1 \times 10^7 - 1 \times 10^8$ cfu. Increased clinical symptoms at 1×10^9 cfu.	About 85% protection in monkeys after 4 doses 1×10^{11} cfu.	Further attenuation may be necessary.	(157, 158)
S. flexneri 2a CVD1203	Deletions in *icsA* and *aroA*.	No symptoms in humans when administered in a single dose of 1×10^6 cfu.	Challenge only performed in guinea pigs, provided 83% protection.	Strain may require further attenuation in order to reduce reactogenicity in humans.	(175, 255, 258)
S. flexneri 2a CVD1207	Deletions in icsA, *sen*, *set* and *guaAB*.	No symptoms in humans when administered in a single dose of up to 1×10^8 cfu.	Challenge only performed in guinea pigs, provided 85% protection.	Immunogenicity in humans may not be sufficient with just one dose.	(176, 256)
S. flexneri 2a SC602	Deletions in *icsA* gene and the *iuc* locus.	Mild diarrhoea and fever in humans at 1×10^4 cfu.	3 out of 7 volunteers challenged experienced mild diarrhoea.	Provides protection to infection with *S. flexneri* 2a. Further attenuation may be required.	(17, 62)
S. flexneri 2a CVD1208	Deletions in *icsA*, *sen*, *set* and *guaAB*	No symptoms in humans when administered in a single dose of up to 1×10^8 cfu.	No challenge performed in humans or monkeys.	Strong antibody response for 1×10^8 cfu dose.	(177)

Table 1.3. Live invasive oral *S. flexneri* vaccines that have been assessed in monkeys or humans. Adapted from Jennison and Verma, 2004

vaccine strain T_{32} carrying a plasmid encoding the *S. sonnei* O-antigen was capable of providing 100% protection against challenge with both *S. flexneri* and *S. sonnei* (316). Similar strategies are being used to produce multiple serotype specific *S. flexneri* vaccines, which are discussed in the following Section.

1.8.6 Multiple serotype protection strategies

The immune response raised against *S. flexneri* is serotype specific, thus vaccination with a live vaccine strain will only provide protection against the homologous serotype. An optimal vaccine for *S. flexneri* would provide protection against all the prevalent serotypes of a particular geographical region. *S. flexneri* serotypes not only share a common O-antigen backbone, but some serotypes also share common type and/or group antigens on their O-antigen (8). As the immune response is primarily directed against the LPS, the antibodies raised against the group or type antigens may be cross-reactive to other serotypes. For example, antibody raised against *S. flexneri* 2a LPS has been shown to cross-react to LPS from serotypes 1a, 2b, 5a and Y, all of which share type or group antigens with serotype 2a (379). It is unclear whether the cross-reactive antibodies can provide protection against infection by a heterologous serotype; further research is required to adequately establish the role of cross-reactive O-antigen epitopes in human immunity against shigellosis.

Animal studies have shown that mixing a number of *S. flexneri* vaccine strains of different serotypes can invoke an immune response with cross-reactive potential. A vaccine cocktail containing serotype 2a and 3a *S. flexneri* strains, administered to guinea pigs, conferred significant protection to challenge by serotypes 1b, 2b, 5b and Y (256). This suggests that by combining a selection of *S. flexneri* serotypes into a vaccine cocktail it may be possible to cross-protect against most *S. flexneri* serotypes. Alternatively, single *S. flexneri* vaccine strains can be engineered to express the O-antigen of more than one serotype. The serotype conversion gene cluster of bacteriophage SfV and the glucosyl transferase gene of bacteriophage SfII, were inserted in tandem into the serotype Y *aroD* vaccine strain, SFL124, to create a strain expressing multiple serotype-specific O-antigen structures on its surface. The resulting strain was able to simultaneously induce a serotype-specific immune response to both serotypes 2a and 5a in mice (148). This strategy could be used to generate

a variety of *S. flexneri* polyvalent vaccine strains, which could be combined into vaccine cocktails designed for specific geographical areas.

1.9 Proteomics

Large-scale DNA sequencing over recent years has resulted in an accumulation of vast amounts of DNA sequences in databases. In this post-genomic era, sequencing of genomes is not sufficient to completely understand the biological functions of organisms. The identification of an open reading frame (ORF) does not necessarily correlate with the production of a functional protein. Thus, the verification of a gene product is an important requirement in the annotation of genomes; this can be achieved by the high-throughput technology of proteomics, which allows the large-scale analysis of cellular protein (392). Furthermore, proteomics allows visualisation of protein modifications that are not apparent from the DNA sequence, such as protein isoforms and post-translational modifications. The protein expression level can also be determined, which is more reliable than studying the mRNA levels that often do not correlate with cellular protein. It is also possible through the use of prefractionation protein extraction techniques to discover the localisation of a protein, which is often difficult to predict from the gene sequence (278). A vast number of bacterial pathogens have been analysed by proteomics including *Heliobacter pylori*, *E. coli*, *Vibrio cholerae*, *Campylobacter jejuni* and *Staphylococcus aureus* (39, 115, 155, 310, 419). The proteome of *S. flexneri* whole cell protein and extracellular protein was published in 2003 (200). Analysis of this proteome confirmed the genomic prediction that *E. coli* and *S. flexneri* are indistinguishable.

A crucial step in visualising a bacterial proteome is obtaining an appropriate protein sample. Proteins in the sample must be denatured, disaggregated and solubilized so as to completely disrupt molecular interactions and to ensure that each spot represents an individual polypeptide. Sample preparation should be as simple as possible to increase reproducibility. Sample solubilisation usually involves resuspension in buffers containing chaotropes, such as urea, to break hydrogen bonds, a reducing agent, such as DTT, to break protein linkages, and a zwitterionic detergent, such as CHAPS, for breaking hydrophobic interactions (107). Alterations can be made to the extraction protocol and solubilisation buffer in order to enrich certain types of proteins (60).

Currently, there is no ideal staining method for proteome analysis, with the most important properties for a stain being high sensitivity, high linear dynamic range, reproducibility and compatibility with post-electrophoretic protein identification procedures. The most common stains include Coomassie blue, silver and fluorescent labelling. Although the main limitation of Coomassie blue staining is its insufficient sensitivity, with no more than a couple of hundred spots visualised on 2-DE gels even with milligrams of sample loaded, it is commonly used as it is readily compatible with protein identification techniques such as matrix associated laser desorption-ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (106). MALDI-TOF MS is the technique of choice for 2-DE as it is sensitive and has the capacity for high-throughput analysis (3).

Proteomics has recently been used for the visualisation of immunoreactive proteins for bacterial pathogens. This technique has been termed immunoproteomics. Instead of staining the 2-DE gels it is possible to electrotransfer the proteins onto PVDF membrane and probe the membrane with patient sera in a Western blot (224). Immunoreactive proteins can be matched to a twin Coomassie-stained gel and consequently identified. This technique has been successful in identifying potential vaccine candidate antigens for a wide range of bacteria including *Staphylococcus aureus*, *Heliobacter pylori* and *Candida albicans* (179, 295, 388). During the course of this thesis, three studies using this approach were published for *S. flexneri* 2457T membrane, soluble and extracellular proteins. These studies used sera raised in immunised mice and rabbits. The antibody response of animals that are not susceptible to shigellosis may not be overly relevant to the understanding of the human immune response to *S. flexneri*. Therefore, immunoproteomics is best performed with sera from natural infection of the appropriate host.

1.10 Knockout strategies

The construction of gene disruptions in bacterial strains is essential for the study of gene function, and particularly useful for the study of virulence genes and the production of attenuated vaccine strains. Early techniques were non-specific, requiring random mutagenesis with elements such as the Tn10 transposon, and have since been replaced with techniques that allow gene targeting and homologous recombination (246, 385). These

strategies include the use of suicide plasmids and group II intron targeting (TargeTron™ system) (296, 318, 422). The efficient construction of gene knockouts in pathogenic strains of bacteria often proves to be elusive (245). Two different strategies were used for the construction of knockouts in *S. flexneri* 2457T in this thesis and are outlined below.

The plasmid, pGP704, which uses the R6K origin of replication, has been designed for use in enterobacteria (232). This plasmid is only able to replicate in bacterial hosts that contain the π protein, encoded by the *pir* gene (169). pGP704 cannot replicate in *Shigella* as it does not possess the *pir* gene and can, therefore, be used to disrupt target genes in *S. flexneri* by cloning a region of the target gene into the multiple cloning site. This strategy has been used to construct a number of knockouts in *Shigella* strains (44, 300).

An alternative knockout system, the lambda red PCR recombination approach, is far more efficient in *E. coli* than suicide plasmids (Figure 2.6) (245). In this technique, the strain of interest must be transformed with an inducible helper plasmid, either pKD46 or pKM208, which expresses the lambda bacteriophage proteins Beta, Exo and Gam, to prevent the bacteria from degrading transformed linear DNA as a defence mechanism and promote efficient recombination of linear DNA with the chromosome (64, 245). A PCR fragment containing an antibiotic cassette that has been amplified with primers containing 40 bp of flanking homology to the targeted gene is introduced into the strain. The ends of the linear DNA can undergo homologous recombination with the target gene producing a double cross over, replacing the target gene with the antibiotic cassette (64). Although efficient in *E. coli* K12, this technique has proved to be highly variable in its success in pathogenic strains of *E. coli* and *S. flexneri* (64, 245, 302).

1.11 Objectives of thesis

Despite over sixty years of research into developing a *S. flexneri* vaccine, there is currently no vaccine suitable for commercial release. The vaccines that have been developed have not achieved an optimal balance between their immunogenicity and reactogenicity. Therefore, vaccine development requires the further identification of ideal attenuation targets and antigens for use in producing a safe and effective vaccine. The broad aim of this thesis is to identify novel immunogenic proteins for *S. flexneri* and investigate their use in

S. flexneri vaccine development, and also to gain further understanding of the pathogenesis of *S. flexneri* by characterising the role of a number of proteins in the virulence and acid resistance of the *S. flexneri* strain 2457T.

The specific objectives of this study are:

1. To identify novel immunogenic proteins for *S. flexneri* by visualising an immunoproteome profile for soluble and membrane proteins of *S. flexneri* 2457T probed with shigellosis patient sera.
2. To characterise the role of a number of these identified immunoreactive proteins in the virulence of *S. flexneri*, by constructing and analysing disruption mutants for variation in their levels of pathogenesis observed in both *in vitro* and *in vivo* virulence assays.
3. To determine the immunogenic and protective potential of a vaccine candidate identified in the immunoproteome study, delivered to mice in a variety of adjuvants that have not previously been assessed for use in *S. flexneri*.
4. To characterise the acid resistance pathways of *S. flexneri* 2457T through acid resistance assays and the construction of gene disruption mutants, as acid resistance most likely contributes to the virulence of *S. flexneri in vivo*.

Chapter 2
Material and methods

Chapter 2

Material and methods

2.1. Bacterial culture conditions and media

Liquid overnight cultures of bacteria were grown in Luria Bretani (LB) broth, containing antibiotics if required, (Appendix A) in a shaking incubator (200 rpm). M9 minimal salts media was used for the growth curves performed in minimal media (Appendix A) (321). Minimal E salts glucose media was used as the acid shock media for acid resistance studies (Appendix A) (386). All *S. flexneri* minimal media cultures were supplemented with 2 μg/mL methionine, tryptophan and nicotinic acid (4). The overexpression experiments required cultures to be grown in YT media (Appendix A). For virulence work, *S. flexneri* cultures were grown overnight at 30 °C, as at 37 °C the plasmid-based virulence genes are expressed, making the virulence plasmid unstable and readily lost from the bacteria. Log phase *S. flexneri* cultures, acid resistance cultures and *E. coli* cultures were grown at 37°C. Bacteria were plated on LB agar plates (Appendix A), containing antibiotics if required. Strains were stored long term in 50% LB: 50% glycerol at –80 °C. The presence of the virulence plasmid was detected by plating cultures on Congo red TSB agar plates (Appendix A) (222).

2.2. Bacterial strains and plasmid vectors

All bacterial strains and plasmids used in this study are described in Tables 2.1, 2.2 and 2.3. *E. coli* JM109 was used for routine cloning with the pBC SK and pBS KS vectors (413). Plasmids pBS KS and pBC SK (Stratagene) (Figures 2.1 and 2.2) were used as the cloning vectors for cloning genes for complementation studies. The M13 forward and reverse primers were used to sequence all cloned genes to ensure sequence accuracy. *E. coli* B150 (SY327 λ pir) was the host strain used for cloning with the suicide vector, pGP704 (232). The suicide vector, pGP704 (Figure 2.3), was used to construct the integration vectors for vaccine development. The overexpression of IpaD was performed with the pGEX 4T-1

Table 2.1. *E. coli* strains used in this study

Strain	Characteristics	Source
JM109	*recA*1 *supE*44 endA1 *hsdR*17 *gyrA*96 relA1 *thi* Δ(*lac-proAB*) *F'* [*tra*D36 *proAB*+ *lacI*^{q} *lacZ* ΔM15]	Yanisch-Perron *et al*, (1985)
MG1655 (B1401)	*E. coli* K-12 F- lambda- *ilvG- rfb*-50 *rph*-1	Blattner *et al*, (1997)
BL21	F- *hsdS gal ompT*	Studier and Moffat, (1986)
SY327λ*pir*	F- *ara*D Δ(*lac pro*) *arg*E *rif*^{R} *rec*A56 *nal*A [λpir]	Miller and Mekanalos, (1988)
B1525	BL21 carrying pNV1326	This study

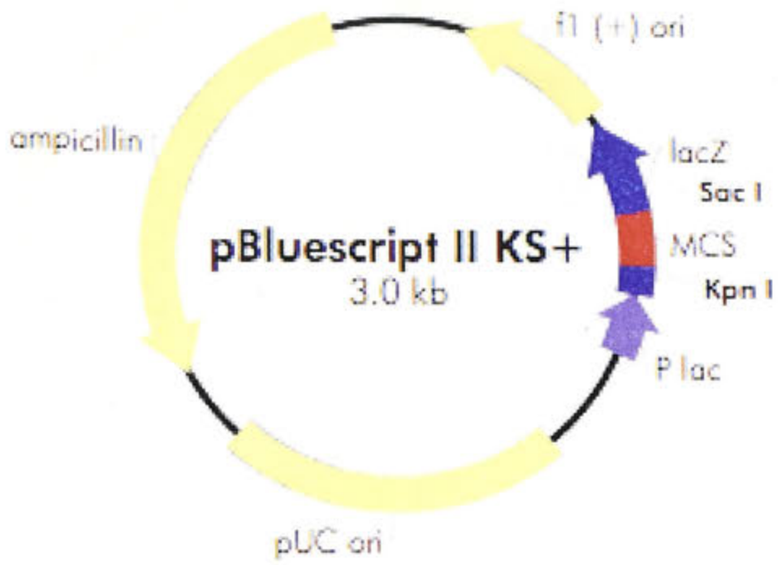
Table 2.2. *S. flexneri* strain used in this study

Strain	Characteristics	Source
SFL1001	*S. flexneri* 2a 2457T, Congo red positive, avirulent in Sereny test	WRAIR
SFL1704	*S. flexneri* 2a 2457T, Congo red positive, virulent in Sereny test	WRAIR
SFL1223	*S. flexneri* 2a 2457T, Congo red positive, 2a, invasion plasmid cured, Congo red negative	A. Maurelli
SFL1617	SFL1001 *mgl* operon disruption mutant, pNV1228 insertion	This study
SFL1621	SFL1001 carrying the helper plasmid, pKD46	This study
SFL1629	SFL1001 *rpoS* disruption mutant, pNV1234 insertion	This study
SFL1641	SFL1001 *gadC* disruption mutant, pNV1206 insertion	This study
SFL1647	SFL1629 carrying pNV1314	This study
SFL1648	SFL1629 carrying pNV1315	This study
SFL1650	SFL1001 *gadB* disruption mutant, *cat* gene insertion	This study
SFL1718	SFL1617 carrying pNV1370	This study
SFL1721	SFL1704 carrying the helper plasmid, pKD46	This study
SFL1723	SFL1650 carrying pNV1389	This study
SFL1726	SFL1704 *udp* disruption mutant, *cat* gene insertion	This study
SFL1727	SFL1704 *S3195* disruption mutant, *cat* gene insertion	This study
SFL1729	SFL1641 carrying pNV1396	This study
SFL1736	SFL1617 carrying pNV1424	This study
SFL1739	SFL1617 carrying empty pBC SK	This study
SFL1772	SFL1704 carrying the helper plasmid, pKM208	This study
SFL1774	SFL1001 carrying the helper plasmid, pKM208	This study

Table 2.3. Plasmids used in this study

Plasmid	Characteristics	Source
pBS II KS	cloning and expression vector, Amp^r	Stratagene
pBC SK	cloning and expression vector, Cm^r	Stratagene
pGEX-4T-1	IPTG inducible expression vector	Amersham Biosciences
pKD3	lambda red PCR KO system, *cat* cassette template plasmid	Datsenko and Wanner, (2000)
pKD46	lambda red PCR KO system, helper plasmid, arabinose induced	Datsenko and Wanner, (2000)
pKM208	lambda red PCR KO system, helper plasmid, IPTG induced	Murphy and Campellone, (2003)
pNV1206	pGP704 carrying 395 bp fragment of *gadC* gene	This study
pNV1228	pGP704 carrying 424 bp fragment of *mglB* gene	This study
pNV1234	pGP704 carrying 281 bp fragment of *rpoS* gene	This study
pNV1314	pBC SK carrying the 0.98 kb 2457T *rpoS* gene	This study
pNV1315	pBC SK carrying the 1.14 kb *E. coli* MG1655 *rpoS* gene	This study
pNV1326	pGEX-4T-1 carrying the 0.75 kb *ipaD* gene	This study
pNV1370	pBC SK carrying the 1.13 kb *mglB* gene	This study
pNV1374	pBS KS carrying the 0.93 kb *udp* gene	This study
pNV1389	pBS KS carrying the 1.55 kb *gadB* gene	This study
pNV1396	pBC SK carrying the 1.80 kb *gadC* gene	This study
pNV1424	pBC SK carrying the 3.7 kb *mgl* operon	This study

f1 (+) origin 135–441
β-galactosidase α-fragment 460–816
multiple cloning site 653–760
lac promoter 817–938
pUC origin 1158–1825
ampicillin resistance (*bla*) ORF 1976–2833

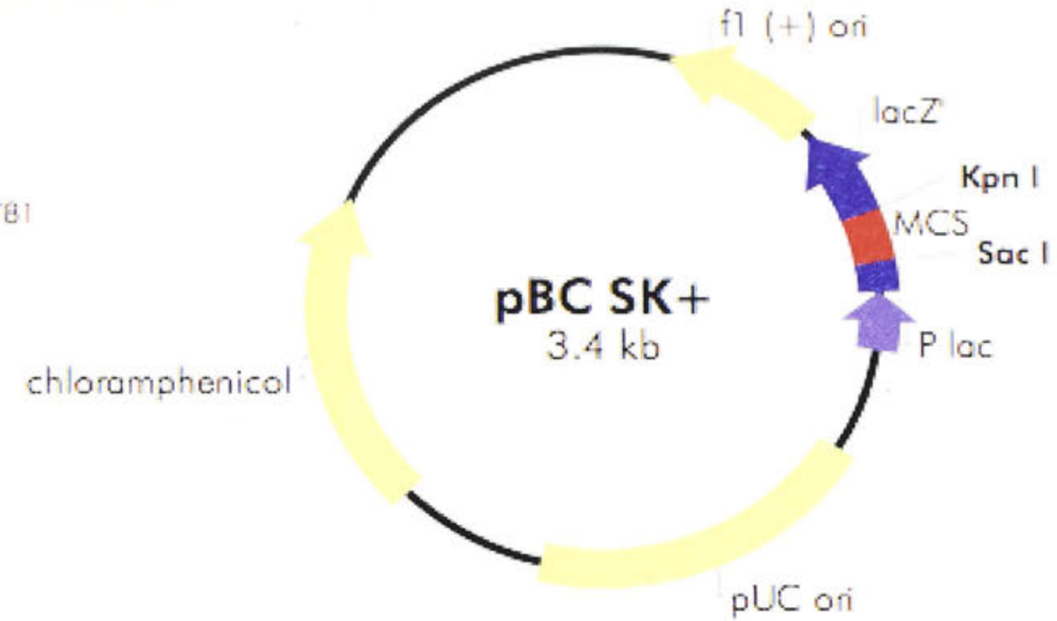


pBluescript II KS (+/–) Multiple Cloning Site Region
(sequence shown 598–826)

could be cleaved

Figure 2.1. Plasmid map of pBS KS, used as a cloning and expression vector in this study. The MCS is located within the N-terminal portion of a β-galactosidase gene fragment, allowing blue-white screening of recombinant plasmids (Adapted from Stratagene).

f1 (+) origin 135–441
β-galactosidase α-fragment 460–816
multiple cloning site 653–760
lac promoter 817–938
pUC origin 1158–1825
chloramphenicol resistance ORF 2125–2781



pBC SK (+/–) Multiple Cloning Site Region
(sequence shown 598–826)

could be shorter

Figure 2.2. Plasmid map of pBC SK, used as a cloning and expression vector in this study. The MCS is located within the N-terminal portion of a β-galactosidase gene fragment, allowing blue-white screening of recombinant plasmids (Adapted from Stratagene).

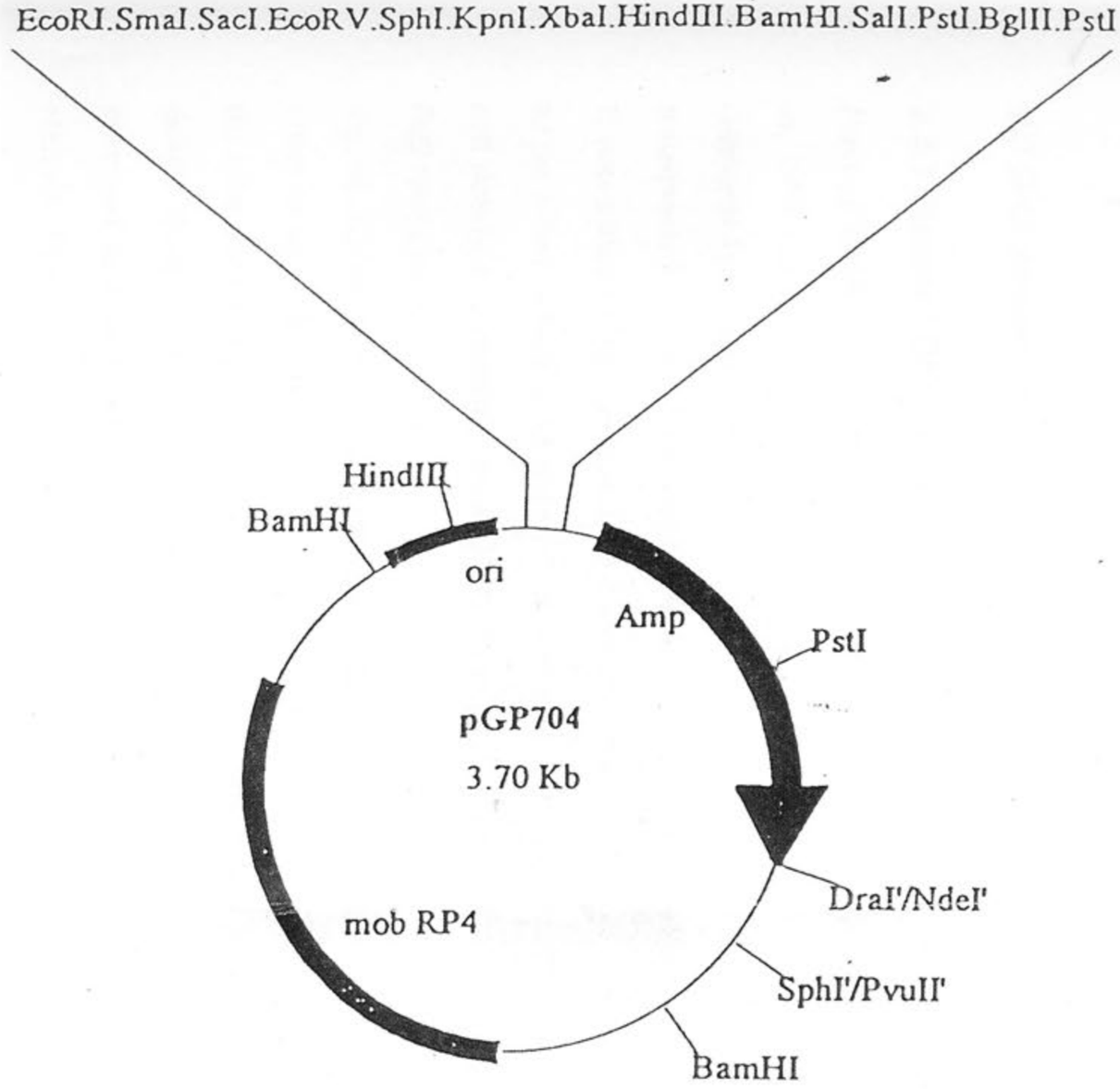


Figure 2.3. Plasmid map of suicide vector, pGP704. The *ori* is the R6K origin, which requires the π protein for replication. The MCS contains unique sites for *Bgl*II, *Sal*I, *Xba*I, *Sph*I, *Eco*RV, *Sac*I and *Eco*RI.

plasmid (Figure 2.4), which was expressed in *E. coli* BL21 (351). Amplification of the *E. coli rpoS* gene was performed with MG1655 (31).

The *S. flexneri* 2457T strains, SFL1001 and SFL1704 were used for all studies. Both strains had the presence of the virulence plasmid confirmed before all virulence assays.

2.3 DNA preparation

2.3.1 Plasmid DNA miniprep

Plasmid DNA was isolated from bacteria using the alkaline lysis method from Sambrook *et al*, 1998 (321). 1.5 mL of an overnight bacterial culture was pelleted in a microcentrifuge (Heraeus Instruments, Biofuge 13) at 13 000 rpm (16 000 g) for 30 seconds. The pellet was resuspended in 100 μL of cold Solution I (Appendix A) with vortexing. 200 μL of Solution II was added to lyse cells and the tubes inverted. 150 μL of ice-cold Solution III was added to the tubes, which were inverted gently and placed on ice for 5 minutes to precipitate the cell debris and chromosomal DNA. The precipitate was spun down by centrifuging at 13 000 rpm (16 000 g) for 5 minutes and the supernatant moved into a new tube. 0.5 μL of 1 mg/mL RNase was added to the tube, which was left at room temperature for 10 minutes, in order to degrade any RNA. An equal volume of 1:1 phenol: chloroform was then added, the tube vortexed and spun at 13 000 rpm (16 000 g) for 2 minutes, in order to extract any debris from the plasmid DNA. The aqueous phase containing the plasmid DNA was removed to a new tube and the DNA precipitated by the addition of two volumes of ethanol. The precipitate was pelleted by spinning at 13 000 rpm (16 000 g) for 5 minutes and washed by a final spin for 2 minutes with 300 μL of 70% ethanol. The DNA pellet was dried under vacuum using the Savant SC100 "Speed Vac" centrifuge, resuspended in 20 μL of Milli-Q water and stored at –20 °C.

2.3.2 Qiagen QIAprep miniprep kit

1.5 mL of overnight cultures were spun down in a microcentrifuge for 1 minute at 13 000 rpm (16 000 g) (Heraeus Instruments, Biofuge 13). The pellet was resuspended in 250 μL of P1 buffer and 250 μL P2 buffer. The tube was inverted until the solution became

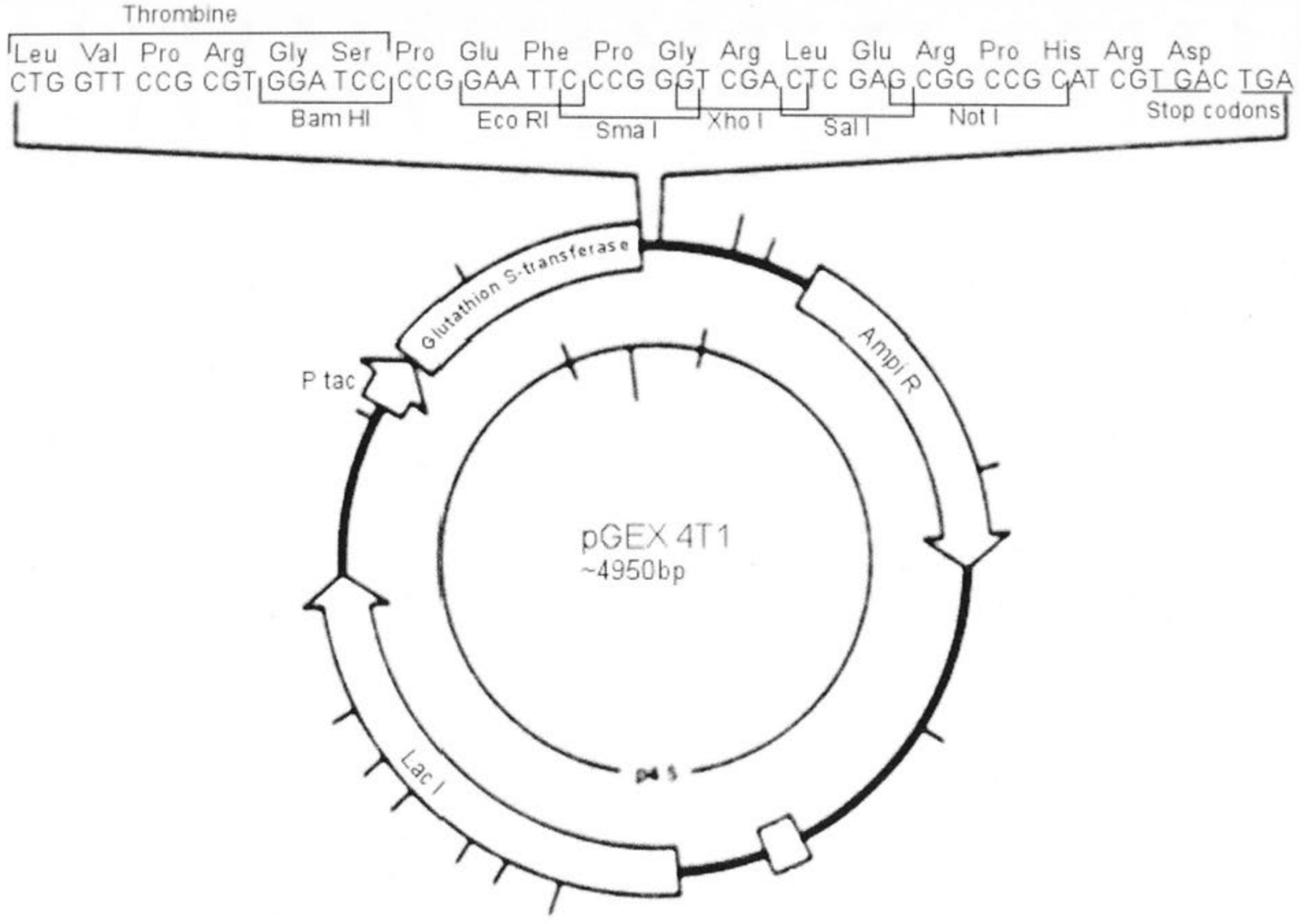


Figure 2.4. Plasmid map of pGEX-4T-1. Shows the reading frame and thrombin cleavage sequence. Expression of the cloned gene is under the control of the *tac* promoter, which is induced by IPTG. The $LacI^q$ repressor prevents expression of the tac promoter in the absennce of IPTG (Amersham Bioscience).

viscous. 350 μL of N3 buffer was added and the solution was gently mixed by inversion. The cell debris was spun down by centrifuging for 10 minutes at 13 000 rpm (16 000 g) for 10 minutes. The supernatant was carefully removed and placed in a QIAprep spin column. The spin column was spun for 1 minute at 13 000 rpm (16 000 g). The flow-through was discarded and 750 μL of PE buffer was added to wash the column. The PE buffer was removed by spinning at 13 000 rpm (16 000 g) for 1 minute and to ensure all buffer had been removed the column was spun once more. The column was then placed in an empty eppendorf tube and the DNA was eluted by the addition of 30 μL of Milli-Q. The DNA was collected by a 1 minute spin at 13 000 rpm (16 000 g) and stored at – 20 °C.

2.3.3 Chromosomal DNA isolation

2 mL of an overnight bacterial culture was spun down in a microcentrifuge at 6 000 rpm (3 300 g) for 10 minutes (Heraeus Instruments, Biofuge 13). An additional 2 mL of culture was then spun down on top of the existing pellet. The resulting pellet was resuspended in 4 mL of TES buffer (Appendix A) and spun down for 5 minutes at 6 000 rpm (3 300 g). The pellet was then resuspended in 400 μL of 25% sucrose, 50 mM Tris solution. 200 μL of 10 mg/mL lysozyme in 0.25 M EDTA was added and the tube left on ice for 20 minutes. The cells were then lysed by the addition of 150 μL of TE buffer and 50 μL lysis buffer (Appendix A). 2 mg of pronase was carefully mixed in to degrade protein. The tube was incubated at 56 °C for 1 hour, cooled to room temperature and extracted 3 times with 800 μl of phenol:chloroform, where the mixture was spun at 6 000 rpm (3 300 g) for 5 minutes and the aqueous phase collected with a wide bore pipette tip. Two additional extractions were performed using 800 μl of chloroform. The chromosomal DNA in the aqueous phase was precipitated by the addition of 1.2 mL of ethanol. The viscous DNA was collected using a glass loop and washed twice in 70% of ethanol. The DNA was dissolved in 200 μL of TE buffer and stored at 4 °C.

2.3.3 Determination of DNA concentration

DNA concentration was determined by spectrophotometric analysis using the NC-1000 spectrophotometer NanoDrop (Biosciences, BioLab). 2 μL of Milli-Q water was used to blank the spectrophotometer before 2 μL of DNA sample was loaded for measurement.

The DNA concentration was determined from the equation:

Ab_{260} = 50 µg/mL = 1

The ratio of Ab_{260}/Ab_{280} was used to determine the purity of the DNA sample. Pure DNA without any contaminating RNA or protein will have a 260:280 absorbance ratio of 1.85.

2.4 DNA separation and purification by electrophoresis

2.4.1 Agarose gel electrophoresis

Agarose gels were prepared using 0.5%-1.5% agarose in 0.5× TBE buffer (Appendix A). Ethidium bromide was added to the gel at a final concentration of 10 µg/mL. Gels were run in 0.5 × TBE running buffer between 40 and 100 V for 2-4 hours. DNA samples were loaded with 1/10 volume of blue loading dye added (Appendix A). The marker used in all gels in this thesis was 500ng of SPP-1 phage DNA/*Eco*RI (Figure 2.5). Samples were run until the blue dye reached the end of the gel and the DNA was then visualized under UV light with a Gel-Doc set-up. Photographs were recorded using the NIH-image program and printed on a thermal printer (Mitsubishi). The sizes of DNA bands can be estimated by comparison to the known sizes of the markers bands.

2.4.2 Gel purification of DNA

DNA bands in the agarose gel were visualized under UV light and the bands of interest were cut out using a scalpel and placed in a weighed eppendorf tube. Gel purification was performed using the QIAquick Gel Extraction Kit (Qiagen). The gel slice and tube were weighed and the weight of the empty tube was subtracted from the total. 3 volumes of buffer QG was added to 1 volume of gel in the tube. The mixture was incubated at 50 °C for 10 minutes, dissolving the agarose gel slice. The solution was transferred into the QIAquick spin columns, placed in a 2 mL collection tube, which was centrifuged in a microcentrifuge for 1 minute at 13 000 rpm (16 000 g). Any flow-through was discarded and an additional 500 µl of buffer QG was added to the column. This was centrifuged for another minute at 13 000 rpm (16 000 g), ensuring that all traces of agarose were removed

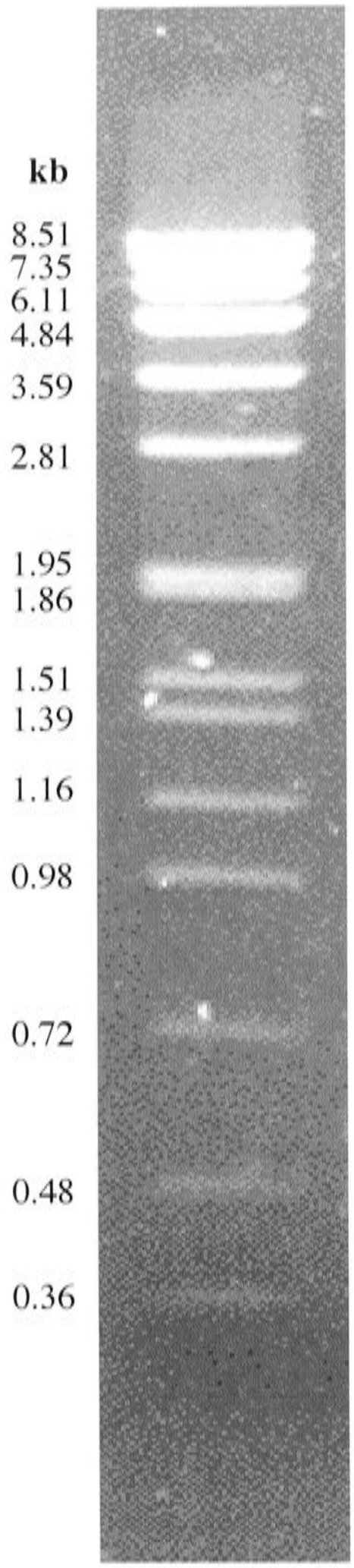


Figure 2.5. Sizes of bands (kb) in the SPP-1 phage DNA/*Eco*RI marker. All agarose gels displayed in this study use the SPP-1 marker (GibcoBRL).

from the sample. The flow-through was discarded and 0.75 mL of buffer PE was added to the column, which was spun for 1 minute at 13 000 rpm (16 000 g). The flow-through was discarded and the column centrifuged for another minute to clean the sample of any residual buffer. The spin column was then placed in a clean eppendorf tube and the DNA eluted by the addition of 30 μL of Milli-Q water. The column was left to sit for 2 minutes and then spun for 1 minute at 13 000 rpm (16 000 g) to collect the resuspended DNA in the bottom of an eppendorf tube. The DNA was stored at –20 °C.

2.5 Polymerase Chain Reaction (PCR)

2.5.1 Amplification of genes for cloning purposes

PCR reactions were performed in 20 μL volumes, using 0.25 units of Taq polymerase or 0.3 units of *Pfu* polymerase (Promega), 0.125 μM primers, 2 μL of 10× Taq Buffer, 0.2 mM dNTPs, 14.1 μL of Milli-Q water and ~ 5 ng of template DNA. The reaction was carried out in the GenAmp2400 Thermal Cycler (Perkin Elmer). The annealing temperature was determined from the primer nucleotide composition using the equation:

Tm = 2*[AT] + 4*[GC]

The final reaction Tm used was 5 °C lower than this calculated Tm. The length of time used in the extension step was based upon the size of the amplified product, where it was assumed that Taq requires 1 minute to amplify 1 kb of DNA and *Pfu* requires 2 minutes per 1 kb. PCR products were stored at 4 °C, until purified using the Qiagen gel extraction kit (Section 2.4.2). Primers used in the reactions are shown in Table 2.4. Primers were designed with the online primer design program, Netprimer (http://www.premierbiosoft.com/netprimer/index.html) and manufactured by Proligo.

2.5.2 Colony PCR

Colony PCR was used to screen for the presence of the virulence plasmid and for screening and sequencing potential gene disruptions in all *S. flexneri* strains. The protocol used was based upon the method of Schuch & Maurelli (1997), where bacterial strains of interest were dilution streaked on LB plates, containing antibiotic if required. Single colonies were picked and resuspended in 25 μL of 0.5 M NaOH and left at room temperature for 30

Table 2.4. Table of primers used in this study. Restriction sites introduced for cloning are underlined.

Primer name	Sequence	Target gene	Comment
M13 Forward	GTAAAACGACGGCCAGT	M13 F site	Binds the M13 Forward site on cloning plasmids, used in this study to sequence all cloned genes.
M13 Reverse	AACAGCTATGACCATG	M13 R site	Binds the M13 Reverse site on cloning plasmids, used in this study to sequence all cloned genes.
Primer 1	GTGTAGGCTGGAGCTGCTTC	pKD3 *cat*	Primer sequence used to amplify the pKD3 *cat* cassette in PCR KO approach, target gene homology was added to this primer sequence.
Primer 2	CATATGAATATCCTCCTTAG	pKD3 *cat*	Primer sequence used to amplify the pKD3 *cat* cassette in PCR KO approach, target gene homology was added to this primer sequence.
UdpF	GTGCAGAGCTGGATGGTAAACCTGTTAT CGTCTGCTCTACGTGTAGGCTGGAGCTG CTTC	*udp*	Forward KO PCR primer for *udp*.
UdpR	TGCACACATGGTCAGCAGAGTTCGAGAT TCCATTTCATAGCATATGAATATCCTCCT TAG	*udp*	Reverse KO PCR primer for *udp*.
OMFP290F	GGGTAAACGTGGCGGTGTGGCGGTTGC ACTGTCTCTTGCCGTGTAGGCTGGAGCT GCTTC	*S3195*	Forward KO PCR primer for *S3195*.
OMFP960R	CCAGTCGGGTGTTATGTGCCTCTCCGTG TACCGTCTGGTCCATATGAATATCCTCC TTAG	*S3195*	Reverse KO PCR primer for *S3195*.
cI	TTATACGCAAGGCGACAAGG	*cat*	Reverse screening primer, binds within pKD3 *cat* cassette.
UdpFscreen	GTATATGTCCAAGTCTGATGTT	*udp*	Forward screening primer binds at the beginning of the *udp* gene.
OMFPFscreen	GAGATTCAGGGAACAACAGAG	*S3195*	Forward screening primer binds at the beginning of the *S3195* gene.
MalQFSac	AGAGCTCACGAAAGTGGCGGTAACG	*malQ*	Forward primer for amplifying *malQ* gene section as control
MalQRXba	TCTCTAGATTTGCCACATCCACCAGC	*malQ*	Reverse primer for amplifying *malQ* gene section as control

Primer name	Sequence	Target gene	Comment
OMFPcompF	ATCTAGAAAGGAAATGCTGATGAAAC	*S3195*	Forward primer for amplifying *S3195* for sequencing across disruption.
OMFPcompR	CAGAGCTCCAACAGAGAATGCTCCC	*S3195*	Reverse primer for amplifying *S3195* for sequencing across disruption.
UdpcompF	ATCTAGAGACTAAACCGATTCACAG	*udp*	Forward primer for amplifying *udp* for complementation and sequencing across disruption.
UdpcompR	ACGAGCTCCTCCTGACGCAACAACT	*udp*	Reverse primer for amplifying *udp* for complementation and sequencing across disruption.
UdpsthnF	GAAGGGATAGAGGTGAAG	*udp*	Forward primer binding at the beginning of the *udp* gene for probing Southern blot.
UdpsthnR	AGACGATAACAGGTTTACC	*udp*	Reverse primer binding at the beginning of the *udp* gene for probing Southern blot.
VirG1	CGGGTACTCAAGAACTTCATT	*icsA*	Forward primer amplifying the *icsA* gene for virulence plasmid screening
VirG2	TTCCGCCAAAATGAGAGTTCC	*icsA*	Reverse primer amplifying the *icsA* gene for virulence plasmid screening
Apy1	CATAATCAAGAGACAAAACGATA	*apy*	Forward primer amplifying the *apy* gene for virulence plasmid screening.
Apy2	CCAGCCTTTCCAGTAATCCC	*apy*	Reverse primer amplifying the *apy* gene for virulence plasmid screening.
MglFSac	AGAGCTCCTGATACTCGCATTGGTG	*mglB*	Forward primer amplifying a 400 bp region of the *mglB* gene.
MglRXba	ACTCTAGACCTTTCAGCAGTACGAAC	*mglB*	Reverse primer amplifying a 400 bp region of the *mglB* gene.
MglBF17	TAACCCTGTCTGCTGTGA	*mglB*	Forward primer binding just upstream from the insertion point in the *mglB* gene.
MglBR664	GATTTTGTTGGCGTTCGG	*mglB*	Reverse primer binding just downstream from the insertion point in the *mglB* gene.
MglBcompF	ACGAGCTCTATCTCAAGCACTACCCT	*mglB*	Forward cloning primer binding just upstream from the start of the *mglB* gene.
MglBcompR	ATCTAGACGGAAGACGGAGTCGT	*mglB*	Reverse cloning primer binding just downstream from the end of the *mglB* gene.

Primer name	Sequence	Target gene	Comment
MglBoperon	GTCTAGATGTAAAACCAGAGACGCA	*mglB*	Reverse cloning primer binding just downstream from the end of the *mglC* gene.
IpaDFGex	TCGGATCCATGAATATAACAACTCTG	*ipaD*	Forward primer amplifying *ipaD* gene in frame with GST tag in pGEX 4T-1.
IpaDRGex	CGTAGTCGACAGTATTATTTACATTATGCA	*ipaD*	Reverse primer amplifying *ipaD* gene in frame with GST tag in pGEX 4T-1.
RpoSF294Sac	AGAGCTCCTTGCGTCTGGTGGTAAA	*rpoS*	Forward primer amplifying an internal segment of *rpoS* for construction of a suicide plasmid.
RpoSR575Xba	CATCTAGATCTCTTCCGCACTTGGTT	*rpoS*	Reverse primer amplifying an internal segment of *rpoS* for construction of a suicide plasmid
RpoSF90	AGTAGAAGAGGAACCCAG	*rpoS*	Forward primer amplifying from the beginning of the *rpoS* gene for screening for disruption.
RpoSR903	AACCTGAATCTGGCGAAC	*rpoS*	Reverse primer amplifying from the end of the *rpoS* gene for screening for disruption.
2457TRpoSSacF	TGAGCTCGGCGGAACCAGGCTTTTG	*rpoS*	Forward cloning primer amplifying *rpoS* from 2457T.
RpoSXbaR	CATCTAGACCTGAATCTGGCGAACAC	*rpoS*	Reverse cloning primer amplifying *rpoS* from 2457T.
MG1566SacF	TGAGCTCCAAGGGATCACGGGTAGG	*rpoS*	Forward cloning primer amplifying *rpoS* from *E. coli* MG1655.
MG1655XbaR	GTTCTAGAGTTGCGTATGGGCGGTAA	*rpoS*	Reverse cloning primer amplifying *rpoS* from *E. coli* MG1655.
GadBKOF	GTCGCATTTCAGATTATCAATGATGAATTATATCTTGAGTGTAGGCTGGAGCTGCTTC	*gadB*	Forward KO PCR primer for *gadB*.
GadBKOR	GTTTCGGGTGATCGCTGAGATATTTCAGGGAGGCTTTGTACATATGAATATCCTCCTTAG	*gadB*	Reverse KO PCR primer for *gadB*.
GadBcompF	CTCTAGACACTTGCTTACTTTATCG	*gadB*	Forward cloning primer amplifying from the beginning of *gadB*.

Primer name	Sequence	Target gene	Comment
GadBcompR	TAGAGCTCCGTTGCTAATGCCAGTGAAC	*gadB*	Reverse cloning primer amplifying from the end of *gadB*.
GadCSacF	CGAGCTCTTACCGTTCTGATGTCCC	*gadC*	Forward primer amplifying an internal segment of the *gadC* gene.
GadCXbaR	CGTCTAGATTTCACCCCTTTACCACC	*gadC*	Reverse primer amplifying an internal segment of the *gadC* gene.
GadCSacpBC	TAGAGCTCAGAACAAAACAGGTGCGG	*gadC*	Forward cloning primer binding at the beginning of the *gadC* gene.
GadCXbapBC	GTCTAGAAATACGACAGCAAGCACG	*gadC*	Reverse cloning primer binding at the end of the *gadC* gene.

minutes. 25 μL 1 M Tris HCl, pH 8.0 and 450 μL of Milli-Q water were then added. 5 μL of this template solution was used in the PCR along with 0.125 μM primers, 2 μL of 10× Taq buffer, 0.2 mM dNTPs, 10.1 μL of Milli-Q water and 0.25 units of Taq polymerase.

Cycle parameters for screening gene disruptions were 95 °C for 5 minutes, 30 cycles of: 95 °C for 30 seconds, annealing temperature as determined in Section 2.5.1 for 30 seconds, 72 °C for extension time as determined in Section 2.5.1, and a final extension time of 72 °C. Parameters for the virulence plasmid genes were taken from the Schuch & Maurelli paper (1997), with the exception of the extension time, which was determined from the amplified fragment size as in Section 2.5.1. Primers used in the reactions are shown in Table 2.4, and were designed and ordered as described in Section 2.5.1. Amplified PCR fragments were visualized on agarose gels.

2.6 Sequencing of cloned genes

All amplified genes used for complementation of disruption mutants were sequenced to ensure that no mutations had been introduced during the PCR steps. PCR amplified fragments spanning the lambda red insertion sites of some disruption mutants were also sequenced. Primers used in the reactions are shown in Table 2.4, and were designed and ordered as described in Section 2.5.1. Approximately, 250 ng of DNA was mixed with 1 μL of Terminator Ready Reaction premix, 4 μL of 5 x Dilution buffer, 1.5 μL of 2.5 μM primer and sterile Milli-Q up to 20 μL. The Terminator Ready Reaction mix and Dilution buffer were purchased from the Biomolecular Resource Facility (BRF), John Curtin School of Medical Research. Sequencing was performed in the GenAmp2400 Thermal Cycler (Perkin Elmer) for 25 cycles of 96 °C, 10 seconds, 50 °C 5 seconds, 60 °C 4 minutes. The reaction was transferred to an eppendorf tube and precipitated with 2 μL of 3 M sodium acetate, 2 μL of 125 mM EDTA and 50 μL of 100 % ethanol. The solution was centrifuged at 13 000 rpm (16 000 g) for 30 minutes, and washed with 3 x 250 μL 70% ethanol washes, the DNA was spun down between each wash with a 2 minute, 13 000 rpm (16 000 g) spin. The DNA pellet was dried under vacuum for 5 minutes using the Savant SC100 "Speed Vac" centrifuge. The tubes were protected from light and stored at - 20 °C.

2.6.1 Sequencing analysis

The reactions were sequenced by the BRF. Sequences were stored and analysed by the Australian National Genomic Information Service (ANGIS)/ Biomanager. Colibase and the MSDB database were used to access sequences of genes of interest (50).

2.7 Cloning techniques

2.7.1 Restriction enzyme digest of DNA

DNA was digested using known cut sites for confirmation of DNA identity. Restriction enzyme sites introduced through PCR were used to create cohesive ends for cloning into vectors. Restriction enzymes from Fermentas were used at concentrations which gave 5-20 units per reaction. The higher number of units was used in digests of chromosomal DNA, which required more enzyme for complete cutting. Digests were generally performed in 20 µL volumes containing 2 µL of the correct 10× buffer, 0.05-1 µg of DNA, enzyme and Milli-Q water. Digests were incubated in a 37°C water bath for 1 hour or 4 hours for chromosomal digests. Digests were inactivated by heating if the enzyme used could be heat-inactivated or by adding 2 µL of loading buffer and immediately running on a 0.7% agarose gel. Digested DNA fragments required for further steps could then be cut out and purified as in Section 2.4.2

When DNA had to be cut with two or more different enzymes, reactions were either performed as double digests using Y Tango buffer (Fermentas) or as two single digests with a precipitation step in between. The DNA was digested with one of the enzymes before 2 volumes of ethanol was added to precipitate the DNA and the tube spun at 13 000 rpm (16 000 g) for 5 minutes. The precipitated DNA was washed in 70% ethanol before the pellet was dried as described in Section 2.1.1. The digested DNA was resuspended in Milli-Q water, the second enzyme and buffer was added and the tube incubated for another hour at 37 °C. Digests were then heat-inactivated and stored at –20 °C.

2.7.2 Ligation of DNA

Vector DNA and inserts were digested with specific enzymes to create cohesive ends and then ligated together to form recombinant plasmids expressing the genes of interest. In order to get efficient recombination, a 3:1 ratio of insert concentration to vector concentration was used in all ligations. Reactions were performed in 15 μL volumes containing digested vector DNA, digested insert DNA, 1 unit of T4 DNA ligase (Promega), 1.5 μL of 10 × ligase buffer and Milli-Q water. Tubes were incubated for 16 hours in a refrigerated water bath at 14 °C. Completed ligation reactions were then stored at –20 °C.

2.8. Transformation of DNA into competent cells

2.8.1 Making electrocompetant cells

Electrocompetant cells are prepared using the method from Dower *et al* (1998) (74). An overnight bacterial culture was diluted 1 in 20 with LB and shaken for 2-3 hours to log phase. 250 mL of cells were transferred to 500 mL polypropylene centrifuge bottles and spun for 10 minutes at 7 000 rpm (8 000 g), 4 °C, in the Sorvall RC 5C Plus Centrifuge, using the SLA3000 rotor. The cell pellet was resuspended in 250 mL of cold Milli-Q water and centrifuged again for 10 minutes. This step was repeated, before the cell pellet was resuspended in 125 mL of Milli-Q and centrifuged again. The resulting cell pellet was then resuspended in 5 mL of 10% glycerol and spun for 10 minutes. Finally the pellet was resuspended in 500 μL of 10% glycerol and 40 μL aliquots were removed to eppendorf tubes, stored at –70 °C. In efforts to increase the competence of cells used in the gene knockouts protocols, the starting volume of cells was increased, extra Milli-Q washes were added and cells were resuspended in a smaller final volume.

2.8.2 Transformation of DNA into electrocompetant cells

Electrocompetant cells were placed on ice and up to 7 μL of DNA suspended in Milli-Q water was mixed in. The cell/DNA mixture was added to an ice-cold Bio-Rad electroporation cuvette and tapped to the bottom. The cuvette was placed in the BioRad Genepulser and a pulse of 25 microfarads, 2.5 kilovolts and 200 ohms was sent through the cells. Immediately after the electroporation, 1 mL of LB was added to the cells and they

were recovered for 1 hr at 37 °C or 30 °C for *S. flexneri* strains. 100 μL of the cells was plated out on a LB plate containing the appropriate antibiotic. The remaining cell suspension was spun down in a bench microfuge, resuspended in 100 μL of LB and plated on a LB plate containing the appropriate antibiotic. Plates were incubated at 37 °C or 30 °C for *S. flexneri* strains.

2.8.3 Determining the efficiency of competant cells

In order to determine the efficiency of a batch of competent cells, 10 ng of pBS KS was transformed into an aliquot of cells as described in Section 2.7.2. 1 μL of the recovered cell suspension was added to 99 μL of LB, plated on a 100 μg ampicillin plate and incubated overnight. The resulting colonies were counted and multiplied by 10^3, to determine the number of transformed cells in the 1mL of original transformed cell suspension. Efficiency was expressed as number of transformed cells per 1μg of DNA, so the figure determined from transforming cells with 10ng of pUC18 was multiplied by 10^2. Efficiency varied depending on the type of cells but generally ranged from 10^6 –10^9 transformed cells/μg of DNA for *E. coli* and 10^4-10^5 cells/μg of DNA for *S. flexneri*.

2.9 Screening methods for cloned plasmids and disruption strains

2.9.1 Antibiotic selection

30 μg/mL of chloramphenicol (Appendix A) added to LB or LB agar was used to select for cells containing the plasmid pBC SK or derivatives of. 100 μg/mL of ampicillin (Appendix A) was used to select for cells containing the plasmid pBS KS and for *pir*$^+$ *E. coli* strains containing the plasmid pGP704. Integrated vaccine strains containing integrated pGP704 derivatives only contain only one chromosomal-based copy of the ampicillin resistance gene, so were selected for with a lower ampicillin concentration of 10 μg/mL. PCR knockout strains, containing an integrated chloramphenicol gene were selected on 30 μg/mL of chloramphenicol.

2.9.2 Blue-white screening

Both the pBS KS and pBC SK plasmids have their polylinkers located within the lacZ' gene fragment, allowing blue-white screening (Figures 2.1 and 2.2). Cells transformed with recombinant plasmids based on pBS KS or pBC SK were selected for on antibiotic plates as above. However, colonies were then patched onto a 100 μg/mL ampicillin or 30 μg/mL chloramphenicol masterplate, which has been spread with 30 μL of 20 mg/mL IPTG and 30 μL of 20 mg/mL of X-Gal. The plate was incubated at 37 °C for 12-16 hours. Any blue colonies were discarded as this suggested they had a functional lacZ protein, indicating that an insert had not been cloned into the polylinker. Colonies, which remained white, suggesting that the lacZ' gene has been disrupted by an insert, were further screened by miniprep and restriction enzyme analysis. Positive clones were further confirmed by sequencing.

2.10 Gene disruption construction in S. flexneri

2.10.1 Suicide plasmid disruption

An internal region of ~400 bp of the gene of interest was amplified by PCR and cloned into the suicide vector, pGP704. Good quality DNA of the targeted suicide plasmid was prepared by the Qiagen QIAprep miniprep kit and eluted in Milli-Q water (Section 2.3.2). 500 ng+ of DNA was electroporated into electrocompetant *S. flexneri* cells (Section 2.8.1) and recovered for at least 2 hours. Cells were spun down and resuspended in 100 μL and plated on LB plates containing 10 μg/mL ampicillin. Plates were incubated at 30 °C for 24-48 hours. Colonies were screened for disruption of the target gene by colony PCR.

2.10.2 PCR lambda red recombination

The *S. flexneri* strain of interest was transformed with the helper plasmid, either pKD46 or pKM208. Electrocompentant cells were prepared of the *S. flexneri* strain containing the helper plasmid as described in Section 2.8.1, except the helper plasmid expression was induced for 2 hours with either 1 mM IPTG (for pKM208) or 100 mM arabinose (for pKD46), after 1 hour of initial growth at 30 °C to ensure that the heat sensitive plasmid was not lost.

The PCR fragment was prepared by amplifying the cat cassette from pKD3 with the P1 and P2 primers from Datsenko and Wanner, (2000), which have been designed to contain 40 bp of the internal sequence of the target gene on the 5' ends (Figure 2.6) (64). The PCR was purified from an agarose gel and treated with DpnI to ensure that all the template plasmid has been removed. 500 ng+ of the PCR fragment was electroporated into the electrocompetant cells of the *S. flexneri* strain, expressing the helper plasmid. Cells were recovered at 37 °C for 3 hours, 500 μL was spun down and plated on a LB plate containing 30 μg/mL chloramphenicol and incubated at 37 °C overnight. 37 °C is used for incubation to ensure loss of the heat sensitive helper plasmid. The remaining 500 μL of transformation mix was left at room temperature overnight and plated onto a LB plate containing 30 μg/mL chloramphenicol the following morning. Colonies were screened for successful disruption by colony PCR.

2.11 Southern hybridisation

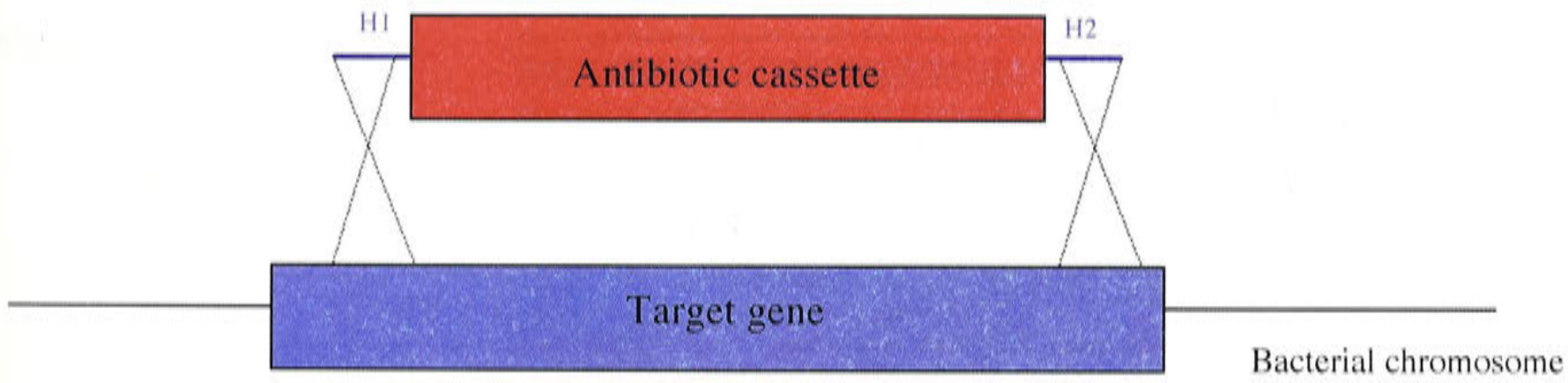
Southern hybridisation was used to check for successful disruption of gene knockouts. Chromosomal DNA was prepared as described in Section 2.3.3, digested with restriction enzymes and electrophoresed on a 0.7% agarose gel along with appropriate controls. The gel was viewed and photographed under UV light as a reference for the autoradiographs.

2.11.1 Alkali blotting of DNA onto nylon membrane

The capillary transfer method was used to blot DNA from the gel onto Hybond-N+ membrane (Amersham Biosciences Pharmacia). The gel was gently shaken in 0.25M HCl for 15 minutes in order to break the DNA into smaller fragments, which increases the efficiency of transfer. The gel was then washed in Milli-Q water and left in denaturing solution (Appendix A) for 30 minutes. Finally, after another rinse in Milli-Q, the gel was placed in neutralizing solution (Appendix A) for 15 minutes. The capillary transfer was assembled as shown in Figure 2.7 and left for at least 16 hours. The membrane was removed and rinsed in 5 ×SSC (Appendix A). The DNA was fixed on the membrane by heating at 80 °C in an oven for 2 hours. The membrane was then wrapped in Glad Wrap and stored at 4 °C until needed.

Step 1. PCR amplify antibiotic cassette using primers containing 40 bp of homology to regions of the target gene (H1 and H2).

Step 2. Transform strain with linear PCR fragment. If strain is expressing Beta, Exo and Gam from the transformed helper plasmid, then double homologous recombination can occur between the H1 and H2 homology regions found in both the PCR fragment and the target gene.



Step 3. Select for successful gene disruption by plating strain on antibiotic plate.

Figure 2.6. Strategy for lambda red PCR recombination system.

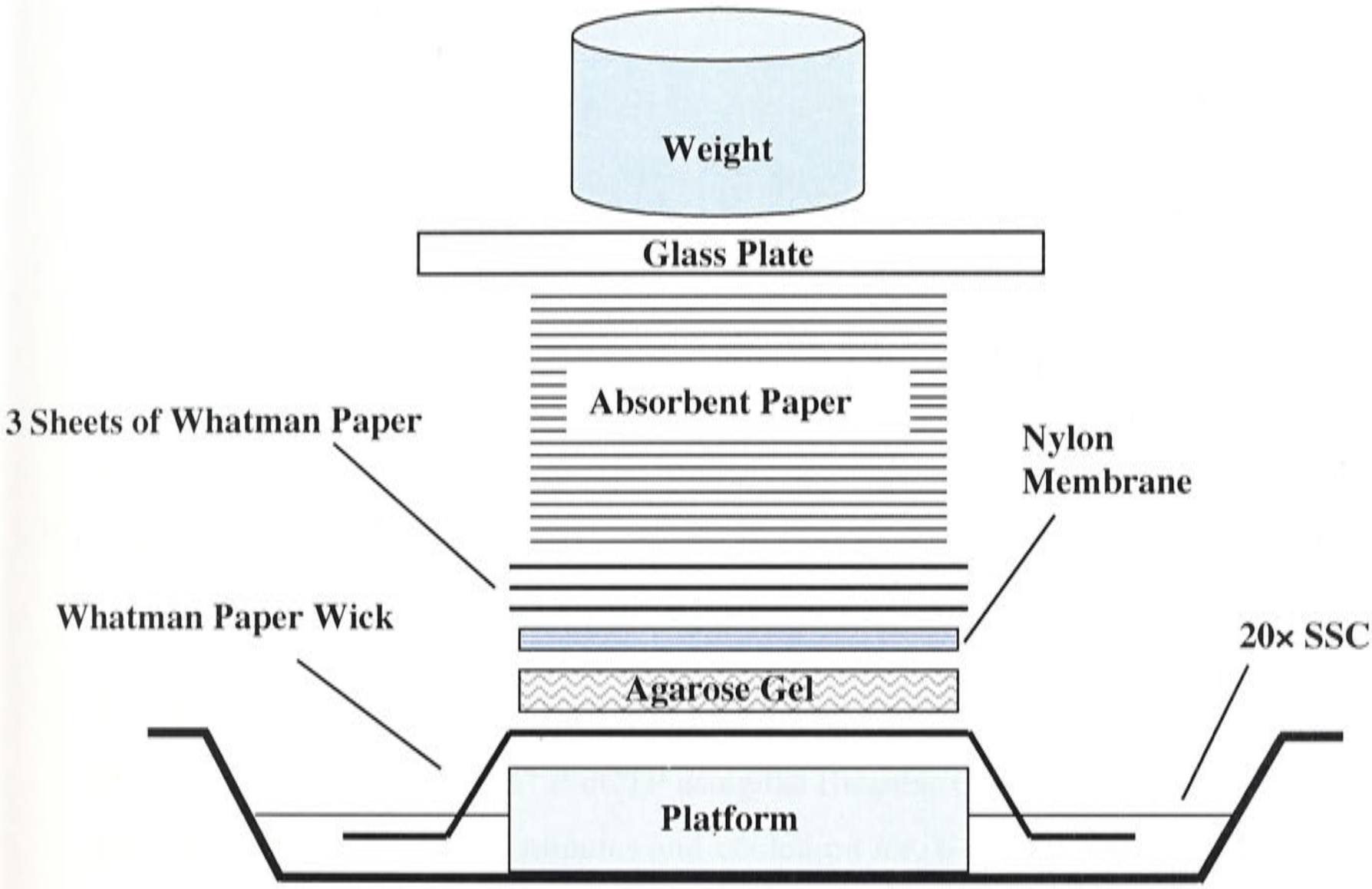


Figure 2.7 The capillary transfer method used to blot DNA onto nylon membrane for Southern hybridisation. The gel is placed upon a moist Whatman paper wick, dipped in a container of 20× SSC, and the membrane is placed on top. Three layers of Whatman paper and a large stack of paper towels is used to draw the 20× SSC through the gel and membrane, transferring the DNA onto the positively-charged membrane.

The agarose gel used in the transfer was incubated in post-stain solution, consisting of Milli-Q and 5 µl of ethidium bromide for 10 minutes, before being checked under UV light for any residual DNA. Gels that showed little to no remaining DNA indicated a successful capillary transfer.

2.11.2 Pre-hybridisation of the membrane

The membrane was rolled up with the DNA side facing inwards and placed in a Hybaid bottle with 50 mL of pre-hybridisation solution (Appendix A). Non-homologous calf thymus DNA was boiled for 5 minutes, rapidly cooled on ice and added to the solution to a final concentration of 100 µg/mL. The bottle was placed in a rotating incubator (Scientronic, HB900) for one hour at 65 °C.

2.11.3 Radiolabelling of the probe

Probe DNA was labelled with ^{32}P-dCTP using the Bresatac Gigaprime labelling kit. 7 µL of probe DNA was boiled for 5 minutes and cooled on ice. 6 µL of decanucleotide solution (containing random primers), 6 µL of nucleotide/buffer cocktail C (containing unlabelled dATP, dGTP and dTTP), 3 µL of ^{32}P-dCTP and 1 µL of Klenow fragment were then added to the denatured probe. This solution was incubated at 37°C for 15 minutes and the reaction was halted by boiling for 5 minutes.

2.11.4 Hybridisation of the radiolabelled probe to the membrane

The denatured probe solution was added to the pre-hybridisation solution in the Hybaid bottle, which was returned to the rotating incubator at 65 °C and left overnight. The next day the pre-hybridisation solution was carefully poured off and 50 mL of 2×SSPE, 0.1% SDS was added to wash unbound probe from the membrane. The bottle was incubated for 10 minutes at 45 °C before the solution was replaced with 50 mL of 1×SSPE, 0.1% SDS and incubated for 15 minutes at 65 °C. Finally, the membrane was rinsed in 0.1×SSPE, 0.1% SDS at 65 °C for 10 minutes. The membrane was removed and wrapped in Glad Wrap.

2.11.5 Autoradiography of the labelled membrane

The membrane was placed in a Kodak film cassette and a piece of Medical X-ray film (Fuiji) was laid over the membrane. The cassette was incubated at –80 °C for the required amount of time, ranging from 5 hours to one week. The film was then removed from the cassette and developed using the Kodak X-OMAT 1000 automated processor.

2.12 Protein extractions

2.12.1 Soluble protein extraction

Bacterial overnight cultures were grown at 30 °C, 1 in 100 dilutions were performed the next day. Cultures were shaken at 140 rpm at 37 °C until an OD of 0.5-0.6 was reached. Cultures were spun for 15 mins at 9000 rpm (14 000 g), 4 °C in the SLA3000 (Sorvall RC 5C Plus Centrifuge). Pellets were resuspended in 50 mL of Wash buffer (Appendix A) and spun as above. Supernatant was discarded and pellets were resuspended in 2.5 mL Lysis buffer (with 25 µL 0.5 M EDTA and 25 µL 0.1 M PMSF added). The cell suspensions were sonicated in a 15 mL falcon on ice for 10 × 10 seconds with 20 second breaks (Branson Sonifier 450). The suspensions were moved to a 7/16 × 13/8 inch Beckman polyallomer tube, another tube containing 2.5 mL buffer was prepared as a balance. Tubes are spun in the cooled buckets of the TLS-55 SB rotor in the Beckman Benchtop Ultracentrifuge at 35 000 rpm (100 000 g) for 1 hr at 10°C. The supernatant was removed and placed in eppendorf tubes with aliquots kept for protein assay. Stored at -80°C.

2.12.2 Membrane protein extraction

Bacterial overnight cultures were grown at 30 °C, 1 in 100 dilutions were performed the next day. 250 mL cultures were shaken at 140 rpm at 37 °C until an OD of 0.5-0.6 was reached. Cultures were spun for 15 mins at 9000 rpm (14 000 g), 4 °C in the SLA3000 (Sorvall RC 5C Plus Centrifuge). Pellets were resuspended in 50 mL of Wash buffer (Appendix A) and spun as above. Pellets were resuspended in 5 mL Tris.HCl pH 7.3, with 2 mg DNase I added. The cell suspensions were sonicated in a 15 mL falcon on ice for 15 ×

15 seconds with 20 second breaks (Branson Sonifier 450). The suspensions were spun down in 30 mL corex tubes, 7 500 rpm (6 000 g) 10 minutes in the SS-34 rotor (Sorvall RC 5C Plus Centrifuge). The supernatant was collected. The pellets were resuspended in 2.5 mL of Tris.HCl, pH 7.3 and suspension was sonicated and spun again. The supernatant was pooled with previous supernatant, mixed with 10 x the volume of 0.1 M $NaCO_3$ and left stirring at 4 °C for 1 hour. The supernatant was spun in 6 9/16 x 3 1/2 polyallomer Beckman tubes in the SW 41Ti rotor, 25 000 rpm for 1 hour, 4 °C, Beckman Ultracentrifuge. The pellets were resuspended in 4 mL of 50 mM Tris.HCl pH 7.3 and moved to 6 7/6 x 2 3/8 pollyallomer Beckman tubes and spun in the 60Ti SW, 30 000 rpm for 20 minutes, 4 °C, Beckman Ultracentrifuge. Pellets were pooled in 1 mL of solubilisation buffer (Appendix A) and stored at – 80 °C.

2.12.3 Extracellular protein extraction

Bacterial overnight cultures were grown at 30 °C, 1 in 100 dilutions were performed the next day. 250 mL cultures were shaken at 140 rpm at 37 °C until an OD of 0.5-0.6 was reached. Cultures were spun for 15 mins at 8000 rpm (10 000 g), 4 °C in the SLA3000 (Sorvall RC 5C Plus Centrifuge). The supernatant was collected and filtered through 0.2 μm filters. 75 mL 30% TCA in cold acetone were added to the supernatant and left on ice for 1 hour. The precipitated proteins were spun down for 10 minutes in the SLA3000 rotor at 8 000 rpm (10 000 g), 4 °C. The pellet was resuspended in 5 mL acetone and spun again and this was repeated once more. The pellets were air dried on the bench for 30 minutes, before being resuspended in 50 μL of PBS. Protein samples were stored at –20 °C.

2.12.3 Protein assay

Protein concentrations were measured using the BioRad-Bradford Protein Assay. BSA fraction V was used to prepare protein standards in seven different concentrations in PBS: 0.2 mg/mL, 0.4 mg/mL, 0.6 g/mL, 0.8 mg/mL, 1.0 mg/mL, 1.2 mg/mL, 1.4 mg/mL. The Biorad Dye Reagent was diluted 1 in 5 in Milli-Q water. 1 mL of the diluted dye was then added to 20 μL of each of the BSA standards. This was left for 7 minutes before being vortexed and placed in a plastic cuvette. The OD's of the samples was measured by a visible light spectrophotometer (Phillips SP6-550) at 595 nm, blanked against 20μL of PBS

containing 1 mL of dye. The OD's were used to construct a standard curve of protein concentration vs. OD. 20 μL of the protein sample was added to 1 mL of dye and left for 7 minutes, before being vortexed and transferred to a cuvette. The OD was read and used to determine the protein concentration of the sample from the standard curve.

2.13 Two dimensional analysis of S. flexneri *proteins*

2.13.1 First dimension focusing

Immobiline DryStrip pH 4-7 11 cm strips (Amersham Biosciences Bioscience) were rehydrated in Rehydration solution (Appendix A). Strips were laid out in grooves of the reswelling tray into 200 μL of Rehydration solution. Paraffin was applied to the strips to create an anaerobic environment and left overnight.

The Amersham Biosciences Multiphor II flatbed electrophoresis system was used. Samples cup loaded at the anode. 1 mg of soluble protein was used and 200μL of the membrane protein preparations were loaded, as the concentration could not be estimated due to incompatibility with the protein assay. Focussing was run at 20 °C, 1 mA, 5 W at the following voltage gradients: 1 min 150 V, 20 min 150 V, 20 min 200 V, 2 hr 300 V, 2 hr on a linear gradient to 3 500 V and 20 hr at 3 500 V.

2.13.2 Running the second dimension

After isoelectric focussing, the strips were treated with equilibration solution I (Appendix A) for 10 min and 10 min in equilibration solution II (Appendix A). Second dimension SDS-PAGE was performed on a horizontal Multiphor II electrophoresis system using precast Excel Gels with a 12-14% acrylamide gradient (Amersham Biosciences). Twin gels were loaded with a soluble protein and a membrane protein 11 cm Immobiline strip. Low molecular range markers (Amersham Biosciences) were loaded between the strips. Electrophoresis was performed at 200V for 1 hr 45 min and 600 V for 4-6 hours, until the bromophenol blue front reached the edge of the gel. Gels were either prepared for immunoblotting or Coomassie blue G250 stained. Commassie blue G250 stain (Appendix A) was left on the gel for 16 hours, destained with 0.1 M Tris-phosphoric acid (pH 6.5) for

2 minutes, 25% methanol for 1 minute and left in 20% ammonium sulphate for 8 hours. The gel was repeatedly stained three times and stored in 20% ammonium sulphate.

2.13.3 Electrotransfer of two dimensional gels

Gel was laid on the film remover (Amersham Biosciences) and moistened with Milli-Q water. The cable is pulled between the gel and backing film, separating the gel. PVDF membrane was laid over the gel, 6 sheets of blotting paper moistened in continuous blotting solution (Appendix A) cut to the dimensions of the gel were laid on top and the bubbles rolled out. Entire sandwich was removed and laid upside down on the moistened graphite plate of the Pharmacia Multiphor II Novablot. Another 6 sheets of blotting paper were laid over the gel and the upper graphite plate was laid on top. The transfer was run at 300 mA, 20% current and maximum voltage of 35 V for 1 hour. Membrane was removed and stored in PBS.

2.13.4 Amido black staining of membranes

Membranes were incubated in 0.1 % amido black for 5 minutes before being destained in detain solution (Appendix A) for 10 minutes. The membrane was scanned to record the image and further destained.

2.13.5 Immunoblotting

Membrane was blocked with 5% skim milk powder/PBS/0.1% Tween-20 overnight at 4 °C. Washed three times in PBS/0.05% Tween-20 for 10 minutes each. The membrane was incubated overnight at 4 °C with 1:100 dilution of pooled sera from five *Shigella flexneri* patients (ICDDRB, Bangladesh) diluted in 1% skim milk powder/PBS/0.1% Tween-20. The membrane was then washed three times with PBS/0.05% Tween-20 for 10 minutes and incubated with 1:100 000 dilution of sheep anti-human Ig-HRP (Chemicon International) in 1% skim milk powder/PBS/0.1% Tween-20 for 1.5 hrs, 4 °C. The membrane was washed with PBS/0.05% Tween-20 three times and twice with PBS, before being incubated for 5 minutes with 8 mL: 8 mL SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) and wrapped in plastic and developed by autoradiography on Hyperfilm

(Amersham Biosciences), exposed for between 1 minute and 5 minutes. Membranes were stripped as per Jungblut and Bumann (153). The membrane was incubated with stripping solution (Appendix A) at 70 °C for 1 hour, washed twice in PBS/0.05% Tween-20 and blocked in 5% skim milk powder/PBS/0.1% Tween-20. Stripped membranes were reprobed as above using sera from five healthy individuals obtained from the Australian Red Cross Blood Bank, for control immunoblots.

2.13.5 MALDI-TOF analysis and searching

Developed films, scanned pictures of the amido black stained membrane and the Coomassie stained twin gel were superimposed and compared using Adobe Photoshop® and transparencies prepared from the images. Protein spots from the Coomassie gel, which aligned to spots on the film, were excised from the gel. Some samples were sent for matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) analysis at the Australian Proteome Analysis Facility (APAF) at Macquarie University. Alternatively protein spots were digested with trypsin for 16 hrs at 37°C and the peptide mixture was mixed (1:1) with a saturated α-cyano-4-hydroxycinnamic acid solution in 30% acetonitrile-0.07% trifluoroacetic acid. 1μL of mixture was spotted onto a sample template plate and mass spectra were obtained at the Research School of Biological Sciences (RSBS) Molecular Biology Facility (Australian National University) with an Omniflex mass spectrometer (Bruker Daltonics, Billerica, USA). Autolysing trypsin peptide 2211.1 Da was used as an internal calibration. Post-analysis data processing was done using XMASS™ software (Bruker Daltonics, Billerica, USA). Searches were performed against the MSDB database with the MASCOT peptide mass fingerprint search engine (Matrix Science, www.matrixscience.com) (286). Searches did not allow a missed cleavage or any modifications and used an error rate of 200-300 ppm. In all significant matches according to the probability Mowse score, which determines if the matches are likely to be random or significant, the top match was a *S. flexneri* protein. Predicted protein sizes and p*I* values were calculated using protein sequences from the MSDB database and the ExPASy p*I*/Mw calculator.

2.14 One dimensional gels of S. flexneri *protein*

2.14.1 SDS-PAGE gels

All one dimensional gels used in this study were 4-20% Tris-Glycine igels (LifeGels). Protein samples were either prepared as in Section 2.12.1 but resuspended in PBS, extracellular protein extractions from Section 2.12.3 or were made from whole cell lysates. Protein or bacterial cells collected by centrifugation, were resuspended in an equal volume of 2 × loading buffer and left at 100 °C for 5 minutes. Protein samples were run on a 4-20% Tris-Glycine igel in running buffer (Appendix A) at 120 V, 30 mA until the loading dye had reached the bottom of the gel. The marker used was the Fermentas Prestained PageRuler ladder (Figure 2.8)

2.14.2 Western blotting

The proteins were transferred by wet blotting with the gel and PVDF membrane placed between a sandwich of 2 sheets of Whatman paper, and a sponge on either side in a BioRad Mini Protean II tank of transfer buffer (Appendix A). The transfer proceeded for 5 hours at 40V at 4 °C. The membrane was blocked overnight, 4 °C in 5% skim milk powder/PBS/0.1% Tween-20. The membrane was washed three times in PBS/0.05% Tween-20 for 10 minutes each and incubated in the primary antibody for 2 hours at 4 °C, washed for 10 minutes in PBS/0.05% Tween-20 three times and incubated in a HRP-conjugated secondary antibody. After three 10 minute washes in PBS/0.05% Tween-20, the signal was developed with the Supersignal West Pico chemiluminescent substrate (Pierce Biotechnology) and captured on Hyperfilm (Amersham Biosciences).

In the patient sera 1D immunoblots, the primary and secondary antibody dilutions were the same as what was outlined in Section 2.13.5. For the RpoS westerns, a rabbit anti-RpoS polyclonal sera was used at a 1 in 6000 dilution (317) and a sheep anti-rabbit IgG HRP (ICN) at a dilution of 1 in 1000. MxiD and MxiJ antibodies raised against a C-terminal peptide in rabbits was used in Westerns at 1 in 1000 dilution (363). IcsA antibody, VRG-N2, was raised in rabbits against residues 82-100 of IcsA and used in Westerns at a 1 in 1000 dilution (359). The secondary antibody in both cases was a 1 in 1000 dilution of sheep anti-rabbit IgG HRP (ICN).

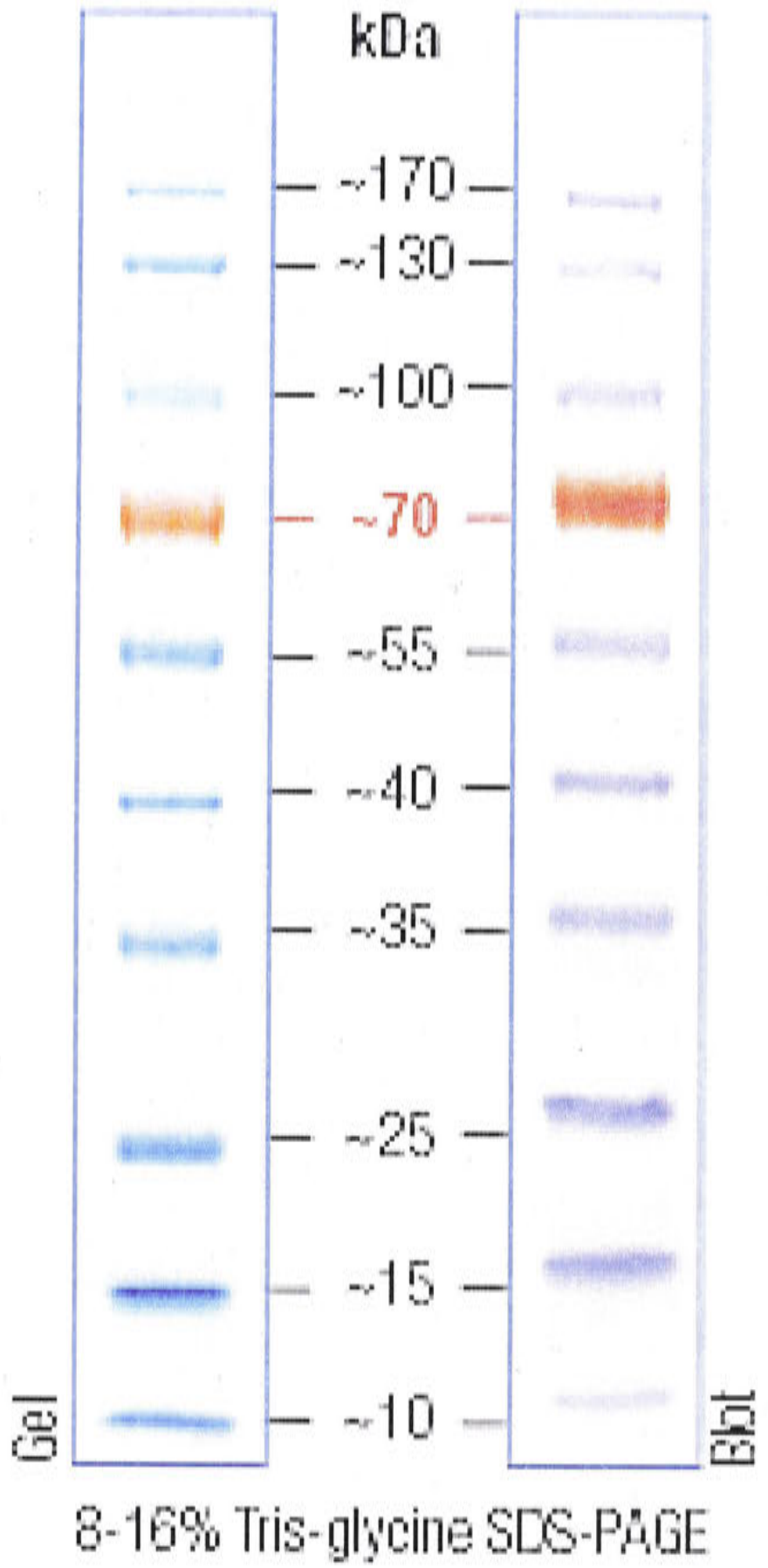


Figure 2.8. The PageRuler Prestained protein ladder (Fermentas) used in 1D SDS-PAGE and Western blotting. Sizes indicated are only approximate, the lot-specific exact marker sizes are provided with each batch of marker. Sizes indicated in the results chapters are from each lot-specific batch and may vary slightly from the figures above (Figure adapted from Fermentas).

2.15 IpaD overexpression

The BL21 strain, overexpressing IpaD from the pGEX plasmid, was grown overnight in YT medium at 30 °C. A 1 in 20 dilution of overnight culture is used to inoculate and grow log phase cultures to OD_{600} of 0.6. 10 mM IPTG is added to the culture to induce expression of the protein and shaken vigorously (200 rpm) for 2 hours. Cells were pelleted in the SLA3000 rotor at 7 000 rpm (8 000 g) for 10 minutes, 4 °C (Sorvall RC 5C Plus Centrifuge). The pellet was resuspended in 1/20th the starting volume of cold PBS containing 10 mg/mL lysozyme in 25 mM Tris.HCl pH 8 and sonicated on ice for 10 x 10 seconds (Branson Sonifier 450). 0.1 % Triton-X was added to the sonicate and incubated on a shaker for 30 minutes. The sonicate was clarified by spinning in the SS-34 rotor at 10 500 rpm (13 000 g) for 10 minutes (Sorvall RC 5C Plus Centrifuge). Meanwhile the glutathione sepharose 4B slurry was prepared by washing in PBS and spinning at 2 500 rpm (500 g) to pellet the beads. The beads were resuspended in PBS to a volume 1/100th of the sonicate and added to the supernatant of the clarified sonicate. The mixture was shaken for 30 minutes, before being poured into a 10 mL column and left to settle for 10 minutes. The column was eluted and washed with 10 sepharose bed volumes of PBS, this was repeated four more times. Thrombin in PBS (Amersham Biosciences) was added to the column, 80 units of thrombin were added for each mL of bed volume. The column was sealed and left for 16 hours at room temperature. Cleaved protein was eluted by adding 1 mL of PBS and catching the eluted solution in eppendorfs. Protein was stored at –20 °C, checked on 1D SDS PAGE and concentration determined by protein assay (Section 2.12.3).

2.16 Growth curves

Growth curves are used to estimate the number of bacteria in a culture by comparing the OD of the suspension to the number of bacteria or colony-forming units (cfu) present in the suspension. Overnight bacterial cultures were diluted 1 in 100 by adding 200µL of culture to 20 mL of LB, this was designated time 0 and the tubes were returned to the 37 °C shaking incubator. At times 0, 60, 90, 120, 150, 180, 210, 240 and 300 minutes, 1 mL of the culture was removed and placed in eppendorf tubes. These tubes were spun at 13 000 rpm (16 000 g) for 2 minutes at 4 °C in order to pellet the cells. The pellets were

resuspended in 1mL of PBS, placed in a microcuvette and the optical density read at 600 nm on a visible light spectrophotometer (Phillips SP6-550), against a PBS blank. 100 μL was also taken from the culture, mixed with 900 μL of PBS and placed on ice. This was used for serial dilutions in PBS up to 10^{-9} and 100 μL of the appropriate dilution was plated on duplicate LB agar plates containing the required antibiotic. The plates were incubated at 37 °C overnight and the colonies counted in order to determine the cfu/mL (colony forming units per mL). The OD measured at each time point were plotted against the cfu/mL to produce a growth curve.

2.17 Autoaggregation assay

Cultures were grown for 24 hours to stationary phase. Their OD_{600} was adjusted to 1.5, 3 mL was placed in clear glass test tubes, sealed with parafilm and left undisturbed for 6 hours. Autoaggregation should be observed as pelleting of cells at the base of the tube (125).

2.18 Catalase assay

The qualitative catalase activity was estimated by dropping 10 μL of 30% H_2O_2 onto individual colonies grown overnight on LB plates at 37°C (352). The rapidity and degree of bubbling was measured for each strain.

2.19 Acid resistance assay

Acid resistance assays were carried out as described by Lin *et al* (1995) (202) and Castanie-Cornet *et al* (1999) in order to study the different pathways (43). Cells were grown overnight in one of several media, including LBG (LB plus 0.4% glucose), buffered LB (100 mM MOPS pH 8 or 100 mM MES pH 5) and minimal salts E glucose (EG) (Appendix A). Glucose was added to promote fermentative growth at pH 5. Cultures at pH 8 did not have glucose added as the resulting fermentative products produced by the bacteria lowered the medium pH from 8 to 5, which was not desired. The addition of the buffers was used to maintain the culture pH during stationary phase growth. 24 hour grown stationary phase cultures were diluted 1:1000 into 5mL of warmed pH 2.5 EG, supplemented where

indicated with 0.012% glutamate and incubated for 2 hrs at 37 °C, 140 rpm. Cultures grown without oxygenation were grown in 5mL bijou bottles with tightly closed lids and 20 rpm shaking. Viable cell counts were determined at 0 and 2 hrs post acid challenge by serial dilution and plating on LB agar plates. At least 3 repetitions were performed for each experiment. Variations to this experiment are outlined in Chapter 7.

2.20 Cell culture

2.20.1 Subculturing

The epithelial cell lines, Baby Hamster Kidney cells (BHK) and HeLa cells were used in the cell culture assays in this study. Both types of cells were grown in 25cm^2 or 75cm^2 tissue culture flasks (Falcon, Becton Dickinson) to 70% confluency. Minimal essential medium (MEM) with 10% FCS and 200 μg/mL of neomycin and streptomycin and 120 μg/mL of penicillin was warmed to 37 °C. The medium in the flasks was poured off and 5 mL of sterile PBS (Appendix A) was added to rinse the cells of any remaining medium. This step was repeated once more before 2 mL of 0.05% Trypsin-0.02% EDTA was added and the flask was returned to the 37 °C incubator for 5 minutes. Once the cells had begun to lift from the flask surface the reaction was halted, by the removal of the trypsin solution and the addition of 4 mL of growth medium to inhibit any remaining trypsin. Gentle pipetting of the medium over the detaching cells was performed to resuspend them in a uniform solution. 10 μl of this solution is taken for counting in a haemocytometer to determine the number of cells per mL (Section 2.20.2). Generally, about 1.5×10^5 cells were used to seed a new 25 cm^2 flask and 1.7×10^6 cells were used to seed a 75 cm^2 flask. The cells were added to a fresh flask containing either 10 ml of MEM/FCS medium for a 25 cm^2 flask or 20 mL for a 75 cm^2 flask. The flasks were returned to the 37 °C 5% CO_2 incubator. Medium was changed every 2 days until the next passage was required.

2.20.2 Counting cells with a haemocytometer

Cell counting was performed with the Improved Neubauer Haemocytometer from Baxter Scientific. The cell suspension was dropped on the edge of the haemocytometer and the solution was pulled under the cover slip by capillary action. The slide was viewed on a light

microscope under ×100 magnification and each of the four corner squares and the centre square were counted. The number of cells in the squares was averaged and this number was multiplied by 10^4 to give the number of cells per mL. The total cells in the original cell suspension can then be worked out by multiplying by the volume of the original solution.

2.20.3 Freezing-down cells

Cells were harvested as previously described and resuspended in 4mL of medium, which was transferred to a 15 mL tube and centrifuged at 1000 rpm (250 g) (Beckman GS-6R Benchtop Centrifuge) for 5 minutes. The subsequent cell pellet was resuspended in 4mL of freezing medium (MEM, 20% FCS, 10% DMSO) and 1 mL aliquots were placed in cold cryotubes (Nunc). The tubes were frozen for 24 hours at –70 °C before being placed in liquid nitrogen. Cells can be thawed at 37 °C and returned to growth medium in a flask when required.

2.20.4 Plaque assay

The plaque assay was performed using the method outlined by Oak *et al*, (1985). 6-well plates were seeded with ~ 6.0 $\times 10^5$ BHK cells per well in 3 mL of cell culture medium. This was grown to confluence over about 2-3 days. Overnight cultures of the bacterial strains being tested were diluted 1 in 100 in LB containing the required antibiotic and shaken at 37 °C for 2 hours until in log phase. 2 aliquots of 1 mL of each culture were removed and spun for 5 minutes at 13 000 rpm (16 000 g), 4 °C. One pellet for each culture was resuspended in 1 mL of PBS. The optical density of the suspension was measured at 600 nm using a visible light spectrophotometer. The number of cells in the suspension was determined using the standard curves previously generated. The remaining pellet for each strain was then resuspended in cell culture medium and diluted to 5×10^4 cfu/mL. 100 µL was removed for serial dilutions and plating to check the exact bacterial count of the inoculum. The suspensions were then supplemented with the appropriate antibiotic to maintain antibiotic selection for any introduced plasmids and placed on ice. Dilutions of the bacterial suspensions were prepared and plated on LB agar plates containing the appropriate antibiotics, in order to determine the exact inoculum. The BHK cells were washed twice in cell culture medium (no antibiotics or FCS). All medium was then

removed and 200 μL of the bacterial suspensions was dropped onto the BHK cell surfaces in duplicate wells. The plates were returned to the 37 °C incubator for 90 minutes and rocked gently every 30 minutes. During the incubation the overlay medium was prepared (Appendix A) and cooled to 37 °C. 3 mL of the medium was gently pipetted into each of the wells and left to set. The plates were incubated in the 37 °C CO_2 incubator and checked daily for signs of plaques. For enhanced visualisation of the plaques, on day 3, 0.01% neutral red was added to more overlay medium and 3 mL was dropped onto the agar layer of each well.

2.20.5 Plaque inactivation assay

The plaque inactivation assay was performed exactly as in Section 2.20.4. However, prior to infection of the BHK monolayers, the bacterial cultures were incubated with a 1 in 20 dilution of the anti-IpaD affinity purified antibody for 45 minutes at 37 °C (395). The control strains were treated exactly the same but did not have antibody added.

2.20.6 Invasion assay

6 well plates were seeded with HeLa cells and grown to confluency over 3 days. Duplicate wells were prepared for each bacterial strain to be tested. Overnight cultures of the bacterial strains being tested were diluted 1 in 100 in LB containing the appropriate antibiotics and incubated for 3 hours at 37 °C. 2 aliquots of 1 mL of each culture were removed and spun for 10 minutes at 13 000 rpm (16 000 g), 4 °C. One pellet for each was resuspended in 1 mL of PBS. The optical density was measured at 600 nm using a visible light spectrophotometer. The number of cells in the suspension was determined using the standard curves previously generated. The remaining pellet for each strain was then resuspended in cell culture medium and diluted to 2.0×10^8 cfu/mL. 100 μL of each was removed for serial dilutions and plating, to check the exact number of bacteria in the inoculum. The remaining bacterial inoculum was supplemented with the appropriate antibiotics to maintain selection for any introduced plasmids. The tubes were kept on ice while the HeLa cells were prepared. The HeLa monolayers were washed twice in PBS and 1 mL of MEM without antibiotics was left in each well for 10 minutes. The medium was removed and 2 mL of the bacterial suspensions was gently applied to duplicate wells for

both an Intracellular plate and a Total bacteria plate. The plates were spun at 3 200 rpm (2 400 g) for 10 minutes at room temperature (Beckman GS-6R Benchtop Centrifuge). The plates were incubated at 37 °C, 5% CO_2 for 2 hours. The plates were then removed and the wells washed twice in 1 mL of PBS to remove unattached bacteria. The wells on the 'Intracellular bacteria' plate were treated with MEM/10% FCS/ 10μg/mL gentamycin, while the wells on the 'Total-associated bacteria' plate were just treated with MEM containing 10% FCS. The plates were incubated for 2 hours in the 37 °C incubator, before the filters were washed three times in PBS. 200 μL of 0.05% Triton X-100 in PBS was added to each well and left at room temperature for 10 minutes to lyse the mammalian cells. 800 μL of LB was added to each well, agitated with a Pasteur pipette and the solution moved to an eppendorf on ice. The bacterial suspensions were serially diluted in PBS and plated on duplicate LB plates, containing the appropriate antibiotic and incubated overnight at 37 °C. The colonies were counted and used to calculate the number of intracellular and total-associated bacteria for each strain.

$$\% \text{ Intracellular Bacteria} = \frac{\text{Average Number of Bacteria counted on Intracellular Plates}}{\text{Number of Bacteria in Inoculum}}$$

$$\% \text{ Adhered Bacteria} = \frac{\text{Average Number of Bacteria counted on Total Bacteria Plates}}{\text{Number of Bacteria in Inoculum}}$$

$$\% \text{ Invasion} = \frac{\text{Average Number of Bacteria counted on Intracellular Plates}}{\text{Number of Bacteria counted on Total Bacteria Plates}}$$

2.20.7 Survival assay

HeLa monolayers were prepared in 6 well plates as described in Section 2.20.6. Overnight cultures of the bacterial strains being tested were diluted 1 in 100 in LB containing the appropriate antibiotics and incubated for 3 hours at 37 °C. 2 aliquots of 1 mL of each

culture were removed and spun for 10 minutes at 13 000 rpm (16 000 g), 4 °C. One pellet for each was resuspended in 1 mL of PBS. The optical density was measured at 600 nm using a visible light spectrophotometer. The number of cells in the suspension was determined using the standard curves previously generated. The remaining pellet for each strain was then resuspended in cell culture medium and diluted to 2.0×10^8 cfu/mL. 100 µL of each was removed for serial dilutions and plating, to check the exact number of bacteria in the inoculum. The remaining bacterial inoculum was supplemented with the appropriate antibiotics to maintain selection for any introduced plasmids. The tubes were kept on ice while the HeLa cells were prepared. The HeLa monolayers were washed twice in PBS, 1 mL of MEM without antibiotics was left in each well for 10 minutes. The medium was removed and 2 mL of the bacterial suspensions was gently applied to duplicate wells for the Time 0, 120, 240 and 360 timepoints. The plates were spun at 3 200 rpm (2 400 g) for 10 minutes at room temperature (Beckman GS-6R Benchtop Centrifuge). The plates were incubated at 37 °C, 5% CO_2 for 90 minutes. The plates were then removed and the wells washed twice in 1 mL of PBS to remove unattached bacteria. 1 mL of MEM/10% FCS/ 10µg/mL gentamycin was added to each well and incubated for 30 minutes. The media was then removed from the Time 0 well and the cells washed three times in 1 mL of PBS. 200 µL of 0.05% Triton X-100 in PBS was added to each well and left at room temperature for 10 minutes to lyse the mammalian cells. 800 µL of LB was added to each well, agitated with a Pasteur pipette and the solution moved to an eppendorf tube on ice. The bacterial suspensions were serially diluted in PBS and plated on duplicate LB plates, containing the appropriate antibiotic and incubated overnight at 37°C. This process was repeated for the Time 120, 240 and 360 timepoints. The percentage of intracellular bacteria was determined as in Section 2.20.6.

2.21 Microscopy

2.21.1 IcsA immunofluorescence

Overnight bacterial cultures were diluted 1 in 100 into LB and grown for 2 hours at 37 °C. 10 mm round microscope slides were boiled in 0.1 M HCl for 5 minutes and air-dried. Slides were placed in the wells of a 24 well plates and 100 µL of 0.01% poly-D-lysine was placed on each slide for 5 minutes before being pipetted off and left to dry. 5 mL of

bacteria was spun down at 13 000 rpm (16 000 g) for 5 mins, 4 °C. The pellet was resuspended in 200 μL of 2% paraformaldehyde solution and left for 20 minutes. The cells were spun down and washed twice in 200 μL of PBS. The cells were resuspended in 200 μL of PBS and pipetted on to the coverslip. The bacteria were spun down onto the coverslip at 800 rpm (100 g) for 10 minutes (Beckman GS-6R Benchtop Centrifuge). The bacterial drop was removed and 200 μL was pipetted onto the coverslip for 2 minutes and pipetted off. The wash in PBS was repeated. The primary antibody was prepared as a 1 in 100 dilution of the rabbit VRG-N2 IcsA antibody in PBS/10% FCS (359). This was applied to the slides for 1 hour, 37 °C. The antibody was pipetted off and the slide washed twice in PBS. The secondary antibody, a FITC sheep anti-rabbit Ig (Chemicon) was diluted 1 in 80 in PBS/10% FCS and left on the slides for 30 minutes. The antibody was removed, the slides washed twice in PBS and left to dry. The slides were removed from the wells with tweezers and placed bacteria-side down on microscope glass slides on a 3μL drop of Mowiol-488. The edges of the round slide were sealed with clear nail polish and left to dry. The bacteria were visualized using the Axiovision "Apotome" Zeiss light microscope with the x 62 objective.

2.21.2 IcsA immunogold labeling and electron microscopy

The protocol used for imunogold labeling was modified from Giron *et al* (1991) (101). A 1 in 100 dilution of overnight cultures were used to grow log phase cultures for 2 hours at 37 °C. 10 mL were spun down at 3 200 rpm (2 400 g) (Beckman GS-6R Benchtop Centrifuge) and resuspended in 20 μL of PBS. 15 μL of the cell suspension was dropped onto a carbon coated 200 mesh grid and the bacteria were allowed to settle for 20 minutes. The grids were constructed by the electron microscopy unit (EMU) at the Research School of Biological Sciences. Three grids were used for each strain to allow for breakages, and positive and negative control strains were included. The bacteria were blocked by inverting the grids into drops of 0.4% BSA in PBS and left for 10 minutes. The grids were transferred to 10μL drops of the primary antibody, which was a 1 in 20 dilution of the rabbit VRG-N2 IcsA antibody in PBS/0.4% BSA. The grids were incubated for 90 minutes before being washed in 6 drops of PBS. The secondary antibody was an anti-rabbit IgG conjugated to 10 nm gold particles (Bristish BioCell International), which was diluted 1 in 10 in PBS/0.4% PBS.

The grids were incubated in 10μL drops of the secondary antibody for 2 hours, before being washed 4 times in PBS and twice in Milli-Q and left to dry overnight. Electron microscopy was performed at the EMU. Grids were viewed under a transmission electron microscope (Hitachi H-7100FA) at x60 000 magnification.

2.22 Animal experiments

All animal work was performed under approval of the Australian National University Animal Experimentation Ethics Committee.

2.22.1 Sereny test

Overnight cultures of the bacterial strains being tested were diluted 1 in 100 in LB containing the appropriate antibiotics and incubated for 3 hours at 37 °C. 2 aliquots of 1 mL of each culture were removed and spun for 10 minutes at 13 000 rpm (16 000 g), 4 °C. One pellet for each was resuspended in 1 mL of PBS. The optical density was measured at 600 nm using a visible light spectrophotometer. The number of cells in the suspension was determined using the standard curves previously generated. The cell suspensions were prepared to the appropriate number of cells in PBS; for testing SFL1001 and SFL1617 this was a high dose of 1 x 10^9 cfu as SFL1001 was very attenuated, for testing SFL1617 and SFL1736 this was 1 x 10^8 cfu in order to observe a difference between the strains, and for SFL1704 and SFL1727, which were based on a highly virulent strain, 1 x 10^7 cfu was used. Guinea pigs from the John Curtin School of Medical Research Animal Services, weighing between 150-250 g were used for the experiments. 10 μL of bacterial suspensions were dropped into each eye of the guinea pig and was gently massaged into the conjunctiva. The guinea pigs were observed over 24, 48 and 72 hours and the level of symptoms displayed were scored blindly. The scoring system used was that from Hartman *et al*, 1991 (120). 0 for a normal eye, 1 for mild conjunctivitis, 2 for moderate conjunctivitis with purulence and 3 for severe conjunctivitis with severe purulence. A score of 2 or above is considered a positive Sereny reaction

2.22.2 Mice immunisation with liposomes and blood collection

The vaccine strains immune induction was tested using the mouse pulmonary model (van de Verg et al., 1995). 5 six-week old Balb/c female mice were immunized for each vaccine strain (Animal Resource Centre, WA). 100 μL of blood was collected from the tail at day 0. Mice were restrained and injected subcutaneously with 200 μL of liposome preparations containing 100 μg of IpaD protein or 100 μg of IpaD protein in PBS. The mice were boosted in the same manner on Days 7 and 14. Blood was collected from the tail on days 14 and 34. The blood samples were spun down at 13 000 rpm (16 000 g) for 5 minutes at 4°C to pellet the red blood cells. The supernatant (serum) was collected in a new eppendorf tube and stored at –20°C.

2.22.3 Challenge of gamma inulin, cochleates and aluminium hydroxide immunised mice

6 8-10 weeks Balb/c female mice were immunised with IpaD in gamma inulin, cochleates and aluminium hydroxide intramuscularly or intranasally in Adelaide, Australia by Vaxine Pty Ltd. They were boosted on Day 14. After the final blood sample on day 41, the mice were sent to ANU, Canberra, Australia. On day 60, overnight cultures of SFL1704 were diluted 1 in 100 in LB containing the appropriate antibiotics and incubated for 3 hours at 37 °C. 2 aliquots of 1 mL of each culture were removed and spun for 10 minutes at 13 000 rpm (16 000 g), 4 °C. One pellet for each was resuspended in 1 mL of PBS. The optical density was measured at 600 nm using a visible light spectrophotometer. The number of cells in the suspension was determined using the standard curves previously generated. The cell suspensions were prepared to the appropriate number of cells in PBS. The mice were then lightly anaesthetised with ether and 20μL of bacterial suspension containing ~2× 10^8 cfu were dropped onto the external nares. The mice were monitored for 120 hours post challenge and the degree of sickness was scored. Mice were scored as healthy if they appeared bright, groomed and active, mildly sick if they showed some lethargy, hunching and roughed coat and severely sick if they were immobile, acutely hunched and highly ruffled.

2.23 ELISA

ELISA plates (Nunc-Immuno F96 Maxisorp) were coated with 100 μL of 5 μg/mL IpaD protein in carbonate buffer or 100 μL of 10 μg/mL *S. flexneri* 2a LPS in carbonate buffer (Appendix A), and stored at 4 °C overnight. The following day, the coating was removed and the plates washed twice with 0.05% Tween-20 in PBS. The wells were blocked with 200 μL of 5% skim milk in PBS and left for 1 hour at room temperature. The plates were then washed in 0.05% Tween-20 in PBS three times. The absorbed serum was further diluted to 1:20 in 1% milk /PBS. 100 μl was added to the first well in duplicate rows. The following seven wells had 1% milk/PBS added in preparation for the serial dilutions of the sera. 50 μL of sera from the first well in each row was then removed and added to the second well, already containing 50 μL of 1% milk/PBS. This was pipetted up and down before 50 μL was transferred to the third well and mixed, this was continued until the final dilution of 1:2560 (well no. 8) was reached. The extra 50 μL from this well was removed and discarded, leaving all wells with a final volume of 50 μL. The plates were incubated for 2 hours at room temperature. The plates were then washed three times with 0.05% Tween-20 in PBS. HRP-conjugated sheep anti-mouse IgG (Chemicon) was diluted 1 in 1000 in 1% milk/PBS and 50 μL was added to each well and left at room temperature for 1 hour. The plates were then washed 5 times in 0.05% Tween-20 in PBS. 100 μL of colour reagent (Appendix A) was added and left for 20 minutes for the yellow colour to develop. The peroxidase reaction was halted by the pH change from adding 50 μL of H_2SO_4. The OD was read on an automated plate reader (Bio-tek Instruments, EL-340) at 490 nm. OD was plotted against the inverse dilutions of the sera, to determine the titer of specific antibody at each time point.

2.24 Statistical analysis

All assays were performed with at least three repeats and are presented with the mean and standard error. Statistical differences between strains were determined using t-tests. The statistical difference between numerous vaccine groups in Chapter 6 was analysed with an ANOVA test.

Chapter 3
Immunoproteome analysis of *S. flexneri* 2457T soluble and membrane proteins

Chapter 3

Immunoproteome analysis of *S. flexneri* 2457T soluble and membrane proteins

3.1 Introduction

The majority of shigellosis cases in the Asian, African and Central American regions are caused by *Shigella flexneri* (178). Bacteria are transmitted via the faecal oral route and require as few as 100 organisms to cause disease. *S. flexneri* penetrates the epithelial layer of the large intestine, invading and spreading throughout the intestinal epithelial cells, inducing both a mucosal and systemic immune response. A number of proteins are known to be important for these invasion steps, particularly the products of the *mxi-spa* and *ipa* loci located on the virulence plasmid (229, 333).

The immune response against *S. flexneri* appears to be predominately humoral and it remains unclear whether the host's cellular immunity plays any protective role (401). The humoral immunity appears to be protective against reinfection, generally against the homologous serotype (79, 149, 227). Serotype-specific protection occurs because both the mucosal and systemic immune responses seem to be primarily directed against the lipopolysaccharide (LPS), the structure of which differs between the serotypes. Besides the LPS, *S. flexneri* proteins that do not show serotype-specificity have been identified as immunogenic during *S. flexneri* infection of higher primates. The invasion plasmid antigens (Ipa) are antigenic in both monkey and human studies, and are capable of inducing a protective immune response in mice and guinea pigs when delivered as a multiprotein complex (112, 156, 263, 374). Additionally, some major outer membrane proteins of 30-35 kDa are recognised by the mucosal immune response of *Shigella* patients and are protective in guinea pigs, mice and rabbits (1, 263, 410).

The escalation in antibiotic resistance of *S. flexneri* isolates is adding increased strain on the limited health services of developing countries. Consequently, the World Health Organisation has prioritised the development of a safe and effective vaccine against *S.*

flexneri (178). However, despite promising results with attenuated live vaccine strains, the current vaccine candidates are either not sufficiently attenuated or not sufficiently immunogenic (62, 176), suggesting that the identification of additional attenuation targets or protective antigens is warranted.

In order to identify such targets in *S. flexneri*, a profile of immunogenic proteins would be a useful tool. To date, a proteomic study of detectable immunoreactive proteins from natural *Shigella* infection of humans has not been reported. It is possible to identify antigens from two-dimensional gels using immunoproteomics; where proteins of a particular pathogen are separated by two-dimensional electrophoresis (2-DE), blotted onto membranes and probed with infected-patients sera. Subsequent matching and identification of any reactive protein spots generates an immunoproteome for the organism in question. This technique was initially used to visualise the antigens of *Borrelia garinii* and has since been used for a number of microorganisms including *Staphylococcus aureus*, *Heliobacter pylori* and *Candida albicans* (154, 179, 295, 388). Similar studies have recently been performed with *S. flexneri* protein probed with sera from immunised mice or rabbits (282, 414, 416). However, shigellosis is not a natural disease of either mice or rabbits and it remains unclear what use immunoproteome data from lab animals will be in the study of human *Shigella* infection. Consequently, the generation of an immunoproteome for *S. flexneri* with sera from human shigellosis patients would ultimately be a desirable tool for future vaccine and virulence research. In this Chapter, immunoproteomics is utilised to visualise and identify immunogenic *S. flexneri* 2457T serotype 2a soluble and membrane proteins, which are reactive to sera from *S. flexneri* infected patients.

3.2 Results

3.2.1 Soluble protein profile

Soluble protein preparations were prepared for the *S. flexneri* 2457T strain, SFL1001, and separated on p*I* 4-7 Immobiline strips. The focused strips were separated by 2D SDS PAGE on two 12-14% acrylamide gels for each of three repeats. Figures 3.1A, 3.2A and 3.3A show the Coomassie blue G250-stained 2-DE gels of *S. flexneri* membrane and soluble protein preparations. Approximately 150 soluble protein spots were visualised from ~1 mg

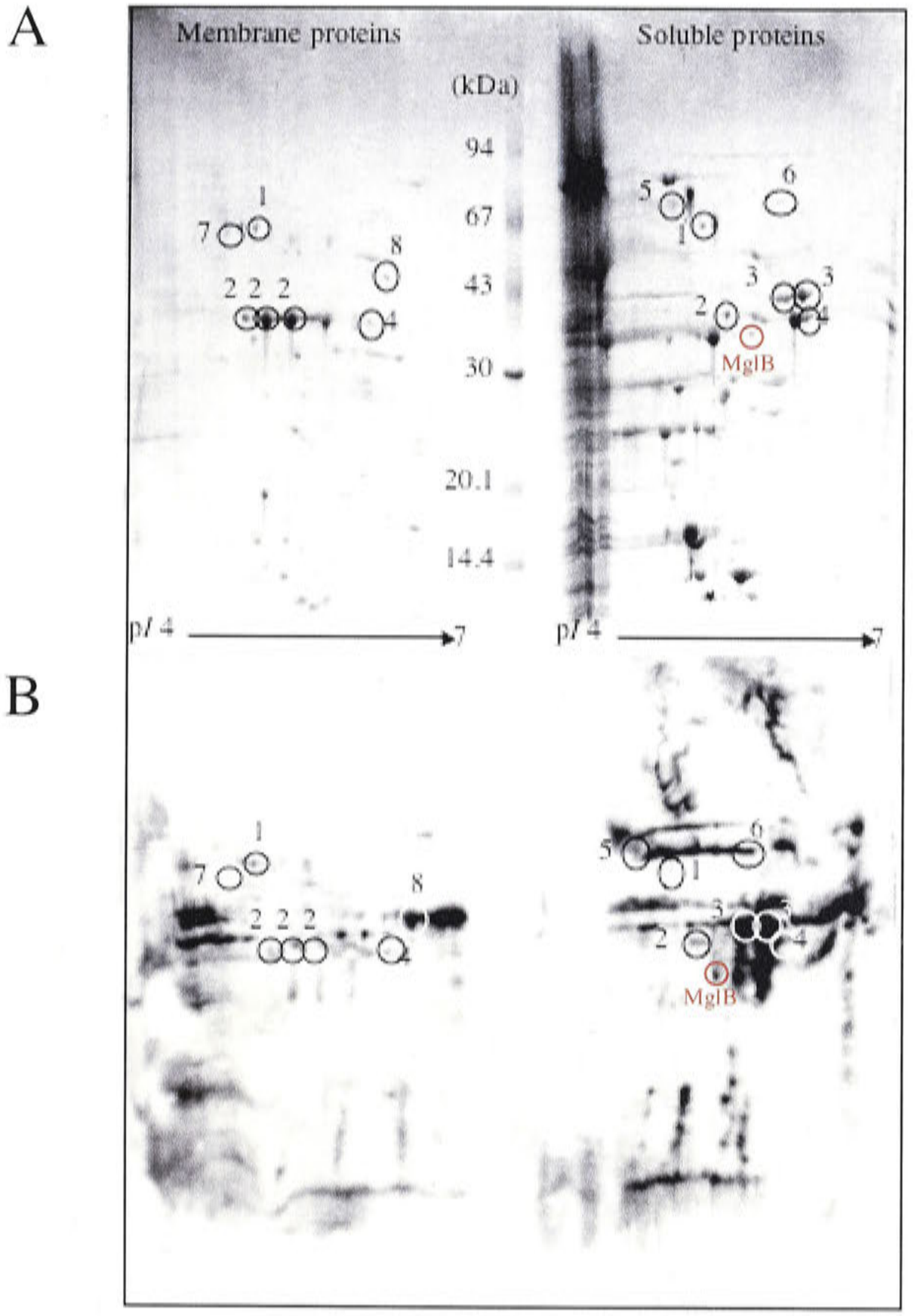


Figure 3.1. Immunoproteome analysis of Repeat 1. **A.** Coomassie blue-stained 2D gel of *S. flexneri* membrane and soluble protein preparations. **B.** Immunoblot of *S. flexneri* membrane and soluble protein preparations. Circles indicate immunoreactive proteins successfully matched and identified on the Coomassie gel (A). Numbers correspond to Table 3.1. Proteins named in red were only identified in one repeat and are not included in the table.

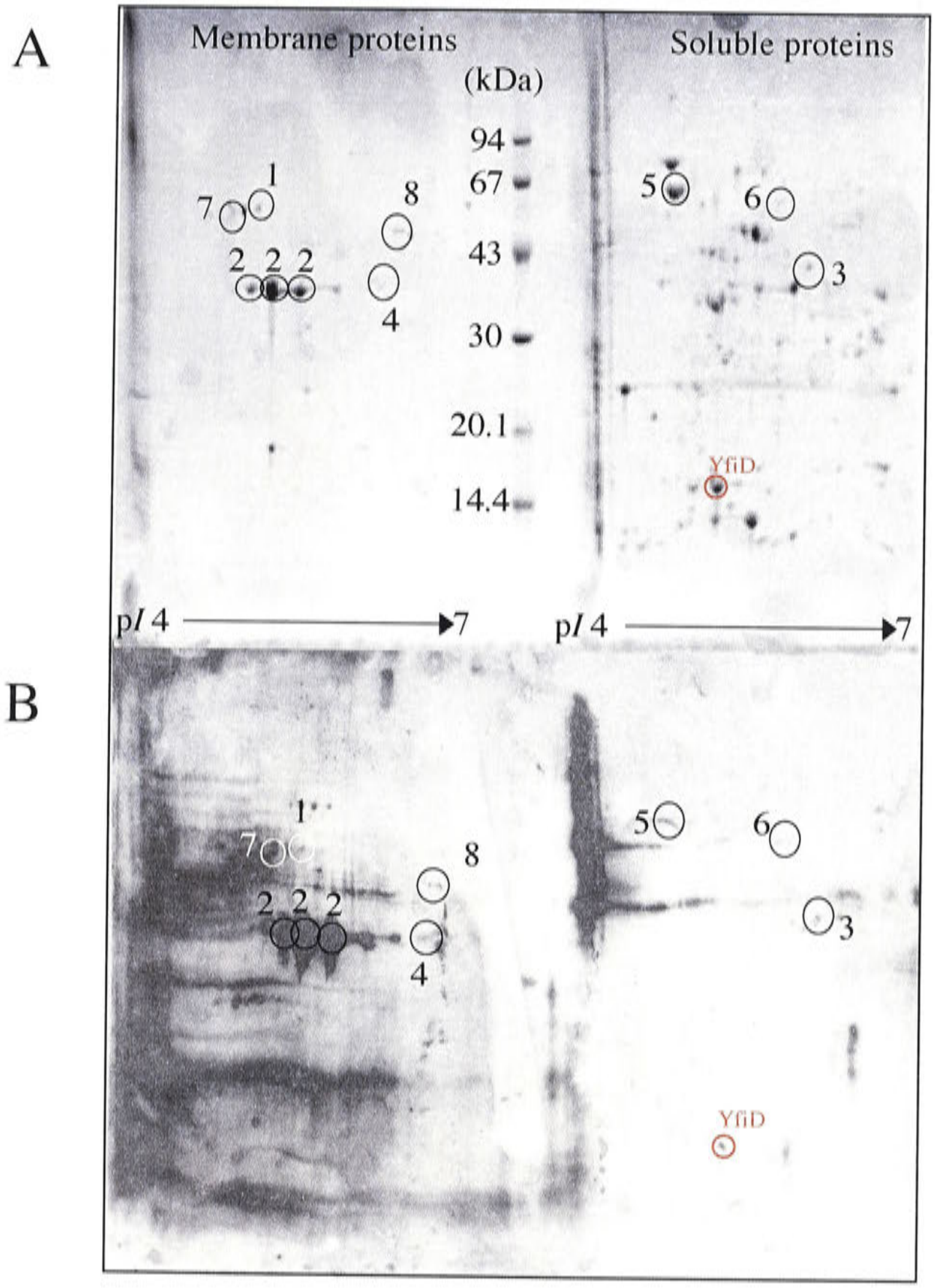


Figure 3.2. Immunoproteome analysis of Repeat 2. **A.** Coomassie blue-stained 2D gel of *S. flexneri* membrane and soluble protein preparations. **B.** Immunoblot of *S. flexneri* membrane and soluble protein preparations. Circles indicate immunoreactive proteins successfully matched and identified on the Coomassie gel (A). Numbers correspond to Table 3.1. Proteins named in red were only identified in one repeat and are not included in the table.

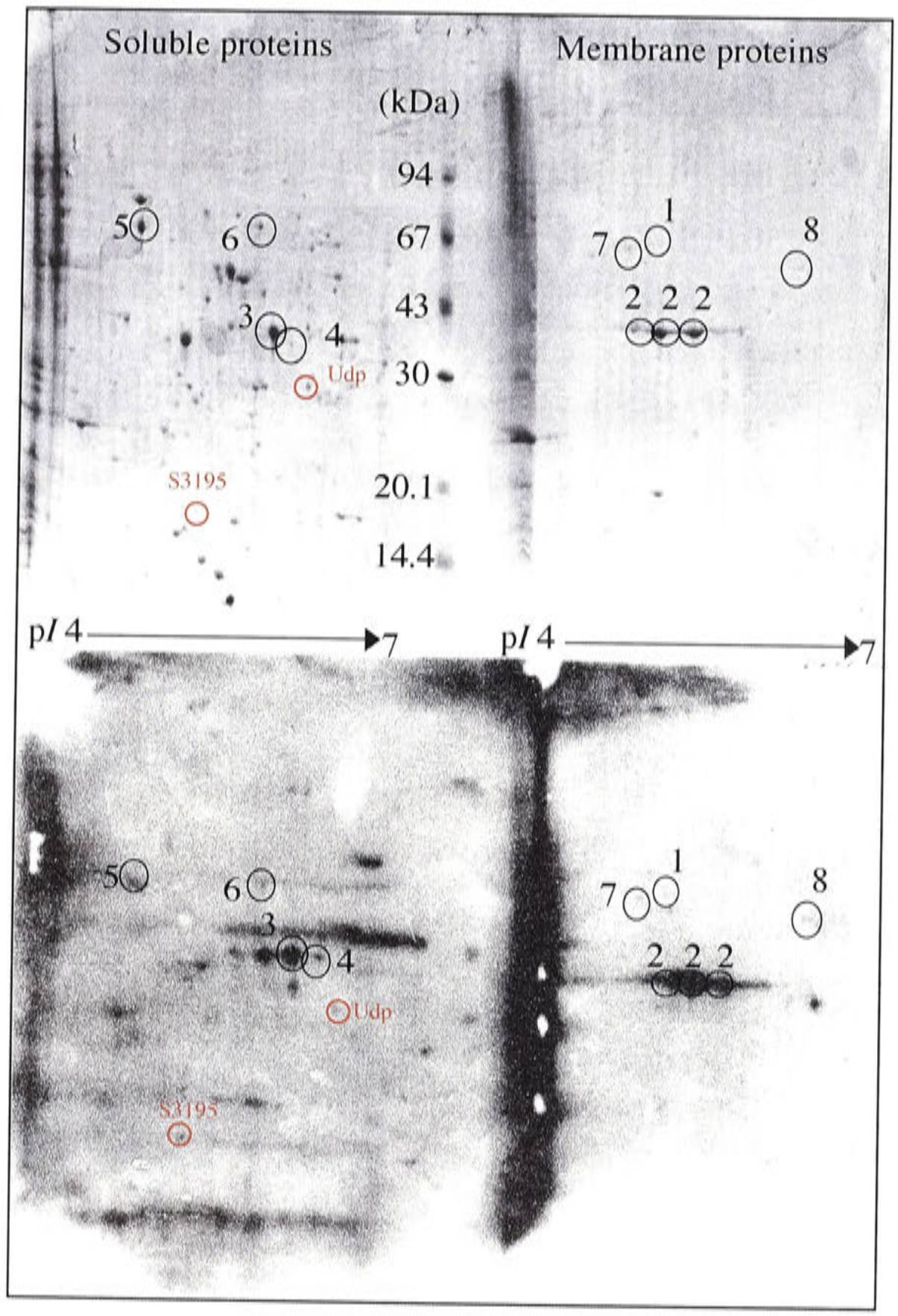


Figure 3.3. Immunoproteome analysis of Repeat 3. **A.** Coomassie blue-stained 2D gel of *S. flexneri* membrane and soluble protein preparations. **B.** Immunoblot of *S. flexneri* membrane and soluble protein preparations. Circles indicate immunoreactive proteins successfully matched and identified on the Coomassie gel (A). Numbers correspond to Table 3.1. Proteins named in red were only identified in one repeat and are not included in the table.

of soluble protein preparations applied to the Coomassie-stained gels. The twin gel was used for electroblotting onto PVDF membrane and membranes were transiently stained with amido black to aid in subsequent spot matching to the Coomassie-stained gel. The membranes were probed with sera from *S. flexneri* patients and a map of immunoreactive proteins was visualised by chemiluminescence. The pattern of immunogenic proteins is shown on the films in panel B of Figures 3.1, 3.2 and 3.3. The immunoreactive protein spots 1, 2, 3, 4, 5, and 6 on the film could be aligned to a corresponding protein spot on the Coomassie-stained gel. Four proteins could only be identified as immunoreactive in one repeat (red circles, Figures 3.1, 3.2, 3.3). All aligned proteins were selected for identification by peptide mass fingerprinting.

3.2.2 Outer membrane protein profile

It was necessary to separately visualise the outer membrane proteins of *S. flexneri,* as the extraction conditions and buffer used in the soluble extraction are not amenable to the purification and solubilisation of many membrane proteins. Outer membrane protein extractions of SFL1001 were isoelectrically focused in p*I* 4-7 Immobiline strips and separated by 12-14% 2D SDS PAGE. One gel for each repeat was stained by Coomassie blue G250, shown in Figures 3.1A, 3.2A and 3.3A. Approximately 25 proteins were visualised for each of the three membrane protein preparations. The films displaying the immunoreactive membrane proteins to *S. flexneri* patient sera are shown in panel B of Figure 3.1, 3.2 and 3.3. It was possible to match immunogenic spots 1, 2, 4, 7 and 8 on the film to the corresponding protein spots on the twin Coomassie-stained gels in three independent repeats.

3.2.3 Probing with control sera

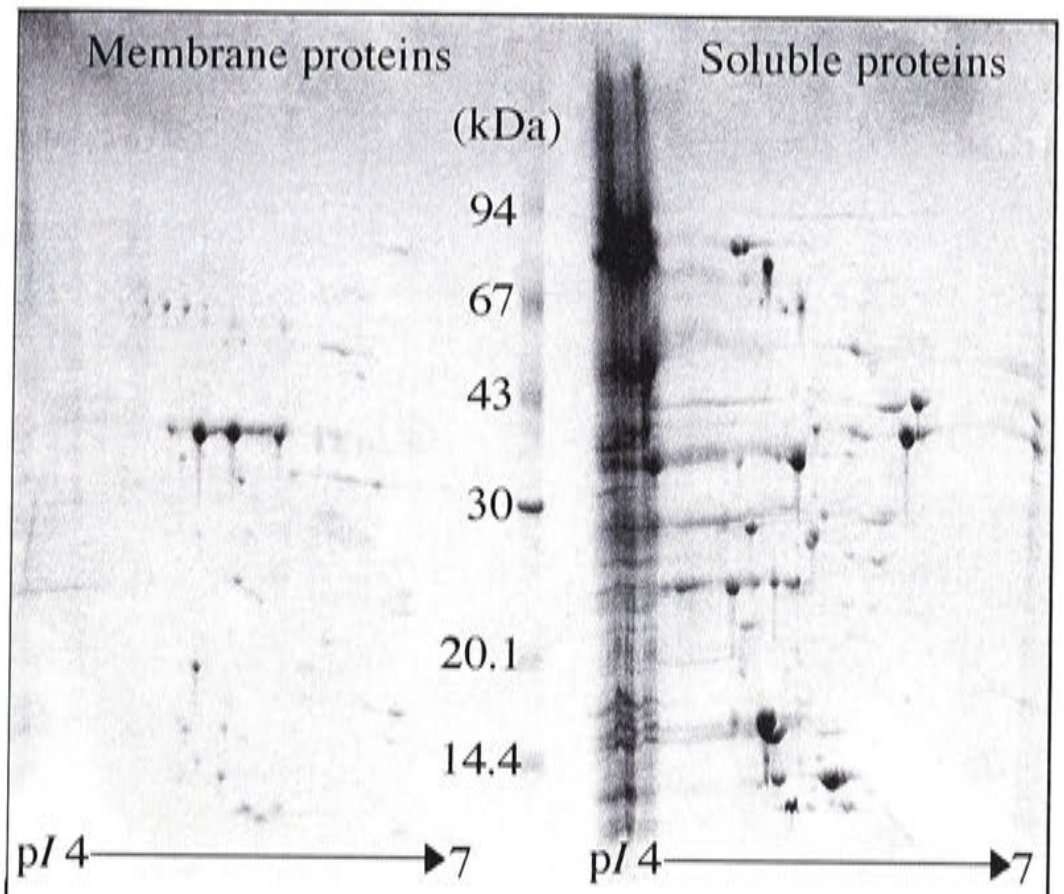
Control sera from Australian Red Cross Blood Bank volunteers was used to determine if the immunoreactive proteins identified in this study were recognised by *Shigella*-specific antibodies in the patient sera or by cross-reactive antibodies produced against common proteins of the gut microflora. The control sera are from Australia, which has a very low incidence of shigellosis, thus, the sera should not contain antibodies raised against *S. flexneri* (275). However, some proteins are common amongst enteric bacteria, so it is

possible that the volunteers will have reactive antibodies to *Shigella* proteins homologous to other gut bacteria proteins. By probing the immunoblots with the sera, it is possible to determine if the immunoreactive proteins identified by the *S. flexneri* patient sera are cross-reactive enteric proteins, or are immunoreactive proteins likely to be specific to *Shigella*.

The membranes from all three repeats were stripped and reprobed with sera from the Australian Red Cross Blood Bank healthy volunteers. The resulting films had very little signal and did not display any spots, which could be aligned to a visible *S. flexneri* protein spot on the Coomassie-stained gel, and thereby could not be analysed by peptide mass fingerprinting. A representative control immunoblot, performed on the membrane used in repeat 1, is shown in Figure 3.4. Therefore, the proteins recognised by the antibodies in the *S. flexneri* patient sera do not appear to be cross-reactive immunoreactive proteins but specific to a *S. flexneri* induced immune response.

However, it was expected that a number of *S. flexneri* proteins would be recognised by the control sera; it is possible that the large 2-DE membrane size and low protein concentrations of the 2-DE immunoblots were too stringent for the possible weaker binding of cross-reactive antibodies in the control sera. The control sera were tested against *S. flexneri* and *E. coli* protein separated by 1D SDS PAGE, as the protein bands are far denser and more concentrated than the separated spots on a large 2-DE blot. Although only 40 μg of protein was separated on the 1D SDS PAGE gel, the protein is only separated over an area of 2 cm^2, whilst the protein on the 2-DE gel was separated over an area of 221 cm^2, meaning the density of each protein is much lower in the 2-DE blots than the 1D SDS-PAGE gels. The control sera did recognize a number of bands in both *E. coli* and *S. flexneri* protein 1D Western blots, indicating that sera was immunoreactive to *S. flexneri* proteins, but requires higher protein concentrations than what was used for the 2-DE blots (Figure 3.5A). Furthermore, the *Shigella* patient sera were also used in a 1D immunoblot with *S. flexneri* and *E. coli* protein (Figure 3.5B). The *Shigella* patient sera recognised many more proteins and bound more strongly to the *S. flexneri* protein, indicating that the majority of antibodies present in the sera are *Shigella* specific.

A



B

Figure 3.4. Control immunoblot of *S. flexneri* protein with uninfected sera. **A.** Coomassie blue-stained 2D gel of *S. flexneri* membrane and soluble protein preparations. **B.** Immunoblot of *S. flexneri* membrane and soluble protein preparations. No immunoreactive proteins were identified.

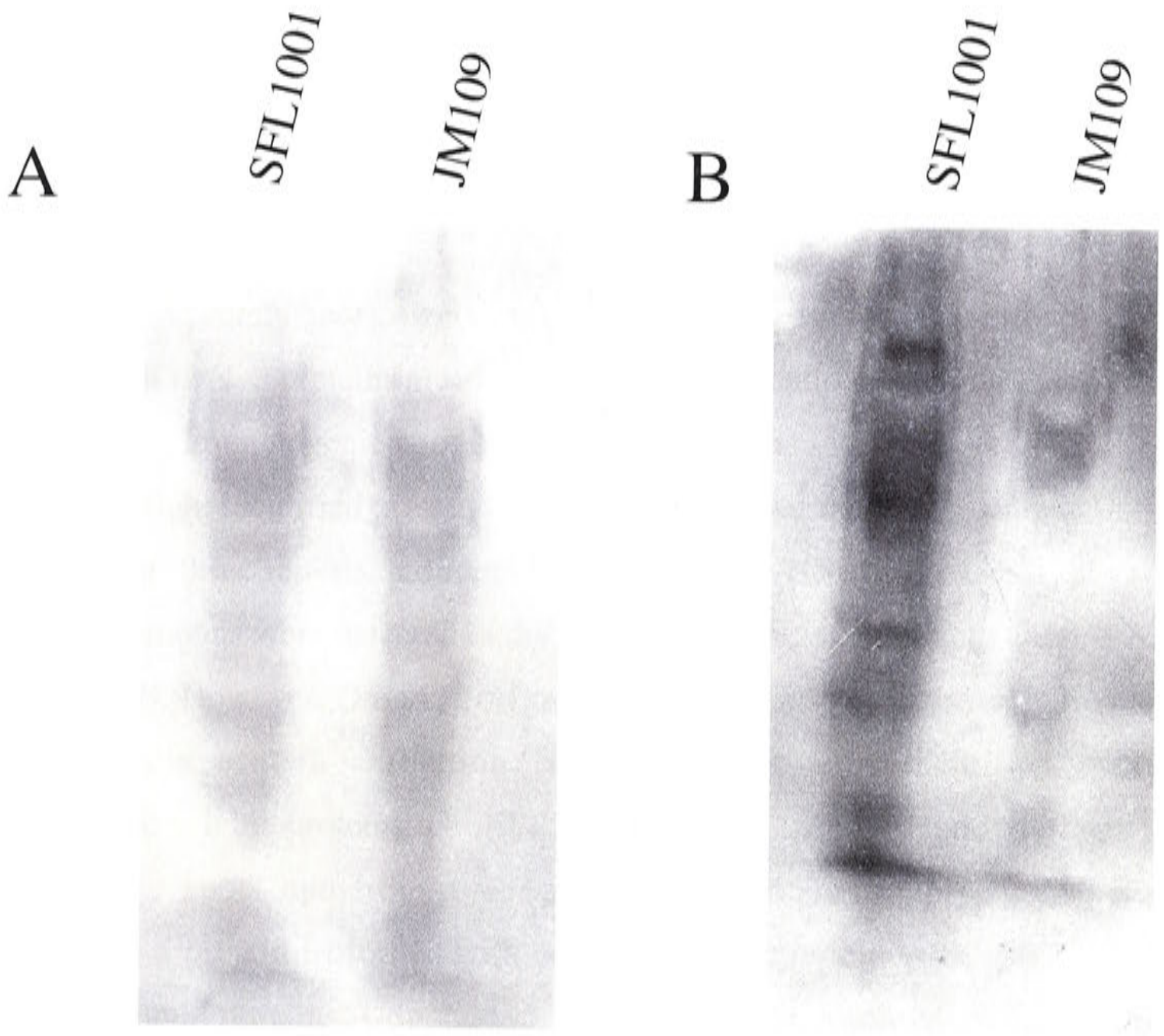


Figure 3.5. Immunoblotting of *Shigella* patient and control sera. *E. coli.* SFL1001: *S. flexneri* 2457T 2a strain, and the *E. coli* JM109 strain. **A.** Control 1D immunoblot for *S. flexneri* and *E. coli*. Membrane was probed with sera from uninfected healthy volunteers. Both *E. coli* and *S. flexneri* possess some proteins that are seroreactive to the control sera. **B.** *Shigella* 1D immunoblot for *S. flexneri* and *E. coli*. Membrane was probed with sera from *Shigella* patients. The sera recognises many more *S. flexneri* proteins than *E. coli.*

Identical protein loading?

3.2.4 Identified immunoreactive proteins

Protein spots corresponding to an immunogenic spot from the immunoblot film were excised from the gels and submitted to in-gel tryptic digestion and MALDI-TOF analysis. The peptide mass fingerprints of these proteins were searched against the MSDB database using the MASCOT peptide mass fingerprinting search engine (Matrix Science), as described in Chapter 2 (286). The significant protein matches, according to their Mowse score ($p<0.05$), were in all cases, a *S. flexneri* protein.

Of the aligned protein spots, twelve proteins were successfully identified by peptide mass matching. All twelve proteins are shown as both the protein spot on the 2-DE gel and as the corresponding immunogenic spot on the matching films in Figures 3.1, 3.2 and 3.3. Table 3.1 lists the eight identified proteins from the soluble and membrane preparations that were detected in all three repeats and identified by MALDI-TOF from at least two gels. Five of these eight proteins were detected in the membrane protein extraction: OmpA, AnsB, TolC, Ggt and TolB. However, OmpA, TolC and AnsB were also detected as immunogenic spots in the soluble protein extraction. Three proteins were identified exclusively as immunogenic soluble proteins: GroEL, Spa33 and IpaD. OmpA and IpaD were detected as immunogenic spots numerous times in the one protein sample, suggesting they are occurring as a protein isoforms, with each isoform retaining its antigenic characteristics. The four soluble proteins, Udp, S3195, YfiD and MglB were only detected in one repeat and were not included in the final short list of proteins, which required identification in all three repeats.

3.3 Discussion

3.3.1 Known immunogenic proteins identified for *S. flexneri*

A total of eight proteins were successfully identified in three separate 2-DE gel immunoblots with a pool of *S. flexneri* patient sera. Of the eight immunogenic proteins, only two are known to be immunoreactive for *S. flexneri*: IpaD and OmpA.

IpaD is a secreted protein essential for entry into epithelial cells and has been reported to be antigenic in a number of *S. flexneri* studies (112, 197, 262, 264, 380). However, it remains

Spot no.	Accession number (Genbank)	Name	Description	Observed p*I*/Mw (kDa)	Predicted p*I*/Mw (kDa)	Coverage (%)	No. of peptides matched	Mowse Score
1	GI:30042642	TolC	outer membrane channel	5.10/65.0	5.35/53.8	17	13	187
2	GI:38000008	OmpA	outer membrane protein A (fragment)	5.00-5.50/42.0	5.47/35.3	38	8	104
3	GI:13310620	IpaD	37 K membrane antigen	5.80/40.0	5.65/36.6	39	9	128
4	GI:30042535	AnsB	periplasmic L-asparaginase	6.10/38.0	5.95/36.8	22	13	87
5	GI:17380384	GroEL	60 kDa chaperonin, protein Cpn60	4.80/68.0	4.85/57.3	18	7	83
6	GI:32307045	Spa33	surface presentation of proteins antigen	5.80/68.0	5.67/33.4	23	4	59
7	GI:30065275	Ggt	gamma-glutamyltranspeptidase	5.00/65.0	5.46/61.8	15	4	61
8	GI:30040341	TolB	Involved in the tonB-independent uptake of group A colicins	6.50/46.0	7.03/46.0	18	6	68

Table 3.1. Table of *S. flexneri* immunoreactive proteins, detected in three repeats, identified by MALDI-TOF analysis. The observed p*I* and molecular weight of protein was calculated from the protein spot's position on the Comassie blue G250 2D gel. The experimental p*I* and molecular weight (kDa) of the protein was calculated from MSDB database and the ExPASy p*I*/Mw calculator. The percentage coverage indicates the percentage of the protein which is covered by the matching peptides. The number of peptides from the MALDI-TOF analysis matched for each significant identification are indicated. The mowse score is a measure of the probability that the match is not a random match ($p<0.05$), generally a score over 57 is significant. All scores listed were significant.

unclear whether anti-IpaD antibodies contribute to protective immunity in humans (229). All four Ipa proteins have previously been reported to be immunogenic in natural infection; yet IpaA, IpaB and IpaC were not detected as antigens in this immunoproteome study (262). A two-dimensional proteome map of *S. flexneri* 2457T generated by Liao *et al* (2003) was also unsuccessful in detecting any of the Ipa proteins besides IpaD (200). Likewise, all three previous immunoproteomic studies for *S. flexneri* were unsuccessful in detecting any of the Ipa protein series as immunogenic (282, 414, 416). It is possible that IpaA, IpaB and IpaC are not present in the 2-DE gels at sufficient protein concentrations for detection by the Coomassie blue G250 staining. The number of *S. flexneri* protein spots visualised on the Coomassie blue G250 gels is quite low, possibly due to insolubility problems during the protein extractions or low resolution of the pI 4-7 Immobiline strips used. A number of immunogenic spots detected on the immunoblot X-ray film could not be matched to a corresponding Coomassie-stained spot, suggesting that some proteins are simply present in concentrations not detectable by Coomassie blue G250. It would be possible to improve the resolution of the 2-DE gels by using silver staining, which increases the sensitivity of protein detection by an order of magnitude over Coomassie blue, but often produces inferior results with MALDI-TOF analysis (29). Alternatively, fluorescent stains that are readily compatible with MALDI-TOF could increase the sensitivity of protein detection. Fluorescent staining was not possible during this study as a fluorescent scanner was not available, however, the Australian National University has recently purchased one, so fluorescent staining could be utilised in future work.

OmpA is a 35 kDa outer membrane protein and was identified multiple times during the immunoproteome analysis. The multiple spots for OmpA and IpaD differ in their p*I* value, which is suggestive of post-translational modifications that have caused a charge change to the protein. Multiple charge variants of OmpA have previously been reported for *E. coli* and *Porphyromonas gingivalis*, although the precise modification or protein conformational change responsible is unknown (236, 383). There is no mention in the literature of any known post-translational modifications for IpaD. OmpA is most likely a constituent of the major outer membrane proteins described as immunoreactive in *Shigella* patients and thus, is known to be immunogenic (262). Furthermore, OmpA has been the subject of a recent

vaccine study, which found that it was capable of inducing a protective immune response in rabbits, providing significant protection against challenge with virulent *S. flexneri* (243).

3.3.2 Six novel immunogenic proteins identified for *S. flexneri*

The other six proteins identified in three repeats have not previously been shown to be reactive to antibodies found in patients with natural *S. flexneri* infection. However, the *E. coli* L-asparaginase II (AnsB) protein which shares 99.1% aa identity with *S. flexneri* AnsB, and the highly conserved chaperonin GroEL found in a number of bacterial species, have been reported as antigenic (53, 425). The remaining four proteins are potential candidates for *Shigella*-specific antigenic and immunisation studies and could ultimately be useful in the development of new *Shigella* vaccines. However, being immunogenic does not necessarily mean that an antigen is capable of producing a protective immune response, with as little as 2.5% of antigens displaying protective properties (38). Alternatively, because these antigenic proteins are clearly expressed during invasion of the host as they are recognised by the host immune response, they may be important for pathogenesis. Any such proteins may be potential virulence factors and could be used in attenuation of live vaccine strains.

Membrane and surface associated proteins are often immunogenic due to their likelihood of being exposed to the host's immune response. A number of the immunogenic proteins identified in this study are membrane or surface displayed proteins. Three outer membrane proteins were identified as immunogenic, OmpA, TolC and TolB. TolC plays a role in outer membrane permeability and has been used as an epitope carrier in *Salmonella*; while TolB is involved in the import of colicins and acts as a drug efflux protein (182, 347, 360). Spa33 is a surface displayed protein involved in the regulation of IpaB and IpaC secretion from the cell, an important virulence step during *S. flexneri* invasion (336). Although Spa33 was identified as a significant match by MASCOT with the peptide mass fingerprints produced from the corresponding protein spot, the observed molecular weight is inconsistent with the predicted mass. Spa33 is observed as a 68 kDa protein in Figures 3.1, 3.2 and 3.3, but the predicted size is 33.4 kDa. This size discrepancy may be due to Spa33 interacting with itself to form a dimer or possibly forming a complex with another protein,

which was not detected in the consequent peptide mass fingerprinting. There is no evidence in the literature for Spa33 forming homodimers, although it is possible that Spa33 interacts with Spa32, which is predicted to be a protein of 35.6 kDa (336).

Often periplasmic proteins are immunogenic, particularly when they are found extracellularly where they are exposed to the host's immune response. In this study, two metabolic periplasmic enzymes, Ggt and AnsB, were identified; gamma glutamyltranspeptidase (Ggt) is a periplasmic enzyme, essential for the utilisation of gamma-glutamyl peptide as an amino acid source and L-asparaginase II (AnsB) is a high affinity periplasmic enzyme induced by anaerobiosis (117, 353). Both AnsB and Ggt were identified from the membrane extraction of *S. flexneri* proteins. It is possible that the membrane preparation contains some contaminating soluble proteins. The protein sequence of Ggt contains a signal peptide and has 99.7% aa identity to *E. coli* K12 Ggt, which also contains a signal peptide and localises to the periplasm (354). However, the mammalian form of Ggt is linked to the plasma membrane and *Neisseria meningitidis* Ggt associates with the inner membrane; suggesting that the presence of *S. flexneri* Ggt in the membrane fraction may not simply be inefficient separation of membrane proteins from soluble proteins, but perhaps due to a membrane association of the protein.

Three of the four proteins that were identified as immunoreactive in only one repeat, Udp, S3195 and YfiD, have never been reported as immunogenic for any bacterial species. MglB has previously been identified as immunoreactive in the *S. flexneri* rabbit antisera immunoproteome study of extracellular proteins (415).

3.3.3 Control immunoblots showed *Shigella* specificity of immunoreactive proteins

Control immunoblots using sera from five healthy individuals, were performed with each of the three membranes. It can be assumed that these five individuals have previously been exposed to commensal gut microflora, including *E. coli* of which *Shigella* is a clone (299). Consequently, the control sera should contain antibodies reactive to *E. coli* and other commensal bacterial proteins, which may potentially recognise homologous *Shigella* proteins (283). Thus, the control immunoblots can be used as a baseline, where any

Shigella proteins recognised by the control sera are most likely reacting with antibodies raised against the commensal gut microflora. Consequently, immunogenic proteins, detected only in the *Shigella* immunoblots and not in control blots, can be considered part of a *Shigella*-specific immune response. The control immunoblots did not generate any spots that aligned to the eight *S. flexneri* protein spots from the *S. flexneri* patient immunoblots, suggesting that the eight antigenic proteins identified are reacting to anti-*Shigella* specific antibodies in the *Shigella* patient sera. The overall lack of reactivity of the control sera to *S. flexneri* protein preparations was unexpected as many *S. flexneri* proteins share significant homology to commensal microflora proteins, so a level of immune recognition in the control sera was anticipated (292). The stripping protocol has been used prior to this study with no problems. It is possible that the potentially reactive protein species were too low in concentration and density on the membrane; especially as a 1D blot with dense bands of *S. flexneri* protein did yield some reactive proteins, all cross-reactive to *E. coli*, indicating that they are common antigens.

3.3.4 Significance of this study

This is the first *S. flexneri* proteomic study of the immunoreactive proteins expressed during natural *Shigella* infection of humans. A similar study has been performed using immunised mice sera in 2-DE gel immunoblots of *S. flexneri* outer membrane protein and soluble protein preparations. In that study, 13 seroreactive proteins were identified, none of which correspond with the proteins identified in this study (282). Another group has visualised immunoproteomes for *S. flexneri* protein probed with sera from immunised rabbits (282, 414, 416). Four proteins, GroEL, MglB, OmpA and TolC identified as immunoreactive in this study were also detected in their work (415). As *S. flexneri* does not naturally infect or cause shigellosis-like symptoms in mice or rabbits, and the experimental animals were not immunised mucosally, it remains unclear how relevant the seroreactive proteins identified in the previous *S. flexneri* immunoproteome studies are to the natural human anti-*S. flexneri* immune response.

Further analysis of the *S. flexneri* immunoproteome profile with human sera should be performed. There was some variation observed across the gels with four proteins Udp, S3195, MglB and YifD, only being matched as immunogenic in one repeat each. The same

sera was used in all three immunoblots, suggesting that the variation has occurred during the three separate protein extractions and/or in electrophoresis and immunoblotting conditions. To avoid temporal variation in the protein samples, all future repeats should be performed simultaneously. Furthermore, the 2-DE separation and visualisation techniques, used in this study, could be improved, in order to identify more immunoreactive proteins. Higher resolution p*I* strips could help "zoom in" on acidic or basic proteins specifically and improve their focusing. As previously mentioned, more sensitive detection stains could be used rather then Coomassie blue. Fluorescence staining can detect protein spots that are of a concentration too low to be stained by Coomassie blue G250 (208). However, the MALDI-TOF identification may become a limiting step, as the ability to produce quality peptide mass fingerprints decreases relative to the sample concentration (423).

3.4 Conclusion

In this study, eight proteins with reactivity to sera from patients with *S. flexneri* infection were identified in three repeats using immunoproteomics. These proteins did not show seroreactivity to control sera from healthy volunteers, suggesting that their immunoreactivity is specific to *S. flexneri* infection. Six of these identified proteins have not previously been reported as immunogenic in *S. flexneri* natural infection. These immunoreactive proteins could be novel candidates for vaccine development. Additionally, such proteins have great potential for roles in virulence, as it seems likely they are expressed by the bacteria during the infection of the host, making them prospective attenuation targets for live vaccine construction. The application of a number of these proteins to *S. flexneri* vaccine research is the subject of further chapters of this thesis.

Chapter 4
The role of Udp and S3195 in *S. flexneri* 2457T virulence

Chapter 4

The role of Udp and S3195 in *S. flexneri* 2457T virulence

4.1 Introduction

Proteins expressed during the invasion steps of the host are often important to a pathogen's virulence. These proteins are frequently immunogenic as they are present within the cell or upon the cell surface during the steps of invasion, and are consequently seen by the host's immune response. Thus, immunogenic proteins may also be important to the bacterium's virulence.

The shortlist of immunogenic proteins generated for *S. flexneri* in Chapter 3 are not only interesting to study from a vaccine antigen perspective, but, as they could also possibly be candidates for roles in *Shigella* pathogenesis, they are also relevant to study from a virulence perspective. The discovery of new virulence genes for *S. flexneri* not only sheds light on the pathogenesis of shigellosis but also offers the possibility of new attenuation targets with which to develop live vaccine strains. The development of live attenuated vaccine strains has had reasonable success in *S. flexneri* thus far. These strains are often aromatic mutants carrying a deletion in a gene that reduces the replication of the strain sufficiently to prevent disease symptoms, but not enough that invasion and stimulation of the immune response cannot occur. More recently virulence genes required for intracellular survival and intercellular spread have also been used to create vaccine strains (161, 176). However, it is often extremely difficult to achieve equilibrium between the safety and efficacy of the strain; an attenuation target may not weaken the bacterium adequately or conversely, it may over-attenuate so that a protective immune response is not induced. Studies to find the most suitable attenuation candidates for *S. flexneri* are ongoing. Consequently, the discovery of new virulence genes is a valuable contribution to research into the production of improved attenuated *S. flexneri* vaccines.

As shigellosis is only a disease of humans and higher primates, there is no simple intestinal animal model available. Adult mice are resistant to oral doses of *Shigella* and whilst it is

possible to generate histologically detectable intestinal inflammation in orally inoculated newborn mice, this model has not yet been widely used in virulence studies (90). Classic *Shigella* virulence studies often involve the Sereny test performed in guinea pigs and cell culture experiments such as the plaque assay (266, 339). The Sereny test requires *Shigella* inoculation into the eye of a guinea pig, where the degree of *Shigella* invasion can be quantitated by the severity of keratoconjunctivitis that develops. Non-virulent forms of *S. flexneri* are asymptomatic in the Sereny test. A number of cell culture assays can also be used for assessing the virulence of *S. flexneri*; the plaque assay which measures intercellular invasion and spread, the invasion assay which detects cell entry, and the survival assay which determines the percentage of *Shigella* survival within the epithelial cell. By screening the immunogenic proteins selected in this Chapter with a combination of these techniques, it was hoped that a new *S. flexneri* virulence factor(s) would be identified and characterised.

4.2 Results

4.2.1 Selection of *S. flexneri* 2457T virulence candidates

The immunoproteome screen, performed in Chapter 3, identified 8 proteins that were detected in three repeats of 2D-E immunoblotting. These 8 proteins were then considered for use in this virulence study of *S. flexneri*, where selection was based on any literature available on the protein, the proposed function, and homology of the protein amongst other bacterial species. Proteins selected were to be relatively uncharacterised in *S. flexneri* and preferably not involved in general housekeeping pathways that show high homology across many species of bacteria. Using this selection criteria, 6 of the 8 protein candidates were not chosen for further virulence examination; IpaD and Spa33 are *Shigella* specific proteins but have previously been shown to be important for the invasion of *S. flexneri* (293, 336), while OmpA, TolC, TolB and GroEL are highly conserved amongst a wide range of bacterial species and have been extensively studied in *E. coli* (41, 166, 171, 390).

AnsB, a periplasmic L-asparaginase, which converts asparagine into aspartate and ammonia, was chosen due to its proposed metabolic function in amino acid catabolism which might be sufficient to reduce bacterial fitness in some way (147). Likewise, the

gamma-glutamyltranspeptidase, Ggt, was selected as a suitable candidate due to its role in amino acid metabolism and studies in *Helicobacter pylori* that show that *H. pylori* Ggt is a virulence factor involved in colonisation (54, 225).

Furthermore, during the immunoproteome analysis described in Chapter 3, a number of proteins were identified as immunoreactive in only one of the three repeats. Although not included in the final list of immunoreactive proteins for *S. flexneri*, which required identification in all 3 repeats, this list of proteins was also considered for this virulence study, and a further 4 proteins were chosen for virulence analysis. These proteins included YfiD, a formate acetyltransferase whose homologue is involved in stress response in *E. coli*, where disruption of the gene results in a microaerobic growth defect (411). The D-galactose periplasmic binding protein, MglB, was also selected as it has been shown to be important in *E. coli* glucose scavenging in conditions of micromolar glucose (69). Since the level of glucose available in the intestine is believed to be micromolar, it is possible that the scavenging pathway that MglB belongs to may contribute to the survival of *S. flexneri* during invasion (89).

Uridine phosphorylase (Udp) was also chosen for assessment as a virulence candidate. Udp catalyses the reversible phosphorolysis of uridine to uracil and ribose-1-phosphate, and is considered a key enzyme in the pyrimidine salvage pathway of *E. coli*. Udp cleaves pyrimidine nucleosides for use as cellular nitrogen and carbon sources; catabolism of pyrimidine nucleosides allows turnover of RNA and the reuse of pyrimidine bases (37, 273). Disruption of this salvage pathway when the cell is exposed to a nutrient and energy-source limited environment could be sufficient to attenuate *S. flexneri* during host cell invasion.

Finally, a protein known as S3195, notated as an outer membrane fluffing protein was also chosen for this study (50). S3195 shows 100% amino acid identity to a protein in *S. flexneri* 3136, named Sap. Both Sap and S3195 have 88% identity to the *E. coli flu* gene product known as Antigen 43 (50). Antigen 43, an autotransported outer membrane protein involved in cell aggregation in *E. coli*, is often encoded by multiple alleles in wild type clinical isolates suggesting it plays a role in virulence or in phase-variable immune evasion

for *E. coli* ML308-225 (167). The function of Sap/S3195 in *S. flexneri* has not been determined.

4.2.2 Initial virulence analysis in SFL1001

In order to study the role of the 6 short-listed proteins in *S. flexneri* virulence, a knockout approach was taken. Disruption mutants were constructed in each of the 6 genes using two simultaneous approaches; a single stranded PCR insertion method and homologous integration with a suicide vector (64, 232). It was necessary to use numerous approaches as the production of gene knockouts in wild type *Shigella* has proven to be inefficient and difficult.

Shigella flexneri 2457T, known as SFL1001 was chosen as the parent strain in which to construct the mutants. SFL1001 was checked for the virulence plasmid by plating on Congo red TSB agar plates before being used as the parent strain for all gene disruptions. All six genes were then successfully disrupted by either the PCR or suicide approach in SFL1001. However, it was determined during a Sereny test that SFL1001 was avirulent *in vivo*, as no symptoms were produced in any guinea pigs even with a high 10^9 cfu dose. This was unexpected as the SFL1001 strain had been used for *in vitro* invasion assays previously and was invasive. Consequently, this strain and the 6 disruption mutants constructed in this background were not suitable for use in the planned virulence study. SFL1001 and the *mglB* mutant constructed in this background are further discussed in Chapter 5.

4.2.3 Construction of disruption mutants in SFL1704

S. flexneri 2457T was sourced from another *Shigella* laboratory that had recently confirmed their strain to be virulent by Sereny test. This strain was named and stored as SFL1704. Congo red TSB plating confirmed that SFL1704 possessed the virulence plasmid and colony PCR using the *apy* and *virG* primer sets detected and amplified the *apy* and *icsA* genes found on the virulence plasmid (Figure 4.1).

The construction of knockouts in *Shigella flexneri* has proven to be extremely difficult and time-consuming. The knockouts produced in the avirulent SFL1001, mentioned above, took

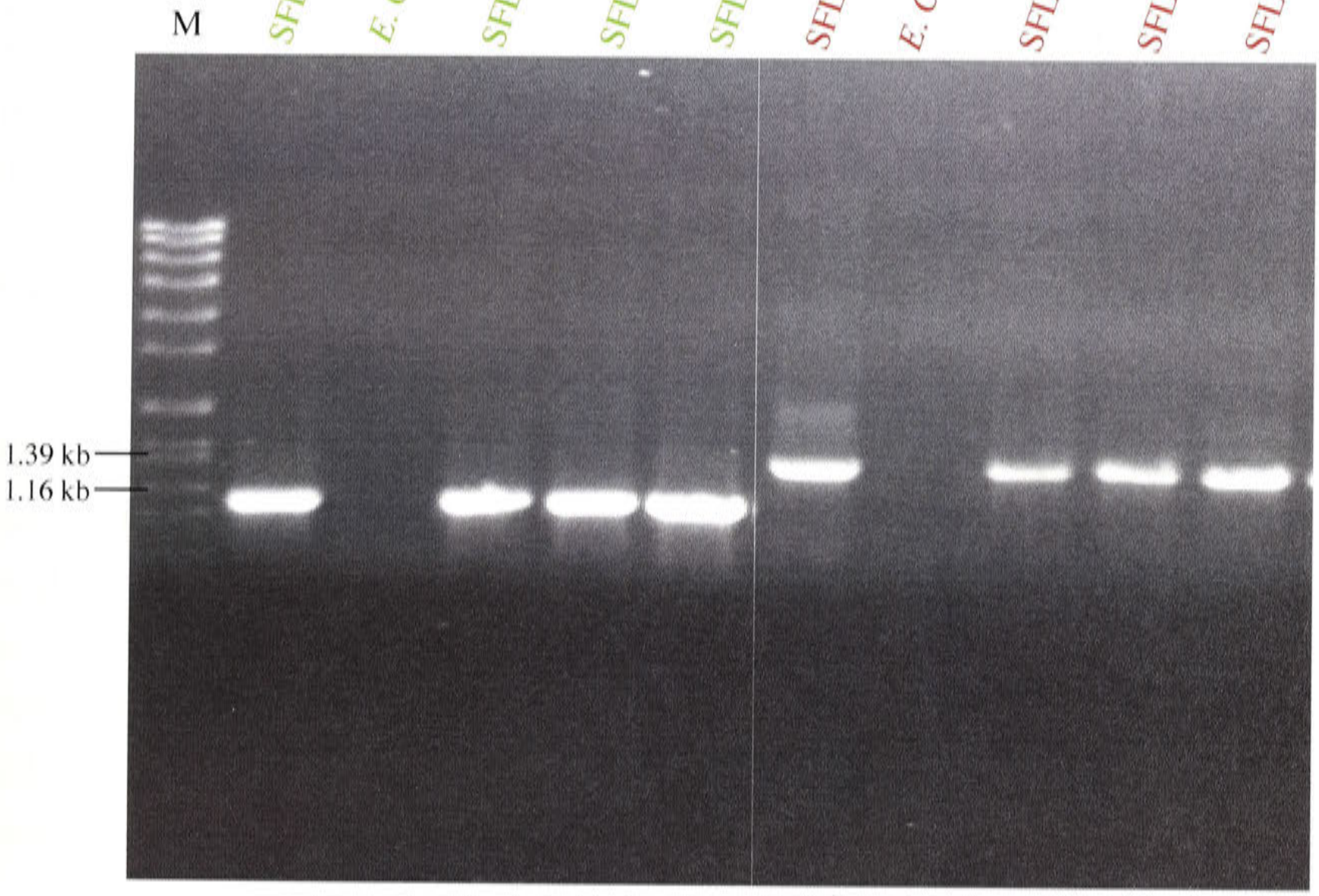


Figure 4.1. Colony PCR to confirm presence of virulence plasmid in *S. flexneri* strains. M: SPP-1 *Eco*RI marker (sizes in Figure 2.5). Some marker sizes are indicated. SFL1704: parent strain, *E. coli* JM109: negative control, SFL1726: *S3195* mutant, SFL1727: *udp* mutant, SFL1732: *udp* mutant with *udp* complemented on plasmid. Apy (in green) primers amplify the *apy* gene from the virulence plasmid, 1.1 kb, IcsA (in red) (VirG) primers amplify the *icsA* gene from the virulence plasmid, 1.4 kb.

nearly one year to produce. The same gene disruptions were attempted in SFL1704 using three different knockout approaches. As mentioned previously, both a PCR knockout approach and suicide vector technique were employed. Two variations to the PCR knockout method were utilised, the arabinose induced pKD46 helper plasmid was introduced into SFL1704 to make SFL1721, and the IPTG induced pKM208 helper plasmid was used to generate SFL1772 (64, 245). Despite the variety of techniques applied, it proved extremely difficult to produce gene disruptions for all 6 short listed proteins in SFL1704 in a reasonable timeframe. Only two gene disruptions were successfully produced for SFL1704 during this project, a knockout for *udp*, named SFL1726, and for *S3195*, named SFL1727. These two genes were the subject of the remainder of this study.

4.2.4 Confirming SFL1726 and SFL1727 as disruption mutants

Both SFL1726 and SFL1727 were constructed with the PCR knockout approach with pKD46 (64). PCR amplified fragments of the *cat* cassette containing 40 bps of flanking *udp* or *S3195* sequence, constructed with the primer sets UdpF/UdpR and OMFP290F/OMFP960R, were electroporated into SFL1721 cells and plated on chloramphenicol LB agar plates to select for integrants. Plates were incubated at 37 °C in order to lose the heat sensitive pKD46 helper plasmid.

Chloramphenicol resistant colonies were initially screened by colony PCR using the reverse cI primer from Datsenko *et al* (2000), which binds within the *cat* gene. When combined with a forward primer that binds at the start of the targeted gene, amplification should only occur when the fragment containing the *cat* gene has been inserted into the target gene. PCR bands were produced for *S3195* in SFL1726, using the primer OMFPFscreen and for *udp* in SFL1727, using the primer udpFscreen, and not in the control, SFL1704 (Figure 4.2). Additionally, PCR fragments spanning the target genes were amplified and analysed by sequencing with the primer sets OMFPcompF/OMFPcompR and udpcompF/udpcompR. In both SFL1726 and SFL1727 the disrupted gene was fused to the *cat* gene sequence at the expected junction point (Figure 4.3 and Appendix B). SFL1727 was studied more thoroughly in this Chapter and therefore was further confirmed as a correct knockout by Southern blot (Figure 4.4).

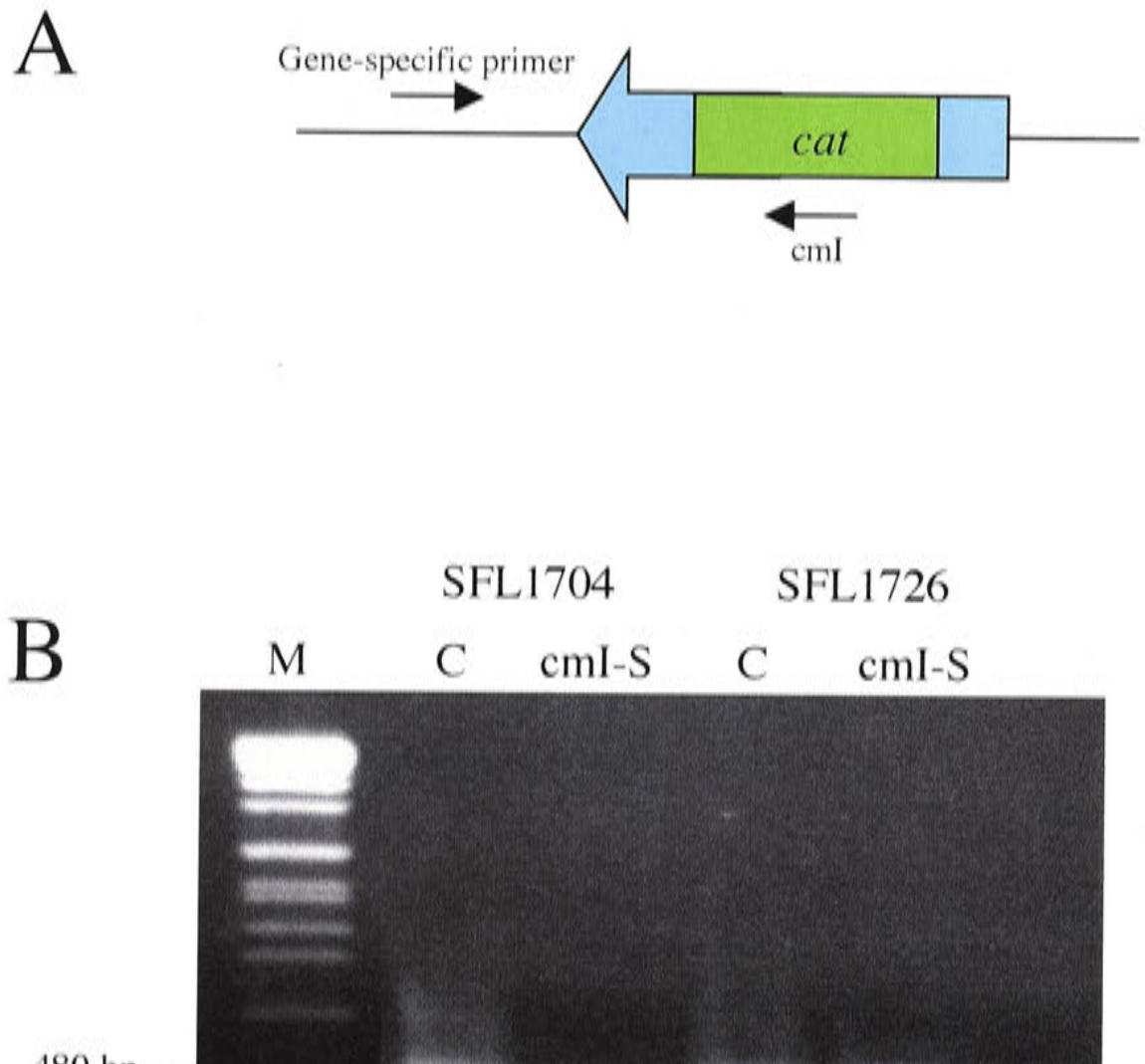


Figure 4.2. PCR screen for *cat* gene insertion into target gene. SFL1704: parent strain, SFL1726: *S3195* mutant, SFL1727: *udp* mutant. M: SPP-1 *Eco*RI marker (sizes in Figure 2.5). Some marker sizes are indicated. **A.** Amplification with cmI screening primer will only occur when *cat* gene is inserted in target gene. **B.** The absence of amplification from control strain, SFL1704 shows the specificity of primers. Control *malQ* PCR is used to show the integrity of SFL1704 template. C: control PCR using the primers MalQFSac and MalQRXba produces 400 bp, cmI-S: PCR with cmI/S3195 primer OMFPFscreen, 530 bp. **C.** cmI-U: PCR with cmI/udp primer UdpFscreen, 420 bp. C: control PCR, *malQ* 400 bp

A

S3195 homology

5' TTGCCTCCGAACTGGCCCGCGCACAGGGTAAACGTGGCGGTGTGGC
GGTTGCACTGTCTCTTGCCGTGTAGGCTGGAGCTGCTTCGAAGTTCC 3'

pKD3 homology

B

Udp homology

5' CCGGTTAAGCTGGCATCTCACCGCGAATTCACTACCTGGCGTGCAGAG
CTGGATGGTAAACCTGTTATCGTCTGCTCTACGTGTAGGCTGGAGCT
GCTTCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAA 3'

pKD3 homology

Figure 4.3. Forward sequencing of SFL1726 and SFL1727 gene insertion points. **A**. SFL1726 *S3195* sequence (red) showing 40 bp OMFP290F primer sequence (bold/underlined). pKD3 *cat* cassette sequence (black) at expected nucleotide location of *S3195* shows that disruption of *S3195* has been successful. **B.** SFL1727 *udp* sequence (red) showing 40 bp udpF primer sequence (bold/underlined). pKD3 *cat* cassette sequence (black) at expected nucleotide location of *udp* shows that disruption of *udp* has been successful. The complete forward and reverse sequence for *udp* and *S3195* are in Appendix B.

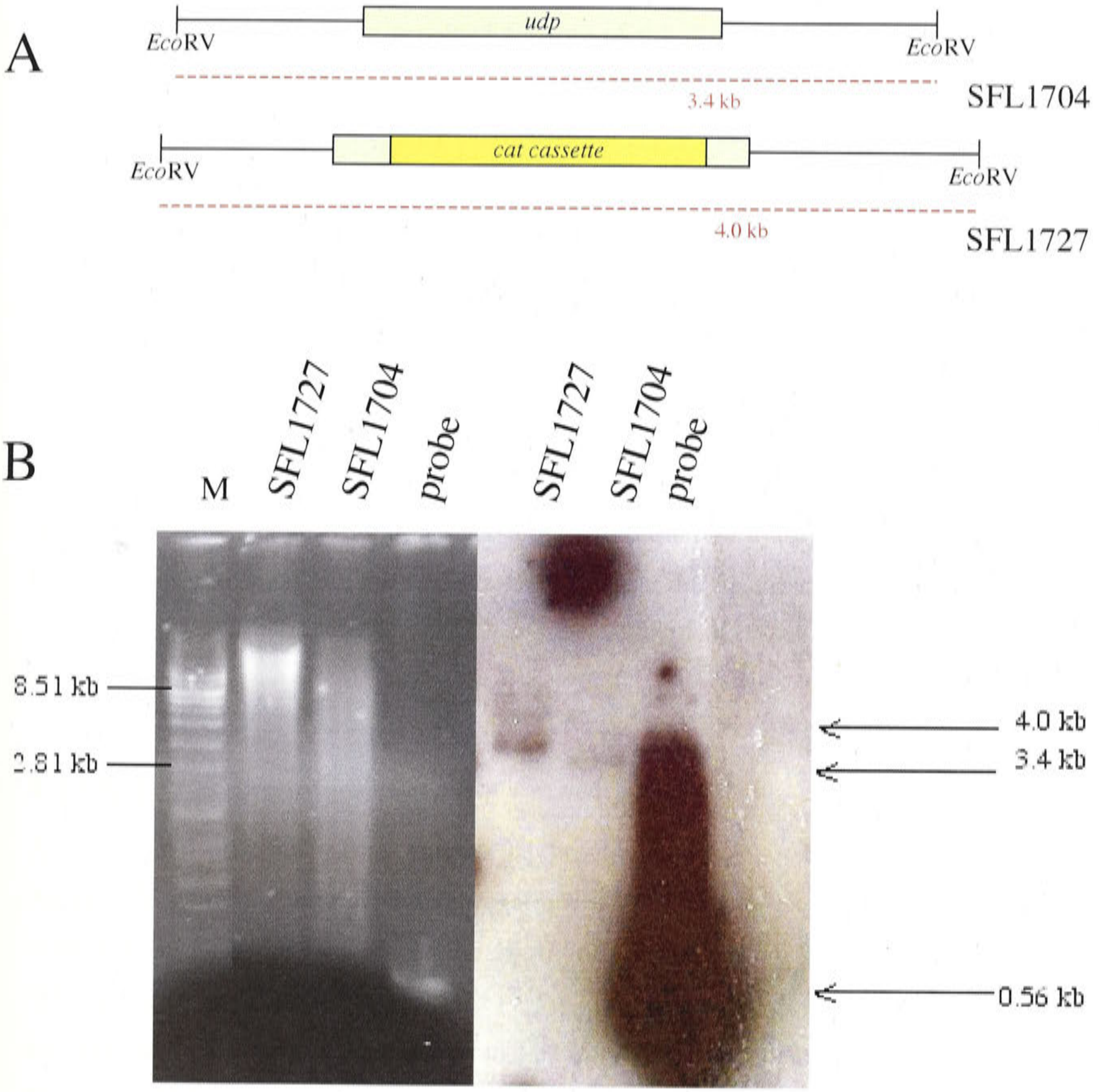


Figure 4.4. A. Insertion pattern of the *cat* cassette in *udp* with an *Eco*RV digest. **B.** Southern blot of SFL1727 to confirm insertion of 1.1 kb *cat* cassette into *udp*. SFL1704 is the parent strain. M: SPP-1 *Eco*RI marker (sizes in Figure 2.5). Some marker sizes are indicated. The probe is a 560 bp PCR fragment of the start of the *udp* gene amplified with the primers UdpSthnF and UdpSthnR. *Eco*RV digest should result in a 3.4 kb fragment recognised by the probe. Exchange of *udp* DNA with the *cat* cassette should generate a 4.0 kb fragment which binds the probe. The agarose gel is shown on the left-hand side and the film on the right-hand side.

SFL1726 and SFL1727 were screened for loss of the heat sensitive pKD46 by duplicate plating onto ampicillin agar plates. The presence of the virulence plasmid was checked with both Congo red plating and colony PCR, with the VirG and Apy primer sets (Figure 4.1). Although neither *udp* nor *S3195* are located within operons, the *cat* cassette carries a RBS at the end of it to ensure that the expression of downstream genes will not be interrupted.

The inserted *cat* cassette was maintained in SFL1726 and SFL1727 by always growing cultures in media containing chloramphenicol. Stability testing was performed on these strains, where the cells were grown in the absence of chloramphenicol for 6 hours and both strains were found to be over 99% stable. However, even if the *cat* insertion is lost, the target gene will not be functional as the gene sequence between the two regions of homology used to integrate the cassette has been lost during the double crossover process of integration.

4.2.5 SFL1726 phenotype testing

As mentioned previously, the Antigen 43 protein of *E. coli* is a homologue of S3195. Antigen 43 confers bacterial autoaggregation, which can be visualised as the settling of cells from static liquid cultures. Deletion of Antigen 43 from *E. coli* results in the loss of the autoaggregation phenotype (315). To determine if SFL1704 is capable of autoaggregation, and whether deletion of S3195 eradicates this phenotype, an aggregation test was carried out. After 24 hours, no aggregation was detected for either SFL1704 or SFL1726, and the optical densities did not significantly differ (Figure 4.5). Therefore, S3195 does not play a role in conferring an autoaggregation phenotype for *S. flexneri* cells. It remains unclear what function S3195 might have in *Shigella*.

4.2.6 Plaque assays of SFL1726 and SFL1727

The production of plaques in a plaque assay performed in an epithelial cell line, such as BHK, requires *Shigella* cells which are capable of cellular invasion, survival within the cell cytoplasm and intercellular spread. A deficiency at any of these virulence steps can be detected by either a reduction in plaque numbers or in the production of smaller plaques.

SFL1704 SFL1726

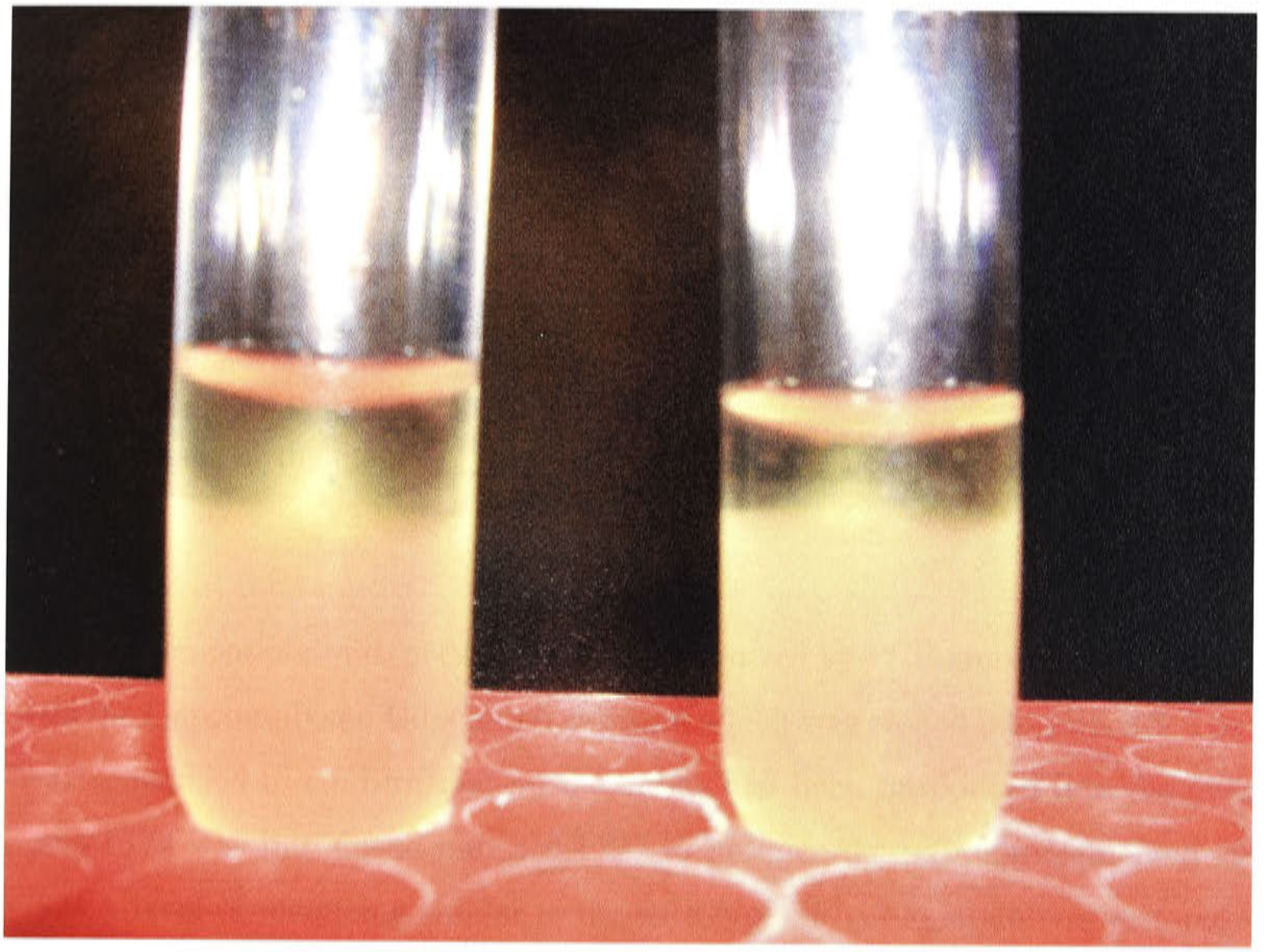


Figure 4.5. Autoaggregation test for SFL1704: parent strain and SFL1727: *S3195* mutant. Neither strain displayed any autoaggregation, which is detected as cells forming a tight pellet on the bottom of the tube after 24 hours in static culture.

This makes plaque assays a useful screen for changes in the virulence properties of *S. flexneri*. For this reason, SFL1726 and SFL1727 were initially analysed by plaque assay.

There was no change in the number or appearance of plaques formed by SFL1726 compared to the wild type SFL1704 strain (Figure 4.6). Thus, the disruption of *S3195* had no detectable influence on the invasion and intercellular spread of *S. flexneri* in epithelial cell culture. Consequently, it seems that S3195 does not play an important role in the virulence of *S. flexneri* and was not continued in this virulence study.

SFL1727 showed a significant reduction in the number of plaques formed in the plaque assays according to a t-test with SFL1704 (Figure 4.6). There was no observable difference in the size or shape of the plaques indicating that the process of intercellular spread has not been affected.

The *udp* complement plasmid, pNV1374, was constructed by PCR amplification of the *udp* gene with the UdpcompF and UdpcompR primers, which was cloned into pBS KS. The *udp* gene was sequenced to confirm that no mutations had been introduced into the gene by PCR. pNV1374 was introduced into SFL1727 to produce the *udp* complemented strain, SFL1732. A level of plaqueing similar to the wild type SFL1704 strain was restored with the complementation of the *udp* mutation in SFL1732.

4.2.7 Invasion assays of SFL1727

The SFL1727 *udp* mutant was further assessed in cell culture assays to narrow down its possible virulence defect. In order to test for any reduction in invasion of epithelial cells, invasion assays in HeLa cell monolayers were performed. An invasion assay measures the number of bacteria that have entered the cytoplasm of epithelial cells during a four hour infection process. Additionally, the assay can be used to measure the total number of infecting bacteria, which include not only the internalised bacteria but also the external bacteria that have adhered to the epithelial cell in the process of invasion. The ratio of the intracellular bacteria to total bacteria can be used to determine the total percentage of invasion.

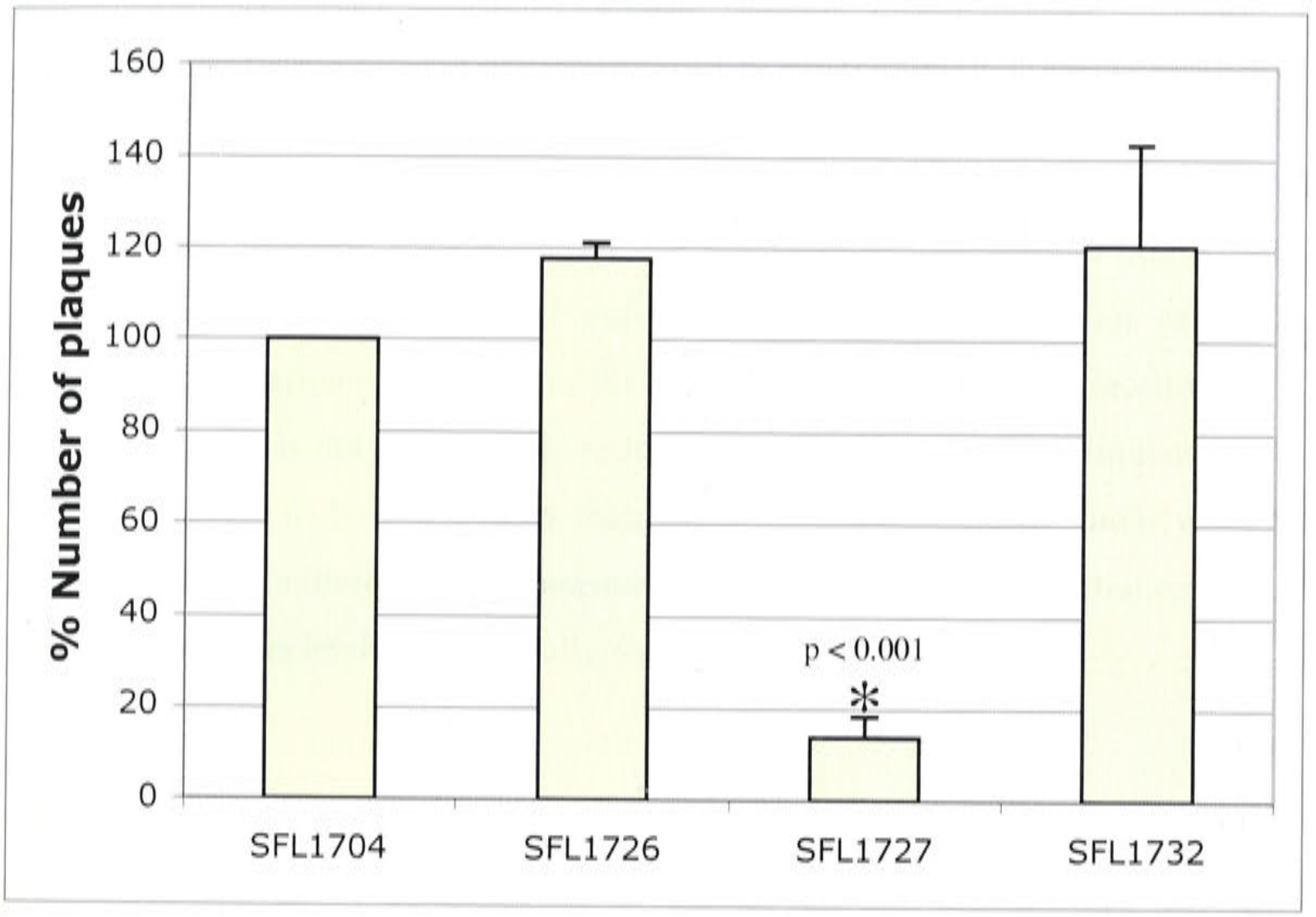


Figure 4.6. Plaque assay results for SFL1727 and SFL1726. Plaque numbers are expressed as a percentage of the number of plaques formed by the parent strain SFL1704. SFL1726: *S3195* mutant, SFL1727: *udp* mutant, SFL1732: *udp* mutant complemented with a plasmid based copy of *udp*. Results are the mean of three independent repeats with standard error. SFL1727 is significantly different to SFL1704 according to a t-test.

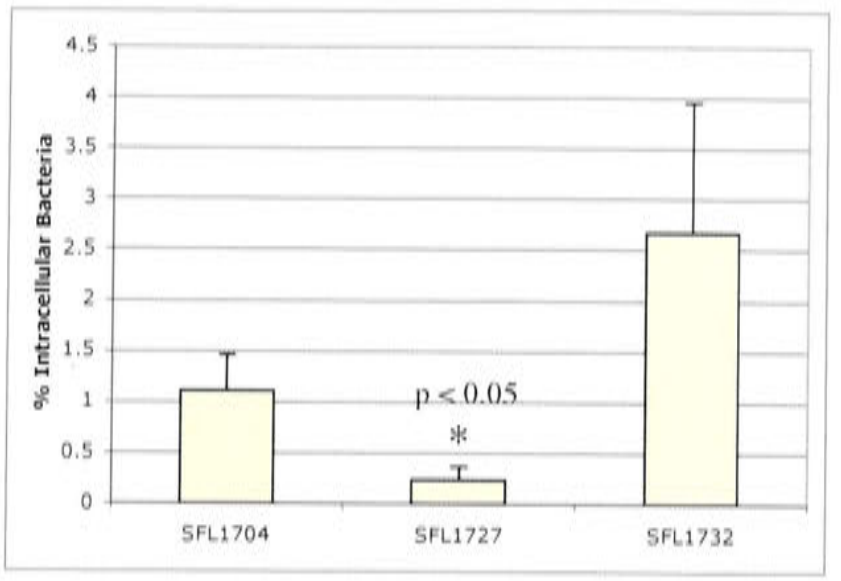
SFL1727 was significantly reduced in the number of intracellular bacteria when compared to the parent strain, SFL1704 by a t-test (Figure 4.7A). The level of invading bacteria could be restored to high levels when *udp* was returned to the *Shigella* cell by complementation on a plasmid in SFL1732. However, there was no significant difference in the number of total bacteria measured for SFL1727 and SF1732 in comparison to SFL1704 according to a t-test (Figure 4.7B). This suggests that SFL1727 is not defective in its ability to adhere to epithelial cells. Furthermore, the overall percentage of invasion was not determined to be significantly different between the parent strain, SFL1704, the *udp* mutant, SFL1727 and the *udp* complement, SFL1732 (Figure 4.7C). Therefore, the invasion assay data suggests that there is a deficiency in the number of SFL1727 cells present intracellularly, but that the actual invasion is not significantly reduced. Thus, it seems that the mutation of *udp* causes SFL1727 cells to be reduced in their ability to survive intracellularly, as SFL1727 is capable of cell adherence and invasion, but once inside the epithelial cell cytoplasm, the bacteria numbers begin to drastically reduce.

4.2.8 Survival assays of SFL1727

To determine if SFL1727 cells are unable to survive and replicate within an epithelial cell, it is possible to perform an *in vitro* survival assay in HeLa cells. The bacteria are left to infect cell monolayers for 90 mins before being treated with gentamycin to destroy all extracellular bacteria, defined as time 0, and the numbers of intracellular bacteria are counted until 6 hours post gentamycin treatment. An invasive strain of *S. flexneri* should show an increase in intracellular numbers from time 0 until approximately 4 hours post-treatment. After 4 hours the epithelial cells, which are loaded with *Shigella* will begin to lyse, releasing bacteria into the extracellular media containing gentamycin. This behaviour results in a slight decrease in intracellular bacteria numbers for the 6 hour time point as the exposed bacteria are killed by the gentamycin. A strain defective in intracellular survival should not display increasing numbers of intracellular bacteria.

As predicted, SFL1727 displayed reduced intracellular survival in cell culture (Figure 4.8). The numbers of intracellular SFL1727 bacteria were significantly reduced in comparison to SFL1704 and SFL1732 counts from 4 hours post treatment onwards (t-test, $p<0.05$). Once again, the provision of a plasmid based *udp* gene was sufficient to restore the virulent

A



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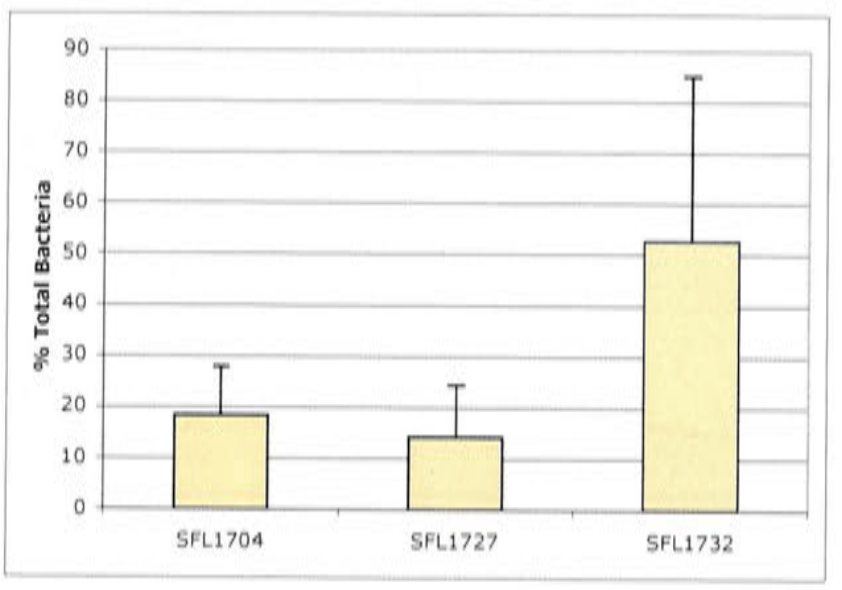


C

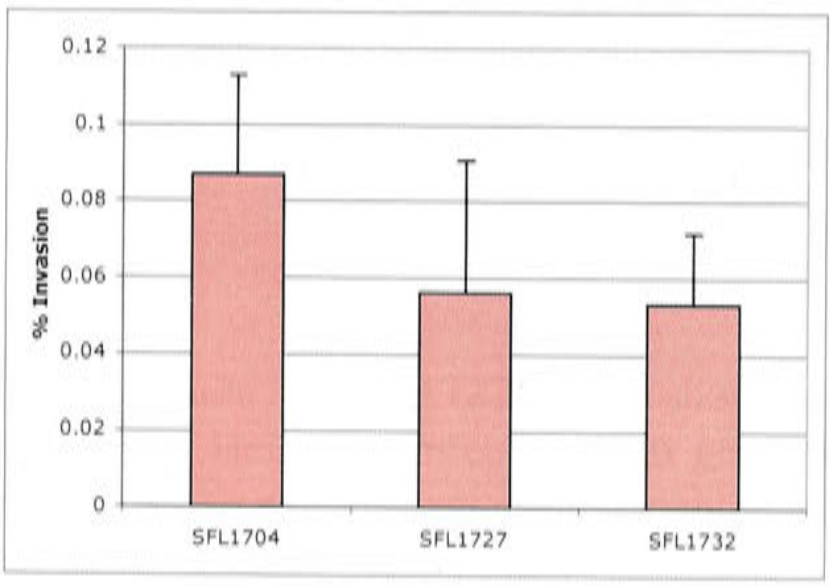


Figure 4.7. Invasion assay results for SFL1727. SFL1727: *udp* mutant, SFL1732: *udp* mutant complemented with a plasmid based copy of *udp*. **A.** Number of intracellular bacteria as a percentage of the infecting inoculum. **B.** Number of intracellular and adherent (total) bacteria as a percentage of the infecting inoculum. **C.** Percentage invasion where the total number of bacteria are divided by the intracellular numbers. Results are the mean of three independent repeats with standard error. SFL1727 is significantly different to SFL1704 in the numbers of intracellular numbers according to a t-test.

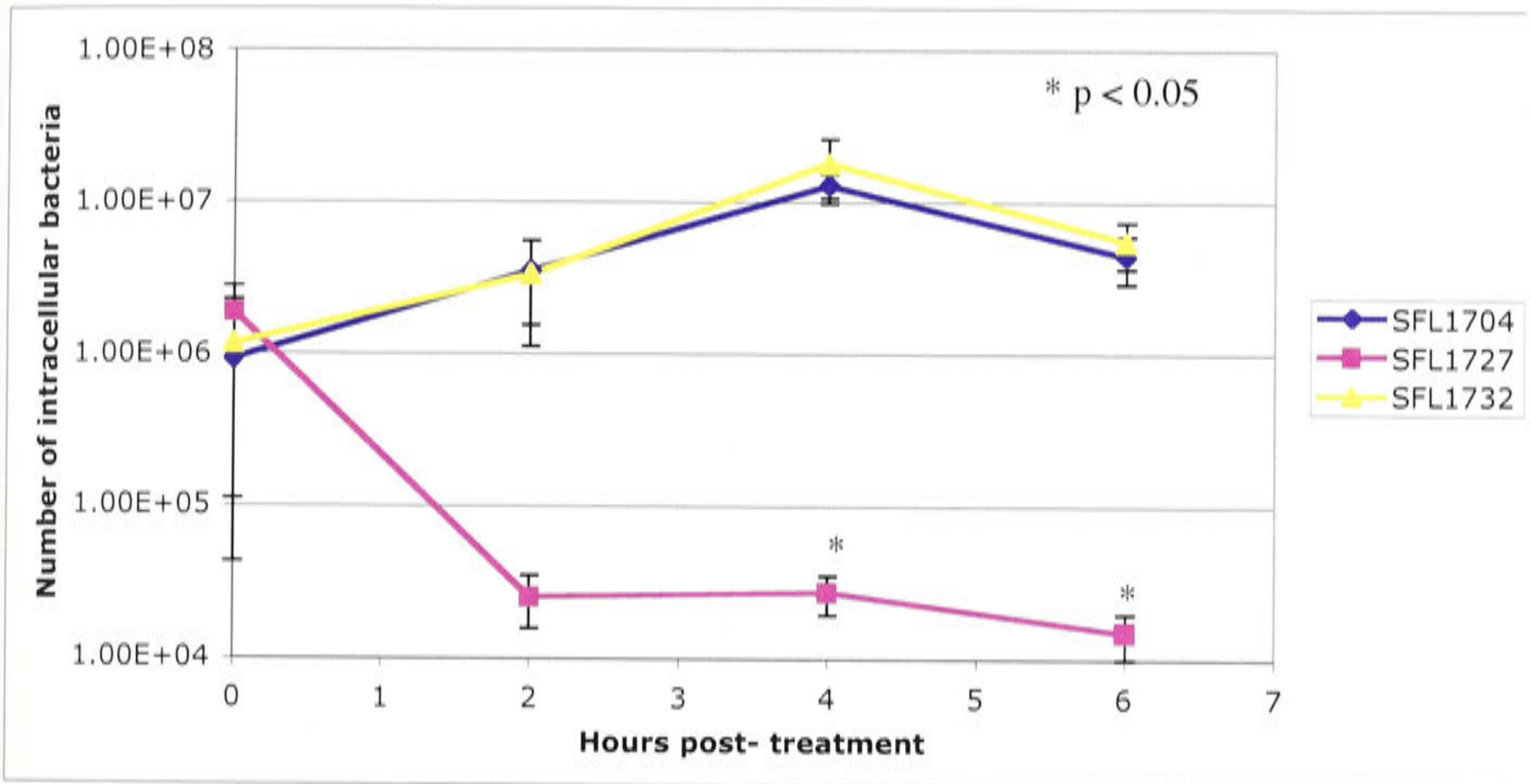


Figure 4.8. Survival assay results for SFL1727. Graph shows the number of intracellular bacteria found over 6 hours of HeLa cell infection after gentamycin treatment at time 0. SFL1727: *udp* mutant, SFL1732: *udp* mutant complemented with a plasmid based copy of *udp*. Results are the mean of three independent repeats with standard error. SFL1727 is significantly different to SFL1704 and SFL1732 at 4 and 6 hours post-treatment according to t-tests.

phenotype to the *udp* mutant cells. As discussed above, the expected growth pattern for intracellular survival of *Shigella* was observed in this assay and a slight reduction in intracellular bacteria numbers was detected for SFL1704 and SFL1732 at the 6 hour time point.

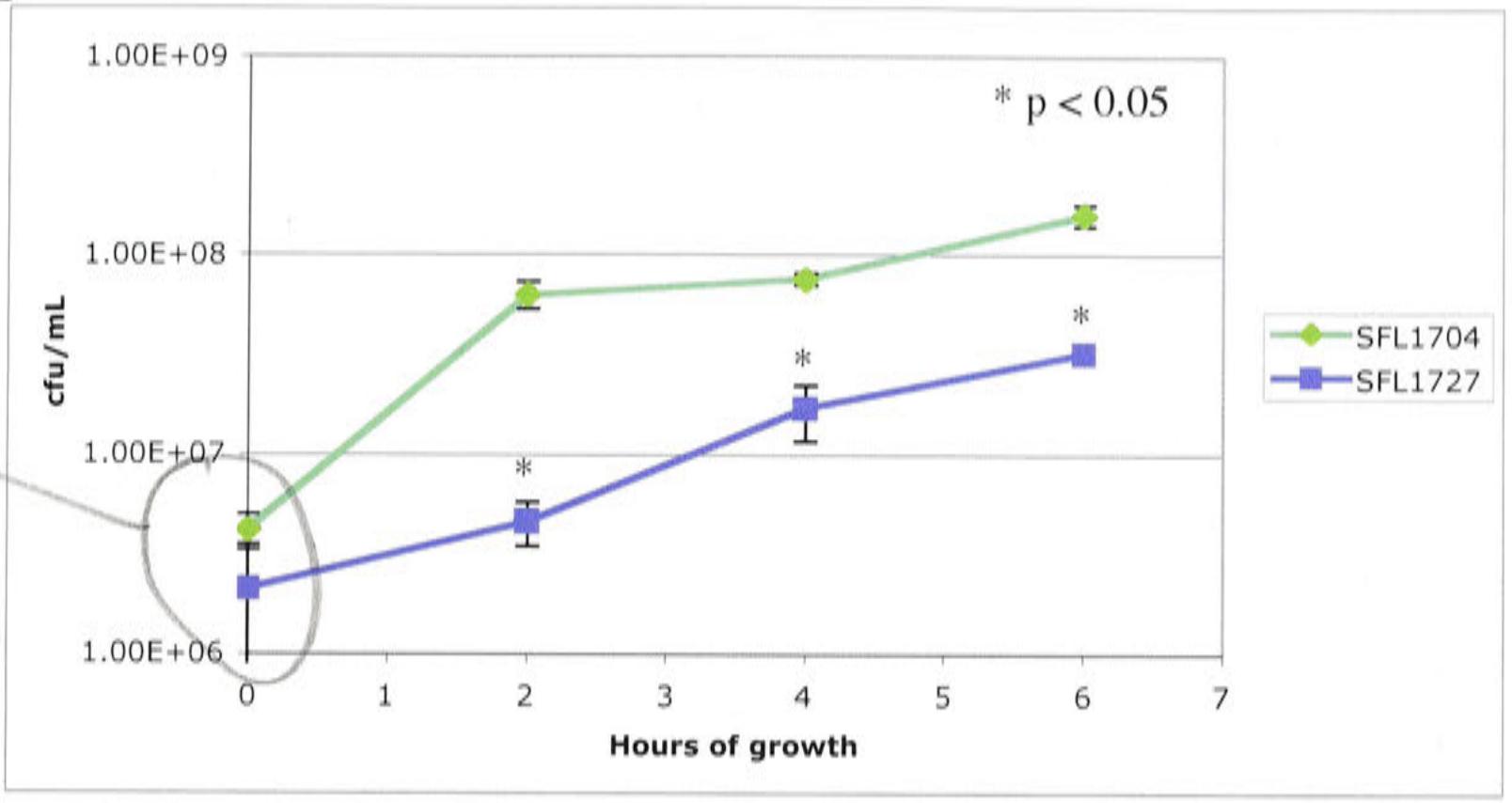
4.2.9 Growth of SFL1727 in minimal media

The defect in intracellular survival of the *udp* mutant, SFL1727, could possibly be the result of the strain's inability to catabolise nucleosides as carbon and nitrogen sources when encountering nutrient stress during replication within epithelial cells. There was no observable difference in the log phase growth of SFL1727 compared to the parent strain, SFL1704, when grown in the complex LB medium, during construction of growth curves. However, in order to detect a growth deficiency for SFL1727 it may be necessary to place the cell under nutrient stress. SFL1727 and SFL1704 cells were grown for 6 hours in M9 minimal salts medium containing only the *Shigella* growth factors methionine, tryptophan and nicotinic acid as carbon and nitrogen sources (Figure 4.9A). A significant reduction in the growth rate of SFL1727 was observed when compared to SFL1704 (t-test, $p<0.05$). Interestingly, it was possible to prevent this growth reduction for SFL1727 when the minimal media was supplemented with 0.5% glucose as a plentiful carbon source (Figure 4.9B). These growth curves support the hypothesis that the *udp* disruption leaves *Shigella* cells attenuated in growth during conditions of low carbon and nitrogen availability.

4.2.10 *in vivo* Sereny testing of SFL1727

in vitro tests are commonly used for *Shigella* virulence testing as the only natural animal shigellosis infections occur in monkeys. The use of monkeys in animal experiments is extremely expensive and ethically difficult. Alternatively, since 1957, the Sereny test in guinea pigs has been an accepted animal model experiment for evaluating *Shigella* virulence (339). In the Sereny test the invasion and multiplication of *Shigella* within the corneal epithelium causes keratoconjunctivitis, which closely mimics the invasion process in the intestinal epithelium. The degree of severity of keratoconjunctivitis can be rated according to the severity of the symptoms and the speed of disease development. The scoring system ranges from 0 for a normal eye, 1 for mild conjunctivitis, 2 for moderate

A



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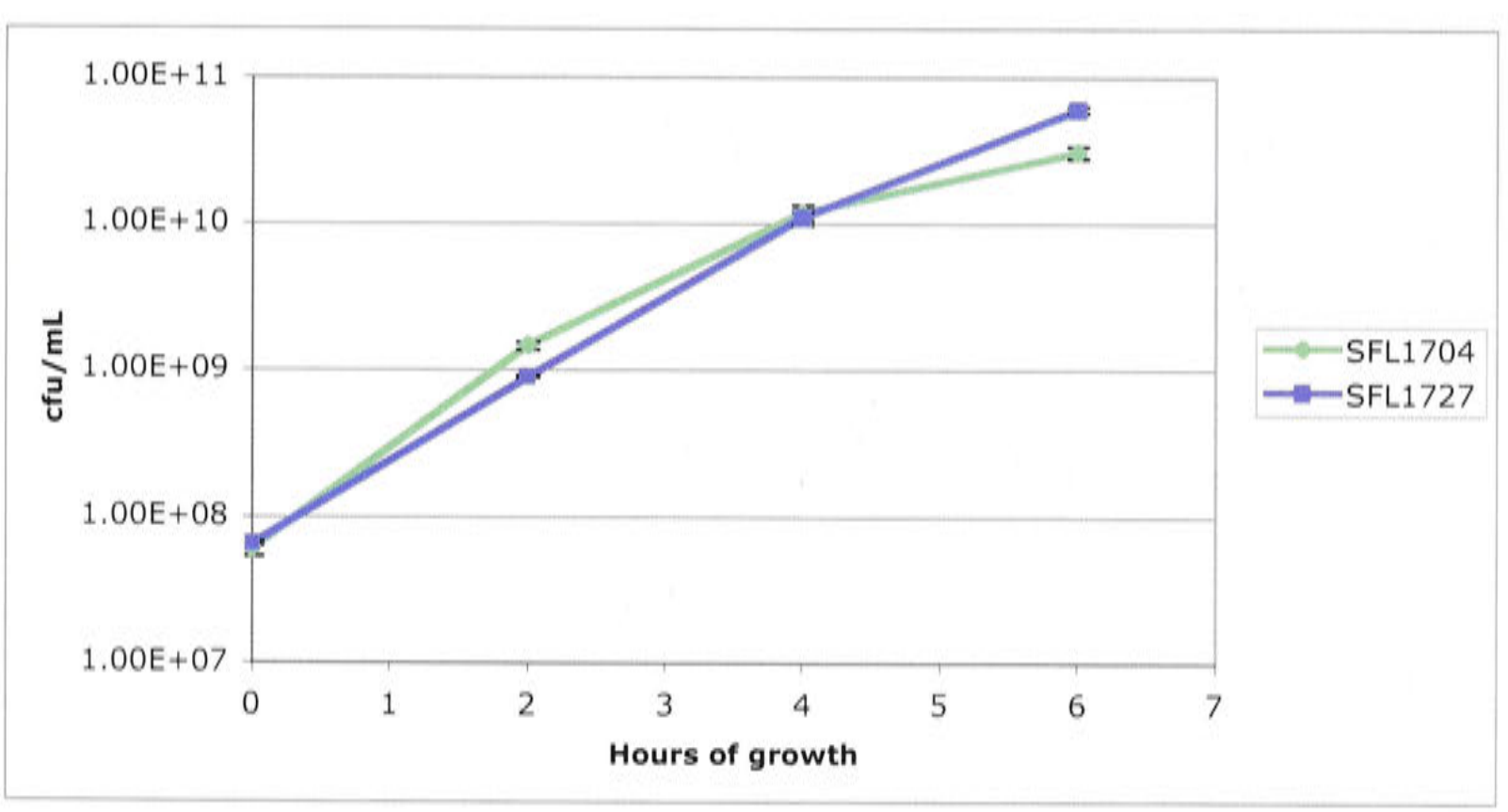


Figure 4.9. Minimal growth testing of the *udp* mutant, SFL1727. **A.** Growth of SFL1727 and parent strain, SFL1704 in M9 minimal salts media. SFL1704 is significantly different to SFL1727 according to a t-test. **B.** Growth of SFL1727 and parent strain, SFL1704 in minimal M9 salts media supplemented with 0.5% glucose as a carbon source. There is no significant difference in the cfu/mL of SFL1704 and SFL1727 according to a t-test.

conjunctivitis with purulence and 3 for severe conjunctivitis with severe purulence (120). A score of 2 or above is considered a positive Sereny reaction.

An *in vivo* model is advantageous in virulence testing because it provides a more accurate indication of the ultimate virulence of a strain as it tests for a phenotype in the presence of an active immune system and with various cell types that form the correct multicellular structures. In order to assess the degree of attenuation the *udp* disruption produces in *S. flexneri,* 10 guinea pig eyes per strain were infected with SFL1704 and SFL1727. The state of each eye was scored for keratoconjunctivitis at 24, 48 and 72 hours post infection (Figure 4.10). There was no detectable difference in the severity or onset of symptoms between the *udp* mutant, SFL1727, and SFL1704. By 48 hours post infection all 10 eyes for each strain had developed full-blown keratoconjunctivitis. Thus, there is no obvious attenuation caused by the *udp* mutation in an animal model of *Shigella* infection. Consequently, despite the virulence defects measured in cell culture assays for SFL1727, disruption of *udp* does not have a significant effect on the *in vivo* virulence of *S. flexneri.*

4.3 Discussion

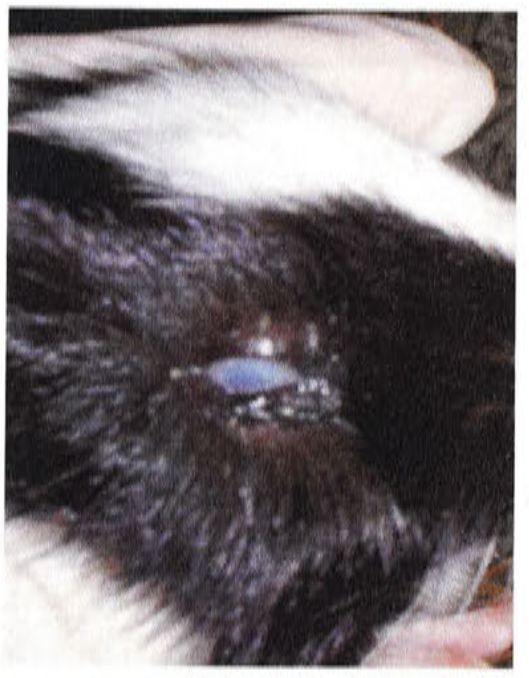
4.3.1 Construction of knockouts in wildtype *Shigella*

The efficiency of constructing knockouts in bacteria is rarely reported. Generally only the construction of successful knockouts are mentioned in the literature. It is understood however, that pathogenic species are more difficult and time consuming in which to produce gene disruptions (245). Mutants are most often constructed in *Shigella* strains using variations of the suicide vector approach (121, 318).

During the course of this virulence study six different gene knockouts needed to be constructed in the pathogenic strain 2457T of *S. flexneri.* Initially, the suicide vector approach was used with SFL1001, where 300+ bp of target gene sequence is cloned into the suicide vector pGP704 to drive integration of the plasmid into the desired gene. This approach worked rapidly for one gene, *mglB*, but was not successful for the other five genes, despite repeated attempts. A number of optimisation attempts were made including changing the length of homology to target genes to 600+ bp, increasing the competency of

A

negative



positive

B

Strain	24 hrs	48 hrs	72 hrs
SFL1704	2/10	10/10	10/10
SFL1727	3/10	10/10	10/10

Figure 4.10. Sereny testing of the *udp* mutant, SFL1727. **A**. Scoring for Sereny test, infection with virulent *Shigella* will cause keratoconjuctivitis, defined as a positive response. **B.** Result of the Sereny test for SFL1704 and SFL1727. 10 guinea pig eyes were infected for each strain and scored for a positive reaction up until 72 hours after infection.

the SFL1001 cells, varying the concentration of ampicillin in the selection plates, raising the purity and concentration of the plasmid DNA, and increasing the transformation recovery time. Despite these attempts, only the knockout in *mglB* could be produced in SFL1001 with this approach.

To speed up the efficiency of knockout production, the lambda red-mediated PCR system was also used. This technique requires pKD46-based expression of bacteriophage *bet*, *gam* and *exo* genes in order to mediate the recombination of single stranded DNA within the *Shigella* cell. This approach has previously been described as highly variable in its efficiency for generating gene knockouts in *S. flexneri*, with 0-250 resistant colonies typically produced with 0-100% of these being the correct knockout (302). Another *Shigella* study found that they were only able to produce a mutation in one of four targeted genes (135). Similar results were obtained in this study, knockout attempts would often yield zero colonies or resistant colonies, which when screened, did not have the correct insertion. Troubleshooting techniques attempted included the immediate use of fresh competent cells, longer induction of the helper plasmid gene expression, using high concentrations of PCR product for transformation, increasing the flanking homology length on the PCR *cat* cassette, reversing the orientation of the *cat* gene, and extended transformation recovery times.

The PCR knockout approach eventually yielded the other five mutants chosen for this study. However, during Sereny analysis of these strains it was determined that the parent SFL1001 strain was in fact avirulent and that the constructed gene knockouts were not suitable for virulence studies. It is not clear how SFL1001 had become avirulent; the creation of the laboratory glycerol stock had occurred many years before. However, the attenuation did not seem to have occurred during this project as the original high-use laboratory glycerol strain was tested and also found to be avirulent. It is well known that laboratory maintained strains are subject to genetic instability and that great care must be taken during preservation, storage and during any manipulation steps (55, 199). SFL1001 is further discussed in Chapter 5.

An alternative *S. flexneri* 2457T strain, SFL1704, was obtained from another laboratory that had recently confirmed the strain's virulent nature by Sereny test. Construction of the six knockouts was attempted in SFL1704 using both the suicide vector approach and the lambda-red PCR technique. It proved extremely difficult to produce any knockouts in this background. No positive integrants could be produced for any gene using pGP704 in SFL1704 despite trying all the optimisation techniques listed previously. Once again, the lambda-red PCR approach proved to be more efficient for constructing *Shigella* knockouts as two positive gene disruptions were produced for *S3195* and *udp*. However, despite repeated attempts using two different helper plasmids in SFL1704, pKD46 and pKM208, no more knockouts could be generated during this project.

It remains unclear why some genes are more readily disrupted than others by lambda-red PCR integration. Murphy and Campellone, (2003) suggest that both the position and orientation of the *cat* gene in the chromosome are important (245). It is possible that the neighbouring transcripts may read into the *cat* gene, influencing its expression, or that the *cat* gene may become unstable. They demonstrated that reversing the orientation of the cat insertion in a particular gene actually increased the efficiency of integration 10-fold. However, the reversal of *cat* gene orientation for the target genes in this project did not increase knockout production. It is possible that particular locations in the chromosome are more conducive to maintaining the *cat* gene insertion and expression. In both the *udp* and *S3195* knockouts many of the upstream and downstream genes have the same orientation as the target gene. Murphy and Campellone, (2003) believe that having the *cat* insertion collinear with the surrounding genes may contribute to insertion stability (245). As to why it was possible to eventually generate all six knockouts in SFL1001 whilst it was not in SFL1704 is unknown. It is possible that the four additional knockouts produced in SFL1001 occurred simply by chance or perhaps that even when strains have the same background, the higher the strain's virulence, the more difficult it is to mutate.

Due to the difficulties and time-constraints encountered in the engineering of knockouts for the gene candidates, it was not possible to assess all six genes in this virulence study. MglB is the subject of Chapter 5, however, the final three virulence candidates *ansB*, *ggt* and *yfiD* still remain to be studied. There are alternative knockout approaches not investigated in this

project due to time constraints, which could be used for these genes, such as the Sigma-Aldrich TargeTron gene knockout system or the use of temperature sensitive suicide plasmids (287, 318).

4.3.2 Disruption of *S3195* in *S. flexneri*

S3195 was successfully disrupted in the SFL1704 chromosome by insertion of a *cat* cassette. The disruption was confirmed by PCR using a *cat* specific primer, and also by sequencing, which detected the correct fusion point between *S3195* and *cat* gene sequence. The function of S3195 in *S. flexneri* is unknown. The closest homologue that has been characterised is Antigen 43 from *E. coli*, which has 88% identity to S3195 (50). Antigen 43 is a self-recognising adhesin which confers bacterial cell autoaggregation (167). In order to determine if S3195 also played a role in autoaggregation, static liquid cultures were prepared and checked for settling of cells after 24 hours. It was found that *S. flexneri* 2457T does not possess an autoaggregation phenotype as no cell settling was observed. Therefore, it can be concluded that S3195 plays no role in autoaggregation for *S. flexneri* 2457T. Interestingly, however, the *S3195* mutant, SFL1726, did display smaller colonies on agar plates than the parent strain, SFL1704, a characteristic which has been reported for *E. coli* strains which are Antigen 43 negative (122). Thus, it is possible that S3195 does share some functional properties with Antigen 43.

Klemm *et al*, (2004) also proposed that some variants of Antigen 43 have lost their autoaggregation properties and are instead optimised as adhesins for mammalian cells, where they may have a role in virulence (167). S3195 does not appear to contribute to the virulence of *S. flexneri* 2457T, as SFL1726 did not display any reduction in its ability to form plaques when tested in plaque assays. The plaque assay is an excellent screen for *S. flexneri* virulence as a plaque can only be formed by bacteria which are able to adhere to the epithelial cell surface, internalise, escape from the phagosomal membrane, replicate in the cytoplasm and spread to neighbouring cells. A defect at any point in this process will either reduce the plaque formation efficiency or produce irregular sized and shaped plaques. Therefore, the disruption of *S3195* did not produce any defect in *S. flexneri* virulence as the mutant was unaffected in its ability to form plaques. For this reason, S3195 appears to be inessential for *S. flexneri* pathogenesis and was not further characterised.

4.3.3 Disruption of *udp* in *S. flexneri*

A disruption mutant was constructed in the *udp* gene of SFL1704. The correct insertion of the *cat* cassette into the *udp* region was confirmed by PCR with a *cat* specific primer and sequenced to determine the correct junction between the *cat* and *udp* sequence. Furthermore, a Southern blot was used to confirm the change in size of the *udp* gene caused by the insertion of the *cat* cassette. The *udp* mutant, SFL1727, was complemented with a full size copy of *udp* to create SFL1732, which was used in the virulence assays to show that any defect in virulence was the cause of the *udp* mutation and could be directly restored with a functional copy of *udp*.

The *udp* mutant, SFL1727, was significantly reduced in its ability to form plaques in plaque assays performed in BHK cells. Plaqueing levels similar to the parent strain, SFL1704, were restored in SFL1732. As there was no change in the size of plaques between SFL1704 and SFL1727, the virulence defect caused by *udp* disruption does not appear to be related to the cells ability to spread intra- and intercellularly. To further characterise what invasion step Udp is involved in it was necessary to perform additional cell culture assays.

The invasion assay not only measures the number of bacteria which have entered the cytoplasm over a total infection time of four hours, but also the ability of bacteria to adhere to the epithelial cell surface during the early stages of cell entry. SFL1727 was significantly reduced in the number of intracellular bacteria measured, but did not display any differences to SFL1704 in the total number of bacteria, which is a measurement of both intracellular bacteria and adherent bacteria. The differences observed in the percentage of intracellular bacteria do not generally affect the total count as the intracellular percentages are so low compared to those measured for the total bacteria. Therefore, it is possible to see a difference in the intracellular counts but no difference in the total counts, indicating that there is no reduction in the numbers of adherent bacteria for SFL1727. Invasion assay data is often expressed as a percentage of invasion, which is the number of intracellular bacteria divided by the number of total bacteria. According to this equation, there is no difference in the percentage invasion between SFL1704 and SFL1727. Thus, although the intracellular numbers of bacteria are significantly reduced for SFL1727, it seems that there is no defect

in bacterial adherence and entry. A possible explanation for this is that the SFL1727 cells are reduced in their intracellular survival and replication, which would result in lower numbers of cytoplasmic bacteria than SFL1704 over the four hours of invasion.

4.3.4 Udp contributes to *S. flexneri* intracellular survival *in vitro*

Survival assays were performed to determine if intracellular survival and replication was deficient in SFL1727. These assays allow invasion for 90 minutes before all extracellular bacteria are killed by gentamycin treatment and the numbers of intracellular bacteria are monitored over six hours. In support for the invasion assay findings, SFL1727 does not appear to have a defect in invasion, since at time 0, which is after 90 minutes of invasion, there are similar numbers of intracellular bacteria to SFL1704 and SFL1732. However, 2 hours later there was a rapid decrease in SFL1727 intracellular numbers, which were significantly reduced in comparison to SFL1704 and SFL1732 counts after 4 hours. It is therefore evident from these cell culture studies that Udp may play some role in the intracellular survival and replication of *S. flexneri* within epithelial cells.

Udp is a uridine phosphorylase which catalyses the reversible phosphorolysis of uridine to uracil and ribose-1-phosphate. This is described as key step in the salvage of pyrimidines, where uridine is broken up and reincorporated into nucleotides. Thus, SFL1727 is not auxotrophic for uridine, as it is not defective in the synthesis of uridine, which is a separate pathway, but it should be unable to recycle or break down any uridine. Exogenous pyrimidine nucleosides are taken up by bacteria from the growth medium and are broken down and used as carbon and nitrogen sources (274, 369). The breakdown of uridine in bacteria requires Udp (151). It is possible that the intracellular growth defect observed for SFL1727 is caused by the bacteria's inability to utilise pyrimidine nucleosides as carbon and nitrogen sources during periods of active growth such as during intracellular replication and spread.

In order to determine if SFL1727 is reduced in its growth when exposed to low nutrient conditions, growth curves were produced for SFL1727 and SFL1704 in minimal media, which contained very little carbon and nitrogen sources. As it is not possible to grow wild type *Shigella* in minimal media without supplementation of methionine, tryptophan and

nicotinic acid, SFL1704 and SFL1727 were grown in M9 minimal salts media containing 2 μg/mL methionine, tryptophan and nicotinic acid, which would only provide low levels of carbon and nitrogen (4). The growth of SFL1727 was significantly reduced in comparison to SFL1704 during 6 hours of log phase growth. However, the addition of 0.5% glucose to provide a rich carbon source to the M9 minimal salts media restored the SFL1727 growth rate to that of SFL1704. Therefore, it seems that the scavenging of pyrimidine nucleosides as a carbon and nitrogen source may aid in the active growth rate of *S. flexneri* 2457T during periods of limited nutrient availability, but is of little consequence when carbon and nitrogen sources are readily available. There are a number of studies of intracellular bacteria which suggest that nucleoside salvaging is important during host cell interactions (92, 235, 274). Very little information is available on which metabolites are required for intracellular replication of *S. flexneri* and whether these are in limited availability within the host cytoplasm. However, it seems that nucleosides are readily available in the cytoplasm of human cells (45, 373).

4.3.5 *in vivo* testing of SFL1727, *udp* mutant

Although cell culture is incredibly useful for characterising a virulence defect in *S. flexneri*, the ultimate verification for a virulence candidate is the phenotype produced *in vivo*. The *Shigella* infection process is complex and cannot be properly studied using homogenous cell populations, which do not receive or produce the external signals derived from the interactions of various cell types and from the activation of the immune response. Therefore it is essential to validate any virulence data produced from *in vitro* models with *in vivo* tests. The Sereny test is often used to evaluate the virulent phenotype of *Shigella* species (45, 132, 272). SFL1727 and SFL1704 were tested for pathogenesis by the Sereny test in 10 guinea pig eyes per strain, and there was no difference in the disease causing abilities between SFL1727 and SFL1704. Both strains induced keratoconjunctivitis in all 10 eyes by 48 hours post infection, and there was no difference in the severity of the symptoms or time of onset. Therefore, whatever virulence defect was observed in cell culture was not sufficient to prevent disease forming in an *in vivo* model.

The *in vitro* studies performed in this study suggest that Udp contributes to the intracellular survival and replication of *S. flexneri* 2457T. However, it is not essential for survival and

replication as it is still possible to measure intracellular bacteria in both the invasion and survival assay, and although the number of plaques was significantly reduced, plaques were still formed by the strain. The attenuation of SFL1727 was sufficient to be detected in cell culture assays, but it was not possible to extrapolate whether this attenuation is strong enough to notably retard the strain's pathogenicity *in vivo*, therefore, requiring an *in vivo* assay to be performed. Clearly, the growth defect observed for SFL1727 did not disadvantage the strain sufficiently to prevent disease forming in the Sereny animal model. Thus, the true extent of an attenuation in *S. flexneri* cannot be understood until animal studies are performed. There are numerous examples in the literature where *S. flexneri* genes are reported as virulence factors based solely on cell culture data, and like in this study, other reports show that mutants attenuated in cell culture are not in animal tests (131, 133, 342). This highlights the need for cell culture assay data to be carefully considered when used for virulence analysis, and such data should only be used for characterisation of the particular virulence defect, not as the determinate measurement of a strain's virulence phenotype.

Unfortunately, no suitable virulence candidates for use as vaccine attenuations were identified in this study. Although Udp appears to play some role in the intracellular survival and replication of *S. flexneri*, it is not suitable for use in vaccine development as the attenuation is not sufficient to prevent disease formation *in vivo*. However, perhaps an *udp* deletion could be useful in balancing the virulence of vaccine strains whose attenuation is not adequate to prevent disease symptoms. The slight additive attenuation possibly offered by *udp* may satisfactorily reduce the virulence of the double mutant whilst not weakening the strain so dramatically that a protective immune response is not invoked.

4.4 Conclusion

In this Chapter, a number of immunogenic proteins were considered for virulence assessment. In order to determine their role in *S. flexneri* virulence, a knockout approach was taken. Unfortunately, the construction of mutants in the pathogenic SFL1704 strain proved to be time-consuming and difficult. The insertion mutants successfully constructed in *S3195* and *udp* were assessed initially for any change in virulence properties by plaque assay. The *S3195* mutant, SFL1726, did not display any reduction in virulence and was not

further characterised. SFL1727, which has a disruption in *udp*, appeared to have a defect in intracellular growth and replication according to the plaque, invasion and survival assay data. This reduction in cell growth and replication was not sufficient to reduce the virulence of SFL1727 in the animal model, the Sereny test. This result suggests that *udp* is not a suitable candidate for single mutation vaccine attenuation studies, but may be useful in balancing the safety and efficacy of other attenuated vaccine strains as a secondary mutation.

Chapter 5
Novel regulation of surface IcsA by the Mgl operon in an avirulent strain of *S. flexneri* 2457T

Chapter 5

Novel regulation of surface IcsA by the Mgl operon in an avirulent strain of *S. flexneri* 2457T

5.1 Introduction

S. flexneri is capable of exploiting the host cell's actin assembly machinery to propel through the host cell cytoplasm, forming extracellular protrusions through which it can penetrate the neighbouring cell (102). This intra- and intercellular spread are important characteristics of *S. flexneri* pathogenesis and is driven by the essential outer membrane protein, IcsA. IcsA is expressed on one pole of the bacterium and this unipolar distribution is required for intracellular movement (25). IcsA interacts with the host protein N-WASP, which stimulates host cell actin polymerisation (356, 357). The actin nucleation and polymerisation form a comet tail at the IcsA pole of the bacterium propelling it through the cytoplasm.

IcsA is expressed as a 116 kDa protein that is exported by the Sec machinery across the inner membrane (355). IcsA is a member of the autotransporter family and is thought to mediate its own transport across the outer membrane (144). The asymmetric location of IcsA is generated by its transport being targeted exclusively to one pole (313, 324). The exact mechanism of this targeting is not understood, however secretion of IcsA by the Sec machinery may only occur at the pole, even though the Sec machinery is distributed circumferentially on the cell (35). This polar targeting of IcsA appears to be mediated by two small regions in the protein, one located at the N-terminus (region 1) and one in an internal region (region 2). The polar protein target(s) with which these regions interact are completely unknown; it seems likely that region 1 plays an initial role in binding a target and that region 2 may be responsible for maintaining polar targeting once the protein is folded (49). It is possible to produce polar localisation of IcsA when it is expressed in *E. coli*, indicating that the components involved may not be *Shigella* specific (324).

MglB, a galactose binding periplasmic protein, was detected as an immunogenic protein in two of the three immunoproteome repeats performed in Chapter 3. Periplasmic solute binding proteins are often identified as immunoreactive and are highly abundant proteins in the cell (98). MglB has not been studied in *S. flexneri* but shares the same gene placement in *E. coli* where it is the first in the *mglBAC* operon, which is cotranscribed as a polycistronic mRNA and cleaved (82). MglB not only acts as a high affinity galactose binding protein that delivers galactose to the MglA and MglC membrane components of the ABC transport system, but also as a chemoreceptor and a protein chaperone (334). Once MglB has bound substrate it interacts with the Trg chemotaxis receptor which mediates chemotaxis to galactose (334). The Trg chemoreceptor is another asymmetrically expressed protein located at only one bacterial pole (210).

5.2 Results

5.2.1 Construction of *mglB* mutant, SFL1617

As mentioned in Chapter 4, during the initial construction of virulence candidates in SFL1001, a *mglB* mutant was successfully produced using the suicide vector approach. A 424 bp internal region of *mglB* sequence, amplified by the primers MglFSac and MglRXba, was inserted into pGP704, to produce pNV1228. This plasmid was transformed into SFL1001 and an ampicillin resistant colony was produced. The sequence of pGP704 is not known, so it is not possible to screen for insertion using a primer that binds within pGP704 to amplify across the insertion. Instead, screening primers, MglBF17 and MglR664, from either side of the insertion region were used to amplify a 400 bp fragment in insertion negative strains; the positive strain does not amplify a PCR fragment, as the plasmid insertion is too large for the PCR amplification conditions (Figure 5.1). The positive strain was named SFL1617 and further analysed by Southern blot to confirm the correct disruption of *mglB* (Figure 5.2).

The insertion of pNV1228 into *mglB* may create a polar knockout for the entire *mgl* operon, so SFL1617 was complemented with pNV1424, a pBC SK based copy of the *mgl* operon amplified with the MglBcompF and MglBoperon primers, to create SFL1736. The presence

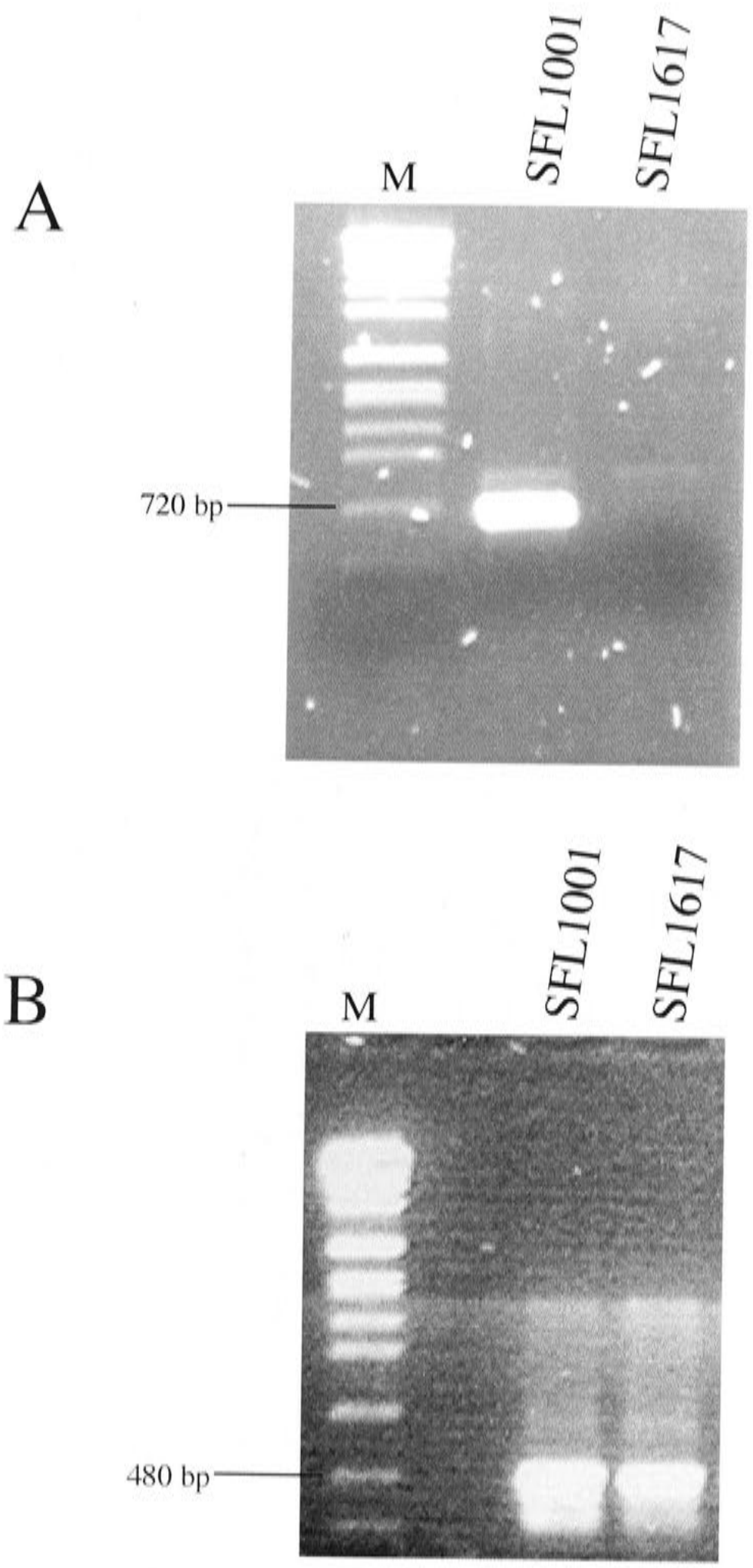
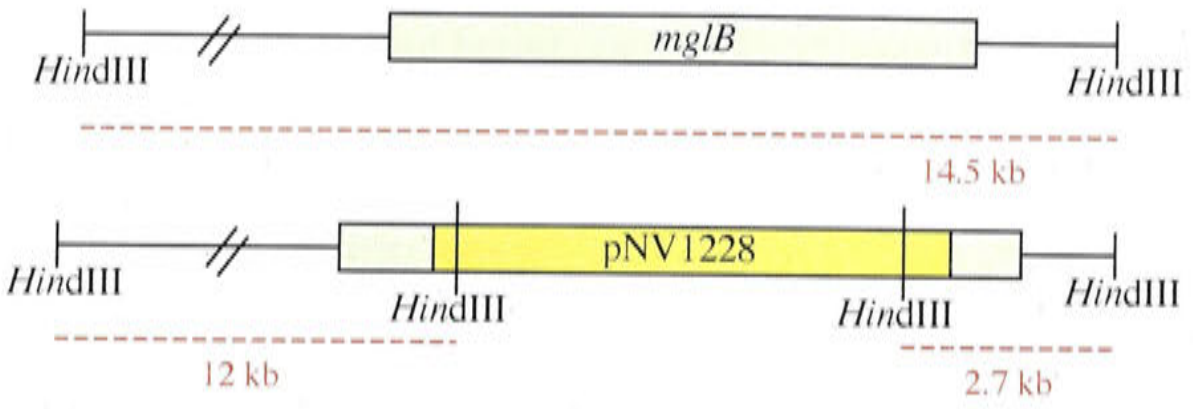


Figure 5.1. PCR screen for the insertion of pNV1228 into the *mglB* gene of SFL1001. SFL1001: avirulent 2457T strain used to construct SFL1617, SFL1617: *mglB* mutant. **A.** Amplification with the mglF17 and mglR664 screening primers will not occur when the *mglB* gene has been disrupted by pNV1228. A PCR fragment of 650 bp is expected in insertion negative strains. **B.** Control *malQ* PCR band of 400 bp, produced with primers, MalQFSac and MalQRXba, was used to show the integrity of the template DNA. M: SPP-1 *Eco*RI marker (sizes in Figure 2.5). Some marker sizes are indicated.

A



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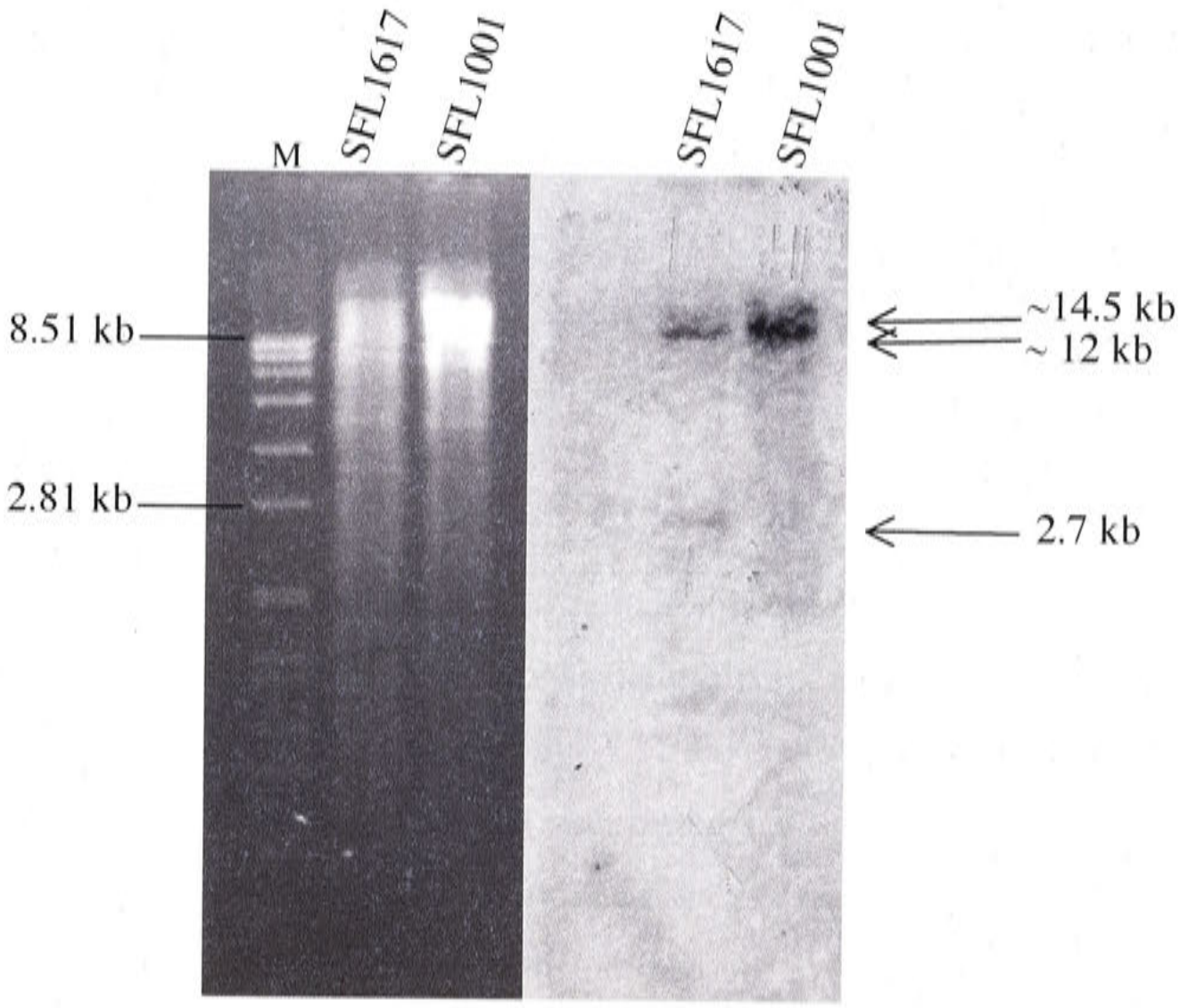


Figure 5.2. A. Insertion pattern of pNV1228 into *mglB* with a *Hin*dIII digest. The Southern probe is able to bind to both fragments of the *mglB* gene in SFL1617 **B.** Southern blot of SFL1617 to confirm insertion of pNV1228 into *mglB*. SFL1001 is the avirulent 2457T strain used to construct SFL1617. M: SPP-1 *Eco*RI marker (sizes in Figure 2.5). Some marker sizes are indicated. The probe is a 424 bp PCR fragment from the start of the *mglB* gene, amplified with the primers MglFSac and MglRXba. *Hin*dIII digest should produce a 14.5 kb band recognised by the probe in SFL1001. Integration of pNV1228 generates two bands which bind the probe, 12 kb and 2.7 kb. The large band sizes cannot be determined accurately as the marker does not exceed 8.5 kb. The agarose gel is on the left-hand side and the film is on the right-hand side.

of the virulence plasmid in all strains was confirmed by Congo red plating and colony PCR using primers against the plasmid based *apy* and *icsA* genes (Figure 5.3).

5.2.2 Stability testing of SFL1617

pGP704 based insertion mutants have been reported as 100% stable in *Shigella* in the absence of selective pressure over 48 hours of growth (108). SFL1617 was grown for 24 hours in the absence of ampicillin and dilution plated onto LB agar plates. 100% of these colonies were able to grow on ampicillin plates indicating that the inserted plasmid is stable in its chromosomal location.

5.2.3 Sereny testing of SFL1001 and SFL1617

mglB was originally disrupted in SFL1001 to determine if it was involved in *S. flexneri* virulence, as it was hypothesised that it's role in high affinity galactose uptake could be important during intracellular survival and replication (69).

SFL1001 and SFL1617 were administered to 10 guinea pig eyes for each strain, to assess the virulence of the strains in the Sereny test. It was determined that SFL1001 was avirulent in this assay. Both Congo red plating and colony PCR have shown that SFL1001 has the virulence plasmid, so it is not obvious what has occurred genetically in the strain to render it negative in the Sereny test. Although the 10 guinea pig eyes were negative for SFL1001, despite a high 10^9 dose being administered, all 10 guinea pig eyes infected with SFL1617 had positive Sereny reactions by 24 hours post infection and remained positive over the 48 and 72 hour timepoints. Therefore, it seemed that the *mglB* mutation had increased the virulence of the SFL1001 strain.

To confirm that the increased virulence observed for SFL1617 was actually caused by the disruption of the *mgl* operon, the *mgl* complemented strain SFL1736 was also analysed by Sereny test. It was hoped that complementation of the *mgl* disruption would reduce the strain's virulence *in vivo*, indicating that the *mgl* operon is playing a role in modulating *Shigella* virulence. 10^8 cfu of SFL1617 and SFL1736 were inoculated into 10 guinea pig eyes each. Each eye was scored for keratoconjunctivitis disease symptoms over 24, 48 and

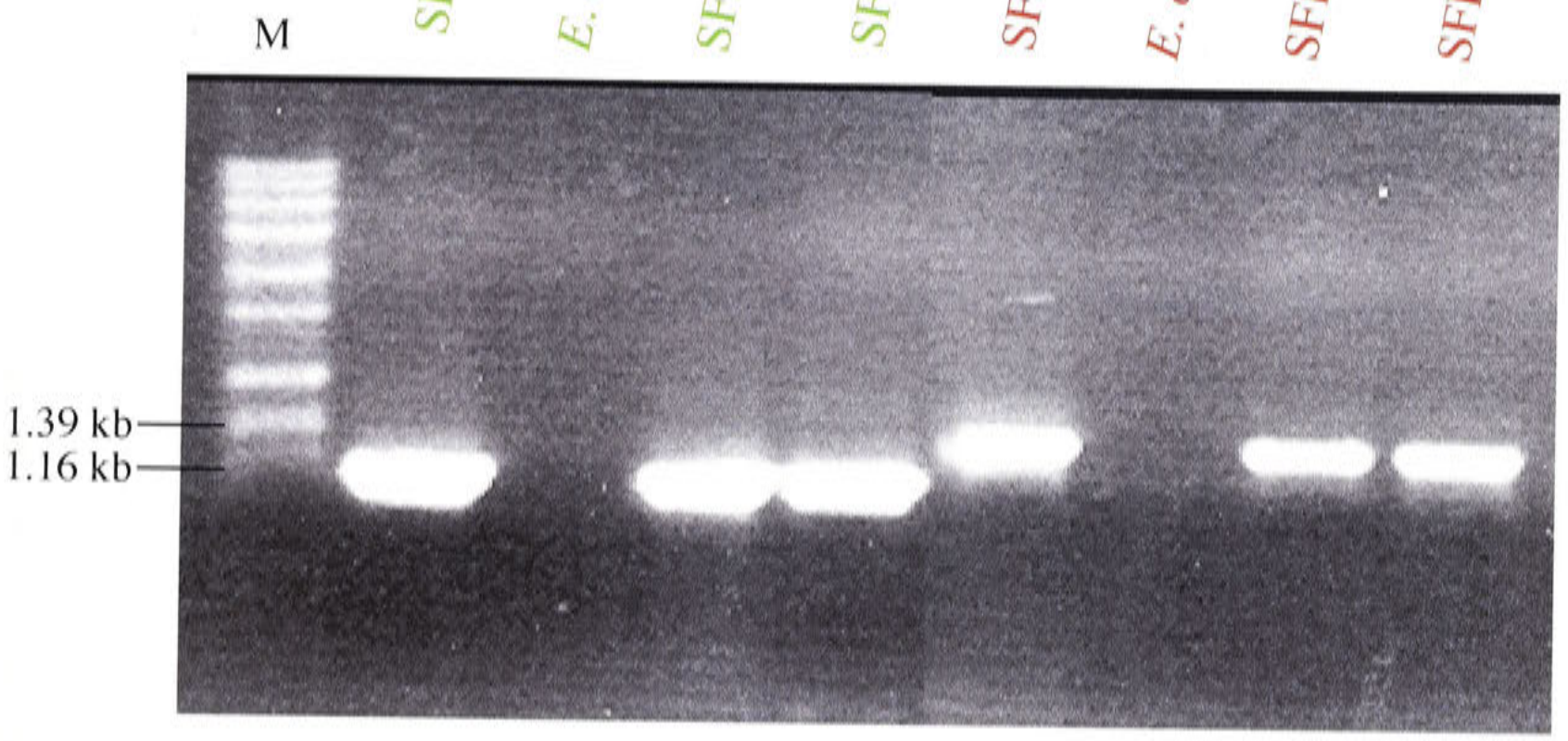


Figure 5.3. Colony PCR to confirm presence of virulence plasmid in *S. flexneri* strains. M: SPP-1 *Eco*RI marker (sizes in Figure 2.5). Some marker sizes are indicated. Apy (in green) primers amplify the *apy* gene from the virulence plasmid, 1.1 kb, IcsA (in red) VirG primers amplify the *icsA* gene from the virulence plasmid, 1.4 kb. SFL1001: parent strain. SFL1617: *mglB* mutant, *E. coli* JM109: negative control, SFL1736: *mglB* mutant with *mgl* operon complemented on plasmid, pNV1424.

72 hours post infection (Figure 5.4). SFL1617 produced positive reactions for all 10 eyes by 48 hours post infection, whereas SFL1736 had only produced one positive reaction by 48 hours and by 72 hours the final positive count was 3 out of 10. Thus, there does seem to be a considerable reduction in the *in vivo* virulence phenotype of SFL1736, suggesting that the *mgl* operon may contribute adversely to the virulence of *Shigella*.

5.2.4 Plaque assay of SFL1001 and SFL1617

In order to confirm the Sereny test results, plaque assays were performed for SFL1001, SFL1617 and SFL1736. Additionally, SFL1617 was also transformed with empty pBC SK to create SFL1739, for use as a control to demonstrate that the burden of carrying the pBC SK plasmid is not causing the detrimental effect on the virulence of SFL1736. In an effort to determine if it is the entire *mgl* operon that is required to reduce virulence or simply *mglB*, pBC SK carrying just the *mglB* gene, amplified with the MglBcompF and MglBcompR primers, was used to complement SFL1617, creating SFL1718.

SFL1001 was not capable of producing plaques in BHK monolayers (Figure 5.5). The *mglB* mutant, SFL1617, produced high numbers of plaques that were normal in size and shape (Figure 5.6). SFL1718, the *mglB* complement, did not significantly differ to SFL1617 in the number of plaques produced. However, the *mgl* operon complement strain, SFL1736, was significantly reduced in the number of plaques produced per assay in comparison to SFL1617, according to a t-test. Furthermore the plaques formed by SFL1736 were much smaller and more irregularly shaped then SFL1617; the average size of a plaque for SFL1617 was 1.9 ± 0.2 mm and for SFL1736 was 1.0 ± 0.1mm (Figure 5.6). SFL1739 behaved similarly to SFL1617 in both plaque numbers and size and shape, indicating that the reduction in virulence seen in SFL1736 is not simply caused by reduced fitness from carrying the pBC SK plasmid.

The plaque morphology observed in these assays suggest that SFL1736 may be reduced in it's ability to spread intercellularly as the plaques are smaller then normal, which is indicative of reduced spread between cells. To further distinguish the exact virulence defect displayed by SFL1001 and SFL1736, invasion assays were used to establish if SFL1001 and SFL1736 are reduced in their abilities to bind and enter epithelial cells.

Strain	24 hrs	48 hrs	72 hrs
SFL1617	3/10	10/10	10/10
SFL1736	0/10	1/10	3/10

Figure 5.4. Table of positive Sereny reactions for SFL1617 and SFL1736. 10 guinea pig eyes were infected for each strain and scored for a positive reaction up until 72 hours after infection. SFL1617: *mglB* mutant, SFL1736: *mglB* mutant complemented with a plasmid based copy of *mgl* operon, pNV1424.

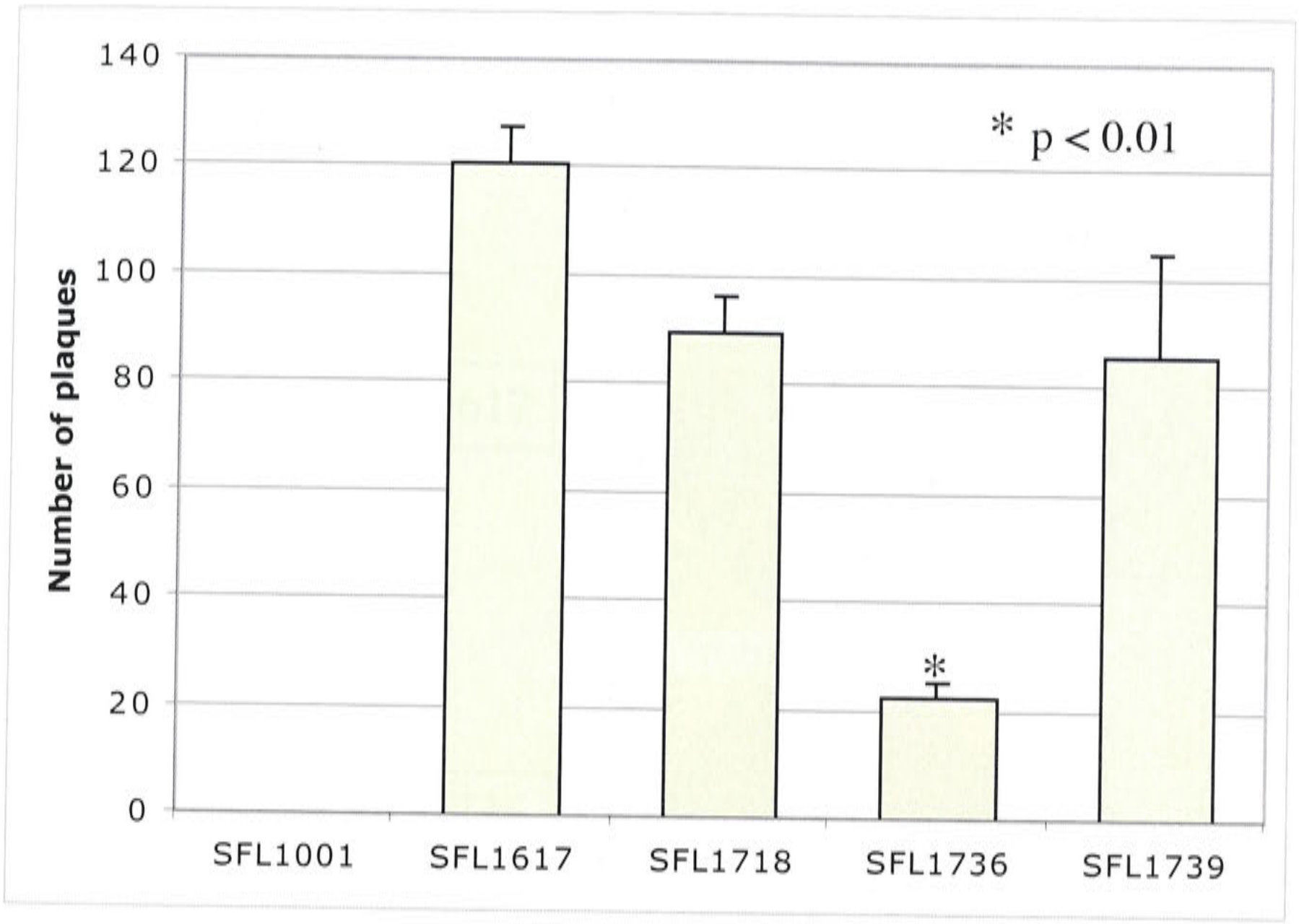
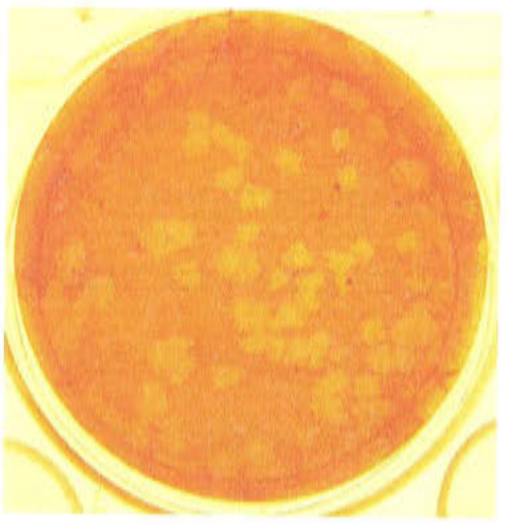


Figure 5.5. Plaque assay results for the *mglB* mutant, SFL1617. SFL1001: parent strain, SFL1617: *mglB* mutant, SFL1718: *mglB* mutant complemented with a plasmid based copy of *mglB* gene, pNV1346, SFL1736: *mglB* mutant complemented with a plasmid based copy of *mgl* operon, pNV1424, SFL1739: SFL1617 with an empty pBC SK vector. Results are the mean of three independent repeats with standard error. SFL1736 significantly differs in plaque numbers in comparision to SFL1617, according to a t-test.

SFL1001

SFL1617



SFL1736

SFL1739

Figure 5.6. Plaque assay pictures showing plaque shape and size. SFL1001: parent strain was not able to plaque in BHK cells, SFL1617: *mglB* mutant produced normal sized plaques, SFL1736: *mglB* mutant complemented with a plasmid based copy of *mgl* operon on pNV1424, produced small plaques. SFL1739: *mglB* mutant with empty pBC SK showed normal plaques.

5.2.5 Invasion assays of SFL1001, SFL1617 and SFL1736

SFL1001, SFL1617, SFL1736 and SFL1739 were all assessed in invasion assays. The wildtype 2457T strain, SFL1704, used in Chapter 4 was included as a positive control for normal levels of adherence and invasion.

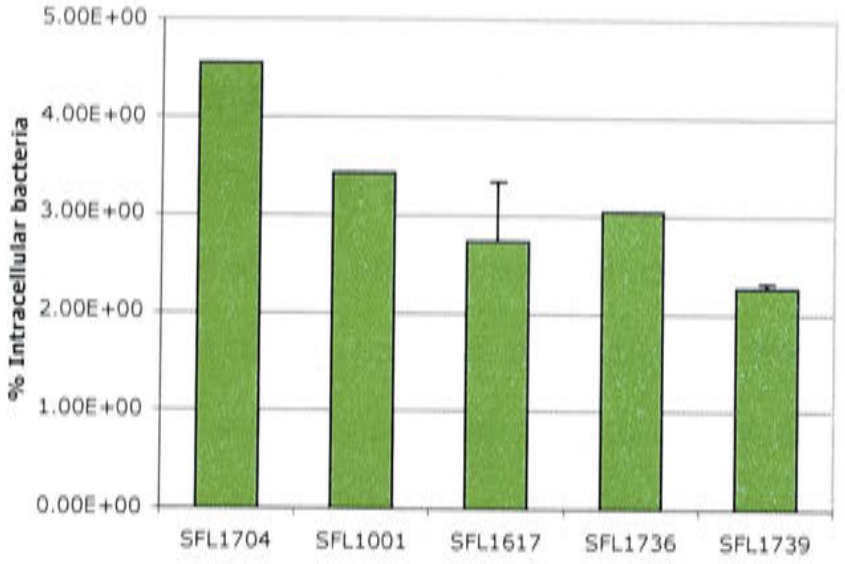
Interestingly, SFL1001 was capable of invading epithelial cells and adhering to their surface at the same levels as SFL1704 (Figure 5.7). Therefore, the virulence defect present in SFL1001 is not involved in the adherence and cell entry steps of epithelial cell invasion. Likewise the *mgl* complement, SFL1736, was not reduced in its invasion levels or its capacity to adhere to epithelial cells. In fact none of the strains differed significantly in their invasive properties to SFL1704, according to t-tests.

The data from the plaque and invasion assays indicates that the defect in virulence observed for SFL1001 and SFL1736 *in vivo* appears to occur during intra- and intercellular spread of the bacteria, as they are not impaired in cell adherence and invasion, but their abilities to form normal plaques is significantly reduced.

5.2.6 Western blot analysis of MxiG and MxiJ

There are two major virulence factors that are important for intra- and intercellular spread in *S. flexneri*; the IcsA protein and the type III secretion system, which delivers the effectors required for lysing the host cell membrane during intercellular spread (338). Twenty Mxi-Spa proteins make up the type III secretion system essential for *S. flexneri* invasion and intercellular spread in epithelial cells. Antibodies against MxiG and MxiJ were available during this study (363). The MxiG and MxiJ proteins form the base of the type III needle complex and are both important in the functioning of the type III secretion system (7, 335). The genes for the *mxi* proteins are located on the virulence plasmid in an operon. Any defect in the regulation and expression of the *mxi* operon should be detectable by analysing the protein production of MxiG and MxiJ, as the operon is co-transcribed, where a defect in *mxi* expression would indicate that the strain is reduced in intra- and intercellular spread because it is deficient in type III secretion (152).

A



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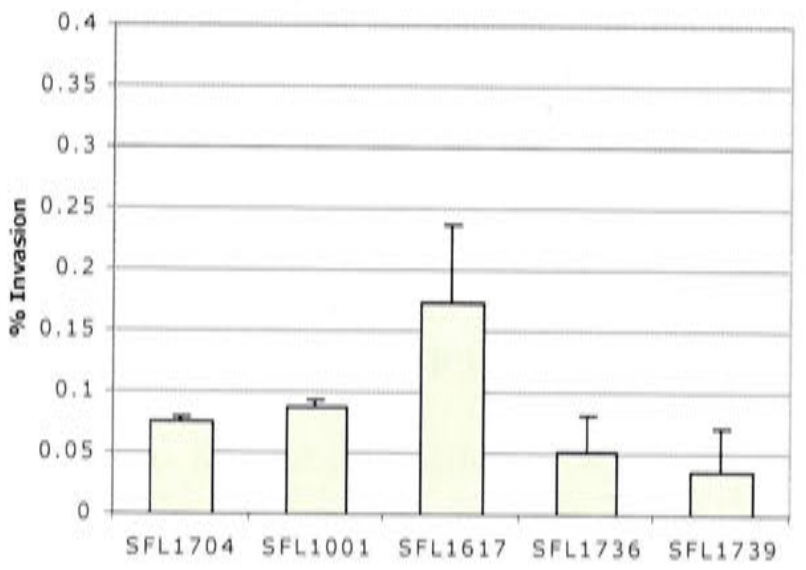


Figure 5.7. Invasion assay results for the *mglB* mutant, SFL1617. There are no significant differences in the invasive properties of the strains in comparison to SFL1704, according to t-tests. SFL1704: virulent 2457T strain, SFL1001: parent strain, SFL1617: *mglB* mutant, SFL1736: *mglB* mutant complemented with a plasmid based copy of *mgl* operon, pNV1424. SFL1739: *mglB* mutant with empty pBC SK. Results are the mean of three independent repeats with standard error.

Western blots were performed on soluble protein extracts of SFL1704, SFL1001, SFL1617 and SFL1736. Equal amounts of protein for each strain were separated by SDS-PAGE, transferred to PVDF and probed with MxiG and MxiJ antibody from Tamano *et al*, (2000) (363) (Figure 5.8). There was no obvious, intense disparity in the levels of the 43 kDa MxiG and the 27 kDa MxiJ protein between the strains. Therefore, the reduction in virulence observed for SFL1001 and SFL1736 in the plaque assays and Sereny test does not seem to be caused by reduced levels of type III secreton protein, as the proteins are expressed in all strains at similar levels as visualised by Western blot.

The soluble protein preparations were also visualised on a Coomassie blue SDS-PAGE gel to confirm that protein loading was even (Figure 5.9). Interestingly, there are two overexpressed protein bands for SFL1736, which are most likely products of the *mgl* complement plasmid, pNV1424. Band number 1 is running at the correct size, 24 kDa, to be the chloramphenicol acetyltransferase protein, whilst band number 2 is the size expected for the MglB or MglC protein, ~36 kDa, confirming that Mgl protein is being expressed from pNV1424. It is most likely that from the *mgl* operon, MglB is present in this protein extraction as it is a soluble periplasmic protein, unlike the ~56 kDa MglA and ~36 kDa MglC, which are membrane-associated proteins that would not be purified in the conditions used to extract the cellular protein.

5.2.7 Western blot of soluble and secreted IcsA

IcsA is essential for *S. flexneri* intra- and intercellular spread; strains defective in IcsA are either unable to form plaques in cell culture or form small plaques (326). Therefore, it seemed prudent to compare the protein levels of IcsA in SFL1001, SFL1617 and SFL1736 to determine if they differ. Soluble protein extracts and extracellular protein TCA precipitations were prepared for SFL1704, SFL1001, SFL1617 and SFL1736. Equal amounts of protein for each strain were separated by SDS PAGE and blotted onto PVDF membrane. Membranes were probed with anti-IcsA antibody, VRG-N2, directed against residues 82-100 of IcsA (Figure 5.10) (359).

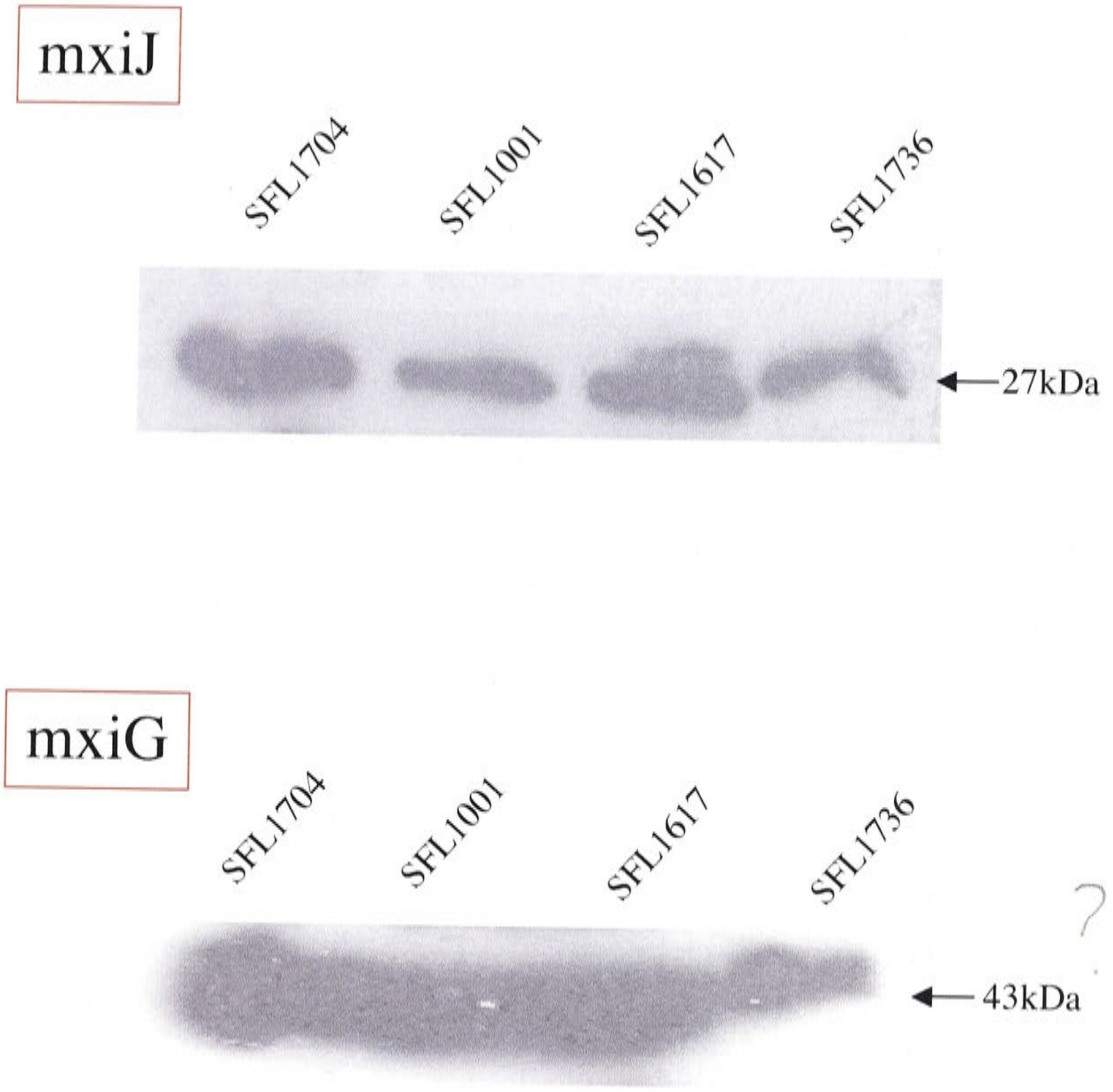


Figure 5.8. MxiG and MxiJ Westerns of soluble protein for SFL1704: virulent 2457T strain, SFL1001: avirulent 2457T strain used to construct SFL1617, SFL1617: *mglB* mutant and SFL1736: *mglB* mutant complemented with a plasmid based copy of *mgl* operon, pNV1424. Similar levels of proteins were detected for all strains. Approximate sizes were determined from the PageRuler Prestained Protein Ladder (Fermentas).

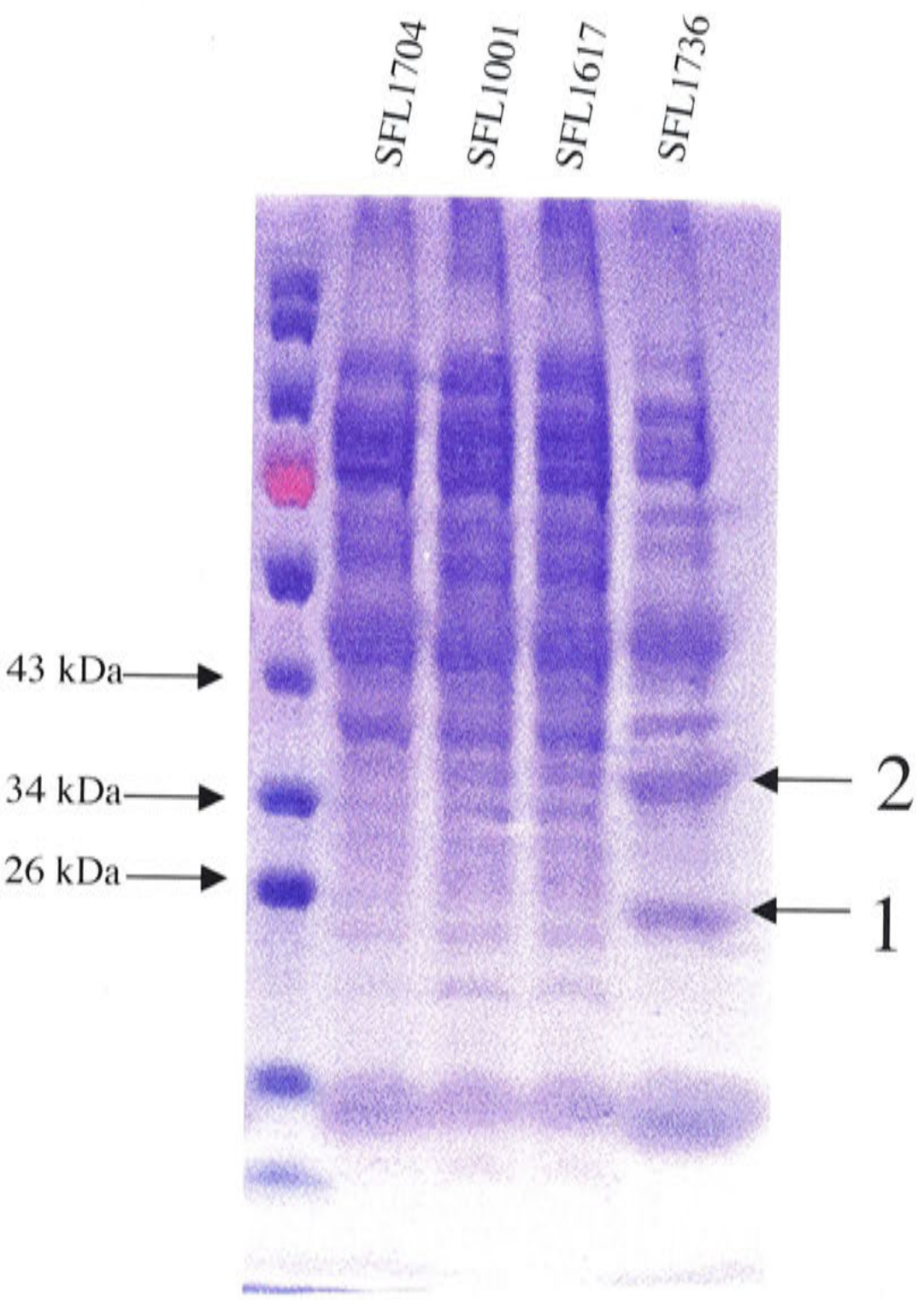


Figure 5.9. Coomassie blue stained SDS-PAGE gel of soluble protein of SFL1704: virulent 2457T strain, SFL1001: avirulent 2457T strain used to construct SFL1617, SFL1617: *mglB* mutant and SFL1736: *mglB* mutant complemented with a plasmid based copy of *mgl* operon, pNV1424. **1:** protein band of correct size, 24 kDa, to be chloramphenicol acetyltransferase expressed from pNV1424. **2:** protein band of correct size to be MglB or MglC, 36 kDa, expressed from pNV1424. Approximate sizes of the closest Fermentas PageRuler Prestained Protein Ladder bands are shown.

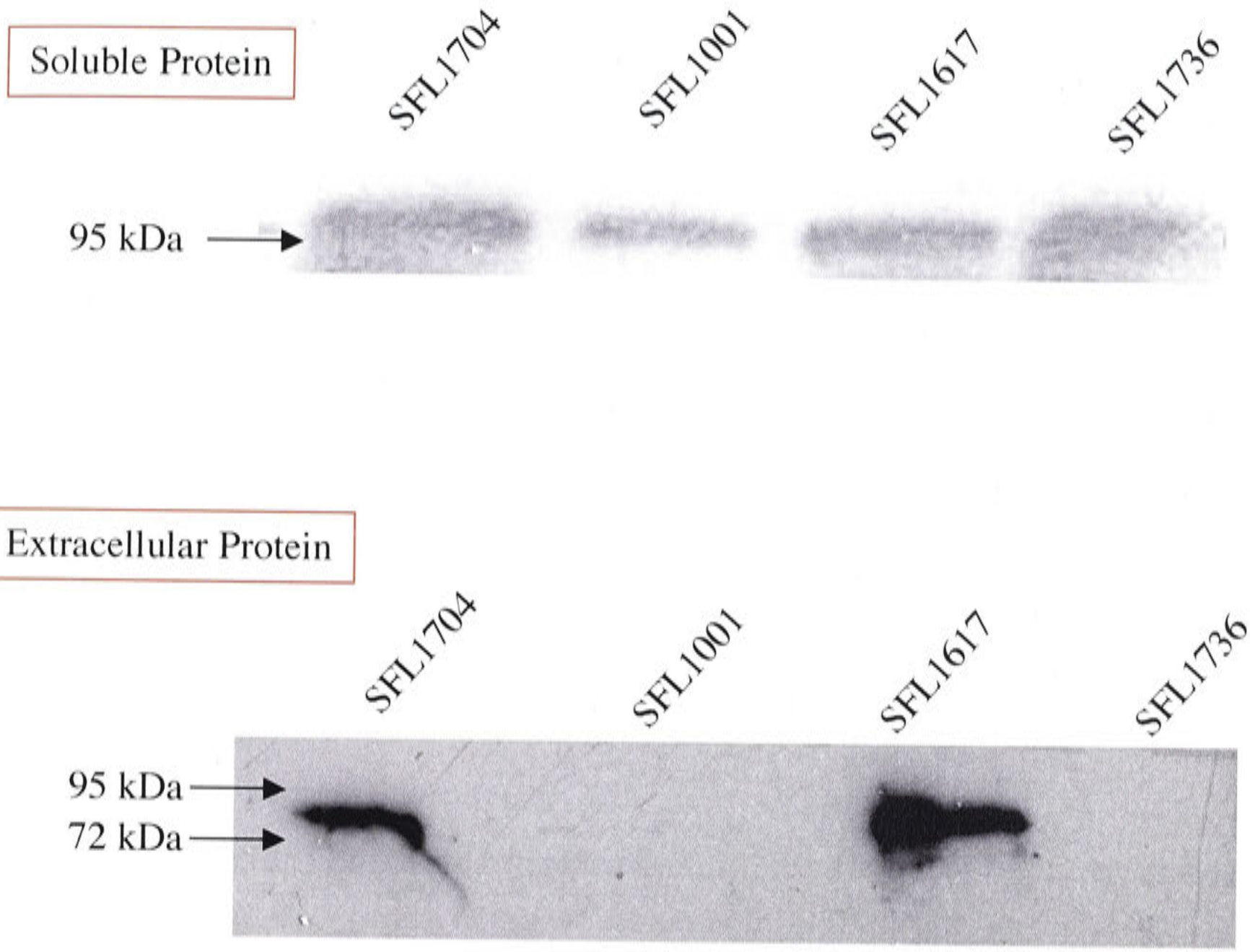


Figure 5.10. IcsA Western blots for soluble protein and extracellular protein. Full size IcsA is 116 kDa and the cleaved secreted IcsA is 85 kDa. SFL1704: virulent 2457T strain, SFL1001: avirulent 2457T strain used to construct SFL1617, SFL1617: *mglB* mutant, SFL1736: *mglB* mutant complemented with a plasmid based copy of *mgl* operon, pNV1424. Approximate sizes of the closest PageRuler Prestained Protein Ladder (Fermentas) bands are shown.

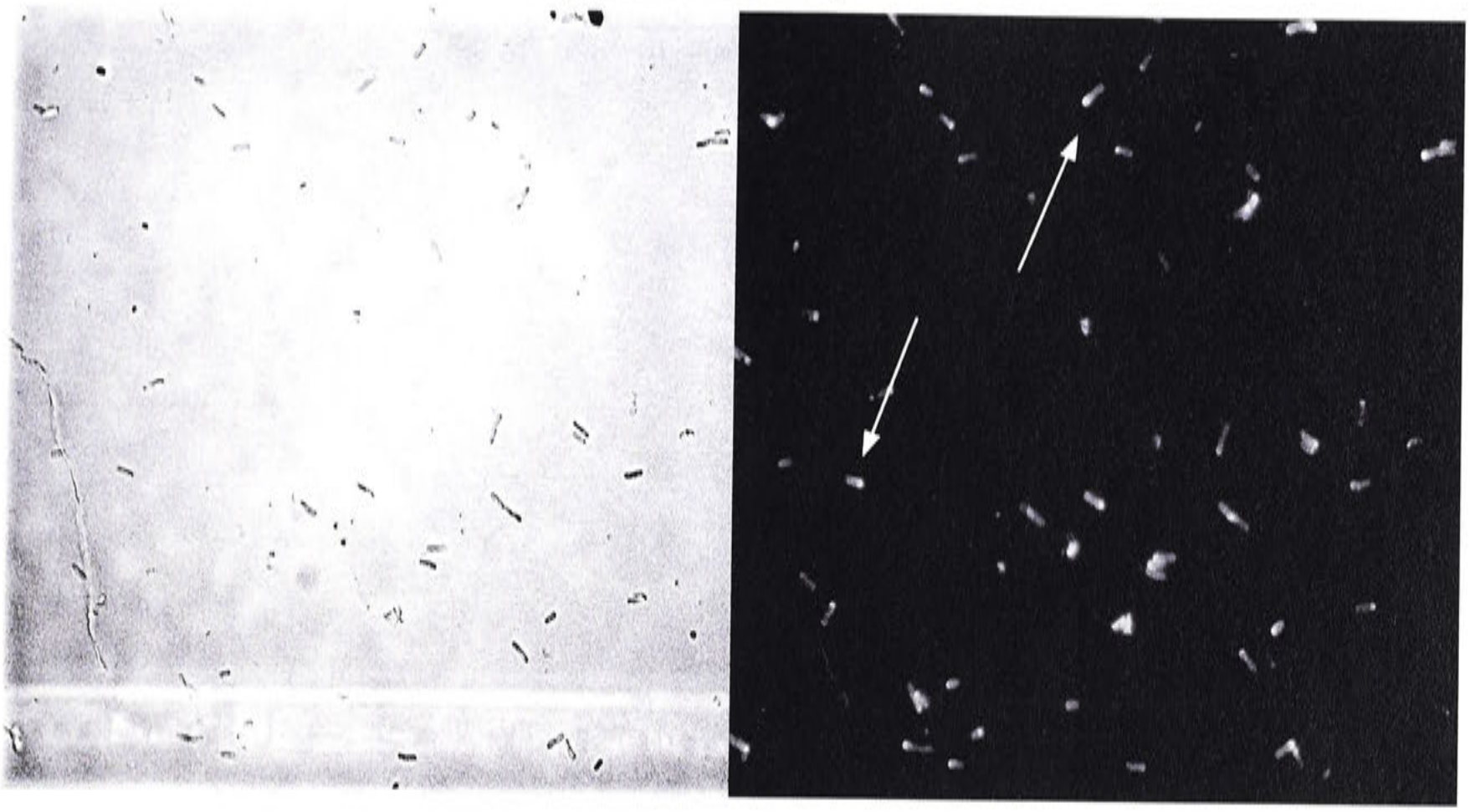
IcsA is produced and transported from the cytoplasm as a 116 kDa protein; once exposed on the cell surface it is cleaved by IcsP and a 85 kDa portion is released into the extracellular environment (355). This portion contains the region recognised by the VRG-N2 anti-IcsA antibody. Therefore, if cleavage is occurring normally on the cell surface, the extracellular protein preparation will contain the 85 kDa IcsA protein product. A strong IcsA band was detected for all strains in the soluble protein blot, indicating that full size IcsA is being produced, even for the reduced virulence strains SFL1001 and SFL1736 (Figure 5.10). However, cleaved IcsA was not detected in the extracellular medium of SFL1001 and the *mgl* complement, SFL1736, while it was present for the *mglB* mutant, SFL1617 and SFL1704. Thus, SFL1001 and SFL1736 are expressing IcsA but it is either not cleaved when it reaches the cell surface, due to a fault in the protein's cleavage site or in the protease, IcsP, or alternatively it never reaches the cell surface to be cleaved by IcsP. It is necessary to visualise IcsA on the outer membrane of the cell in order to determine whether it is reaching the cell surface. IcsA can be detected on the surface of the bacterial cell using microscopy techniques.

5.2.8 IcsA analysis by microscopy

IcsA distribution can be visualised with immunofluorescent labelling viewed under a light microscope (240). It is possible to resolve the asymmetric position of IcsA as the labelling is predominately located on one pole of the bacteria.

SFL1704 was included in the anti-IcsA antibody, VRG-N2, immunofluorescence labelling as a positive control for normal IcsA distribution and cleavage. IcsA was detected predominately at one pole of each SFL1704 cell as expected (Figure 5.11). SFL1617 showed the same polar distribution and intensity of IcsA labelling as SFL1704, whilst SFL1001 and SFL1736 had very little polar IcsA, visible on only one or two cells per microscope image (Figures 5.11 and 5.12). The total number of cells in three microscope images was counted for each strain and the proportion displaying polar IcsA determined. The percentage of polar labelled cells was similar for SFL1704 and the *mglB* mutant SFL1617, while there was a significant decrease in the number of IcsA labelled cells for SFL1001 and the *mgl* complement SFL1736 according to t-tests with SFL1704 (Figure 5.13).

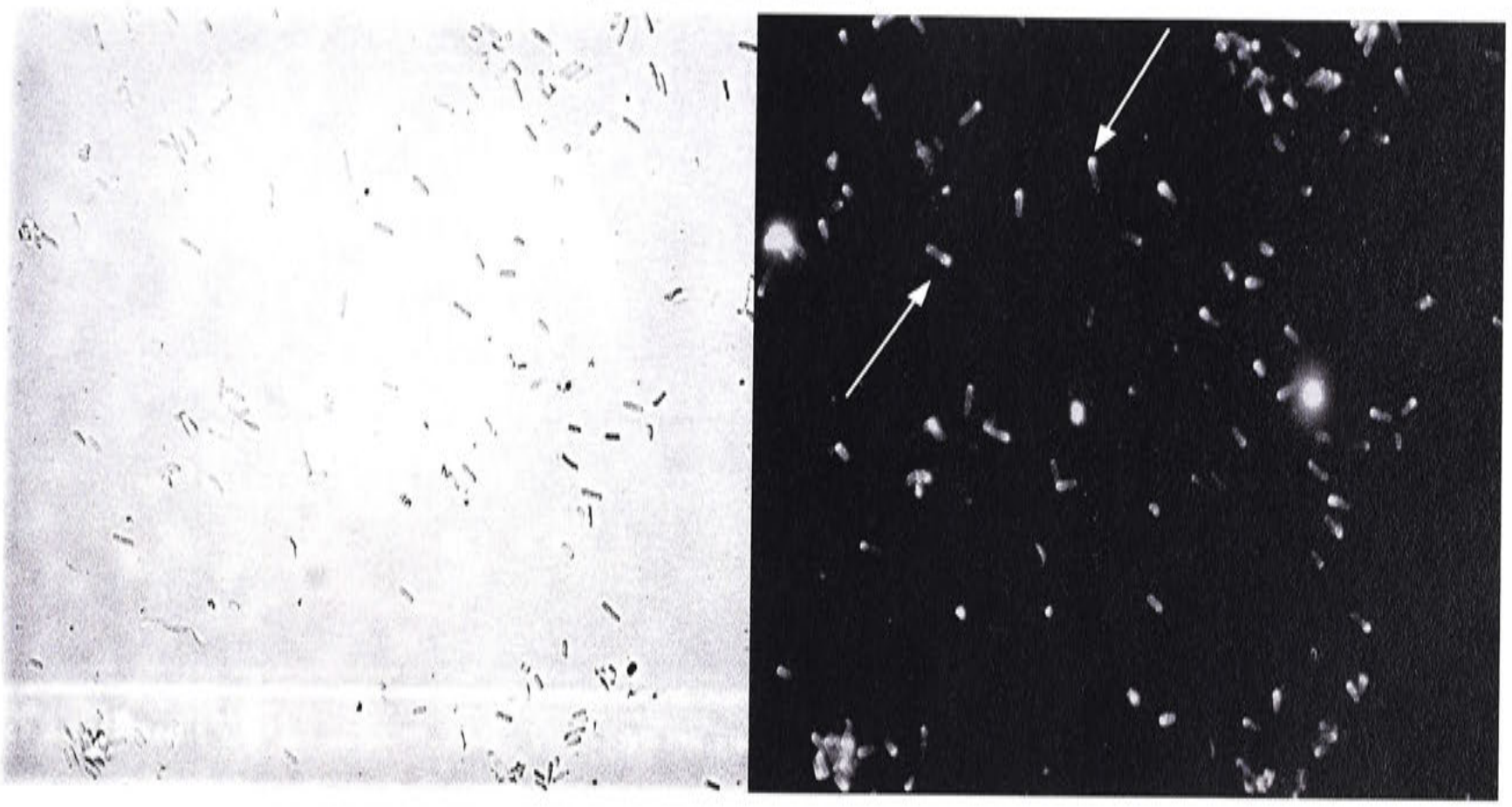
SFL1704



SFL1001

Figure 5.11. IcsA immunofluorescence for SFL1704: virulent 2457T strain, SFL1001: avirulent 2457T strain used to construct SFL1617. Examples of polar staining are indicated by arrows. For each strain the phase contrast image is on the left-hand side and the immunofluorescent image is on the right-hand side.

SFL1617



SFL1736

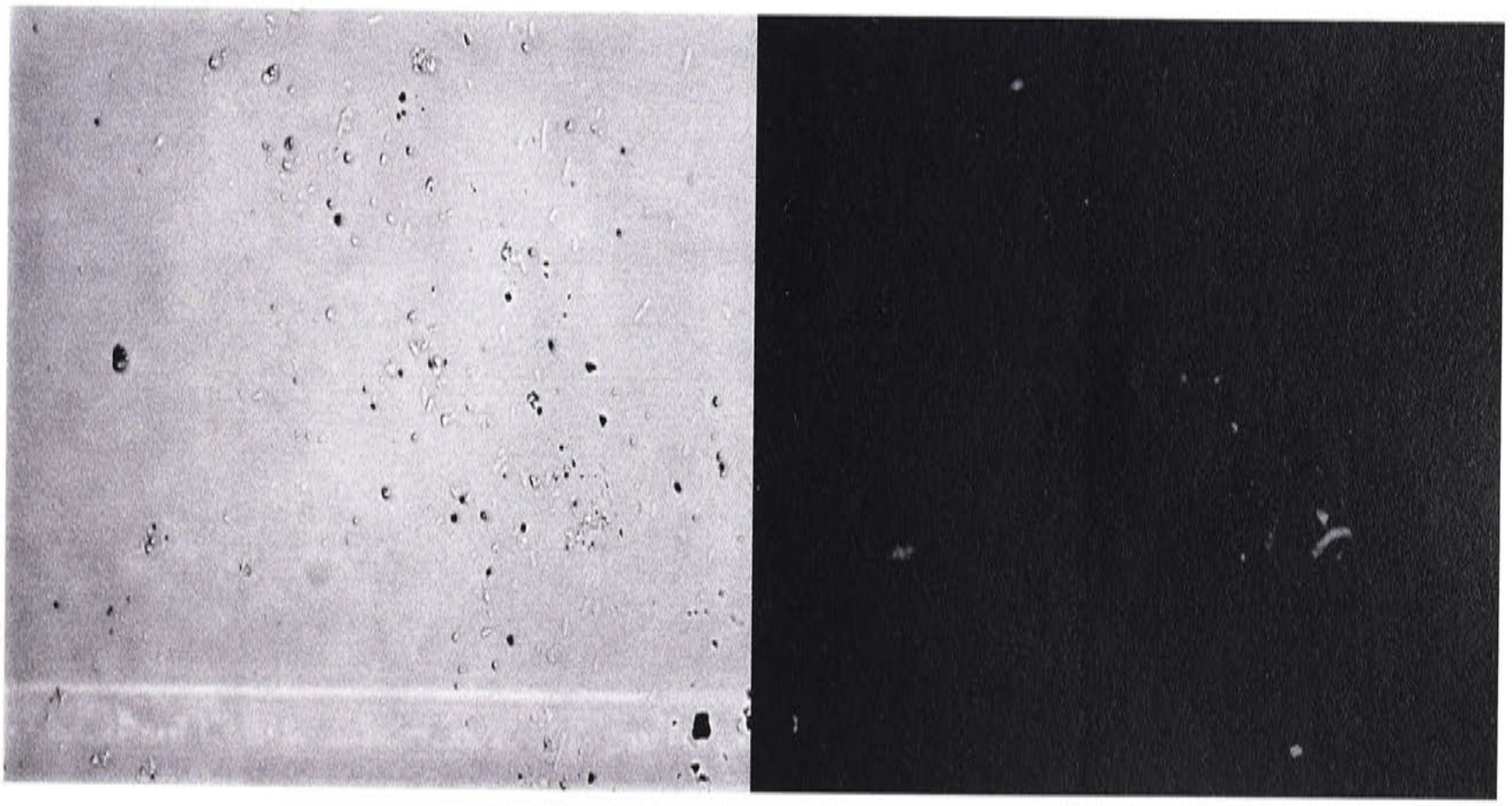


Figure 5.12. IcsA immunofluorescence for SFL1617: *mglB* mutant of SFL1001, SFL1736: *mglB* mutant complemented with a plasmid based copy of *mgl* operon, pNV1424. Examples of polar staining are indicated by arrows. For each strain the phase contrast image is on the left-hand side and the immunofluorescent image is on the right-hand side.

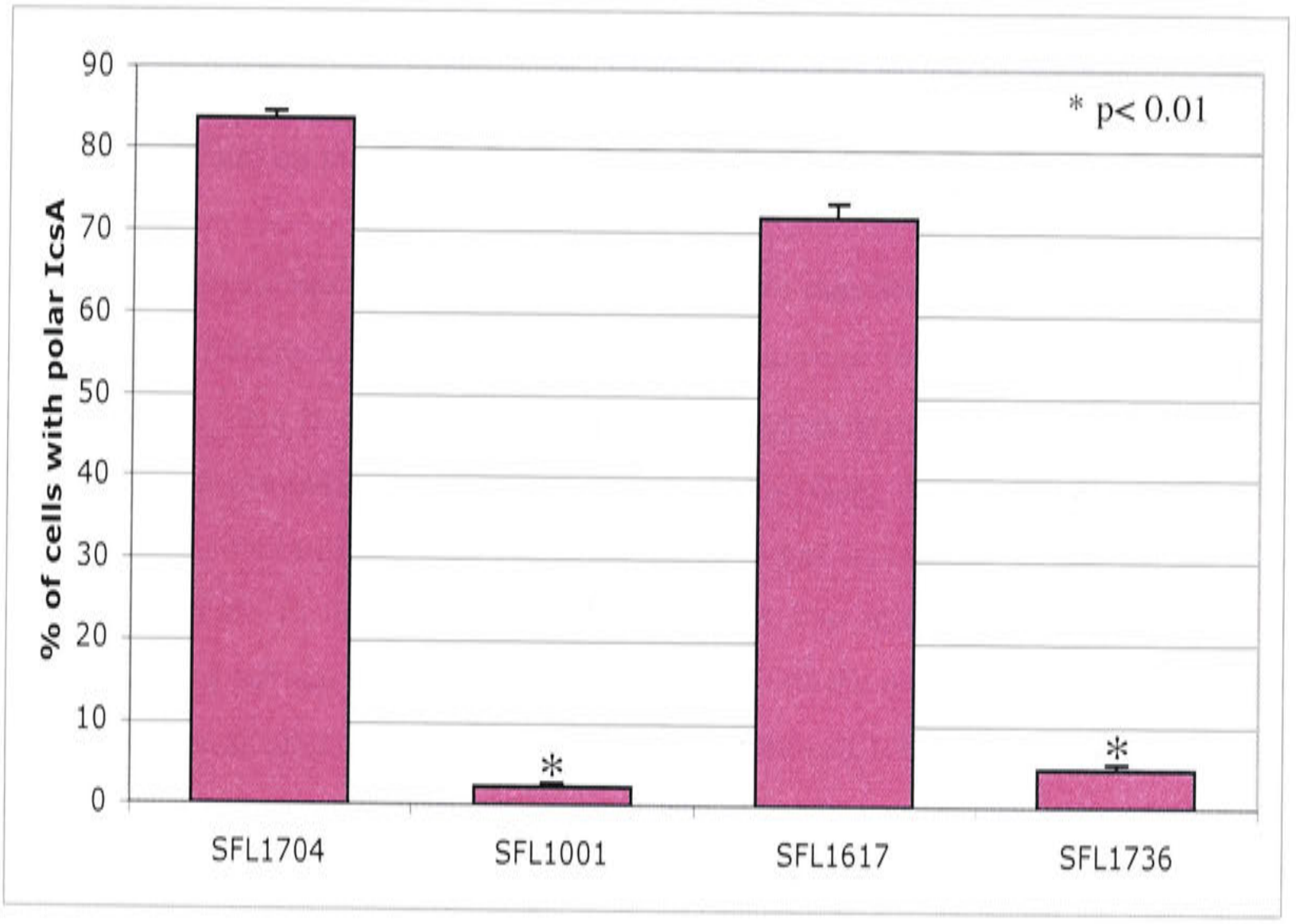


Figure 5.13. Percentage of cells per image with polar IcsA immunofluorescence staining. SFL1704: virulent 2457T strain, SFL1001: avirulent 2457T strain used to construct SFL1617, SFL1617: *mglB* mutant of SFL1001, SFL1736: *mglB* mutant complemented with a plasmid based copy of *mgl* operon, pNV1424. Results are the mean of three images per strain with standard error. SFL1001 and SFL1736 are significantly reduced in polar IcsA in comparison to SFL1617 according to t-tests.

To confirm the finding that IcsA is significantly decreased on the surface of SFL1001 and SFL1736, immunogold labelling of the strains was also performed and visualised by electron microscopy. SFL1223 was included as a negative control for IcsA staining, as the virulence plasmid has been cured from this strain so it cannot express IcsA (Figure 5.14). A polar cap of IcsA could be visualised for SFL1704 cells, likewise SFL1617 cells also displayed a polar distribution of gold particles on the cell surface (Figures 5.15 and 5.16). Neither SFL1001 nor SFL1736 had strong polar binding for IcsA and very few gold particles were bound on the cells (Figures 5.15 and 5.16).

Two different microscopy techniques have both shown that SFL1001, which is reduced in virulence in the Sereny test and plaque assay, has minimal levels of IcsA present on the outer membrane. This finding is consistent with the phenotype displayed by SFL1001, as IcsA mutants are dramatically impaired in the plaque assay and Sereny test but display normal invasion levels (324). The disruption of the *mgl* operon in SFL1617 appears to result in an increase in the strain's virulence, whilst also returning the outer membrane levels of IcsA to a normal level. The complementation of the *mgl* operon in SFL1736 has a negative effect on virulence and also reduces the amount of IcsA present on the surface of the cell.

5.3 Discussion

5.3.1 SFL1001 is impaired in intra- and intercellular spread

It was determined during this work that the *S. flexneri* 2457T strain, SFL1001, had become avirulent. The strain had been stored in glycerol for over ten years and prior to this study had only once been used in any type of virulence assay, which was an invasion assay. It has been shown that preserved *Shigella* strains become avirulent with time, with over 95% of strains being identified as avirulent after 10 years of storage (55). Avirulence is most often caused by molecular instability of regions of the virulence plasmid (297).

SFL1001 was capable of invasion in invasion assays, suggesting that the type III secretion system and the Ipa proteins necessary for cellular penetration are functional. Furthermore,

SFL1223

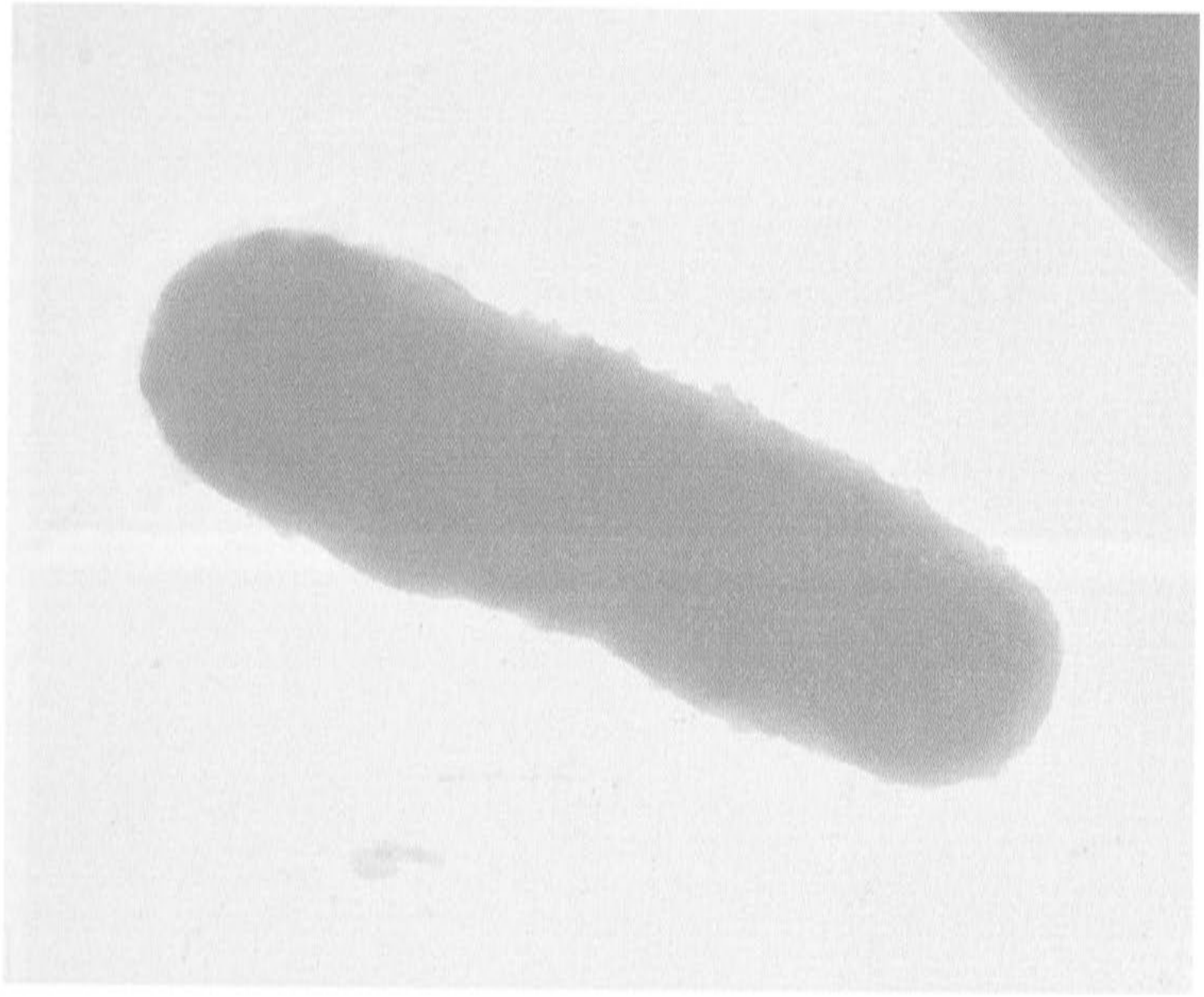
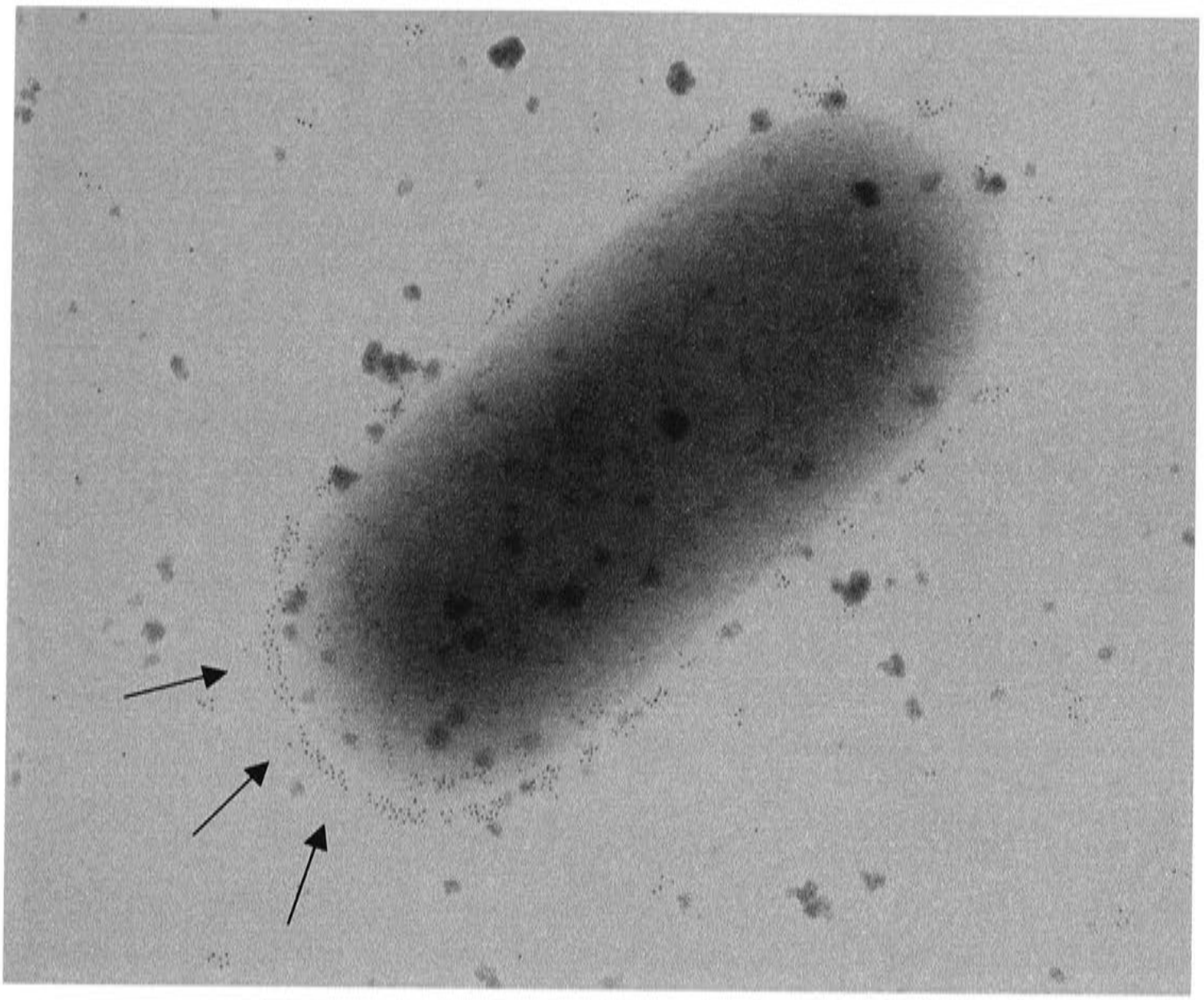


Figure 5.14. Immunogold staining for IcsA on SFL1223: virulence plasmid cured strain of *S. flexneri* 2a. Negative control for IcsA staining. X 60 000 magnification.

SFL1704



SFL1001

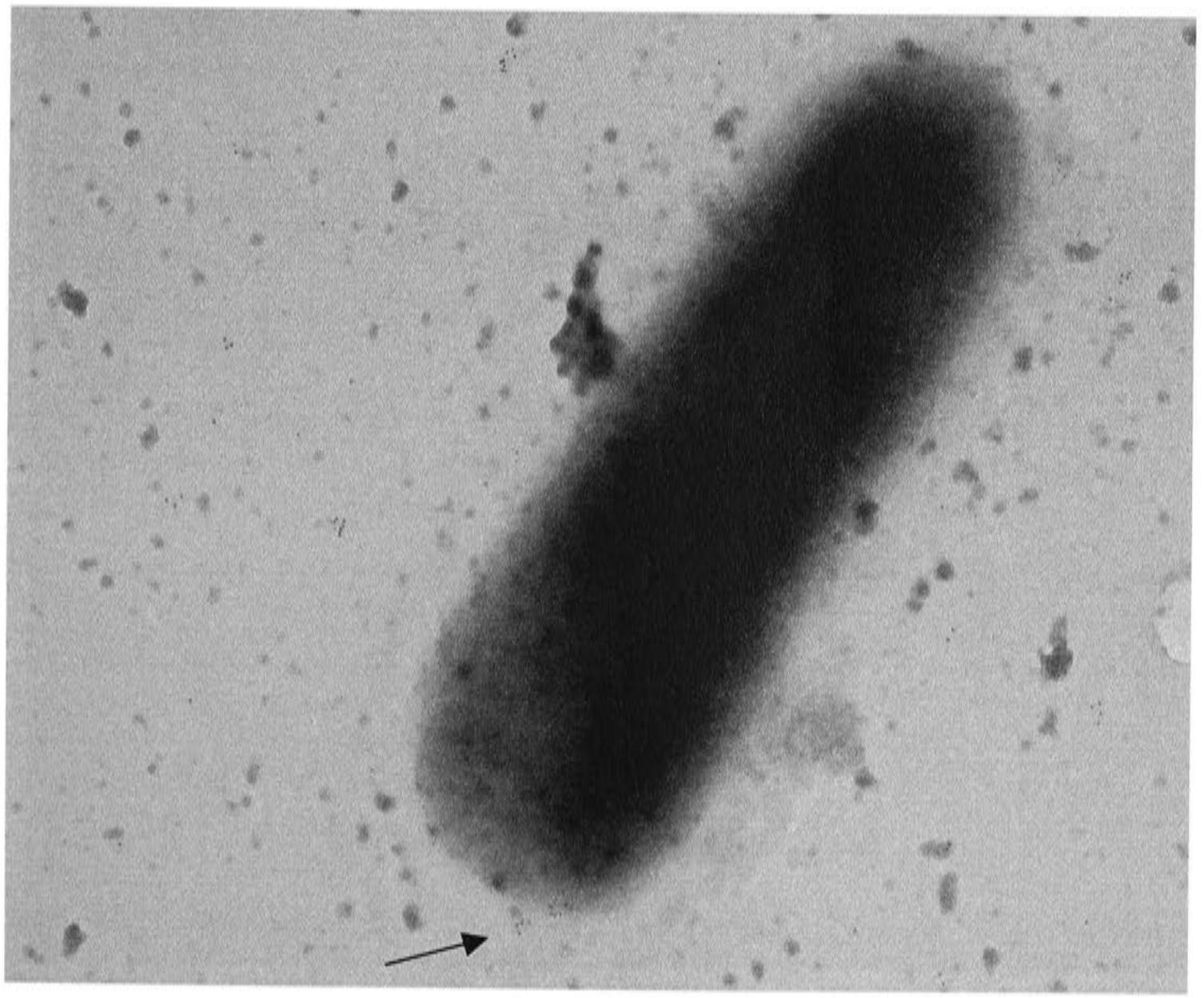


Figure 5.15. Immunogold staining for IcsA on SFL1704: virulent 2457T strain and SFL1001: avirulent 2457T strain used to construct SFL1617. Arrows indicate polar gold particles. X 60 000 magnification.

SFL1617

SFL1736

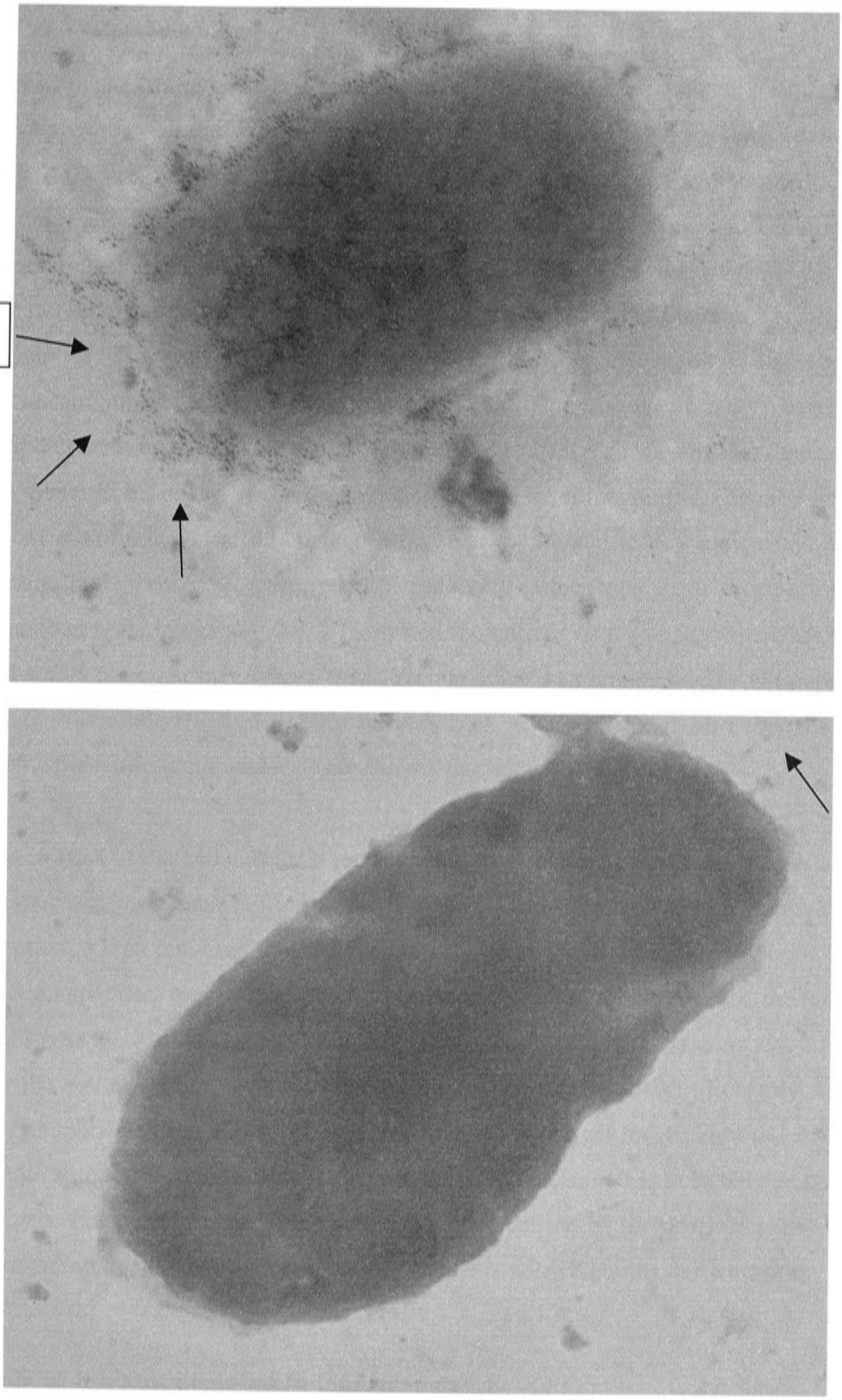


Figure 5.16. Immunogold staining for IcsA. SFL1617: *mglB* mutant, SFL1736: *mglB* mutant complemented with a plasmid based copy of *mgl* operon, pNV1424. Arrows indicate polar gold particles. X 60 000 magnification.

Western analysis of the MxiG and MxiJ proteins showed that they are expressed normally in SFL1001, suggesting that the structural proteins of the type III secretion system are not impaired. SFL1001 was not able to form plaques in cell culture monolayers, and was avirulent in the *in vivo* Sereny test. This behaviour suggests that the strain is deficient in intra- and intercellular spread, which is mediated by cell surface IcsA. It was determined by Western blot, immunofluorescent and immunogold microscopy that SFL1001 has a greatly reduced amount of IcsA on the cell surface. Western blotting also showed that the cytoplasmic concentration of IcsA is similar to that of SFL1704, a pathogenic 2457T strain, demonstrating that there is no defect in the expression levels of IcsA. It appears that there is a blockage in the transport of IcsA from the cytoplasm to the outer membrane, although not a complete restriction in IcsA trafficking as a little outer membrane IcsA was detected in both the immunofluorescence microscopy and the electron microscopy. IcsA transport is not well understood at the molecular level. However, the current model suggests that IcsA is transported across the inner membrane by the Sec apparatus and targeted to the old pole, possibly through interaction with a protein intermediate, where it inserts into the outer membrane by its β-domain and translocates to the cell surface (35, 355).

The reduction in IcsA at the cell surface does not seem to be caused by mutation in either the IcsA signal or target sequence regions as the IcsA levels can be immediately restored by the *mgl* disruption, which indicates that the protein itself is not defective. There may be a defect in the Sec apparatus, preventing IcsA from leaving the cytoplasm. However, the Sec transport pathway is a common pathway used to secrete a huge variety of proteins and it is not clear how the *mgl* disruption would reverse a defect in the apparatus (35). Recently, an apyrase gene, *phoN2*, has been shown to be important for the correct localisation of IcsA, possibly through an IcsA protein-protein interaction (332). Therefore, it is possible that the reduction in IcsA transport observed in SFL1001 could be caused by the down-regulation in the levels of an intermediate protein required for the IcsA polar targeting and transport.

5.3.2 The role of the Mgl proteins in IcsA transport

It was unexpected that the disruption of the *mgl* operon generated a virulent phenotype in SFL1617. However, it is not entirely uncommon for a gene disruption to increase the virulence of *S. flexneri*, both *icsP* and *shiA* mutants cause more severe pathogenesis than

their parent strain (140, 341). The *mglB* mutant was capable of generating plaques in BHK cell monolayers and a positive Sereny reaction in guinea pigs. Whilst the cytoplasmic levels of IcsA did not differ to SFL1001, it was determined by microscopy that IcsA was present on the SFL1617 cell surface at a vastly higher concentration than SFL1001 and, in fact, in comparable amounts to the wild type 2457T strain, SFL1704. This finding suggests that the removal of *mgl* protein from the cell in some way allows cytoplasmic IcsA to reach the outer membrane.

Interestingly, the complement of the *mgl* operon on a plasmid in SFL1736 was sufficient to reduce the virulence of the strain in both plaque assays and the Sereny test. Furthermore the outer membrane levels of IcsA were reduced to similar amounts as SFL1001. Therefore, it seems that the *mgl* operon actually has a negative effect on *S. flexneri* virulence. Complementation with just the *mglB* gene in SFL1718 did not reduce virulence in the plaque assay. However, it is not clear whether the *mglB* transcript would be stable when it is not cotranscribed with *mglAC*. It is possible that the entire operon is not required to reduce virulence, but is required for complete expression of the one gene that is important. The complemented strain, SFL1736, was never quite as attenuated in virulence as SFL1001; the Coomassie-stained 1D SDS-PAGE gel demonstrated that at least MglB is being expressed from the plasmid, pNV1424, however it may not be 100% stable in the cell culture and Sereny assays, as antibiotic selection is absent during intracellular growth.

The results presented in this Chapter suggest that the *mgl* operon plays a role in regulating the transport of IcsA from the cytoplasm to the outer membrane. It is not yet clear just how this may be occurring but it is possible to develop a number of hypotheses based on known characteristics of the *mgl* proteins, which could be confirmed in further work.

The *mgl* operon produces a high affinity galactose ABC transport system, made up of MglA and MglC that are inserted in the inner membrane. MglB acts as a periplasmic galactose binding protein, which binds galactose and delivers it to the transport components. Interestingly, MglB is also a chemoreceptor that interacts with the Trg chemotaxis receptor found in the inner membrane (116). MglB binding to the Trg receptor induces the formation of polar clusters of chemoreceptor complexes in order to amplify the

chemotactic signal (210). Therefore, it may be possible that the MglB induced aggregation of chemoreceptors at the bacterial pole could interfere with the polar transport of IcsA across the inner membrane. However, in this hypothesis it is not clear why this effect would be so severely amplified in SFL1001, such that IcsA is not transported at reasonable efficiency to the cell surface until MglB has been removed from the cell, as in SFL1617. It could be possible that in SFL1001 the levels of chemoreceptor aggregation have been dramatically increased by a regulatory defect resulting in high receptor protein levels or increased sensitivity to chemo-activation, which would result in increased production of polar chemoreceptor complexes.

Alternatively, MglB will presumably be found towards the poles of the bacteria if it must interact with polar localised chemoreceptor proteins. Therefore, there may be a bias for MglA and MglC membrane complexes to also locate to the pole. Perhaps the presence of these membrane complexes may be sufficient to impede polar IcsA transport in SFL1001. In this case, SFL1001 may possess a regulatory defect that results in large amounts of MglA and MglC accumulating within the membrane.

It should also be noted that MglB appears to be transported by the Sec apparatus across the cytoplasmic membrane in *E. coli* (82). The IcsA protein, therefore, may share the Sec apparatus for transport across the cytoplasmic membrane with MglB. As previously mentioned, it seems likely that the polar transport of IcsA uses protein interactions to guide IcsA to the pole of the bacteria and it is believed that these protein interaction(s) may initially occur in the cytoplasm prior to Sec transport. It is not known what protein(s) are involved. MglB also functions as a chaperone capable of binding proteins in either its substrate bound or unbound conformation (312). Perhaps MglB in its chaperone capacity interacts and binds with an unknown protein component of IcsA polar transport, competing with IcsA for the protein partner and therefore, for transport with the Sec apparatus. A previous study has shown that co-expression of a polar located *Lactococcus* protein, LtrA, actually interferes with the polar location of IcsA on the cell surface of *E. coli*, which the authors proposed was due to competition for a common binding partner (421).

The competitive binding of MglB to a possible IcsA protein transport partner may act as a regulatory step for the amount of outer membrane IcsA displayed by the cell. IcsA found on the cell surface only remains functional for a short period of time before it is cleaved by IcsP, so perhaps it is useful to regulate the amounts of IcsA present on the cell surface when it is not necessarily needed. In periods of low galactose availability, such as during host invasion when the high affinity binding of MglB is important, it is possible that MglB binds galactose preferentially over proteins, allowing IcsA to be transported to the cell surface for use in intra- and intercellular spread within host cells. This regulatory effect may only be minor in a normal virulent *S. flexneri* cell, however in SFL1001 the expression of the IcsA transport protein partner may be significantly lowered; a scenario particularly feasible if the gene is located in a virulence gene region, since these are most likely to suffer alterations in virulent strains stored for long periods (55). If the protein partner is reduced in SFL1001 then it is possible that the majority of it will be bound by the highly abundant MglB protein, leaving little to assist in IcsA transport (312). Therefore, the disruption of *mglB* in SFL1001 would allow IcsA to reach the outer membrane, such as what is observed for SFL1617. Furthermore, the complementation of MglB would result in high amount of MglB protein available to bind the protein partner and impede IcsA transport in SFL1736, restoring the low outer membrane IcsA phenotype.

5.3.3 Further work on the role of the *mgl* operon in regulation of cell surface IcsA

The most pertinent question raised by this work is whether *mgl* disruption would have any effect on the virulence or IcsA cell surface levels in a virulent *S. flexneri* strain, or whether the phenotype described in this Chapter is specific to the altered virulent state of SFL1001. Numerous attempts were made within this project to develop disruption mutants for the *mgl* operon in SFL1704, a virulent 2457T strain. As described in Chapter 4, both the suicide vector and dual PCR integration methods were used in parallel in SFL1704. It was hoped that as the insertion of pNV1228 into SFL1001 had been successful, that it would be possible to readily insert the same suicide vector into the *mgl* operon in the SFL1704 background. Unfortunately, despite a number of resistant colonies being produced, screening did not yield a correct insertion. Similarly, both pKM208 and pKD46 were used as helper plasmids in lambda red-mediated PCR knockout attempts on SFL1704. It was

intended to not only produce a polar knockout for the *mgl* operon but to also generate a non-polar knockout in order to determine if disruption of just *mglB* was sufficient for the increase in virulence, and possibly IcsA transport. The optimisation attempts discussed in Chapter 4 were implemented, but due to the time constraints of this project it was not possible to successfully produce a *mgl* mutant in SFL1704. It seems that there is an increased difficulty in producing allelic exchange knockouts in a virulent *S. flexneri* strain, like SFL1704, in comparison to an avirulent strain such as SFL1001. It is unclear why this may be, although virulent strains can be inherently more difficult to transform efficiently, which may contribute to the lower efficiency of recombination (34, 245, 407). As discussed in Chapter 4, there are alternate targeted disruption techniques that may be useful for ultimately constructing mutants for the *mgl* operon in SFL1704. The mutants, once constructed, could be analysed for any increase in virulence in plaque assays and the Sereny test, and IcsA distribution could be analysed again by microscopy. Immunogold microscopy would be useful to determine quantatively if the levels of surface IcsA increase when the *mgl* operon is disrupted in SFL1704.

To explore whether MglB and the Trg chemoreceptor play a role in impeding IcsA transport, as hypothesised, it would be ideal to produce a Trg disruption mutant and determine, by microscopy, if removal of Trg also restores IcsA transport in a SFL1001 background. It would also be interesting to investigate the possible protein-binding partners of both MglB and IcsA. According to literature searches, it seems IcsA and MglB have never had their protein interactions analysed. It would be possible to search for protein-protein interactions for these proteins using a technique such as the Stratagene BacterioMatch® II Two-Hybrid System. IcsA and MglB may both interact with a common protein, suggesting that the hypothesis that MglB competes with IcsA for this binding partner during high galactose conditions is feasible.

5.4 Conclusion

The *mgl* operon was disrupted in the avirulent strain, SFL1001, creating SFL1617. Interestingly, the disruption of this operon in SFL1617 produced a virulent phenotype in plaque assays and the Sereny test. The complementation of the *mgl* operon in SFL1736 significantly reduced the virulence displayed by this strain in the same assays. It was

determined that none of the strains were defective in adherence to, and invasion of epithelial cells suggesting that the decease in virulence in SFL1001 and SFL1736 was due to a defect in intra- and intercellular spread. Western analysis showed that the strain's expression levels of the type III secretion proteins, MxiG and MxiJ, and the cytoplasmic concentration of IcsA were all normal. However, a Western blot of extracellular IcsA suggested that there was a marked decrease in IcsA being cleaved from the cell surface in SFL1001 and SFL1736. Immunofluorescent and immunogold microscopy showed that SFL1001 and SFL1736 had very little cell surface IcsA, which explains the observed virulence deficiencies. The *mgl* operon appears to be impeding the passage of IcsA to the cell surface, possibly by preventing IcsA from either passing through the cytoplasmic membrane at the pole, or by binding a protein transport partner, thereby blocking its interaction with IcsA.

Chapter 6
Development of *S. flexneri* IpaD subunit vaccines

Chapter 6

Development of *S. flexneri* IpaD subunit vaccines

6.1 Introduction

The cost of treating shigellosis with antibiotics is unrealistic for developing countries where the disease is most prolific. As the immune response stimulated by infection can be protective against reinfection, vaccination is a viable option against *S. flexneri*. An ideal vaccine for shigellosis must meet certain requirements: the immunity raised needs to be long-lasting, the vaccine cheap to manufacture, induce minimal side effects and be simple to administer as children in developing countries will be the main recipients (149).

Since the 1940s, a number of candidate vaccines for *S. flexneri* have been developed. Unfortunately, none have yet been successful enough for field release. Invasive live vaccines are increasingly being developed for *S. flexneri* as they deliver antigen to the mucosal immune response, which appears to be important in the generation of protective immunity against *S. flexneri* (290). A number of such strains have produced encouraging results in human trials (161, 175, 176). However, even with these promising vaccine strains an ideal balance has not been struck between the strength of immunogenicity induced and the degree of symptoms produced in the human subjects. Often, in order to prevent a live vaccine strain producing any detrimental side effects in the volunteers, it is necessary to administer a lower dose of the strain, which can be too low to provoke a sufficiently strong immune response (149).

The use of subunit vaccines can avoid the safety issues associated with live vaccine strains. Traditionally *S. flexneri* LPS has been used in subunit vaccine studies, as it is a major target for the host's immune response. For example, LPS has been fused to carrier proteins and delivered parenterally to volunteers. These preparations are safe in humans and are capable of inducing strong serum antibody responses; however, the protective capabilities of these vaccines are yet to be explored (11, 279). Furthermore, LPS has been complexed to

meningococcal outer membrane proteins and proteosomes, which is well tolerated in humans and capable of inducing significant antibody levels (97).

It is not clear whether cellular immunity plays any role in protective immunity against *S. flexneri* as T-cell deficient mice are still completely protected against challenge with wild type *Shigella* (401). Therefore, vaccine studies for *Shigella* generally only concentrate on systemic and mucosal antibody responses, which have both been shown to be important for immunity (306, 400). Immunity to *S. flexneri* appears to be serotype-specific as it is generally directed against the serotype-specific structure of the LPS (79, 96). However, as many different serotypes of *S. flexneri* are prevalent in a geographical area at any one time, it would be advantageous to develop vaccines that are capable of protecting against heterogenous serotypes simultaneously. It may be possible to do this by utilising immunogenic proteins of *S. flexneri* that are common to all serotypes, in a subunit vaccine.

The immunogenicity of a subunit vaccine can be greatly improved with the use of an optimal adjuvant or delivery system. *Shigella* antigens have never been administered in liposomes. It is possible to load liposomes with adjuvants and ligands with which to target and enhance particular immune cell responses (378). Thus, it would be interesting to determine if liposomes are a potent delivery system when coupled with *S. flexneri* protein antigens. Additionally, other adjuvants yet to be tested with *S. flexneri* protein, include gamma inulin and cochleates (218, 288). Gamma inulin is a plant carbohydrate, which activates the alternative complement pathway and has been used to boost the immune response against a hepatitis B surface antigen and tetanus toxoids (344). Cochleates are a stable phospholipid-calcium precipitate, which have been used to induce high serum IgG titres against influenza glycoproteins (218).

The protein IpaD was detected in the immunogenic screen performed in Chapter 3. IpaD is an invasion plasmid antigen, expressed from the *S. flexneri* virulence plasmid and is specific to *Shigella* species (40). The type III secretion system-mediated secretion of IpaD is required for epithelial cell membrane disruption and bacterial invasion (293). IpaD has previously been shown to be immunogenic both in natural and experimental *Shigella* infections (197, 264). It remains unclear whether the immune response directed against

IpaD offers any protection against reinfection, although an invasin complex preparation known as Invaplex, which contains IpaB, C and D as well as *S. flexneri* LPS is able to protect mice and guinea pigs against *Shigella* challenge (374). IpaD was chosen as a subunit candidate for this vaccine adjuvant, because it is a *Shigella*-specific protein, is immunogenic in natural infection and may be able to contribute to serotype independent protective immunity.

6.2 Results

6.2.1 Plaque inactivation assay for IpaD

A plaque assay can be used as a measure of *S. flexneri* invasion, where a reduction in invasion can be observed as a decrease in the number of plaques formed (266). Antibodies against IpaD are produced during shigellosis but it is unclear whether these anti-IpaD antibodies offer any protection against *Shigella* infection (197). In order to determine if an antibody capable of binding IpaD has any capacity to reduce *S. flexneri* invasion, plaque inactivation assays were performed.

SFL1704, a wild type *S. flexneri* 2a strain, was incubated with an affinity-purified polyclonal anti-IpaD antibody, raised against a C-terminal peptide of IpaD (395). The treated cells were used in plaque assays alongside untreated SFL1704 cells and the number of plaques was measured (Figure 6.1). There was a significant decrease in the number of plaques formed by the anti-IpaD treated SFL1704 cells in comparison to the untreated SFL1704 cells, according to a t-test ($p<0.01$). There was no change in plaque size or shape indicating that only epithelial cell invasion was affected by IpaD neutralization (Figure 6.2).

These results indicate that IpaD is a suitable candidate for use in subunit vaccine studies since antibody neutralisation of IpaD has a detrimental effect on the invasive phenotype of *S. flexneri*. This suggests that an immune response raised against IpaD by a subunit vaccine could potentially preclude *S. flexneri* from entering epithelial cells, thereby preventing the development of shigellosis.

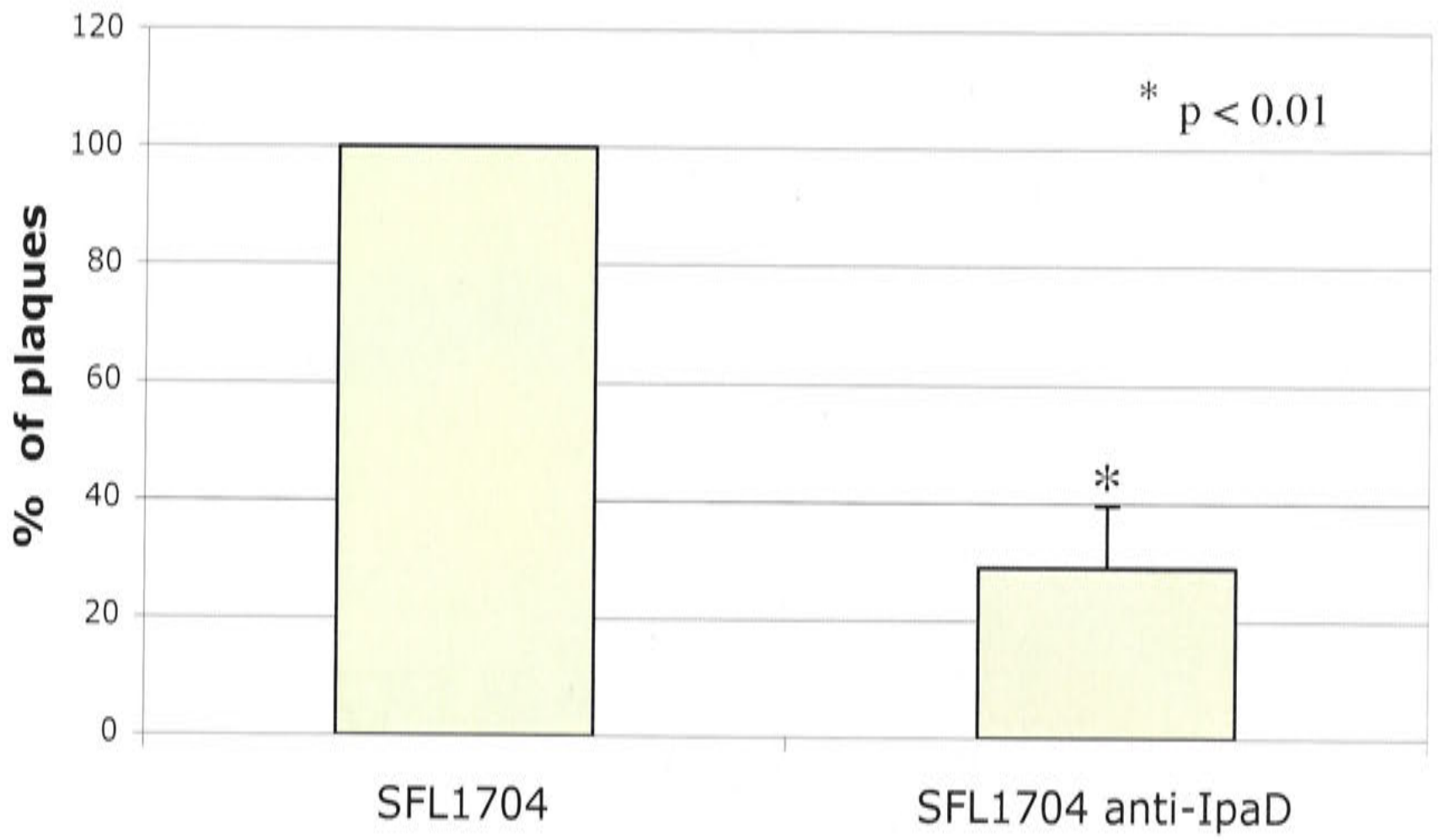
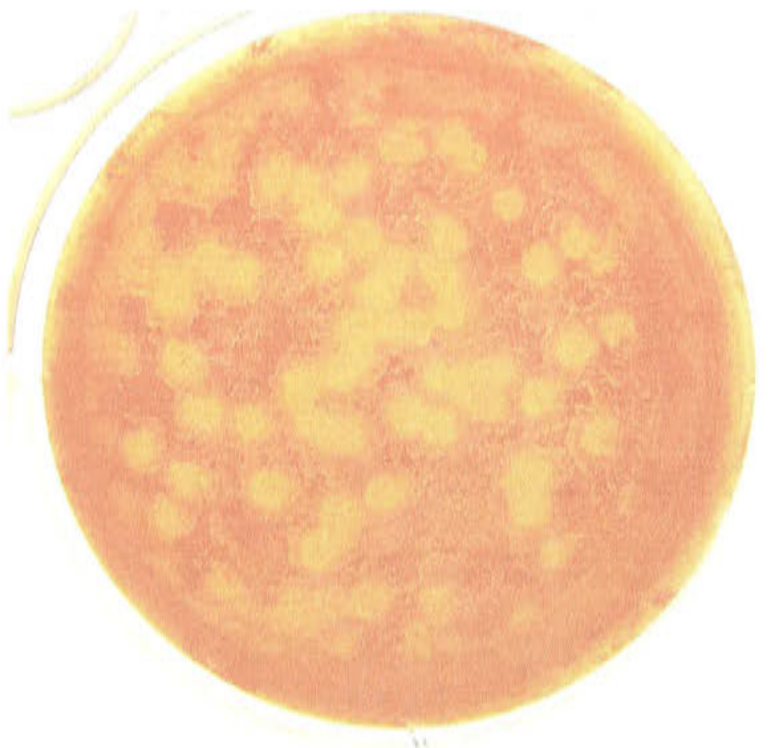


Figure 6.1. Plaque inactivation assay results for SFL1704 cells treated with anti-IpaD antibody. Plaque numbers are expressed as a percentage of the number of plaques formed by untreated SFL1704. Antibody inactivation of IpaD significantly reduces the number of plaques formed by SFL1704 according to a t-test. Results are the mean of three repeats with standard error.

SFL1704



SFL1704
Anti-IpaD
treated

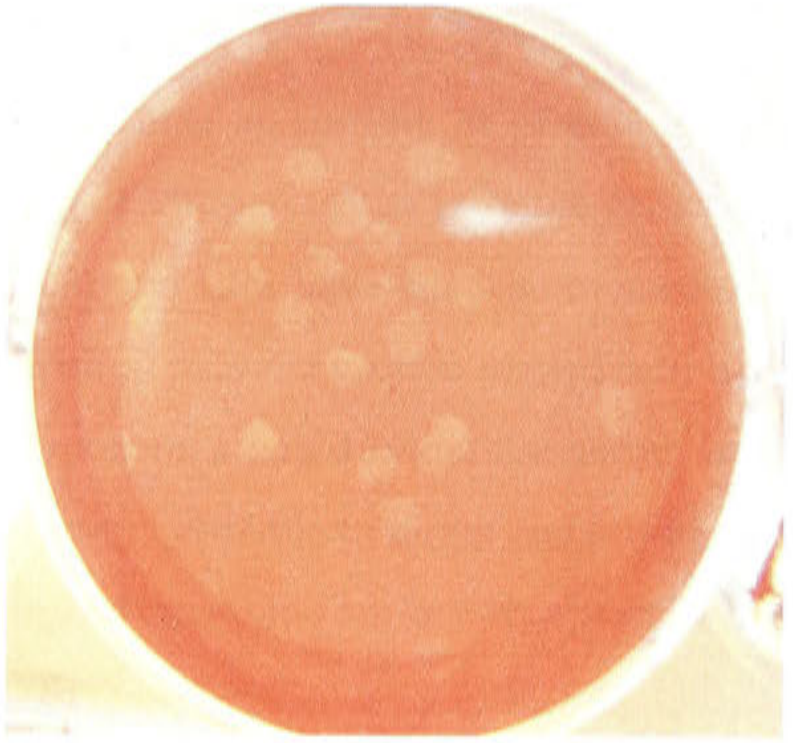


Figure 6.2. Plaque inactivation assay photos of SFL1704 cells treated with anti-IpaD antibody. Antibody treatment reduces the number of plaques but has no effect on the size or shape of plaques.

6.2.2 Overexpression of IpaD

IpaD was cloned into pGEX-4T-1 using the primers IpaDFGex and IpaDRGex, creating pNV1326 in B1525. pNV1326 was sequenced to ensure that the gene was in frame with the N-terminal GST tag. GST-IpaD protein expression was induced with IPTG for 3 hours and proteins extracted by sonication and centrifugation. The tagged protein was purified from cellular protein by binding the GST tag to a glutathione sepharose 4B column. Contaminating protein was removed by repeated washes with PBS and the IpaD protein cleaved from the GST tag by incubation with thrombin. IpaD was eluted from the column into sterile PBS and the protein size and purity was checked by SDS PAGE stained with Coomassie blue. Figure 6.3 shows protein from each step of the purification and demonstrates that the purified IpaD protein is clean and free from high concentrations of contaminating cellular protein. This protein was used in the vaccine studies of this Chapter.

6.2.3 Liposome vaccine study with IpaD

Liposomes not only act as adjuvants, but also as carriers of protein antigens capable of protecting and delivering large amounts of antigen to the immune cells of the host (261). Although proteosome studies with *S. flexneri* LPS have been quite successful, there is no mention in the literature of any trials with *S. flexneri* protein loaded into liposomes (97). It would be interesting to determine if liposomes could boost the serum immune response raised against IpaD protein. Furthermore it is possible to target antigen-loaded liposomes to particular immune cells by including ligands that bind surface receptors of dendritic cells or MHC class II expressing cells (377, 378).

IpaD was chosen as the *S. flexneri* antigen for assessment of the viability of liposomes in *S. flexneri* vaccine development. Six different vaccine groups were designed, which are listed in Figure 6.4A and designated A-F. *S. flexneri* 2a LPS was included in two of the groups to determine if LPS boosts the antibody response to IpaD. The ligand CD4 was also used in this trial to investigate if directing the liposomes to MHC class II expressing cells would increase the immune response, and finally a single chain full length variable Ab fragment (ScFv), which binds CD11c on dendritic cells, was included to target liposomes to dendritic cells. IpaD in PBS was used as a baseline immunisation to determine if liposomes are capable of inducing a stronger antibody response then the protein alone. The purified IpaD,

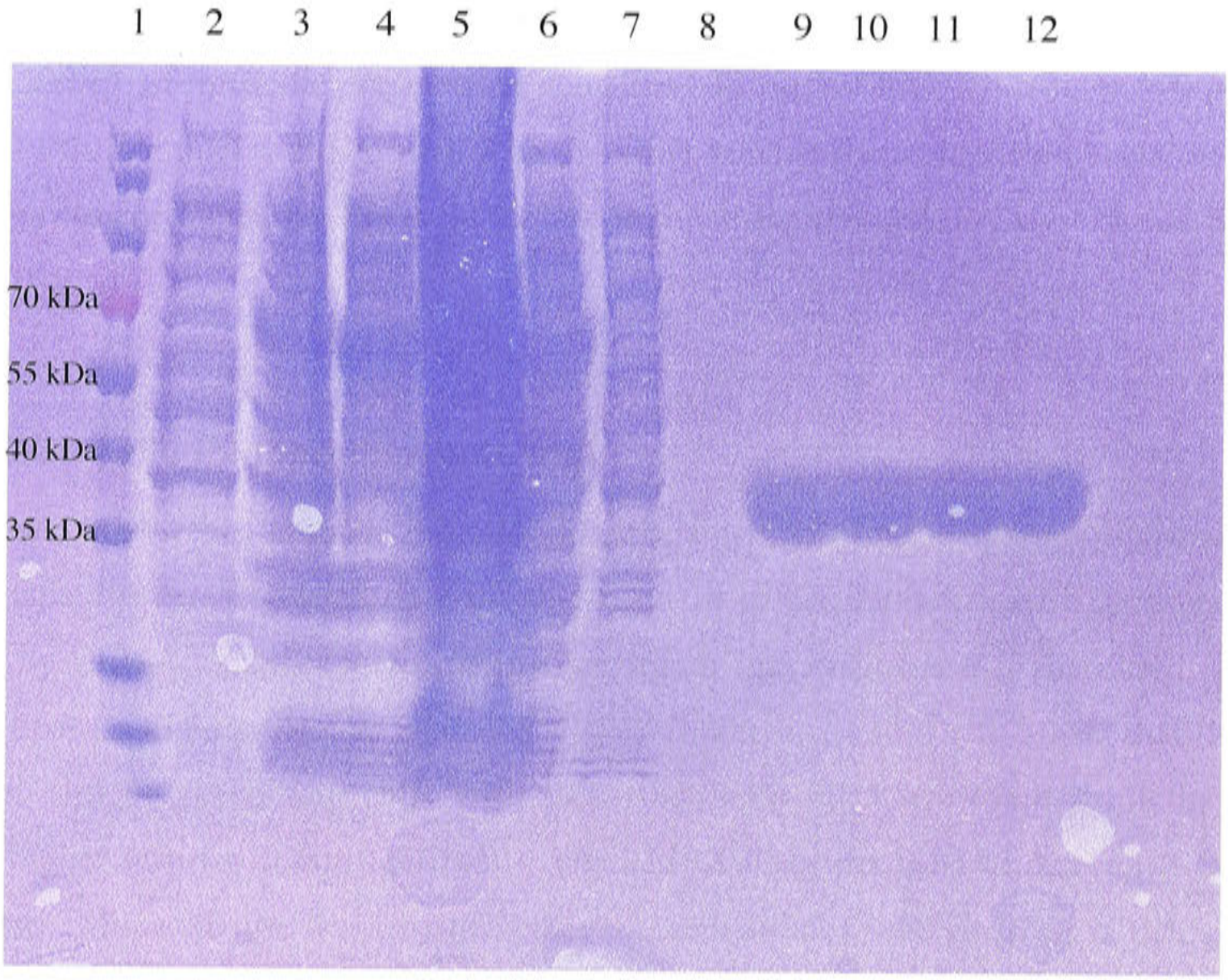


Figure 6.3. Protein overexpression for IpaD from B1525. Lane 1: Fermentas Prestained PageRuler Marker, Lane 2: uninduced B1525 whole cell lysate, Lane 3: induced B1525 whole cell lysate, Lane 4: sonicate supernatant, Lane 5: sonicate pellet, Lane 6: wash 1, Lane 7: wash 2, Lane 8: wash 3, Lanes 9-12: trypsin cleaved IpaD elute. IpaD is a 37 kDa protein.

ligand and liposome preparations were made by Dr Joe Altin, School of Biochemistry and Molecular Biology, Australian National University.

5 female Balb/c mice were used per vaccine group and were inoculated subcutaneously with either 200 μL of liposome preparations containing 100 μg of IpaD protein, or 100 μg of IpaD in PBS. The mice were boosted with the same vaccine preparations on Day 7 and Day 14. Sera was collected on Day 0 as the pre-immune sample and on Days 14 and 34 (Figure 6.4B).

6.2.4 ELISA results for IpaD liposome trial

In order to study the serum antibody response raised against IpaD for the different liposome vaccine groups, the sera collected on Days 0, 14 and 34 of the immunisation experiment was tested by ELISA against IpaD protein. The serum IgG response was measured, as serum IgG appears to be protective against *S. flexneri* infection, and it was hoped that the targeting of antigen presenting cells with CD4 and ScFv (Cd11c) would result in a detectably higher titre for serum IgG (400). The ELISA data was used to determine the antibody titres, where the titre was the highest inverse sera dilution with an OD_{490} of 0.5.

There was minimal antibody titre detected against IpaD for the Day 0 sera samples (Figure 6.5). All six groups had produced IgG antibodies against IpaD by Day 14, which had significantly increased from Day 0 according to t-tests ($p<0.05$). The antibody titres had increased 10 fold by Day 34 and were also significantly increased in comparison to the Day 0 pre-immune antibody titres (t-tests, $p<0.01$). There was no significant difference between the antibody titres of each group for any day according to ANOVA analysis. Therefore, the delivery of IpaD within liposomes containing immune cell targeting ligands and LPS did not significantly increase the antibody titre above that produced against IpaD protein alone.

The anti-LPS IgG response was measured for groups B (liposomes, IpaD and LPS) and D (liposomes, IpaD, LPS and CD4). Group F (IpaD protein in PBS) was also included to determine if there was any contaminating LPS present in the IpaD protein preparation, which would artificially boost the immune response raised against the IpaD protein alone (Figure 6.6). There was only a minimal titre of anti-LPS IgG detected for group F, which

A

Liposome Delivery Trial Groups

A. IpaD + Liposome preparation

B. IpaD + LPS + Liposome preparation

C. IpaD + CD4 + Liposome preparation

D. IpaD + LPS + CD4 + Liposome preparation

E. IpaD + ScFv (CD11c) + Liposome preparation

F. IpaD in PBS

B

Day	
0	First bleed, First injection
7	Second injection
14	Third injection, Second bleed
34	Third bleed

Figure 6.4. Outline of liposome vaccine trial. **A**. Vaccine groups for liposome trial with IpaD. **B.** Immunisation schedule. The trial vaccine groups were delivered subcutaneously to 5 mice per group.

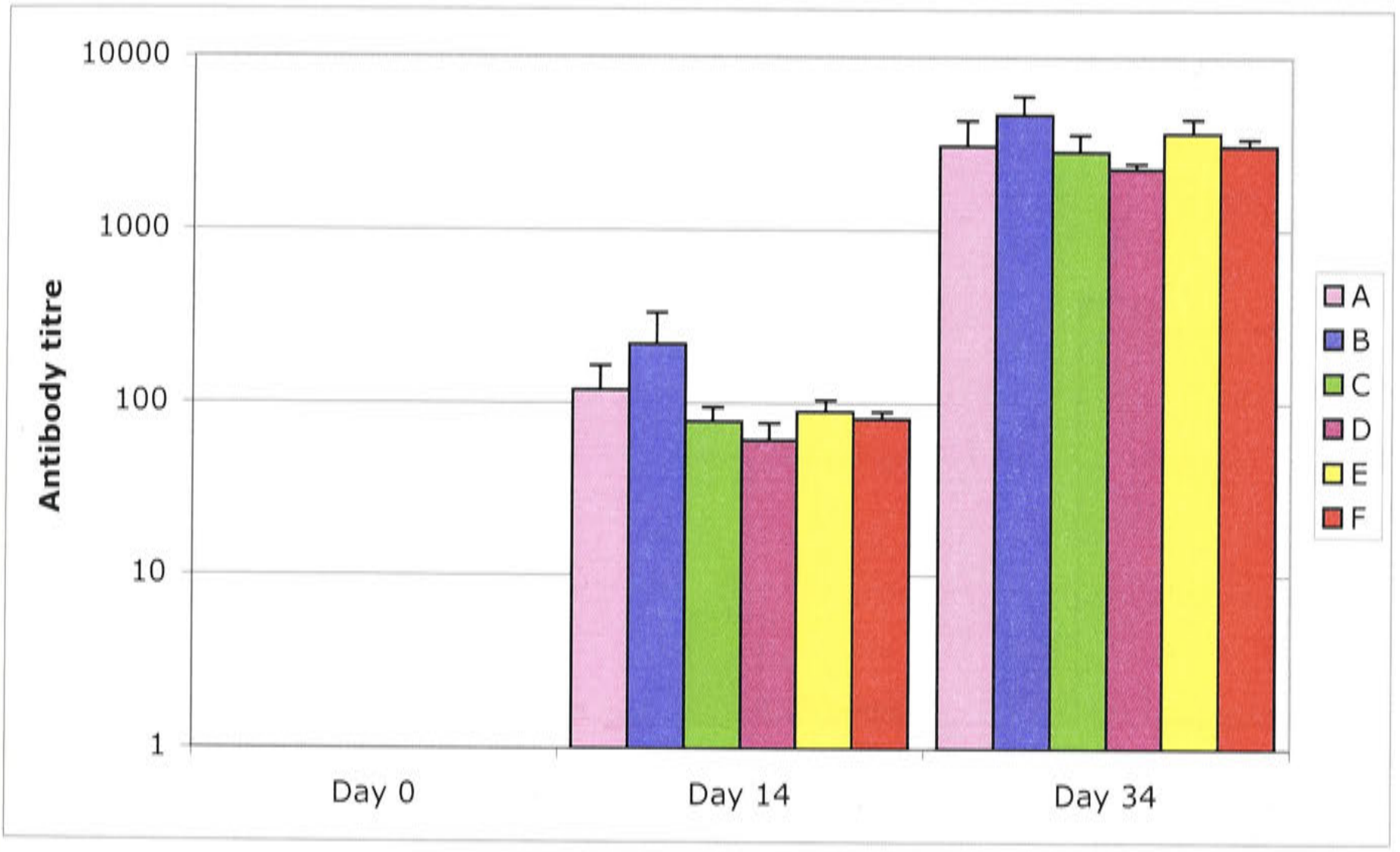


Figure 6.5 IgG antibody titre for IpaD liposome groups A-F with standard error. There is no significant difference between the antibody titres of each group on Day 14 and 34 according to ANOVA analysis. All groups' titres were significantly increased on Day 14 and Day 34 in comparison to Day 0 according to t-tests, Day 14 $p<0.05$, Day 34 $p<0.01$. Titre is expressed as the highest inverse dilution of sera with an OD_{490} of 0.5. Group A: IpaD + liposomes, Group B: IpaD + LPS + liposomes, Group C: IpaD + CD4 + liposomes, Group D: IpaD + LPS + CD4 + liposomes, Group E: IpaD + ScFv (CD11c) + liposomes, Group F: IpaD in PBS.

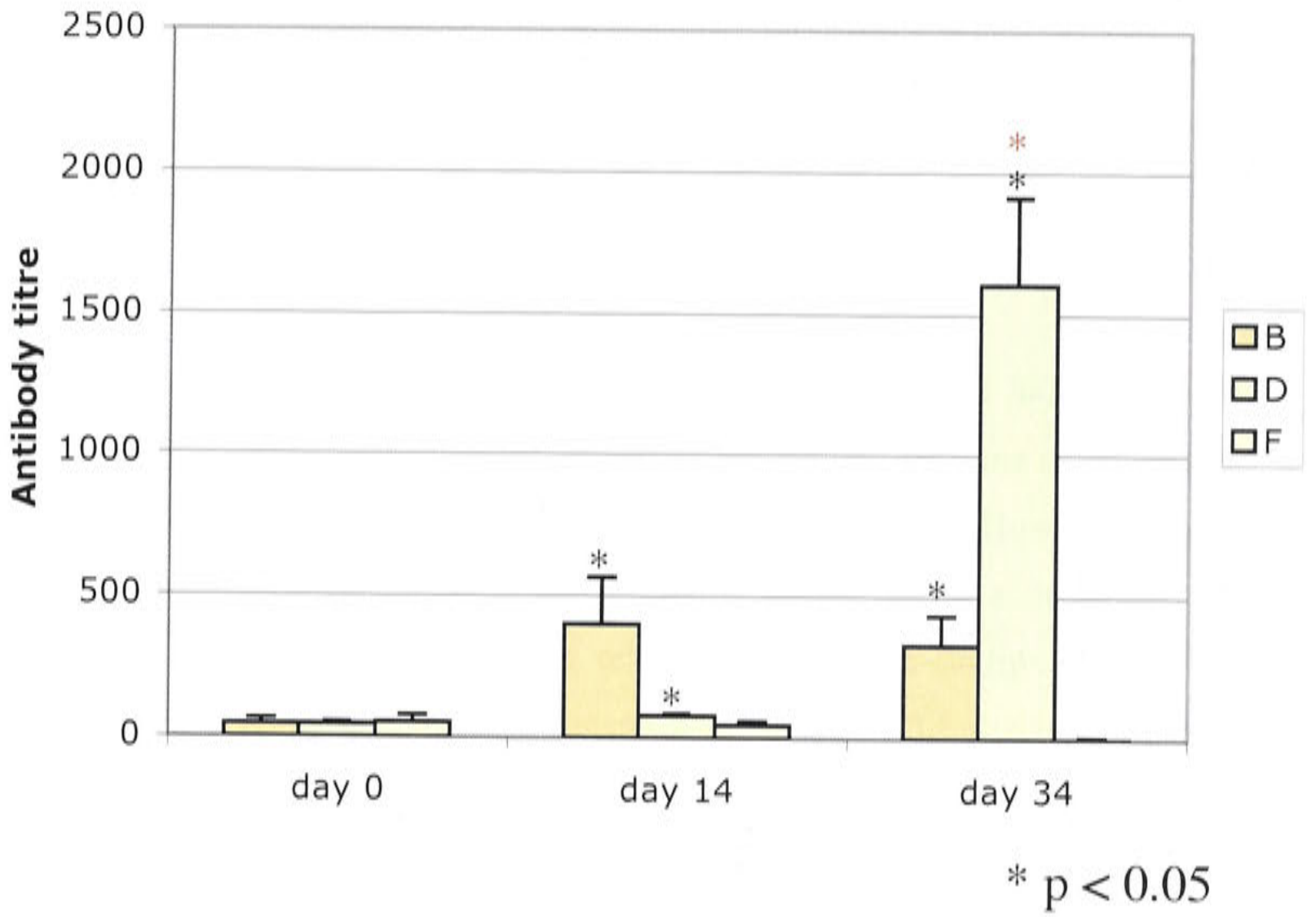


Figure 6.6. IgG LPS antibody titre for IpaD liposome groups B, D and F with standard error. There was a significant increase in the titre for groups B and D on Day 14 and Day 34 in comparison to Day 0 according to t-tests. There was no significant change to the titre for group F. On Day 34, group D's anti-LPS titre is significantly higher than group B (indicated by red asterix). Titre is expressed as the highest inverse dilution of sera with an OD_{490} of 0.1 Group B: IpaD + LPS + liposomes, Group D: IpaD + LPS + CD4 + liposomes, Group F: IpaD in PBS.

did not increase after immunisation, demonstrating that the IpaD protein sample was not contaminated with LPS. Both group B and D had a significant increase in their anti-LPS IgG titres after immunisation (t-test, $p<0.05$), indicating that the liposomes had successfully delivered LPS to the immune response. Interestingly, the Day 34 anti-LPS titre for group D was significantly increased in comparison to the titre for group B on Day 34 (t-test, $p<0.05$). Group D received IpaD and LPS in liposomes coupled with CD4 to target MHC class II expressing cells, while group B only received IpaD and LPS in liposomes. It is therefore possible that by targeting antigen presenting cells with CD4, the liposome preparation has generated a significantly stronger immune response against the LPS. Unfortunately, the same effect was not observed for the anti-IpaD IgG response for group D.

The use of liposomes with LPS and the incorporation of CD4 and ScFv (CD11c) as targeting molecules did not significantly increase the murine IgG immune response against IpaD in comparison to protein delivered in just PBS or liposomes. However, it can be concluded that IpaD is highly immunogenic when delivered as a subunit vaccine subcutaneously in a mouse model. Further research on the use of liposomes was not performed during this study due to time constraints. Section 6.3.1 discusses additional experiments, which would help determine if liposomes could be a useful adjuvant for *S. flexneri* subunit vaccines.

6.2.5 Gamma inulin, alum and cochleates as adjuvants in IpaD immunisation

The following experiment was performed in collaboration with Vaxine Pty Ltd and the vaccine preparation and ELISA results were produced by Vaxine Pty Ltd and are not presented in this thesis.

The adjuvant systems, gamma inulin and cochleates, which have not previously been used in *S. flexneri* vaccine studies, were chosen for assessment with IpaD. Gamma inulin, a carbohydrate derived from the plant roots of the *Compositae* family, is a relatively new adjuvant that is a potent activator of both the cell-mediated and humoral arms of immunity (344). Gamma inulin has been deemed safe for use in humans, a classification that most adjuvants have not yet received (344). Cochleates are highly stable lipid based vaccine

carriers formed by calcium precipitation of phospholipids, creating a rolled up bilayer sheet that can be loaded with protein antigen (331). Cochleates have been shown to generate strong mucosal immune responses, which would be potentially useful for a *S. flexneri* vaccine (218).

These two adjuvants were used in an adjuvant trial of IpaD alongside aluminium hydroxide, which is a well-characterised adjuvant and the only adjuvant currently approved for use in licensed vaccines for humans in the U.S.A (18). 20 μg of IpaD protein was used in each vaccine formulation per mouse. The vaccine groups and immunisation schedule are outlined in Figure 6.7. Two different formulations of gamma inulin, D1 and PG2 were used.

The incorporation of the IpaD protein, produced during this study, into each adjuvant preparation was performed by Professor Petrovsky's research group at Vaxine Pty Ltd. The immunisation of the mice, sera and saliva collections and ELISA analysis were also performed by Vaxine and will not be discussed in this thesis. Once all immunisations and bleeds were complete, the mice were sent to the Australian National University for challenge with virulent *S. flexneri*.

Why not show this and acknowledge?? commercial?

6.2.6 Challenge of IpaD gamma inulin, aluminium hydroxide and cochleate immunised mice

In this pilot study, the immunised mice were intranasally challenged with 2×10^8 cfu of SFL1704, a virulent *S. flexneri* serotype 2a strain, on Day 60. It was expected that this would be a lethal dose as the literature reports 10^7 cfu is sufficient for most *Shigella* strains to cause death in intranasally infected mice (381, 400). SFL1704 was shown to be virulent *in vivo* by Sereny test in Chapter 4; however, the challenge dose did not cause lethality in any of the mice groups. It was possible to score the degree of sickness produced by *S. flexneri* challenge in each of the mice. Mice were scored as healthy if they appeared bright, groomed and normal, as mildly sick if they showed some lethargy, hunching and roughed coat, and mice which were immobile, acutely hunched and had a high degree of ruffled coat were classified as severely sick. Mice were observed up until 120 hours post challenge.

mouse strain?

A

Intramuscular groups

1. IpaD (20 μg/mouse) in 100 μL saline
2. IpaD (20 μg/mouse) in gamma inulin (D1)
3. IpaD (20 μg/mouse) in gamma inulin (PG2)
4. IpaD (20 μg/mouse) in aluminium hydroxide
5. IpaD (20 μg/mouse) in cochleates
6. Cochleates alone

Intranasal groups

7. IpaD (20 μg/mouse) in cochleates
8. Cochleates alone

B

Day	
0	1st injection
14	2nd injection
27	2nd Bleeding
28	3rd injection
37	1st Saliva
41	3rd Bleeding

Figure 6.7. Adjuvant trial for IpaD **A.** Vaccine groups for gamma-inulin and cochleates trial with IpaD. **B.** Immunisation schedule. Delivered intramuscularly or intranasally to 6 mice per group

Results are shown for the mice scores at 24 and 72 hours post challenge in Figures 6.8 and 6.9. By 120 hours after challenge, symptoms had resolved in all the mice groups.

24 hours after challenge, a number of IpaD immunised mice displayed mild symptoms, but only one mouse, from the PG2 gamma inulin group, had signs of severe sickness, while two mice each from the intramuscular and intranasal control cochleate groups were severely sick (Figure 6.8). By 72 hours after challenge, all IpaD immunised mice were healthy except one mouse from the D1 gamma inulin group, which was still showing some mild symptoms (Figure 6.9). The control cochleate groups, in comparison, had a number of mice still displaying severe symptoms, suggesting that the IpaD mice had cleared the infection more rapidly then the control mice. Although this experiment is only a preliminary examination of the IpaD protective immune response, it appears that some protection has been conveyed by immunisation with IpaD protein and immunisation with gamma inulin, cochleates and aluminium hydroxide. This suggests that these adjuvants and the protein, IpaD, warrant further examination for use in *S. flexneri* vaccine development.

6.3 Discussion

6.3.1 Attempting to boost the immune response against IpaD in *S. flexneri* with liposomes and immune targeting

IpaD has been detected as an immunogenic protein in natural infections of shigellosis. An immunoproteome screen in Chapter 3 showed that shigellosis patients from Bangladesh produce serum antibodies that strongly recognise IpaD protein (197, 198). It is unclear whether the immune response raised against IpaD offers any protection against reinfection. According to the literature, IpaD has never been studied as a single candidate for *S. flexneri* subunit vaccine development. An extracellular protein-LPS complex, termed Invaplex, capable of inducing significant protection against *S. flexneri* challenge in mice and guinea pigs, has been shown to contain IpaD (374). However, the exact protein components of this extracted complex have not been defined and it has not been determined which proteins are responsible for inducing protective immunity.

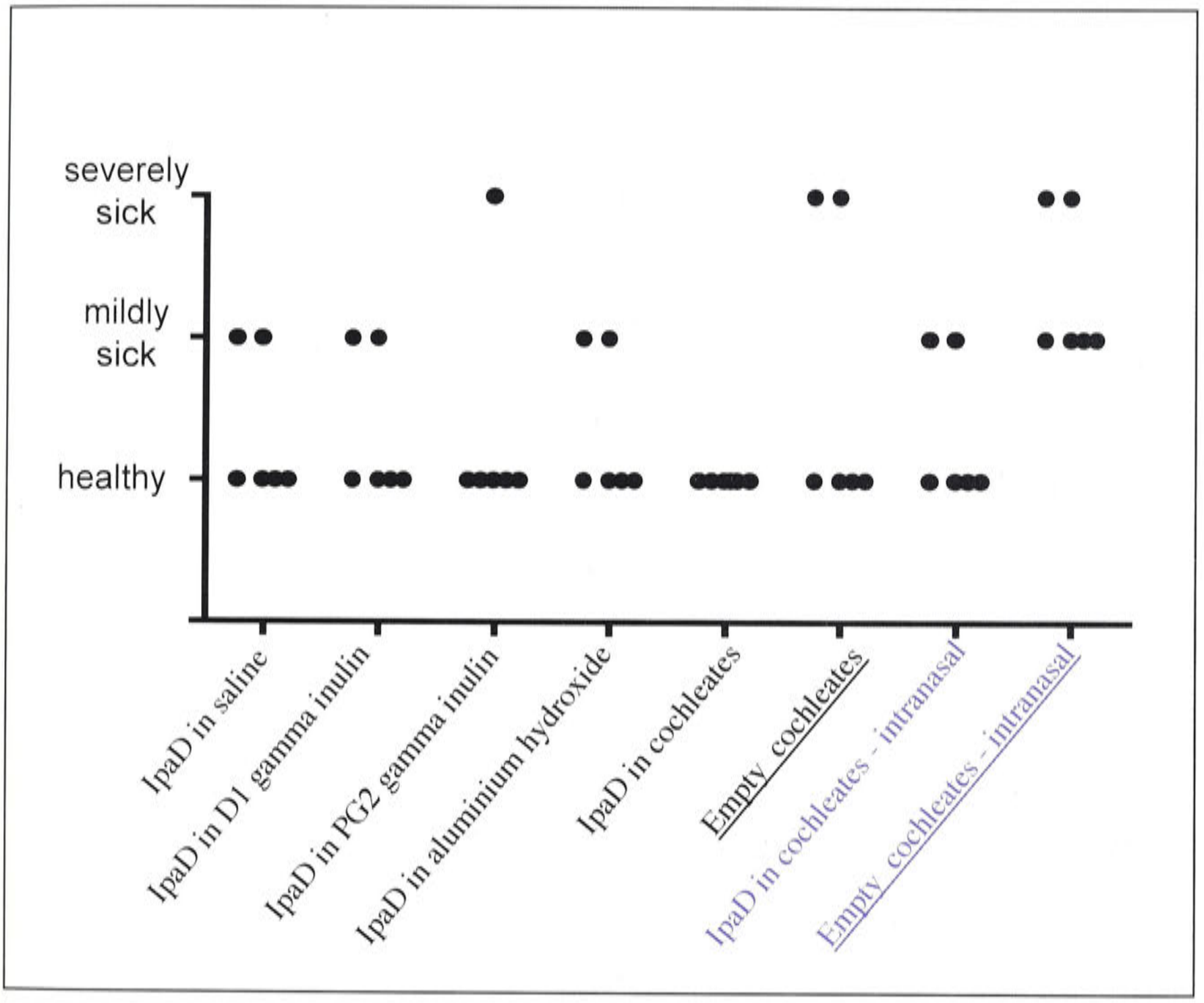


Figure 6.8. Challenge data for vaccine groups for gamma-inulin and cochleates trial with IpaD. Mice were challenged with wild type *S. flexneri* on Day 60 and assessed for symptoms 24 hours post-infection. Mice were scored as either healthy, showing mild symptoms or severe symptoms. Control mice groups are underlined. Intranasally inoculated groups are in blue.

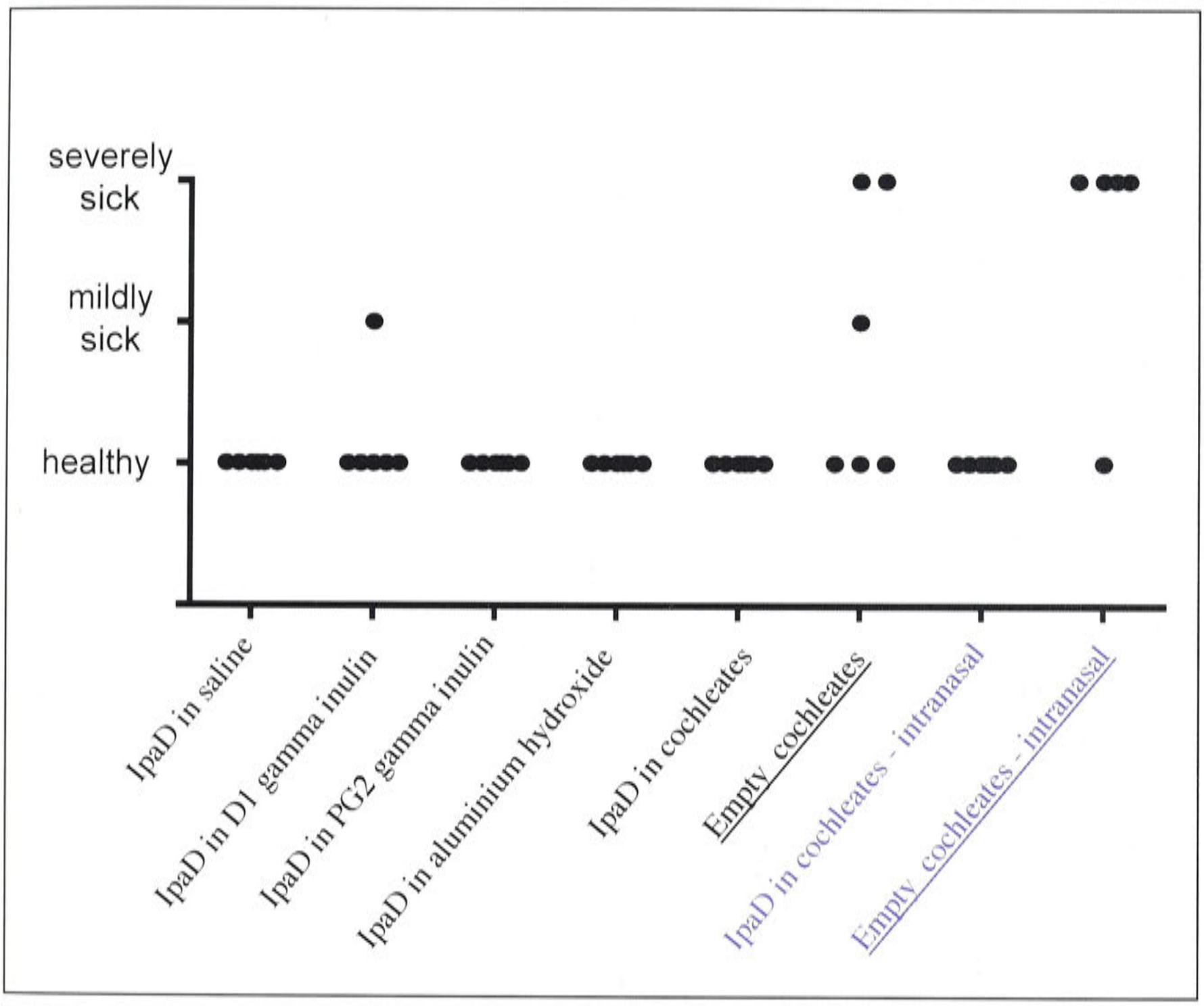


Figure 6.9. Challenge data for vaccine groups for gamma-inulin and cochleates trial with IpaD. Mice were challenged with wild type *S. flexneri* on Day 60 and assessed for symptoms 72 hours post-infection. Mice were scored as either healthy, showing mild symptoms or severe symptoms. Control mice groups are underlined. Intranasally inoculated groups are in blue.

The literature does not contain any references to the previous use of liposomes for *S. flexneri* subunit vaccine development. However, proteins for bacterial species such as *Streptococcus pyogenes*, *Neisseria meningitidis* and *Vibrio cholerae* have been delivered in liposomes, resulting in strong humoral responses and often significant levels of protection (46, 113, 136). In this study, liposomes were loaded with IpaD protein and also with *S. flexneri* 2a LPS to elucidate if the inclusion of LPS would boost the immune response to IpaD. Two immune targeting strategies were also employed, CD4 was incorporated into the liposomes in order to direct the antigen towards antigen presenting cells, which express the CD4 receptor, MHC class II (377). It was hoped that this would increase the antigen presentation to CD4+ T cells, which would in turn boost the Th2 antibody response towards IpaD. The single chain full-length variable Ab fragments (ScFv) recognised by the CD11c receptor found on dendritic cells, was also included with the liposomes, in the hope that this would target the liposomes towards to dendritic cells, increasing the chances that dendritic cells are activated by IpaD, producing a more potent immune response (378).

Mice were immunised subcutaneously, boosted twice to ensure a strong immune response was generated, and the serum IgG response raised was measured. Subcutaneous injection ensures that the numerous dendritic cells located within the skin will be activated and can migrate to the peripheral tissue to activate a serum antibody response (303). The IgG antibody response appears to be important for *S. flexneri* immunity as strong serum responses are always detected in patients after natural infection, and $IgA^{-/-}$ mice can still be protected against *S. flexneri* infection in what appears to be an IgG dependent manner (197, 314, 400).

A significant serum IgG response was raised against IpaD for all the different vaccine groups by Day 14 and this was increased 10 fold by Day 34; however, according to ANOVA analysis there was no significant difference between the antibody titres of each vaccine group. Therefore, delivery of IpaD in liposome preparations does not significantly increase the antibody response over the response generated against protein delivered in PBS. Furthermore, the inclusion of the targeting ligands, CD4 and ScFv anti-CD11c did not amplify the IgG response against IpaD. These results were disappointing; it was hoped that the adjuvant properties of liposomes, the immune targeting conveyed by the ligands and the

protection offered to the internalised protein by liposomes would boost the anti-IpaD antibody response. A previous study has shown that targeting of meningococcal protein in liposomes to dendritic cells enhanced the antibody response compared to the response induced against non-targeted liposomes (10). However, it should be noted that this is only a very preliminary investigation of the use of liposomes in *S. flexneri* vaccine development and further work is required to determine if liposomes are effective immune modulators for IpaD. It is possible that mucosal immunisation would deliver the liposomes to the Peyer's patches of the gut, inducing a local mucosal response, which may be heightened by the adjuvant and targeting properties of the liposome preparations. Additionally, in further experiments, the serum antibody response should be measured over a longer period of time after immunisation, as the liposome induced IgG response may be a more sustained response then that produced against the protein alone. Alternatively, sometimes it is simply possible that a particular protein antigen does not work effectively with a certain adjuvant formulation; for example, the delivery of cholera toxin in liposomes had no increased adjuvant effect in rats, when compared to toxin delivered alone (288, 294).

Immunising adjuvant capacity of IpaD??

6.3.2 Incorporation of CD4 into IpaD liposomes boosted anti-LPS IgG

The anti-LPS IgG titre was also determined for mice immunised with IpaD liposome preparations and LPS (group B) and for mice immunised with IpaD liposomes, LPS and CD4 (group D). Mice only immunised with IpaD protein (group F) were also screened for the presence of any anti-LPS IgG in order to establish if the strong anti-IpaD IgG response raised against just the protein had been boosted by contaminating LPS in the protein preparation. The antibody titre against LPS for group F was not significantly raised suggesting that the protein preparation was relatively free of LPS. Thus, it seems that the IpaD protein is a highly immunoreactive protein for *S. flexneri*, even in the absence of an adjuvant. IpaD is known to contain a number of B-cell epitopes along the amino terminal half of the protein, which may be responsible for activating the strong IgG response (375).

Interestingly, the titre of anti-LPS IgG for group D was significantly higher than the titre for group B on Day 34. It is possible that the inclusion of CD4 in the liposomes of group D has increased the efficiency of delivery of LPS to the MHC class II expressing B-cells,

thereby enhancing the antibody response. It is not clear why CD4 did not simultaneously increase the IgG response to IpaD in group D. It is possible that the T-cell independent nature of LPS as an antigen may be responsible; once CD4 has delivered LPS containing liposomes to MHC class II expressing B-cells, no other signals are required to activate the B-cell to produce antibody (193). Protein antigens, on the other hand, require processing and peptide display by MHC class II molecules to activate CD4+ T-cells and further co-stimulatory signals are required before B-cells will become activated (76). It should also be noted that B-cells are not the most efficient antigen presenting cell (340). Thus, it seems that the targeting of liposomes to CD4 expressing cells such as B-cells was successful in increasing the efficiency of antigen recognition in the case of the T-cell independent antigen, LPS, but not the efficiency of antigen processing for the protein antigen, IpaD.

6.3.3 Liposomes as adjuvants for *S. flexneri*

It is not possible to conclude from this preliminary experiment whether liposomes are a promising adjuvant for *S. flexneri*. As previously discussed, further experimentation using mucosal delivery, and investigation to determine whether liposomes induce a more prolonged immune response are required. Furthermore, challenge experiments might reveal if protective immunity is improved by liposome delivery. Plaque inactivation assays were attempted to quantitate the scale of protective antibody present in the sera from each group, but the assays were not continued as serum induces the type III secretion system to release the Ipa proteins, increasing the efficiency of invasion in antibody-treated cells (230). The plaque inactivation assays performed in Section 6.2.1 were performed using an affinity-purified anti-IpaD antibody, so no serum was present to interfere with the assay.

Although delivery of proteins by liposomes avoids the safety issues associated with live vaccine strains, it is possible that liposomes are not an ideal adjuvant delivery system for use against shigellosis. A shigellosis vaccine will ultimately be used in developing countries, where a vaccine must be cheap, easy to store and simple to administer. Liposomes are highly unstable at room temperature, cannot be lyophilised, must be manufactured fresh and it is difficult to achieve quality assurance across batches (288). Thus, it is imperative to continue assessing adjuvants for use with *S. flexneri* that better fulfil the requirements for a shigellosis vaccine.

6.3.4 Challenge of IpaD, gamma inulin, aluminium hydroxide and cochleate immunised mice

Gamma inulin and cochleates have not previously been used as adjuvants for *S. flexneri* antigens. Both adjuvants are highly stable, relatively simple to produce and are capable of inducing both humoral and cell-mediated immunity (218, 344). Furthermore, gamma inulin has been approved for use in humans. In this study, mice were immunised both intramuscularly and intranasally with cochleates containing IpaD and also empty cochleates as a control. Intranasal immunisation was included as cochleates are highly efficient at stimulating local mucosal immune responses (218). Two different formulations of gamma inulin mixed with IpaD were administered to mice intramuscularly and the well characterised adjuvant, aluminium hydroxide was also included.

This work was performed as a collaboration with the commercial company Vaxine Pty Ltd as an initial investigation into the suitability of gamma inulin and cochleates for *S. flexneri* vaccine development with IpaD. The ELISA analysis performed on the immunised mice will not be discussed here, except to note that higher IgG antibody titres were raised against IpaD protein delivered intramuscularly for all types of adjuvant in comparison to the IpaD protein alone, and that intranasal delivery of cochleates containing IpaD stimulated production of an IgA response.

The immunised mice were challenged with wild type *S. flexneri*. Preliminary dose optimisation with 10^7 cfu of *S. flexneri* in five Balb/c mice suggested that SFL1704 required a higher dose for lethality. Thus, for the challenge of the IpaD immunised mice, 2 x 10^8 cfu was administered, however, although some mice showed severe sickness, none of the cochleate control groups died. It is possible that the administration of empty cochleates to these mice was sufficient to stimulate a non-specific immune response, which offered enough protection to prevent *S. flexneri* infection causing death. The experiment should have included a control group of mice that were not immunised, but unfortunately this appropriate control was overlooked at the time. However, the cochleate control mice were noticeably sicker than the IpaD immunised mice, so it was possible to score the severity of symptoms caused by the *S. flexneri* challenge. The results suggested that immunisation

with IpaD allowed the mice to recover from infection earlier; by 72 hours post infection all mice immunised with IpaD, besides one, did not display any symptoms of disease, whilst a number of the cochleate control mice were still very sick. Therefore, it seems that murine antibody directed against IpaD is capable of producing a degree of protective immunity by neutralising *S. flexneri* infection. This *in vivo* finding is supported by data generated in the *in vitro* plaque inactivation assays, where the binding of the purified anti-IpaD antibody to *S. flexneri* cells significantly reduced the plaque forming abilities of the strain.

Although conclusions should not be drawn from this single experiment, where the challenge administered was sub-lethal and the appropriate controls were not included, it appears that systemic IgG raised in intramuscularly immunised mice and the mucosal IgA raised in mucosally immunised mice contributed to the protection observed. This correlates with the literature, which reports that mice expressing anti-*Shigella* serum IgG in the absence of IgA, and that mice expressing anti-*Shigella* IgA from grafted hybridoma's are both protected from *S. flexneri* challenge (290, 400). Furthermore, the antibody inactivation plaque assay performed in this study demonstrated that antibody neutralisation of IpaD can reduced the virulence of *S. flexneri*. This finding correlates with a recent study, which has reported that IpaD antibody neutralisation reduces *S. flexneri* invasion in the *in vitro* invasion assay (86). Thus, not only is IpaD highly immunogenic but the antibodies generated possibly provide protection against reinfection. This makes IpaD a highly relevant and promising subunit vaccine candidate for *S. flexneri* vaccine development and research is on going with this protein.

6.3.5 Further vaccine development based on findings in this study

The results presented here suggest that IpaD has potential as a protein candidate for *S. flexneri* subunit vaccine development. However, further research is required to determine the degree of protection conveyed by the anti-IpaD antibody response. Further IpaD vaccine trials with the promising adjuvants, gamma inulin and cochleates, should concentrate on more detailed analysis of the potential protective immunity induced. Furthermore, the purity of IpaD was only checked by SDS-PAGE and Coomassie staining, which is not sensitive enough to determine if there are very small concentrations of contaminating protein or LPS. Future experiments should confirm that the immunogenicity

observed for IpaD was not due to contaminants from the preparation, by further purifying the protein with high performance liquid chromatography (HPLC).

The appropriate lethal dose for SFL1704 should be determined so that intranasal challenge of immunised mice can be more effective. An additional mucosal immunisation could also be included, where in a modification of the Sereny test, it is possible to immunise guinea pig eyes, challenge the eye with wild type *S. flexneri* and measure the degree of keratoconjunctivitis produced (120). Additionally, as the IpaD immunised mice challenged in this study recovered more rapidly than the control cochleate immunised mice, it would be interesting to confirm, in future experiments, that IpaD immunised mice clear the bacterial load from their lungs more efficiently by performing bacterial counts from the lungs of sacrificed mice.

Because IpaD is found in all serotypes of *S. flexneri*, it is possible that IpaD protective immunity may offer protection against heterogenous serotypes simultaneously. Therefore, IpaD could be useful in the development of a multi-serotype protective vaccine for *S. flexneri*. Since the challenge experiment in this study was only performed with *S. flexneri* serotype 2a, in further experiments it would be interesting to determine if immunity induced against IpaD would protect against challenge by various heterogeneous serotypes.

The work performed in this study suggests that IpaD may be a useful candidate for *S. flexneri* subunit vaccine development. Subunit vaccines often combine a number of antigens into the one vaccine preparation, thereby provoking multiple protective immune responses simultaneously (72, 265). In future work, IpaD could be combined with other *S. flexneri* subunit candidates to determine if increased protection can be generated with combinations of antigens. IpaD may prove to be a valuable asset to the development of effective subunit combination vaccines for *S. flexneri*.

6.4 Conclusion

The immunogenic protein, IpaD, was assessed for its suitability as a *S. flexneri* subunit vaccine candidate in two different studies with a number of adjuvant delivery systems. Although these studies are only preliminary, there were some favourable results suggesting

that IpaD is not only highly immunogenic, but in a mouse model it may raise protective immunity against *S. flexneri*. Due to these promising results, IpaD will be further studied as a *S. flexneri* vaccine candidate. Gamma inulin, aluminium hydroxide, cochleates and liposomes were tested with IpaD to determine if they are suitable adjuvants for use with *S. flexneri*. Liposomes did not induce an anti-IpaD IgG response any stronger than that raised against the protein alone. Furthermore, with the complicated production, difficulty in storage and probable associated high costs of administering them in developing countries, it is unlikely that liposomes are a suitable delivery vehicle for *S. flexneri*. Gamma inulin, aluminium hydroxide and cochleates were also examined for their abilities to boost the anti-IpaD antibody response in mice. Both adjuvants increased the anti-IpaD titres in comparison to IpaD protein alone. The mucosal and systemic anti-IpaD immune response generated in this immunisation experiment was sufficient to increase the efficiency with which mucosally challenged mice resolved *S. flexneri*–induced illness. According to this study, IpaD and the adjuvants gamma inulin, aluminium hydroxide and cochleates may be promising prospects for *S. flexneri* vaccine development.

Chapter 7
Characterisation of the acid resistance pathways of *S. flexneri* 2457T

Chapter 7

Characterisation of the acid resistance pathways of *S. flexneri* 2457T

7.1 Introduction

Acid resistance is defined as an *in vitro* inoculum survival of ≥10% after two hours of acid exposure at pH 2.5 (105). Acid resistance has been extensively studied in *E. coli*, whilst the acid resistance phenotype of *S. flexneri* has been described in fewer instances. Studies have shown that *S. flexneri* possesses at least two acid resistance pathways that seem to be similar to two of the three pathways identified in *E. coli*. Acid resistance pathway 1 is a stationary phase, acid induced, glucose repressed oxidative pathway, while acid resistance pathway 2 is a stationary phase, glutamate dependent, acid resistance (GDAR) pathway (202).

The GDAR pathway, which is induced by growth in mildly acidic conditions (pH 5) and in fermentatively grown cells, is a glutamate decarboxylase system consisting of two homologous decarboxylase enzymes, *gadA* and *gadB*, and an antiporter *gadC* (126, 202, 397). This system appears to act by mopping up protons leaking into the bacterial cytosol through the decarboxylation of glutamate to gamma-aminobutyric acid (GABA). GABA is then exchanged for external glutamate by the antiporter, GadC, thereby maintaining the cytoplasm's pH homeostasis, reversing the cell's membrane potential to create an internal positive charge and gradually alkalising the extracellular medium (42, 311). Thus, this pathway is dependent on glutamate being present in the acid shock media (202). A number of genes involved in regulating this pathway have been identified in *E. coli*, revealing a complex network of regulation (130, 221).

The GDAR pathway appears to be highly effective at acid protection in both *E. coli* and *S. flexneri* (43, 397). However, it is still unclear how important the oxidative system is to *S. flexneri's* acid resistance phenotype as so little is understood about the pathway. The mechanism and major components of the *Shigella* oxidative system are not yet known,

besides some regulatory proteins. It has been shown in *E. coli* that the alternate sigma factor, RpoS, and the cyclic AMP receptor protein (CRP) play a role in control of this pathway (43, 397). The *Shigella* oxidative system, characterised in strain 3136, displays similar properties to the oxidative pathway in *E. coli*, including a requirement for complex media, oxidative growth, acid induction and repression of the pathway by glucose (202). Recently, it has been shown that a *fur* mutant in the *S. flexneri* serotype 2a strain, SA100, is defective in the oxidative system. This defect appears to be due to the constitutive expression of a small regulatory RNA molecular, RyhB, in the absence of Fur regulation, repressing the transcription of YdeP, an oxidoreductase that reduces acidic metabolic products in the cell (270).

In this Chapter, the acid resistance pathways of the *Shigella flexneri* serotype 2a strain, 2457T, have been characterised. This strain is a well characterised *Shigella* strain, which has been widely used in virulence studies (403). It is not known what contribution the acid resistance pathways of 2457T make to the virulence of this strain, as they have not been previously characterised. Prior acid resistance studies have used either *S. flexneri* strain 3136, SA100 or M25-8A (270, 346, 398). The known GDAR gene components are present in the 2457T sequence (403). However, there is a key difference between 3136, the most characterised *S. flexneri* strain, and the sequenced 2457T strain; it has been shown by Small *et al* (1994) that 3136 possesses an intact *rpoS* gene, producing a 330 amino acid protein (346). Instead, the 2457T sequence has a frameshift, 675 bp into the *rpoS* sequence, creating a truncated protein of 255 amino acids (403). It is unclear whether this truncated RpoS retains its function as a major regulator of stationary phase growth genes and whether this has implications for the regulation of the acid resistance pathways of *S. flexneri* 2457T.

7.2 Results

7.2.1 The glutamate dependent acid resistance pathway of SFL1001

The acid resistance experiments are based on the methods and conditions of Lin *et al*, (1995) and Castanie-Cornet *et al*, (1999) (43, 202). Table 7.1 shows the GDAR pathway characteristics for the *S. flexneri* 2457T strain, SFL1001. To examine the GDAR pathway cells were grown overnight to stationary phase at either pH 5 or 8, in the presence or

Condition	Adaptation medium	pH 2.5 Challenge medium	% Survival	std error ±
1	LBG (pH 5) WO	EG + Glt	88.6	3.90
2	LBG (pH 5)	EG + Glt	37.9	8.90
3	LB (pH 8) WO	EG + Glt	3.30	0.39
4	LB (pH 8)	EG + Glt	<0.001	0
5	EG (pH 5) WO	EG + Glt	52.0	9.90
6	EG (pH 5)	EG + Glt	5.87	1.60

Table 7.1. Table of *S. flexneri* 2457T strain, SFL1001, acid resistance for the GDAR pathway. Adaptation involved overnight growth in medium listed. LB: Luria broth, LBG: LB with 0.4% glucose, EG: minimal E salts glucose, Glt: glutamate added at 0.012%, WO: growth without oxygenation. Challenge was a 1:1000 dilution of overnight culture into pH 2.5 medium for a period of 2 hours, survival is stated as a percentage of the inoculum. Results are the mean of 3 experiments with standard error.

absence of oxygen, and acid shocked at pH 2.5 in minimal E salts glucose media (EG) containing 0.012% glutamate. pH 8 cultures do not contain glucose as the consequent fermentative growth lowers the growth medium to pH 5 overnight. Acid induction was displayed by the strain at pH 5, as there was very little acid survival detected for cultures grown at pH 8, whilst cultures grown overnight at pH 5 were capable of surviving the acid exposure (Table 7.1, condition 2 versus 4). Additionally, SFL1001 exhibited increased resistance to acid exposure when grown in culture without oxygenation (Table 7.1, condition 1 versus 2 and condition 3 versus 4). Fermentative conditions in the presence of limited oxygen are known to induce GDAR in *E. coli*, and are also a known stimulator of resistance in *S. flexneri* 3136 (26, 201). Finally, GDAR resistance in *S. flexneri* 2457T is not dependent on overnight growth in complex media, as acid resistance was detected when the strain was grown overnight in EG pH 5 (Table 7.1, conditions 5 and 6). The negative control for the GDAR pathway, which is acid shock in EG pH 2.5 without glutamate is included in Table 3.2 and will be discussed in the following sections.

7.2.2 The oxidative acid resistance pathway of SFL1001

The glutamate-independent oxidative acid resistance pathway detected for SFL1001 is outlined in Table 7.2; cells were grown overnight to stationary phase and acid shocked at pH 2.5 in EG. Buffers are included in the overnight medium to maintain the exact pH levels. Similar to *E. coli*, *S. flexneri* 2457T's oxidative system protects cells at pH 2.5 in minimal media in the absence of glutamate (Table 7.2, condition 1) (202). There is a clear requirement for oxidative growth conditions as resistance was not present in cells grown without oxygenation (Table 7.2, condition 1 versus condition 2). Furthermore, as observed by Lin *et al* (1995) for *S. flexneri* 3136, the oxidative pathway of *S. flexneri* 2457T requires overnight growth in complex media and cannot be detected for cells grown overnight in minimal media at pH 5 (Table 7.2, condition 4) (202).

It has been reported for *E. coli* that the oxidative pathway does not require acid induction, by overnight growth at pH 5 to induce resistance, but that the lack of resistance observed during growth at pH 8 is due to the presence of a secreted inhibitor (43). Movement of the cells into fresh media is sufficient to remove the inhibitor and restore acid resistance (43). In this study, the *S. flexneri* 2457T strain's oxidative pathway appears to display acid

Condition	Adaptation medium	pH 2.5 Challenge medium	% Survival	std error ±
1	LB-MES (pH 5)	EG	54.9	5.80
2	LB-MES (pH 5) WO	EG	0.53	0.13
3	LB-MOPS (pH 8)	EG	<0.001	0
4	EG (pH 5)	EG	<0.001	0
5	LB-MES (pH 5) + Glt	EG	57.0	8.70
6	LB-MOPS (pH 8) + Glt	EG	<0.001	0
7	LB-MOPS (pH 8) + fresh EG	EG	<0.001	0
8	LBG (pH 5)	EG	59.4	10.4
9	LBG (pH 5) WO	EG	<0.01	0

Table 7.2. Table of *S. flexneri* 2457T strain, SFL1001 acid resistance for the oxidative acid resistance pathway. Adaptation involved overnight growth in medium listed. LB: Luria broth, LBG: LB with 0.4% glucose, EG: minimal E salts glucose, WO: growth without oxygenation. Glt: 5.9 mM glutamate was added to overnight cultures immediately prior to acid challenge. fresh EG: cells were grown overnight in the media indicated, before being transferred to fresh EG (pH 7). Challenge was a 1:1000 dilution of overnight culture into pH 2.5 medium for a period of 2 hours, survival is stated as a percentage of the inoculum. Results are the mean of 3 experiments with standard error.

induction, where resistance is detectable when cells are grown overnight at pH 5 but not at pH 8 (Table 7.2, condition 1 versus condition 3). To determine whether this behaviour was due to the production of an inhibitor, as reported for *E. coli*, cells grown overnight at pH 8 were transferred to fresh EG (pH 7) prior to acid challenge, as performed by Castanie-Cornet *et al* (1999) (43). This treatment did not generate acid resistance in these cells (Table 7.2, condition 7), suggesting that the lack of resistance in pH 8 grown cells of *S. flexneri* 2457T is not due to an inhibitor, but that oxidatively grown cells require acid induction in order to display acid resistance in EG pH 2.5. Additionally, Castanie–Cornet *et al* (1999) demonstrated that the addition of 5.9 mM glutamate to cells grown overnight at pH 8 could activate oxidative pathway acid resistance (43). Table 7.2, conditions 5 and 6 show that the addition of 5.9 mM glutamate to SFL1001 stationary cells had no effect on the levels of acid resistance.

7.2.3 Detection of non-glucose repressed, glutamate-independent acid resistance in SFL1001

Many previous studies of *S. flexneri* and *E. coli* have stated that the oxidative resistance pathway is in fact glucose repressed and not observed in cells grown to stationary phase in media containing glucose (201, 202). It should be noted that the glucose contained within the acid shock media, EG, does not appear to play a role in the glucose repression, suggesting that it is the stationary phase growth in the presence of glucose that is important. In those studies, it appears that overnight growth in LBG at pH 5 suppresses the oxidative pathway sufficiently, such that no protection is observed when cells are acid shocked in EG pH 2.5. The absence of glutamate in the acid shock media should prevent the GDAR pathway from operating, making these acid test conditions essentially a negative control for both pathways. However, as shown in Table 7.2, condition 8, nearly 60% cell survival is possible for *S. flexneri* 2457T in these conditions. When cells are grown similarly at pH 5 in LBG but with limited oxygenation, which favours the GDAR system, there is no acid resistance detected (Table 7.2, condition 9). This result seems to suggest that the response is due to the oxidative pathway, which prefers oxygenated growth and can operate in acid shock minimal media without glutamate supplementation. If this is the case, the pathway is not repressed by the cells' overnight fermentative growth in glucose-containing media. As *S. flexneri* 2457T requires growth factor supplementation in minimal media, it was

necessary to determine if the presence of the supplements could be contributing to this novel resistance. Similar resistance was seen when EG pH 2.5 was not supplemented with the *S. flexneri* growth supplements, either methionine, tryptophan or nicotinic acid, demonstrating that the resistance was not dependent on the presence of these growth supplements (Figure 7.1). This resistance, observed in cells grown to stationary phase overnight in LBG pH 5 and acid shocked in EG pH 2.5, will be referred to as non-glucose repressed, glutamate-independent acid resistance. To determine the contribution of either the oxidative or GDAR pathway to the non-glucose repressed, glutamate-independent pathway, mutants were constructed in acid resistance-related genes known to contribute to the GDAR and oxidative pathways of *S. flexneri* 3136.

7.2.4 The role of RpoS in SFL1001 acid resistance

It is believed that the oxidative acid resistance pathway is RpoS dependent, where disruption of *rpoS* results in a loss of the resistance phenotype in both *E. coli* and *S. flexneri* cultures acid shocked in unsupplemented minimal media (43, 397). Furthermore, RpoS appears to be important, but not essential, for operation of the GDAR pathway in *S. flexneri* (346, 397). However, in *S. flexneri* 2457T, the *rpoS* gene contains a frameshift mutation resulting in a truncated protein missing the last 89 amino acids (Figure 7.2) (403). The *rpoS* gene of SFL1001, cloned into pNV1314 with the primers 2457TrpoSSacF and RpoSXbaR, was sequenced by the universal M13 forward and reverse primers to confirm the presence of the frameshift mutation in SFL1001, and aligned to the full-size *rpoS* gene sequence of *S. flexneri* 301 published by Small *et al* (Figure 7.2) (346).

To determine whether this altered RpoS protein was still important for the acid resistance pathways of *S. flexneri* 2457T, in particular the non-glucose repressed, glutamate-independent resistance, an *rpoS* mutant was produced for SFL1001. A suicide plasmid, pNV1234, was constructed by inserting an internal region of the 2457T *rpoS* gene, amplified with the primers RpoSF294Sac and RpoSR575Xba, into pGP704. pNV1234 was inserted into the *rpoS* gene of SFL1001 to create the strain, SFL1629. This *rpoS* mutant strain had the *rpoS* disruption complemented with a plasmid-based copy of the *S. flexneri* 2457T *rpoS* gene, by pNV1314 to create SFL1647, and also with a copy of the full-sized *E. coli rpoS* from MG1655, amplified with the primers MG1655SacF and MG1655XbaR,

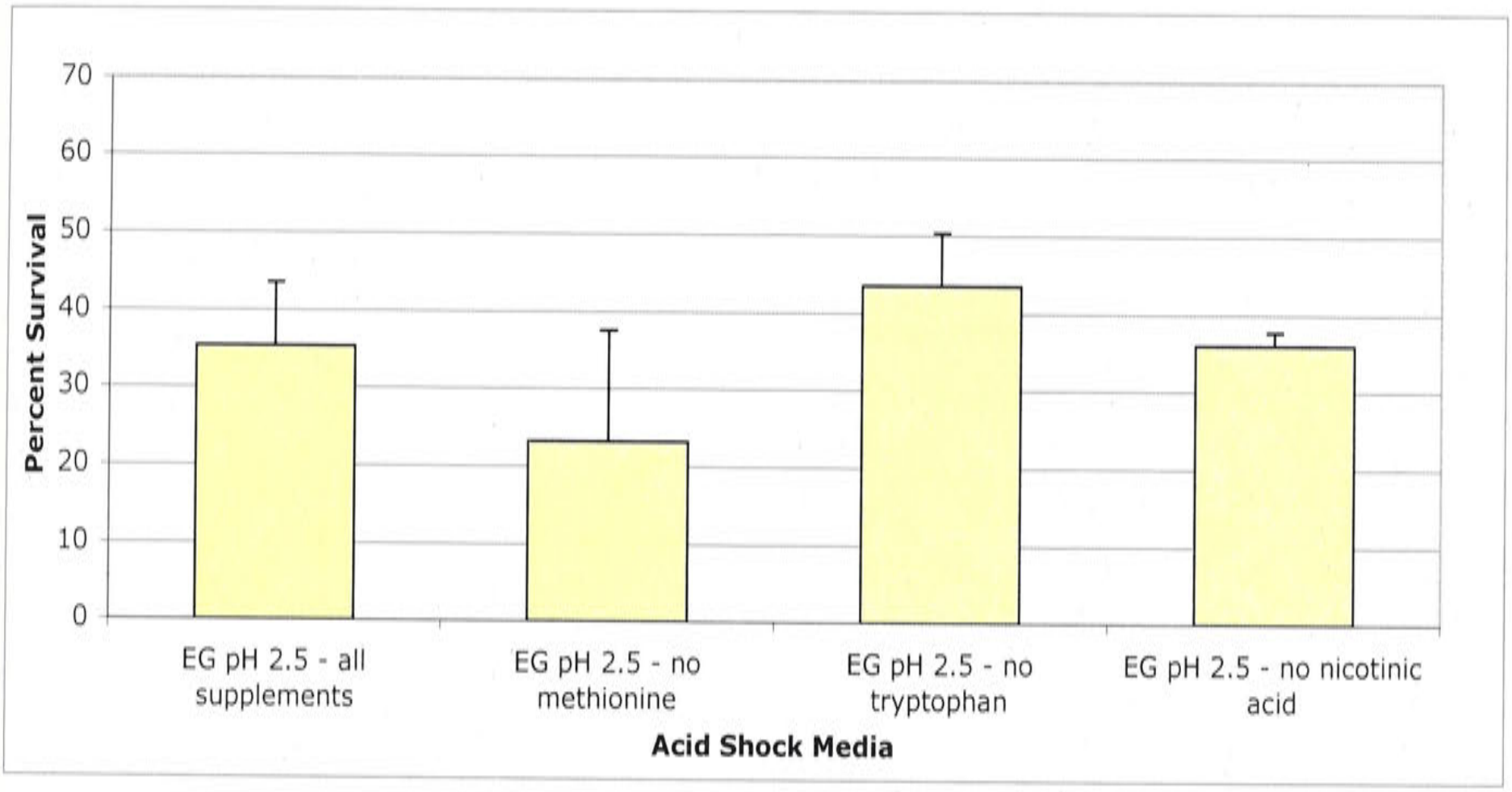


Figure 7.1. Effect of acid shock media growth supplements on the non-glucose repressed, glutamate-independent acid resistance pathway of SFL1001. The supplements methionine, tryptophan and nicotinic acid in EG pH 2.5 have no effect on the non-glucose repressed acid resistance of SFL1001. There is no significant reduction in the level of acid resistance when each supplement is removed from the acid shock media for cells grown overnight in LBG pH 5. Results are the mean of three experiments with standard error.

A

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S. flexneri 301     601 caactggataagccagttgatgacgtcagccgtatgcttcgtcttaacga 650
                        ||||||||||||||||||||||||||||||||||||||||||||||||||||
S. flexneri 2457T   601 caactggataagccagttgatgacgtcagccgtatgcttcgtcttaacga 650
                                .         .         .         .         .
S. flexneri 301     651 gcgcattacctcggtagacacccegctgggtggtgattccgaaaaagcgt 700
                        |||||||||||||||||||||||| ||||||||||||||||||||||||||
S. flexneri 2457T   651 gcgcattacctcggtagacacccc.ctgggtggtgattccgaaaaagcgt 699
                                .         .         .         .         .
S. flexneri 301     701 tgctggacatcctggccgatgaaaaagagaacggtccggaagataccacg 750
                        ||||||||||||||||||||||||||||||||||||||||||||||||||||
S. flexneri 2457T   700 tgctggacatcctggccgatgaaaaagagaacggtccggaagataccacg 749
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B

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  1  MSQNTLKVHDLNEDAEFDENGVEVFDEKALVEEEPSDNDLAEEELLSQGATQRVLDATQL
 61  YLGEIGYSPLLTAEEEVYFARRALRGDVASRRRMIESNLRLVVKIARRYGNRGLALLDLI
121  EEGNLGLIRAVEKFDPERGFRFSTYATWWIRQTIERAIMNQTRTIRLPIHIVKELNVYLR
181  TARELSHKLDHEPSAEEIAEQLDKPVDDVSRMLRLNERITSVDTPWVVIPKKRCWTSWPM
241  KKRTVRKIPRKMTI*SRASSNGCSS*TPNSVKYWHVDSVCWGMKRQHWKM*VVKLASPVN
301  VFARFRLKACAVCAKSCKRRG*ISKRCSAS
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Figure 7.2. A. Nucleotide sequence of *S. flexneri* 2457T *rpoS* gene, sequenced from SFL1001, aligned with the *S. flexneri* 301 published sequence of *rpoS* (NCBI accession number P35540). The SFL1001 2457T *rpoS* sequence correlates with the published 2457T sequence, where there is missing a G at 675 bp. **B.** *S. flexneri* 2457T RpoS predicted truncated protein sequence. The red amino acids are 100% homologous to the sequence of *S. flexneri* 301 RpoS. The frameshift results in the altered amino acids indicated by the blue underlining, creating a stop codon (indicated by first asterix), 255 amino acids into the protein.

generating pNV1315 in SFL1648. A Western blot was performed with whole cell lysate to determine whether the truncated RpoS is in fact, expressed by SFL1001 and also to confirm the successful disruption of *rpoS* in SFL1629 (Figure 7.3). A truncated RpoS of approximately 30 kDa was detected for SFL1001, which was not found in SFL1629, confirming that the gene disruption had been successful in SFL1629. The full-sized RpoS of *E. coli* MG1655 was observed as a 38 kDa band. RpoS protein of the respective sizes, 30 kDa and 38 kDa, were observed for the complemented strains SFL1647 and SFL1648 as expected. Although roughly the same number of cells were loaded on the 1D SDS-PAGE gel for each strain, there was a large discrepancy in the expression levels of RpoS. The plasmid based RpoS levels were higher, as would be expected as there are multiple copies of the gene present within each cell, with the SFL1647 expression of RpoS being extremely high for unknown reasons.

Acid resistance assays were performed on the strains grown in LB (MES) pH 5 and LBG pH 5 and challenged in EG pH 2.5 or EG pH 2.5 supplemented with 0.012% glutamate (Figure 7.4). The *rpoS* disruption did not have a significant effect on either the GDAR (Condition 3, Figure 7.4) or oxidative resistance pathway (Condition 2, Figure 7.4), as SFL1629 did not display any attenuation in acid resistance. Likewise, there was no change in the non-glucose repressed, glutamate-independent resistance (Condition 1, Figure 7.4). Clearly, the truncated RpoS of *S. flexneri* 2457T has little to no importance in the regulation of the 2457T acid resistance pathways.

7.2.5 Activity of SFL1001 truncated RpoS measured by catalase assay

To determine the activity of the truncated RpoS of SFL1001 it is possible to measure the activity of RpoS using a rapid semi-quantitative catalase assay, where the release of oxygen is graded from the level of bubbling produced by a colony when a drop of hydrogen peroxide is placed upon it (352). Catalase, otherwise known as hydrogen peroxidase II (HPII), catalyses the catabolysis of water and oxygen from hydrogen peroxide and is regulated by RpoS (365). An RpoS mutant will display a reduced level of bubbling, although some bubbling will still be observed as the cell possesses a second hydrogen peroxide (HPI) that is not regulated by RpoS (352). The results of this study's catalase assay show that the *E. coli* strain MG1655, which possesses a full-sized (330 amino acid)

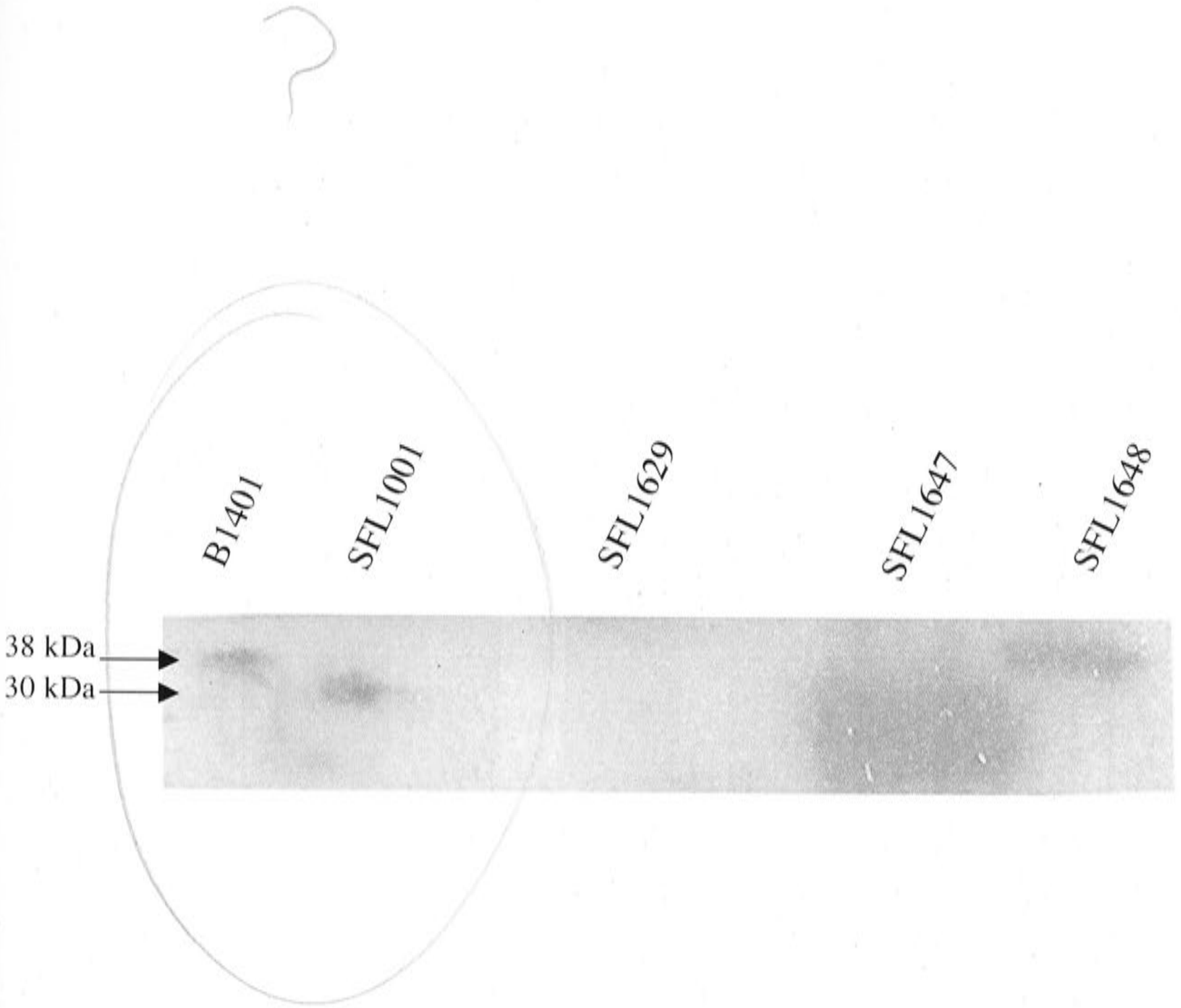
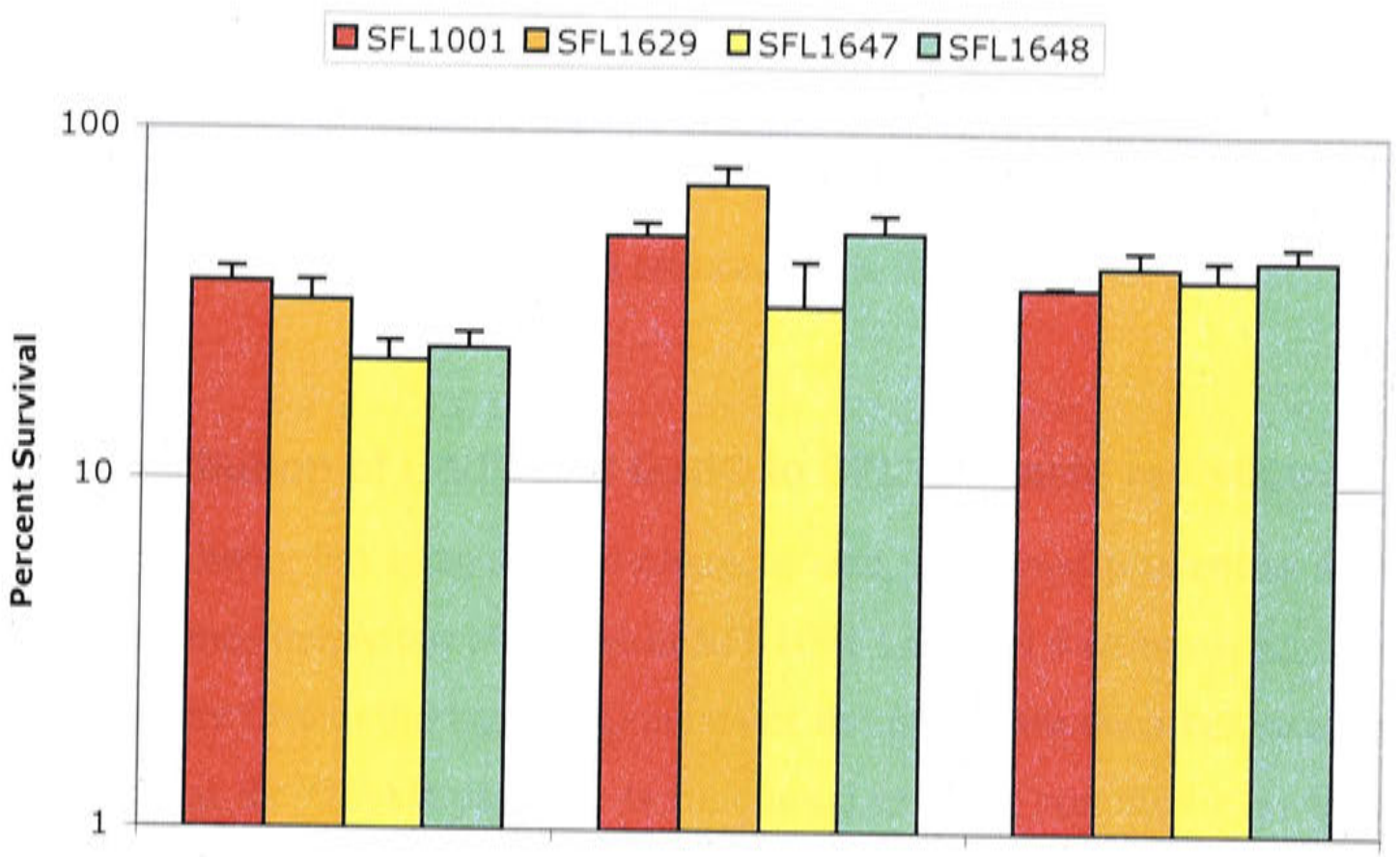


Figure 7.3. Western blot of RpoS disruption from *S. flexneri* 2457T strain, SFL1001. B1401: *E. coli* MG1655 with full sized RpoS, SFL1001: *S. flexneri* 2457T with truncated RpoS, SFL1629: 2457T with disrupted RpoS, SFL1647: SFL1629 with *S. flexneri* 2457T truncated *rpoS* in pNV1314, SFL1648: SFL1629 with *E. coli* MG1655 full-sized RpoS in pNV1315. The expected size for full size RpoS is 38 kDa and 30 kDa for 2457T truncated RpoS. Approximate band sizes were determined from the closest protein ladder bands of the Fermentas prestained PageRuler.



Overnight	LBG (pH 5)	LB-MES (pH 5)	LBG (pH 5)
Acid Shock	EG	EG	EG + 0.012% Glt
Condition	1	2	3
Pathway	Non-glucose repressed glutamate independent	Oxidative	Glutamate dependent acid resistance

Figure 7.4. Effect of RpoS disruption from *S. flexneri* 2457T strain, SFL1001, on the acid resistance pathways. Cells were grown overnight in the media indicated and diluted 1:1000 into the acid challenge media, survival is stated as a percentage of the inoculum. Red bars, SFL1001: 2457T strain, Orange bars, SFL1629: *rpoS* disruption mutant, Yellow bars, SFL1647: SFL1629 complemented with *S. flexneri rpoS* in pNV1314, Green bars, SFL1648: SFL1629 complemented with full sized *E. coli rpoS* in pNV1315. Results are the mean of four experiments with standard error.

RpoS, has a rapid, strong bubbling phenotype, while *S. flexneri* 2457T, which contains a truncated (255 amino acid) RpoS, displays reduced oxygen production (Table 7.3). Furthermore, the disrupted *rpoS* mutant, SFL1629, was only able to slowly decompose the H_2O_2. SFL1647, the 2457T truncated *rpoS* complement, was also severely attenuated in catalase activity, while the *E. coli* full-sized *rpoS* complement created vigorous bubbling for *S. flexneri*, similar to that observed for MG1655. Therefore, the reduction in SFL1001 catalase activity in comparison to the catalase activity displayed by the full-sized *E. coli* RpoS, suggests that the truncated RpoS is reduced in its function.

7.2.5 The contribution of GadB and GadC to SFL1001 acid resistance

As the GDAR pathway has been so well characterised, it seemed prudent to determine if the unusual resistance behaviour observed in SFL1001 LBG pH 5 cultures, acid shocked in EG pH 2.5, had any link to the key components of the glutamate decarboxylase system. It seems unlikely that the GDAR pathway is functional in these conditions, as the pathway has an absolute requirement for glutamate to be present in the acid shock medium. Disruption mutants of *gadB* and *gadC* were produced in SFL1001; the suicide plasmid approach was used initially and was successful for *gadC*. However, the *gadB* mutant required construction of a non-polar knockout so as to not disrupt the downstream *gadC* gene. The lambda red PCR integration system, with the primers GadBKOF and GadBKOR, was used to disrupt the *gadB* gene in a non-polar fashion, as the inserted cat cassette carries a RBS to ensure downstream gene expression (64). The Southern blot in Figure 7.5 shows the correct location of the chloramphenicol cassette in the *gadB* gene for SFL1650. *gadB* was also amplified by the GadBcompF and GadBcompR primers and sequenced with GadBcompF and GadBcompR to further confirm the disruption point (Appendix B). The suicide plasmid, pNV1206, constructed with the primers GadCSacF and GadCXbaR, was inserted into the *gadC* gene in the 2457T chromosome, producing the *gadC* mutant, SFL1641. The correct insertion and disruption of the *gadC* gene was confirmed by Southern blot (Figure 7.6). SFL1641 and SFL1650 were complemented with plasmid-based copies of *gadC* and *gadB*, pNV1397 and pNV1389 respectively, to give the strains SFL1729 and SFL1723.

Strain	RpoS protein	Rate of bubbling
E. coli MG1655	Full size, 330 aa	Vigorous
SFL1001	Truncated, 254 aa	Slow
SFL1629	Disrupted	Very slow
SFL1647	Disrupted, complemented with copy of SFL1001 RpoS	Very slow
SFL1648	Disrupted, complemented with copy of MG1655 RpoS	Vigorous

Table 7.3. Catalase activity for SFL1001 and the *rpoS* disruption mutant, SFL1629. Strains were assayed for catalase activity by dropping H_2O_2 onto individual colonies and measuring oxygen production by the rate of bubbling. SFL1001: 2457T strain, SFL1629: *rpoS* disruption mutant, SFL1647: SFL1629 complemented with truncated *S. flexneri rpoS* in pNV1314, SFL1648: SFL1629 complemented with full sized *E. coli rpoS* in pNV1315. Observations were the same in all four colonies tested per strain.

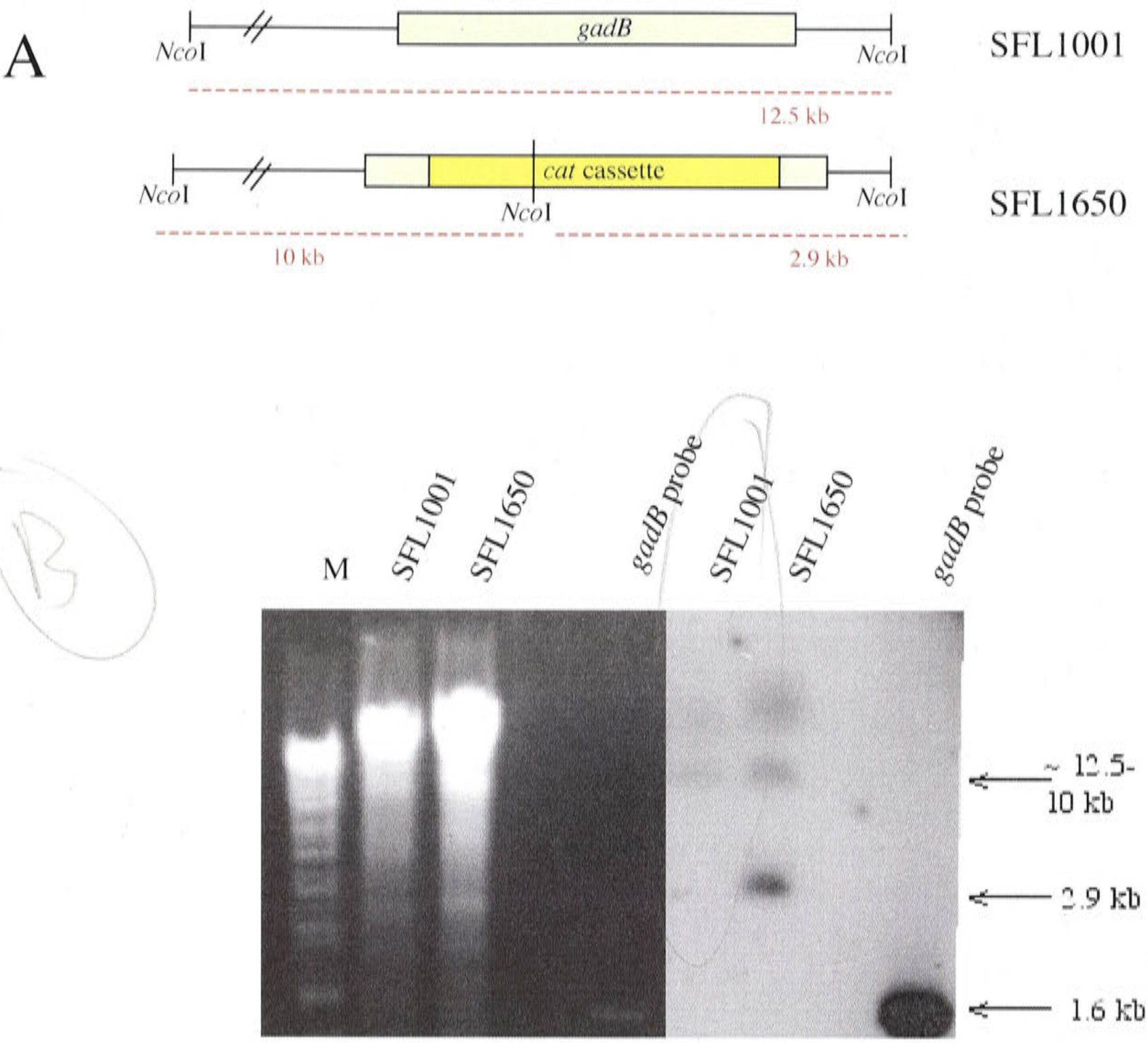
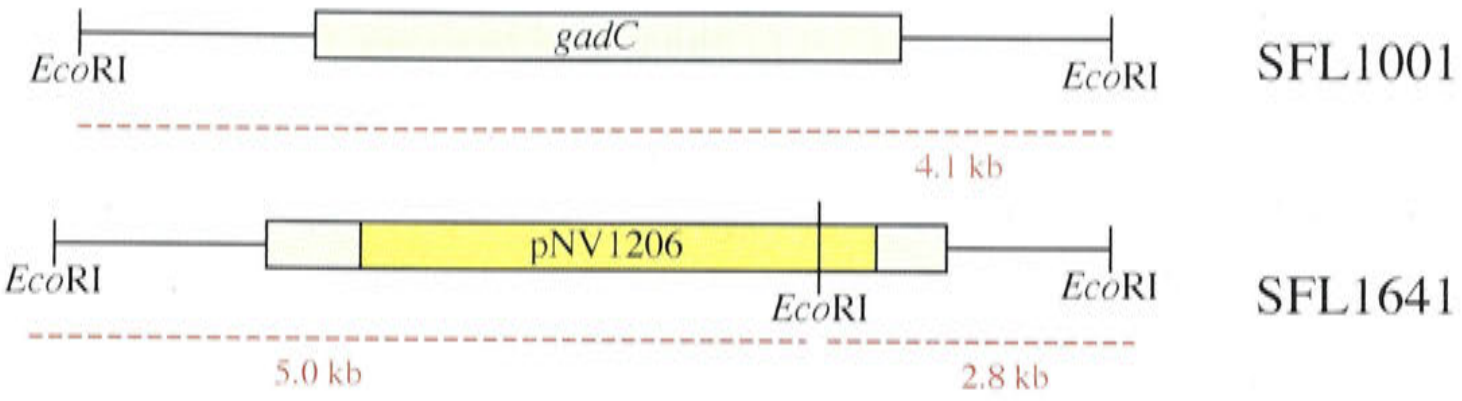


Figure 7.5. A. Insertion pattern of the *cat* cassette in *gadB* with a *Nco*I digest. The Southern probe is able to bind to both fragments of the *gadB* gene in SFL1650 **B.** Southern blot showing insertion of 1.1 kb *cat* cassette into *gadB* mutant, SFL1650. M: SPP-1 *Eco*RI marker (sizes in Figure 2.5). Some marker sizes are indicated. SFL1001: *Nco*I digest of *S. flexneri* 2457T, SFL1650: *Nco*I digest of *S. flexneri* 2457T *gadB* mutant. Probe is 1.6 kb DNA segment spanning *gadB* amplified with the primers gadBcompF and gadBcompR. The probe should recognise a 12.5 kb band in SFL1001 and insertion of *cat* cassette into *gadB* should generate 10 kb and 2.9 kb *Nco*I bands recognised by the probe. The large band sizes cannot be determined accurately as the marker does not exceed 8.5 kb. Image of the agarose gel is on the left-hand side and the image of the film is on the right-hand side. As the large bands have not run evenly and there is uncut DNA binding the probe, this Southern blot is further supported with sequencing data showing the correct *cat* insertion in *gadB* for SFL1650 (Appendix B).

A



B

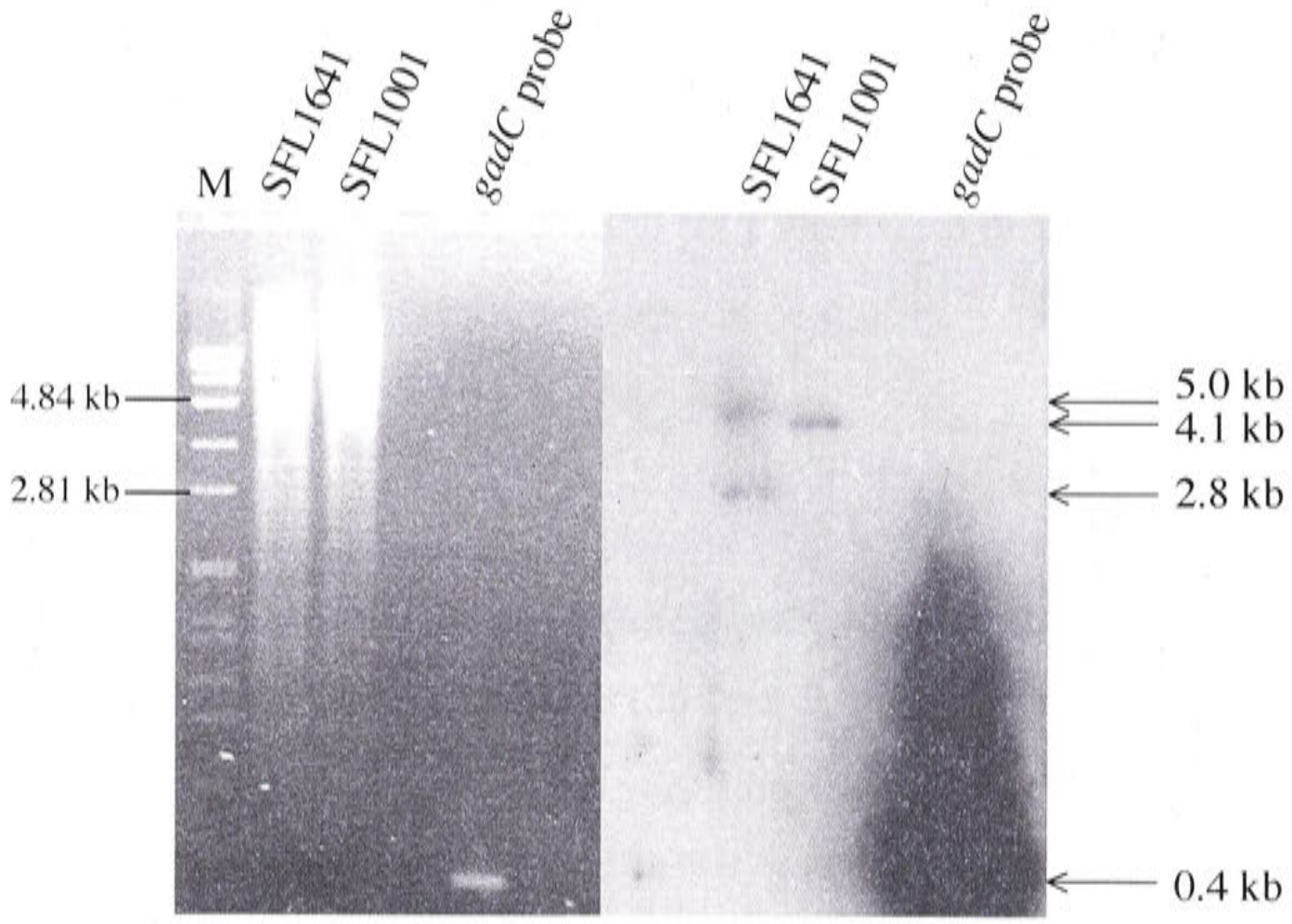


Figure 7.6. A. Insertion pattern of pNV1206 into *gadC* with an *Eco*RI digest. The Southern probe is able to bind to both fragments of the *gadC* gene in SFL1641 **B.** Southern blot showing insertion of pNV1206 into *gadC* in SFL1641. M: SPP-1 *Eco*RI marker (sizes in Figure 2.5). Some marker sizes are indicated. SFL1001: *Eco*RI digest of *S. flexneri* 2457T, SFL1641: *Eco*RI digest of *S. flexneri* 2457T *gadC* mutant. Probe is a 0.4 kb DNA segment spanning an internal region of *gadC*, amplified with the primers gadCSacF and gadCXbaR. *Eco*RI digestion of SFL1001 produces a 4.1 kb band, correct insertion of pNV1206 into *gadC* generates 2 bands of 2.8 kb and 5 kb, recognised by the probe. Image of the agarose gel is on the left-hand side and the image of the film is on the right-hand side.

Acid resistance assays were performed on all strains as shown in Figures 7.7 and 7.8. Both the *gadB* and *gadC* mutants displayed a change to their GDAR phenotype. Both strains' survival in EG pH 2.5, with 0.012% glutamate added (Condition 3, Figures 7.7 and 7.8), were significantly reduced in comparison to SFL1001 (t-tests, $p < 0.01$). Complementation of each gene disruption was sufficient to restore the acid resistance. The loss of GDAR in a *S. flexneri gadB* mutant, which still has a functional *gadA* gene, was also seen by Waterman and Small (2003) (398). There was no change to resistance levels of the oxidative pathway (Condition 2, Figures 7.7 and 7.8), as expected, or in the unusual resistance phenotype for the *gadB* and *gadC* mutants (Condition 1, Figures 7.7 and 7.8), suggesting that the key components of the GDAR pathway are not contributing to the glutamate-independent non-glucose repressed resistance observed in *S. flexneri* 2457T. Interestingly, this resistance, which is observed in LBG pH 5 cultures shocked in EG pH 2.5, did not prevent the loss of acid resistance for *gadB* and *gadC* mutants in LBG pH 5 challenged in EG 2.5 containing 0.012% glutamate (Condition 3, Figures 7.7 and 7.8).

7.3 Discussion

7.3.1 The GDAR and oxidative acid resistance pathways of *S. flexneri* 2457T are similar to the pathways of *S. flexneri* 3136 and *E. coli*

The acid resistance phenotype appears to be very important to intestinal pathogens. Bacteria must survive exposure to gastric acid in the stomach and volatile fatty acids produced within the intestine (99). This ability to survive acidic conditions encountered during passage through the host is most likely a contributing factor to the very low infectious dose of *S. flexneri*. *S. flexneri* 2457T has been widely studied in virulence assays and challenge work and is known for its virulent phenotype, however it has never been examined from an acid resistance perspective (403). This study detected two acid resistance assays, the GDAR and oxidative systems which have previously been characterised in *S. flexneri* 3136 (202).

The GDAR system observed for *S. flexneri* 2457T behaved the same as the GDAR pathway of *S. flexneri* 3136 (202); the pathway was acid induced, where cultures required growth at pH 5 to generate measurable acid resistance in acidified EG supplemented with 0.012%

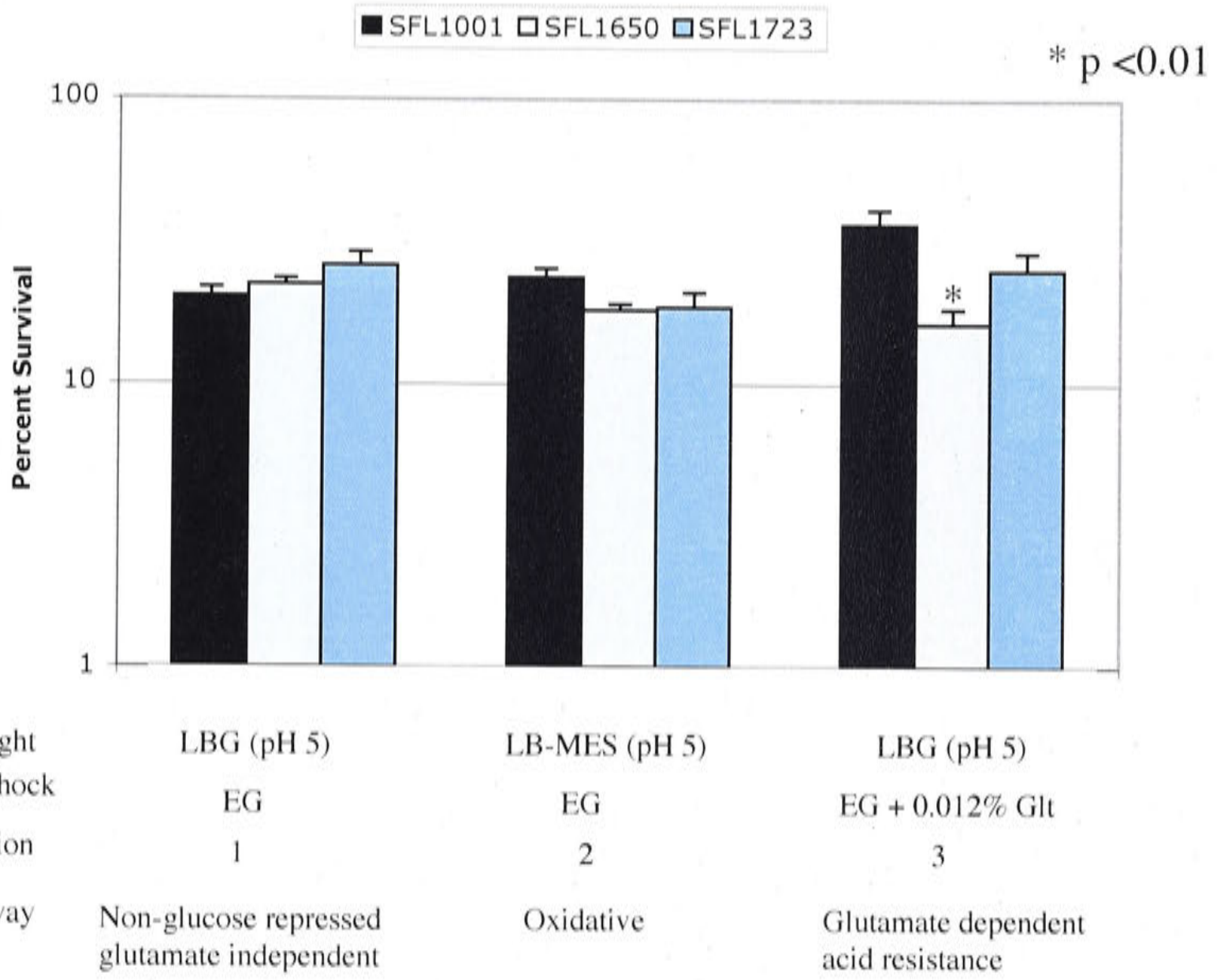


Figure 7.7. Acid resistance for SFL1650, *gadB* mutant. Cells were grown overnight in the media indicated and diluted 1:1000 into the acid challenge media, survival is stated as a percentage of the inoculum. Black bars, SFL1001: 2457T, Pale blue bars, SFL1650: *gadB* mutant, Bright blue bars, SFL1723: *gadB* mutant complemented with *gadB* in pNV1389. Results are the mean of three experiments with standard error. SFL1650 was significantly reduced in acid resistance for condition 3, when compared to SFL1001 by t-test ($p<0.01$).

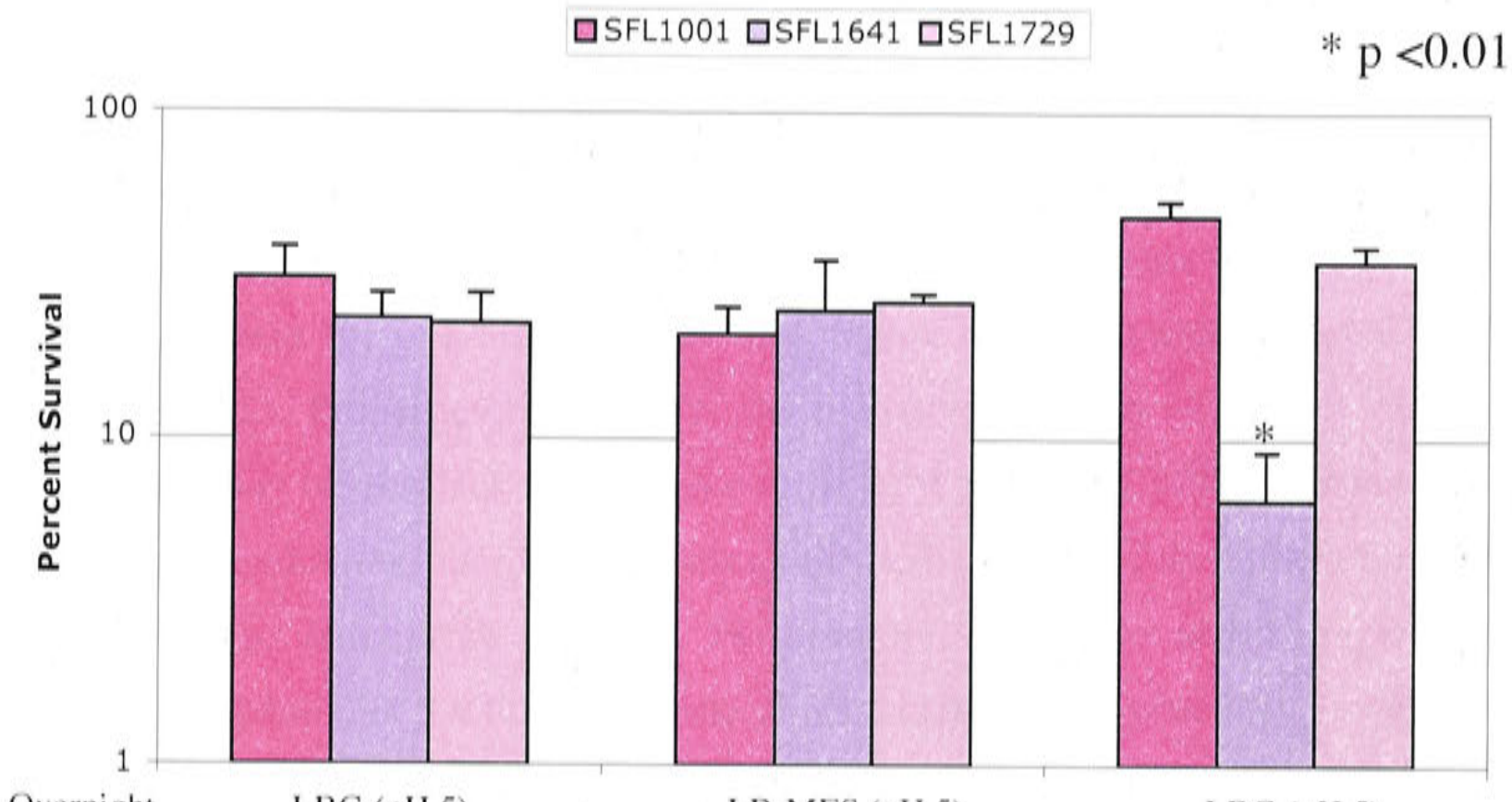


Figure 7.8. Acid resistance for SFL1641, *gadC* mutant. Cells were grown overnight in the media indicated and diluted1:1000 into the acid challenge media, survival is stated as a percentage of the inoculum. Dark pink bars, SFL1001: 2457T, Purple bars, SFL1641: *gadC* mutant, Pale pink bars, SFL1729: *gadC* mutant complemented with *gadC* in pNV1397. Results are the mean of three experiments with standard error. SFL1641 was significantly reduced in acid resistance for condition 3, when compared to SFL1001 by t-test ($p<0.01$).

glutamate. Likewise, the pathway showed anaerobic induction, with higher resistance observed for cultures grown in conditions of limited oxygen availability. Additionally, the GDAR pathway was capable of operating in cells grown in minimal media at pH 5, demonstrating that the system has no requirements for complex media during overnight growth.

The oxidative system was also present in *S. flexneri* 2457T. Cultures grown in MES buffered LB overnight were capable of surviving exposure to acidified EG in the absence of glutamate. The pathway did not operate in cells grown without oxygenation and showed an absolute requirement for overnight growth in complex media, as also reported for *S. flexneri* 3136 (202). Properties reported for the oxidative pathway of *E. coli* were not detected in this study (43). A lack of resistance at pH 8 for *S. flexneri* 2457T was not due to the presence of an inhibitor, but instead seems simply to be due to a requirement for acidic growth in order to induce the pathway. Furthermore, glutamate activation was not observed, as the addition of 5.9 mM glutamate to pH 8 cells did not generate any oxidative resistance.

7.3.2 SFL1001 has a non-glucose repressed, glutamate-independent acid resistance pathway

In contrast to the behaviour reported for both *E. coli* and *S. flexneri* 3136, it was determined that 2457T is capable of displaying resistance when grown in LBG at pH 5 and acid shocked in EG pH 2.5 without glutamate supplementation. According to the literature, these acid shock conditions are effectively a negative control for both the GDAR and oxidative pathways (43, 201, 202). The GDAR system should not be able to operate under these conditions, as there is no glutamate present in the acidified EG. Likewise, the oxidative system should be repressed by the presence of glucose in the overnight growth medium. When the test cultures were grown in LBG pH 5 without oxygenation there was a substantial drop in the resistance, suggesting this glutamate-independent, non-glucose repressed pathway is induced by oxidative growth. It must be noted that although it was determined in this thesis that SFL1001 is attenuated in some virulence assays, this defect does not appear to be responsible for the unusual acid resistance reported here, as SFL1704, a virulent strain of 2457T, also displayed this acid resistance phenotype in EG pH 2.5.

Thus, the non-glucose repressed glutamate-independent acid resistance appears to be *S. flexneri* 2457T specific. Interestingly, this pathway was also recorded, but not discussed, by de Jonge *et al* (2003) (68). In their case, *E. coli* 0157 grown in LBG pH 5 showed substantial survival in EG pH 2.5. However, no explanation was made for this unusual resistance. Whether this pathway is novel for *S. flexneri* 2457T and certain *E. coli* strains, or if the oxidative pathway is functional in the presence of glucose, remains unclear. To further clarify this unusual acid resistance phenotype, mutants for *S. flexneri* 2457T were constructed in genes known to be important to the oxidative and GDAR pathways, respectively.

7.3.3 *S. flexneri* 2457T RpoS is truncated and possible reduced in activity

As previously mentioned, this glutamate-independent, non-glucose repressed resistance is induced by oxidative growth. Consequently, to explore whether this resistance is from the known oxidative pathway, which is not being repressed by glucose as expected, a disruption was constructed in the *rpoS* gene. RpoS is considered essential for the oxidative pathway to operate, and although also involved in the GDAR pathway, it is not essential as resistance can still be detected for the GDAR system in an *rpoS* mutant in *E. coli*, and resistance is still present for *S. flexneri* 3136 at pH 5 (43, 202, 346). The *S. flexneri* 2457T *rpoS* mutant did not show a reduction in the non-glucose repressed glutamate independent acid resistance observed for LBG pH 5 cultures. However, there was also no defect or significant reduction in either the GDAR or oxidative pathway for the *rpoS* knockout. It appears that the *rpoS* gene does not play an essential role in the regulation of any acid resistance pathway in *S. flexneri* 2457T. Interestingly, this may be connected to the observation that 2457T possesses a truncated RpoS protein, caused by a frameshift mutation within the gene.

This study has shown that the RpoS protein missing the last 89 amino acids is still produced in 2457T by Western blot analysis, but it is unclear whether it is still functional. The truncation means the protein is missing the end of the fourth domain of the conserved σ^{70} family structure, which is involved in –35 promoter recognition of target genes (207). The loss of the fourth region of *RpoS* in *E. coli* is sufficient to render the protein effectively inactive (271, 352). In an attempt to determine if the 2457T RpoS is functional, a catalase

assay was performed. *S. flexneri* 2457T was clearly reduced in its catalase activity when compared to *E. coli* MG1655, which carries a full-sized RpoS. This activity was also reduced in the *rpoS* disruption mutant, SFL1629, and reduced in the *rpoS* complement, SFL1647, carrying the 2457T *rpoS* gene. It was possible to create strong catalase activity in *S. flexneri* 2457T by expressing the full-sized *E. coli* RpoS in SFL1648. These results seem to suggest that the *S. flexneri* RpoS truncated protein is at least reduced in its activity, although further research is required to confirm this.

The complementation of the *rpoS* disruption with a plasmid expressed, full-size RpoS from *E. coli* MG1655 did not increase acid resistance for 2457T. It may be that the loss of RpoS activity has forced 2457T to develop alternative regulation for its acid resistance pathways, and this may explain why there was no reduction in the acid resistance observed for the *rpoS* deletion in SFL1001.

7.3.4 GadB and GadC are important for the GDAR pathway but not for non-glucose repressed, glutamate-independent acid resistance

In order to clarify any possible role of the GDAR system in the novel glutamate-independent non-glucose repressed acid resistance, disruptions were also constructed in the *gadB* and *gadC* genes. GadB is a glutamate decarboxylase, responsible for the conversion of intracellular glutamate to GABA. A non-polar mutation was constructed for *gadB* to maintain expression of *gadC*, which lies downstream from *gadB*. The *gadB* knockout showed a significant reduction in GDAR acid resistance but was not reduced sufficiently to be classified as acid sensitive (< 10% survival). This is most likely due to the presence of the functional glutamate decarboxylase isoform, *gadA*, which is located elsewhere in the chromosome. Castanie-Cornet *et al*, (1999) demonstrated that in *E. coli* K12 only one Gad isoform is required for measurable acid resistance (43). Conversely, Waterman and Small, (2003) showed that a *gadB* mutant in *S. flexneri* M25-8A was completely attenuated in the GDAR pathway (398). The *gadB* disruption had no effect on the non-glucose repressed, glutamate-independent pathway in *S. flexneri* 2457T. The complementation of the *gadB* mutant with a plasmid-based copy of *gadB* was sufficient to restore the reduced acid resistance levels for the GDAR pathway, demonstrating that the non-polar mutation had been successful in maintaining the downstream *gadC* expression.

A *gadC* disruption was also constructed for *S. flexneri* 2457T. As expected, this strain became acid sensitive for the GDAR pathway, and *gadC* plasmid complementation restored the acid resistance. However, the *gadC* mutant was still capable of surviving exposure to EG pH 2.5 after growth in LBG pH 5. Thus, it seems that this unusual acid resistance pathway is perfectly operational when the GDAR pathway has been disabled and does not seem to be linked to this system. Interestingly, the non-glucose repressed, glutamate-independent pathway did not rescue the *gadB* or *gadC* mutants, which had been rendered deficient or reduced in acid resistance under the GDAR growth conditions. The non-glucose repressed, glutamate-independent pathway is functional when the *gadB* and *gadC* mutants are grown in LBG pH 5 media and shocked in EG pH 2.5. However, when the cells were grown as above and acid shocked in EG pH 2.5 containing glutamate, the pathway does not appear to contribute any resistance, since if this was the case the *gadB* and *gadC* related drops in acid resistance would not have been observed. It may be possible that this unusual pathway is in some way repressed by glutamate.

7.3.5 RpoS is not essential for SFL1001 acid resistance

It still remains unclear whether the oxidative pathway is contributing to this novel resistance in a non-glucose repressible manner. It was hoped that the previously reported oxidative pathway's absolute dependence on RpoS could be used to determine if the oxidative system is responsible for this non-glucose repressible, glutamate-independent resistance. In contrast, it appears that RpoS is not important to the *S. flexneri* 2457T acid resistance phenotype. RpoS, the sigmaS subunit of RNA polymerase, is strongly induced on entry into the stationary phase and is the main regulator of the stress response in *E. coli* (402). The loss of RpoS from *E. coli* strains, both laboratory and natural, is quite common (165). It appears that loss of RpoS can actually convey a selective advantage for *E. coli*; where *rpoS* deficiency may be advantageous under conditions of nutrient deprivation, may increase amino acid scavenging ability in stationary phase cells and even give cells competitive advantage in mouse colonisation experiments (13, 94, 424). In this case, *S. flexneri* 2457T has not lost expression of *rpoS* but instead produces a truncated protein detectable by Western blot, which may have reduced activity as demonstrated in a catalase

assay. It seems that *S. flexneri* 2457T has adapted its acid resistance regulation and is operational in the absence of RpoS.

7.3.6 Further analysis of the non-glucose repressed, glutamate-independent acid resistance displayed by SFL1001

The acid resistance of SFL1001 includes an unusual acid resistance phenotype, which has also been reported for *E. coli* 0157 (67). This phenotype is not related to the operation of the GDAR, as it does not display a dependence on glutamate in the acid shock medium or require GadB and GadC. It is not clear whether this resistance is the oxidative pathway operational in the presence of glucose, or if an entirely independent pathway is present. Little is known about the oxidative pathway of *S. flexneri*, however, in a recent study, a *fur* mutant of *S. flexneri* SA100 was found to be defective in the oxidative pathway (270). It would be interesting to determine if a *fur* mutant of *S. flexneri* 2457T would also be defective in oxidative acid resistance and, furthermore, determine if disruption of oxidative resistance also eradicates the novel non-glucose repressed, glutamate-independent acid resistance. Despite repeated attempts to construct a *fur* mutant in *S. flexneri* 2457T, with both the suicide vector and lambda red PCR knockout approach, a *fur* knockout could not be produced during this study. Further attempts should be made to produce a *fur* knockout, with alternative disruption approaches such as the Sigma-Aldrich TargeTron gene knockout system or the use of temperature sensitive suicide plasmids (287, 318).

To identify additional genes involved in the non-glucose repressed, glutamate-independent acid resistance of SFL1001 a screening approach could be taken. A genomic library of SFL1001 could be constructed and expressed in an *E. coli* strain that does not possess any resistance when grown in the presence of glucose and acid shocked in EG pH 2.5. It is possible that if the *S. flexneri* 2457T gene(s) required for this phenotype lie close together in the chromosome, then at least one of the library constructs could convey the non-glucose repressed resistance in EG pH 2.5 to *E. coli*. Through subsequent subcloning and sequencing the genes responsible could be identified from the library clone.

7.4 Conclusion

This study is the first report on the stationary phase acid resistance pathways of *S. flexneri* 2457T. Two acid resistance systems, the glutamate-dependent (GDAR) and the oxidative pathways, previously reported for *E. coli* and *S. flexneri* 3136, were both detected in *S. flexneri* 2457T. Furthermore, *S. flexneri* 2457T cells grown overnight under fermentative growth conditions and acid shocked in minimal medium in the absence of glutamate, an acid test often described as a negative control for both pathways, were capable of surviving acid challenge. It is unclear whether this resistance is due to the oxidative pathway operating in a non-glucose repressible manner, or if a novel pathway is present in *S. flexneri* 2457T. The construction of *gadB* and *gadC* mutants ruled out any contribution by the GDAR pathway, whilst further characterising the GDAR properties of *S. flexneri* 2457T. Interestingly, study of the role of *rpoS* in the oxidative and the unusual acid resistance phenotype revealed that the frameshift present in the *rpoS* gene results in expression of a truncated RpoS protein, which may be reduced in activity and is not essential for the acid resistance phenotype of *S. flexneri* 2457T

Chapter 8
General Discussion

Chapter 8

General discussion

8.1 Summary

This study used a proteomic approach to screen for immunoreactive proteins in *S. flexneri* 2457T. Eight proteins from soluble and membrane protein extractions were identified as immunogenic in three repeats, of which, six have never been reported as targets of the immune response in *S. flexneri* natural infection. This is the first immunoproteomic study of *S. flexneri* using sera from shigellosis patients.

The proteins Udp and S3195 were identified during the immunoproteome analysis but were not included in the final protein list as they were only seen in one of the three repeats. They were analysed for their roles in the virulence of *S. flexneri* by constructing knockouts in the *udp* and *S3195* genes, and evaluating any change in the strains' virulence phenotype with cell culture assays. Disruption of *S3195* had no effect on the strain's ability to invade epithelial cells and form plaques, indicating that S3195 is not important in the pathogenesis of *S. flexneri*. The disruption of *udp*, however, significantly reduced the number of plaques formed by the strain, SFL1727. Subsequent cell culture analysis of SFL1727 suggested that the virulence defect was caused by reduced survival within the epithelial cell's cytoplasm. Udp may be important for *S. flexneri's* ability to scavenge pyrimidine nucleosides, as carbon and nitrogen sources, during periods of active growth in conditions of low carbon and nitrogen availability. However, this growth defect was not sufficient to prevent SFL1727 from causing disease in the *in vivo* Sereny test. Thus, although Udp may contribute to the intracellular survival of *S. flexneri*, it is not an essential virulence factor and is not a significant candidate for the attenuation of live vaccine strains.

The *S. flexneri* 2457T strain, SFL1001, was avirulent in both the Sereny test and in plaque assays. However, it was determined that disruption of the *mgl* operon in SFL1001, creating SFL1617, produced a virulent phenotype capable of producing disease in the Sereny test and plaques in cell culture. The complementation of the *mgl* operon, in SFL1736, reduced

the virulence of the strain in these assays. The virulence defect in SFL1001 and SFL1736 was determined to be in intra- and intercellular spread, as the strains' invasive phenotype was normal. Western blotting of the IcsA protein, a crucial protein for *S. flexneri* intra- and intercellular spread, revealed that although the cellular levels of IcsA are normal for SFL1001 and SFL1736, the extracellular IcsA levels for these two strains were dramatically reduced. Immunofluorescent and immunogold microscopy was used to show that the extracellular levels of IcsA were reduced in SFL1001 and SFL1736 because very little IcsA is transported to the outer membrane of the bacterial cell. The *mgl* knockout, SFL1617, displayed similar levels of surface IcsA to the wild type virulent strain, SFL1704, indicating that by eliminating *mgl* operon expression from the cell, IcsA is capable of reaching the outer membrane. These findings suggest that the *mgl* operon is preventing cytoplasmic IcsA from reaching the cell surface. Further research is required to determine the mechanism by which the Mgl proteins could be impeding IcsA transport.

The immunogenic protein, IpaD, was considered as a potential candidate for *S. flexneri* subunit vaccine development in two preliminary adjuvant studies. IpaD was loaded into liposomes and a number of different molecules were also included, such as LPS, the CD4 receptor and an anti-CD11c single chain antibody. CD4 and anti-CD11c were used to determine if, by targeting the IpaD-liposomes to antigen presenting cells, the anti-IpaD immune response could be amplified. The liposome preparations did not induce a stronger anti-IpaD IgG response than that raised against IpaD in PBS when delivered subcutaneously. Gamma inulin, cochleates and aluminium hydroxide were also used to deliver IpaD in a mouse model. All three adjuvants boosted the anti-IpaD antibody titres higher than those raised against the IpaD protein in saline. Furthermore, the anti-IpaD immune responses generated against IpaD delivered with gamma inulin, cochleates and aluminium hydroxide were sufficient to increase the recovery from *S. flexneri*-induced illness in mucosally *S. flexneri* challenged mice. Therefore, IpaD and the gamma inulin, cochleates and aluminium hydroxide adjuvants may offer promising developments in the search for a multi-serotype protective *S. flexneri* subunit vaccine.

The stationary phase acid resistance pathways of *S. flexneri* 2457T were characterised for the first time in this study. The glutamate-dependent (GDAR) and the oxidative resistance

pathways were identified in the *S. flexneri* 2457T strain, SFL1001. Interestingly, *S. flexneri* cells grown overnight in LBG pH 5 and acid challenged in EG pH 2.5 without glutamate were resistant to the acid shock; this resistance profile was termed non-glucose suppressed, glutamate-independent resistance. These test conditions are described as a negative control for the GDAR and oxidative pathways in *E. coli*. Knockouts were constructed in the GDAR genes, *gadB* and *gadC*, and the *rpoS* gene, which is crucial for *S. flexneri* 3136 oxidative acid resistance. None of these knockouts were important for the non-glucose repressed, glutamate-independent resistance of SFL1001; it remains unclear what proteins are contributing to this resistance and further work is required to identify candidate proteins. The RpoS protein of *S. flexneri* 2457T was shown by Western blot to be a truncated protein, which appears to be reduced in its activity in a catalase assay. It appears that this truncated RpoS is not important to any of the *S. flexneri* 2457T acid resistance pathways, suggesting that *S. flexneri* 2457T has evolved an alternate regulatory mechanism for its acid resistance phenotype.

8.2 Immunoproteome analysis of S. flexneri *2457T protein*

Immunoproteome analysis of seroreactive bacterial proteins has been described as a powerful tool for the identification of novel bacterial antigens in pathogens and as a foundation for rational vaccine design (388). Immunoproteomics has been used extensively in *H. pylori* and in a number of pathogens including *Staphylococcus aureus*, *Francisella tularensis*, *Chlamydia pneumoniae* and *Borrelia garinii* (38, 93, 124, 154, 170, 179, 224, 388). *S. flexneri* has previously been submitted to immunoproteome analysis, but only with sera raised in experimental animals, which are not susceptible to shigellosis (282, 415, 416). This study is the first reported for *S. flexneri* that has studied the immunoproteome that is recognised by sera from natural infection. Proteins recognised by the human anti-*S. flexneri* immune response are far more relevant to the development of a vaccine against *S. flexneri*, as it is a disease that only manifests in humans and higher primates.

The immunoproteome analysis revealed that some proteins were recognised more strongly than others, independently of the protein spots' concentration, indicating that it is possible to detect both minor and major antigens by this technique (162). IpaD and OmpA were clearly major immunogens, producing a strong response in all three repeats. The sera of the

five *S. flexneri*-infected patients used in this study were pooled, due to the limited volume of sera that could be obtained. Therefore, it is not clear whether each immunoreactive protein is patient-specific or common to all five infections.

Proteomics offers many advantages to the screening for vaccine candidate proteins; it is possible to have a "global" view of the proteins present in the cell, protein post-translational modifications can generally be visualised, and the immunogenic profile of multiple proteins is determined simultaneously (75). However, it should be remembered that a significant portion of a proteome cannot be detected in a 2-DE study; in particular 2-DE rarely identifies low-abundance and hydrophobic proteins, while alkaline proteins are difficult to resolve in the first dimension (392). Therefore, low abundance proteins important to *S. flexneri* pathogenesis may not be detected by immunoproteome screening in the absence of enrichment (75). Very few *S. flexneri* proteins were detected by Coomassie stain in this study; only 150 protein spots were visualised for the soluble protein extraction. It is estimated that there is more then 4000 proteins expressed by *S. flexneri*, and although all would not be expected to be expressed during the extraction conditions, the number visualised on the 2-DE gels represents extremely poor coverage of the complete *S. flexneri* proteome (403). While a number of potentially useful proteins were still identified, it would be ideal to continue probing the *S. flexneri* proteome for immunogenic proteins.

It is possible to improve the resolution of the 2-DE gels; for example, modifications to the 2-DE procedure can be used to "zoom" in on particular protein groups, such as using narrow range immobilised pH gradients for focusing the first dimension, increasing the visibility of proteins which run within that particular pI range (61). Furthermore, protein sample preparation can use prefractionation techniques to split proteins into cellular compartments, such as cytosolic, secreted, cytosolic membrane and outer membrane protein extractions (60, 348). Difficult proteins to resolve, such as basic proteins can also be enriched by specific protein extraction techniques (14). A combination of these techniques would improve the resolution of proteins observed in this study, and would ultimately result in further immunogenic proteins being identified for *S. flexneri*.

The immunoproteomic technique has a distinct advantage for screening *S. flexneri* for vaccine candidates since the proteins detected are likely to be immunogenic across all *S. flexneri* serotypes, as the majority of proteins are common across the serotypes (249, 403). As up to four different serotypes of *S. flexneri* are generally prevalent in the one endemic area, an ideal *S. flexneri* vaccine would offer protection simultaneously against all the prevailing serotypes. Therefore, the findings from this immunoproteomic study may contribute to the development of novel multi-serotype protective vaccines. In this thesis, one of the identified proteins, IpaD, was further assessed as a prospective component of *S. flexneri* subunit vaccines, and a number of the proteins were considered for their use as attenuation targets for live vaccine strains. In future work, Spa33 would be an excellent candidate for vaccine assessment as it is a *Shigella*-specific protein and an important component of the type III secretion system. It is possible that a neutralising anti-Spa33 antibody response could have protective capabilities, as Spa33 is an essential component of the type III secretion system (238). Thus, the proteins identified during this study are promising additions to the list of potential antigens that could be used in the development of new multi-serotype protective vaccines for *S. flexneri*.

8.4 Disruption of Udp causes adverse in vitro *virulence effects which are not detected* in vivo *for* S. flexneri

Bacterial proteins that are recognised by the host's immune response must be expressed during invasion of the host. Therefore, they may be important in the virulence of the bacterium. Virulence proteins are ideal candidates for attenuating bacterial strains to create live vaccine strains; for example, IcsA has been used as an attenuation in the promising *S. flexneri* vaccines, CVD1203, CVD1207 and SC602 (62, 175, 176). Two immunogenic proteins, Udp and S3195, were considered for their use as vaccine attenuations in this study. Disruption of S3195 did not have any adverse effects on the virulence of *S. flexneri* in the initial screen by plaque assay and thus was discarded as a vaccine attenuation candidate.

Disruption of Udp significantly reduced the survival of intracellular *S. flexneri* in cell culture assays. It seems that the virulence defect observed is caused by a reduction in the strain's ability to scavenge and recycle pyrimidines, possibly for their use as carbon and

nitrogen sources during periods of high growth and low nutrient availability. Nucleoside phosphorylases, such as Udp, are considered sugar-activating enzymes which yield phosphorylated pentoses without ATP expense, a mechanism that allows bacteria to utilise pyrimidines from tissues and living cells for growth (369). Catabolism of pyrimidines is increased when the bacterium's primary sugar source has been exhausted (369). The growth curves performed in this study suggested that in the absence of glucose as a plentiful energy source, *S. flexneri* uses Udp to scavenge pyrimidines for energy, as the *udp* disruption reduced the strain's ability to grow in the absence of glucose. It is important to note that the defect is not caused by an auxotrophy for uridine as the synthesis pathways are independent of Udp (369).

Despite the growth deficiency observed for the *udp* mutant *in vitro*, there were no adverse effects observed in the disease-causing abilities of the strain *in vivo*. It is possible that the discrepancy between the *in vitro* and *in vivo* results is caused by the difference in the cell types; little is known about the nutrient availability within eukaryotic cells and it may be possible that there are increased carbon and nitrogen sources available in the cytoplasm of guinea pig conjunctiva cells, which are not found in human epithelial secondary cell lines. It is well known that established cell lines experience genetic instability and may differ from the primary cell source (216). However, perhaps the scavenging of pyrimidines is involved in providing energy for *S. flexneri* replication *in vivo* but is not essential. Although the survival of the *udp* mutant is reduced in the *in vitro* survival assay, large numbers of bacteria are still maintained within the cytoplasm, most probably enough to cause damage to the epithelial layer, generating the symptoms of shigellosis. Therefore, this finding demonstrates the caution with which *in vitro* data should be considered for pathogens. It is not possible to predict if an *in vitro* defect is significant enough to manifest as a reduction in virulence *in vivo*.

The *udp* mutant cannot be used as an attenuated live vaccine strain, which was the goal of this study, as it is not attenuated *in vivo*. However, it is possible that there is a slight fitness deficiency conveyed by the reduced intracellular replication of the *udp* mutant *in vivo* that is not distinguishable in the Sereny test. A competition assay could be used to directly measure any reduction in virulence relative to the wildtype strain (343). A slight virulence

deficiency caused by *udp* disruption might be useful as a secondary mutation in an attenuated vaccine, which is not sufficiently weakened. In such a case, it is necessary to reduce the reactogenicity of the vaccine without overly reducing the invasive nature, to ensure that a strong protective immune response is still induced. Double vaccine mutants may accomplish the elusive balance between the safety and efficacy of attenuated *S. flexneri* strains. For example, the addition of a secondary mutation in the guanine auxotroph CVD1204, to create CVD1205, reduced the degree of keratoconjunctivitis induced in guinea pigs (257). Furthermore, additional mutations in the enterotoxin genes of CVD1205, were used to create CVD1207, generated a vaccine suitable for testing in human volunteers (176). Therefore, further work could reveal that Udp may be a useful attenuation candidate for delicately manipulating the reactogenicity and immunogenencity of attenuated *S. flexneri* strains currently under development.

8.5 The role of the mgl *operon in* S. flexneri *intra- and intercellular spread*

It was determined during this study that disruption of the *mgl* operon from the avirulent *S. flexneri* strain, SFL1001, produced a detectable virulent phenotype. Complementation of the mutation confirmed the contribution of the *mgl* genes, as a significant decrease in the strain's virulence was observed for the complemented strain. Western blotting and microscopy analysis revealed that SFL1001 had very little IcsA on the outer membrane, and that *mgl* disruption restored normal IcsA levels to the SFL1001 cell surface. Western blotting demonstrated that the intracellular levels of IcsA were similar between SFL1001 and the *mgl* mutant, SFL1617, indicating that SFL1001 was not reduced in IcsA expression but simply in the movement of IcsA to the outer membrane.

It is not unknown for a study to identify a gene that has an adverse effect on the bacterium's virulence. Disruption of the *grvA* gene in *Salmonella enterica* increased the virulence of the strain in a mouse model (128). Furthermore, a study in *Salmonella enterica* serovar *typhimurium* identified a mutation that resulted in increased intracellular growth, allowing the mutant strain to out-compete the parent strain *in vivo* (85). In *Shigella*, it has been determined that the introduced expression of lysine decarboxylase attenuates virulence by producing cadaverine, which interferes with *Shigella* enterotoxin activity (223). It is possible that the suppression of cell surface IcsA by the *mgl* operon acts as a protein-level

regulation of IcsA. Cell surface IcsA is constantly cleaved by outer membrane-located IcsP and once cleaved, IcsA is no longer functional. Steinhauer *et al* (1999) propose that during exponential growth phase some IcsA must be protected from cleavage, possibly by being located subcellularly (350). Therefore, *mgl*-related regulation of IcsA reaching the surface may ensure that there is always subcellular IcsA available as an immediate supply of full-length functional IcsA, able to reach the cell surface once intercellular spread is required.

It is not yet clear how the Mgl proteins may be impeding the process of IcsA transport in SFL1001. However, this study has proposed a number of hypotheses in Section 5.3.2, two of which will be expanded on with figures in this discussion (Figure 8.1).

In Hypothesis 1, it is proposed that the Mgl proteins are acting to exclude the Sec machinery from the polar region of the bacterial cytoplasmic membrane (Figure 8.1B). This would most likely be occurring due to the MglB-mediated activation of the Trg chemoreceptors, producing clusters of Trg chemoreceptors in the cytoplasmic membrane at the bacterial pole (210, 334). However, it is also possible that the MglA and MglC membrane complex is associated with the polar region of the cytoplasmic membrane, so it is close to the polar MglB –galactose complexes for transport. These Trg and/or Mgl complexes may block up the polar cytoplasmic membrane, preventing the Sec machinery from operating efficiently at the pole. Removal of the Mgl proteins from the cell, thereby preventing MglB-mediated Trg clustering and MglAC complexes forming, would allow the Sec machinery unimpeded access to the polar cytoplasmic membrane region. It is not clear why the Mgl proteins have such a strong influence on IcsA transport in the avirulent SFL1001 strain. It is possible that SFL1001 has a regulatory defect, which creates an unusually high amount of Trg or Mgl protein, or perhaps that the chemoreceptors in SFL1001 have increased sensitivity to activation, resulting in increased blocking of IcsA. It would be interesting to determine, by real-time PCR, if SFL1001 expresses larger amounts of Mgl or Trg protein.

Hypothesis 2 proposes that MglB and IcsA competitively bind the same protein partner and that in SFL1001 MglB is out-competing IcsA for the binding partner, effectively impeding IcsA transport (Figure 8.1C). It has been proposed that IcsA interacts with protein

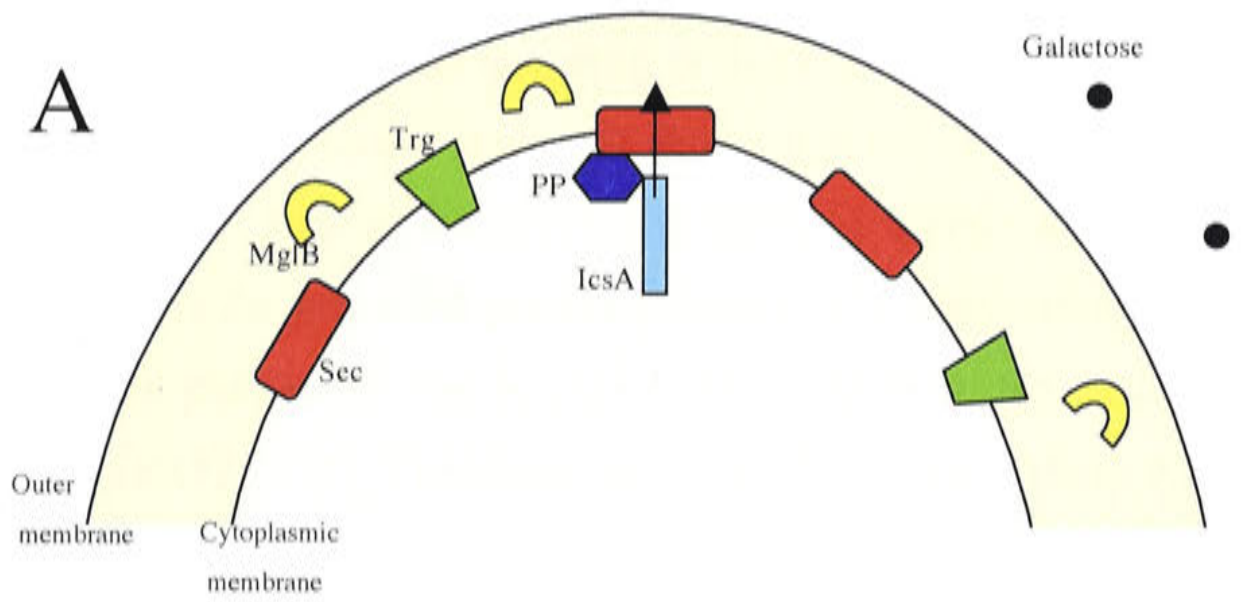
Figure 8.1. Hypothesised models for Mgl-mediated blocking of cellular IcsA reaching the outer membrane in SFL1001.

A. **Transport of IcsA from cytoplasm to periplasmic space.** IcsA is most probably bound by a unknown protein partner (labeled as PP), which directs IcsA to the Sec machinery located at the old pole of the bacterium. IcsA is translocated across the cytoplasmic membrane by the Sec machinery only at the poles. It is not clear if the unknown protein partner is transported across with IcsA and dissociates in the periplasm.

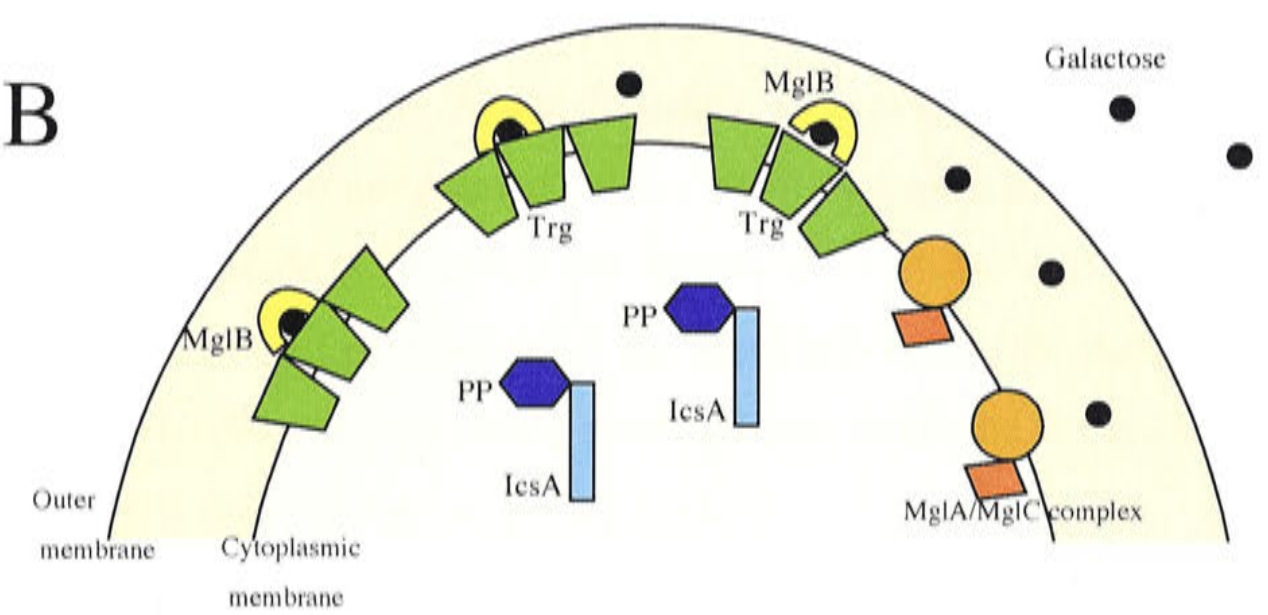
B. **Hypothesis 1:** Binding of MglB to galactose stimulates MglB to bind and activate the Trg chemoreceptors, which form clusters at the bacterial pole. This clustering may block the polar Sec machinery, reducing the amount of polar Sec machinery that is available for the protein partner (PP) and bound IcsA. The membrane complex of MglA and MglC may also associate towards the pole to be available to MglB, possibly further excluding Sec machinery at the pole. By removing the Mgl proteins from the cell, such as in SFL1617, the Sec machinery will no longer be blocked from accessing the polar area of the cytoplasmic membrane and IcsA polar transport will be restored.

C. **Hypothesis 2:** MglB acts as a protein chaperone and may bind the protein partner (PP) of IcsA. PP may be transported through the Sec machinery with IcsA to the periplasm and be recycled back to the cytoplasm or may remain on the cytoplasmic side. MglB may bind the periplasmic PP so that little is available to return to the cytoplasm or cytoplasmic MglB may be responsible for binding PP. MglB is also transported by the Sec machinery. IcsA cannot be directed to the polar Sec machinery for transport in the absence of the polar targeting PP. By removing MglB from the cell, such as in SFL1617, PP would be free to interact with IcsA and resume polar transport.

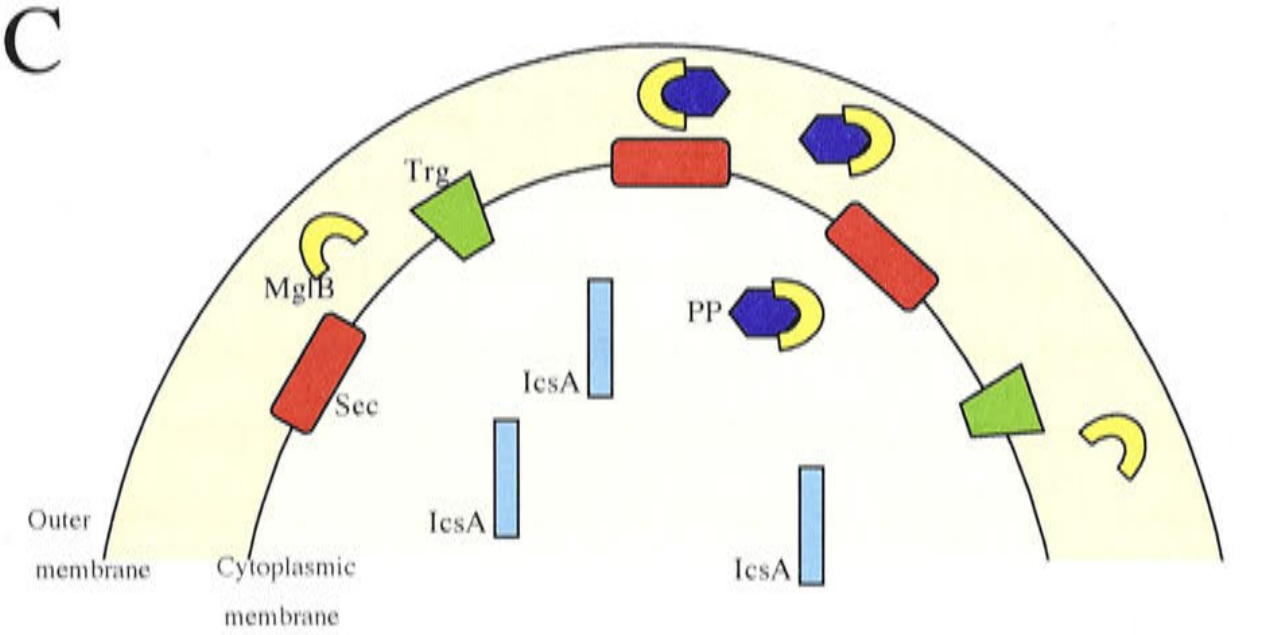
A


Galactose
Trg
PP
MglB
IcsA
Sec
Outer membrane
Cytoplasmic membrane

B


Galactose
MglB
Trg
Trg
MglB
PP
IcsA
PP
IcsA
MglA/MglC complex
Outer membrane
Cytoplasmic membrane

C


Trg
MglB
Sec
IcsA
PP
IcsA
IcsA
Outer membrane
Cytoplasmic membrane

partner(s), which directs the polar targeting of IcsA through the Sec machinery at the bacterial poles (49, 324). A recent study has shown that the apyrase gene, *phoN2*, may be important for the correct localisation of IcsA, possibly by a protein-protein interaction, but it is not clear if this is the proposed protein partner (332). Interestingly, MglB is not only known as a protein chaperone, but is also transported from the cytoplasm by the Sec apparatus in *E. coli* (82, 334). Therefore, IcsA and MglB may share a targeting protein partner, which is responsible for directing both proteins to the Sec machinery. In SFL1001, MglB may be out-competing IcsA for the protein partner, either in the cytoplasm prior to targeted transport, or in the periplasm if the protein partner is co-transported with IcsA and recycled back to the cytoplasm. As discussed previously, this protein binding interaction may not be exclusive to SFL1001, but may be involved in regulating how much IcsA is present on the cell surface in all *S. flexneri* strains. However, in SFL1001, the levels of surface IcsA appear to be "over-regulated" to the point that only minimal IcsA is reaching the cell surface, until the Mgl proteins are removed from the cell. It is possible that SFL1001 has either increased expression of the *mgl* operon, or that the protein partner is down regulated in SFL1001, leaving only a small amount available for binding by IcsA and MglB, for which MglB may be out-competing IcsA.

There are a number of experiments that can be performed to confirm either of the two hypotheses, many of which were outlined in Section 5.3.3. It would be useful to analyse the transcriptional levels of the *mgl* and *trg* genes in SFL1001 by real-time PCR to determine if they are unnaturally high, and therefore, likely to be contributing to the IcsA suppression observed for SFL1001. Furthermore, a Trg knockout in SFL1001 would indicate if removal of the Trg chemoreceptor clusters from the bacterial pole restores Sec-mediated IcsA transport to the surface. The protein-protein interactions of both IcsA and MglB could be explored with the Stratagene BacterioMatch® II Two-Hybrid System. It would be interesting to use this technique to determine if apyrase does interact with IcsA, as has been proposed by Santapaola *et al* (2006) (332). It should be possible to conclude from this technique whether IcsA and MglB interact with a common protein. If IcsA and MglB share a protein partner, it would be relevant to establish if MglB is targeted to the pole by this protein interaction by raising antibodies against the MglB protein and using them in microscopy localisation experiments. Finally, it is crucial that the role of the Mgl proteins

in IcsA transport be determined for a virulent strain of *S. flexneri*. At this stage it is not clear whether the Mgl regulation of IcsA is peculiar to SFL1001, which is avirulent and lacks surface IcsA. During this study, numerous attempts were made to produce *mgl* operon and non-polar *mglB* mutants in the virulent strain, SFL1704, but neither could be produced within the time-constraints of the project. However, there are other knockout techniques that were not tested in this study, including the use of temperature sensitive suicide plasmids and the TargeTron gene knockout system that could assist in the production of knockouts in SFL1704 (287, 318). The cell surface levels of IcsA should be monitored for the *mgl* mutant of SFL1704 by microscopy and Western blotting to determine if the *mgl* deletion results in higher levels of outer membrane IcsA. Furthermore, to examine if a *mgl* mutation in SFL1704 increases the virulence of the strain, such as what was observed for SFL1001, plaque assays and Sereny tests could be performed. The findings would confirm if the Mgl operon plays a role in the regulation of cell surface levels of IcsA in all *S. flexneri* strains, or just the avirulent strain, SFL1001.

8.6 IpaD as a S. flexneri *subunit vaccine with different adjuvants*

8.6.1 Subunit vaccines for *S. flexneri*

IpaD is a known immunoreactive protein from natural *S. flexneri* infection and was identified as a major antigen in the immunoproteome study performed in this thesis (197, 262). IpaD protein was purified and used in preliminary *S. flexneri* subunit vaccine studies to assess the efficiency of liposomes, gamma inulin and cochleates as subunit adjuvants. Subunit vaccines have not been extensively studied in *S. flexneri* and most studies have only focused on delivering LPS to the immune response (11, 56, 97). Recent studies have shown that an undefined complex of Ipa proteins and LPS, termed Invaplex, is highly immunogenic without added adjuvant and invokes protective immune responses against *S. flexneri* in both guinea pigs and mice models (156, 265). Protein subunit vaccines have a number of properties that make them ideal for *S. flexneri* vaccine development; they are non-invasive, which means they avoid the safety issues associated with live vaccines, proteins are generally not serotype-specific, meaning subunit vaccines have the capabilities to be simultaneously protective against multiple serotypes, they allow the flexible

incorporation of different "protective" antigens and they contain adjuvants to boost, enhance and manipulate the immune response (145, 149).

To develop a successful subunit vaccine it is necessary to find an optimal adjuvant. Adjuvants can protect the protein during delivery, increase cellular infiltration to the injection site, directly promote the uptake of the protein by antigen presenting cells and modulate and enhance the immune response produced (261). The most appropriate adjuvant for a candidate antigen is often difficult to predict, and adjuvant selection remains rather "hit-and-miss" for bacterial pathogens.

8.6.2 Liposomes as adjuvants for IpaD

Liposome vesicles consist of an external lipid bilayer and an aqueous inner compartment. They are advantageous as adjuvants as they allow the encapsulation of antigens, the integration of targeting devices and their hydrophobic nature allows them to insert into cell membranes (88, 191). The insertion of targeting molecules into liposomes is a rational approach for improving the delivery of antigens to antigen presenting cells, and a number of strategies have been formulated. Liposomes can be engineered to express mannose ligands on their surface to target C-type lectin receptors found on dendritic cells, or similarly, liposomes can be loaded with phosphatidylserine (PS), which is recognised by the PS receptor on antigen presenting cells (10). These approaches were successful in improving the antibody response against the meningococcal protein antigen, PorA (10).

In this study, CD4 was incorporated into liposomes, to direct them to interact with MHC class II expressing cells, as well as an ScFv antibody fragment directed against the CD11c receptor found on dendritic cells. Both strategies in previous studies successfully delivered liposomes to the targeted cells (377, 378). It was hoped that by targeting antigen presenting cells, in particular the most efficient antigen presenting cell, dendritic cells, that the anti-IpaD antibody response would be boosted by an increased CD4 T-cell response. However, there was no significant improvement observed in the serum IgG titre in comparison to untargeted liposomes. It is possible that the immune response induced in the targeted liposome-immunised mice may be more prolonged in its response. It would have been interesting to have extended the sera collection further than Day 34, particularly as the last

boost had only occurred on Day 14; it is possible that with a longer experiment, a more sustained IgG response may be detected for the targeted liposomes than the untargeted liposomes. Furthermore, a T-cell activation assay could be included in the analysis to determine if the targeting molecules are actually increasing the amount of liposomes reaching antigen presenting cells, therefore boosting the antigen presenting cells activation of T-cells.

Although liposomes did not produce any stronger immune response than IpaD protein alone, it is possible that the subcutaneous delivery of liposomes is not an optimal route for adjuvant delivery of IpaD. In future work, it would be interesting to determine if mucosal delivery of IpaD in liposomes boosts the anti-IpaD immune response. After oral administration, liposomes can be detected within M-cells. However, often there is inefficient activation of the gut-associated lymphoid tissue (GALT), possibly because the majority of liposomes are being trapped in the mucosal layer and are not reaching the mucosal inductive sites (118). Nevertheless, a number of liposome vaccines for bacterial enteric pathogens, delivered mucosally, have been successful in inducing strong antibody responses, suggesting that a vaccine experiment with mucosal delivery of IpaD-liposomes may be warranted (47, 118, 364).

This trial of IpaD and liposomes is not the first study to report insignificant increases in antigen serum titres for antigen delivered in liposomes. Both meningococcal and cholera vaccine trials have also reported that some liposome formulations did not enhance the immune response to a protein antigen (66, 294). Furthermore, liposomes may not be ideal for the development of a *S. flexneri* vaccine; liposomes are unstable at room temperature, cannot be lyophilised and are not quick and inexpensive to manufacture - factors that are essential for a vaccine to be used in world's least developed areas (189).

8.6.3 Gamma inulin, cochleates and aluminium hydroxide adjuvants for IpaD

The adjuvants, gamma inulin, aluminium hydroxide and cochleates are stable and easy to manufacture, indicating that they may be suitable candidates for *S. flexneri* subunit development (218, 288, 344). Aluminium adjuvants are approved for use in human vaccines and are known to stimulate a strong type 2 immune response, which would be

useful in immunity against *S. flexneri* (129). Gamma inulin stimulates both type 1 and type 2 immune responses and is both safe and non-toxic in humans (344). Cochleate formulations are highly immunogenic and capable of inducing strong long-lasting mucosal responses (218). In the pilot study performed in this thesis, all three adjuvants were found to induce a strong anti-IpaD serum IgG response, and intranasally delivered cochleates also produced an anti-IpaD IgA response. Challenge of the IpaD immunised mice revealed that the anti-IpaD response was sufficient to increase the clearance time of *S. flexneri* from the mouse lung. Further work should be performed with these adjuvants as they may offer promising alternatives for the development of a safe and effective *S. flexneri* vaccine.

In both vaccine trials performed in this thesis, IpaD delivered without adjuvant was determined to be highly immunogenic. Furthermore, the mice immunised with IpaD alone were able to clear the *S. flexneri* infection as rapidly as the mice co-immunised with adjuvant. Protein antigens delivered in saline typically produce weak and transitory immune responses (137). The preliminary experiments performed in this thesis used GST purified IpaD protein and the purity was only determined by Coomassie staining on SDS PAGE gels. Although the protein was very concentrated and any contaminating material would be at a comparatively very low level, it is imperative that future IpaD vaccine work uses further purified IpaD, by techniques such as HLPC, to confirm that the immunogenicity detected in the mice immunised with IpaD protein alone is not directed against contaminating material (146). However, a number of proteins have been reported to be immunogenic when delivered without adjuvant, including bacterial heat shock proteins, malarial proteins and the PspA protein from *Streptococcus pneumoniae* (36, 185, 214). IpaD is also a component of the highly immunogenic Invaplex complex, which is not only a promising vaccine for *Shigella* but has been assessed for its use as an adjuvant (156, 265). Based on the previous findings that IpaD is immunoreactive, and the results presented in this thesis that the anti-IpaD immune response assists in the clearance of *S. flexneri* infection, IpaD is a promising candidate for future subunit vaccine research.

8.7 Acid resistance pathways of S. flexneri *2457T*

8.7.1 Non-glucose repressed glutamate independent acid resistance

Analysis of the acid resistance pathways of the *S. flexneri* 2457T strain, SFL1001, revealed that the strain exhibited a novel acid resistance phenotype. SFL1001 cells grown overnight in LBG pH 5 were capable of surviving exposure to EG pH 2.5 without glutamate supplementation. These growth and acid shock conditions are described as a negative control for both the GDAR and oxidative pathways in *E. coli,* as the presence of the glucose in the overnight growth medium suppresses the oxidative pathway, and the absence of glutamate in the acid shock medium prevents the GDAR pathway from operating (201, 202). Hence, this novel phenotype of *S. flexneri* 2457T was termed non-glucose repressed, glutamate independent acid resistance. The same resistance has also been reported for *E. coli* 0157, but was not discussed by the authors (68). Knockouts in the GDAR genes, *gadB* and *gadC* of SFL1001 revealed that the GDAR pathway is not involved in the non-glucose repressed, glutamate independent resistance. It was not possible to determine if the oxidative resistance pathway is generating this resistance, as an *rpoS* mutant of SFL1001 was not attenuated in any of its acid resistance pathways (discussed below). However, the non-glucose repressed, glutamate independent resistance was improved in cells grown in oxygenated conditions, suggesting that the pathway at least favours an oxidative environment, and thus may be the oxidative pathway operating in the presence of glucose.

It is not clear how the oxidative pathway operates in *E. coli* or *Shigella* as it is poorly characterised and no hypotheses on its mechanism have been proposed. Studies have shown that RpoS is essential for the oxidative pathway to function, that the pathway is repressed by growth in glucose-containing medium and that expression in *E. coli* requires CRP (43). A recent study has shown that the Fur protein is important to the oxidative resistance pathway of *S. flexneri* SA100, as it represses *ryhB*, allowing the *ydeP* gene to be expressed. YdeP encodes a putative oxioreductase, with homology to formate dehydrogenase (270). Oglesby *et al* (2005) propose that YdeP provides oxidative acid resistance by removing acidic formate from the cell by converting it to the less acidic products of formate, carbon dioxide and dihydrogen (270).

It seems likely that the oxidative resistance pathway might consist of numerous cell "stress survival" strategies, such as the removal of acidic products from the cytoplasm to maintain a homeostatic cytosolic pH, the use of chaperones to bind and protect proteins during acid exposure, by changing the membrane composition of the cell, an increase in the activity of the TCA cycle to utilise the high proton potential, or the use of proteins that repair acid-induced damage to macromolecules (19, 30, 270, 349, 418). Therefore, it is possible that the non-glucose repressed, glutamate-independent resistance observed in SFL1001 is a combination of a number of these mechanisms, which are no longer regulated by glucose-repression. Since Fur is a known regulator of the oxidative pathway in *S. flexneri* SA100, it would be ideal to confirm the contribution of the oxidative resistance in this non-glucose repressed, glutamate-independent resistance by testing a *fur* mutant of SFL1001 for loss of resistance. A mutant could not be constructed for *fur* during this project, due to time constraints, but should be attempted in further work, as it should establish the contribution of the oxidative pathway. Furthermore, as previously discussed, genomic library screening could be used to search for the genes responsible for the non-glucose repressed, glutamate-independent resistance in SFL1001. However, this screening may not be useful if there are multiple genes contributing, as suggested above, or if the genes are separated on the chromosome. Alternatively, a transposon insertion library could be constructed in *S. flexneri* and strains screened for the loss of resistance. Any strains with an altered phenotype could be sequenced to determine the gene involved.

Finally, *S. flexneri* 2457T is known as a highly virulent strain of *S. flexneri* and is used in pathogenesis studies for this reason (403). It would be interesting to determine if the additional non-glucose repressed, glutamate-independent resistance, observed in this study for *S. flexneri* 2457T, contributes to the highly pathogenic nature of the strain by increasing bacterial survival in the stomach during host invasion. It is possible that this resistance is an extra level of protection for *S. flexneri* 2457T during infection. SFL1001 could have it's level of acid resistance directly compared to other *S. flexneri* strains, such as 3136, that do not exhibit the non-glucose repressed, glutamate-independent resistance. To increase the physiological relevance of the experiment, the acid resistance tests could be performed in simulated gastric fluid, which would demonstrate which of the strain's acid resistance pathways better contribute to bacterial survival during human infection (418).

8.7.2 The role of RpoS in *S. flexneri* 2457T acid resistance

It was also determined during this work that the acid resistance pathways of *S. flexneri* 2457T operate independently of RpoS. An *rpoS* mutant of SFL1001 was not altered in its acid resistance phenotype for any of the pathways. This was unexpected, as RpoS is described as being essential for operation of the oxidative pathway in *S. flexneri* 3136 (26). However, the RpoS protein of *S. flexneri* 2457T, unlike the RpoS protein of *S. flexneri* 3136, is truncated by 89 amino acids due to a frameshift mutation in the gene (346, 403). It was determined in this study that the truncated RpoS protein is expressed, but it appears reduced in its activity according to a catalase assay. It appears that in *S. flexneri* 2457T, alternative regulation is used to control the acid resistance pathways, although it is unclear whether this change in regulation occurred before or after the *rpoS* gene became mutated. A recent study has found that in *E. coli* strains divergence in the genes that are RpoS-regulated may be quite common (27). However, all previous reports on the loss of *rpoS* in *E. coli* and *S. flexneri* have shown that the oxidative pathway is consequently reduced in acid resistance, making this the first study to demonstrate *rpoS*-independence for this pathway (43, 259, 298).

It is thought that nearly 10% of *E. coli* genes are under direct or indirect control of RpoS. Although RpoS plays an important role in the global gene expression under starvation conditions, paradoxically it is commonly lost from both laboratory and natural strains (165). Expression of large numbers of genes under the control of RpoS probably creates a significant metabolic load on the cell and this may drive the loss of RpoS from strains (51). *rpoS* mutants of *E. coli* are actually more virulent in mouse colonisation experiments and have increased amino acid scavenging abilities (13, 424). It is possible that the loss of RpoS activity in *S. flexneri* 2457T contributes to the virulence of the strain, however because virulent strains must also be able to survive exposure to the acidic environment of the stomach during infection, the strain has evolved alternative regulation for its acid resistance pathways. Therefore, the RpoS-independent nature of the acid resistance pathways may be important for the low dose required and high virulence of *S. flexneri* 2457T.

8.8 Future work

The experiments that should be performed to continue the work presented in this thesis have previously been discussed in detail in each Chapter's discussion and also in the above final discussion. A summary of these experiments is presented below.

Immunoproteome analysis of *S. flexneri* 2457T

- Prepare extractions of *S. flexneri* 2457T protein to improve the concentrations of low abundance and difficult proteins to purify, such as secreted, inner membrane and basic protein extractions.
- Separate proteins on narrow range p*I* Immobiline strips to improve the resolution of proteins within those p*I* ranges.
- Use fluorescent stains with the 2-DE gels to increase the sensitivity of protein detection.

multiple serum samples.

Virulence gene identification for attenuation candidates for *S. flexneri* live vaccine development

- Construct mutants for the remaining immunogenic virulence candidates *ansB*, *ggt* and *yfiD*, using strategies such as the TargeTron technique or temperature sensitive suicide plasmids.
- Test the *udp* mutation as a secondary mutation in attenuated *S. flexneri* strains.

The role of the *mgl* operon in *S. flexneri* intra- and intercellular spread

- Construct a *mgl* mutant in SFL1704 and determine by microscopy and Western blot if the IcsA levels are altered.
- Construct a *trg* mutant in SFL1001 and determine by microscopy and Western blot if the IcsA levels are increased on the surface.
- Perform real time PCR on MglB and Trg in SFL1001 to determine if they are elevated in comparison to a virulent strain such as SFL1704.
- Investigate the protein-protein interactions of MglB and IcsA with the BacterioMatch® II Two-Hybrid System.
- Raise antibodies against MglB for use experiments to determine the location of the protein within the cell.

IpaD subunit vaccine development and adjuvant assessment

- Deliver IpaD liposomes to mice mucosally and test for a sustained immune response by increasing the sera collection time points.
- Purifying IpaD by HPLC for use in further mouse vaccine trials of gamma inulin, cochleates and aluminium hydroxide.
- Improve the mouse challenge experiment for IpaD subunit vaccines by raising the dose to a lethal dose, determining the bacterial load in the lungs by dilution plating and challenging with different serotypes of *S. flexneri*.

The acid resistance pathways of *S. flexneri* 2457T

- Construct a *fur* mutant in SFL1001 to disrupt the oxidative acid resistance pathway and determine if the non-glucose repressed, glutamate-independent resistance is also disrupted.
- Construct a genomic library of SFL1001, express in *E. coli* and screen for resistance under the non-glucose repressed, glutamate-independent test conditions, to attempt to identify the genes involved. OR construct a transposon library in *S. flexneri* 2457T and screen for a loss of resistance.

8.9 Conclusion

Proteomics was used to visualise the immunoproteome for *S. flexneri* 2457T using sera from five patients. Eight proteins were identified as immunogenic across three repeats. Six of these proteins have never been reported as immunogens for *S. flexneri*. These proteins are potential vaccine candidates and possible virulence factors. One immunogenic protein, Udp, is important in the scavenging and recycling of pyrimidines and it was determined by *in vitro* tests that this might contribute to the replication of *S. flexneri* within the host cell cytoplasm. However, disruption of the *udp* gene was not sufficient to reduce the virulence of *S. flexneri in vivo*, indicating that *udp* is not an appropriate single attenuation target for vaccine development. Furthermore, the *mgl* operon was found to block the movement of IcsA from the cytoplasm to the outer membrane of SFL1001, suggesting that the Mgl proteins may play a regulatory role in IcsA surface display for *S. flexneri*. IpaD was identified in the immunoproteome analysis and was previously known to be

immunoreactive in shigellosis patients. Subcutaneous delivery of IpaD in liposomes to mice did not boost the anti-IpaD IgG response any higher than what was raised against the protein alone. As such, liposomes may not be a suitable adjuvant for use with *S. flexneri* protein. However, the adjuvants gamma inulin, cochleates and aluminium hydroxide all induced strong antibody responses against IpaD, which was capable of clearing a *S. flexneri* infection faster than mice not immunised with IpaD. These adjuvants and the protein antigen, IpaD, offer promising progress towards the development of a safe and effective *S. flexneri* vaccine. The acid resistance pathways of *S. flexneri* 2457T were characterised and the known GDAR and oxidative pathways detected. A novel resistance phenotype was also detected, termed non-glucose repressed, glutamate-independent resistance, which is not related to the GDAR pathway according to tests performed with strains carrying mutations in the *gadB* and *gadC* genes. It seems likely that this resistance is related to the oxidative pathway, operating in the presence of glucose. Furthermore, it was discovered that *S. flexneri* 2457T expresses a truncated RpoS protein, which is reduced in activity and does not appear to be important for the acid resistance pathways. This is the first report of a *S. flexneri* strain that does not require RpoS for regulating any of its acid resistance pathways.

Appendix A
Buffers, solutions and media

Appendix A

Buffers, solutions and media

Bacterial Culture

LB Broth
1% w/v tryptone
0.5% w/v yeast extract
0.5% w/w NaCl

LB Broth Agar
1.5 % Bacto-agar to LB broth

YT Medium
1.6% w/v tryptone
1% w/v yeast extract
0.5% w/v NaCl

Minimal E salts glucose
In 400 mL
0.08 g $MgSO_4.7H_2O$
0.8 g citrate
4 g K_2HPO_4.anhydrous
1.4 g $NaNH_4HPO_4.4H_2O$

M9 Minimal Salts Medium
20% 5 x M9 salts stock solution
0.1 % 1 M $MgSO_4$
0.01 % 1 M $CaCl_2$
0.4% w/v glucose

5x M9 Salts
In IL
64 g $Na_2HPO_4.7H_2O$
15 g K_2HPO_4
2.5 g NaCl
5 g NH_4Cl

Congo red TSB agar plates
3% w/v tryptic soy broth
0.15% 4 M NaOH
1.5% w/v agar
- Autoclave and add 1% filter sterilised 1% w/v Congo red solution

Ampicillin
100 mg/mL stock

- 1 g of ampicillin powder added to 10 mL Milli-Q water. Filter sterilize.
- Store aliquots at –20°C.

Chloramphenicol
30 mg/mL stock

- 300 mg of chloramphenicol powder added to 10 mL ethanol.
- Protect from light.
- Store aliquots at –20°C.

Plasmid Miniprep

Solution I
50 mM glucose
25 mM Tris.Cl (pH 8.0)
10 mM EDTA (pH 8.0)

- autoclave, store at 4°C.

Solution II
0.2 M NaOH
1% w/v SDS

- make fresh.

Solution III
3 M CH_3COOK
2 M glacial acetic acid

- autoclave, store at 4°C.

Caesium Chloride Maxiprep

Solution I
50 mM glucose
20 mM Tris.HCl (pH 8.0)
10 mM EDTA

- autoclave, store at 4°C.

Solution II
0.2 M NaOH
1% w/v SDS

- make fresh.

Solution III
3 M CH_3COOK
2 M acetic acid

- autoclave, store at 4°C.

TE Buffer
100×
10 mM Tris.HCl (pH 7.5)
1 mM EDTA (pH 8.0)

- pH to 8.0, autoclave.
- dilute to 1× in autoclaved Milli-Q.

Chromosomal Preparation

TES Buffer
50 mM Tris.HCl (pH 8.0)
5 mM EDTA (pH 9.0)
50 mM NaCl.

- autoclave.

Lysis Buffer
5% w/v SDS
50 mM Tris.HCl (pH 8.0)
62 mM EDTA

- autoclave.

Gel Electrophoresis

0.5 × TBE
45 mM Tris.HCl
45 mM boric acid
1 mM Na_2EDTA

Southern Hybridisation

20×SSC
3 M NaCl
0.3 M tri-sodium citrate

- autoclave.

20×SSPE
3.6 M NaCl
0.2 M NaH_2PO_4
0.02 M EDTA (pH 7.7)

- pH to 7.4 and autoclave.

Denaturing Solution
1.5 M NaCl
0.5 M NaOH

Neutralising Solution
1.5 M NaCl
0.5 M Tris.HCl
1 mM Na_2EDTA

Pre-hybridisation Solution
5× SSPE
5× Dendhardts Solution
0.5% w/v SDS
- make up fresh.

Total Protein Extraction

Wash Buffer
68 mM NaCl
3 mM KCl
1.5 mM KH_2PO_4
9 mM NaH_2PO_4
- autoclaved and stored at 4°C.

Lysis Buffer
8 M urea
4% w/v CHAPS
1% w/v DTT
0.8% 3/10 ampholytes
35 mM trizma
- store at - 20 °C.

Membrane Protein Extraction

Wash Buffer
68 mM NaCl
3 mM KCl
1.5 mM KH_2PO_4
9 mM NaH_2PO_4
- autoclaved and stored at 4°C.

Solubilisation Buffer

2% w/v ASB-14
1% w/v DTT
7 M urea
2 M thiourea
0.5% 3/10 ampholytes
- store at -20 °C

Two Dimensional Analysis

Rehydration Solution
8 M urea
0.5% w/v CHAPS
0.2% w/v DTT
0.52% 3/10 ampholytes
1% bromophenol blue
- filtered and stored at – 80 °.

Equilibration Solution I
40% v/v glycerol
0.05 M Tris-HCl pH 6.8
6 M urea
2% w/v SDS
2% w/v DTT

Equilibration Solution II
40% v/v glycerol
0.05 M Tris-HCl pH 6.8
6 M urea
2% w/v SDS
2% w/v iodoacetamide
0.005% bromophenol blue

Coomassie Blue G250 Stain
0.1% Coomassie blue G250
20% methanol
10% ammonium sulfate in 2% phosphoric acid

Continuous Blotting Solution
0.04 M glycine
0.05 M Tris base
0.04 % w/v SDS
20% methanol
- make fresh

Amido Black Stain
0.1% w/v amido black
10% acetic acid
10% methanol

Destain Solution
10% acetic acid
10% methanol

Stripping Buffer
2% w/v SDS
0.67% 2-me
67 mM Tris pH 6.7

<u>One Dimensional SDS-PAGE Gels</u>

2 x Loading Buffer
100 mM Tris-HCl [pH 6.8]
4% w/v SDS
0.2% glycerol
2.5% β-mercaptoethanol
0.2% bromophenol blue
- stored at –20 ° C

Running Buffer
1.92 M glycine
0.25 M Tris base
1% w/v SDS

Transfer Buffer
50 mM Tris base
384 mM glycine
0.1% w/v SDS
20% methanol
- make fresh

<u>Cell Culture</u>

PBS
10×
8% w/v NaCl
0.2% w/v KCl
1.44% w/v Na_2HPO4
0.24% w/v KH_2PO_4
- pH to 7.4, autoclave.

Plaque Assay

Overlay Medium
5% FBS
20 μg/mL gentamycin
0.5% w/v agarose
0.45% w/v glucose

- make to volume with cell culture medium at 37°C. Use immediately.

ELISA

Carbonate Coating Buffer
50 mM Na_2CO_3

- pH to 9.6.

Phosphate Citrate Buffer
2.55g citric acid
3.65g Na_2HPO_4

- make up to 500mL with Milli-Q water and autoclave.

Colour Reagent
10 mL phosphate-citrate buffer
4 mg o-phenylenediamine
8 μL H_2O_2

Appendix B
Sequences

Appendix B

Sequences

Figure B.1. Forward sequence of *S3195* disruption from SFL1727, PCR amplified with OMFPcompF and OMFPcompR and sequenced with the OMFPcompF primer. The yellow shaded sequence has 100% homology to *S3195*, the blue shaded sequence matches pKD3 sequence beginning with the underlined Primer 1 sequence.

```
  1  TAATCTGGAATCCTGGCTACAGGCTGGTATGGAATCACATTACGGGCGCTTTCGTGGTTG
 61  CCTCCGAACTGGCCCGCGCACAGGGTAAACGTGGCGGTGTGGCGGTTGCACTGTCTCTTG
121  CCGTGTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAA
181  TAGGAACTTCATTTAAATGGCGCGCCTTACGCCCCGCCCTGCCACTCATCGCAGTACTGT
241  TGTAATTCATTAAGCATTCTGCCGACATGGAAGCCATCACAAACGGCATGATGAACCTGA
301  ATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATAATATTTGCCCATGGTGAAAACG
361  GGGGCGAAGAAGTTGTCCATATTGGCCACGTTTAAATCACAACTGGTGAAACTCACCCAG
421  GGATTGGCTGAGACGAAAAACATATTCTCAATAAACCCTTTAGGGAAATAGGCCAGGTT
```

Figure B.2. Reverse sequence of *S3195* disruption from SFL1727, PCR amplified with OMFPcompF and OMFPcompR and sequenced with the OMFPcompR primer. The yellow shaded sequence has 100% homology to *S3195*, the blue shaded sequence matches pKD3 sequence beginning with the underlined Primer 2 sequence.

```
  1  TGGGCGGGTTTTGGTGCCGGGTGACGGTTGCAGCTGTACTGGTAACCAGTGCACCGCCCG
 61  TGTTCTGGGTGACATCACACTTTATTTACCGCCGGCATTCACCTGCAGCTTTCCTTTCTG
121  CGTTGATGGTGGTATGCGCGGCAGTTCCTCCTTCCTTAATCACCTGCCAGCCATCACGGT
181  TTATCAGCGTCTCTGTTGCCGTGCCACCGTTGTGTACATACTGGTTACCCCCCTCCAGTC
241  GGGTGTTATGTGCCTCTCCGTGTACCGTCTGGTCCATATGAATATCCTCCTTAGTTCCTA
301  TTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCGGCGCGCCTACCTGTGACGGAA
361  GATCACTTCGCAGAATAAATAAATCCTGGTGTCCCTGTTGATACCGGGAAGCCCTGGGCC
421  AACTTTTGGCGAAAATGAGACGTTGATCGGCACGTAAGAGGTTCCAACTTTCACCATAAT
481  GAAATAAGATCACTACCGGGCGTATTTTTTGAGCTGTCGAGATTTTCATGAGCTAAGGAA
541  GCTAAAATGGAGAAAAAAATCACTGGATATACCACCGTTGATATATCCCCAATGGCATCG
601  TAAAAGAACATTTTTGAGGCATTTCAGTCAGTTGCTCAATGTACCCTATAACCCAGACCG
661  TTCAGCTGGGATATTTACGGCCTTTTTTAAAGACCATAAAAGAAAAATAAGCACAGTTTAA
721  TCCGGCCTTTATTCAC
```

Figure B.3. Forward sequence of *udp* disruption from SFL1726, PCR amplified with UdpcompF and UdpcompR and sequenced with the UdpcompF primer. The yellow shaded sequence has 100% homology to *udp*, the blue shaded sequence matches pKD3 sequence beginning with the underlined Primer 1 sequence.

```
   1  CAAAGTTGGTTTTTTGTCCCCAAGTCTGATGTTTTTCATCTCGGCCTCACTAAAAACGAT
  61  TTACAAGGGGCTACGCTTGCCTTCGTCCCTGGCGACCCGGATCGTGTGGAAAAGATCGCC
 121  CCGCTGATGGATAAGCCGGTTAAGCTGGCATCTCACCGCGAATTCACTACCTGGCGTGCA
 181  GAGCTGGATGGTAAACCTGTTATCGTCTGCTCTACGTGTAGGCTGGAGCTGCTTCGAAGT
 241  TCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCACTTAAATGGCGCGCCT
 301  TACGCCCCGCCCTGCCACTCATCGCAGTACTGTTGTAATTCATTAAGCATTCTGCCGACA
 361  TGGAAGCCATCACAAACGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCG
 421  CCTTGCGTATAATATTTGCCCATGGTGAAAACGGGGGCGAAGAAGTTGTCCATATTGGCC
 481  ACGTTTAAATCAAAACTGGTGAAACTCACCCAGGGATTGGCTGAGACGAAAAACATATTC
 541  TCAATAAACCCTTTAGGGAAATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAA
 601  TATATGTGTAGAAACTGCCGGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACGTT
 661  TCAGTTTGCTCATGGAAAACGGTGTAACAAGGGTGAACACTATCCCATATCACCAGCTCA
 721  CCGTCTTTCATTGCCATACGTAATTCCGGATGAGCATTCATCAAGCGGGCAAGAATGTGA
 781  ATAAAGGCCGGATAAAACTTGTGCTTATTTTTCTTACGGTCTTAAAAGGCCGTAATATCC
 841  AGCTGAACGGTCTGTATAGGTACATTGAGCAACTGACTGAAATGCTCAAATGTTCTTTAC
 901  GATGCCATTGGAATATCAACGTGGATATCCAGGAATTTTTTCTCATTTAAGCTCCTAGCT
 961  CCTGAAATCTCGACAACTCAAAAAATACGCCGGTAGGATCTTATTCATATGGTGAAGTGA
1021  ACTCTACGTGCGATCAACGTCCAATTCGCAAAGTGCCAGGCTCCCGATCAAAGAACCAGA
1081  ATTTTATTCTGGAAAGGACTTCGTCCAAGGAGGGCCGCGAAAGTCTTAACTTCTAAGAAA
1141  TAGGAACTTCGAAATAGAACCTAGGGAATTCCTATGCCTTGAAAGGGAAATTCCGAACTT
1201  TCTGCTGAGACAACTTGCGTGGCGGGCAAGACACG
```

Figure B.4. Reverse sequence of *udp* disruption from SFL1727, PCR amplified with UdpcompF and UdpcompR and sequenced with the UdpcompR primer. The yellow shaded sequence has 100% homology to *udp*, the blue shaded sequence matches pKD3 sequence beginning with the underlined Primer 2 sequence.

```
   1  CCCATTCCCCCCCCTTTATAAGGCCGGAACGCTGTTCGGCCTTCAGACAGGAGAAGAGAA
  61  TTACAGCAGACGACGCGCCGCTTCCACCACGATTTTCACCGCATGGCTTTCGGTTTGTTT
 121  CATCGTCTCAGCATTCGGGATCTCTTGCTGGGTGCGGTTAACGATAACACCCGCTACCAT
 181  ACCGGCACGCAGGCCCTGACTTGCACACATGGTCAGCAGAGTTCGAGATTCCATTTCATA
 241  GCATATGAATATCCTCCTTAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGA
 301  ACTTCGGCGCGCCTACCTGTGACGGAAGATCACTTCGCAGAATAAATAAATCCTGGTGTC
 361  CCTGTTGATACCGGGAAGCCCTGGGCCAACTTTTGGCGAAAATGAGACGTTGATCGGCAC
 421  GTAAGAGGTTCCAACTTTCACCATAATGAAATAAGATCACTACCGGGCGTATTTTTTGAG
 481  TTGTCGAGATTTTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAAATCACTGGATATACC
 541  ACCGTTGATATATCCCAATGGCATCGTAAAGAACATTTTGAGGCATTTCAGTCAGTTGCT
 601  CAATGTACCTATAACCAGACCGTTCAGCTGGATATTACGGCCTTTTTAAAGACCGTAAAG
 661  AAAAATAAGCACAAGTTTTATCCGGCCTTTATTCACATTCTTGCCCGCCTGATGAATGCT
 721  CATCCGGATTACGTATGGCAATGAAAGACGGTGAGCTGGTGATTTGGGATAGTGTTCACC
 781  CTTGTTACACCGTTTTCCATGAGCAAACTGAAACGTTTTCATCGCTCTGGAGTGATACCA
 841  CGACGATTTCCGGCAGTTCTAACATATATCGCAAGATGTGGCGTGTACCGTGAAAACCTG
 901  GCCTATTTCCTTAAGGGTTAATGAAAAATGGTTTTCGTCCAGCCATTCCTGGGTGAGTTT
 961  CACGGTTTTGATTAAACGTGCAATATGACACTTCTTCGCCCCGTTTCACCATGGGCAAAT
1021  TTATCCCAGGCACAAGGGGTGATGCCGCTGGGATTCAGTTCTACTGCCTGTTGAAGGCTT
1081  CTTGCGGGAAGTTTATGATATTCACACGACTTCGAATAAAGGAAGGGCGGGGGTAAGGCG
1141  CCCTATTAGGGAGAGTCTCTTATCCCAAGTTCCTTTCTTCTAAAAAGTATGGAACTCTGG
1201  AACGGCTCGCGCGTCGCGGTAGCGAGACAGTATACCCGCCGTTTCACCTCCACGTGCTCG
1261  CGAGCCTCACC
```

Figure B.5. Forward sequence of *gadB* disruption from SFL1650, PCR amplified with gadBcompF and gadBcompR and sequenced with gadBcompF. The yellow shaded

sequence has 100% homology to *gadB*, the blue shaded sequence matches pKD3 sequence beginning with the underlined Primer 2 sequence.

```
1      TATCGGTTTAACGCATGCATAGATCGCTTTAAACGATTTAAAAGGTATGTTTAAAGCTGT
61     TCTGCTGGGCAATACCCTGCAGTTTCGGGTGATCGCTGAGATATTTCAGGGAGGCTTTGT
121    ACATATGAATATCCTCCTTAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGA
181    ACTTCGGCGCGCCTACCTGTGACGGAAGATCACTTCGCAGAATAAATAAATCCTGGTGTC
241    CCTGTTGATACCGGGAAGCCCTGGGCCAACTTTTGGCGAAAATGAGACGTTGATCGGCAC
301    GTAAGAGGTTCCAACTTTCACCATAATGAAATAAGATCACTACCGGGCGTATTTTTTGAG
361    TTGTCGAGATTTTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAAATCACTGGATATACC
421    ACCGTTGATATATCCCAATGGCATCGTAAAGAACATTTTGAGGCATTTCAGTCAGTTGCT
```

Figure B.6. Reverse sequence of *gadB* disruption from SFL1650, PCR amplified with gadBcompF and gadBcompR and sequenced with gadBcompR. The yellow shaded sequence has 100% homology to *gadB*, the blue shaded sequence matches pKD3 sequence beginning with the underlined Primer 1 sequence.

```
  1    TTATTAATTTTTGCTTTTTTTATGAAAAAATATCATTCCAACAATATCTCACGATAAATAA
 61    CATTAGGATTTTGTTATTTAAACACGAGTCCTTTGCACTTGCTTACTTTATCGATAAATC
121    CTACTTTTTTAATGCGATCCAATCATTTTAAGGAGTTTAAAATGGATAAGAAGCAAGTAA
181    CGGATTTAAGGTCGGAACTACTCGATTCACGTTTTGGTGCGAAGTCTATTTCCACTATCG
241    CAGAATCAAAACGTTTTCCGCTGCACGAAATGCGCGACGATGTCGCATTTCAGATTATCA
301    ATGATGAATTATATCTTGAGTGTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGA
361    GAATAGGAACTTCGGAATAGGAACTTCATTTAAATGGCGCGCCTTACGCCCCGCCCTGCC
421    ACTCATCGCAGTACTGTTGTAATTCATTAAGCATTCTGCCGACATGGAAGCCATCACAAA
481   CGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATAA
```

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