Recombinant antibodies against *Clostridium difficile* Toxin A

College of Medical Veterinary and Life Sciences
School of Life Sciences

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Sabir M.Shakir Almdni

Supervisor: Dr R. Aitken

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Abstract

Clostridium difficile is a major cause of nosocomial intestinal infection. The pathogen possesses two potent toxins, Toxin A and Toxin B, both of which contribute to diarrhoea, intestinal inflammation and tissue damage. Antibiotics are effective against the disease, however around 20 % of patients on treatment relapse after the termination of antibiotic therapy. The binding of Toxin A to a receptor on human intestinal epithelial cells initiates disease: this is considered the starting point from which the toxin elicits its effect. One feature of the carboxy-terminal domain of Toxin A is the presence of repeating units of amino acids that form a series of binding sites able to recognise disaccharides and trisaccharides on glycolipid and glycoprotein receptor molecules. Antibody response against the toxin can protect against *C. difficile* disease and efforts to generate vaccines have focused upon the carboxy-terminal, receptor binding domain.

The aims of this project were to use phage display to isolate recombinant antibodies against those features of the carboxy-terminal domain of Toxin A thought to be responsible for receptor-binding and to assess if the antibodies were capable protecting against the action of Toxin A.

Using published crystallographic data that has shown the interaction of Toxin A and trisaccharide, a region of about 113 amino acids from the carboxy-terminal region of Toxin A was expressed as a fusion to maltose-binding protein. The MBP fusion protein was expressed, purified on amylose resin, and characterised. The fusion protein was then used to isolate single chain antibodies from the Tomlinson libraries of scFvs, a synthetically diversified

phage display library of single scaffold human antibodies. Conventional bio-panning methods were used in which the MBP fusion protein was bound to a plastic surface and the phage display libraries were pre-mixed with native MBP to inhibit the isolation of anti-MBP antibodies. Progressive enrichment of scFvs through 3 rounds of selection was observed. Those scFvs that showed strongest reaction against the target protein in ELISA but failed to react with native MBP were sequenced, expressed as soluble antibodies and purified on nickel chelating columns. While the resulting panel of scFvs showed similarities of sequence, none were identical. All were reactive with native, full-length Toxin A and appeared to bind to conformational (nonlinear) epitopes. Cross-reaction with Toxin B from *C. difficile* was also evident. A panel of truncation mutants were generated from the MBP fusion protein and using these in ELISA with the scFvs, reactivity appeared to be directed to features of a long repeat sequence of Toxin A.

To assay whether the isolated scFvs possessed biological activity of significance, *in vitro* and *in vivo* protection assays were established. For experiments *in vitro*, the action of Toxin A upon cultured Vero cells was studied. Native Toxin A triggered a conversion of the cells from stellate to rounded morphology. When cells were exposed to 100 ng of toxin, this effect was evident within 60 minutes; at a 10-fold lower dose, the minimum quantity to which a response was detectable, virtually all cells had undergone rounding within 2 h. When individual scFvs were mixed with 10 ng of Toxin A prior to addition to Vero cells, there was a consistent delay in cytopathic activity that extended to 5 h. In this assay, the percentage of cells that had retained their stellate morphology 5 h post-challenge was dependent on the scFv used. To quantify the potency of this neutralising activity, the amount of each scFv required to achieve 50%

protection during a 2 h challenge period was established. This revealed 3.5-fold difference between the most and the least effefctive scFv. The most potent scFv was used in an *in vivo* assay in which Toxin A was administered to the ligated intestinal loops of rats. Again, protective activity was evident.

Overall, phage display technology enabled the assembly of a panel of scFv antibodies against the putative receptor binding site in the carboxy-terminal domain of Toxin A from *C. difficile*. The scFvs were able to protect against the cytopathic activity of Toxin A *in vitro* and *in vivo* and proposals are made about how these observations could be taken forward in a model of *C. difficile* infection that best mimics the human disease.

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Author’s declaration

This thesis is the original work of the author except where otherwise stated.

Sabir M.Shakir Almdni

Abbreviations

BoNT: Botulinum neurotoxin

bp: Base pair

BSA: Bovine serum albumin

CDAD: *C. difficile* associated disease

CDR: Complementarity determining region

Cfu: Colony forming units

CROPS: Combined repetitive oligopeptides

DNA: Deoxyribonucleic acid

dNTP: Deoxyribonucleotide triphosphate

EDTA: Ethylenediaminetetraacetic acid

EF: Edema factor

FHC: *Clostridium botulinum* neurotoxin F

g: Gram

h: Hour

HRP: Horseradish peroxidase

IPTG: Isopropyl-ß-D-thiogalactopyranoside

kb: Kilobase

kDa: kilodalton

LE: Lethal factor

LR: Long repeat

M: Molar

MBP: Maltose binding protein

mg: Milligram(s)

min: Minute(s)

ml: Millilitre

mM: Millimolar

OD: Optical density

PA: Protictive antigen

PBS: phosphate buffered saline

PAGE: polyacrylamide gel electrophoresis

PCR: polymerase chain reaction

SDS: sodium dodecyl sulphate

Sec: Second

scFv: single chain antibody fragment

SR: Short repeat

TMB: Trimethyl borate

µl: Micro litre

Chapter One Introduction

1.1 Overview

Clostridium difficile is a gram-positive, anaerobic, spore-forming bacillus that is responsible for the development of antibiotic-related diarrhoea and colitis (Bartlett, 1994, Kelly et al., 1994 A, Rupnik et al., 2009).

C. difficile was first described in 1935 (Hall and O'toole, 1935) as a component of the faecal flora of healthy newborns. Initially, the organism was not thought to be pathogenic and was named "difficile" because of its slow growth and difficulty in culture. Even though early investigators noted that the bacterium produced a potent toxin, the role of *C. difficile* in antibiotic-associated diarrhoea and pseudomembranous colitis was not described until the 1970s. (Bartlett et al., 1977, Bartlett et al., 1978, Larson et al., 1977)

C. difficile is the now the most common cause of hospital-acquired antibiotic-related diarrhoea in many countries and is a major cause of morbidity and mortality, particularly in elderly patients on broad-spectrum antibiotic therapy (Kelly and LaMont, 1998). This pathogen is responsible of about one fifth (20%) of the cases of antibiotic associated diarrhea, three quarters of antibiotic associated colitis, and more importantly almost all the cases of life threatening pseudomembranous colitis (Bartlett, 1994). There are estimated to be more than three millions cases annually of pseudomembranous colitis in the United States of America alone (Bartlett, 1992, Mylonakis et al., 2001) which costs the health care more than $1.1 billion (Kyne et al., 2002) *C. difficile*-associated disease

(CDAD) causes noticeable morbidity, increased financial burdens on hospitals and prolonged hospitalisation for patients (Silva, 1989).

Symptoms may range from mild dairrhoea, low grade fever and leukocytosis to a very serious disease associated with fulminant colitis leading to a high mortality rate (Wilcox et al., 2003). The reasons for such variation in symptoms or asymptomatic infection are not fully clarified but it is thought that mild symptoms of CDAD are associated with elevated serum titers of IgG against Toxin A, one of the important virulence factors (Warny et al., 1994, Kyne et al., 2001). Reports from England and Wales showed a six-fold increase in *C. difficile* infection in the three years starting from 1990 (Settle and Wilcox, 1996). Several possible explanations for this include the development of better methods for detection of the disease, high frequency use of antibiotics that promote CDAD and increasing contamination of hospital environments with the spores of *C. difficile* (Voth and Ballard, 2005).

Since 2001, a hypervirulent clone III NAP/ 027 has emerged that produces elevated levels of toxin and is antibiotic-resistant and this is now widely isolated (Pepin et al., 2005 A, Loo et al., 2005). Hypervirulent strains are able to cause higher severity of disease, recurrence, morbidity and mortality and has caused prominent outbreaks in North America and Europe (Warny et al., 2005, Greco et al., 2006). The pathogen is a very persistent nosocomial pathogen and its transmission in hospital wards and staff accommodation is associated with contamination with the spores (Bartlett, 1981). In some cases wards or even hospital closure are essential for the control of the infection (Cartmill et al., 1994). With the increased use of broad-spectrum antibiotics such as amoxicillin,

cephalosporins and clindamycin during the last three decades, CDAD has become a foremost clinical problem (Bartlett, 1981).

In contrast to several other bacterial infections, exposure to antibiotic is essential for *C. difficile* to exert its effect. Almost all antibiotics, together with vancomycin (Hecht and Olinger, 1989) and cancer chemotheraputics are able to encourage CDAD (Anand and Glatt, 1993). To combat the infection, most patients respond well initially to treatment with metronidazole and vancomycin but relapse remains common. Alternative treatments, including good structural data on some of the most important virulence factors are beginning to show promise (Greco et al., 2006). Even so, antibiotics remain one of the very few treatment options for CDAD. With the persistent increase in antibiotic resistance, the development of novel non-antibiotic tools that target important virulence factors like the major protein toxins are extremely advantageous (Bebbington and Yarranton, 2008, Rasko and Sperandio, 2010).

1.2 Pathogenesis

The pathogenicity of *C. difficile* in mammals was first described in germ-free rat that developed transient diarrhea after inoculation (Hammarstrom 1969 (by Sten Hammarstorm; Peter Perlmann; Bengt E. Gustafsson and Rutger Lagercrantz (*from the Wenner-Gren institute for Experimental Biology, University of Stockholm, and the Department of Germfreee research, Karolinska Institute, Stockholm, Sweden*)). The pathogen in its vegetative state dies rapidly when exposed to air but the spores can survive up to 5 months in the environment (Yassin et al., 2001) and can withstand drying, heat, and many disinfectants (Wilcox et al., 2003).

The pathogen exerts its pathological effects by colonising luminal surfaces of the colon and secreting two high molecular weight exotoxins, Toxin A (TcdA) and Toxin B (TcdB) (Lyerly et al., 1985, Kim et al., 1987, Babcock et al., 2006, Lyras et al., 2009). Adherence of the pathogen to the intestinal epithelium via surface layer proteins is believed to be a crucial stage in gut colonisation (Calabi et al., 2002) but rather less is known about this process than the mode of action of TcdA and TcdB.

Figures 1.1 and 1.2 show schematic representations of these virulence factors. TcdA and TcdB can be divided into three functional regions as shown – regions that carry enzymic activity, the capacity for translocating part of the toxin to the cytsol of the target cell, and receptor-binding. The latter features possess multiple peptide motifs that form a series of carbohydrate binding sites. The structural genes are carried in a pathogenicity locus that includes regulatory functions and the gene for a holin, required for efficient release of the toxins from the bacterial cell (Figure 1.2).

TcdA and TcdB differ in length, Toxin A being 2710 amino acids long whereas Toxin B is somewhat shorter at 2366 amino acids. They share a common mode of action being monoglucosytransferases, and sequence alignment reveals 48% identity and 68% similarity with repeating motifs evident in their carboxy terminal regions (Figures 1.1, 1.2, 1.3).

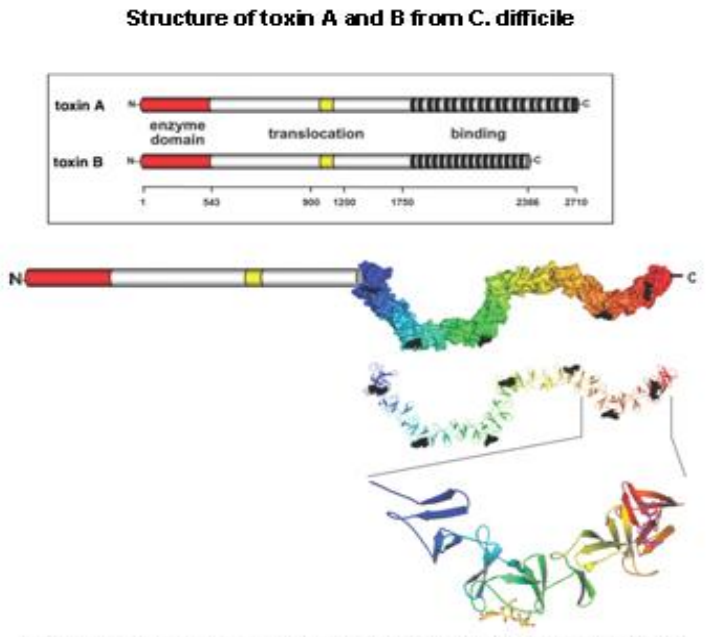

Figure 1-1 Schematic representation of TcdA and TcdB structure
The three dimensional representation of a toxin protein presents receptor carbohydrates as black or ball and stick structures.

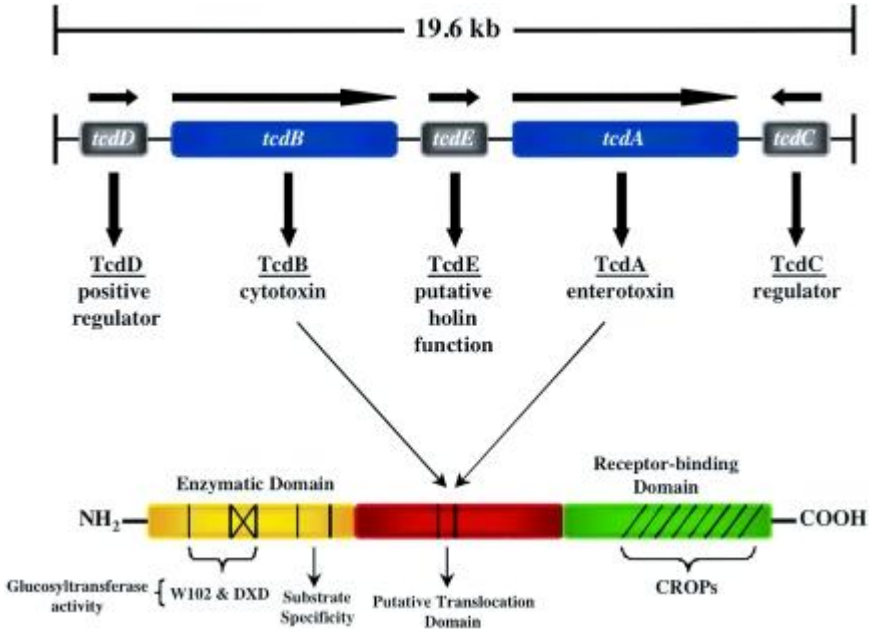

Figure 1-2 Schematic genetic representation of TcdA and TcdB

Studies show that quorum sensing molecules plays an important role in transcriptional regulation of toxin production (Lee and Song, 2005). Both toxins are responsible for the virulence that is associated with tissue damage. Toxin A is the primary mediator of intestinal tissue damage (Lyerly et al., 1982, Lyerly et al., 1985). This is a potent enterotoxin with slight cytotoxic activity, while Toxin B is an extremely potent cytotoxin. Toxins A and B possess glycosyltransferase activity and cause an extensive amount of tissue damage to the gut mucosa (Faust et al., 1998).

Translocation of the toxins across the cell membrane is a prerequisite for their actions because their substrates – the small GTPases Rho, Rac and Cdc42 - are intracellular (von Eichel-Streiber et al., 1996). These undergo monoglucosylation at threonine 37 and threonine 35, respectively. GTPases are key cellular regulatory proteins and irreversible inactivation as a consequence of glucosylation disrupts important cell signalling pathways that are essential for transcriptional regulation, apoptosis, cytoskeleton integrity and finally the barrier function of the colonic epithelium (Jank et al., 2007 A).

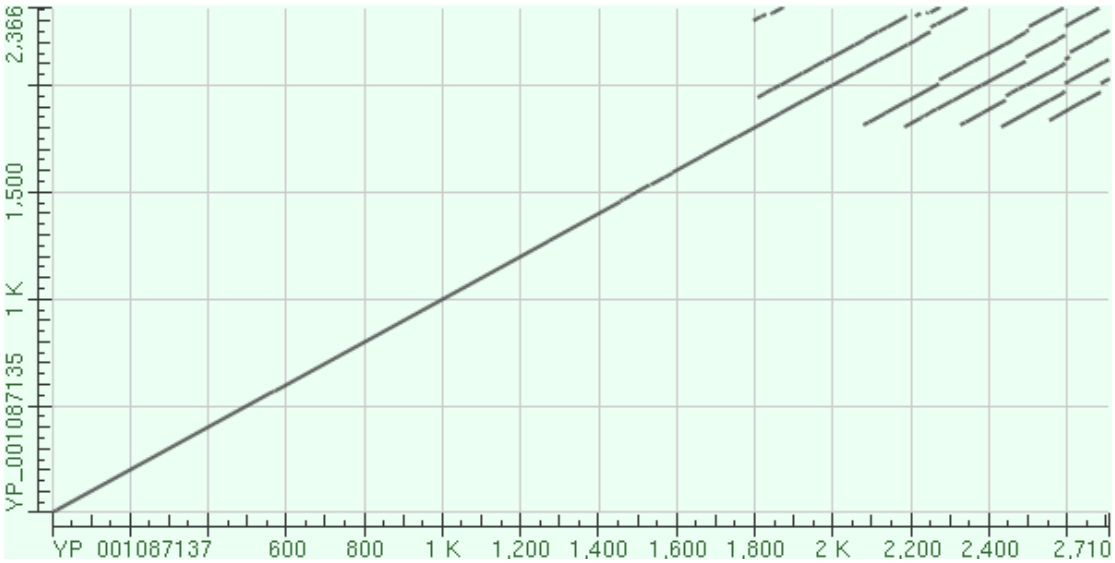

Figure 1-3 Alignment of the protein sequences of TcdA and TcdB
Protein sequences (TcdA, NCBI accession number YP_001087137, x axis; TcdB, NCBI accession number YP_001087135, y axis; all sequences from C. difficile strain 630) were aligned by pairwise BLAST using the BLOSUM80 matrix with an expected threshold setting of 10 and a wordsize of 3. The dotplot identifies sequence identities and similarities summing to 48% and 66% respectively with repetitive peptide motifs evident in both proteins at their carboxy termini (top right of the dotplot).

Toxins that act upon intracellular targets generally follow a four-phase process: (a) binding to the cell surface and internalisation by endocytosis (Papatheodorou et al., 2010) or some other pathway; (b) intra-vesicular processing followed by translocation of (at minimum) the catalytic domain across the vesicular membrane (Giesemann et al., 2008); (c) enzymatic modification of an intracellular target(s) (Reineke et al., 2007); (d) local and systemic changes arising from damage to the target molecule(s) (Schroeder, 2005).

Toxin A does not appear to be dependent upon the action of other virulence factors (Bartlett, 1994) which gives it the flexibility to attack damaged or intact tissues. Reports propose that Toxin B has only modest effects on the host when administered orally to experimental animals in the absence of Toxin A, and lethal effects only become apparent after Toxin A has acted upon the intestine (von Eichel-Streiber et al., 1996). As Toxin A triggers cell retraction, rounding and shrinkage (Hecht et al., 1988) as a result of the loss of filamentous F-actin and the accumulation of G-actin (Dillon et al., 1995, Pothoulakis and Lamont, 2001), tight junctions are disrupted along with the barrier function of the epithelium (Chang et al., 1979, Fiorentini et al., 1990) and leading to the typical diarrhea that characterises CDAD (Poxton et al., 2001). Loss in integrity of the epithelium provides access for Toxin B to the basolateral surfaces of epithelial cells, thereby adding to pathological change. The fluid exudate in the intestine consists of serum and cellular components that reflect a strong inflammatory response, an element that is not seen in other enterotoxigenic infections (Tucker et al., 1990). For example, Toxin A-induced fluid secretion is accompanied with epithelial cell damage and neutrophil infiltration, unlike cholera toxin and the *E. coli* enterotoxins which trigger intestinal secretion without causing intestinal infiltration (Pothoulakis and Lamont, 2001).

While these changes lead to diarrhoea, inflammation and the neutrophil infiltration that accompanies it is critical in the development of pseudomembranous colitis. Neutrophil infiltration is thought to take place following to the loss of epithelial integrity. Direct neutrophil-Toxin A interaction was reported as an essential step for neutrophil infiltration (Kelly et al., 1994 A).

There is also evidence that sensory neurons are regulated by Toxin A. This was supported by studies of substance P, an immunoreactive peptide that is linked to sensory neurons in the epithelium (Mantyh et al., 1996). Substance P is thought to activate neutrophils via the activation of macrophages and the release of TNF-α. Substance P was also shown to mediate mast cells activation and degranulation. Hence, it may be also be involved in the pathogenesis of *C. difficile*-induced colonic inflammation.

Triggering of apoptosis is another important effect of TcdA on intestinal cells, mast cells, and endothelial cells (Mahida et al., 1996, Alcantara et al., 2001, Carneiro et al., 2006). Though apoptosis is an essential natural process that corrects cell populations by managing growth and death, it can be triggered by extracellular factors leading to damage and dysfunction of tissues. This damage is noticed in the form of cell shrinkage, damage nucleic acids and paradoxically, impaired inflammation activity(Kroemer et al., 1995). Caspase-3 is involved in TcdA-induced apoptosis (Carneiro et al., 2006, Solomon et al., 2005) as is activation of caspase-6 (Carneiro et al., 2006). The originator caspase-8 represents the extrinsic pathway for apoptosis that can be activated by transmembrane death receptors such as the Fas receptor. Caspase-9, representing the intrinsic pathway, is activated by an apoptosome containing cytochrome *c* released from damaged mitochondria (Boatright and Salvesen, 2003). Both initiator caspase-8 and caspase-9 are known to cleave procaspase-3 into the activated caspase-3. Cytochrome c is released from mitochondria in response to treatment with TcdA. As a consequence, caspase-9 becomes activated (Brito et al., 2005, Kim et al., 2005, Le et al., 2005).

The strong inflammatory response seen during *C .difficile* infection is linked to the production of IL-6, IL-8, IL-1ß and TNF-α by macrophages (Flegel et al., 1991) that can be activated by exposure to Toxins A and B (Linevsky et al., 1997). These factors up-regulate the expression of neutrophil and endothelial adhesion receptors, guide neutrophils to the vascular endothelium, promote diapedesis and tissue infiltration (Strieter et al., 1994). This provides a link between macrophage activation and neutrophil infiltration in the pathogenesis of *C. difficile* (Linevsky et al., 1997). The damaging effect of Toxin A in the intestine can be minimised by inhibition of cytokine synthesis (Triadafilopoulos et al., 1989). This finding shows the role of cytokines as important proinflammatory mediators in the pathophysiology caused by the virulence factors of *C. difficile*. Toxin A can stimulate sensory neurones and elicit degranulation of mast cells causing the production of other inflammatory mediators (Kelly et al., 1994 A). It also stimulates rat intestinal epithelial cells to release macrophage-inflammatory protein-2 (MIP-2). The importance of MIP-2 is that it is a potent neutrophil chemoattractant and leads to neutrophil activation and attraction which as detailed, is a feature of the clinical impact of Toxin A release (Castagliuolo et al., 1998 A). Moreover, it has been reported that Toxin A can act directly as a chemotactic factor for neutrophils and peripheral blood monocytes (Pothoulakis and Karmeli, 1993). Although these cells are unlikely to be exposed directly to significant amounts of the toxin in the peripheral circulation, the situation may be different in areas of colonic damage (Linevsky et al., 1997). Inflammation may also be exacerbated by down regulation of other activities. For example one study has reported that Toxin A leads to activation of adenosine, an enzyme that can down-regulate immune responses by acting as an anti-inflammatory agent (Cavalcante et al., 2006).

These findings show how the pathogenesis of *C. difficile* enterocolitis is influenced by neutrophils (Pothoulakis, 1996), the inhibition of Rho GTPases and apoptotic pathways (Gerhard et al., 2008).

While the importance of Toxins A and B is undisputed, it has been repeatedly reported that pseudomembranous colitis and nosocomial CDAD can be caused by Toxin A negative, Toxin B positive strains (A^-/B^+) (Kato et al., 1998, Limaye et al., 2000, Johnson et al., 2001). These strains were distinguished by deletions in *tcdA* (Sambol et al., 2000). It is possible that these deletions create variants of the toxin with equivalent potential to cause disease initiation and the pathogenecity of A^-/B^+ strains was very similar to A^+/B^+ when measured by fluid secretion in rabbit intestinal loops (Borriello et al., 1992). These variants were found to be comparable in potency to *C. sordellii* where the lethal toxin contributes to disease (Drudy et al., 2007, Soehn et al., 1998). Several outbreaks with A^-/B^+ *C. difficile* have been documented. Alfa and colleagues (Alfa et al., 2000) reported an outbreak in a Canadian hospital in 1998 that continued for a period of three months. The diagnostic protocol was changed from an ELISA to detect Toxin A to a cell cytotoxicity assay as the first attempts failed to detect the A^-/B^+ strains. A second outbreak of A^-/B^+ was reported in the Netherlands between 1997-1998. The introduction of strategic infection control protocol plus the substitution of clindamycin as a modification of the surgical prophylactic antibiotic policy, resulted in the outbreak subsiding (Kuijper et al., 2001). Sato et al (Sato H and Kato H, 2004) reported a third outbreak of A^-/B^+ in 10 patients in a cancer hospital in Japan. Drudy et al (Drudy et al., 2007) reported a fourth outbreak of A^-/B^+ that resulted in 73 cases in one Dublin hospital.

Investigators in Korea have screened samples of loose or watery stools from patients showing symptoms of CDAD, accumulating samples in 2004 and 2005. The group reported about 40% of strains of *C. difficile* were A^-/B^+ and about 50% were A^+/B^+ suggesting the frequency of the unusual phenotype may be high. Samples were characterised by PCR (Shin et al., 2008). In addition, Kim and colleagues screened 462 Korean *C. difficile* isolates and found 77.5% B^+ while 21.4% were A^- and A^+/B^+ were found in only 9 cases (Kim et al., 2008).

Interestingly, Marwah and colleagues have reported A^-/B^+ of ribotype 017 in Scotland (Bakri et al., 2009).

In contrast, Sambol and colleagues 2000 (Sambol et al., 2000) reported as few as 7 isolates from 5000 as A^-/B^+, a much lower frequency than documented in some studies. This is consistent with findings mentioned earlier that in the absence of Toxin A, Toxin B has has relatively modest effects on the intestine. These contradictory findings suggest that there is a very significant variation in the occurrence of A^-/B^+ in different parts of the world. These findings raise some interesting questions; in the absence of Toxin A, does Toxin B play a more active role than was previously thought and does the loss of Toxin A represent a selection event that takes place within the host (Voth and Ballard, 2005).

1.3 Epidemiology

Infection occurs when a susceptible person ingests *C. difficile* spores. The spores then colonise the large intestine and the production of toxins leads to colitis and diarrhoea. The normal bacterial flora of the intestinal and anti-toxin antibodies developed over time protect the healthy adult (Kyne et al., 2000). The infection is most common in elderly, hospitalised adults exposed to

antibiotics or cancer chemotherapy that either deplete the normal microflora or the patient's immune system respectively. About 70% of healthy newborns and infants around 12-18 months old may be colonised with *C. difficile* without developing clinical symptoms, again illustrating the importance of the normal microflora in preventing the development of disease (Larson et al., 1982, Viscidi et al., 1983) although it is also possible that young children lack the receptors required for toxin binding (Eglow et al., 1992).

Kim and his group (Kim et al., 2008 A) reported 4895 cases of CDAD between 2001 and 2006, in children's hospitals in the USA, a rate of 4.4 to 6.5 per 10,000 patients-days. The median age of the children with CDAD was 4 years.

While *C. difficile* is best-recognised as a nosocomial pathogen, patients in the community are also at risk, albeit at a considerably lower rate than those who are hospitalised. Reports shows that community *C.difficile* infection in the United States was 7.7 cases out of 100,000 people in the year, of which 35% had not received antibiotic therapy within the previous 42 days (Hirschhorn et al., 1994). Recent studies conducted by the Centers for Disease Control and Prevention (CDC) reported similar community rates, but with higher severity of the disease (CDC 2008).

1.4 Risk factor

Several factors may increase the risk of developing CDAD. The key factor is antibiotic therapy with certain agents, coupled with advanced age (more than 65 years), serious underlying illness, an institutional setting and immunodeficiency due to AIDS or chemotherapy (Samore et al., 1994). Use of ampicillin or

amoxicillin, cephalosporins, fluoroquinolones, and clindamycin are most often linked with the infection but almost all antibiotics including metronidazole have been associated with infection. This is despite the observation that many antibiotics show activity against *C .difficile*, at least *in vitro* (Pepin et al., 2005). Cancer chemotherapy may have a similar pre-disposing effect towards infection with *C. difficile* (Larson et al., 1982, Viscidi et al., 1983) and contamination of common services such as hospital facilities, staff accommodation, and rehabilitation facilities are also considered important factors (Pepin et al., 2005). This is may be because of the increased spore concentration from staff and infected persons. Elderly persons are highly susceptible and prone to the development of severe disease (Carignan et al., 2008). Pepin and colleagues have observed that people older than 65 years old have a 10 fold higher risk of developing *C. difficile* during an outbreak than younger people with similar risk factors (Pepin et al., 2005 A).

Ethnicity is an important risk factor related to *C. difficile* infection (Redelings et al., 2007) and inflammatory bowel disease is the most common superimposed infection (Mylonaki et al., 2004, Issa et al., 2008).

1.4.1 Toxin function

Toxin A is an intracellular toxin with effects that arise from the binding of UDP-glucose and glucosylation of the small GTP-binding proteins Rho, Rac, and Cdc42 (Aktories and Just, 1995, Just et al., 1995). Following receptor binding and internalisation into the host cell cytosol, the toxin elicits its specific effects through modulation of the physiology of the host cell. Studies show that cells exposed to toxins experience disruption of the actin cytoskeleton (Thelestam and Bronnegard, 1980, Wedel et al., 1983, Mitchell et al., 1987). Glucosylation

of the target proteins at threonine at 37 and threonine at 35 deactivates the small GTPases (Chardin et al., 1989, Paterson et al., 1990) leading to inhibition of the signal transduction pathways controlled by these proteins (Just et al., 1995, Prepens et al., 1996) and destruction of the cytoskeleton, transcription of some stress-activated protein kinases, and a drop in synthesis of phosphatidylinositol 4,5 bisphosphate which contributes to the loss of cell polarity (Mackay and Hall, 1998). In the absence of the acceptor protein, Toxin A hydrolyses UDP-glucose to UDP and glucose (Ciesla and Bobak, 1998). This is similar to the ADP-ribosyltransferase action of toxins. For example, cholera toxin hydrolyses NAD to nicotinamide and ADP-ribose in the absence of acceptor proteins. The internalization of Toxin A is summarized in Figure 1.4.

The small GTPases Rho, Rac, and Cdc42 share analogous mechanisms of regulation and control the actin cytoskeleton (Hall, 1990). Rho and its relatives bind GTP thereby converting to the “on” state. Their GTPase activity then hydrolyses GTP to GDP, resulting in conversion from the “on” to the “off” state. This fluctuation is controlled by guanine exchange factors and GDP dissociation inhibitors that block the interaction between Rho and guanine exchange factors (Zhou et al., 1998). Dissociation of GDP from Rho allows interaction with guanine exchange factors, leading to and further round of GTP binding, activation, and initiation of the regulatory cascade. Furthermore, through activation of Rho-kinase by other signalling proteins, Rho contributes to the control of stress fibre formation (Fujisawa et al., 1998). In contrast, the activity of Rac and Cdc42 are directed more towards the formation of lamellipodia and filopodia which are important for movement of cells (Nobes and Hall, 1995).

As detailed elsewhere, Toxin A is able to stimulate many inflammatory changes in the intestine. It has been reported that the stimulation of chemokine production is triggered by the transcription factor NF-$_k$B. The activation of NF-$_k$B in the early stage of *C. difficile* pathogenesis is accompanied by release of reactive oxygen species and activation of mitogen-activated kinases. It is thought that neutrophil infiltration is stimulated by this pathway (Pothoulakis and Lamont, 2001). It has also been reported that cellular infiltration may occur before the Rho glucosylation, suggesting an independent pathway of action that requires more investigation

1.4.2 Toxin expression regulation

The external signals that modulate toxin expression by *C. difficile* remain unclear. Investigations *in vitro* have shown that toxin expression may be enhanced by stress (including the presence of antibiotics) and catabolite suppression (Dupuy and Sonenshein, 1998). Given the role of antibiotic therapy in initiating pseudomembranous colitis, early studies focused on the influence of various antimicrobials on toxin production and found that sub-inhibitory levels of penicillin and vancomycin enhanced toxin production in continuous cultures of *C. difficile* (Onderdonk et al., 1979).

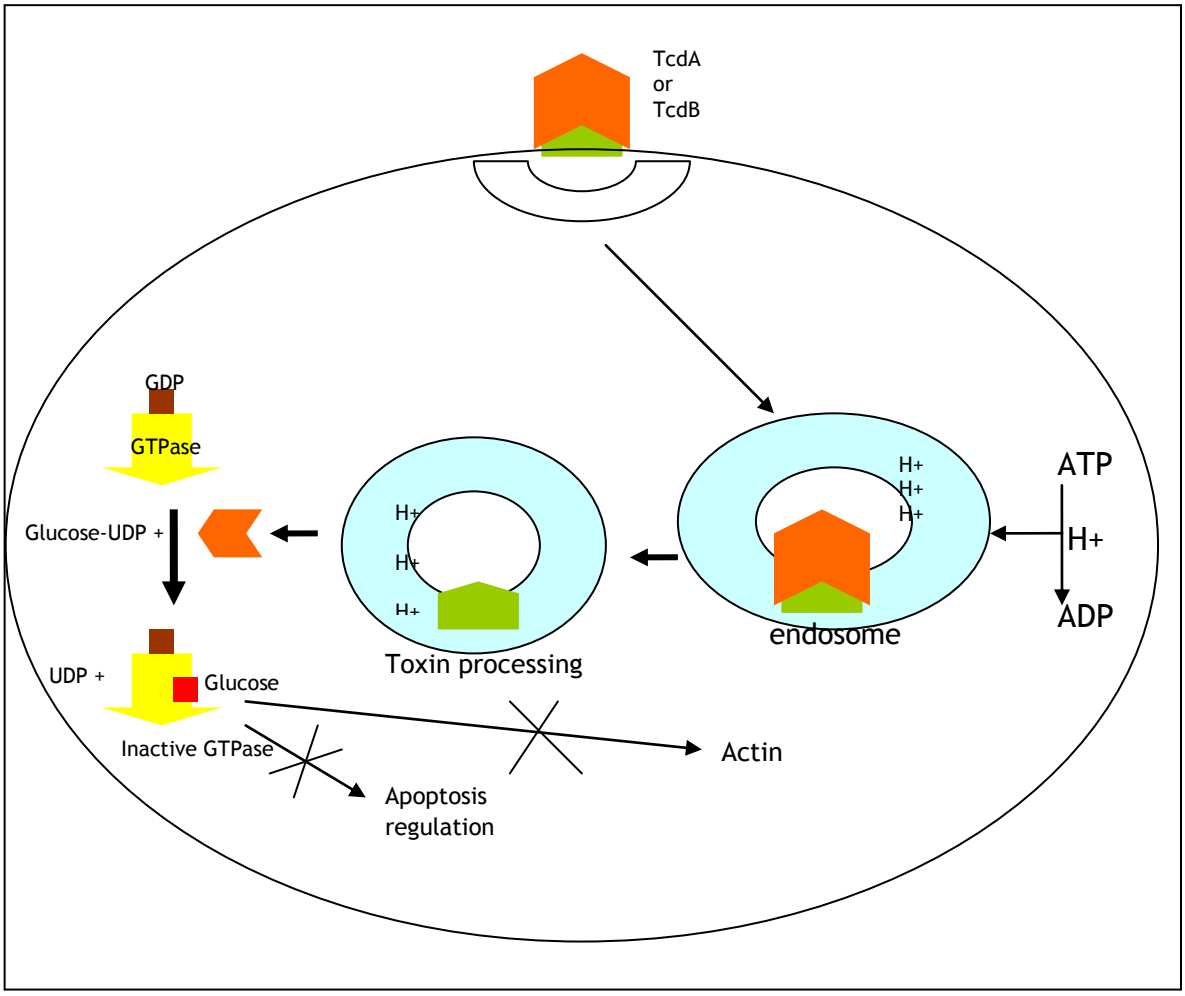

Figure 1-4 Schematic representation of the proposed mechanism of action of *C. difficile* Toxins A and B.

Toxins A and B (orange) bind to receptors (Galß1-4GlcNac for TcdA; green) on the surface of target cells and are then endocytosed. Acidification of endosome exposes hydrophobic regions of the toxin allowing their insertion into the vesicle membrane. The toxins form pores, and the amino-terminal domain is translocated into the cytosol. The location of toxin processing is unknown. In the cytosol, the toxins utilise UDP-glucose to glucosylate target GTPases (yellow). Glucosylation causes: (1) collapse of the actin cytoskeleton resulting in increased permeability of tight junctions in the intestinal epithelium; (2) disruption of the regulation of apoptosis regulation. The net effects are colitis, diarrhoea and tissue necrosis. (Jank et al., 2007 A)

While it is interesting to speculate that antibiotics could act as specific inducers of toxin expression, numerous studies have failed to reach this conclusion and thus, sub-inhibitory levels of antibiotics may be just one of several ways in which *C. difficile* might encounter a stress signal and be stimulated to produce toxin.

Nutrient availability is likely to be another. Decreasing concentrations of biotin in defined medium have been shown to enhance toxin production (Yamakawa et al., 1996). Interestingly, it was reported that a 64-fold and a 35-fold increases in TcdB and TcdA production took place when *C. difficile* was grown in a medium containing 0.05 nM biotin (Yamkawa et al., 1994). Moreover, some investigators have explored links between purine biosynthesis or amino acid availability and toxin production (Karlsson et al., 1999, Maegawa et al., 2002). Recently, the contribution of TcdE to the production of Toxins A and B was investigated by Olling and colleagues. The inactivation of TcdE in *C.difficile* 630 showed no effect on toxin expression and release (Olling et al., 2012).

Overall, the factors that regulate toxin expression remain poorly understood, (Voth and Ballard, 2005).

1.5 Strains of importance

While much of the basic work carried out on Tcd A and TcdB has been based on the properties of one strain, VPI 10463, this may only represent around 20% of clinical isolates. There are now 22 different toxinotypes of *C. difficile* described (Geric et al., 2004). Those strains can be further subdivided based on restriction length polymorphisms (RFLPs) in the toxin genes, reflecting variations, deletions, and duplications within the pathogenicity locus. Although RFLP analysis has revealed that the heterogeneity of Toxin A is much less than that of Toxin B, deletions have been discovered in *tcdA*. In addition, it has also been observed that Toxin A variants have arisen that represent hybrids formed among different Clostridial strains.

The different toxinotypes of *C. difficile* are assigned Roman numbers I to X according to subtle difference variations in the genes at the pathogenicity locus. Strain VPI 10463 was designated as toxinotype 0 (Table 1.1 and 1.2). No association has yet been established between strains and the nature of disease (Table 1.2). It has been speculated that molecular typing might be a useful tool not only in establishing toxinotypes and variants among *C. difficile* strains, but also in diagnosis and the prediction of the virulence of clinical isolates (Rupnik et al., 1998).

Some strains are known to be of more significance than others due to their epidemic nature or virulence properties. For example, the 027 strain isolated in Canada has been associated with a number of very important outbreaks and seems to create greater severity of illness than the classical *C. difficile* strains. It has been revealed that some isolates of 027 release 20 times more Toxin A and B than other *C. difficile* strains, a property that has obvious implications for patient health and the costs of treatment (Razavi et al., 2007, Cookson, 2007).

Toxino-type	*Hin*cII and *Acc*I restriction pattern for B1 fragment[x]	*Hin*dIII and *Rsa*I restriction pattern for B3 fragment	*Eco*RI restriction pattern for A3 fragment	*Nsi*I restriction pattern for PL2 fragment	Standard strain	No. of strains found
0	1	1	1	1	VPI 10463	172
I	1	1	4d	1	EX 623	2
II	1	1	3d	1	AC 008	1
III	4	4	2	2	44027	6
IV	2	2	2	3i	55767	4
V	3	3	8	4i	SE 881	1
VI	3	3	5d	4i	51377	4
VII	3	3	6d	4i	57267	1
VIII	5	1	7d	3i	1470	25
IX	5	4	2	4i	51680	2
X	5	4	NA	4i	8864	1

Table 1-1 RFLP characteristics used for typing of toxin genes

X Restriction patterns were analysed and a number given to each profile; NA, not amplified; d, deletion; i, insertion (Rupnik et al., 1998).

Strain	Toxino-type	PFGE type	Serotype	*tcdB*	*tcdA*	Date of isolation (D.M.Y)	Patient birth date (yr)	Diagnosis
38544	I	NT	C	+	+	1.7.1991	1944	PMC
EX 623	I	8	C	+	+	19.11.1990	Unknown	AAD
AC 008	II	9	A14	+	+	7.4.1994	Unknown	AAD
SE 844	III	2	A1	+	+	10.4.1995	1966	AAD
SE 847	III	2	A1	+	+	10.4.1995	1959	AAD
EX 482	III	2	A1	+	+	2.1.1990	1920	AAD
45129	III	2	A1	+	+	7.9.1992	1939	AAD
35004	III	2	A1	+	+	16.11.1989	1932	PMC
44027	III	ND	A1	+	+	15.6.1992	1939	PMC
55538	IV	5a	A1	+	+	12.5.1995	1991	AAD
55767	IV	5b	A1	+	+	12.6.1995	1944	AAD
40067	IV	5b	A1	+	+	14.10.1991	1990	AAD
7701	IV	6	A5	+	+	23.7.1985	Unknown	AAD
SE 881	V	10	A15	+	+	30.8.1995	1951	AAD

48489	VI	3	A15	+	+	9.4.1993	1931	PMC
51377	VI	4	A15	+	+	11.2.1994	1926	PMC
BR 071	VI	3	A15	+	+	3.7.1995	Unknown	AAD
39696	VI	4	E6	+	+	19.9.1991	1980	AAD
57267	VII	4	E6	+	+	21.12.1995	1946	AAD
1470	VIII	7a	F	+	–	21.12.1981	1981	A. S
20376	VIII	7b	X	+	–	8.7.1988	1986	A. S
51680	IX	11a	A16	+	+	17.3.1994	1933	AAD
SE 938	IX	11b	A16	+	+	5.1.1996	1931	AAD
8864	X	12	unknown	+	–	Unknown	Unknown	Unknown

Table 1-2 *C. difficile* strains with variant toxin genes

NT, non-typeable; ND, not done; AAD, antibiotic associated disease; PMC, pseudomembranous colitis (Rupnik et al., 1998).

1.6 Diagnosis

Many diagnostic tests for *C. difficile* focus upon production of Toxin A, a combination of Toxins A and B, or the presence of common antigen. More than a dozen antibody-based tests for *C.difficile* are commercially available; sensitivity ranges from 71% up to 94% and specificity between 92.5% and 98% (Rath et al., 1988, Whittier et al., 1993). The common antigen assayed is glutamate dehydrogenase (GDH), a product that is conserved by both toxigenic and non-toxigenic isolates.

A wide range of *in vitro* tests are also available using bacterial culture, assay in tissue culture, and antibody-based tests for toxins or the common antigen (Wilkins and Lyerly, 2003). The most sensitive and specific test available is an *in vitro* cytotoxicity assay in which samples are pre-incubated with a neutralising antibody to demonstrate the specificity presence of *C. difficile* toxin (Kelly and LaMont, 1998, Kelly et al., 1994). As some important clinical isolates are A^-B^+, it is very important to use a method that detects both Toxins A and B. Several commercial ELISA assays have been developed that meet this objective (Lyerly et al., 1998).

The tissue culture cytotoxicity assay is 100-1000 fold more sensitive than ELISA in detecting Toxin B and can also detect Toxin A. Hence, this assay is considered the "gold standard" (Peterson et al., 1996, Bouza et al., 2001). This assay system can detect as little as 10 pg of toxin in stool specimens and has a sensitivity of approximately 94 -100% and a specificity of 99% (Kelly and LaMont, 1998, Kelly et al., 1994). However, disadvantages include a lack of standardisation, technical complexity, and slow turnaround time which ranges from 24 h to 4 days. Typically, infection is confirmed by demonstrating the

presence of toxin in fresh or refridgerated stool samples. Cultured human cells such as fibroblasts are exposed to sterile faecal filtrates and cytopathic activity re-tested in the presence of neutralising anti-toxin. The absence of cytotoxin in a single stool sample dose not exclude *C. difficile* infection; at least three stool samples taken at 12 h intervals are required.

In light of the limitations outlined, enzyme immunoassay has been developed to increase the speed of diagnosis but these methods generally lack sensitivity to low toxin concentrations. Noting that limitation, several studies have demonstrated that immunoassays offer significant advantages in terms of cost and ease of performance (Merz et al., 1994, Staneck et al., 1996, Vargas et al., 1997, Lyerly et al., 1998, O'Connor et al., 2001, Turgeon et al., 2003) but new tests are needed for the reliable diagnosis of CDAD. One promising approach is to use the polymerase chain reaction to detect *C. difficile* DNA in stool samples (Morelli et al., 2004). Real time PCR methods in particular offer rapid diagnosis of *C. difficile* infection with 100% specificity and 94% sensitivity (van den Berg et al., 2006) but these tests are not universally available and some investigators have questioned reliability (Reddymasu et al., 2006). The presence of elevated neutrophil counts and levels of C-reactive protein can be used to assess the severity of *C. difficile* colitis. This is supported by *in vitro* studies showing that *C. difficile* toxins stimulate the synthesis of acute phase proteins (Mazuski et al., 1998).

One example of the power of DNA-based diagnostics comes from a study using a new real-time PCR assay for testing 1368 stool samples taken from patients between July 2004 through April 2006. As the final validation of the investigation, 350 inpatients were prospectively interviewed for clinical findings

for 365 episodes of diarrhoeal illness. Test results and clinical criteria were used to assess the performance of 4 assays. With an assay turnaround time of under 4 h, real-time PCR was found to be more sensitive and equally rapid with enzyme immunoassay, and was seen as a feasible laboratory alternative option for detection of toxigenic *C. difficile* (Peterson et al., 2007).

1.7 Treatment and therapeutics

CDAD patients may respond to the simple termination of the antibiotic agent, but in many cases, treatment with metronidazole or vancomycin may be required. *C. difficile* strains that are resistant to both antibiotics have been reported (Peláez et al., 2002, Gerding, 2005). Although patients may react positively to these antibiotic therapies and make an initial recovery, relapse is common as the antimicrobial agents kill the normal bacterial flora and once the colon has been injured, it becomes more susceptible to recurrence of the disease. Relapse rates are around 20%, prolonging illness, the complications of treatment and impacts for the hospital environment (Wilkins and Lyerly, 2003).

Numerous new therapeutic approaches have been developed that aim to block the activities of toxins from *C. difficile*. Passive immunisation with antisera directed to TcdA and TcdB decreases the intestinal injury caused by *C. difficile* infection and more detailed structural understanding of the interaction between TcdA and its carbohydrate receptor provides a realistic foundation for improving the efficiency of the treatment and the development of novel approaches (Greco et al., 2006).

1.7.1Antibiotic therapy

Broad spectrum antibiotics therapy disrupts the normal flora equilibrium in the intestine leading to overgrowth of resident or incoming *C. difficile* which accordingly release the toxins that result in CDAD (Nord and Edlund, 1990). While termination of the offending antibiotic or substituting it to a less potent compound is usually adequate to decrease the symptoms in mild cases of CDAD along with electrolyte replacement (McFarland et al., 2000), it is ironic that the standard approach to treatment of mild or severe cases of CDAD is further use of antibiotics, the two most effective drugs being metronidazole and vancomycin. Most patients respond to these two antibiotics, but 20% of patients relapse after the termination of antibiotic treatment (Fekety et al., 1997) and many will experience several rounds of relapse (Buggy et al., 1987, Jobe et al., 1995). Antibiotics like bacitracin, fusidic acid, rifampicin and teicoplanin are also reported as first choice of antibiotics in the management of CDAD. However, high relative toxicity, the cost of these antibiotics and the elevated rate of the pathogen load in the stool following to the treatment, leaves metronidazole and vancomycin as first and second choices of antibiotics for CDAD (McFarland, 2005, Young et al., 1985). Various strategies have been employed for the treatment of the disease – normalisation of the intestinal microflora and the use of combination of antibiotics are examples - but they show limited efficacy (Buggy et al., 1987) and it has been found that some *C. difficile* strains carry antibiotic resistance genes (Roberts et al., 1994).

Courses of antibiotic therapy in recurrent cases can be extended to months or even years, in some cases, as the probability of recurrence increases at each round of relapse (Fekety et al., 1997, McFarland et al., 1999). Some

reports have reported that relapse was often as a result of the termination of antibiotic therapy (Wilcox et al., 1998, Tang-Feldman et al., 2003). The authors discovered that 50% of recurrent infections were due to relapse with the original strain that caused the first infection. New strains of *C. difficile* were blamed for the other 50% of recurrence. Termination of antibiotics may take into consideration factors like age, health status and the severity of the illness. Moreover, the decision to stop antibiotic treatment may be due to the return to normal of faecal cultures. Relapse may arise when spores germinate in the susceptible intestine. Metronidazole has been found to be very effective in the majority of CDAD but it can lead to the emergence of resistance and the compound does possess relatively high toxicity (Peláez et al., 2002, Beloosesky et al., 2000). Vancomycin appears to be an effective and relatively safe treatment for reinfection with very limited side effects (Jarvis, 1998) but the appearance of vancomycin resistant bacterial strains is of particular concern (Watanabe et al., 1997). Moreover, metronidazole, vancomycin or other choices of antibiotics do not allow recovery of intestinal normal flora, perhaps the best innate defence against enteric pathogens like *C. difficile*. This has been addressed by the introduction of microorganisms like *Saccharomyces boulardii* and lactobacillus species to the intestine. Some studies have shown this can lead to a significant reduction in relapse rates but this was only achieved when probiotics were combined with vancomycin or metronidazole treatment (Surawicz et al., 2000).

Alternative antibiotics include the macrocyclic antibiotic fidaxomicin, a compound that shows about 8 times greater activity against clinical isolates than vancomycin *in vitro* (Finegold et al., 2004, Ackermann et al., 2004) and *in vivo* (Louie et al., 2011). The compound is active against the hypervirulent

NAP1/B1/027 strains. This increased activity against *C. difficile* strains goes along with minimal systemic absorption (Shue et al., 2008) leading to high concentrations of antibiotic in the gut and low activity against the normal bacterial flora *in vitro* (Ackermann et al., 2004, Finegold et al., 2004) and *in vivo* (Louie et al., 2009). These properties make fidaxomicin a promising antibiotic for therapy against *C. difficile* infection.

Two clinical trials have compared fidaxomicin to vancomycin for the treatment of *C. difficile* infection. Fidaxomicin was shown to cure without recurrence at a higher rate than vancomycin (Hardesty and Juang, 2001, Mullane and Gorbach, 2011) and it has been approved by the United States Food and Drug Administration for the treatment of *C. difficile* infection.

Other therapeutic protocols like bowel irrigation or the use of ion exchange resins have been outlined in some reports. Although some show promising findings, these investigations were either carried out on a small scale lacking placebo controls, or antibiotics were still required to achieve success (Mogg et al., 1982, Persky and Brandt, 2000). The sole use of antibiotics for control of *C. difficile* infection also fails to limit the exposure to pathogens in hospital environments. Personal hygiene specially hand washing and the isolation of CDAD cases along with antibiotic therapy are essential issues in controlling the disease (Valiquette et al., 2004).

1.7.2Non-antibiotic therapy

Immunotherapy

New therapeutic approaches for CDAD such as active immunisation through vaccination and passive immunotherapy have been used to target the major virulence factors, TcdA and TdcB.

Active vaccination against Toxins A and B has been used to protect animals against *C. difficile* infection but the vaccination of human patients is still some way off. Kotloff and colleagues have investigated the safety and immunogenicity of Toxoids A and B vaccines in humans (Kotloff et al., 2001). This group found that the both toxoids were tolerated without serious side effects and triggered strong serum (IgG) and faecal (IgA) responses in more than 90% and 50% of subjects respectively. However, the significance of IgG and IgA responses against Toxins A and B in human infection is still incompletely understood. These findings have been supported by the work of Sougioultzis and colleagues. They have reported work with 3 patients with recurrent CDAD. Subjects received intramuscular injections with a *C. difficile* vaccine containing Toxoid A and B proteins. Two of the three cases showed 3 to 4-fold elevation of serum IgG against TcdA and serum IgG against TcdB rose 52-fold in one case, 20-fold in the other. All three cases had required continuous oral vancomycin therapy for 7-22 months to control CDAD but the termination of this treatment following vaccination did not result in further recurrence (Sougioultzis et al., 2005).

Stubbe and his group have clarified that dimeric IgA is more efficient against Toxin A than either monomeric IgA or IgG immunoglobulins that share the same variable domain. The scientists anticipated that the neutralisation

efficiency of dimeric IgA and its prolonged duration of protection might be due to the avidity of dimeric IgA (Stubbe et al., 2000). This is relevant when considering the passive therapeutic options. Salcedo and colleagues have reported the use of human Igs pooled from healthy donors for treatment against *C. difficile*. The immunoglobulin therapy was provided intravenously to patients with severe pseudomembranous colitis that was not responsive to antibiotic therapy. In spite of the initial success of this protocol in controlling disease, diarrhoea recurred four weeks post infusion (Salcedo et al., 1997). Kyne and co-workers have also reported a successful treatment of chronic, recurrent *C. difficile* colitis with pooled immunoglobulins (Kyne et al., 2001). Immunotherapy has been recommended as a routine treatment for recurrent CDAD when antibiotics treatment has failed (Murphy et al., 2006) but the limited availability of toxin specific immunoglobulin along with the cost of the treatment imposes a restriction to the wider use of this approach in CDAD (Wilcox, 2004).

Investigations on animals have observed that the protective effect of anti-toxin antibodies is related to *in vitro* neutralisation of the toxins and not to the quantitative level of antibodies as measured by ELISA. In addition, as the immune response requires time to develop, active immunisation with toxoids may be not the best prophylactic or therapeutic treatment for hospitalised patients and for people at high risk (Corrado et al., 1990). The risk of undesirable immune interactions and inflammation are important concerns. Hence, passive immunisation might be a more appropriate therapeutic choice.

Animal studies have evaluated these ideas. Only some have concluded that immunotherapy for CDAD can be effective after the disease has become established (Kink and Williams, 1998). Extensive attempts have been made to

establish active immunisation schemes that can protect against infection (Kim et al., 1987, Libby et al., 1982, Lyerly et al., 1991, Corthier et al., 1991). One group has reported that hamsters could be immunised with toxoids generated using formaldehyde. The immunising antigens were made from Toxins A or/and Toxin B. Although immunisation stimulated high antibody titres, vaccination against Toxin A or Toxin B alone failed to protect against the fatal consequences of the disease (Libby et al., 1982). More recently Aboudola and his colleagues have confirmed this finding and showed that high levels of antibodies failed to neutralise the toxin when tested in a cytotoxicity assay *in vitro* (Aboudola et al., 2003).

Different findings have been reported by other groups. In one study, 80% protection was achieved when toxoids derived from TcdA and TcdB were used together to immunise hamsters. Interestingly, immunisation with Toxin B alone resulted in more protection than Toxin A alone (Fernie et al., 1983). Kim et al. have suggested that further investigations are needed to resolve these discrepancies. This group also suggested that Toxin B might have less significance than Toxin A in the pathogenesis of ileocecitis because immunisation against Toxin A was necessary to protect hamsters against antibiotic-associated *C. difficile* disease (Kim et al., 1987). Also, immunisation of the animals against Toxin B failed to protect from effect of Toxin A (Kim et al., 1987). This was supported by later studies which showed that the administration of Toxin B alone in high concentration to the intestinal loop did not show damaging effects. This conflicts with earlier reports suggesting equivalent roles for Toxins A and B in the disease in hamsters (Lyerly et al., 1985). The administration of both Toxins A and B together to the intestinal tract in low, non-lethal doses lead to the development of the disease and death suggesting a synergy of action in

which Toxin B appears to be dependent upon Toxin A, but is more aggressive in its effects.

Kink and Williams (Kink and Williams, 1998) have investigated the effect of avian antibodies against TcdA and TcdB in a hamster infection model. Fragments from the carboxy-termini of each toxin were used to raise the antibodies. Anti-Toxin A was sufficient to provide protection against disease when administered before infection with *C. difficile*, but protection against diarrhoea, weight loss and death was only partial if the passive therapy was administered after infection. This finding is consistent with earlier work (Kim et al., 1987).

Lyerly and colleagues (Lyerly et al., 1991) have used bovine antibodies against *C. difficile* toxins to evaluate passive immunisation in hamsters. Although antibodies failed to clear CDAD after the onset of the disease, the group was able to report protection against disease, raising the prospect of a novel, high volume source for persons at high risk of *C. difficile* infection. In bovine colostrum, IgG is the dominant antibody class whereas IgA is the main immunoglobulin in human colostrum. Kelly et al. prepared a bovine Immunoglobulin concentrate from the colostral milk of Holstein cows immunised with *C. difficile* toxoids. This had the ability to neutralise TcdA and TcdB when tested *in vitro* and in animal models. The group has suggested the use of bovine immunoglobulin concentrate as an oral therapy for CDAD and colitis. For human studies, immunoglobulins were combined with an anti-acid. The IgG survived passage through the full length of the intestinal tract (Kelly et al., 1996, Kelly et al., 1997, Warny et al., 1999). As a specific immunotherapy, anti-toxin would

not be expected to affect the normal bacterial flora of the colon, a property that might also contribute to reduced or elimination of *C. difficile* recurrence.

Passive protection using murine monoclonal antibodies against TcdA has been investigated by Corthier et al. The study investigated if passive transfer of neutralising anti-TcdA monoclonals could protect mice. Three monoclonal antibodies were selected upon the capacity to bind the repeating units at the carboxy-terminus of Toxin A. Each of the selected antibodies protected against *C. difficile* disease when administered intravenously to mice (Corthier et al., 1991). For passive therapy in human subjects, a second antibody against TcdB might be required as Toxins A and B do not share neutralising epitopes. A further issue of potential importance is the existence of *C. difficile* strains of different toxinotypes (Giannasca and Warny, 2004).

A distinctive study has proposed the use of recombinant fragments from the repetitive receptor-binding domain of Toxin A for passive or active vaccination depending on the manner of administration. In a passive application, it was envisaged that the recombinant fragments would bind to receptors on epithelial cells and then block the action of Toxin A. As an active immunogen, the recombinant fragment could be delivered to animals or human subjects to trigger a protective immune response. The effectiveness of the recombinant fragment to protect against TcdA was examined and the ability to protect against the Toxin A toxicity *in vitro* and *in vivo* was demonstrated (Sauerborn et al., 1997). More recently, a group has reported the first DNA vaccine against *C. difficile* Toxin A. A synthetic gene carrying the receptor binding domain of TcdA was created, optimised for expression in human cells. The group tested the construct *in vitro* and expression of the recombinant proteins. The construct was

administered to mice by injection and as well as developing anti-TcdA antibodies, the animals were protected against challenge with native TcdA (Gardiner et al., 2009).

In conclusion, research is developing several strategies for CDAD management. This includes re-establishment of the normal microflora, development of toxin binding resins or polymers, and approaches to protect patients by active or passive immunisation (Bauer et al., 2009, Leffler and Lamont, 2009, óHoro and Safdar, 2009).

From these studies, there is a clear requirement for alternatives to antibiotic therapy for CDAD. Work has shown the potential of anti-toxin antibodies to serve as therapeutics and many lines of investigation have shown that antibodies against the receptor-binding domain of Toxin A can be protective. While active immunisation has potential, the numbers of patients at risk of CDAD and their age profiles suggest that orally-administered passive immunotherapy might have significant advantages.

Therefore the primary aims of this project are to isolate and characterise antibodies against the receptor-binding domain of TcdA, the hypothesis being that antibodies directed against appropriate features of the protein will have the capacity to neutralise the native toxin. These antibodies will be obtained by recombinant methods using phage display, the rationale being that recombinant human antibody fragments produced in bacterial culture will not suffer the limitations of availability, immunogenicity or cost associated with immunoglobulins from human donors, animals or expression in mammalian cells *in vitro*.

1.8 Antibodies

In response to foreign material, B-lymphocytes of the mammalian immune system produce immunoglobulins which can recognise and bind to these immunogens. These proteins are known as antibodies.

Immunglobulins have a common basic structure but they can interact with different antigens due to the differences in sequence in regions of their variable domains that are known as complementarity determining regions (CDRs). The massive range of CDR diversity permits recognition of a very wide range of protein antigens and non-peptide molecules such as phospholipids, sugar moieties or even metals (Bosslet et al., 1991, Amoroso et al., 2003) Each antibody chain consists of a variable (V) region that defines its antigen-binding properties and the constant (C) region that interacts with effectors cells and effector molecules like the proteins of the complement cascade.

The association of two heavy and and two light chains in the basic structure of an immunoglobulin creates through the association of variable domains and their CDRs two identical antigen-binding sites. The characteristics of these two sites does not seem to depend to a significant extent on the C region (Weir et al., 2002). The actual antigen-binding sites are formed from the residues contributed by CDRs from the heavy and light chains (Presta et al., 1993). Both “lock and key” (Amit et al., 1986) and “induced fit” models (Sheriff et al., 1996, Van Regenmortel, 2002) have been used to describe recognition of antigen by antibody. The induced-fit mechanism has the potential to create antibodies that are multi-specific and polyreactive. It has been suggested that usually about 5-10 residues make significant contributions to the binding energy (Van Regenmortel, 2002).

The C region of a native antibody contributes three essential effector functions: initiation of antibody-dependent cell cytotoxicity through interaction with receptors on immune effector cells; activation of complement with consequent effects on activation and recruitment of phagocytes; transport of the immunoglobulin to different body compartments and secretion in tears and milk (Janeway and Travers, 2001). In addition, the C region modulates *in vivo* stability (Bogard et al., 1989, Van Regenmortel, 2002, Weir et al., 2002, Lobo et al., 2004) although serum half-life and binding to immunoglobulin-binding proteins (*eg* Protein G) are influenced by domains outwith the C region. The hinge region of an antibody provides flexibility in bivalent interaction with antigen and activation of effector functions (Chan et al., 2004, Van Regenmortel, 2002).

When antibody-based therapies were pioneered, antibodies were created from murine sources to take advantage of monoclonal technologies. However, when administered to patients, these antibodies elicited human anti-mouse antibody responses leading to loss of therapeutic effect and potential problems of hypersensitivity. In an attempt to overcome these problems, humanised chimeric antibodies were generated that were 60-70% human in sequence but with the antigen specificity of the original mouse construct. First generation chimerics carried mouse V regions and human C regions. Reports have shown that adverse responses in human patients to chimeric antibodies were rare and some chimeric antibodies have been developed as therapeutics (Brekke and Sandlie, 2003, van Dijk and van de Winkel, 2001).

Whilst improvements were achieved in this way, these proteins were further optimised with the development of highly humanised antibodies through CDR-

grafting, the replacement of human CDR sequences with residues observed in the original mouse antibody (Jones et al., 1986) and hence its antigen specificity (Van Regenmortel, 2002, Roque et al., 2004). After manipulation, around 90% of the recombinant antibody is human in sequence. In CDR grafting, one main difficulty is that the affinity of the antibody might not be as high as the original mouse antibody. Accordingly, it is essential to use a human antibody framework with high sequence homology to the original murine immunoglobulin to minimise the loss of affinity from sub-optimal conformation in the CDRs (Shearman et al., 1991).These antibodies possess approximately the same immunogenicity as complete human antibodies.

To capitalise upon the monoclonal technologies that are available for rodents, particularly mice, a recent innovation has been to use mice that have an impaired or inactivated immunoglobulin response but carry repertoires of human heavy and light chains in their genome. These transgenic mice can be immunised with any target and the resulting antibody response can be captured and immunised by creating hybridomas that produce complete functional human monoclonal antibodies (Fishwild et al., 1996). Human chromosomal fragments containing the immunoglobulin heavy chain and k light chain loci were introduced to a mouse after inactivation of the endogenous humoral response. Several human antibodies generated from transgenic mice are currently in clinical trails (van Dijk and van de Winkel, 2001). One further strategy described the “trimera” mouse (Reisner and Dagan, 1998). The animal’s immune system is disabled by exposure to radiation. Then bone marrow from as SCID mouse is transplanted to generate a partially reconstituted immune system that can accept foreign cells or tissue. The B cell from a human source is then engrafted and monoclonal antibodies are produced. Antibodies against Hepatitis B

produced in the Trimera mouse are currently under clinical trials (Dagan and Eren, 2003).

Other antibody formats have also emerged to meet the needs of diagnosis and therapy. F_V fragments are non-covalent heterodimers of V_H and V_L domains. Stabilisation of these small proteins has been achieved by incorporation of a hydrophilic flexible peptide linker to tether the domains together and thereby generate single chain Fv proteins (scFvs) (Roque et al., 2004, Van Regenmortel, 2002). These scFvs are 25-30 kDa in size, typically carrying a polypeptide linker of at least 12 residues. Shorter linkers (5-10 residues) constrain the interaction of V_H and V_L domains sufficiently to prevent intramolecular association but this can occur such that the V_H of one protein interacts with the V_L domain of another and *vice versa*, creating a bivalent dimer (diabody; about 60 kDa) or trimers (tribody; about 90 kDa). Disulfide-free scFv molecules are relatively stable and useful for intracellular applications ("intrabodies"; (Van Regenmortel, 2002, Roque et al., 2004, Dall'Acqua and Carter, 1998). The smallest of the antibody fragments is the minimal recognition unit that can be derived from the peptide sequences of a single CDR (Roque et al., 2004, Van Regenmortel, 2002).

The modification and manipulation of naturally existing proteins including antibodies has been made possible by advances in molecular biology. This has lead to the development of antibody engineering techniques so that antibody-coding sequences can be recovered from the mRNA from spleen, lymphocytes and hybridomas source using appropriate oligonucleotide primers (Maynard and Georgiou, 2000). This has facilitated the development of a huge range of recombinant engineered antibody molecules for research, diagnosis, and therapy

with specificities out of reach of conventional antibody technology (Boss et al., 1984, Winter and Milstein, 1991, Kontermann and Müller, 1999, George et al., 1994).

This has the capacity to create large libraries of immunoglobulin sequences from which antibodies of interest (*eg* antibodies that will bind a particular target) must then be extracted. Phage display has emerged as one crucial technology platform that makes this possible.

1.9 Phage display

Phage display technology involves the introduction of foreign sequences into the gene for a phage capsid protein. When the cloned genetic sequence is expressed, the foreign peptide is "displayed" on the phage surface (Arap, 2005). This simple principle was established in the 1980s by George Smith (Smith, 1985) working with the filamentous phage, a virus that replicates and assembles without killing the bacterial host cell. Since the innovative work of Smith, phage display technology has matured into a widely-used technique for selecting from molecular libraries peptides and proteins with defined properties.

One crucial principle underlying all phage display systems is the physical linkage of the properties of a polypeptide (its phenotype) with its coding sequence (its genotype). Genotype-phenotype coupling (Bradbury and Marks, 2004) occurs during phage assembly in the bacterial cell when DNA including sequence for the foreign peptide or protein and the viral protein to which it is fused is packaged into the viral particle; the fusion protein is then incorporated into the phage particle (Endemann and Model, 1995) Hence, selection for the foreign peptide simultaneously captures its coding sequence.

The most popular phage coat proteins exploited in phage display are the major coat protein pVIII and one of the minor components, pIII. The modified gene – the gene for the phage coat protein and the foreign, fused sequence – can be carried in the complete viral genome along with all other genes required for the replication and assembly of the filamentous phage (Cwirla et al., 1990, Scott and Smith, 1990, Kay et al., 1993, Petrenko et al., 1996) or inserted as an additional copy of the coat protein (McLafferty et al., 1993, Haaparanta and Huse, 1995). The former vector system produces phage that present only the fusion protein, the latter yields phage bearing a mixture of wild type and the fusion coat proteins on the same phage particle (Paschke, 2006).

The filamentous phage possess single stranded DNA genomes and hence there are technical difficulties in isolating and manipulating viral genomic DNA. For these reasons, phage display systems that utilise phagemid vectors have come to dominate the field (Marks et al., 1991, Hoogenboom et al., 1991, Barbas et al., 1991, Breitling et al., 1991, Söderlind et al., 1992). A phagemid bears a plasmid origin of replication and selectable marker(s) but in addition, carries the gene fusion (foreign sequence and the gene for the viral coat protein) and sequence from a viral intergenic region that confers upon it the capacity for single-stranded DNA to enter the packaging pathway during viral replication (Russel and Model, 1989). Since the phagemid carries insufficient information for translation of all viral proteins required for viral assembly, super-infection with helper phage is required. The replication and packaging machinery supplied by the helper phage acts on the phagemid DNA and on the helper phage genome itself. Therefore, two distinct types of phage particles with different genotypes can be produced from cells bearing phagemid and helper phage DNA: those carrying the phagemid genome; those carrying the helper phage genome. Phage

particles containing the helper phage genome are useless in phage display processes because they do not contain the foreign sequences of interest (Russel et al., 1986, Vieira et al., 1987). In practice, helper phage are generally created with packaging defects to reduce this potential complication, or are modified in other ways that enable them to be eliminated from the selection process.

In "antibody phage display", antibody coding sequences are recovered from immunised, non-immunised (naïve) lymphocytes (Amersdorfer et al., 2002b) or even from synthetic constructs (Rauchenberger et al., 2003) and fused with a phage coat gene. Full length antibodies are too large for successful display: instead, smaller antigen-binding proteins are used.

1.9.1Antibody formats

Antibodies displayed at the phage surface can be in the form of scFvs, Fab fragments (Marks et al., 1991, McCafferty et al., 1990), Fvs or single domain antibodies (McCafferty et al., 1990). These small antibody fragments retain the capability to interact with antigen and hence can be isolated on the basis of their capacity to bind to antigen coated surfaces (McCafferty et al., 1991, Kang et al., 1991). Figure 1.5 shows different formats of antibodies. Single domain antibodies have achieved popularity in commercial applications, an example being the library created by Domantis (Stockwin and Holmes, 2003, Holt et al., 2003). Fv antibodies suffer the drawback that they dissociate into their V_H and V_L components at low protein concentrations and under physiological conditions. Recombinant Fab antibodies represent the nearest equivalent to natural immunoglobulins and have been very widely used in phage display (Hoogenboom, 1997). The group at the Scripps Institute, in particular, have had great success in

creating immune libraries of Fab antibodies for studies of infectious diseases (Skerra and Pluckthun, 1988, Zhang et al., 2006, Bowley et al., 2007).

The most popular format of recombinant antibody used in phage display is the single chain antibody (scFv). The scFv consists of cloned heavy V_H and light V_L domains linked by a peptide sequence and brings together into a stable protein the minimal structural components required for antigen binding (Huston et al., 1988, Maynard and Georgiou, 2000). It has been found that the linker does not obstruct the antigen binding site, impair domain folding, or block domain association. Also, these small structures can be expressed in bacterial systems, they are stable and they are very effective in binding to their targets (Sheets et al., 1998). scFvs can be assembled from a wide range of starting materials as detailed below and using phage display, any antibody from a large library can then be selected on its ability to bind to a particular antigen (Vaughan et al., 1996).

1.9.2Affinity maturation

In vivo, immunoglobulins undergo somatic hypermutation to increase the affinity of their interaction with antigenic targets. In this process, antibody sequences are diversified, leading to the generation of variants from the original primary immunoglobulin. These variants may show an enhanced affinity towards the target. This process can be mimicked *in vitro* (Adams and Schier, 1999).

The use of phage display to select antibodies from recombinant libraries typically recovers a combination of low and high affinity antibodies. If the affinity of antibodies selected in this way requires improvement, this can be achieved by generating a secondary phage library in a process that imitates the

immune system. There are different approaches. Modifying the V gene by mutagenesis is one of the most popular approaches. This has been done by introducing substitutions at random through the sequence (Hawkins et al., 1993). Variants created in this way can be screened by phage display against low antigen concentrations to isolate antibodies with higher affinities to that of the original construct (Hawkins et al., 1992). Accordingly the power of phage display can be applied to libraries of antibodies that include several possible specificities, and equally to variants of an antibody in which the specificity is the same.

1.9.3Antibody libraries

Phage can thereby display a large – potentially very large – number of different antibody sequences. Antibodies can be extracted from these complex mixtures by a range of methods, the simplest being through capture to a surface coated with a target molecule of interest (Paschke, 2006). Other approaches to the creation of a diverse library of antibodies can include methods that mimic somatic hypermutation involved in a natural immune response (Gram et al., 1992). Displaying antibody molecules on the surface of bacteriophage particles in this way enables selection for specific molecules from a diverse pool such as a library (McCafferty et al., 1990). Each phage particle contains the gene for the displayed antibody and hence attachment of phage to a target molecule through a specific antibody interaction recovers the coding sequence for that antibody. This technique has thus been to isolate antigen specific antibodies from un-immunised human libraries (Marks et al., 1991) and from immunised mouse libraries (Clackson et al., 1991). Antigen specific antibodies were also selected from semi-synthetic combinatorial antibody libraries (Barbas et al., 1992).

The creation of large antibody libraries for phage display has provided a source of antibodies to nearly any antigen even to those targets such as self antigens that are not naturally immunogenic (Griffiths et al., 1993, Griffiths et al., 1994, de Wildt et al., 1996) or cell surface proteins (de Kruif et al., 1995 A, de Haard et al., 1999, Sheets et al., 1998).

In general terms, two kinds of libraries can be created: naïve or immune. Naïve libraries are derived from natural, unimmunised sources (Marks et al., 1991, Vaughan et al., 1996, Sheets et al., 1998, de Haard et al., 1999, Sblattero and Bradbury, 2000), synthetic human V genes (Griffiths et al., 1994, de Kruif et al., 1995, Knappik et al., 2000), or shuffled V genes (Soderlind et al., 2000). Immune libraries are created from immunised subjects (Amersdorfer et al., 2002b, Barbas et al., 1991, Zebedee et al., 1992, Williamson et al., 1993) or mice (Orum et al., 1993, Ames et al., 1994, Ames et al., 1995) and have a strong bias towards antibodies of certain specificity, although they have also been used to select antibodies against antigens which were not used in the immunisation process (Williamson et al., 1993). Moreover, synthetic libraries can be created in which single or small numbers of antibody sequences are diversified *in vitro* (Rauchenberger et al., 2003).

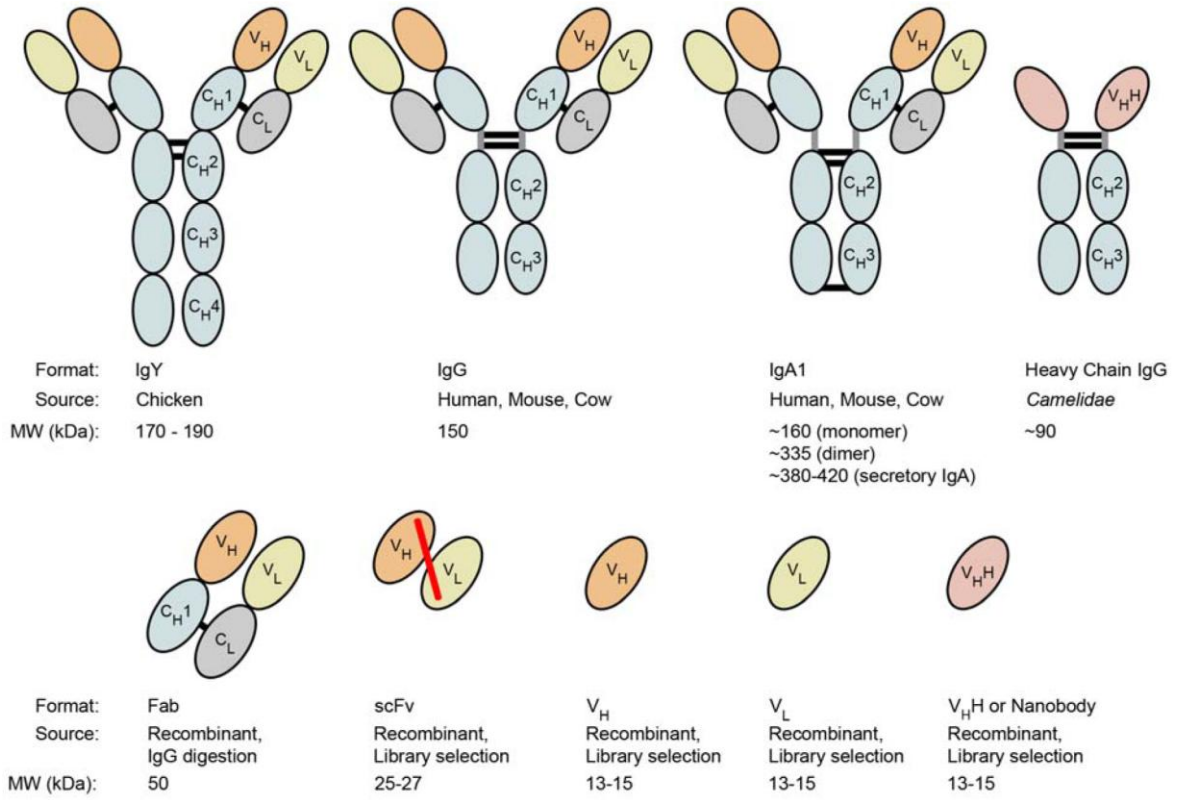

Figure 1-5 Schematic representation of different antibody formats. Figure taken from the work of Hussack and Tanha (Hussack and Tanha, 2010).

1.9.4Naïve libraries

Naïve libraries are also termed non-immune libraries. The diversity created by antibody rearrangement is captured in cDNA isolated from naïve B-cells, sometimes pre-selected for expression of IgM or IgD reflecting their naïve status (Glockshuber et al., 1990). This means that antibodies can be selected against a wide range of antigens including self antigens (Marks et al., 1991) or toxins (Cardoso et al., 2000). As antibodies produced by the starting B-cells have not undergone antigen-driven somatic hypermutation, they may be of lower affinity compared to the antibodies isolated from immune libraries. To resolve this difficulty, larger libraries are often formed to increase the possibility of isolating higher affinity antibodies (Griffiths et al., 1993, Marks et al., 1991, Perelson, 1989). These resources can be used to isolate antibodies against a very wide range of targets.

1.9.5Immune libraries

Immune libraries utilise the ability of the immune response to respond to challenges such as infection, vaccination or experimental immunisation and to develop an antigen-specific repertoire (Clackson et al., 1991). B-cells from lymphoid cells or tissue (Welschof et al., 1995) of human or animal origin from a subject challenged with a target antigen can be the source of the initial material for these libraries (Cai and Garen, 1995, Barbas et al., 1993). In light of this, the library will be biased in favour of the target antigen and so the size of the library may not need to be large because the immune system has already encountered the target antigen. A great advantage of immune library is that the antibody repertoire is rich with high affinity antigen specific antibodies (Clackson et al., 1991). An important disadvantage of this type of library is that it will likely lack the capacity to yield antibodies against self antigens or targets that the donor has not encountered before.

1.9.6Synthetic libraries

Synthetic libraries are known as “single pot libraries” because antibodies against any target can be isolated if the libraries are big enough (Nissim et al., 1994). An attempt is made to imitate the immune system by generating diversity after the rearrangement of V, D, and J segments using *in vitro* methods (Greg, 1998). The natural immune response will select antibodies that carry somatic mutations in the CDRs (Rajewsky, 1996). Hence, it is to these sequences that diversification is directed *in vitro*.

Hoogenboom was amongst the first to apply these concepts to the creation of antibody phage display libraries (Hoogenboom and Winter, 1992)

along with Barbas (Barbas et al., 1992). In these studies, synthetic diversity was created in the CDRs especially CDR3 and its adjacent framework regions. In another early study, Griffiths and colleagues reported control over the choice of framework sequences (Griffiths et al., 1994). While its is the CDRs that mediate interaction with antigen, some antibody scaffolds – the supporting sequences of the heavy and light chain V domains - may be better suited to the integration of random sequences in the CDRs. The choice of particular scaffolds can also overcome problems associated with the expression of recombinant antibodies in bacterial systems (Knappik et al., 2000). The construction of the Human Combinatorial Library (HuCAl R GOLD) was one of the most innovative advances in the development of synthetic diversified display libraries. In this resource, all six CDRs were diversified upon master frameworks generated from consensus sequences of functional human antibody genes. The library contained more than 10^{10} clones. Furthermore, the use of human framework sequences opened the possibility of using antibodies from these antibodies in human therapy (Hoogenboom, 1997). In this library, antibodies were not displayed as fusions to a phage coat protein. Instead, they were bound to phage by disulfide bonds offering a convenient option for phage elution (htpp://www.morphosys.com).

1.9.7The choice of phage coat protein

The most commonly chosen coat protein for these applications is pIII, the minor coat protein that is present in three to five copies at the tip of the filamentous phage virion. The protein is responsible for binding to the F pilus on susceptible strains of *E. coli* and it has a three domain structure separated by glycine rich regions (Hoogenboom, 1997, Lubkowski et al., 1998). The amino-terminal domain is essential for the penetration of phage into the bacterial host

by virtue of its interaction with the carboxy-terminal domain of the periplasmic protein TolA (Lubkowski et al., 1999, Karlsson et al., 2003). The neighbouring domain binds the primary receptor of phage infection, the F pilus. Without this binding event, interaction with TolA is blocked but contact with the pilus releases this blockade during the infection process (Riechmann and Holliger, 1997). Although peptides have been displayed between these domains (Smith, 1985), addition of fusion proteins at the amino-terminus, after the leader sequence, has become the site of choice for the display of antibodies and other foreign proteins.

Other approaches to phage display have used the major coat protein pVIII. This is present in 2700 copies per phage particle. However, comparisons have shown pIII to be the much more efficient regardless of its lower copy number (Kretzschmar and Geiser, 1995).

1.9.8 Some limitations of phage display

Some of the strengths of phage display include the capacity to create antibody libraries of human sequences and to quickly and efficiently extract from these, antibodies with affinity for a target molecule. Although this represents considerable advantages over hybridoma technology, some limitations still exist. First, library construction requires that large samples of DNA of diverse sequence need to be transformed into bacteria and inefficiencies in this step limit the size of the library. Secondly, loss of components of the library can occur if particular sequences impair the growth of bacteria, phage stability or replication, or prove to be toxic for *Escherichia coli*. Furthermore, difficulties may be encountered in recovering phage that carrying antibodies with very high affinity for the target (Schier and Marks, 1996).

1.9.9Selection in antibody phage display

Selection is the process carried out to isolate from a library those phage clones that carry antibodies capable of binding to a specific target. Various procedures can be followed for selection but the most common approach involves two steps: panning and screening. Panning is carried out by adsorbing a target molecule onto a plastic surface. The coated surface is then challenged with virus comprising the antibody library and those phage that fail to bind are washed away. Those that adhere are recovered and replicated to increase their number (Clackson et al., 1991, Kang et al., 1991, Marks et al., 1992). Whilst this is simple in principle, purified target is required and high affinity antibodies may be difficult to rescue because of the avidity effect. In other approaches, the antigen can be biotinylated, phage and target allowed to interact in suspension, and complexes then captured to magnetic beads coated with streptavidin or streptavidin coated plastic surfaces (Hawkins et al., 1992). Other methods include passing phage libraries through columns to which the target antigen has been attached (Hawkins et al., 1992), selection on fixed prokaryotic cells, eukaryotic cells or even tissues (Bradbury et al., 1993, Parsons et al., 1996, Van Ewijk et al., 1997).

After incubating the phage libraries with the target and washing out non-adherent clones, virus are eluted. There are different elution methods. Some scientists (Kang et al., 1991 A, Roberts et al., 1992) have used a single aliquot or gradients of acidic buffers to disrupt the interaction between phage displayed antibody and its target. Others (Griffiths et al., 1993) have used chaotropic agents like dithiothreitol when biotin is linked to the target antigen by a disulphide bridge or basic solutions such as triethylamine (Marks et al., 1992). In

addition, some have used enzymatic digestion at an engineered site located between the antibody and pIII to cleave the fusion protein and release the virus from the selecting surface (Ward et al., 1996). Competition with high antigen concentration has also been reported as an elution method (Clackson et al., 1991).

The effectiveness of panning can be influenced by washing frequency, the stringency of washing, and the concentration of the immobilised target molecule. Virus that display antibodies of low affinity will not remain attached to the selecting surface if subjected to violent washing. Accordingly, it is essential to use a controlled number of washing cycles. On the other hand, high affinity antibodies may be best isolated after aggressive washing. The same principles are also applied to the immobilised target antigen concentration. High concentrations of immobilised target antigen may lead to the isolation of low affinity binders while lower concentration may favour the isolation of virus that display antibodies of high affinity.

Normally, effective isolation can be achieved with two to five cycles of selection, washing, elution, amplification and re-application to the selecting surface. However, the degree of enrichment achieved after each round of selection is an important factor which can be used to decids how many rounds should be used. Normally, 5 to 1000 fold enrichment after each round of selection is expected (Griffiths et al., 1993, Marks et al., 1991). Figure 1.6 summarises the selection process.

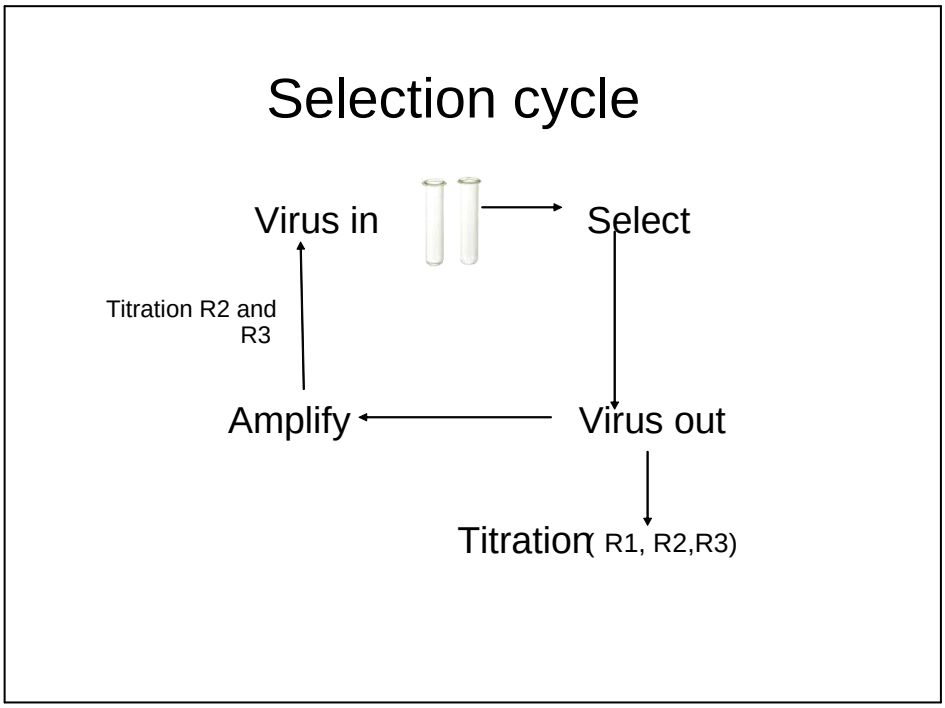

Figure 1-6 Steps in a phage display selection cycle.
Phage were selected against the target protein that coated to the surface of the immune tube, the unbond phages were washed off and the bond pahges were eluted and amplified before been selected again aginst the target protein (this process known as first round of selection). Phages were undergoing three rounds of selection. Recovered and amplified phages were titrated to know the number of pahges eluted after each round and the number of phage selected at the beginning of each round.

1.9.10 Screening of phage displayed antibodies and antibody expression

Given that the selection process works to enrich for phage that display antibodies with the desired properties, a mixture of phage variants with differing properties are typically recovered after each round. Large numbers of clones can be screened to identify the clones of interest. The most commonly used screening procedure is an ELISA in view of its speed, specificity and capacity for high throughput (Marks et al., 1991), although other methods such as flow cytometry have been described (Zaccolo et al., 1997).

The copy number of antibodies displayed at the phage surface has an important impact on screening. Systems that favour monovalent display allow stringent screening whereas the display multiple of multiple copies of the

antibody may create avidity effects. Phagemid systems that incorporate an amber codon between the antibody and pIII sequences (Lowman et al., 1991, Hoogenboom et al., 1991) allow convenient expression of the isolated antibody fragment, and attached tags (*eg* c-myc and / or a his-tag) can facilitate detection or purification (Marks et al., 1991).

The antibodies carried by clones of interest must be expressed on a large scale and at higher yield and purity to allow further characterisation. High protein expression of antibodies with the right folding and function can be achieved depending upon the conditions used (Skerra and Pluckthun, 1988, Better et al., 1988). Several approaches have been used to meet these goals. The first approach has been to express soluble antibodies in the periplasm of *E. coli*. This can lead to successful expression of recombinant antibodies through the activity of periplasmic disulfide-forming proteins DsbA, DsbB, and DsbC. The second approach is the production of soluble recombinant antibodies which accumulate in the periplasm or culture medium through the use of specialised vectors with strong promoters (Colcher et al., 1990, Gibbs et al., 1991). The third approach is to induce the production of cytoplasmic inclusion bodies (Knappik and Plückthun, 1995) the yield of recombinant antibody can also depend on a significant degree upon the *E. coli* host (Strachan et al., 2002).

1.10 Overall aims of the project

Whilst a number of reports document clinical disease caused by strains of *Clostridium difficile* that are unable to produce Toxin A, the consensus remains that TcdA is an significant virulence factor that plays an important role in the pathogenic process. This is supported by a number of observations, notably the many publications showing that immunisation with toxoids or recombinant

proteins containing sequences from TcdA can protect against infection (Corthier et al., 1991, Demarest et al., 2010, Hussack and Tanha, 2010, Hussack et al., 2011, Lowy et al., 2010, Lyerly et al., 1986, van Dissel et al., 2005). Recent studies in this area have used a spore challenge model in hamsters, one of the best experimental models of human infection with *C. difficile*, and have shown conclusively that vaccination using sequences from the receptor-binding domain of TcdA can trigger protective antibody responses (Tian JH, 2012). The receptor binding domain contains numerous repeating peptide motifs (Figures 1.1, 1.2, 1.3) and recent investigations have developed a detailed understanding of the structure of this part of Toxin A and the nature of its interaction with receptor (Ho et al., 2005, Greco et al., 2006). The region is structurally complex, there appear to be multiple sites at which receptor interaction is possible, yet it is also clear that antibodies against epitopes in this carboxy-terminal region of Toxin A have the capacity to neutralise the action of the toxin. Human antibodies with these properties have therapeutic potential and better understanding of which regions best stimulate protective immunity would inform the design of potential vaccines.

Therefore the project aims to isolate recombinant human antibodies against defined features in the receptor-binding domain of Toxin A, to characterise them, and to assess their capacity to protect against the toxin *in vitro* and *in vivo*. The goals of these investigations will be to see if recombinant human antibodies have therapeutic potential and if so, which features of TcdA are the target of toxin-neutralising reagents.

Chapter Two Protein expression and purification

2.1 Introduction

2.1.1 Toxin A binding to its receptor

Recently Pruitt and colleagues revealed the 3D structure for TcdA and mapped the organisation of its functional domains. The TcdA molecule has a pincher-like head which is the central hydrophobic domain, plus long and short tails which are the carboxy and amino terminal domains, involved in receptor binding and catalysis respectively (Pruitt et al., 2010). The carboxy-terminal domain of Toxin A carries a series of repetitive sequence units which are thought to represent binding sites for carbohydrates carried by receptor molecules on the host intestinal epithelium. In structural studies, these motifs have been shown to recognise the trisaccharide Galα1-3Galß1-4GlcNAc and the disaccharide Galß1-4GlcNac. One of the first clues to toxin action came from an analysis of the carboxy-terminal part of the toxin, where so-called combined repetitive oligopeptides (CROPS) were identified (Florin and Thelestam, 1983) these consist of 32 short repeats (SRs) of 15-21 residues and 7 long repeats (LRs) of 30 residues. The repeat sequences appear to be unique among extra-cellular bacterial proteins. Structural analysis of TcdA has revealed that 4 of the short repeat sequences and 1 copy of the long repeat forms a distinct unit that has the capacity to bind carbohydrate (Ho et al., 2005, Greco et al., 2006). Figure 2.3 shows a schematic representation of this unit. Recently, TcdA mutants have been produced from which these CROPS have been deleted (Olling et al., 2012). Interestingly, these proteins retain the ability to enter cells in culture, suggesting that there may be multiple receptors and internalisation pathways. However, the capacity of these features to bind carbohydrate (Jank et al.,

2007), the ability of the isolated carboxy-terminal domain to bind to cells (Sauerborn et al., 1997, Ward et al., 1999), and the toxin-neutralising capacity of antibodies directed towards this part of TcdA and their ability to protect against infection with *C. difficile* (Ward et al., 1999 A, Lowy et al., 2010) (Kink and Williams, 1998, Babcock et al., 2006, Permpoonpattana et al., 2011) demonstrates that they are involved in receptor-binding and serve an important role in the function of Toxin A.

2.1.2 General strategy

The strategy for this phase of the project was to clone, express and purify a recombinant fusion protein carrying TcdA sequences implicated in receptor binding. From crystallographic studies (Ho et al., 2005, Greco et al., 2006), attention focussed upon a discrete unit comprising a series of four short peptide sequences and a single longer motif that are each repeated multiple times in the carboxy-terminal domain of TcdA. These sequences are organised as SR1-LR-SR2-SR3-SR4 (Figure 2.3). Further objectives were to derive a series of truncation mutants to enable mapping of epitopes of anti-TcdA antibodies. The maltose-binding protein of *Escherichia coli* (MBP) was chosen as the fusion partner for these TcdA sequences.

2.1.3 Maltose-binding protein

A variety of bacterial proteins have been commonly used as carriers for the construction of fusion protein. These include maltose-binding protein (MBP) (Smith and Johnson, 1988). Recombinant MBP is expressed abundantly by *E. coli* and the protein has become popular for studies in research and industry (Kane and Hartley, 1988). MBP fusion proteins retain the ability to bind with high

affinity to amylose and this facilitates purification by amylose resin chromatography and elution with moderate concentrations of maltose (Maina et al., 1988). These elution conditions generally have little impact upon the folding or other properties of the fusions protein. This provides a simple system with the capacity to deliver high yields and high degrees of purity (Ferenci and Klotz, 1978, Kellermann and Ferenci, 1982). The use of MBP fusion methods is also well-suited to expression in *E. coli* systems (Graäf et al., 1999).Further, MBP has been shown to possess chaperone functions that can improve the solubility of fused sequences and their folding in *E. coli* (Bach et al., 2001).

2.1.4Aims for the experiments

The aims for this phase of the project were to clone, express and purify MBP fusion proteins carrying TcdA sequences thought to be associated with receptor binding and from these, to create a series of truncation mutants to facilitate epitope mapping later in the project.

2.2 Materials and methods

2.2.1 Construction, expression and purification of maltose-binding protein (MBP) fusions

Native maltose binding protein (MBP), and a series of fusions to maltose binding protein were created as targets for the isolation of recombinant antibodies against the attached C-terminal receptor binding domain of *C. difficile* Toxin A, and defined regions within this sequence. Other truncated fusions were created for analysis of the specificity of recombinant antibodies. Fusions comprised the putative carbohydrate binding motif of Toxin A (Figure 2.1; sequences SR1, LR, SR2, SR3 and SR4), a shorter sequence made up of the long-repeat (LR) and short-repeat 2 (SR2) sequences (MBP-LR-SR2 fusion), and other truncated maltose binding protein fusions.

2.2.1.1 Confirmation of sequence

To confirm the sequences of native MBP or the fusions, the relevant *E.coli* strains were inoculated onto TYE agar plates containing 100 µg/ml ampicillin and incubated overnight at 37 ^{o}C. The next day, a single colony from each culture plate was inoculated into 3 to 5 ml of 2x TY liquid medium containing 100 µg/ml ampicillin and incubated overnight at 37 ^{o}C in a shaking incubator (150 rpm). The next day, bacteria were harvested from the cultures by centrifugation at 12000 x g for 3 min. DNA was extracted from both cultures using a miniprep kit (QIA miniprep, QIAGEN, UK). The manufacturers' recommended protocol was carefully followed. Samples of plasmid DNA were eluted from QIAprep columns using the elution buffer provided with the kit. DNA samples were stored at 4 ^{o}C until sent for sequencing. MBP fusions were created in the fusion vector pCG806

(di Guan *et al.*, 1988; Appendix 6.9). In this vector, the sequence fused to the 3' terminus of the reading frame for MBP could be characterised using the M13 forward primer. Sequencing was carried out at the Sir Henry Wellcome Functional Genomics Facility, University of Glasgow. Chromas LITE version 2.01 software was used for data analysis.

2.2.1.2 Primer design for putative binding site of TcdA-MBP fusion

Forward and reverse primers were designed for amplification of the coding sequence for the putative carbohydrate binding motif from the 3' region of *tcdA* and insertion to pCG806 to create an in frame fusion to *malE*. The amplified product (339 base pairs) included sequences coding for SR1, LR, SR2, SR3 and SR4 (Figure 2.1) that together comprise about 113 amino acids. Crystalography has shown that this region is sufficient for binding the carbohydrate receptor and hence, scFvs against this TcdA sequence may be able to block binding of Toxin A to cells. The forward primer (Table 2.1) was designed from the *tcdA* sequence of *C. difficile* strain 630 with addition of a restriction site for *Bam*HI. The reverse primer was designed by adding a restriction site for *Hin*dIII downstream from a stop codon. The restriction sites were chosen and positioned to allow cloning into the appropriate reading frame in pCG806. For both primers, 21 bases of *tcdA* sequence were incorporated (Table 2.1).

Nucleotide and amino acid sequence of SR1, LR, SR2, SR3, SR4 TcdA to be cloned
Ggt tat aaa act att gat aat aaa aat ttt tac ttt aga aat ggt tta cct cag ata gga G Y K T I D N K N F Y F R N G L P Q I G Gtg ttt aaa ggg tct aat gga ttt gaa tac ttt gca cct gct aat acg gat gct aac aat V F K G S N G F E Y F A P A N T D A N N Ata gaa ggt caa gct ata cgt tat caa aat aga ttc cta cat tta ctt gga aaa ata tat I E G Q A I R Y Q N R F L H L L G K I Y Tac ttt ggt aat aat tca aaa gca gtt act gga tgg caa act att aat ggt aaa gta tat Y F G N N S K A V T G W Q T I N G K V Y Tac ttt atg cct gat act gct atg gct gca gct ggt gga ctt ttc gag att gat ggt gtt Y F M P D T A M A A A G G L F E I D G V Ata tat ttc ttt ggt gtt gat gga gta aaa gcc cct ggg I Y F F G V D G V K A P G SR1 LR SR2 SR3 SR4

Figure 2-1 Sequence of the TcdA carbohydrate binding motif used for fusion to MBP. Colours are used to indicate SR and LR sequences according to the key beneath the Figure.

Forward	**GG GGG GAT CCT GGT TAT AAA ACT ATT GAT AA**
Reverse	**AAA AAG CTT CTA CCC AGG GGC TTT TAC TCC ATC**

Table 2-1 Sequence of the forward and reverse primers used to amplify the *tcdA* sequence shown in Figure 2.1.

Red shows the sequence of the restriction sites used for cloning, blue indicates the stop codon incorporated into the reverse primer, green indicates a random sequence of nucleotides lying 5' to the restriction sites.

2.2.1.3 Polymerase chain reaction (PCR)

Forward and reverse primers (Table 2.1) were used to amplify the target sequence from genomic DNA of *C. difficile* strain 630. Genomic DNA was provided by Ali Nazari, another student in the laboratory. Primers (Sigma-Aldrich) were rehydrated using sterile distilled water to prepare stock solutions of 100 mM.

The PCR reaction was set up to a total volume of 50 µl the reaction comprising 2.5 µl forward and 2.5 µl reverse primers from stock solutions of 100mM, 4 µl $MgCl_2$ from a stock solution 3.0 mM, 1 µl dNTP mix at 10 mM, 10 µl of 5x GoTaq buffer, 0.5 µl GoTaq polymerase (Promega, USA) and 4 µl of template DNA (*C.*

difficile 630 genomic DNA). Genomic DNA was omitted from negative control reactions. Positive control reactions used other forward and reverse primers known to generate a 750 bp product from *C. difficile* genomic DNA. The volume was then increased to 50 µl with sterile distilled water.

Amplification was performed using a Hybaid thermal cycler (Hybaid Ltd, Middlesex, UK). Conditions used were initial denaturation at 94 ºC for 3 min, followed by 30 cycles of denaturation at 94 ºC for 1min, annealing 55 ºC for 1 min, extension at 72 ºC for 1 min, and a final extension step at 72 ºC for 10 min. On completion, PCR reactions were stored at 4 ºC or used immediately.

- 94 ºC for 5 min 1 cycle
  - 94 ºC for 50 s
  - 52 ºC for 50 s
  - 72 ºC for 1.5 min

  } 30 cycles
- 70 ºC for 10 min 1 cycle
- Stored at 4 ºC

Figure 2-2 Standard PCR reaction conditions

PCR reactions were analysed on a 1% (w/v) agarose gel prepared using 1x Tris-Acetate-EDTA (TAE) buffer and 6 µl of SYBER Safe DNA gel stain (Invitrogen) added to each 100 ml of the agarose solution. Samples from each reaction were mixed with 10x BlueJuice gel loading buffer (Invitrogen) before loading along with 1 Kb and 100 bp DNA ladders (Invitrogen) were used as a DNA molecular weight standards. The gel was run at 1-5 volts/cm until the marker dye had migrated a suitable distance. Gels were visualised using a UV transilluminator. An image of the gel was printed and stored electronically for further analysis.

2.2.1.4 Preparation of pCG806 and target insert

An *E.coli* strain carrying the MBP fusion vector pCG806 was inoculated on TYE agar containing 100 µg/ml ampicillin and incubated overnight at 37 °C. Next day, a single colony from the culture plate was transferred to 3 to 5 ml of 2x TY liquid medium containing 100 µg/ml ampicillin. The culture was then incubated overnight in a shaking incubator at 37 °C. The next day, plasmid DNA was extracted using a miniprep kit (QIA prep-miniprep, QIAGEN, UK) using the protocol recommended by the manufacturer.

PCR product amplified from *C. difficile* genomic DNA was purified using commercial reagents (QIAquick PCR purification kit, QIAGEN, UK) and eluted from the purification column using 50 µl of elution buffer (10 mM Tris-HCl, pH 8.5) provided with the kit.

Purified plasmid DNA of pCG806 plasmid (Appendix 6.9) and the purified PCR product were digested with *Bam*HI and *Hin*dIII restriction endonucleases in preparation for ligation. Reactions were set up with 1 µl of reaction buffer E, 1 µl of each restriction enzyme (Promega, USA), 5 µl of either pCG806 or target insert and sterile distilled water to a final volume of 10 µl. Reaction tubes were incubated at 37 °C for 3 h. Restriction enzymes used and their target sequences are shown in Table 2.2. Reactions were terminated by incubation at 70 °C for 20 min.

The DNA from each reaction was precipitated to concentrate it in preparation for ligation. Reaction tubes were transferred to ice and one tenth of the reaction volume of 3M sodium acetate was added, followed by double the original volume of absolute ethanol. Tubes were then incubated overnight at -20 °C and the next day, centrifuged at 13000 rpm for 20 min. Supernatants were

carefully removed and the tube contents were washed with 1 ml of 70% ethanol. Tubes were centrifuged again at 13000 rpm for 20 min before removal of the supernatants and drying at room temperature. The precipitated DNA was dissolved in 10 µl sterile distilled water. The concentration of vector and insert fragments were estimated using a Nanodrop instrument.

2.2.1.5 Ligation and transformation

Vector and insert fragments were ligated using T4 DNA ligase (Promega and New England Biolabs). Ligation reactions were performed in a total volume of 21 µl. As the relative sizes of insert and vector fragments were about 1:10, about 90 ng of insert and the same amount of the vector fragment were sampled for each reaction. Then, 10 µl of 2x ligase buffer and 1 µl of T4 DNA ligase were added and the reaction volume was increased to 21 µl with sterile distilled water. After brief centrifugation, tubes were incubated at room temperature for 5 min and the ligation mixture was chilled on ice and used immediately for transformation or stored at -20 ^{o}C in preparation for this step.

A vial of commercially prepared *E. coli* DH5α (Invitrogen) was thawed on wet ice. Fifty µl aliquots of cells were transferred to sterile chilled polypropylene tubes before addition of 2 µl of ligation mixture or control plasmid and slow, gentle mixing with a pipette tip. The tubes were then incubated on ice for 30 min. Cells were then heat-shocked for about 45 sec at 42 ^{o}C in a water bath without shaking. After chilling on ice for 2 min, 900 µl of pre-warmed SOC was added to each reaction and the mixtures were incubated at 37 ^{o}C for 1 h in a shaking incubator (225 rpm). After this, 50 µl of each transformation mixture was spread on TYE agar plates containing 100 µg/ml ampicillin and 1% glucose and incubated overnight at 37 ^{o}C.

Colonies were re-plated to medium supplemented with X-gal to help distinguish between transformants likely to carry the insert and those that likely did not. About 10 µl of the transformed cells were plated on TYE agar containing 100 µl/ml of ampicillin and 1% glucose supplemented with 40 µl of 100 mM IPTG and 40 µl of 100 mM X-gal. Plates were incubated overnight at 37 ^{o}C. After this, some blue colonies (likely to lack the insert) and several white colonies (those

likely to carry the *tcdA* insert) were picked and grown overnight at 37 ^{o}C in 3 to 5 ml of 2x TY for plasmid extraction.

Restriction Enzyme	Target sequence	Source
*Bam*HI	5'....G GATC C....3' 3'....C CTAG G....5'	Promega, USA
*Hin*dIII	5'....A AGCTT....3' 3'....TTCGA A....5'	Promega, USA

Table 2-2 Restriction enzymes and their target sequences

2.2.1.6 Analysis of transformants

Plasmid DNA was extracted from colonies with a white phenotype and hence likely to carry the *tcdA* insert. As before, a commercial kit (QIA prep-miniprep, QIAGEN, UK) was used for isolation of plasmid DNA. DNA samples were digested with *Hin*dIII and *Bam*HI endonucleases as mentioned earlier and reaction products were analysed by electrophoresis on 1% agarose gel to check for the presence of the intended insert.

From this, two clones were chosen for further analysis. Plasmid DNA from these clones was used as template for PCR with primers used to amplify the original target from *C.difficile* 630 genomic DNA. This attempted to confirm the presence of the intended target sequence. PCR reactions were performed in a total volume of 50 µl, each reaction containing 10 µl of reaction buffer, 4 µl of $MgCl_2$, 0.5 µl of Go Taq polymerase enzyme, 1 µl of dNTPs at 10 mM, 2.5 µl of each primer at 100 mM, and 4 µl of plasmid DNA as a template. The total volume was increased to 50 µl using sterile distilled water. Reaction conditions were as described earlier. PCR products were analysed on 1% agarose gels.

2.2.1.7 Sequencing

Plasmid DNA was purified from *E. coli* DH5α clones of likely to carry *malE-tcdA* fusions as described earlier. Sequencing was performed using M13 primer by staff at the Sir Henry Wellcome Functional Genomics Facility, University of Glasgow. Chromas LITE version 2.01 software was used for data analysis.

2.2.1.8 Bacterial growth, induction and expression of the native MBP and fusions proteins

E. coli bacteria transformed with the MBP fusion carrying the putative binding site of TcdA, bacteria that carry the sequence of the first MBP-TcdA fusion and bacteria carrying only MBP were grown in 2x TY at 37 ^{o}C with shaking until the OD at 600 nm reached 0.2. Then the cultures were induced with IPTG (Melford, UK) to a final concentration of 1mM and allowed to grow for a further 4 h at 30 ^{o}C with shaking. The cultures were then centrigued at 3300 x g at 4 ^{o}C for 30 min. The supernatant was discarded and bacterial pellet was re-suspended in PBS.

The harvested bacterial cells were lysed by sonication (Status US200, Philip Harris Scientific, Germany) using 6 cycles of 20 sec at 20% sonication power with 30 sec intervals of cooling on wet ice. Supernatants were collected after centrifugation of the bacterial lysate at 10000 x g at 4 ^{o}C for 5 min. Supernatants were then stored at -20 ^{o}C or used immediately for analysis.

2.2.1.9 Purification of the proteins using affinity chromatography

Proteins were purified from the cleared bacterial lysates using affinity chromatography on amylose resin (New England BioLabs). This exploited the capacity of MBP to bind to the amylose resin matrix.

Amylose resin was washed with 5 column volumes of column buffer (20 mM Tris-HCl, pH 7.4, 0.2 M NaCl and 1 mM EDTA) before each use in preparation for binding. The supernatant from each bacterial lysate was loaded to the column, allowed to flow through and then recycled for 3 times to maximise protein binding to the resin. Unbound protein was collected and kept at -20 ^{o}C. The amylose resin was then washed with 10 column volumes of column buffer to remove proteins loosely associated with the matrix. Bound proteins were then eluted using maltose buffer.

Optimisation experiments were carried out to identify the most appropriate concentration of maltose for elution of native MBP and MBP-TcdA fusion proteins. The concentration of maltose in the elution buffer was increased stepwise through 15, 25, 50 and 100 mM. Proteins eluted at each step were stored at -20 ^{o}C.

The resin was regenerated by washing with 3 column volumes of distilled water, 3 column volumes of 0.1% SDS, 1 column volume of distilled water and prepared for the next run using 5 column volumes of column buffer. Between runs, the amylose resin was stored in 20% ethanol at 4 ^{o}C.

2.2.1.10 SDS-PAGE analysis of purified proteins

The purified proteins were characterised by running SDS-PAGE with standard procedures. Polyacrylamide gels were cast with a 10% separating layer and a stacking layer of about 1cm. Samples of 13 µl were taken for each sample, boiled with 7 µl of sample buffer for 10 min at 95 ^{o}C and 15 µl of each boiled sample was loaded to the polyacrylamide gel. Electrophoresis was carried out in a running buffer of 25 mM Tris, 0.2 glycine and 0.1% w/v SDS using 100 V. Separation was generally accomplished in 1 h. Each gel was transferred to clean plastic or glass container and soaked with about 3-5 volumes of Commassie

Brilliant Blue stain (2.5 % stain, 50 % methanol, 10 % acetic acid and 40 % water) for 1 h with agitation. The stain was then removed and saved as it could be reused several times before replacement. Each gel was washed by incubation in destain solution (5 % methanol, 7.5 % acetic acid and 90 % water) overnight with agitation.

2.2.1.11 Electroblotting of the purified proteins

After electrophoresis, proteins were blotted from SDS-PAGE gels to nitrocellulose membrane sing a standard protocol for Western blotting. Two fibre pads, 4 pieces of filter paper cut to the same size as the pads and a sheet of nitrocellulose membrane were all soaked in transfer buffer. The soaked nitrocellulose membrane was placed on the gel and covered with two filter papers and one fibre pad on each side. The arrangement was placed into a blotting cassette and transferred to BioRad electrophoresis tank. The tank was filled with transfer buffer and stirred with a small magnetic stirrer. Western blotting was carried out at 100 V for 1 h in a cold room. The membrane was then removed and stained with Ponceau red dye (Sigma, UK) to demonstrate successful transfer. After confirmation of protein transfer, the membrane was washed 3 times with distilled water to wash off the stain and was blocked with 2% marvel milk in PBS for 2 h. The membrane then was washed 3 times with PBS to wash away the blocking solution. The membrane was probed with 1/5000 rabbit anti-MBP antibody (New England Biolabs) in PBS or blocking buffer for 1 h at room temperature. The membrane was washed 3 times with 0.1 % Tween 20 (Sigma-Aldrich, USA) in PBS and probed with 1/5000 HRP conjugated anti-rabbit antibody (Sigma-Aldrich, USA) in PBS or blocking buffer for 1 h at room temperature. Finally the membrane was washed 3 times with the same washing

solution and developed with the TMB substrate (Promega Corporation) for 5-15 min. TMB reaction was stopped by washing the membrane with distilled water.

2.2.1.12 Protein assay

A bicinchoninic acid (BCA) protein assay Kit (Pierce, USA) was used to determine the concentrations of the purified proteins. BCA reagents provide an accurate measurement of protein concentration for most samples of protein and hence are suitable for measurement of protein concentrations in complex mixtures like whole cell lysates or simple mixtures that might arise from affinity chromatography.

BSA samples of known concentrations were prepared along with the working assay solution. Two ml of the working solution was added to samples of 0.1 ml aliquots of each BSA standard or the purified proteins. The colorimetric reaction tubes were incubated at 37 ^{o}C for 30 min and then were measured at 562 nm using UNICAM UV/Vis spectrophotometer. Protein concentrations were calculated by constructing a calibration with the standards.

2.2.2 Construction of truncation mutants from the putative binding site of TcdA-MBP fusion

Site-directed mutagenesis was used to introduce defined mutations. The methods were first introduced by Michael Smith in 1978 and can now be achieved conveniently using PCR with primers that contain the desired mutation. After PCR amplification using mutagenic primers (Figure 2.3), the original template plasmid DNA was eliminated by digestion with the *Dpn* I restriction enzyme (Promega, USA) which is specific for methylated DNA, and hence DNA that has been generated *in vivo*. Mutated plasmid DNA will be preserved as it is

unmethylated as a consequence of synthesis *in vitro*. The mutated plasmid can then be transformed to *E. coli* for analysis and protein expression.

2.2.2.1 Construction of MBP-SR1, MBP-LR, MBP-SR2 and MBP-SR3

The plasmid mutagenesis technique was used to create a series of MBP-TcdA fusions carrying stop codons just after SR1, LR, SR2 or SR3 sequences in the putative binding site of the TcdA-MBP fusion. The protocol also incorporated a restriction site that could be used to identify mutated plasmids.

2.2.2.1.1 Primers design

Forward and reverse primers were designed to amplify the plasmid carrying the putative binding site of TcdA-MBP fusion sequence (Table 2.3). Primers were designed to create stop codon and an additional restriction site just after the targeted sequence in *tcdA* (Figure 2.3). For the first mutant, the last 12 bases of the SR1 sequence were selected and then a TGA stop codon was created plus a *Sal* I restriction site. These modifications replaced the first 9 bases of the LR sequence. Primers for truncation of translation after the other TcdA repeats were designed using the same approach.

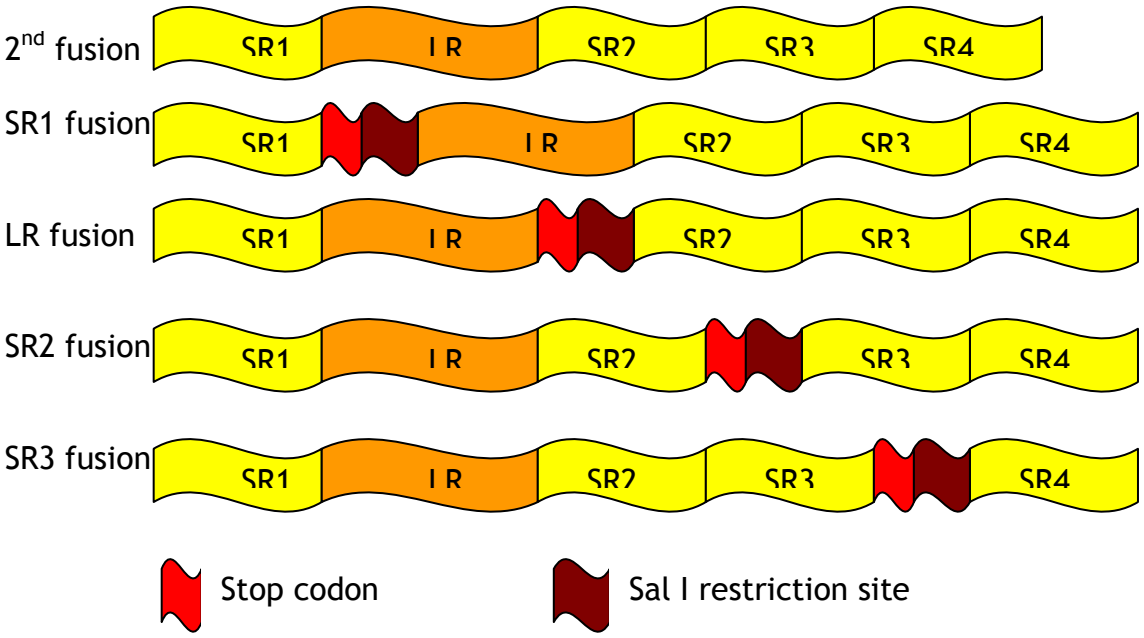

Figure 2-3 Schematic representation to illustrate the approach for plasmid mutagenesis.

The principle for PCR-plasmid-mutagenesis is to mutate the plasmid carrying the putative binding site of TcdA fusion and create a stop codon after each of the repeat sequences.

SR1 for.	TTACCTCAGATATGAGTCGACAAAGGGTCTAAT
SR1 rev.	ATTAGACCCTTTGTCGACTCATATCTGAGGTAA
LR for.	TGAGTCGACCATTTACTTGGAAAAATATAT
LR rev.	GTCGACTCAATTTTGATAACGTATAGC
SR2 for.	ACTTGAGTCGACACTATTAATGGTAAAG
SR2 rev.	TGTCGACTCAAGTAACTGCTTTTGAAT
SR3 for.	GGTTGAGTCGACGAGATTGATGGTGTTATATATTTCTTTGG
SR3 rev.	CTCGTCGACTCAACCAGCTGCAGCCATAGC

Sal I stop codon SR1 LR SR2 SR3 SR4

Table 2-3 Sequences of forward and reverse primers used for plasmid mutagenesis

2.2.2.1.2 Preparation of the putative binding site of TcdA-MBP fusion plasmid

E.coli carrying the TcdA-MBP fusion sequence was inoculated on TYE plate containing 100 µg/ml ampicillin and incubated overnight at 37 °C. The following day, 3-5 ml of 2X TY liquid medium was inoculated with a single colony and incubated overnight at 37 °C. The method for DNA extraction was as described.

2.2.2.1.3 PCR plasmid mutagenesis

Forward and reverse primers (Table 2.3) were used to amplify the putative binding site of TcdA-MBP fusion plasmid using a PCR-plasmid-mutagenesis protocol. Each reaction tube received 10 µl reaction buffer, 4 µl magnesium chloride, 1 µl of each forward and reverse primers at 125 ng/ml, 1µl of dNTP mix at 10 mM, 1 µl of the template at 50 ng/ml and 1 µl of Taq polymerase to the test and positive controls. The volume then was increased to 50 µl using sterile distilled water. Template was omitted from negative control reactions. Cycling parameters were: one cycle of 95 °C for 30 sec, sixteen cycles of 95 °C for 30 sec, 55 °C for 1 min and 68 °C for 10 min, and finally one cycle of 72 °C for 7 min then hold at 4 °C (Figure 2.4). After cycling, PCR product was stored at -20 °C or analysed immediately by electrophoresis on 1% agarose gels.

- 95 ºC for 30 sec 1 cycle
  - 95 ºC for 30 sec
  - 55 ºC for 1 min
  - 68 ºC for 10 min

  16 cycles
- 72 ºC for 7 min 1 cycle
- Stored at 4 ºC

Figure 2-4 PCR-Plasmid-mutagenesis reaction conditions

PCR products were purified using a QIAquick PCR purification kit (QIAGEN, UK) as mentioned earlier. The purified PCR products were then treated with *Dpn* I restriction endonuclease to digest the template DNA. Digestion reaction was carried out as mentioned earlier and products were stored at -20 ^{o}C if not immediately used for transformation.

2.2.2.1.4 Transformation of the mutated plasmid and analysis of transformants

Amplified plasmids were transformed to *E. coli* DH5α competent cells. Samples of 5 µl of the *Dpn* I treated plasmids were transformed to 50 µl of *E. coli* DH5α as mentioned earlier. Two µl of putative binding site of TcdA-MBP fusion plasmid was transformed to 50 µl of competent cell as a positive control. Transformed bacteria were recovered by adding 900 µl of pre-warmed SOC medium and incubated at 37 ^{o}C for 1 h in a shaking incubator at 225 rpm. Cells for both tests and positive control were then plated on TYE agar containing 100 µg/ml ampicillin and grown overnight at 37 ^{o}C. Two µl of *E. coli* DH5α cells was plated to the medium with and with out antibiotic as additional controls and grown overnight at 37 ^{o}C.

Colonies were screened by colony PCR technique using forward and reverse primers annealing to the termini of the *tcdA* sequence and by digestion of plasmid DNA with *Bam* HI, *Hind* III and *Sal* I restriction enzymes to confirm

successful mutagenesis. Sites for *Bam* HI and *Hind* III exist at the termini of the *tcdA* sequence while *Sal* I was inserted with stop codon through mutagenesis. Digestion products were analysed by 1% agarose gel electrophoresis to confirm the presence of *Sal* I restriction site and the sizes of DNA fragments.

2.2.2.1.5 Sequencing

Plasmid DNA from selected clones of *E. coli* DH5α that carried mutated sequences were extracted using Qiagen reagents as mentioned earlier. Sequencing was performed using M13 primer at the Sequencing Service, School of Life Sciences, University of Dundee. Chromas LITE version 2.01 software was used for data analysis.

2.2.2.1.6 Expression and purification

E. coli bacteria transformed with the mutated plasmids were grown in 2x TY at 37 ^{o}C with shaking until the OD at 600 nm reached 0.2. Cultures were then induced with IPTG (Melford, UK) to a final concentration of 1mM and growth continued for 4 h at 30 ^{o}C in a shaking incubator. The cells were then pelleted at 3300 x g at 4 ^{o}C for 30 min. Supernatants were discarded and pellets were re-suspended in PBS.

Harvested bacterial cells were sonicated as described earlier and proteins were purified from supernatants of bacterial lysates using affinity chromatography on amylose resin. Proteins purification was carried out exactly as mentioned earlier with elution using 15 mM maltose. Eluted purified proteins were stored at -20 ^{o}C if not immediately used.

2.2.2.1.7 SDS-PAGE analysis and Electroblotting

Purified proteins were characterised by running SDS-PAGE and Western blotting with anti-MBP antibodies as described earlier.

2.2.2.1.8 Protein assay

Protein concentrations were measured using bicinchoninic assay reagents as mentioned earlier. Proteins concentrations were calculated by comparison with BSA standards.

2.3 Results

2.3.1 Expression and purification of maltose-binding protein (MBP)

At the outset of this phase of the project, DNA sequences were confirmed for native MBP and an MBP-TcdA fusion carrying the LR-SR2 region from the C-terminus of *C. difficile* Toxin A, the first MBP-TcdA fusion to be constructed. MBP and this MBP-TcdA fusion were expressed, purified and characterised. MBP proved valuable as a control protein and as a size marker for differentiation of the sizes of a range of other MBP-TcdA fusions. It was also important that this protein was available in good yield for the isolation of recombinant antibody libraries for pre-incubation and elimination of scFvs directed against MBP. The initial fusion protein was also used to optimise purification methods and for differentiation of sizes of other fusion proteins. In a second construction, MBP was fused to the full, putative binding site of TcdA spanning repeats SR1 to SR4 (Figure 2.1). This was to be used for selection of scFvs antibodies against the crucial part of the TcdA receptor-binding domain. Finally, truncated MBP-TcdA fusions were constructed which differed not in the length of the *tcdA* sequence, but the position of stop codons at the end of each repeat sequence (Figure 2.3). The sequences of these newly constructed MBP-TcdA fusions were confirmed and the recombinant proteins were then expressed, purified and characterised. Truncated fusions will be used for epitope mapping of anti-TcdA scFvs.

2.3.1.1 MBP sequences

The sequences of MBP and the first MBP-TcdA fusion were collected using M13 forward primer that anneals to a sequence downstream of the multiple cloning site in the vector pCG806 (Appendix 6.9). MBP nucleotide sequence (Figure 2.5) was translated to amino acids (Figure 2.6) and checked by BLAST against databases (Figure 2.7) confirming the sequence and reading frame for translation of MBP. The presence of the coding sequence of the first MBP-TcdA fusion sequence (LR-SR2) was confirmed by sequencing of plasmid DNA (Figure 2.8 Panel A). LR and SR2 sequences were flanked by *Sac*I and *Bam*HI restriction sites; translation of the sequence to amino acids is shown in Panel B.

2.3.1.2 Analysis of the amplified target insert and pCG806

Forward and reverse primers were designed (Table 2.1) and used to amplify the complete receptor-binding domain of TcdA from *C. difficile* 630 genomic DNA using standard PCR protocols. The size of the amplification product was analysed by electrophoresis on a 1% agarose gel (Figure 2.9) confirming that the size was as predicted (Figure 2.1). In Figure 2.9, the PCR product was loaded in lane 2 between 1 kb (lane 1) and 100 bp DNA ladders (lane 3) and the band was confirmed as being of the size expected (339 bp). A positive control PCR gave a product of the predicted size (750 bp; lane 4) confirming the quality of the genomic DNA template was sufficient for the intended purpose. In lane 5, a negative control PCR in which water replaced the DNA template confirmed the specificity of the reaction; only primers could be seen on the gel (Figure 2.9).

```
atgaaaataaaaacaggtgcacgcatcctcgcattatccgcattaacgac
gatgatgttttccgcctcggctctcgccaaaatcgaagaaggtaaactgg
taatctggattaacggcgataaaggctataacggtctcgctgaagtcggt
aagaaattcgagaaagataccggaattaaagtcaccgttgagcatccgga
taaactggaagagaaattcccacaggttgcggcaactggcgatggccctg
acattatcttctgggcacacgaccgctttggtggctacgctcaatctggc
ctgttggctgaaatcaccccggacaaagcgttccaggaca agctgtatc
cgtttacctgggatgccgtacgttacaacggcaagctgattgcttacccg
atcgctgttgaagcgttatcgctgatttataacaaagatctgctgccgaa
cccgccaaaaacctgggaagagatcccggcgctggataaagaactgaaag
cgaaaggtaagagcgcgctgatgttcaacctgcaagaaccgtacttcacc
tggccgctgattgctgctgacggggttatgcgttcaagtatgaaaacgg
caagtacgacattaaagacgtgggcgtggataacgctggcgcgaaagcgg
gtctgaccttcctggttgacctgattaaaaacaaacacatgaatgcagac
accgattactccatcgcagaagctgcctttaataaaggcgaaacagcgat
gaccatcaacggcccgtgggcatggtccaacatcgacaccagcaaagtga
attatggtgtaacggtactgccgaccttcaagggtcaaccatccaaaccg
ttcgttggcgtgctgagcgcaggtattaacgccgccagtccgaacaaaga
gctggcgaaagagttcctcgaaaactatctgctgactgatgaaggtctgg
aagcggttaataaagacaaaccgctgggtgccgtagcgctgaagtcttac
gaggaagagttggcgaaagatccacgtattgccgccaccatggaaaacgc
ccagaaaggtgaaatcatgccgaacatcccgcagatgtccgctttctggt
atgccgtgcgtactgcggtgatcaacgccgccagcggtcgtcagactgtc
gatgaagccctgaaagacgcgcagactcgtatcaccaagtaa
```

Figure 2-5 Native MBP DNA sequence.

Plasmid DNA carrying the sequence of full MBP was extracted and sequenced using M13 forward primer. M13 forward primer anneals to a sequence downstream of the multiple cloning site in the cloning vector pCG806. Initial triplet shows the initiation codon, the final triplet forms the TAA stop codon at the end of the *malE* coding sequence.

```
M  K  I  K  T  G  A  R  I  L  A  L  S  A  L  T  T  M  M  F  S  A  S  A  L  A  K
I  E  E  G  K  L  V  I  W  I  N  G  D  K  G  Y  N  G  L  A  E  V  G  K  K  F  E
K  D  T  G  I  K  V  T  V  E  H  P  D  K  L  E  E  K  F  P  Q  V  A  A  T  G  D
G  P  D  I  I  F  W  A  H  D  R  F  G  G  Y  A  Q  S  G  L  L  A  E  I  T  P  D
K  A  F  Q  D  K  L  Y  P  F  T  W  D  A  V  R  Y  N  G  K  L  I  A  Y  P  I  A
V  E  A  L  S  L  I  Y  N  K  D  L  L  P  N  P  P  K  T  W  E  E  I  P  A  L  D
K  E  L  K  A  K  G  K  S  A  L  M  F  N  L  Q  E  P  Y  F  T  W  P  L  I  A  A
D  G  G  Y  A  F  K  Y  E  N  G  K  Y  D  I  K  D  V  G  V  D  N  A  G  A  K  A
G  L  T  F  L  V  D  L  I  K  N  K  H  M  N  A  D  T  D  Y  S  I  A  E  A  A  F
N  K  G  E  T  A  M  T  I  N  G  P  W  A  W  S  N  I  D  T  S  K  V  N  Y  G  V
T  V  L  P  T  F  K  G  Q  P  S  K  P  F  V  G  V  L  S  A  G  I  N  A  A  S  P
N  K  E  L  A  K  E  F  L  E  N  Y  L  L  T  D  E  G  L  E  A  V  N  K  D  K  P
L  G  A  V  A  L  K  S  Y  E  E  E  L  A  K  D  P  R  I  A  A  T  M  E  N  A  Q
K  G  E  I  M  P  N  I  P  Q  M  S  A  F  W  Y  A  V  R  T  A  V  I  N  A  A  S
G  R  Q  T  V  D  E  A  L  K  D  A
```

Figure 2-6 Native MBP amino acids sequence.

Native MBP DNA sequence in Figure 2.5 was translated to amino acids sequence.

```
maltose ABC transporter periplasmic protein
[Escherichia coli O157:H7 EDL933] (Over 10 PubMed links)
Score =  802 bits (2072),  Expect = 0.0, Method: Compositional
matrix adjust.
Identities = 396/396 (100%), Positives = 396/396 (100%), Gaps =
0/396 (0%)

Query  1    MKIKTGARILALSALTTMMFSASALAKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIK  60
Sbjct  1    MKIKTGARILALSALTTMMFSASALAKIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIK  60

Query  61   VTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTW  120
Sbjct  61   VTVEHPDKLEEKFPQVAATGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTW  120

Query  121  DAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEP  180
Sbjct  121  DAVRYNGKLIAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEP  180

Query  181  YFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKNKHMNADTDYSIAE  240
Sbjct  181  YFTWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKNKHMNADTDYSIAE  240

Query  241  AAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKE  300
Sbjct  241  AAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPFVGVLSAGINAASPNKE  300

Query  301  LAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIP  360
Sbjct  301  LAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEELAKDPRIAATMENAQKGEIMPNIP  360

Query  361  QMSAFWYAVRTAVINAASGRQTVDEALKDAQTRITK  396
Sbjct  361  QMSAFWYAVRTAVINAASGRQTVDEALKDAQTRITK  396
```

Figure 2-7 Result of BLAST of MBP amino acid sequence

MBP amino acid sequence in Figure 2.6 was checked against databases using BLAST. Note the complete identity of the query sequence with the subject sequence.

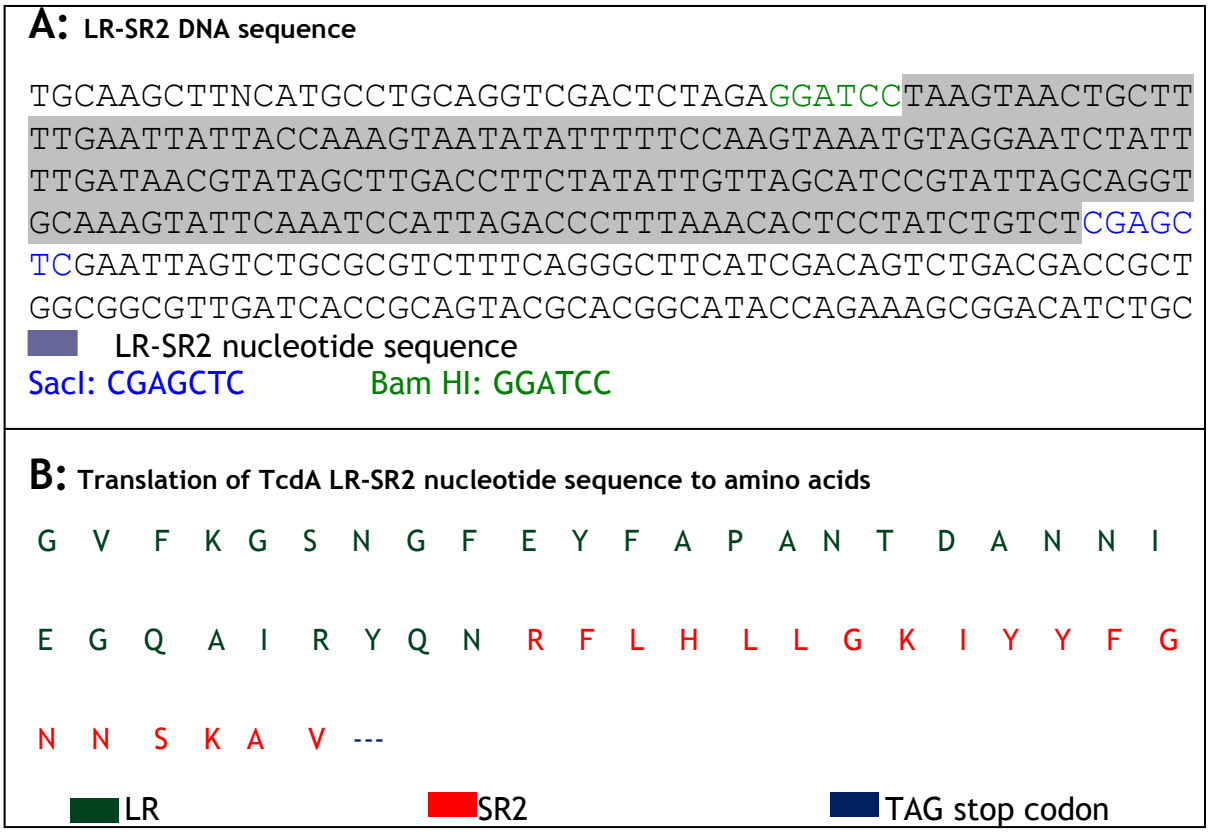

Figure 2-8 Sequence analysis of the first MBP-TcdA fusion to be constructed.

Plasmid DNA carrying the fusion was sequenced. Colour key under Panel A show the restriction sites and the DNA sequence of the first MBP-TcdA fusion. Panel B shows the translation of DNA sequence in Panel A to amino acids. Colour key under Panel B shows amino acids sequence of LR and SR2 repeats, and stop codon. LR and SR2 amino acid sequences were identical to that shown in Figure 2.1.

The cloning vector pCG806 and purified PCR product were digested with *Bam*HI and *Hin*dIII restriction endonucleases and again, analysed on 1% agarose gel to confirm sizes (Figure 2.10).

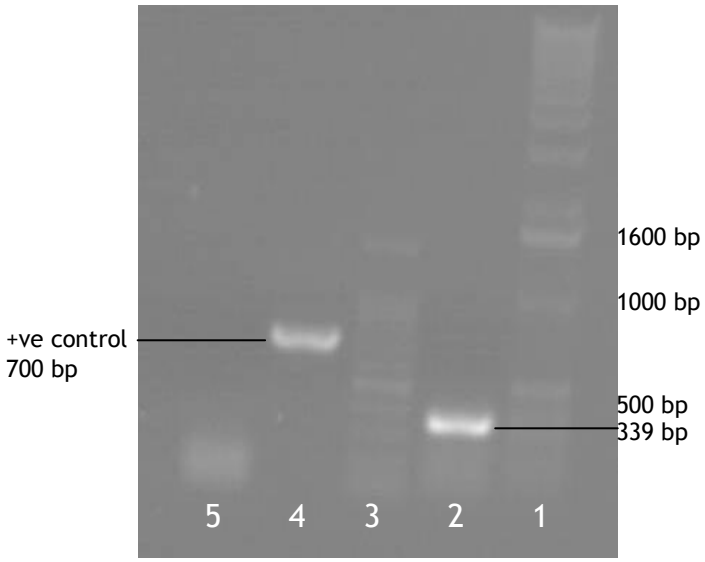

Figure 2-9 Analysis of PCR products of the amplified sequence.
Lane 1 shows 1kb DNA ladder, lane 2 shows PCR product of the target sequence encoding the carbohydrate-binding sequence (Figure 2.1), predicted to be 339 bp in length. Lane 3 shows 100 bp DNA ladder. Lane 4 shows a positive control reaction and lane 5 shows the negative control

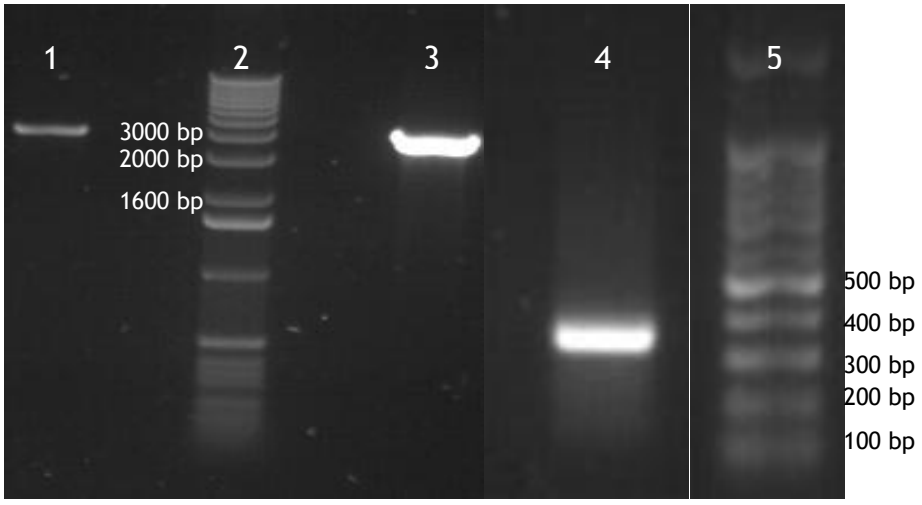

Figure 2-10 Analysis of target insert and the vector after digestion with *Bam* HI and *Hind* III.
Lanes 1 and 3 shows pCG806 vector after digestion with *Bam* HI and *Hin*d III. Lane 2 shows 1kb DNA ladder. Lane 4 shows PCR product of the target insert after digestion with the same restriction enzymes that been used for the cloning vector. Lane 5 shows 100 bp DNA ladder. Size of the digested PCR product was predicted to be 339 bp.

Digested pCG806 and the target insert were concentrated by precipitation and their DNA concentrations were estimated by Nano drop to be 909 ng/µl (pCG806) and 968 ng/µl (PCR product). As the relative sizes of insert and vector were about 1:10, about 90 ng of insert and the same amount of the vector fragment were prepared for the ligation reaction. Vector and insert fragments were ligated using T4 DNA ligase and transformed to *E. coli* DH5α. Transformants were selected on TYE agar plates containing 100 µg/ml ampicillin.

2.3.1.3 Screening and analysis of transformants

X-gal indicator plates were used in an attempt to distinguish between transformants carrying the insert and those that did not. A few blue colonies (likely to lack the insert) and several white colonies (those likely to carry the *tcdA* insert) were characterised by extracting plasmid DNA and restriction analysis with *Bam* HI and *Hind* III (Figure 2.11). Release of the insert at the expected size (339 bp) was detected.

Following confirmation of the presence of the cloned *tcdA* sequence in two clones, amplification from these two clones was carried out using standard PCR protocol and primers used to recover the target insert from *C. difficile* 630 genomic DNA (Figure 2.12). PCR from the first candidate clone showed a strong band at the predicted size of 339 bp (lane 1). Template DNA was seen in a reaction using original cloning vector DNA as template – a band at about 2.5 kb and a band from unused primers at a lower molecular weight (lane 2). A negative control containing primers and water in place of a DNA template showed a primer band (lane 3), and a further negative control PCR with DNA from the clone under test and no primers showed no bands at all (lane 4). PCR using DNA from the second candidate clone again showed a band around the predicted size of 339 bp (lane 5). In a positive control reaction, *C. difficile* 630

genomic DNA was amplified with the same primer set. This PCR showed a band at the predicted size of 339 bp and a fainter primer band (lane 6). A 1kb DNA ladder (lane 7) enabled estimation of the size of PCR products.

2.3.1.4 DNA sequencing

Plasmid DNA was isolated from bacteria known to carry the recombinant plasmid and it was sequenced using the M13 primer. Putative receptor-binding sequence of TcdA-MBP fusion sequence (Figure 2.13) was confirmed with distal sequences corresponding to the reading frame for MBP. The reading frames for MBP and TcdA were in frame so that expression of an intact fusion protein could be expected.

2.3.1.5 Bacterial growth, induction and expression of MBP and MBP-fusion proteins

E. coli bacteria transformed with plasmids for expression of MBP, the first MBP-TcdA fusion or the second, larger TcdA-MBP fusion were grown and induced with IPTG. Proteins were released from cells by sonication and purified from lysates.

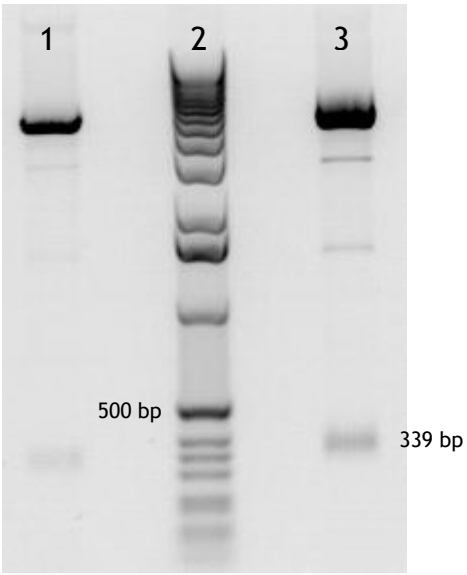

Figure 2-11 Agarose gel electrophoresis of restriction fragments from of clones predicted to carry the *tcdA* gene fusion.

Plasmid DNA was extracted from candidate colonies and digested with *Bam* HI and *Hind* III restriction enzymes. Note the band in lane 3 and a fainter band in lane 1 between 300 and 400 bp. These bands represent the expected sizes of the cloned insert around 339 bp. Larger molecular weight bands around 4.5 kb are the digested vector. 1 kb DNA ladder was loaded in lane 2 for size identification.

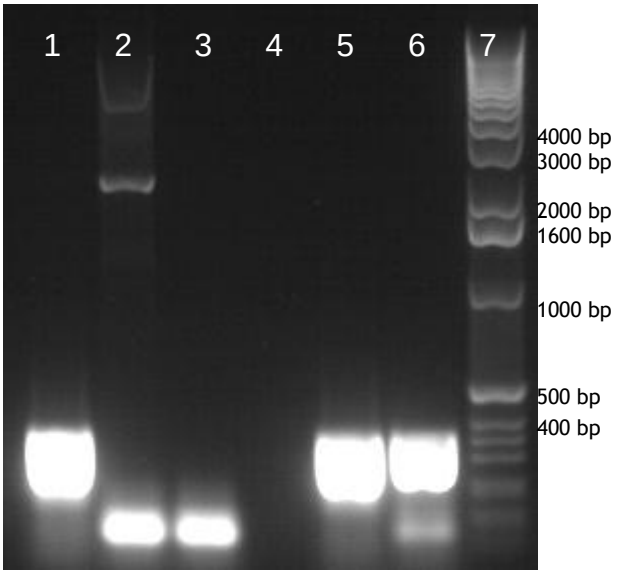

Figure 2-12 Agarose gel electrophoresis analysis of the amplified inserts from the recombinant plasmids.

Amplifications were carried out with primers used to recover the target sequence from *C. difficile* genomic DNA. DNA from one candidate clone was used as template (lane 1) or, as a control, the original pCG806 vector lacking insert (lane 2). DNA was omitted from the reaction as a further control (lane 3). DNA from the same candidate clone used in lane 1 was template while the primers were omitted as negative control (lane 4). DNA from another clone was used as template (lane 5) and *C. difficile* genomic DNA was used as template to provide a positive control (lane 6). 1kb DNA ladder was loaded to lane 7. Size of the target insert in lanes 1, 5 and 6 was predicted to be 339 bp.

A: Putative binding site DNA sequence.

ATCATCAAGGGGCTTTTACTCCATCACACCAAAGAAATATATAACACCATCA
ATCTCGAAAAGTCCACCAGCTGCAGCCATAGCAGTATCAGGCATAAAGTAAT
ATACTTTACCATTAATAGTTTGCCATCCAGTAACTGCTTTTGAATTATTACC
AAAGTAATATATTTTTCCAAGTAAATGTAGGAATCTATTTTGATAACGTATA
GCTTGACCTTCTATATTGTTAGCATCCGTATTAGCAGGTGCAAAGTATTCAA
ATCCATTAGACCCTTTAAACACTCCTATCTGAGGTAAACCATTTCTAAAGTA
AAAATTTTTATTATCAATAGTTTTATAACCAGGATCCCCGGGTACCGAGCTC
GAATTAGTCTGCGCGTCTTTCAGGGCTTCATCGACAGTCTGACGACCGCTGG
CGGCGTTGATCACCGCAGTACGCACGGCATACCAGAAAGCGGACATCTGCGG
GATGTTCGGCATGATTTCACCTTTCTGGGCGTTTTCCATGGTGGCGGCAATA
CGTGGATCTTTCGCCAACTCTTCCTCGTAAGACTTCAGCGCTACGGCACCCA
GCGGTTTGTCTTTATTAACCGCTTTCCAGACCTTCATCAGTCAGCAGATAGT
TTTCGAGGACTCTTTCGCAGCTCTTTGTTCGGACTGGCGGCGTTATACCTGC
GCTCAGCACGCACGAACGCTTGGATGGTTGACCCTTGAGGTCGCAGTACCGT
TACACCATAATTCACTTTGCTGGTGTCGATGTTGACCTATGCCCACGGACGG
TTGATGGTCATCGCTTGTTTCGCTTTATTAAAGGCAGCTTCTGC

Bam HI Putative binding site of TcdA (SR1-LR-SR2-SR3-SR4)

B: Blast of the translated sequence in panel A.

```
>  ref|YP_001087137.1|  toxin A [Clostridium difficile 630]
emb|CAJ67494.1|  Toxin A [Clostridium difficile]
Length=2710
GENE ID: 4914076 tcdA | toxin A [Clostridium difficile 630]
(10 or fewer PubMed links)
Sort alignments for this subject sequence by:
E value  Score  Percent identity
Query start position  Subject start position
Score =  222 bits (565),  Expect = 5e-66, Method: Compositional
matrix adjust.
Identities = 106/106 (100%), Positives = 106/106 (100%), Gaps =
0/106 (0%)
Query  1     GYKTIDNKNFYFRNGLPQIGVFKGSNGFEYFAPANTDANNIEGQAIRYQNRFLHLLGKIY  60
Sbjct  2595  GYKTIDNKNFYFRNGLPQIGVFKGSNGFEYFAPANTDANNIEGQAIRYQNRFLHLLGKIY  2654

Query  61    YFGNNSKAVTGWQTINGKVYYFMPDTAMAAAGGLFEIDGVIYFFGV  106
Sbjct  2655  YFGNNSKAVTGWQTINGKVYYFMPDTAMAAAGGLFEIDGVIYFFGV  2700
```

Figure 2-13 Sequence analysis of putative binding site from the TcdA-MBP fusion

Panel A shows SR1, LR, SR2, SR3 and SR4 sequences and *Bam* HI restriction site as indicated by colour coding. The sequence downstream from *Bam* HI is the nucleotide sequence of MBP. Panel B shows BLAST analysis of the translated nucleotides sequence of SR1, LR, SR2, SR3 and SR4. Amino acid sequence identity with the receptor-binding domain of *C. difficile* 630 TcdA is evident.

2.3.1.6 Purification of MBP and MBP-fusions using affinity chromatography

Purification was carried out using amylose resin affinity chromatography. Purified proteins were eluted with 25 mM maltose and 15 mM maltose for MBP and MBP-TcdA fusions respectively. MBP and MBP-TcdA fusions were then characterised by SDS-PAGE and Western blotting.

2.3.1.7 SDS-PAGE electrophoresis

Purified proteins were characterised by SDS-PAGE electrophoresis on 10% gels (Figure 2.14). The difference in size between native MBP (40 kDa) and the first MBP-TcdA fusion (42.5 kDa) is evident in Panel A and in Panel B, step wise differences can be seen between native MBP, the first MBP-TcdA fusion and the second larger TcdA fusion (52 kDa) that was sequenced (Figure 2.13). All purified fractions showed a single band with minimal evidence of contaminants from the bacterial lysates at significant concentrations.

2.3.1.8 Electroblotting

Western blotting was carried out immediately after the SDS-PAGE electrophoresis to test for the presence of the MBP carrier protein. Patterns were similar to those observed from Coomassie staining in Figure 2.14: a positive signal was evident from native MBP at 40 kDa, whereas the first MBP-TcdA fusion carrying only LR-SR2 was evident at a slightly higher molecular weight (about 42.5 kDa); positive reaction with the anti-MBP antibodies was also seen for the TcdA-MBP fusion carrying SR1-LR-SR2-SR3-SR4 (about 52 kDa) (Figure 2.15). In several instances, reactive bands can be seen at lower molecular weights perhaps reflecting the presence of proteolytic digestion products or the presence of MBP from the genomic copy of the gene for MBP.

2.3.1.9 Protein assay

Bicinchoninic acid assay was used to determine the concentrations of the purified proteins. The purified sample of native MBP contained protein at 380 ng/µl, the protein concentration of the first MBP-TcdA fusion was 250 ng/µl and the putative binding site TcdA-MBP fusion protein was 350 ng/µl.

2.3.1.10 Construction of truncation mutants of MBP-TcdA fusion proteins

Truncated MBP-TcdA fusion proteins were constructed using the longer fusion carrying the putative binding site of TcdA and MBP. Plasmid mutagenesis technique was used to create MBP fusions carrying only SR1, SR1-LR, SR1-LR-SR2 or SR1-LR-SR2-SR3. These MBP-TcdA fragments will be used in a later phase of the project for epitpe mapping of scFvs against TcdA sequences.

2.3.1.11 PCR-plasmid-mutagenesis for SR1, LR, SR2 and SR3

Forward and reverse primers were designed (Table 2.3) and used to amplify and mutate key sequences in the TcdA-MBP fusion by introducing a stop codon and *Sal* I restriction site just after each repeat in the TcdA sequence.

Figure 2.16 shows gel analysis of PCR-plasmid-mutagenesis products created using SR1 forward and reverse primers.

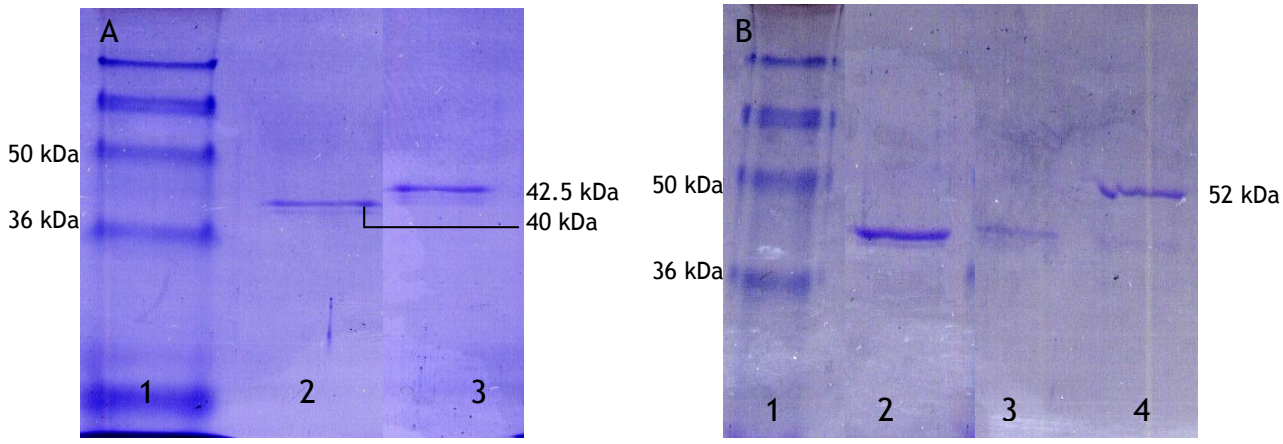

Figure 2-14 SDS-PAGE analysis of MBP, the first and the second MBP-TcdA fusions. SDS-PAGE gels were stained with Commassie Brilliant Blue. Note the band at 40kDa which represents native MBP (lane 2 in Panel A and Panel B). The band around 42.5kDa represents the first MBP-TcdA fusion that carries LR and SR2 (lane 3 in Panel A and Panel B). The band around 52kDa represents of the larger TcdA-MBP fusion containing sequences SR1 to SR4 (lane 4 in Panel B). SeeBlue Plus2 protein size ladder was loaded to lane 1 in each panel.

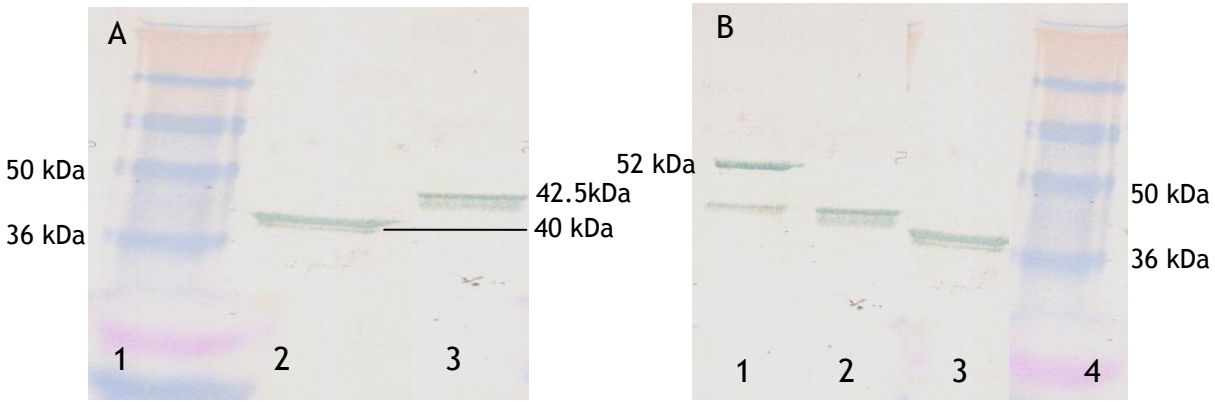

Figure 2-15 Western blot analysis of MBP, the first and the second MBP-TcdA fusions. Purified MBP, the first MBP-TcdA fusion and the larger TcdA-MBP protein were blotted to nitrocellulose and probed with anti-MBP and anti-rabbit antibodies. Lane 2 in Panel A and lane 3 in Panel B show bands of 40kDa representing native MBP. Lane 3 in Panel A and lane 2 in Panel B show bands of 42.5kDa for the first MBP-TcdA fusion. Lane 1 in Panel B shows a band of 52kDa for the TcdA-MBP fusion carrying SR1 to SR4. Lane 1 in Panel A and 4 in Panel B show SeeBlue Plus2 protein ladder.

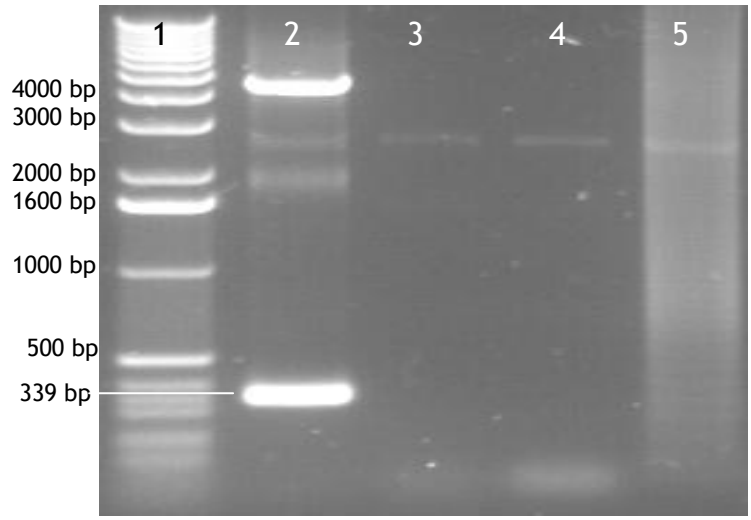

Figure 2-16 Agarose gel electrophoresis of PCR-plasmid-mutagenesis for SR1
Lane 1 is a 1kb DNA ladder. Forward and reverse primers used to amplify the coding sequence for the putative binding site of TcdA were employed as positive control (lane 2). A band of 339bp can be seen. Taq polymerase enzyme was omitted from the reaction as a negative control and to compare the DNA band from the test to confirm the plasmid amplification (lane 3). Products from a reaction using an annealing temperature of 53 ^{0}C was used for test optimisation (lane 4) and the DNA from the amplification using 55 ^{0}C as the annealing temperature was loaded to lane 5.

A positive control reaction (lane 2) confirmed that the cycling conditions would support amplification and a reaction lacking enzyme (lane 3) revealed the presence and concentration of the template for the mutagenesis reaction. Primers were incompletely incorporated at an annealing temperature of 53 ^{o}C (lane 4); better incorporation was achieved at 55 ^{o}C (lane 5), with slight suggestion that the yield of plasmid DNA was greater in PCR reactions (lanes 4 and 5) than in the control (lane 3).

Similar procedure was used to incorporate a stop codon after the LR repeat (Figure 2.17).

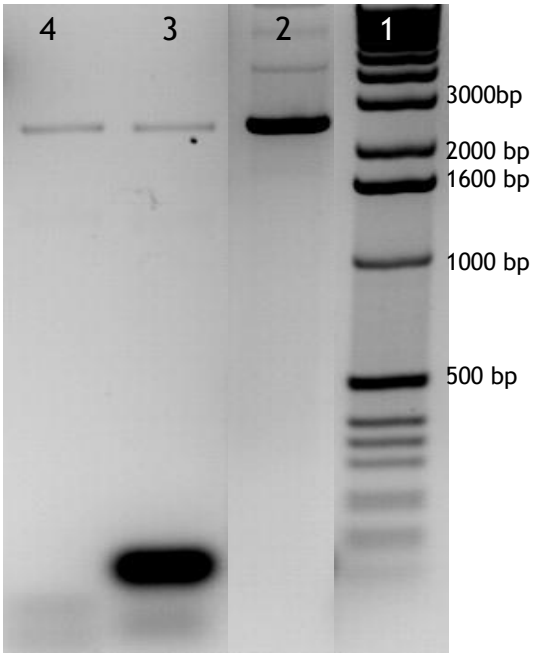

Figure 2-17 Agrose gel electrophoresis analysis of the PCR-plasmid-mutagenesis test for LR.

Lane 1 is 1kb DNA ladder, lane 2 shows template as a control for size comparison, lane 3 shows negative control test with no polymerase enzyme and lane 4 shows PCR-plasmid-mutagenesis test using LR forward and reverse primers

Primers were evident in the control reaction that lacked polymerase (lane 3); these were incorporated in the mutagenesis reaction (lane 4) suggesting that the reaction was successful.

Forward and reverse primers for SR2 and SR3 (Table 2.3) were used to amplify the TcdA-MBP coding sequence and to incorporate stops codons Figure 2.18) as planned (Figure 2.3) with better evidence of success in these reactions.

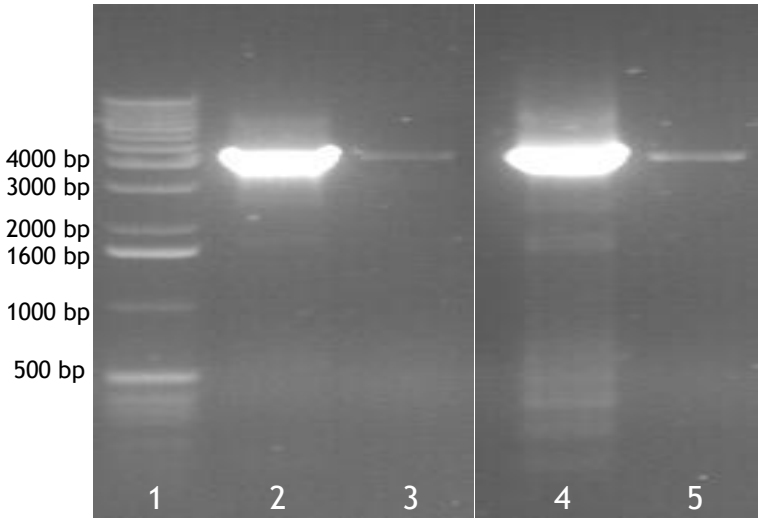

Figure 2-18 Agrose gel electrophoresis analysis of the PCR-plasmid-mutagenesis test for SR2 and SR3.

Amplifications were carried out using the PCR-plasmid-mutagenesis protocol using primers for SR2 (lane 2) and SR3 (lane 4). Polymerase enzyme was omitted from the reaction as negative control for SR2 and SR3 (lanes 3 and 5). One kb DNA ladder is shown in lane 1.

2.3.1.12 Transformation of the mutated plasmids

Amplified DNA from SR1, LR, SR2 and SR3 PCR-plasmid-mutagenesis reactions were purified and then treated with *Dpn*I restriction endonuclease to digest the parental DNA template as mentioned earlier. DNA was then transformed to *E.coli* DH5α using the TcdA-MBP fusion plasmid as a positive control. The positive control plate yielded good numbers of transformants. Several colonies were recovered from plates selecting for SR1, LR, SR2 and SR3 mutation. These cells were taken forward for further analysis.

2.3.1.12.1 Screening and analysis of the transformants

Candidate colonies were initially screened by colony PCR and those that produced amplification products of the expected size were further analysed by endonuclease digestion with enzymes including *Sal*I to confirm successful mutagenesis.

In initial testing, colonies from the SR1 mutation reaction were screened using SR1 forward primer and reverse primers. The expected size of the

amplification product was 290 bp. This is evident in Figure 2.19. Plasmid DNA was then extracted from a candidate clone and analysed by digestion with *Bam*HI, *Hind*III or *Sal*I restriction enzymes (Figure 2.20). Sites for *Bam*HI and *Hind*III were predicted to exist at the termini of *tcdA* sequence, while *Sal*I was inserted along with a stop codon just after the SR1 sequence through mutagenesis. The plasmid DNA was linearised by *Bam*HI and *Hin*dIII as predicted (lanes 3 and 4) and digestion with *Sal*I (lane 5) confirmed that the mutagenesis reaction was successful.

Colonies transformed with mutated plasmid DNA for SR2 or SR3 were screened by colony PCR technique using a forward primer that annealed to the 5' terminus of the *tcdA* sequence and SR2 or SR3 reverse primers. The expected size of amplification products was around 220 and 290 bp for SR2 and SR3 respectively. PCR products were analysed by agarose electrophoresis (Figure 2.21), showing evidence of success in most cases.

Plasmids DNA from colonies that showed positive reactions for SR2 and SR3 were extracted and analysed by double digestion with *Bam* HI and *Sal* I restriction enzymes to assess if mutagenesis had been successful (Figure 2.22). It was predicted that successful mutation of the SR2 sequence would place a *Sal*I restriction site 220 bp from *Bam*HI; a fragment of this size can be seen at the right hand side of Figure 2.22. Similarly, a fragment at the left hand side of the Figure indicates successful mutation of the SR3 sequence (290 bp fragment).

Transformants from mutation reactions with the LR repeat were screened by colony PCR technique using LR forward primer and reverse primers. The expected size of this fragment was around 200 bp and in Figure 2.23, a product of the predicted size can be seen in lane 3. Plasmid DNA was extracted from a

candidate clone that showed positive reaction from LR colony PCR. The extracted plasmid was digested with *Sal* I (Figure 2.24 lane 1) or *Bam* HI (lane 2) to assess for the presence of the *Sal* I *restriction* site. This confirmed the success of mutagenesis.

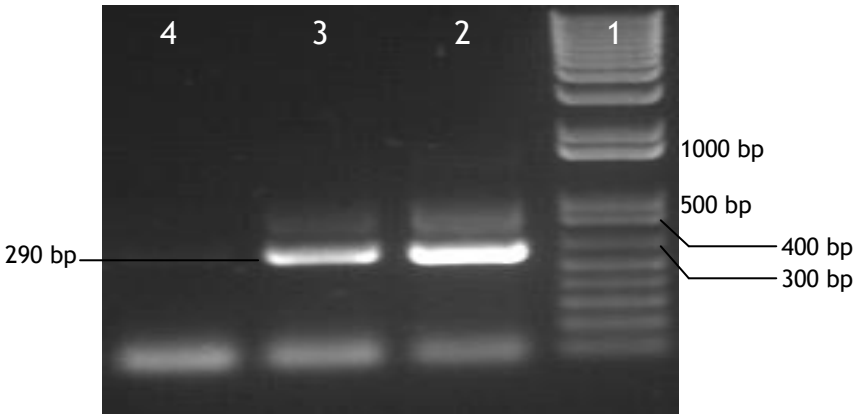

Figure 2-19 Agarose gel electrophoresis analysis of colony PCR for SR1 mutants.
Colony PCR was carried out using standard PCR protocol. Lane 1 shows the 1 kb DNA ladder while lanes 2, 3 and 4 show successful amplification of target sequence at the expected size (290 bp).

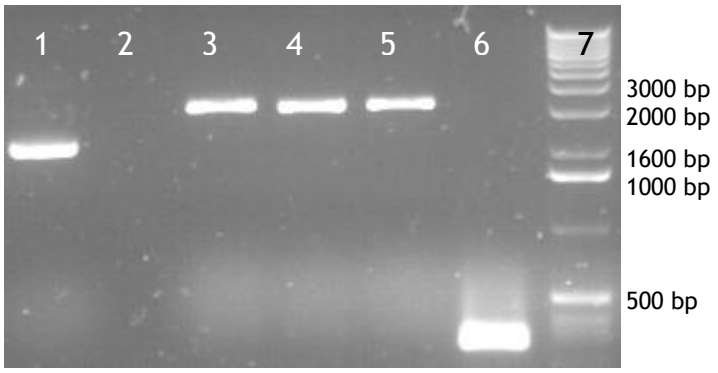

Figure 2-20 Agarose gel electrophoresis analysis of SR1 mutant by restriction digestion.
Uncut plasmid was loaded to lane 1 for comparison with digested samples elsewhere on the gel. A negative control for the PCR that lacked primers was loaded to lane 2. Lanes 3, 4 and 5 shows digestion of plasmid DNA sample with *Bam*HI, *Hin*dIII and *Sal*I respectively. PCR showed a band at the predicted size of 290 bp (lane 6). A 1kb DNA ladder was loaded to lane 7.

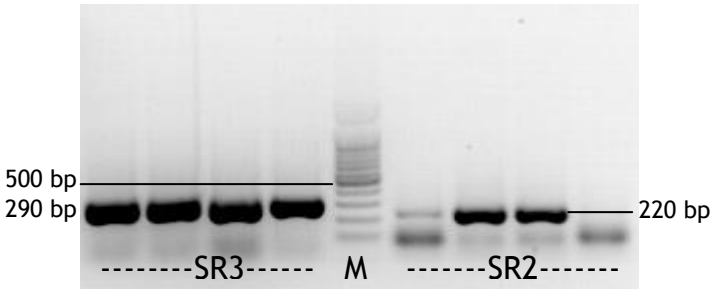

Figure 2-21 Agarose gel analysis of colony PCR product for SR2 and SR3 mutants.
Four lanes at the left show bands of a size (290bp) consistent with the SR3 product. M indicates the 100 bp DNA ladder that was used to facilitate the estimation of product size. Four lanes at the right show bands from the SR2 reaction. The central two show bands of a size (220bp) consistent with the intended product with a fainter band to the left of this series.

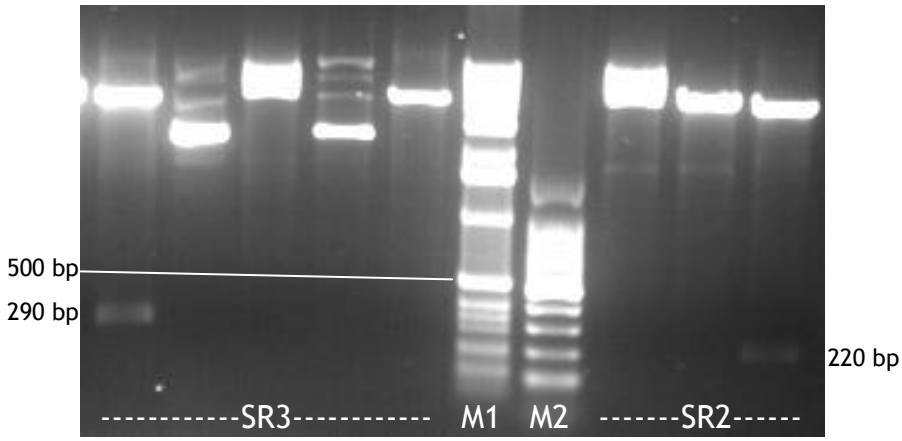

Figure 2-22 Agarose gel analysis of SR2 and SR3 mutants by restriction digestion.
Plasmid DNA from three candidate SR2 mutants were double digested with *Bam*HI and *Sal*I and samples loaded to the right of the gel. M1 shows 1kb DNA markers, M2 100bp markers. Five SR3 samples were digested in the same way and loaded to the left of the gel.

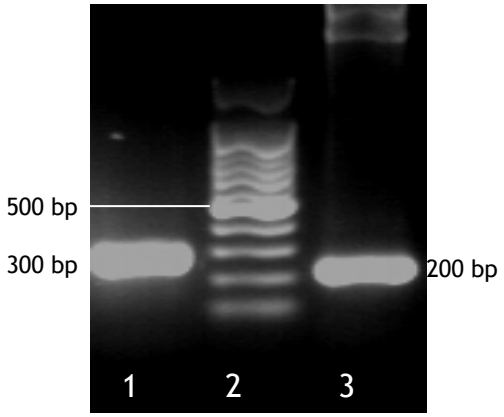

Figure 2-23 Agarose gel electrophoresis analysis of LR mutants.
Colony PCR was carried out using LR forward primer and reverse primers. Lane 1 shows a band around 300 bp that is about 100 bp larger than the expected size. The sample in lane 3 shows better agreement with prediction. Lane 2 was loaded with a 100 bp DNA ladder.

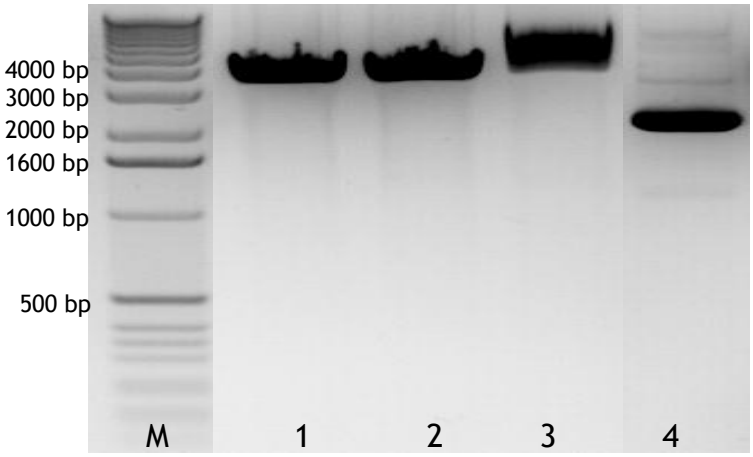

Figure 2-24 Agarose gel analysis for digestion products from treatment with *Sal*I or *Bam* HI. M is the 1 kb DNA ladder. A positive clone from LR colony PCR was digested with *Sal* I (lane 1) or *Bam* HI (lane 2). As a control, the template for the mutagenic PCR was digested with *Bam* HI (lane 3). Lane 4 shows undigested plasmid DNA to aid differentiation between linearise and uncut plasmids.

2.3.1.13 Sequencing

Those candidate clones from PCR-plasmid-mutagenesis for SR1, LR, SR2 and SR3 that showed positive reactions in analysis were sequenced and analysed. Sequencing results showed the presence of stop codons and *Sal*I restriction sites at the expected positions, confirming the success of the mutagenesis reactions. Figures 2.25, 2.26, 2.28 and 2.30 show the nucleotide sequences for SR1, SR2, SR3 and LR respectively.

A: Nucleotide sequence for SR1 mutant

```
TGTTAGCATCCGTATTAGCAGGTGCAAAGTATTCAAATCTATTAGACCCTTTGTCGACTCATATCTGAGGT
AACCCATTTCTAAAGTAAAAATTTTTATTATCAATAGTTTTATAACCGGATCCCCGGGTACCGAGCTCGAA
TTAGTCTGCGCGTCTTTCAGGGCTTCATCGACAGTCTGACGACCGCTGGCGGCGTTGATCACCGCAGTACG
CACGGCATACCAGAAAGCGGACATCTGCGGGATGTTCGGCATGATTTCACCTTTCTGGGCGTTTTCCATGG
TGGCGGCAATACGTGGATCTTTCGCCAACTCTTCCTCGTAAGACTTCAGCGCTACGCACCCCAGCGGTTTG
TCTTTATTAACCGCTTCCAGACCTTCATCAGTCAGCAGATAGTTTTCGAGGAACTCTTTCGCCAGCTCTTT
GTTCGGACTGGCGGCGTTAATACCTGCGCTCAGCACGCCAACGAACGGTTTGGATGGTTGACCCTTGAAGG
TCGGCAGTACCGTTACACCATAATTCACTTTGCTGGTGTCGATGTTGGACCATGCCCACGGGCCGTTGATG
GTCATCGCTGTTTCGCCTTTATTAAAGGCAGCTTCTGCGATGGAGTAATCGGTGTCTGCATTCATGTGTTT
GTTTTTAATCAGGTCAACCAGGAAGGTCAGACCCGCTTTCGCGCCAGCGTTATCCACGCCCACGTCTTTAA
TGTCGTACTTGCCGTTTTCATACTTGAACGCATAACCCCCGTCAGCAGCAATCAGCGGCCAGTGAAGTACG
GTTCTTGCAGTTGAACATCAGCGCGCTCTTACTTTCGCTTTCAGTTCTTTATCAGCGCGGGATCTCTTCCC
AGATTTTTGGCGGTCGGCAGCAG
SalI: GTCGAC
Stop codon: TCA
SR1: nucleotide sequence
Bam: GGATCC
```

B: Translation of SR1 nucleotides to amino acids and BLAST analysis

```
      GGTTATAAAACTATTGATAATAAAAATTTTTACTTTAGAAATGGTTTACCTCAGATATGA
       G   Y   K   T   I   D   N   K   N   F   Y   F   R   N   G   L   P   Q
```

```
I    emb|AM180355.1| D Clostridium difficile 630 complete genome
Length=4290252
Features in this part of subject sequence:
Toxin A
Score =  100 bits (54),  Expect = 5e-19
Identities = 56/57 (98%), Gaps = 0/57 (0%)
Strand=Plus/Plus
Query  1       GGTTATAAAACTATTGATAATAAAAATTTTTACTTTAGAAATGGGTTACCTCAGATA  57
               ||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Sbjct  803625  GGTTATAAAACTATTGATAATAAAAATTTTTACTTTAGAAATGGTTTACCTCAGATA  803681
```

Figure 2-25 Sequence analysis of the SR1 mutant

Colour key under Panel A shows the restriction sites for *Sal*I, *Bam* HI, and stop codon and the SR1 sequence. The sequence downstream from the *Bam* HI site is the coding sequence for MBP. Panel B (upper part) shows translation of the nucleotide sequence of the SR1 mutant. Panel B (lower part) shows BLAST output for the mutated SR1 amino acid sequence.

A: Nucleotide sequence for SR2 mutant

CAAATCCAAAGGGGCTTTTCTCATCAACACCAAAGAAATATATAACACCATCAATCTCGAAAAGTCCACC
AGCTGCAGCCATAGCAGTATCAGGCATAAAGTAATATACTTTACCATTAATAGTGTCGACTCAAGTAACT
GCTTTTGAATTATTACCAAAGTAATATATTTTTCCAAGTAAATGTAGGAATCTATTTTGATAACGTATAG
CTTGACCTTCTATATTGTTAGCATCCGTATTAGCAGGTGCAAAGTATTCAAATCCATTAGACCCTTTAAA
CACTCCTATCTGAGGTAAACCATTTCTAAAGTAAAAATTTTTATTATCAATAGTTTTATAACCAGGATCC
CCGGGTACCGAGCTCGAATTAGTCTGCGCGTCTTTCAGGGCTTCATCGACAGTCTGACGACCGCTGGCGG
CGTTGATCACCGCAGTACGCACGGCATACCAGAAAGCGGACATCTGCGGGATGTTCGGCATGATTTCACC
TTTCTGGGCGTTTTCCATGGTGGCGGCAATACGTGGATCTTTCGCCAACTCTTCCTCGTAAGACTTCAGC
GCTACGGCACCCAGCGGTTTGTCT

SalI: GTCGAC
Stop codon: TCA
SR1-LR-SR2: nucleotide sequence
BamHI: GGATCC

B: Translation of SR2 nucleotides to amino acids

```
 G  Y  K  T  I  D  N  K  N  F  Y  F  R  N  G  L  P  Q  I  G
GGTTATAAAACTATTGATAATAAAAATTTTTACTTTAGAAATGGTTTACCTCAGATAGGA

 V  F  K  G  S  N  G  F  E  Y  F  A  P  A  N  T  D  A  N  N
GTGTTTAAAGGGTCTAATGGATTTGAATACTTTGCACCTGCTAATACGGATGCTAACAAT

 I  E  G  Q  A  I  R  Y  Q  N  R  F  L  H  L  L  G  K  I  Y
ATAGAAGGTCAAGCTATACGTTATCAAAATAGATTCCTACATTTACTTGGAAAAATATAT

 Y  F  G  N  N  S  K  A  V  T  -
TACTTTGGTAATAATTCAAAAGCAGTTACTTGA
```

Figure 2-26 Sequence analysis of the SR2 mutant.

Colour key under Panel A show the restriction sites for *Sal*I, *Bam* HI, and stop codon and the SR1-LR-SR2 sequence. The sequence downstream from the *Bam* HI site is the coding sequence for MBP. Panel B shows translation of the nucleotide sequence of SR1-LR-SR2 to amino acid sequence.

```
>  emb|AM180355.1|  Clostridium difficile 630 complete genome
Length=42902
Sort alignments for this subject sequence by:
E value  Score  Percent identity
Query start position  Subject start position
Features in this part of subject sequence:
Toxin A
Score =  388 bits (210),  Expect = 6e-105
Identities = 210/210 (100%), Gaps = 0/210 (0%)
Strand=Plus/Plus

Query  1       GGTTATAAAACTATTGATAATAAAAATTTTTACTTTAGAAATGGTTTACCTCAGATAGGA 60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  803625  GGTTATAAAACTATTGATAATAAAAATTTTTACTTTAGAAATGGTTTACCTCAGATAGGA  803684

Query  61      GTGTTTAAAGGGTCTAATGGATTTGAATACTTTGCACCTGCTAATACGGATGCTAACAAT 120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  803685  GTGTTTAAAGGGTCTAATGGATTTGAATACTTTGCACCTGCTAATACGGATGCTAACAAT  803744

Query  121     ATAGAAGGTCAAGCTATACGTTATCAAAATAGATTCCTACATTTACTTGGAAAAATATAT 180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  803745  ATAGAAGGTCAAGCTATACGTTATCAAAATAGATTCCTACATTTACTTGGAAAAATATAT  803804

Query  181     TACTTTGGTAATAATTCAAAAGCAGTTACT  210
               |||||||||||||||||||||||||||||
Sbjct  803805  TACTTTGGTAATAATTCAAAAGCAGTTACT  803834
```

Figure 2-27 BLAST output of the SR2 amino acid sequence.

A: Nucleotide sequence for SR3 mutant

```
GGGGCTTTACTCATCACACCAAAGAATATATAACACCATCAATCTCGTCGACTCAACCAGCTGCAGCCAT
AGCAGTATCAGGCATAAAGTAATATACTTTACCATTAATAGTTTGCCATCCAGTAACTGCTTTTGAATTA
TTACCAAAGTAATATATTTTTCCAAGTAAATGTAGGAATCTATTTTGATAACGTATAGCTTGACCTTCTA
TATTGTTAGCATCCGTATTAGCAGGTGCAAAGTATTCAAATCCATTAGACCCTTTAAACACTCCTATCTG
AGGTAAACCATTTCTAAAGTAAAAATTTTTATTATCAATAGTTTTATAACCAGGATCCCCGGGTACCTTA
GTCTGCGCGTCTTTCAGGGCTTCATCGACAGTCTGACGACCGCTGGCGGCGTTGATCACCGCAGTACGCA
CGGCATACCAGAAAGCGGACATCTGCGGGATGTTCGGCATGATTTCACCTTTCTGGGCGTTTTCCATGGT
GGCGGCAATACGTGGATCTTTCGCCAACTCTTCCTCGTAAGACTTCAGCGCTACGGCACCCAGCGGTTTG
TCTTTATTAACCGCTTCCAGACCTTCATCAGTCAGCAGATAGTTTTCGAGGAACTCTTTCGCCAGCTCTT
TGTTCGGACTGGCGGCGTTAATACCTGCGCTCAGCACGCCAACGAACGGTTTGGATGGTTGACCCTTGAA
GGTCGGCAGTACCGTTACACCATAATTCACTTTGCTGGTGTCGATGTTGGACCATGCCCACGGGCCGTTG
ATGGTCATCGCTGTTTCGCCTTTATTAAAGGCAGCTTCTGCGATGGAGTAATCGGTGTCTGCATTCATGT
GTTTGTTTTTAATCAGGTCAACCAGGAAGGTCAGACCCGCTTTCGCGCCAGCGTTATCCACGCCCACGTC
TTTAATGTCGTACTTGCCGTTTTCATACTTGAACGCATAACCCCCGTCAGCAGCAATCAGCGGCAGGTGA
AGTACGGTTCTTGCAGTTGAACATCAGCGCGCTCTACTTTCGCTTCAGTTCTTTTATCAGCGCGGATCTC
TTCCCAGTTTTGGCGGGTCGCAGCCGATCTTTGTATAATCAGCGATACGCTCACAGCGAATCCGTAGCCA
TCAGCTGCGTGTAACGTACGCAATCAAGTACGAACGCTACCTGCACCATGTCGGGATGATTCAGCTCACA

SalI: GTCGAC
Stop codon: TCA
SR1-LR-SR2-SR3: nucleotide sequence
BamHI: GGATCC
```

B: Translation of SR3 nucleotides to amino acids

```
 G  Y  K  T  I  D  N  K  N  F  Y  F  R  N  G  L  P  Q  I  G
GGTTATAAAACTATTGATAATAAAAATTTTTACTTTAGAAATGGTTTACCTCAGATAGGA

 V  F  K  G  S  N  G  F  E  Y  F  A  P  A  N  T  D  A  N  N
GTGTTTAAAGGGTCTAATGGATTTGAATACTTTGCACCTGCTAATACGGATGCTAACAAT

 I  E  G  Q  A  I  R  Y  Q  N  R  F  L  H  L  L  G  K  I  Y
ATAGAAGGTCAAGCTATACGTTATCAAAATAGATTCCTACATTTACTTGGAAAAATATAT

 Y  F  G  N  N  S  K  A  V  T  G  W  Q  T  I  N  G  K  V  Y
TACTTTGGTAATAATTCAAAAGCAGTTACTGGATGGCAAACTATTAATGGTAAAGTATAT

 Y  F  M  P  D  T  A  M  A  A  A  G  -
TACTTTATGCCTGATACTGCTATGGCTGCAGCTGGTTGA
```

Figure 2-28 Sequence analysis of the SR3 mutant

Colour key under Panel A show the restriction sites for *Sal*I, *Bam* HI, and stop codon and the SR1-LR-SR2-SR3 sequence. The sequence downstream from the *Bam* HI site is the coding sequence for MBP. Panel B shows the translation of the nucleotide sequence of SR1-LR-SR2-SR3 to amino acid sequence.

```
>  emb|AM180355.1|  Clostridium difficile 630 complete
Genome Length=4290252
Sort alignments for this subject sequence by:
E value  Score  Percent identity
Query start position  Subject start position
Features in this part of subject sequence:
Toxin A
Score =  510 bits (276),  Expect = 2e-141 Identities = 276/276
(100%), Gaps = 0/276 (0%)
Strand=Plus/Plus

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               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  61      GTGTTTAAAGGGTCTAATGGATTTGAATACTTTGCACCTGCTAATACGGATGCTAACAAT  120
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  803685  GTGTTTAAAGGGTCTAATGGATTTGAATACTTTGCACCTGCTAATACGGATGCTAACAAT  803744

Query  121     ATAGAAGGTCAAGCTATACGTTATCAAAATAGATTCCTACATTTACTTGGAAAAATATAT  180
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  803745  ATAGAAGGTCAAGCTATACGTTATCAAAATAGATTCCTACATTTACTTGGAAAAATATAT  803804

Query  181     TACTTTGGTAATAATTCAAAAGCAGTTACTGGATGGCAAACTATTAATGGTAAAGTATAT  240
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  803805  TACTTTGGTAATAATTCAAAAGCAGTTACTGGATGGCAAACTATTAATGGTAAAGTATAT  803864

Query  241     TACTTTATGCCTGATACTGCTATGGCTGCAGCTGGT  276
               ||||||||||||||||||||||||||||||||||||
Sbjct  803865  TACTTTATGCCTGATACTGCTATGGCTGCAGCTGGT  803900
```

Figure 2-29 BLAST output of the SR3 nucleotide acid sequence

A: Nucleotide sequence for LR mutant

TTACCAAAGTAATATATTTTTCCAAGTAAATG**GTCGAC**TCAATTTTGATAACGTATAGCTTGACCTTCT
ATATTGTTAGCATCCGTATTAGCAGGTGCAAAGTATTCAAATCCATTAGACCCTTTAAACACTCCTATC
TGAGGTAAACCATTTCTAAAGTAAAAATTTTTATTATCAATAGTTTTATAACCA**GGATCC**CCGGGTACC
GAGCTCGAATTAGTCTGCGCGTCTTTCAGGGCTTCATCGACAGTCTGACGACCGCTGGCGGCGTTGATC
ACCGCAGTACGCACGGCATACCAGAAAGCGGACATCTGCGGGATGTTCGGCATGATTTCACCTTTCTGG
GCGTTTTCCATGGTGGCGGCAATACATGGATCTTTCGCCAACTCTTCCTCGTAAGACTTCAGCGCTACG

SalI: GTCGAC
Stop codon: TCA
SR1-LR: nucleotide sequence
BamHI: GGATCC

B: Translation of LR nucleotides to amino acids

```
       G  Y  K  T  I  D  N  K  N  F  Y  F  R  N  G  L  P  Q  I  G
      GGTTATAAAACTATTGATAATAAAAATTTTTACTTTAGAAATGGTTTACCTCAGATAGGA
       V  F  K  G  S  N  G  F  E  Y  F  A  P  A  N  T  D  A  N  N
      GTGTTTAAAGGGTCTAATGGATTTGAATACTTTGCACCTGCTAATACGGATGCTAACAAT
       I  E  G  Q  A  I  R  Y  Q  N  -
      ATAGAAGGTCAAGCTATACGTTATCAAAATTGA
```

```
>  emb|AM180355.1|  Clostridium difficile 630 complete genome
Length=4290252
Sort alignments for this subject sequence by:
E value  Score  Percent identity
Query start position  Subject start position
Features in this part of subject sequence:
Toxin A
Score =  279 bits (151),  Expect = 2e-72
Identities = 151/151 (100%), Gaps = 0/151 (0%)
Strand=Plus/Minus

Query  1       ATTTTGATAACGTATAGCTTGACCTTCTATATTGTTAGCATCCGTATTAGCAGGTGCAAA  60
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  121     AAAATTTTTATTATCAATAGTTTTATAACCA  151
               |||||||||||||||||||||||||||||||
Sbjct  803654  AAAATTTTTATTATCAATAGTTTTATAACCA  803624
```

Figure 2-30 Sequence analysis of the LR mutant

Colour key under Panel A shows the restriction sites for *Sal*I, *Bam* HI, and stop codon and the SR1-LR sequence. The sequence downstream from *Bam* HI sequence is the coding sequence for MBP. Panel B (upper part) shows translation of the nucleotide sequence of the LR mutant. Panel B (lower part) shows BLAST output for the mutated LR amino acid sequence.

2.3.1.14 Expression and purification of MBP- fusions

E. coli bacteria transformed with the MBP-TcdA truncated fusions were grown, induced and sonicated as mentioned earlier. Proteins were purified from the bacterial lysates using amylose resin affinity chromatography. The proteins were eluted using 15 mM maltose.

2.3.1.15 Characterisation of the MBP-fusions by SDS-PAGE and electroblotting

The purified proteins were characterised by SDS-PAGE and Western-blotting. Gels were stained with Commassie Brilliant Blue, while blots were probed with anti-MBP antibody (Figures 2.31, 2.32 and 2.33). To assist the analysis, samples of native MBP were analysed along with of the intact TcdA-MBP fusion and the mutant proteins to highlight the differences in size.

Panel A in Figure 2.31 shows SDS-PAGE and (Panel B) Western blot of native MBP (lane 1 in each Panel), several samples of the SR1 mutant (lanes 2, 3 and 4) and the TcdA-MBP fusion protein (lane 5). Although the SR1 repeat sequence added only modestly to the molecular weight of MBP, the difference is apparent in Figure 2.31.

Figure 2.32 shows that the MBP-LR fusion could be successfully purified. Note that the protein migrated alongside a marker protein intermediate between 39 kDa and 51 kDa, at a molecular weight greater than the MBP-SR1 fusion but less than that of the full length TcdA-MBP fusion (Figure 2.31).

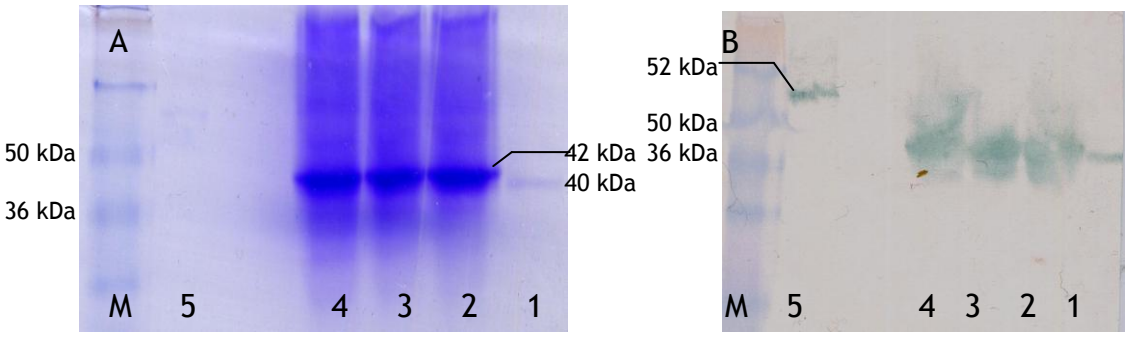

Figure 2-31 SDS-PAGE and electro-blotting analysis of SR1 mutant

Purified proteins from induced cultures expressing MBP, MBP-SR1 and the TcdA-MBP fusion were characterised by SDS-PAGE (Panel A) and by electro-blotting with anti-MBP serum (Panel B).

In both panels, lane 1 shows native MBP, lanes 2-4 show the MBP-SR1 fusion protein, and lane 5 shows ful-length TcdA-MBP fusion. Lane M contains a protein size ladder.

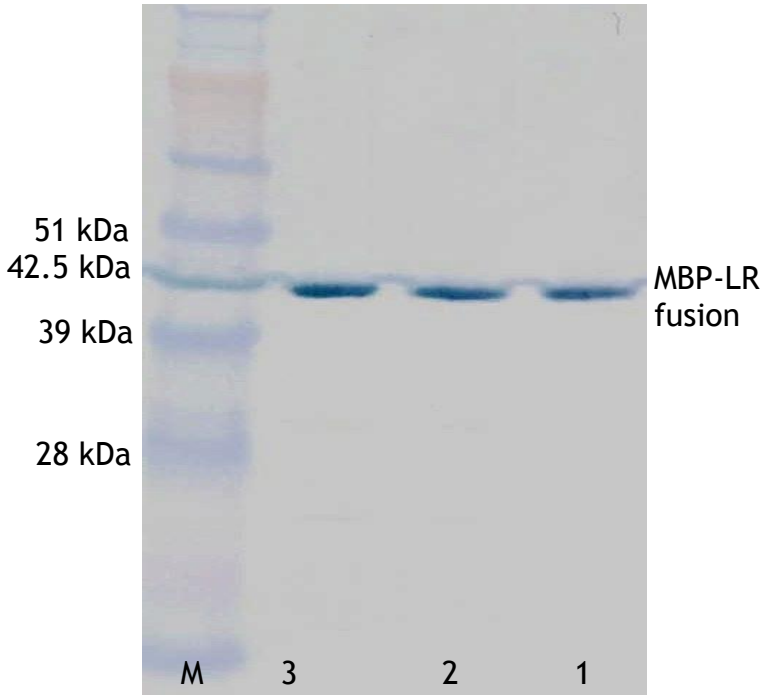

Figure 2-32 Electro-blotting analysis of the MBP-LR fusion protein.

Purified proteins from induced cultures expressing MBP-LR fusion were characterised by electro-blotting with anti-MBP serum. Three fractions of protein eluted from amylose resin (lanes 1, 2 and 3) for analysis. M is the protein size ladder.

For comparison of all fusions carrying defined regions of the TcdA receptor-binding site, MBP-SR1, MBP-LR-SR2 (the first fusion described in this part of the project), MBP-LR, MBP-SR2 and MBP-SR3 were analysed by SDS-PAGE and Western blotting. The fusion proteins were loaded side by side on the gel to show the step-wise difference in size. Figure 2.33 shows the stained gel (Panel A) and the Western blot (Panel B). The gel and blot show the step-wise difference in size between the proteins ranging from MBP-SR1 (lane 1 in each panel) through to the MBP-SR3 fusion (lane 5).

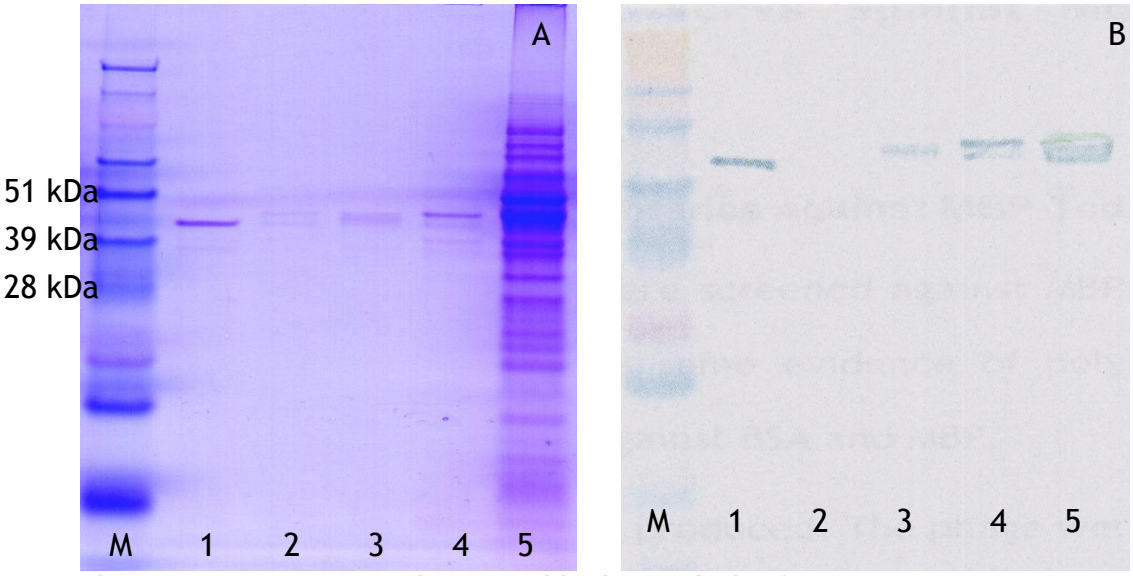

Figure 2-33 SDS-PAGE and Western blotting analysis of MBP-SR1, MBP-LR-SR2, MBP-LR, MBP-SR2 and MBP-SR3 fusion proteins

Purified proteins from induced cultures expressing the fusions were characterised by SDS-PAGE (Panel A) and by electro-blotting with anti-MBP serum (Panel B).

In both panels, lane 1 shows MBP-SR1, lane 2 MBP-LR-SR2, lane 3 MBP-LR, lane 4 MBP-SR2 and lane 5 MBP-SR3. Lane M contains a protein size ladder.

2.4 Discussion

TcdA is produced by pathogenic strains of *Clostridium difficile* that cause antibiotic associated diarrhoea. In this phase of the study, recombinant MBP fusion proteins were constructed to allow later isolation of recombinant antibodies against defined regions of the receptor binding domain of TcdA. A fusion comprising the LR and SR2 repeat sequences was available at the start of the project. This was complemented by construction of a fusion containing all repeat elements (i. e. SR1, LR, SR2, SR3 and SR4). Truncated proteins – MBP-SR1, MBP-SR1-LR, MBP-SR1-LR-SR2, and MBP-SR1-LR-SR2-SR3 - were constructed from this using site directed mutagenesis. Sequences were checked to confirm that constructions were as intended and all proteins were successfully expressed and purified.

Expressing proteins fused to MBP is a well established protocol. In an early study, di Guan and colleagues constructed vectors specifically to facilitate the fusion of peptides and proteins to the C-terminus of MBP (di Guana et al., 1988). These vectors have been used widely and adapted; in one study, staphylococcal enterotoxin A was expressed as a fusion to MBP (Aitken et al., 1994). Fusion of proteins to MBP facilitates purification providing the carrier's affinity for cross-linked amylose is preserved and proteins bound to this affinity matrix can be recovered under mild conditions by competitive elution using maltose solutions. One beneficial characteristic of MBP as a carrier for fusion proteins is that MBP can act as molecular chaperone. This was revealed by Bach and his group when they examined the stability and functionality of a wide panel of scFv antibodies that were fused to MBP and compared their properties with native forms of the same scFvs (Bach et al., 2001). In light of their findings, MBP seems to act as a

molecular chaperone that can enhance the solubility and stability of the fused scFv.

Bossé and colleagues used MBP to facilitate the purification of the small, heat-stable enterotoxin b of *E. coli*. The toxin was fused to MBP and once purified, the mature heat-stable enterotoxin was then cleaved from the MBP. The group were able to detect both the cleaved and uncleaved fusion protein with a monospecific polyclonal rabbit antisera generated against enterotoxin b (Bossé et al., 1993). In this sort of application, yields of protein are important to the overall aims. Acheson et al examined a variety of expression vectors to optimise a method that would give the highest yield of Shiga-like toxin-II B subunit. One particularly successful approach was to fuse SLT-II B to MBP (Acheson et al., 1995).

In other work with bacterial toxins, Weller and colleagues studied the structure and function of Streptolysin O toxin (SLO) (Weller et al., 1996). The group described the cloning of SLO as a fusion to MBP and expression of the construct in *E. coli*. Interestingly, they revealed that cleaved and uncleaved MBP-SLO showed similar haemolytic and cytotoxic activities against human fibroblasts and keratinocytes, illustrating that attachment of the MBP carrier did not interfere with SLO function to a detectable extent. In studies of clostridia, fragments of DNA covering the entire genes for *Clostridium difficile* toxins A and B have been cloned in the expression vector pMALc to create MBP fusions, as well as in pET vectors. Recombinant proteins were purified by affinity chromatography using amylose resin for MBP fusions and nickel chelating columns for His-tag fusions from the pET system. Antibodies were raised against these constructs and then tested for the ability to neutralise the clostridial

toxins in a hamster model. Antibodies against the carboxy-terminal fragments of toxin A and toxin B were most effective in toxin-neutralisation (Kink and Williams, 1998). This illustrates both the value of MBP as a carrier for TcdA sequences in the present study and the importance of the C-terminal, receptor binding domain in toxin neutralisation.

More recently, the binding domain of *Clostridium botulinum* neurotoxin F (FHc) was expressed as a fusion to MBP using *E. coli* as an expression host. MBP-FHc was purified and along with FHc, prepared by cleavage from the MBP carrier, both proteins were examined for their ability to generate protective immune responses against toxin challenge. Both recombinant proteins were able to generate immune response against the toxin after intraperitoneal or intramuscular administration (Holley et al., 2000).

This phase of the study focussed up the construction MBP fusions proteins using features taken from the receptor-binding domain of TcdA, a crucial virulence factor produced by *C. difficile*. One prominent feature of this region of the toxin is the presence of repeating units of amino acids that are believed to form a series of binding sites for receptor molecules on the intestinal epithelium of the host. This makes the toxin a multivalent lectin able to recognise a putative receptor, Galα1-3Galß1-4GlcNAc trisachariде. This receptor is thought to be present on the surface of rabbit erythrocytes, bovine thyroglobulin, a range of Ig and non-Ig molecules in human milk, and on the brush border membranes of hamster ileum (Ho et al., 2005, Tucker and Wilkins, 1991).

In light of this knowledge, the SR1, LR, SR2, SR3, and SR4 sequences were fused to MBP for expression and purification so that in a later stage of the project, scFv antibodies could be isolated against this recombinant protein.

Construction of the fusion proteins was successful as evidenced by SDS-PAGE and Western blotting. The TcdA-MBP fusion was successfully detected at the expected size of 52 kDa on SDS-PAGE and clear differences in size were evident with native MBP, the LR-SR2-MBP constructed in advance of the project start, and the smaller MBP fusions created by site directed mutagenesis (Deng and Nickoloff, 1992). The latter process was accomplished successfully by PCR methods that introduced a unique restriction site so that mutants could be conveniently identified.

SDS-PAGE and Western blotting revealed minor bands suggestive of proteolytic digestion of some of the fusion proteins but overall, this phase of the project accomplished its aims. Potentially, future work could attempt to establish whether receptor-binding activity was retained in these fusion proteins by testing their ability to bind the Galα1-3Galß1-4GlcNAc trisacharide.

Chapter Three Antibody Phage Display

3.1 Introduction

During the past decade, developments in antibody cloning technology have facilitated the development of a huge diversity of recombinant forms of antibody molecules that can be used for research, diagnosis, and therapy. These technological advances have also allowed researchers to create antibodies with specificities that could not be achieved with conventional approaches such as the development of antibodies against highly-conserved "self" sequences, toxic moieties *etc* (Boss et al., 1984, Kontermann and Müller, 1999, George et al., 1994, Winter and Milstein, 1991). Amongst these developments, phage display holds a particularly prominent place.

3.1.1 Principles of phage display

As described earlier, phage display involves the introduction of foreign sequences into genes that encode the capsid proteins of bacteriophage, often the temperate, filamentous phage of *E. coli*. The resulting fusions are expressed and incorporated into the phage such that the foreign sequence is displayed on the phage surface where it is accessible for various experimental purposes (Arap, 2005).

These methods provide powerful technologies for selecting a specific antibody from a diverse antibody pool such as a library. Each phage particle contains the genes for the displayed antibody and hence attachment of phage to a target molecule through a specific antibody interaction recovers the coding sequence for that antibody. This technique has thus been to isolate antigen specific antibodies.

The phage display cycle includes four essential features that are common to all selection platforms: genotypic diversity, genotype/phenotype coupling, selection and amplification (Figure 1.3 in Chapter 0ne) (Bradbury and Marks, 2004). Selection can be executed quickly, efficiently and with high specificity.

3.1.2Recombinant antibody libraries

Many different approaches have been used to create diverse antibody libraries from which useful antibodies can be extracted by phage display. The natural immune system of an individual can be stimulated through vaccination, experimental immunisation or infection so that particular specificities are present in high numbers. For example, Kramer and colleagues have reported the isolation of scFv antibodies able to neutralise rabies virus (Kramer et al., 2005). The phage display libraries in this study were prepared from human vaccinees. Similarly, Moulard et al generated a Fab library from an HIV-positive patient, isolating from this resource an anti-gp120 antibody capable of neutralising a broad range of HIV-1 isolates (Moulard et al., 2002).

While these so-called “immune libraries” have great value, they rely upon the capacity of the host immune system to raise a response, making it very difficult to prepare anti-self antibodies or antibodies against highly toxic targets. Equally, the way in which they are prepared and their modest size makes it unlikely that they contain antibodies against virtually any target molecule. To meet the challenges of a “single pot” library (Nissim et al., 1994), investigators have created libraries from the naïve repertoire or undertaken extensive diversification of natural antibodies *in vitro* to create large resources from which

a very wide range of specificities can be extracted. For example, semi-synthetic libraries have provided scFvs antibodies against conformational epitopes of the heat-labile enterotoxin of *E. coli* (Chung et al., 2008).

3.1.3 Tomlinson libraries

For this study, two libraries constructed by Tomlinson and colleagues at the MRC Centre for Protein Engineering were used (Holt et al., 2000). These are semi-synthetic scFv libraries in which single VH and V kappa framework sequences were diversified *in vitro* by targeting randomised sequence to key residues in the antigen-binding complementarity determining regions (CDRs). Positions were identified from structural studies as being important for the interaction with antigen: specifically, H50, H52, H52a, H53, H55, H56, H58, H95, H96, H97, H98 were diversified in the heavy chain; in the light chain, randomised amino acids were created at positions L50, L53, L91, L92, L93, L94 and L96. To achieve this, nominated codons in library I were altered to DVT (A, G or T; A, C or G; T) and in library J, NNK (any base; any base; G or T) (Holt et al., 2000). It is estimated that each library contains around 1 x 10^8 different scFv specificities and studies have successfully extracted antibodies against a very wide range of targets from these resources.

3.1.4 Aims for the experiments

Using MBP fusion proteins as targets, the aim for this phase of the project was to extract and characterise anti-TcdA antibodies from the Tomlinson phage display libraries using simple selection and amplication methods. These entailed binding the target proteins to a plastic surface, applying the libraries, and extracting those virus capable of adhering to the target-coated surface.

3.2 Materials and methods

3.2.1 Characterisation of plasmids

Bacteria carrying anti-BSA and anti-ubiqutin scFvs were provided as control reagents by MRC Geneservice along with the I & J Tomlinson libraries. All are based upon the pIT2 vector (Appendix 6.10). The bacterial clones were plated on TYE agar containing 100 ug/ml ampicillin and 1% glucose. Bacterial stocks were made from each plate and stored at -70 ^{o}C in 15-20% glycerol. Fresh single colonies were picked from selective plates, and grown overnight in 3-5 ml of 2xTY for isolation of DNA encoding the scFv antibodies. Plasmid DNA was extracted from the overnight culture using a QIA mini prep kit as detailed earlier. Extracted DNA was digested with *Nco*I and *Not*I restriction enzymes to isolate the scFv coding sequence from the pIT2 vector (Appendix 6.10). Reaction mixtures were set up containing 1 µl of *Nco*I (Promega, UK; 10U / µl), 1 µl of *Not*I (Promega, UK; same activity), 1 µl of 10x reaction Buffer D (Promega; 60mM Tris-HCl, PH 7.9, 1.5 M NaCl, 60 mM $MgCl_2$ and 10mM DTT), 5 µl of plasmid DNA and the reaction volumes were increased to 10 µl with sterile distilled water. Reactions were incubated at 37 ^{o}C for 3 h. Products were analysed by electrophoresis at 100 volts for 1h on 1% agarose gels and visualised on a UV transilluminator. Images were captured with a camera system.

3.2.2 Growth of E. coli TG1 bacteria

E. coli TG1 bacteria were provided with the libraries as a glycerol suspension. A secondary stock of TG1 bacteria was prepared by inoculating sample of TG1 to TYE agar containing no antibiotic and incubating overnight at 37 ^{o}C. A single colony was picked from the overnight culture and inoculated into 3-5 ml of 2xTY

liquid medium. The culture was then incubated overnight at 37 ^{o}C in a shaking incubator at 150 rpm. The overnight culture was centrifuged at 3,300 x g for 10 min and the supernatant was removed. The pellet was resuspended in 1 ml of 2xTY liquid medium with 15-20% glycerol and stored at -70 ^{o}C.

3.2.3Growth and titration of KM13 helper phage

A stock of KM13 helper phage was prepared using TG1 bacteria. TG1 bacteria were inoculated on TYE agar and incubated overnight at 37 ^{o}C. Next day, a single colony of TG1 was inoculated into 3-5 ml of 2xTY liquid medium and grown overnight at 37 ^{o}C in a shaking incubator at 150 rpm. The following day, sufficient overnight culture was added to 50 ml of 2xTY to reach an OD at 600 nm of 0.07-0.09 and the suspension was grown at 37 ^{o}C with shaking until the OD reached 0.4. Aliquots of 200 µl of TG1 culture were then infected with 10 µl samples of 100-fold serial dilutions of KM13 helper phage. The mixtures were incubated statically at 37 ^{o}C for 30 min before addition of 3 ml of H-top agar that had been pre-warmed to 42 ^{o}C. The contents were spread on to TYE agar plates pre-warmed to 42 ^{o}C. The medium contained no antibiotics. The plates were allowed to set and then incubated overnight at 37 ^{o}C. Next day, a single plaque was carefully picked and inoculated into 5 ml fresh TG1 culture at an OD of 0.4 (600 nm) and grown at 37 ^{o}C in shaking incubator at 150 rpm for 2 h. The culture was then added to 500 ml of 2xTY and grown with shaking at 150 rpm for 1h at 37 ^{o}C. The temperature was then reduced to 30 ^{o}C, kanamycin was added to a final concentration of 50 µg/ml and the culture was grown overnight. The following day, bacteria were pelleted by centrifugation at 10,800 x g for 15 min, and 400 ml of the supernatant was mixed with 100 ml PEG/NaCl (20% polyethylene glycol 6000, 2.5 M NaCl) to precipitate the phage. The mixture was

incubated on ice for 1 h then centrifuged at 10,800 x g for 30 min. The supernatant was removed and the pellet was resuspended in 8 ml PBS. Two ml PEG/NaCl was added, mixed and incubated on ice for 20 min to re-precipitate the virus. The mixture was centrifuged at 3,300 x g for 30 min and the supernatant was removed. The tube was respun briefly and the remaining dregs of PEG/NaCl were eliminated. The pellet was then resuspended in 5 ml of PBS and spun at 11,600 x g for 10 min in a microcentrifuge tube to pellet any remaining bacterial debris. The supernatant which contained the helper phage was then transferred to a clean tube and stored at 4 ^{0}C (short term) or in 15-20% glycerol (longer term storage at –70 ^{o}C).

The helper phage stock was titrated by the addition of 5 µl of trypsin stock solution to 45 µl of helper phage. The mixture was incubated for 30 min at 37 ^{o}C. One µl of the trypsin-treated phage was added to 1 ml PBS and then five, 100-fold serial dilutions were prepared in 1 ml PBS. Aliquots of 50 µl from each dilution were mixed with 1 ml of freshly prepared TG1 (OD of 0.4). Infected TG1 from each dilution were mixed with 3 ml of H-top agar and plated on TYE agar as described earlier. Plaques were counted to establish viral titre and compared to infections carried out with untreated phage.

3.2.4Growing E. coli HB2151 bacteria

E. coli HB2151 bacteria, a non-suppressor strain used for expression of soluble scFv antibodies were provided with as a glycerol stock with the libraries. A secondary stock of HB2151 was prepared by inoculating a sample to TYE agar containing no ampicillin and incubating overnight at 37 ^{o}C. A single colony was picked from the overnight culture and inoculated into 3-5 ml of 2xTY liquid medium. The culture was then incubated overnight at 37 ^{o}C in a shaking

incubator at 150 rpm. The overnight culture was centrifuged at 3,300 x g for 10 min and the supernatant was removed. The pellet was resuspended in 1 ml of 2xTY liquid medium and stored at -70 ^{o}C in glycerol.

3.2.5Preparation of competent E. coli HB2151

E. coli HB2151 was grown overnight on TYE agar at 37 ^{o}C. The next day, a single colony was inoculated into 5-10 ml of 2xTY and grown overnight at 37 ^{o}C with shaking at 150 rpm. Following this, overnight culture was added to 50 ml of 2xTY broth to reach an OD of about 0.07-0.09. The culture was incubated at 37 ^{o}C in a shaking incubator and growth was monitored until the OD reached 0.5. At this point, 40 ml of culture was sampled and centrifuged at 3,000 rpm for 8 min at 4 ^{o}C. The supernatant was removed and the pellet was resuspended in half the culture volume of cold, sterile 50 mM calcium chloride. The suspension was incubated in an ice-water bath for 20 min. The mixture was then spun as before and the pellet was gently resuspended in one-tenth the original volume of cold, sterile 50 mM calcium chloride to yield the final competent cells. Aliquots of 200 µl of competent HB2151 cells were stored in 15-20% glycerol at -70 ^{o}C.

3.2.6Expression of anti-BSA scFvs

3.2.6.1 Growth of bacteria

E. coli TG1 bacteria carrying an anti-BSA scFv were used for experiments as a positive control. The strain was inoculated on TYE agar containing 100 µg/ml ampicillin and 1% glucose and then incubated overnight at 37 ^{o}C. A single colony was picked from overnight culture and inoculated to 3-5 ml 2xTY liquid culture containing 100 µg/ml ampicillin and 1% glucose and then grown overnight at 37

^{o}C with shaking at 150 rpm. Plasmid DNA was extracted from the overnight culture as described earlier.

3.2.6.2 Transformation of anti-BSA scFv to HB2151

A 50 µl aliquot of freshly prepared, competent *E. coli* HB2151 was transformed with 2 µl of plasmid DNA as described earlier. The bacteria were recovered with 1 ml of SOC medium pre-warmed to room temperature and were incubated with shaking at 225 rpm for 1 h at 37 ^{o}C. A sample of 50 µl of the transformation mix was plated on TYE agar plate containing 100 µg/ml ampicillin and 1% glucose, spread evenly, and was then grown overnight at 37 ^{o}C.

3.2.6.3 Expression of soluble anti-BSA scFv

A single colony was picked from the overnight culture of transformed HB2151 cells and inoculated to 3-5 ml of 2xTY containing 100 µg/ml ampicillin and 1% glucose. The culture was then incubated overnight at 37 ^{o}C with shaking at 150 rpm. The following day, sufficient culture was inoculated to 100 ml of 2xTY containing 100 µg/ml ampicillin and 0.1% glucose in 500 ml flask to achieve an OD of 0.07-0.09. The culture was then grown at 37 ^{o}C in a shaking incubator at 150 rpm until the OD reached 0.9. The culture was then induced with IPTG to a final concentration of 1mM. The temperature of incubation was reduced to 30 ^{o}C and growth was continued overnight with shaking at 150 rpm. The overnight culture was centrifuged at 3000 x g for 10 min and the supernatant containing the recombinant anti-BSA scFv was collected for storage at -20 ^{o}C.

3.2.6.4 Characterisation of soluble anti-BSA antibody

Samples of culture supernatant containing the anti-BSA scFv were analysed by SDS-PAGE and electroblotting exploiting the c-myc tag fused to each scFv (Appendix 6.10). Samples were separated on 10% SDS-PAGE gels and blotted to nitrocellulose membranes as mentioned earlier. Blots were blocked with 2% skimmed milk in PBS and probed with 1/5000 dilution of an anti c-myc antibody and an HRP-conjugate. Signals were developed with TMB substrate and the reaction was stopped by washing the membrane with distilled water.

3.2.7 Tomlinson I and J scFv libraries

Tomlinson I and J libraries were constructed at the MRC Centre for Protein Engineering, Cambridge, UK and obtained from MRC Geneservice. The libraries were supplied as 500 µl aliquots in *E. coli* TG1 preserved in glycerol. Aliquots were kept at -70 ^{o}C prior to processing.

3.2.7.1 Production and titration of Tomlinson I and J phage

3.2.7.1.1 Production of virus

The five hundred µl aliquots of each library were added to 200 ml 2xTY broth containing 100 µg/ml ampicillin and 1% glucose in separate flasks. The cultures were grown with shaking at 150 rpm at 37 ^{o}C until the OD reached 0.4. Fifty ml of each culture were transferred to 250 ml flasks and then infected with $2x10^{11}$ KM13 helper phage. The remaining bacterial cultures were mixed with glycerol and stored at –70 ^{o}C, creating secondary bacterial stocks. Infected cultures were incubated statically at 37 ^{o}C for 30 min then centrifuged at 3000 x g for 10 min. Bacterial pellets were resuspended in separate flasks using 100 ml 2xTY supplemented with 100 µg/ml ampicillin, 50 µg/ml kanamycin and 0.1% glucose,

and then cultured at 30 ^{o}C overnight in a shaking incubator (150 rpm). The following day, cultures were pelleted at 3,300 x g for 30 min. Cold PEG/NaCl (20 ml) was mixed with each supernatant sample (80 ml) and incubated on ice for 1 hr to precipitate phage from each library. The mixtures were then centrifuged at 3,300 x g for 30 min and the supernatants were removed. The tubes were briefly spun and remaining PEG/NaCl was removed. Each pellet was resuspended in 4 ml of PBS and centrifuged at 11,600 x g for 10 min. The supernatant, which contained the library phage, was transferred to clean tubes and stored at -70 ^{o}C in 15-20% glycerol.

3.2.7.1.2 Titration

After production of phage libraries, virus were titrated. As described earlier, TG1 bacteria were grown to an OD 0.4 and then 900 µl aliquots were infected with samples from six, 100 fold serial dilutions of phage produced from each library. Since infection with library phage delivered phagemid DNA from the virus to the bacterial host, infection resulted in conversion from ampicillin sensitivity to ampicillin resistance. Hence, samples of 10 µl were taken from each infection mix and spread evenly on TYE agar containing 100 µg ampicillin and 1% glucose. The plates were incubated at 37 ^{o}C overnight. Antibiotic-resistant colonies were counted next day and the concentrations of I and J phage were calculated.

3.2.7.2 Selection of scFv antibodies against TcdA-MBP fusion proteins

Twenty four individual clones were picked at random from each of the Tomlinson libraries I and J, phage were prepared using the methods described earlier and the virus were screened against native MBP, milk, and BSA to establish whether the libraries contained polyreactive clones at high frequency.

Wells of an ELISA plate were coated with 100 µl of 50 µg/ml of native MBP (purification described earlier), skimmed milk in PBS or BSA. Separate plates were prepared for screening of libraries I and J, each plate carrying 24 wells coated with each target protein. ELISA plates were then incubated overnight at 4 ^{o}C. Next day, the plates were washed 3 times with PBS and then blocked with 200 µl of 2% milk in PBS and incubated at room temperature for 2 h. The plates were washed 3 times with PBS and then about 10^6 cfu (colony forming units) of monoclonal phage from each library diluted into 100 µl PBS were added to the coated wells. Each single clonal stock from each library was tested against the 3 target protein samples. Plates were incubated at room temperature for 1 h to allow phage to bind and then washed 10 times with PBS containing 0.1% Tween-20. One hundred µl of HRP-anti-M13 antibody (GE Healthcare, UK) diluted to 1/5000 was added to each well and the plates were incubated at room temperature for 1 h. They were then washed 3 times with PBS containing 0.1% Tween-20 and reactions were then developed with 100 µl of TMB for 5-15 min at room temperature. The reaction was then stopped with 50 µl of 1 M sulfuric acid.

3.2.7.2.1 Extraction of anti-TcdA scFvs from Tomlinson libraries I and J

Having established that polyreactivity against invidual proteins was not a significant issue, phage display techniques were used with the TcdA-MBP fusion protein to extract scFvs from the libraries that were reactive with the putative Toxin A receptor-binding site. Expression and purification of the target protein was described earlier.

First round of selection

Two immunotubes were coated with 4 ml of purified MBP-TcdA fusion protein containing all the repeat motifs thought to contribute to receptor interaction. Tubes were incubated overnight at 4 ^{o}C. The following day, the coating material was poured off and the tubes were washed 3 times with PBS. The tubes were blocked with 2% skimmed milk in PBS for 2 h at room temperature. During the blooking, approximately 10^{12} cfu phage from libraries I and J were pre-incubated in separate tubes with 5% milk and about 250 µg/ml native MBP to eliminate from later selection those phage with reactivity towards milk constituents and the MBP carrier protein. After these steps, the blocking solution was poured from the selection tubes and they were washed 3 times with PBS. One of the tubes received the pre-incubated phage from library I, while the other tube received the pre-incubated phage from library J. The tubes were then sealed and rotated for 1 h at room temperature to allow attachment of phage bearing reactive scFvs. Following this, the tubes were incubated vertically for a further 1 h at room temperature. The contents of both tubes were discarded and the tubes were washed 10 times with 0.1% Tween-20 in PBS and the excess of the washing solution was shaken out. Those phage bound to the coated tubes were eluted by adding 500 µl of trypsin-PBS (50 µl of 10 mg/ml trypsin stock solution added to 450 µl of PBS) and rotating the tubes for 10 min at room temperature.

Titration and amplification of phage from the first round of selection

As described earlier, *E.coli* TG1 bacteria were grown to an OD of 0.4 and 250 µl of the eluted phage from each library were infected to 1.75 µl of the bacteria. The remaining 250 µl of the viral eluate was kept at 4 ^{o}C. The infected cultures were incubated statically at 37 ^{o}C for 30 min before 100 fold serial dilutions were prepared and 10 µl from each dilution was spread to TYE agar

containing 100 µg/ml ampicillin and 1% glucose. Plates were incubated overnight at 37 ^{o}C so that the titre of the recovered phage ("output") could be established.

The remaining cultures of infected *E. coli* TG1 from each library were centrifuged at 11,600 x g for 5 min and then resuspended in 50 µl of 2xTY broth. The resuspended bacteria were spread on separate TYE plate containing 100 µg/ml ampicillin and 1% glucose. The plates were grown at 37 ^{o}C overnight and then the resulting colonies were scraped into 2 ml of 2xTY. One ml of suspension from each library was stored in 15-20% glycerol at -70 ^{o}C, while 50 µl from each library were used for phage rescue.

Rescue of phage from the first round of selection

The 50 µl samples of bacteria were added to 50 ml of 2xTY containing 100 µg/ml ampicillin and 1% glucose, and grown at 37 ^{o}C with shaking at 150 rpm until the OD reached 0.4. Aliquots of 10 ml from each culture were infected with $5x10^{10}$ KM13 helper phage and were incubated statically at 37 ^{o}C for 30 min to allow infection to take place. Bacteria were then pelleted at 3,000 x g for 10 min and the cells were resuspended in 50 ml of 2xTY containing 100 µg/ml ampicillin, 50 µg/ml kanamycin and 0.1% glucose. Cultures were grown in a shaking incubator at 150 rpm at 30 ^{o}C overnight. Next day, the cultures were spun at 3,300 x g for 15 min. Phage were precipitated from the supernatant of each culture as described earlier. One ml of the amplified phage from each library was sampled for titration as described earlier and later, was used in the second round of selection. The remainder was stored at 4 ^{o}C. Titration plates were stored at 4 ^{o}C so that single colonies could be picked for further, monoclonal analysis.

Second and third rounds of selection

Second and third rounds of selection were carried out in a similar fashion to that described for the first round of selection. Reduced concentrations of the target protein were used for coating the selecting immunotubes to drive the selection of more specific scFvs of higher affinity.

As before, separate immunotubes were coated for screening of libraries I and J. For round two of selection, tubes were coated with the TcdA-MBP fusion protein at 50 µg/ml. Output phage from the first round were added to the coated and blocked tubes. The tubes were washed 20 times with the same washing solution as used in round one. Bound phage were eluted, enriched and titrated as described.

The procedure for the third round of selection was carried out exactly as described except that the concentration of the TcdA-MBP fusion protein was further reduced to 25 µg/ml.

3.2.7.2.2 Screening of phage by polyclonal and monoclonal ELISA

In the selection process used, it is envisaged that phage display will recover a polyclonal population of phage at the end of each round of selection with variable specificity towards the target protein. The ability of these polyclonal phage to recognise the target protein can be tested using ELISA. Using colonies from titration plates, monoclonal viral stocks can also be prepared by infection with helper phage to characterise the properties of individual members of the polyclonal population.

Polyclonal phage ELISA

Output phage recovered from rounds two and three of the selection process were screened for their reactivity against the target protein by ELISA. Four rows

of four wells of an ELISA plate were coated with the target fusion protein in 5 fold dilutions starting from 50 µg/ml in 100 µl PBS. The plate was incubated overnight at 4 ^{o}C. The next day, the plate was washed 3 times with PBS and each well was blocked with 200 µl of 2% skimmed milk in PBS for 2 h at room temperature and then washed 3 times with PBS. Wells in the first row received 100 µl of phage from library I taken from the second round of selection, while wells in the second row received 100 µl from the third round. The third row received 100 µl of phage from library J taken from the second round of selection, while the fourth row received 100 phage from the third round. Using data from titration, all phage samples contained approximately 10^6 cfu. The plate was then incubated at room temperature for 1 h, washed 3 times with PBS containing 0.1% Tween-20 and virus were detected with HRP conjugated anti-M13 antibody as described earlier.

Screening monoclonal phage by ELISA

Titration plates from each round of selection of libraries I and J were used to produce monoclonal phage. Twenty four individual colonies from titration plates were inoculated into 100 µl of 2xTY containing 100 µg/ml ampicillin and 1% glucose using a 96 well culture plate (Nunc, Denmark) for convenience. The plates were grown with shaking at 250 rpm overnight at 37 ^{o}C. The next day, samples of 2 µl were taken from each well and transferred to a second 96 well culture plate, each well containing 200 µl of 2xTY supplemented with 100 µg/ml ampicillin and 1% glucose. The plates were incubated with shaking at 250 rpm at 37 ^{o}C for 2 h. Plates used for overnight culture were used to make a glycerol stocks by adding glycerol to a final concentration of 15-20% and storage at -70 ^{o}C. Helper phage were added to each well of the growth plate using 10^9 in 25 µl of 2xTY containing 100 µg/ml of ampicillin and 1% glucose, per well. The plates

were incubated at 37 ^{o}C in a shaking incubator at 250 rpm for 1 h. The plates were centrifuged at 1,800 x g for 10 min using a plate carrier and the supernatant was aspirated off. Pellets were resuspended in 200 µl of 2xTY containing 100 µg/ml ampicillin and 50 µg/ml kanamycin. Growth then continued at 30 ^{o}C, overnight, in a shaking incubator at 250 rpm. Plates were centrifuged at 1,800 x g for 10 min and 50 µl of the supernatant containing the monoclonal phage stocks was used in screening ELISA.

The wells of two ELISA plates were coated with 100 µl per well of TcdA-MBP fusion at 25 µg/ml as this represented the lowest concentration used for selection. The plates were then washed and blocked as described earlier, and 50 µl of monoclonal phage stock was added to each well along with 50 µl of PBS. The plates were incubated at room temperature for 1 h and then were washed and probed as described earlier.

3.2.7.3 Genetic characterisation of reactive phage identified in ELISA

Clones that were positive in their reaction with the TcdA-MBP fusion in ELISA were characterised by PCR amplification using gIII and LMB3 primers that annealed to sequences flanking the scFv reading frame (Appendix 6.10). PCR amplification was carried out to establish whether full length scFv sequence was present.

3.2.7.3.1 PCR amplification from reactive clones

Plasmid DNA was prepared from about 90 clones that were positive in ELISA assays. DNA was also prepared from positive controls from the I and J libraries, scFvs known to carry full-length scFv sequences. DNA was prepared from bacteria picked from titration plates or from bacterial glycerol stocks laid down during the production of monoclonal phage. Bacteria were initially plated on TYE

agar plates containing 100 µg/ml ampicillin and 1% glucose. They were then grown in 3-5 ml of 2xTY. Plasmid DNA was then extracted using Qiagen mini-prep reagents as described earlier. Using the plasmid DNA as template, scFv sequences were amplified by standard PCR protocol using gIII and LMB3 primers. These were designed to anneal to sequences flanking the scFv insert. PCR reactions were performed in a total volume of 50 µl and the reaction mix used was as mentioned earlier. PCR products were analysed on agarose gels to verify the size of scFv inserts.

3.2.7.3.2 Sequencing

Plasmid DNA was sequenced to confirm the integrity of the scFv reading frame and the diversity of sequence. Plasmid DNA from candidate clones – those that were shown to carry full length scFv inserts by comparison with control clones – was sequenced using the LMB3 forward primer. Sequencing was carried out in Dundee at the College of Life Sciences, University of Dundee. Data was analysed using Chromas Lite Version 2.01.

3.2.7.4 Production of soluble scFv antibodies

E.coli TG1 was used for phage production as the stop codon between the scFv reading frame and gIII, the gene for the minor viral coat protein pIII is suppressed (Appendix 6.10). This results in attachment of the scFv to the viral particle. To produce soluble scFv antibody, phagemid DNA from candidate clones was transformed to *E. coli* HB2151, a non-suppressor strain of *E.coli*.

3.2.7.4.1 Transformation of phagemid DNA to E. coli HB2151

After the analysis of data from sequencing, DNA from candidate clones was transformed to HB2151. A vial of freshly prepared *E. coli* HB2151 was thawed on wet ice, 50 µl aliquots of cells were transferred to sterile chilled polypropylene

tubes and 5 µl of DNA from candidate clones or a control plasmid was added to each tube and mixed gently with a pipette tip. The tubes were then incubated on ice for 30 min before heat-shock for about 45 sec at 42 ^{o}C. The tubes were then chilled on ice for 2 min and 900 µl of SOC pre-warmed to room temperature was added. Cells were incubated at 37 ^{o}C for 1 h in a shaking incubator at 225 rpm. After recovery, 50 µl of each transformation mixture or control was spread on TYE agar plates containing 100 µg/ml ampicillin and 1% glucose and incubated overnight at 37 ^{o}C. Samples of 2 µl of HB2151 competent cells were inoculated on TYE agar with and without ampicillin and the plates were incubated at 37 ^{o}C overnight as additional controls. Additional controls were carried out to confirm that HB2151 were viable and antibiotic sensitive.

3.2.7.4.2 Expression of scFvs in E.coli HB2151

Single clones were picked from each transformation plate and then inoculated to 3-5 ml 2xTY broth and incubated overnight in a shaking incubator at 37 ^{o}C. Next day, 1 ml of overnight culture was added to 100 ml of 2xTY and grown at 37 ^{o}C in a shaking incubator until the OD reached 0.9. Bacterial cultures were then induced with IPTG to a final concentration of 1 mM. The temperature was reduced to 30 ^{o}C and cultures were grown overnight. The following day, cultures were placed on ice for 10 min to cool and were then centrifuged at 3,000 x g for 10 min at 4 ^{o}C. Supernatants were then tested for the presence of scFvs both by ELISA and dot blot.

ELISA test

Several wells on an ELISA plate were coated with 2 fold serial dilutions of the TcdA-MBP fusion protein and blocked with 2% milk as described earlier. scFv samples were added, incubated and the wells were washed 3 times with PBS. scFv binding was detected by probing with 100 µl of anti-c-myc antibody diluted

1/5000. The plates were incubated for 1 h at room temperature before washing 3 times with 0.1% Tween-20 in PBS. After probing with 100 µl HRP-anti-rabbit antibody at 1/5000 for 1 h at room temperature, the wells were then washed as before and developed with 100 µl of TMB substrate for 5-15 min at room temperature. The reaction was the stopped with 50 µl 0f 1 M H_2SO_4.

Dot blot test

Supernatant samples of 10 µl containing each scFvs were blotted on nitrocellulose membrane and left to dry. The membrane was blocked with 2% milk for 2 h at room temperature with agitation. The membrane was washed 3 times with PBS and probed with anti c-myc antibody at 1/5000 for 1 h at room temperature. The membrane was washed 3 times with 0.1% Tween 20 and then probed with anti-rabbit antibody at 1/5000 for 1 h at room temperature. Again, the membrane was washed as before and developed with TMB substrate for 5-15 at room temperature. The reaction was stopped by washing the membrane with water.

3.2.7.4.3 Large scale expression and purification of soluble scFvs

Expression

Candidate scFvs that emerged from dot blot and ELISA analysis were then expressed on large scale and purified to allow further characterisation of their properties. Clones were inoculated to 2xTY and incubated at 37 ^{o}C in a shaking incubator overnight. Next day, sufficient overnight cultures was added to 150 ml of 2xTY, containing 100 µg/ml ampicillin and 1% glucose, to attain an OD of 0.07-0.09. The cultures were then grown at 37 ^{o}C in a shaking incubator until the OD 600 reached 0.9; they were then induced with IPTG to a final concentration of 1 mM. The growth temperature was reduced to 30 ^{o}C for overnight culture. Cultures were then centrifuged at 4,000 x g for 15 min at 4 ^{o}C.

Precipitation

Soluble scFvs were precipitated from the culture supernatants using saturated ammonium sulphate. Eighty percent saturation was achieved by the gradual addition of ammonium sulphate to a final concentration of 561 g/L at 25 ^{o}C (Green and Hughes, 1955). Mixtures were incubated at 4 ^{o}C overnight to allow the precipitation of the scFvs. Next day, the mixtures were centrifuged at 3,300 x g for 15 min at 4 ^{o}C and the pellets were resuspended in about 3.5 ml of water and stored at -20 ^{o}C if not dialysed immediately.

To remove the ammonium sulphate, the concentrated scFvs were dialysed in the binding buffer (0.02 M sodium phosphate, 0.5 M NaCl, and 5 mM imidazole, pH 7.4) that was to be used for affinity purification of the scFvs. The precipitated scFvs were dialysed 6 times in 1L binding buffer for 1 h per buffer change and then in 3L overnight at 4 ^{o}C. The proteins were then stored at -20 ^{o}C if not purified immediately.

Purification by affinity chromatography

The scFvs were purified using a 5 ml HiTrap chelating HP column (GE Healthcare, Amersham Biosciences, UK). The HiTrap column was first washed with 5 column volumes of distilled water to wash away the 20% ethanol preservative. The column was then charged with half a column volume of 0.1 M nickel sulphate before washing with 5 column volumes of distilled water. Finally, the column was washed with 10 column volumes of binding buffer. All scFvs samples were adjusted to pH 7.4 using 10 x binding buffer before loading to the column. The samples were recycled through the column 3 times to maximise binding and then the column was washed with 10 column volumes of binding buffer to remove any unbound protein. The scFvs were then eluted using elution buffer (0.02 M sodium phosphate, 0.5 M NaCl, and imidazole ranging from 0.1 to

0.5M, pH 7.4). Fractions of 5 ml and 25 ml were collected at each imidazole concentration used. Protein concentration in each purified sample was estimated using a BCA assay kit (Pierce, USA). Purified proteins (scFvs) were used for characterisation and further investigation of their properties.

3.2.7.5 Characterisation of properties of the scFvs

3.2.7.5.1 SDS-PAGE and electroblotting analysis of purified scFvs

To check molecular weight against predicted size, purified scFvs were analysed by SDS-PAGE as described earlier. Gels were blotted to a nitrocellulose membrane following the standard protocol for Western blotting as detailed before and the transferred proteins were visualised using Ponceau stain to confirm transfer. The membranes were then blocked with 2% milk for 2 h at room temperature and probed with 1/5000 anti-c-myc antibody, HRP-conjugated antibody and developed with TMB substrate.

3.2.7.5.2 Recognition of TcdA-MBP fusion protein by soluble scFvs in ELISA

The purified soluble scFvs were tested for their ability to recognise the target protein - the TcdA-MBP fusion proteins - using ELISA. Each well of an ELISA plate was coated with 100 µl of 50 µg/ml target protein, while each well of a second plate was coated with 100 µl of each scFv. ELISA plates were coated and blocked with 2% milk as mentioned earlier. The plate coated with fusion protein was probed with 100 µl each scFvs per well, while the plate that coated with antibodies received 100 µl of TcdA-MBP at 50 µg/ml. After 1 h at room temperature, the plates were washed 3 times with 0.1% Tween-20 in PBS. The plate that was coated with the target protein was probed with 1/5000 anti c-myc and HRP conjugated anti-rabbit, while the plate coated with the scFvs was probed with 1/5000 anti-MBP and HRP conjugated anti-rabbit antibody. The plates were then developed with TMB substrate. The binding activity of the scFvs was also tested by preparing 5 fold serial dilutions of the TcdA fusion protein starting from 200 µg/well. The wells were probed with scFvs under test at equal concentrations and binding was detected with anti-c-myc antibody and HRP-conjugated reagents.

3.2.7.5.3 Recognition of native Toxin A by soluble scFvs in ELISA

The ability of the scFvs to recognise the native TcdA was tested in ELISA. An ELISA plate was coated with serially diluted native TcdA in 5 fold dilutions starting from 200 ng/well. The TcdA-MBP fusion was used as positive control and MBP served as a negative control. The plate was washed and blocked with 2% milk as mentioned earlier and all wells then received 100 µl of each scFv. This was incubated for 1 h at room temperature before washing, probing with anti-c-myc antibody and anti-rabbit regents as described earlier.

3.2.7.5.4 Recognition of TcdA-MBP fusion protein by soluble scFvs in Western blotting

The target protein that was used in the selection process – the TcdA-MBP fusion protein - was separated by SDS-PAGE. For separation, 80 µl of the target protein was mixed with 40 µl of SDS-loading buffer and heated for about 10 min at 95 ^{o}C water bath. Samples were then loaded to 4 adjacent lanes of a 10% polyacrylamide along with a protein ladder. The gel was run and blotted to a nitrocellulose membrane using the standard blotting protocol mentioned earlier. The transfer of proteins was confirmed by Ponceau stain and the membrane was then blocked in 2% milk for 2 h at 4 ^{o}C. The membrane was washed 3 times with PBS and the membrane was then divided into 4 pieces, each carrying a sample of the TcdA fusion and a sample of the protein ladder. The membranes were then probed with separate scFvs under test for 1 h at room temperature. The membranes were then washed 3 times with 0.1% Tween-20 in PBS and then were probed with anti-c-myc reagents as described before.

3.2.7.5.5 Recognition of putative binding site of TcdA-MBP fusion by scFvs using immunoprecipitation

Given the results of experiments to this point, the purified scFvs were tested for their ability to detect the target protein in immunoprecipitation assays. One hundred µl of each scFv was mixed thoroughly with 100 µl of the target protein at 100 µg/ml and incubated at room temperature for 2 h. Each tube then received 20 µl of EZ view Red Protein A gel (Sigma, UK), a coloured reagent in which the immunoglobulin-binding Protein A was conjugated to a gel matrix. Mixtures were incubated for a further 2 h at room temperature with shaking. The tubes were centrifuged for 3 min at 11,000 rpm and the supernatants were removed. The gel pellets, identified by their red colour, were washed 3 times by the addition of 1 ml PBS and re-centrifuged. The contents of each tube were mixed with 20 µl of SDS-loading buffer and heated in boiling water for 5-10 min. The contents of the tube were then loaded to duplicate 10% gels for SDS-PAGE analysis. After separation, the gels were blotted using standard Western blotting protocols, and blocked with 2% milk as described before. One blot was probed with anti-c-myc reagents to detect the presence of scFvs. The second blot was probed with anti-MBP reagents to detect the presence of the target fusion protein. Blots were developed with TMB substrate.

3.2.7.5.6 Recognition of native TcdB by soluble scFvs in ELISA

The ability of the purified scFvs to cross-react with Toxin B from *C. difficile* (TcdB) was tested in ELISA by coating 6 wells of an ELISA plate with 100 µl TcdB at 10 µg/ml (test), 100 µl TcdA at 10 µg/ml (positive control), 100 µl BSA at 2% (negative control) and three other blank wells (further controls). This arrangement was repeated for each scFv under test. The plate was coated and blocked as described earlier, then washed 3 times with PBS, and 100 µl of each scFv was added to the test, positive, BSA-coated and one of the additional

controls wells, while the other two controls wells received 100 µl of PBS. The plate was incubated for 1 h at room temperature and it was then washed 3 times with 0.1% Tween-20 in PBS. The test, positive and negative controls and two of the additional controls wells were probed with 100 µl of anti-c-myc reagent, while the last well of the additional controls was received 100 µl of PBS. The plate was incubated for 1h more at room temperature and then was washed as before. All wells then received 100 µl of the HRP-conjugated anti-rabbit antibody. After incubation and washing, signal was developed with TMB substrate.

3.2.7.6 Epitope mapping of anti-TcdA scFvs

Using the TcdA-MBP fusion protein and truncation mutants in which MBP was fused to SR1, SR1-LR, SR1-LR-SR2, and SR1-LR-SR2-SR3 (see earlier descriptions), attempts were made to map the region of TcdA recognised by the most promising scFvs. This was accomplished by ELISA.

3.2.7.6.1 Recognition of TcdA repeat sequences by scFvs using ELISA

To characterise the properties of the 4 most promising ani-TcdA scFvs, wells across a series of rows of an ELISA plate were coated with 100 µl of the truncated fusion proteins and TcdA-MBP, each adjusted to a protein concentration of 50 µg/ml. The plate was coated and blocked with 2% milk as outlined earlier. The plate was washed 3 times with PBS and then single columns were used to test the properties of each of the candidate scFv by adding 100 µl of each scFv and incubating for 1 h at room temperature. The plate was washed 3 times with 0.1% Tween-20 in PBS and all wells received 100 µl of 1/5000 anti-c-myc antibody to detect scFvs binding. The plate was incubated for a further 1

h at room temperature, then washed as mentioned before and signal developed with HRP-conjugated anti-rabbit antibody and TMB substrate.

3.2.8 Cloning of gVIII phage sequence into pIT2 vector

One hypothesis addressed by the project was that multivalent display of scFvs at the surface of phage might enhance the toxin neutralising capacity of anti-TcdA scFvs. Phage display of scFvs as fusions to pIII presented no more than 5 copies of the antibody on the viral capsid. As the number copies of pVIII in capsid is much higher (2700 copies), this part of the project aimed to create phage with the capacity to display scFvs as fusions to pVIII.

3.2.8.1 Preparation of gVIII sequence and pIT2 vector carrying scFv-15

gVIII encoding the phage coat protein VIII (Figure 3.1) was amplified from phage genomic DNA using gVIII forward and reverse primers (Table 3.1). Template DNA was prepared from helper phage by heating an aliquot of the viral stock to 95 ^{o}C and cooling it to 50 ^{o}C several times. PCR was then carried out using the standard PCR protocol (see earlier description). The gVIII reaction received all standard PCR reagents as detailed earlier and 4 µl of extract from denatured phage. Primers were omitted as a negative control. PCR products were then analysed by 1% agarose electrophoresis.

The phage display vector (pIT2; Appendix 6.10) carrying anti-TcdA clone scFv-15 was digested with *Not*I and *Eco*RI restriction endonucleases to excise the gIII sequence. The gVIII PCR product was also digested with these restriction enzymes and the PCR product was ligated into the vector. Digestion of the cloning vector released a fragment of about 1500 bp containing the sequences for His-tag, c-myc and gIII.

gVIII for.	ATCGCGGCCGCAGAGGGTGACCCC
gVIII rev.	ACTGAATTCTTATCAGCTTGCTTT
LMB3	CAGGAAACAGCTATGAC
DPK9 FR1	CATCTGTAGGAGACAGAGTC

Table 3-1 Sequence of forward and reverse primers for amplification of gVIII

Nucleotide sequence of phage gVIII
GAGGGTGACCCCGCAAAAGCGGCCTTTAACTCCCTGCAAGCCTCAGCG ACCGAATATATCGGTTATGCGTGGGCGATGGTTGTTGTCATTGTCGGCG CAACTATCGGTATCAAGCTGTTTAAGAAATTCACCTCGAAAGCAAGCTGA

Figure3-1 Sequence of gVIII.

3.2.8.2 Ligation and transformation

Ligation reactions were performed in a total volume of 21 µl. One µl of the digested pIT2 vector was added to 8 µl of digested gVIII product. Ten µl of 2x ligase buffer and 1 µl of T4 DNA ligase were added. The reaction volume was made up to 21 µl with sterile distilled water. After snap centrifugation, tubes were incubated at room temperature for 5 min then the ligation mixture was chilled on ice and stored at -20 ^{o}C if not used immediately for transformation.

Transformation

Competent *E. coli* TG1 cells were prepared as described earlier and thawed on wet ice. Fifty µl aliquots of cells were transferred to sterile chilled polypropylene tubes for transformation reactions and controls. Then 2 µl of ligation mixture was added to each tube. Transformation was carried out as described earlier and bacteria were spread on TYE agar plates containing 100 µg/ml ampicillin and 1% glucose.

3.2.8.3 Analysis of transformants

Several colonies from the transformation were screened using colony PCR. Crude extracts were prepared from transformants and amplified using forward and reverse primers for gVIII sequence to confirm the presence of the gVIII sequence. Products from colony PCR were analysed on 1% agarose gels.

Single colonies from candidate clones were inoculated to 3 ml of 2xTY, grown and plasmid DNA was then extracted. DNA was digested with *Not*I and *Eco*RI restriction endonucleases as mentioned earlier. Products were analysed by 1% agarose gels to confirm the presence of an insert of the predicted size.

3.2.8.4 Sequencing

Plasmid DNA from candidate clones was extracted using Qiagen reagents as mentioned earlier. Sequencing was performed using DPK9 FR1 (Table 3.1) primer to test for the presence of gVIII sequence downstream from the coding sequence for scFv-15. Sequencing was carried out at the University of Dundee. Chromas LITE version 2.01 software was used for data analysis.

3.2.8.5 Production and characterisation of phage displaying pVIII fusion to scFv-15

Bacteria carrying scFv-15 fused to pIII and 4 clones suspected to carry the scFv-15 sequence fused to pVIII were grown at 37 ^{o}C with shaking in 2xTY until the OD reached 0.4. Cultures were then infected with helper phage and virus were precipitated as described earlier.

Characterisation of phage displaying the scFv15-pVIII fusion was carried out in ELISA using aliquots containing about 1x 10^{6} cfu. Wells of an ELISA plate were coated with 50 µg/ml of the TcdA-MBP fusion protein and 50 µg/ml of MBP as a negative control. Sufficient wells were coated to allow testing of phage displaying the scFv15-pIII fusion, the scFv-pVIII fusion, KM13 helper phage as a negative control and PBS as a further negative control. Phage binding was detected with anti-M13 conjugate and TMB substrate as described earlier.

3.3 Results

3.3.1 Titration of KM13 helper phage stock

KM13 helper phage was grown to produce large quantities of virus that could be used as a stock throughout the project. The helper phage was titrated to determine the concentration of virus in the stock. This was done by counting the number of plaques evident in H-top agar. This is shown in Figure 3.2. From these experiments, the stock was estimated to contain 5 x 10^{17} plaque forming units/ml.

3.3.2 Characterisation of an anti-BSA positive control scFv

A positive control anti-BSA scFv provided with the Tomlinson I and J libraries was expressed and purified. The purified anti-BSA antibody was characterised by SDS-PAGE, blotted to nitrocellulose membrane and probed with anti-c-myc antibody and an HRP conjugate. The blot was then developed with TMB substrate (Figure 3.3). The protein was evident as a single reactive protein at about 30 kDa.

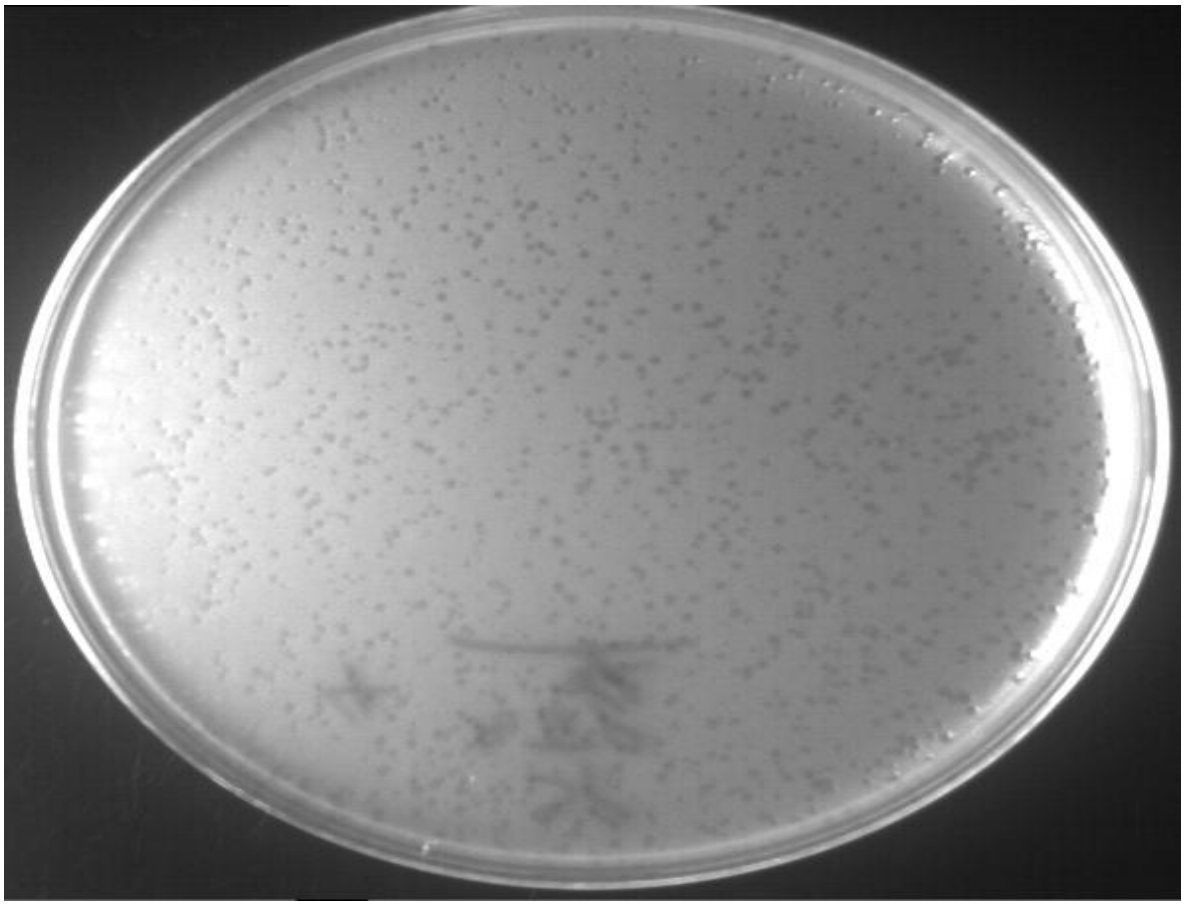

Figure 3-2 Plaques of KM13 helper phage evident in H-top agar on a TYE agar plate.

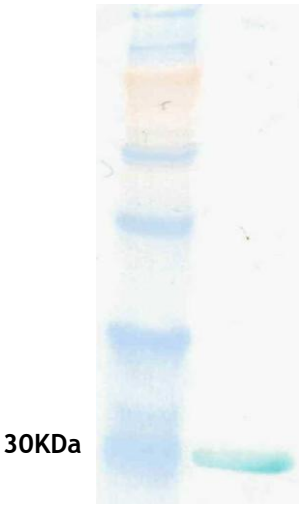

Figure 3-3 Western blotting analysis of anti-BSA scFv
Anti-BSA scFv was expressed in transformed *E. coli* HB2151 cells and purified by nickel chelation chromatography. The eluted antibody was separated by SDS-PAGE (right lane) with pre-stained molecular weight markers (left lane), electroblotted and probed with anti-c-myc and HRP-conjugated antibody.

3.3.3Growing, titration and screening of Tomlinson I and J libraries

Libraries I and J were grown in *E.coli* TG1 according to the protocol provided with the materials and infected with KM13 helper phage. The resulting phage particles displaying each scFv library were precipitated with PEG/NaCl. Diluted phage stocks were used to infect exponentially growing *E. coli* TG1 and after plating to selective medium, colonies were counted to calculate the titre of each phage library. This was estimated to be 3×10^{12} and 1.2×10^{12} cfu per ml for libraries I and J respectively.

Phage libraries were firstly tested for polyreactivity against milk, MBP and BSA in ELISA. Individual phage stocks were prepared and tested in ELISA against these protein samples. This was carried out as in preliminary work with aliquots taken from older phage libraries, polyreactivity (and hence non-specificity) was identified as a problem. In the upper panel of Figure 3.4, it can be seen that individual phage stocks from library I were only occasionally reactive with MBP (top two rows of the ELISA plate) or BSA bottom two rows, whereas reaction with milk proteins was more frequently observed (central section of the plate). The lower panel shows results from library J phage; similar patterns were observed.

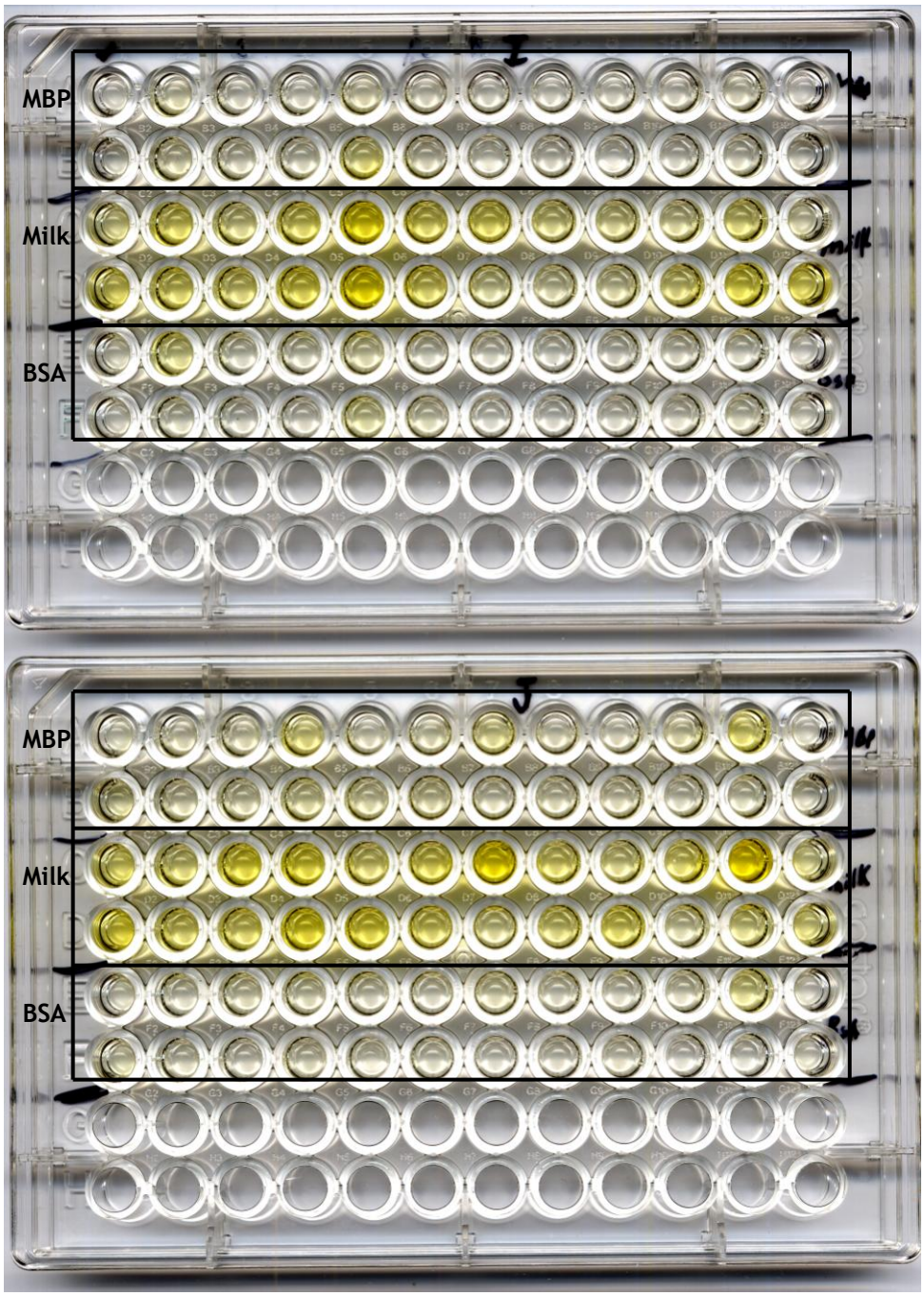

Figure 3-4 Reaction of monoclonal phage stocks from libraries I (upper panel) and J (lower panel) against native MBP, milk and BSA as boxed and indicated to the left.
Twenty four monoclonal phage stocks were prepared from each library and tested against native MBP, milk and BSA in ELISA. Phage were detected by HRP-conjugated anti-M13 antibody and TMB substrate.

3.3.4Selection of phage against TcdA-MBP from Tomlinson I and J libraries

Phage antibodies against the putative receptor-binding site of TcdA were extracted from scFv libraries I and J by 3 successive rounds of selection. The selection strategy was simple: immunotubes were coated with the TcdA-MBP fusion protein as target, and input phage were pre-incubated in 2% milk in PBS and MBP before addition to the coated tubes to minimise the chances that anti-milk or anti-MBP scFvs would be isolated. A progressive reduction of target protein concentrations in the second and third rounds of selection was used to increase the affinity and specificity of the extracted antibodies. Input phage numbers were established by titration and the numbers of virus recovered after each round of selection (output) were also estimated. This enabled the progress of selection to be monitored (Table 3.2), percentage recovery for each round taking account of differing inputs between library and the round of selection. Very low recoveries from each library were evident at round one, unsurprising despite the diversity of the Tomlinson libraries estimated to be over 1×10^8 different scFv specificities by the library's constructors. Phage eluted from round one were amplified to create the input for round 2. At this step, recoveries rose significantly and about 1000 (library I) to 10,000 fold enhancement (library J) was observed. In round 3, more modest gains (30-fold, library I; 150-fold, library J) were recorded. The data in Table 3.2 shows progressive enrichment of the specific phage during selection against the target protein, suggestive that phage display was effective in recovering target-specific scFvs.

Selection round	Tomlinson I library			Tomlinson J library		
	Input	Output	% recovery	Input	Output	% recovery
R1	$3x10^{12}$	$5x10^{4}$	$1.7x10^{-8}$	$1.2x10^{12}$	$5.6x10^{4}$	$4.66x10^{-8}$
R2	$3.65x10^{10}$	$6.7x10^{5}$	$1.8x10^{-5}$	$1.7x10^{9}$	$8.5x10^{5}$	$5x10^{-4}$
R3	$4.6x10^{9}$	$2.9x10^{6}$	$6.3x10^{-4}$	$3.0x10^{8}$	$2.3x10^{7}$	$7.6x10^{-2}$

Table 3-2 Recovery of phage from libraries I and J over three rounds of selection against TcdA-MBP.

Round of selection is shown to the left as R1, R2, and R3. The body of the Table shows the total number of phage from each library that went into ("Input") and were recovered ("Output") from each round of selection. % recovery shows the output value as a percentage of input.

3.3.5Screening selected phage by polyclonal and monoclonal ELISA

ELISA was carried out to assess whether the phage recovered at each round of selection showed specificity towards the target protein. To do this, phage antibody particles recovered from rounds 2 and 3 of selection were examined in ELISA against the recombinant putative binding site of TcdA. In initial experiments, polyclonal phage from I and J libraries that were eluted after rounds 2 and 3 were tested in ELISA against 5 fold serial dilutions of the target protein starting from 50 µg/ml. In this way, the strength of recognition between the scFvs recovered by phage display selection was tested against the target protein sequence. Figure 3.5 suggests that reaction between phage in polyclonal eluates from both libraries were reactive with the target protein. There was also some indication from signals at the lowest concentration of TcdA-MBP tested that reaction with eluates from round 3 was stronger than that from round 2.

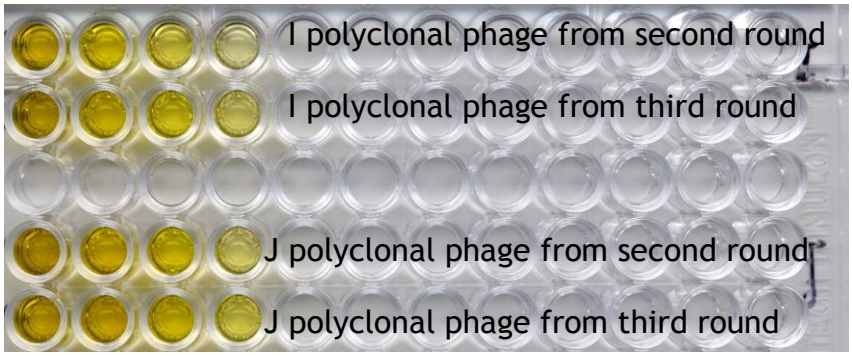

Figure 3-5 Polyclonal ELISA of phage recovered from rounds 2 and 3
Wells of an ELISA plate were coated with 5 fold serial dilutions of the TcdA-MBP fusion protein (left to right; range 50 µg/ml to 400 ng/ml). Samples of phage from library I (upper lanes) and J (lower lanes) eluted after rounds 2 and 3 of selection as designated were added and binding was detected with an HRP-anti-M13 antibody and TMB substrate.

To better define the properties of phage recovered by selection, monoclonal stocks of output phage recovered after rounds 1, 2 and 3 were produced and screened against the recombinant putative TcdA-MBP fusion using ELISA. From the output at each round, for each library, 24 monoclonal phage stocks were produced and tested (Figures 3.6 and 3.7). Reactions that were judged by visual inspection to be strong were counted and the data were tabulated (Table 3.3). With library J, 4 strong positive clones were detected from the 24 stocks that were tested from the output of the first round of selection (Figure 3.6; top section). This increased to 13 from 24 (middle section) and 15 from 24 (lower section) from rounds two and three respectively. As a percentage of the stocks tested, strongly reactive phage represented 16.6, 54.2 and 62.5 % for library J for rounds 1, 2 and 3 respectively (Table 3.3).

This progressive enrichment of target-specific phage was also observed for library I across the three rounds of selection (Figure 3.7). Three positive clones were identified in round one from 24 tested clones, increasing to 9 in round 2 and 13 in round three. The percentages of strong reactive phage were 12.5, 37.5 and 54.2 % of those tested for rounds 1, 2 and 3 respectively 9 (Table 3.3).

Selection was thus judged to have successfully isolated target-specific scFv from the Tomlinson libraries.

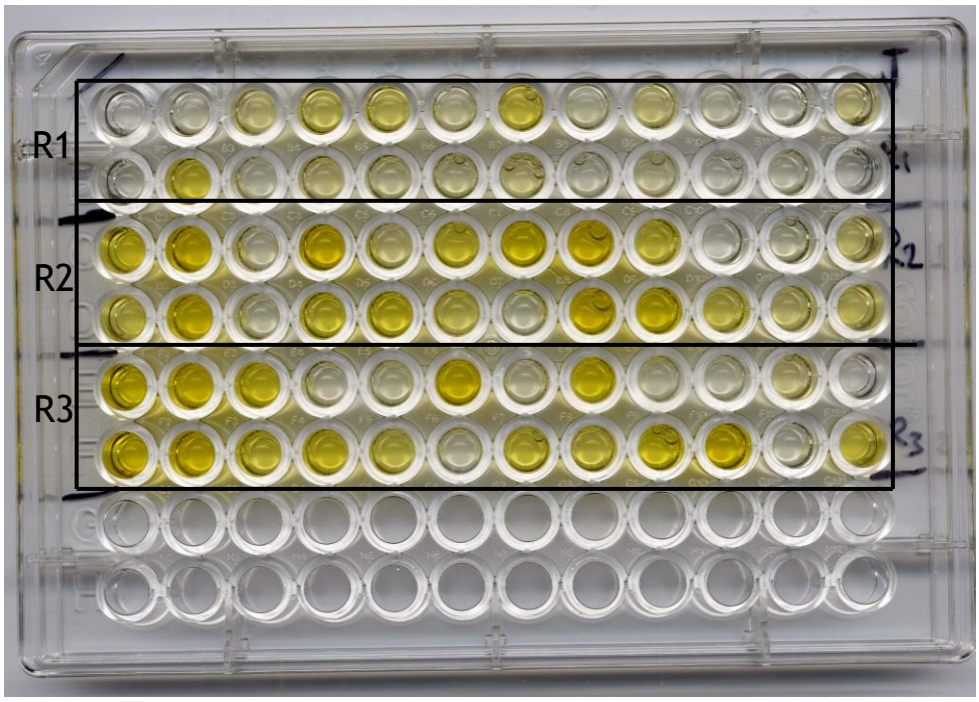

Figure 3-6 Monoclonal phage ELISA with output from library J
Twenty four monoclonal phage stocks were prepared after each round of selection as indicated to the left of the plate. Samples were added to an ELISA plate coated with the recombinant TcdA-MBP fusion. The binding of phage was detected with HRP-conjugated anti-M13 and TMB substrate.

Figure 3-7 Monoclonal phage ELISA with output from library I

Twenty four monoclonal phage stocks were prepared after each round of selection as indicated to the left of the plate. Samples were added to an ELISA plate coated with the recombinant TcdA-MBP fusion. The binding of phage was detected with HRP-conjugated anti-M13 and TMB substrate.

Selection Round	Tomlinson I library			Tomlinson J library		
	Tested	Positive	% Positive	Tested	Positive	% Positive
1st round	24	3	12.5	24	4	16.6
2nd round	24	9	37.5	24	13	54.2
3rd round	24	13	54.2	24	15	62.5

Table 3-3 Recovery of specific phage against TcdA-MBP.

Of the 24 monoclonal phage stocks from Tomlinson libraries I and J libraries tested in ELISA, those judged positive against the target were scored and their percentage calculated as a proportion of the total number screened.

3.3.6 Genetic characterisation of phage against the TcdA-MBP fusion

During library construction, clones arise that lack V_H or V_L components of the scFv reading frame. Bacteria that carry these short inserts can grow more quickly than these with full scFvs and if the antibodies possess affinity for the target, they can emerge from selection. Therefore, genetic characterisation was used to verify the size and integrity of the inserts.

3.3.6.1 Analysis of positive and control clones by PCR

Plasmid DNA was prepared from about 90 clones that were judged positive in ELISA against the TcdA-MBP fusion. DNA was also prepared from the positive control clone against BSA that was provided with the libraries, a scFv that was known to carry a full-length scFv sequence. scFv sequences were amplified from plasmid DNA by a standard PCR protocol using primers that flanked the scFv reading frame. PCR products we analysed by agarose gel electrophoresis. The results (Figure 3.8) showed many PCR products from anti-TcdA-MBP clones were of exactly the same size as the positive controls; this can be seen in lanes 2-10, the positive control being in lane 1. In contrast, two reactions gave products that were smaller in size than the positive controls (lanes 11 and 12) and some reactions showed two bands (lanes 13 to 15). The analysis thus showed that many clones probably carried full length scFv inserts, while others possibly lacked part of the antibody coding sequence.

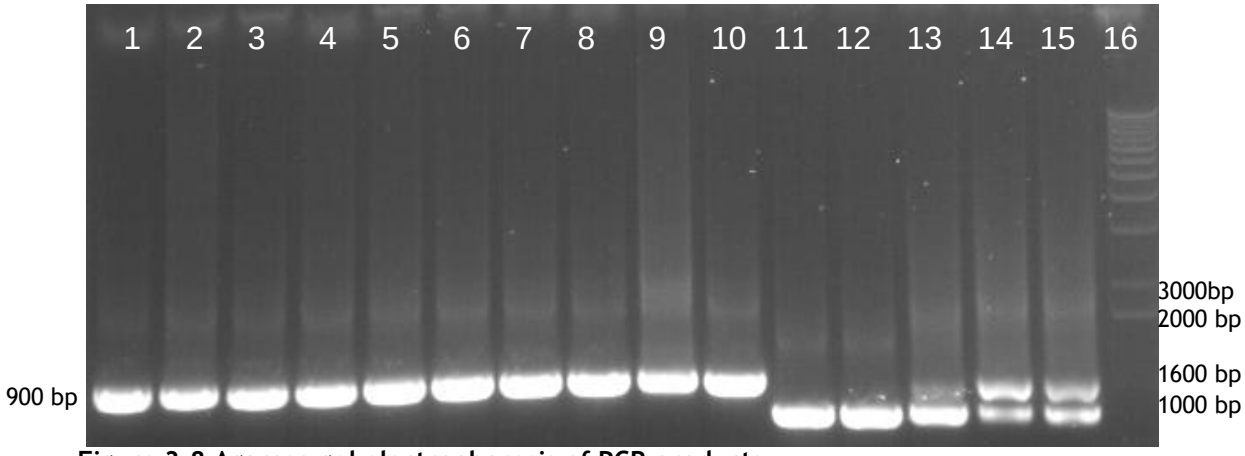

Figure 3-8 Agarose gel electrophoresis of PCR products.
Plasmid DNA from single clones was used as template in a PCR with primers that annealed to sites flanking the scFv insert. Lane 1 shows the positive control reaction from an anti-BSA scFv sequence, lanes 2-10 shows products that align with the positive control sample indicating the presence of full scFv inserts. Lanes 11-12 shows products that are smaller in size than the positive control. Lanes 13-15 shows reactions in which two bands could be observed indicating the possibility of contamination. Lane 16 shows 1kb size ladder.

3.3.6.2 DNA sequencing

To determine the integrity of the full length scFv reading frames identified in Figure 3.8 and their amino acid diversity, plasmid DNA was sequenced using the LMB3 forward primer (Table 3.1). Several clones had the sequence predicted for a full length scFv reading frame but carried a stop codon within the sequence or lacked components of the expected sequence. Four of the clones sequenced appeared to have an intact open reading frame. In reaching these findings, sequence results were initially checked for the presence of the complete V_H-linker-V_L sequence. Figure 3.9 shows that the sequences for clones 1, 14, 15 and 18 are identical for most part as would be expected from clones from the Tomlinson libraries since they are based on single human V_H and V_L frameworks. Figure 3.10 shows the predicted amino acid sequences for the heavy chains and light chains of these 4 clones. Regions of the scFv V_H domains where diversity might exist were within complementarity determining regions (CDR) 2 (residues H50, H52, H52a, H53, H55, H56, H58), and CDR3 (H95, H96, H97, H98). For the

V_L component, diversity might exist in CDR2 (L50, L53) and CDR3 (L91, L92, L93, L94, L96). Focussing upon these residues, the data showed that the selected clones possessed similarities in sequence but were non-identical in the diversified regions of the reading frame (Figure 3.11).

3.3.7Production and purification of soluble scFv antibodies

Those anti-TcdA-MBP scFvs that carried the full length sequence were expressed as histidine tagged fusion proteins in the non-suppressor *E.coli* strain HB2151. The presence of scFv protein in each of the culture supernatants and the reactivity of the scFv protein with the TcdA-MBP fusion protein was confirmed by ELISA and dot blotting (Figures 3.12 and 3.13). Having established this, scFvs were expressed from larger scale cultures, total protein was concentrated using 80% ammonium sulphate, and the scFvs were purified on nickel-chelating columns. To elute, column buffer containing imidazole (200 mM) was used and fractions were collected for further analysis.

1-scFv
ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCGGCCCAGCCGGCCATGGCCGAGGTGC
AGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATT
CACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGGTATT
GCTGCTTCTGGTAATAGTACATCTTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCA
AGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAACTAC
TACTACTTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGCGGTGGAGGCGGTTCAGGCGGAGGT
GGCAGCGGCGGTGGCGGGTCGACGGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAG
ACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATCAGCAGAAACCAGG
GAAAGCCCCTAAGCTCCTGATCTATAGTGCATCCAATTTGCAAAGTGGGGTCCCATCAAGGTTCAGTGGCAGT
GGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCTGAAGATTTTGTAACTTACTACTGTCAAC
AGACTGATGCTACTCCTAATACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGGGCGGCCGCACATCATCA
TCACCATCACGGGGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTGAATGGGGCCGCATAG

14-scFv
ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCGGCCCAGCCGGCCATGGCCGAGGTGC
AGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATT
CACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGGTATT
ACTGATACTGGTACTTCTACAGATTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCA
AGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAATATAA
TTCTGGTTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGCGGTGGAGGCGGTTCAGGCGGAGGT
GGCAGCGGCGGTGGCGGGTCGACGGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAG
ACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATCAGCAGAAACCAGG
GAAAGCCCCTAAGCTCCTGATCTATGGTGCATCCTCTTTGCAAAGTGGGGTCCCATCAAGGTTCAGTGGCAGT
GGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAACTTACTACTGTCAAC
AGAGAGCTTATTCTCCTAATACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGGGCGGCCGCACATCATCA
TCACCATCACGGGGCCGCAGACAAAAACTCATCTCAGAAGAGGATCTGAATGGGGCCGCATAG

15-scFv
ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCGGCCCAGCCGGCCATGGCCGAGGTGC
AGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATT
CACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCATATATT
GCTGATGCTGGTGCTTCTACAAATTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCA
AGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAACTGA
TGCTTCTTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGCGGTGGAGGCGGTTCAGGCGGAGGT
GGCAGCGGCGGTGGCGG*GTCGAC*GGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAG
ACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATCAGCAGAAACCAGG
GAAAGCCCCTAAGCTCCTGATCTATGCTGCATCCACTTTGCAAAGTGGGGTCCCATCAAGGTTCAGTGGCAGT
GGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAACTTACTACTGTCAAC
AGTCTACTTATTCTCCTGCTACGTTCGGCCAAGGGACCAAGGTGGAAATCAAACGGGCGGCCGCACATCATCA
TCACCATCACGGGGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTGAAT*GGGGCCGCA*TAG

18-scFv
ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCGGCCCAGCCGGCCATGGCCGAGGTGC
AGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATT
CACCTTTAGCAGCTATGCCATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCATATATT
TCTAGTTCTGGTGCTAATACAACTTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCA
AGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAACTGA
TAGTGATTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGCGGTGGAGGCGGTTCAGGCGGAGGT
GGCAGCGGCGGTGGCGGGTCGACGGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAG
ACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATCAGCAGAAACCAGG
GAAAGCCCCTAAGCTCCTGATCTATACTGCATCCTATTTGCAAAGTGGGGTCCCATCAAGGTTCAGTGGCAGT
GGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAACTTACTACTGTCAAC
AGTATGCTACTGATCCTAGTACGTTCGGCCAAAGGACCAAGGTGGAAATCAAACGGGCGGCCGCACATCATCA
TCACCATCACGGGGCCGCAGAACAAAAACTCATCTCAGAAGAGGATCTGAATGGGGCCGCATAG

Figure 3-9 Nucleotide sequences of 4 full-length anti-TcdA-MBP scFvs.
The coloured and underlined sequences show restriction sites sequences within the sequence of scFv. The sequences between *Nco*I and *Xho*I (red) represent the heavy chain reading frame, the *Xho*I and *Sal*I sites flank the scFv linker, and light chain coding sequence lies between *Sal*I and *Not*I (blue) sites.

Figure 3-10 Predicted amino acid sequences of 4 full-length anti-TcdA-MBP scFvs. Numbers to the left of the Figure show clone designations and the red font shows amino acids diversified in library construction.

	V_H sequences											V_L sequences						
	CDR2							CDR3				CDR2		CDR3				
clone	50	52	52a	53	55	56	58	95	96	97	98	50	53	91	92	93	94	96
1	G	A	A	S	N	S	S	T	T	T	T	S	N	T	D	A	T	N
14	G	T	D	T	T	S	D	Y	N	S	G	G	S	R	A	Y	S	N
15	Y	A	D	A	A	S	N	T	D	A	S	A	T	S	T	Y	S	A
18	Y	S	S	S	A	N	T	T	D	S	D	T	Y	Y	A	T	D	S

Figure 3-11 Diversity of amino acid sequences in CDRs of selected scFv clones of against TcdA-MBP fusion.

3.3.8 Characterisation of properties of the scFvs

Characterisation of the scFvs expressed in bacterial culture supernatants began with simple detection of the target protein by the scFvs in ELISA. The wells of an ELISA plate were coated with the TcdA-MBP fusion protein, blocked with 2% milk, and each well received 100 µl of supernatant from induced culture of *E. coli* HB2151, allowing scFvs present in the sample the opportunity to bind to the target. scFv antibody binding was then detected with anti-c-myc reagent and HRP-conjugate. The results are shown in Figure 3.12 which shows that soluble scFvs were successfully expressed from all 4 clones under test.

This was independently confirmed by blotting culture supernatants directly to nitrocellulose membrane and detecting the presence of the scFv protein in each sample using again the c-myc tag fused at the carboxy-terminus of the the scFvs (Figure 3.13).

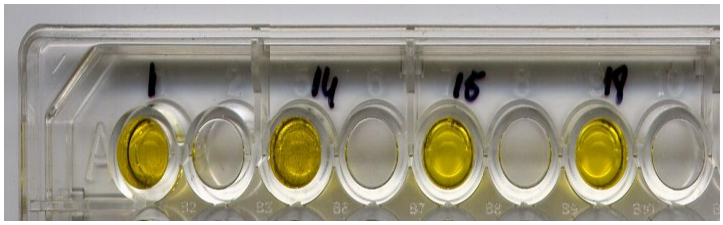

Figure 3-12 Anti-TcdA-MBP ELISA showing using soluble scFvs.
Target protein was coated to alternating wells of an ELISA plate and the binding of scFvs in the supernatant of induced bacterial cultures was detected using anti-c-myc antibody, HRP conjugate and TMB substrate.

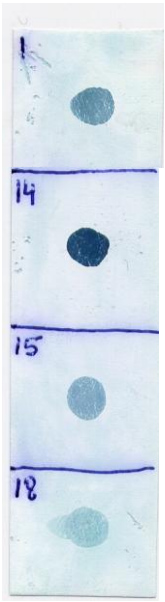

Figure 3-13 Dot blot detection of scFvs in supernatants of induced bacterial cultures.
Supernatants from induced cultures of *E. coli* HB2151 were applied to nitrocellulose membrane and the presence of scFvs was detected with anti-c-myc HRP conjugate and TMB substrate.

3.3.8.1 Electroblotting analysis of the purified scFvs

Given the evidence that soluble scFv protein could be expressed from clones 1, 14, 15 and 18, the antibodies were purified from supernatants prepared from larger induced cultures using nickel affinity chromatography. Western blotting was used to characterise the purified scFvs to determine the size of the protein that been expressed. Results are shown in Figure 3.14. scFv protein was concentrated into a discrete number of fractions and migrated at about 29 KDa when compared with marker proteins run beside the analysed proteins. In some cases (*eg* clone 14, fraction loaded to central lane; clone 15, all fractions analysed), a protein of lower molecular weight was evident that reacted with anti-c-myc reagents. With regards to this undefined component, purity of the purified samples appeared best for clone 1. In terms of yield, clone 18 appeared to yield the least recombinant antibody.

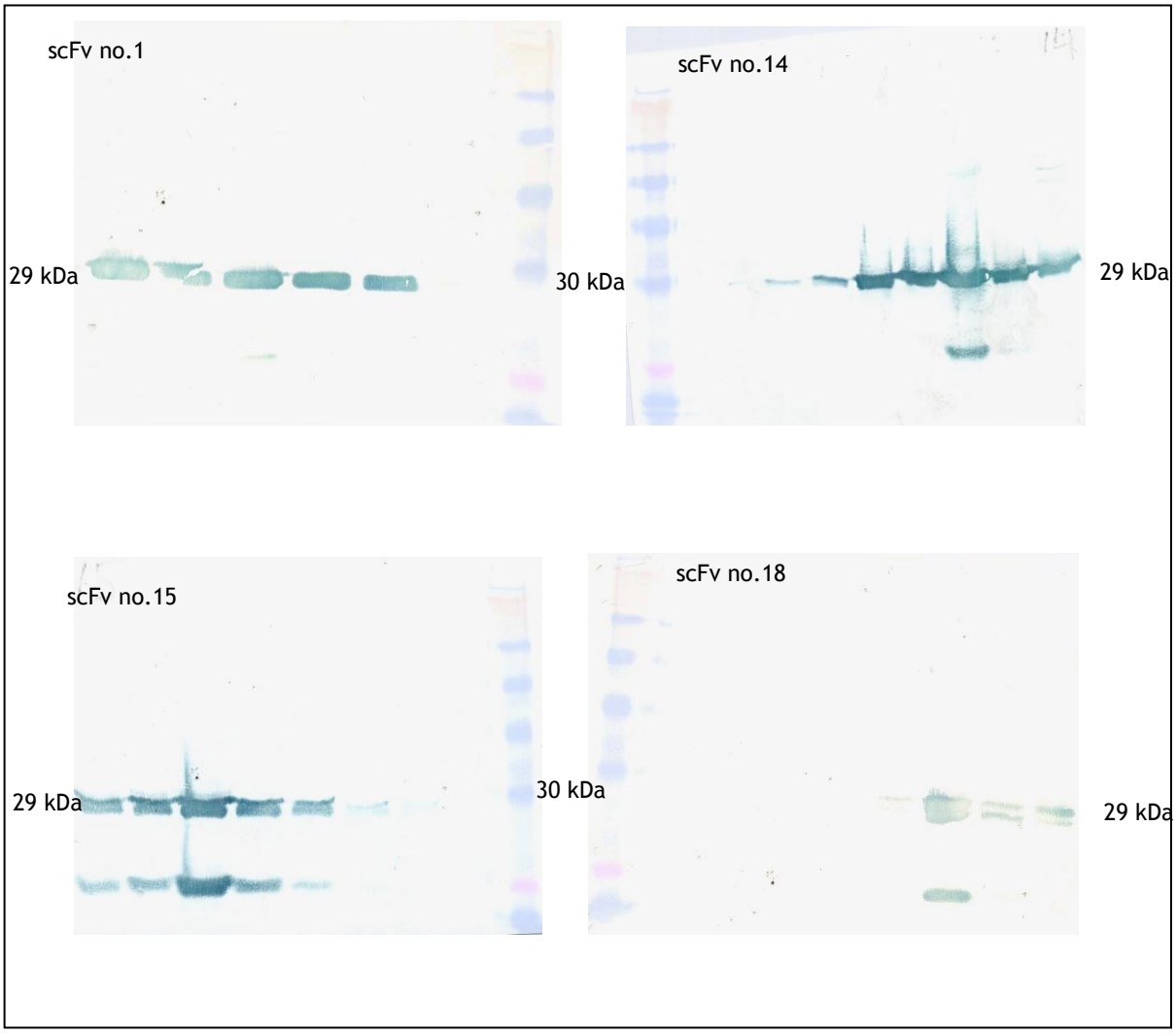

Figure 3-14 Electroblotting analysis of the purified scFvs.
For each clone, fractions eluted from a nickel chelation column were separated by SDS-PAGE, blotted to nitrocellulose membrane and probed with anti-c-myc and HRP-labelled reagents. Lanes at the edge of each panel show the migration of pre-stained molecular weight markers. The position of a 30 kDa marker is noted.

3.3.8.2 Recognition of TcdA-MBP fusion proteins by soluble scFvs in ELISA

The binding ability of the four selected and purified scFvs to bind to the TcdA fusion protein was examined in two ways using ELISA.

In the first assay format, the target fusion protein was coated to the wells of an ELISA plate and the ability of the purified scFvs to bind to the target was tested using anti-c-myc reagents (Figure 3.15 panel B). In the second assay

format, the purified scFvs were coated to the wells of a ELISA plate and the Tcd-MBP fusion protein was added. The capacity of the target protein to bind was tested using anti-MBP reagents (Figure 3.15 panel A). In both cases, the yellow colour of the colorimetric product indicates the positive recognition of the target protein by the purified scFvs, independent of the assay format (Figure 3.15).

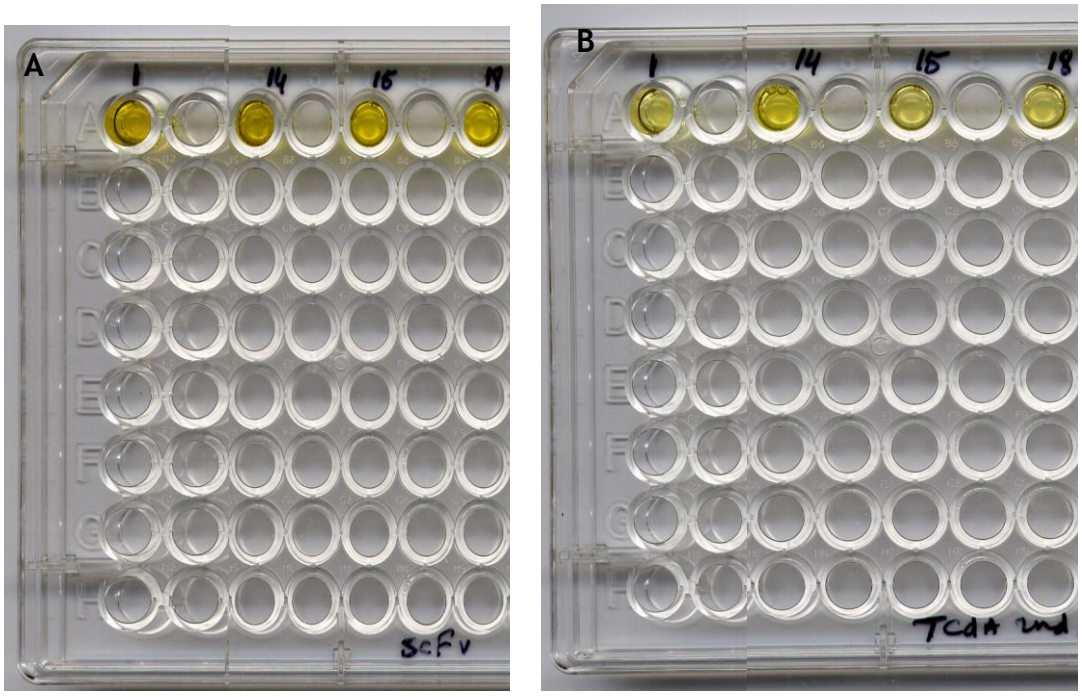

Figure 3-15 ELISA assays of the binding of soluble scFvs to TcdA-MBP fusion protein. Selected soluble scFvs were expressed, purified and characterised. scFvs were challenged to the target protein. In Panel A, scFvs were coated to ELISA plate and the TcdA-MBP fusion protein was added. Binding of the fusion was probed with anti-MBP. In Panel B, the TcdA-MBP fusion protein was coated to the plate and scFvs were then added. Binding of scFvs was probed with anti-c-myc.

To assess the relative binding activities of the scFvs against the target protein, the fusion protein was serially diluted in 5 fold steps over 7 dilutions starting from 200 µg/well and each dilution was coated to wells of an ELISA plate. The purified scFvs were prepared at equal concentrations, each was added to the coated wells and binding was then detected with anti-c-myc antibody and HRP-conjugate. Figure 3.16 shows the results. For scFvs clones 1,

14 and 15, antibody binding to the target protein was detectable from its initial coating (200 µg/well) through to down to the penultimate dilution (64 ng/well). In contrast, the binding of scFv clone 18 was detectable down to 320 ng/well but beyond this lower limit, reactivity was so low it was indistinguishable from background.

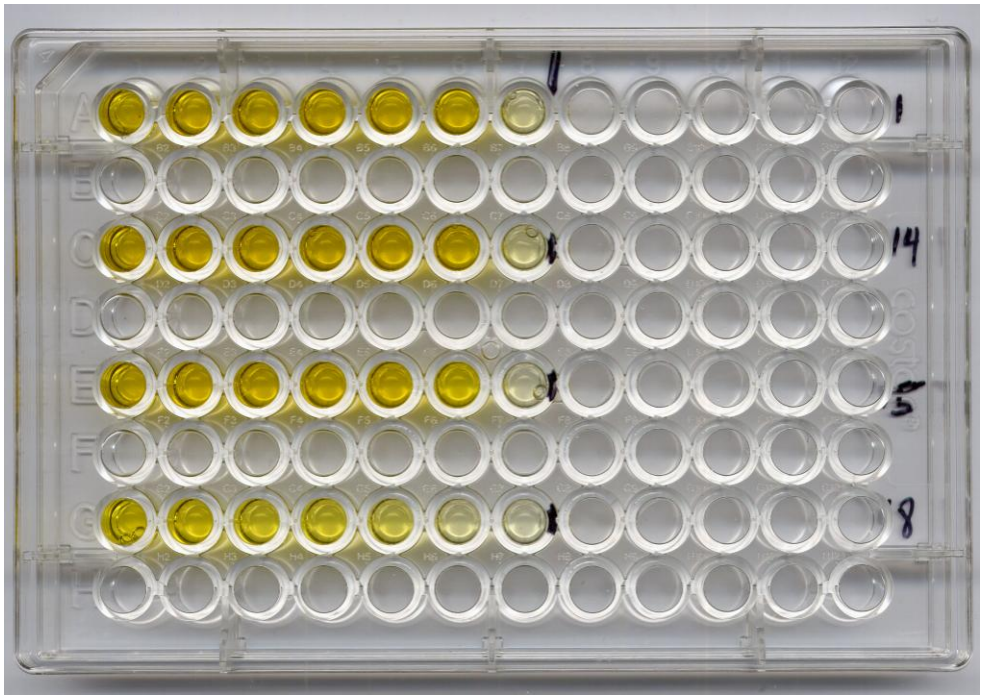

Figure 3-16 Binding of soluble scFvs to decreasing concentrations of TcdA-MBP fusion protein in ELISA.

TcdA-MBP fusion protein was coated to rows A, C, E and G at seven concentrations, decreasing 5-fold from 200 µg/well (column 1). Soluble scFv (clones as indicated on the right) were adjusted to the same concentration and applied to coated wells. scFv binding was detected with anti-c-myc reagents.

3.3.8.3 Recognition of native Toxin A by the scFvs using ELISA

The strategy for isolation of scFvs from the Tomlinson libraries attempted to divert selection away from the MBP carrier through use of a pre-incubation step with free, MBP protein, and hence attempted to enrich for anti-TcdA specificities. However, it remained possible that scFvs isolated in this way might recognised epitopes present in the TcdA-MBP fusion protein but absent in native TcdA. To assess if this was the case, the capacity of the four scFvs to bind to native TcdA was assessed by ELISA. To do this, the native toxin was diluted and applied to the wells of an ELISA plate from 200 ng/well over four, 5-fold dilutions. The TcdA-MBP fusion protein and native MBP were used as positive and negative controls respectively. The wells received equal concentrations of the scFvs and binding of the recombinant antibody was detected with anti-c-myc reagents. Figure 3.17 shows that the purified scFvs were able to recognise the native TcdA in ELISA across all dilutions of toxin. The strength of signal from each of the scFvs was comparable between the TcdA-MBP fusion and native toxin. For scFv clone 14 (second row), faint binding to MBP was detected but signal was substantially lower than for interactions with native or recombinant forms of Toxin A.

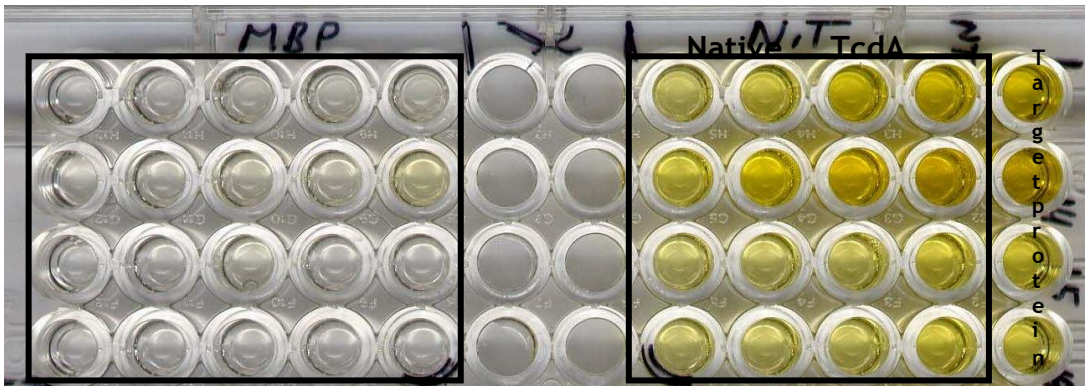

Figure 3-17 Binding of soluble scFvs to native Toxin A using ELISA.
Wells were coated with MBP (left area), dilutions of native Toxin A (right area; toxin diluted from right to left in 5-fold dilutions from 200 ng/well in the first column), or TcdA-MBP fusion protein (final column on the right). Soluble scFvs were applied at fixed concentration (from top, clones 1, 14, 15, 18) and binding was detected with anti-c-myc reagents.

3.3.8.4 Recognition of putative binding sites on TcdA-MBP using Western blotting

Western blotting was used to examine the ability of the purified scFvs to recognise TcdA epitopes after treatment with SDS-PAGE and heating. Samples of the TcdA-MBP fusion protein were separated on SDS-PAGE and blotted to a nitrocellulose membrane. The blot was divided into a set of strips and each one was probed with one of the purified scFvs. Binding of the scFvs to the target protein on the blot was detected with monoclonal anti-c-myc, HRP-conjugated antibody and TMB substrate. None of the scFvs were able to recognise the target protein after treatment with SDS and heat. This suggests that the specific epitope for binding to scFvs were lost through unfolding of the protein in preparation for SDS-PAGE. To take forward the analysis, the interaction between purified scFvs and target proteins was instead studied by immunoprecipitation of the target protein in its folded state.

3.3.8.5 Recognition of putative binding sites on TcdA-MBP using immunoprecipitation

Each scFv was incubated with the target protein and immune complexes were captured to gel-bound Protein A for separation on duplicate SDS-PAGE gels. Proteins on each gel were transferred to nitrocellulose membranes. One membrane was probed with anti-MBP reagents to detect the presence of the target protein; the other membrane was probed with anti-c-myc reagents to detect the presence of the scFv under analysis. Results show that all purified scFvs were able to recognise the original target protein, the TcdA-MBP fusion, in the immunoprecipitation assay. Data are shown in Figure 3.18. Panel A shows the presence of the target protein as detected with anti-MBP antibody, while Panel B shows the presence of scFvs. Capture of the scFvs to Protein A is evident from the prominent band detected at around 30 kDa (Panel B) though for clone 18, an additional signal is evident at high molecular weight (right hand lane). Recognition of the TcdA-MBP protein in each reaction is evident from the sharp band in Panel A, migrating at a molecular weigh greater than 50 kDa though the reaction for scFv clone 1 (lane at the left adjacent to the markers) is faint.

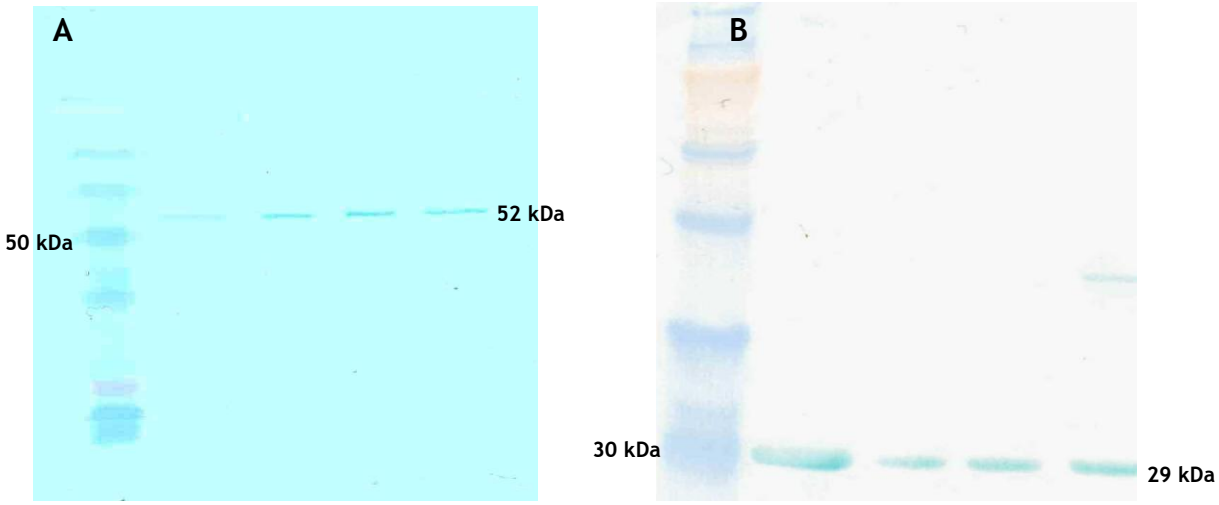

Figure 3-18 Immunoprecipitation of TcdA-MBP fusion protein by soluble scFVs.
In each panel, the left hand lane shows the migration of pre-stained molecular weight markers. Immunoprecipitation reactions with soluble scFvs clones 1, 14, 15 and 18 are shown in adjacent lanes. Immune complexes were captured to Protein A gel, separated by SDS-PAGE and blotted. In Panel A, the blot was probed with anti-MBP reagents. In Panel B, the blot was probed with anti-c-myc reagents.

3.3.8.6 Recognition of native TcdB by scFvs using ELISA

Given that the scFvs were reactive with recombinant and native forms of TcdA, further experiments assessed for reactivity with another major virulence protein from *C. difficile*, TcdB. TcdA and TcdB possess similar but non-identical sequences in their carboxy-termini (see Introduction Figure 1.3). The assay was carried out in ELISA by coating native TcdB, and native TcdA to wells of an ELISA plate, along with BSA as a negative control and uncoated wells to exclude the possibility that cross reactivity of the scFvs or anti-c-myc or HRP-conjugated antibodies with the plastic of the plate would confound the result. scFvs were added and wells were probed with anti-c-myc and HRP-conjugate. Results in Figure 3.19 show that all scFvs were reactive with TcdB and reaction with TcdA was confirmed. BSA and all the additional controls failed to generate significant signal in the assay.

To assess the basis for this cross-reaction, the protein sequence of TcdA used in the MBP-TcdA fusion (Figure 2-1) was compared with the protein sequence of TcdB in a pairwise BLAST. Dotplot data derived from this alignment revealed significant similarities (Figure 3.20). Whilst the most obvious similarities existed in the carboxy-terminal region of TcdB, others were noted more towards the amino terminus. Cross-reaction of anti-TcdA scFvs with Toxin B is therefore most likely to arise because of reaction with similar or identical epitopes present in the carboxy termini of the two clostridial toxins.

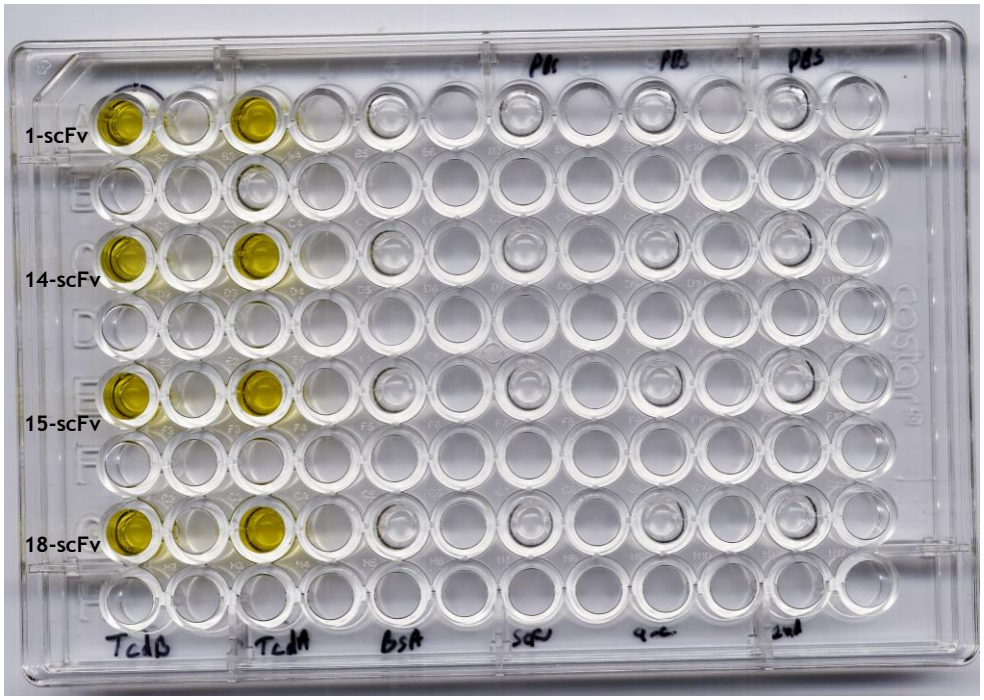

Figure 3-19 ELISA against native TcdB.
Native TcdB was coated to alternating rows in the left hand column (column 1). TcdA was added to column 3, with BSA in 5 and no target protein in columns 5, 7, 9 and 11. The four purified scFvs were used to probe wells in rows A, C, E and G (clones 1, 14, 15 and 18 respectively) through to column 7. Binding was detected with anti-c-myc (all well through to column 9) and HRP conjugate (all wells through to column 11).

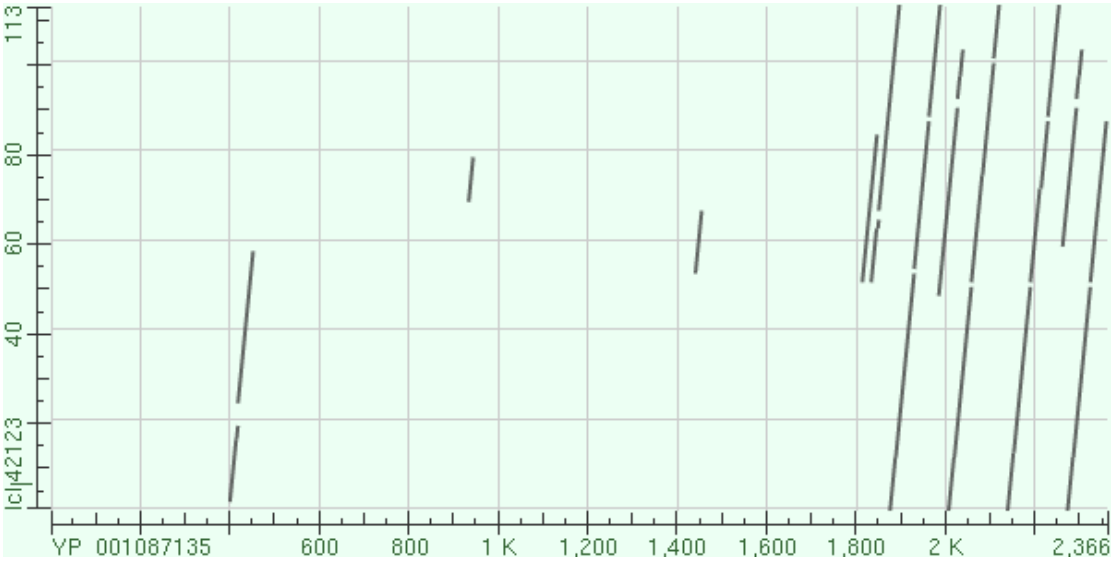

Figure 3-20 Alignment of TcdB and TcdA sequence carried in the MBP-TcdA fusion protein
Protein sequences (TcdB, NCBI accession number YP_001087135, x axis; TcdA sequence as shown in Figure 2-1, y axis) were aligned by pairwise BLAST using the BLOSUM62 matrix with an expected threshold setting of 10 and a wordsize of 3. The dotplot identifies short sequence identities and similarities occurring most frequently but not exclusively in the carboxy-terminal region of TcdB (right side of the dotplot). The best match reaches 41% identity and 62% similarity.

3.3.9Epitope mapping

In order to identify the epitopes recognised by the anti-TcdA scFvs, a series of truncated TcdA-MBP fusion proteins were created which carried defined but incomplete components of the TcdA sequence. The ability of the scFvs to react with these truncation mutants was evaluated in ELISA.

3.3.9.1 Recognition of SR1, LR, SR2 and SR3 by the scFvs using ELISA

The ability of the scFvs to detect the panel of truncated TcdA-MBP proteins was tested in ELISA. Wells were coated with MBP fusions carrying the SR1 repeat alone, SR1 and LR sequences, or progressively longer stretches of sequence taken from the putative receptor-binding domain of TcdA. After blocking with milk, each scFv was tested for reaction with the fusions, the binding of the

recombinant antibodies being detected with anti-c-myc antibody and HRP-conjugate. Results in Figure 3.21 show that scFvs 1, 14, 15 and 18 were unreactive with an MBP fusion protein carrying SR1 alone, but that addition of LR restored scFv binding. Further extension of the TcdA sequence by addition of SR2 and SR3 maintained reactivity for all of the scFvs under test in ELISA.

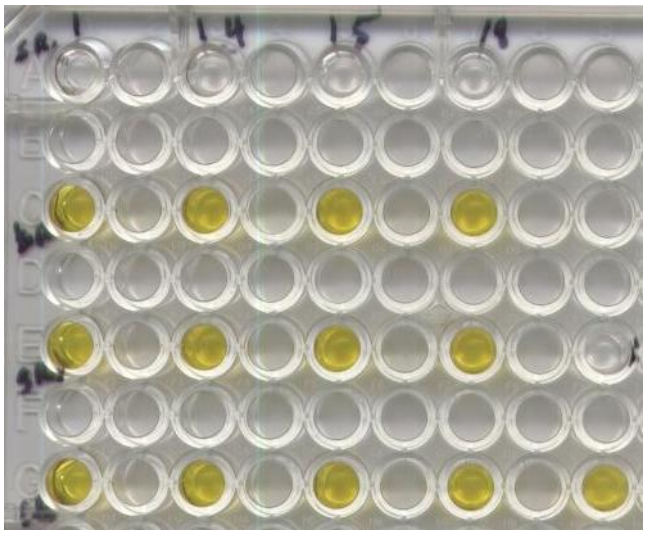

Figure 3-21 Epitope mapping of scFvs against SR1, LR, SR2 and SR3 in ELISA.

MBP-fusions of SR1, LR, SR2 and SR3 were coated to rows A, C, E and G. BSA and MBP were coated to G9 and E9 as positive and negative controls. . The four purified scFvs were used to probe wells in rows A, C, E and G (clones 1, 14, 15 and 18 respectively as indicated at the top) through to column 7. Anti BSA was used to probe wells G9 and E9. Binding was detected with anti-c-myc and HRP conjugate (all wells through to column 9). All wells were developed with TMB.

In light of this finding scFvs 1, 14, 15 and 18 plus anti-c-myc and anti-rabbit-HRP conjugated antibodies were further tested for their ability to bind the plastic of ELISA plate. This was done to confirm the findings revealed in Figure 3-21. Figure 3-22 shows that scFvs, anti-c-myc and anti-rabbit antibodies were unreactive to the plastic of the ELISA plate. However, a positive control anti-BSA scFv was reactive to BSA while, the same, anti-BSA antibody was unreactive with MBP as a negative control.

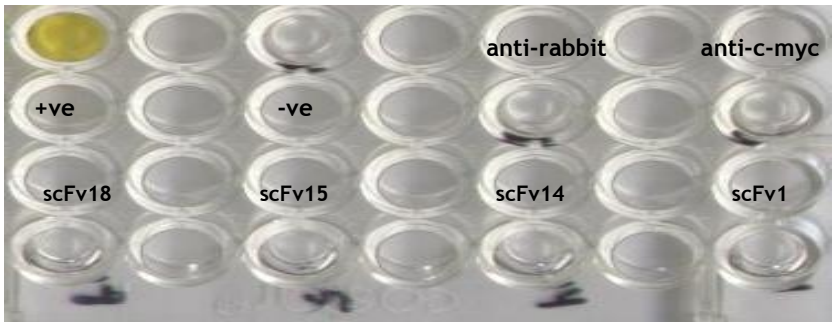

Figure 3-22 Binding of scFvs, anti-c-myc and anti-rabbit HRP conjugated antibodies to the plastic of ELISA plate.
The bottom row of four wells (labelled scFvs 1, 14, 15 and 18) and the two wells at the top right (labelled as anti-rabbit and anti-c-myc) were coated with PBS. BSA and MBP were coated to the left area of the top row, labelled as positive and negative controls. The bottom row was probed with the scFvs and then probed with anti-c-myc along the top right well. All wells except control wells were probed with anti-rabbit along with the well labelled as anti-rabbit. Control wells were probed with anti-BSA and then anti-c-myc followed by HRP conjugate and then all wells were developed with TMB.

3.3.10 Cloning of pVIII phage protein into pIT2 vector

While experiments to this point demonstrated that anti-TcdA scFvs could be isolated from the Tomlinson libraries, it was recognised that the project's ultimate goal – assessment of whether these antibodies possessed the ability to neutralise the biological activity of the toxin – might demand a different set of characteristics (*eg* aggregation of TcdA to inhibit entry to the target cell or higher avidity interaction with the toxin than could be achieved with a monovalent scFv). Presentation of the scFvs in a multivalent format was developed to enable these issues to be addressed. To accomplish this, fusion of a scFv to the phage coat protein pVIII was attempted since pVIII is present on the phage coat in much higher numbers (2700 copies) than the normal capside protein used for phage display (pIII; 5 copies per virus particle).

3.3.10.1 Analysis of digested pIT2 vector carrying anti-TcdA scFv15 and amplified gVIII

Forward and reverse primers were used to amplify the gene for pVIII from phage genomic DNA using a standard PCR protocol. The product was analysed by agarose gel electrophoresis and the results (Figure 3.23) showed that an amplification product was obtained of around the size expected for gVIII (180 bp).

The cloning vector pIT2 had already been used for successful phage display of scFvs as fusions to pIII. The vector carrying the anti-TcdA scFv clone 15 was digested with *Not*I and *EcoR*I restriction endonucleases to excise the gIII sequence as a 1500 bp fragment as shown in Figure 3.24. After preparation of the PCR product carrying pVIII coding sequence with the same restriction enzymes (Figure 3.24, lane 6), the DNA was ligated into the vector.

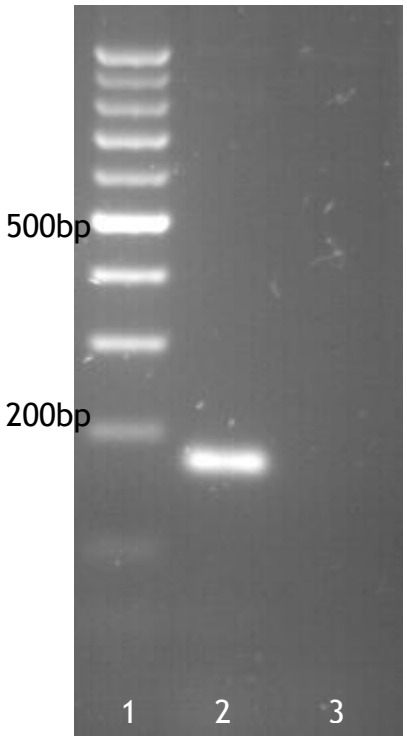

Figure 3-23 Agarose gel analysis of gVIII amplification
The PCR product at 180bp (lane 2) and a negative control sample (lane 3) were loaded to a 1% agarose gel along with DNA markers (lane 1).

3.3.10.2 Ligation and transformation

Digested pIT2 vector and the insert carrying pVIII sequence were ligated and transformed into competent *E. coli* TG1 cells. Transformants were screened by colony PCR using forward and reverse primers for the pVIII coding sequence. This was predicted to generate a PCR product of 180 bp; Figure 3.25 shows bands in lanes 4 to 6 that identified candidate clones for further analysis.

3.3.10.3 Restriction analysis of candidate transformants

Candidate clones that showed preliminary evidence for the presence of the pVIII coding sequence were grown and plasmid DNA was prepared from liquid cultures. Plasmid DNA was then digested with *Not*I and *Eco*RI restriction endonucleases to excise the small insert thought to be present. Products were analysed by agarose gel electrophoresis (Figure 3.26). Samples loaded to lanes 4, 5 and 6 show clear evidence of excision of a small DNA fragment consistent with the predicted size of the pVIII reading frame with a much larger vector fragment evident at the top of the gel. The analysis was less clear-cut for the sample loaded to lane 3 where faint bands can be seen between 500 bp and 1 kb, and a fragment migrating at less that 200 bp is faint. The results shown in Figure 3.26 reveal that for at least some of the transformants, replacement of the pIII coding sequence with pVIII adjacent to that for scFv 15 had taken place.

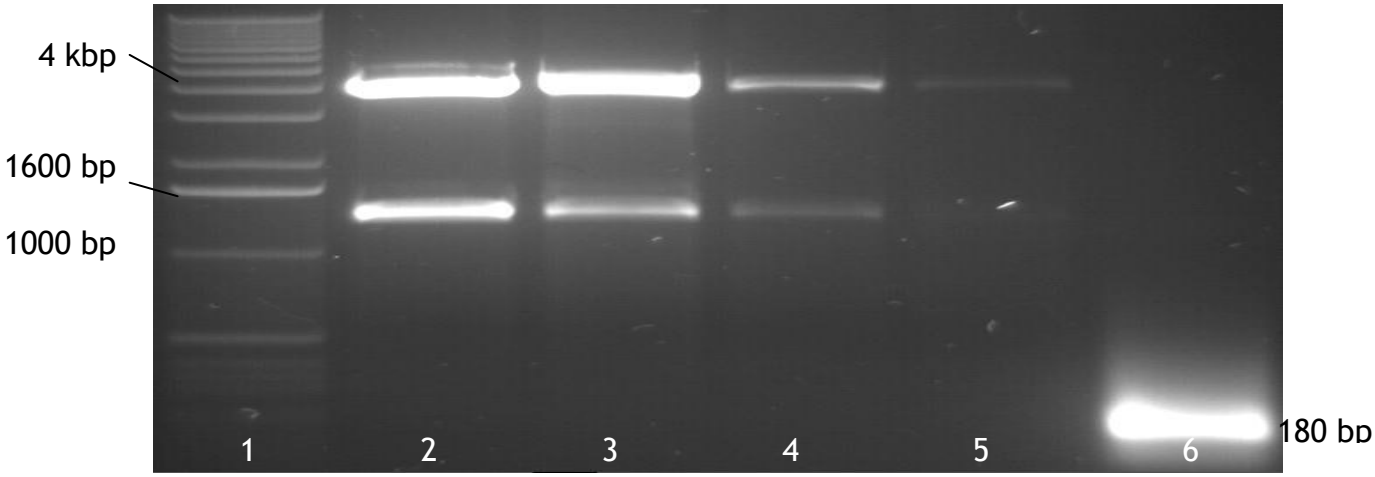

Figure 3-24 Agarose gel analysis of digestion products of plasmid DNA and PCR product
The cloning vector and amplified gVIII sequence were digested with *Not*I and *Eco*RI restriction endonucleases and loaded to a 1% agarose gel (lanes 2 – 5: pIT2 vector containing scFv clone 15 sequence; lane 6: amplified pVIII sequence). A set of 1 kb DNA markers were loaded to lane 1 to enable the size of products to be checked.

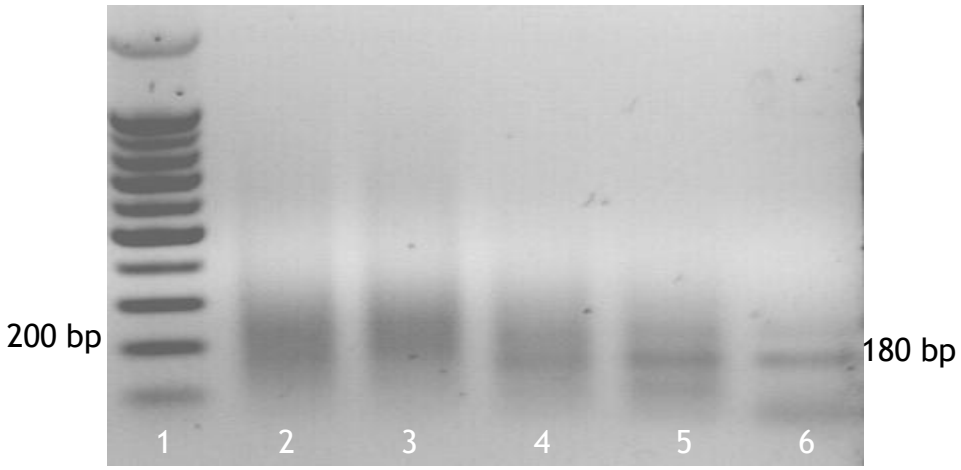

Figure 3-25 Agarose gel analysis of colony PCR products
Single transformants were picked and crude DNA extracts were used as template for PCR reactions for presence of the pVIII coding sequence (lanes 2 to 6). A 100 bp DNA ladder was loaded to lane 1 to enable estimation of the size of amplification products.

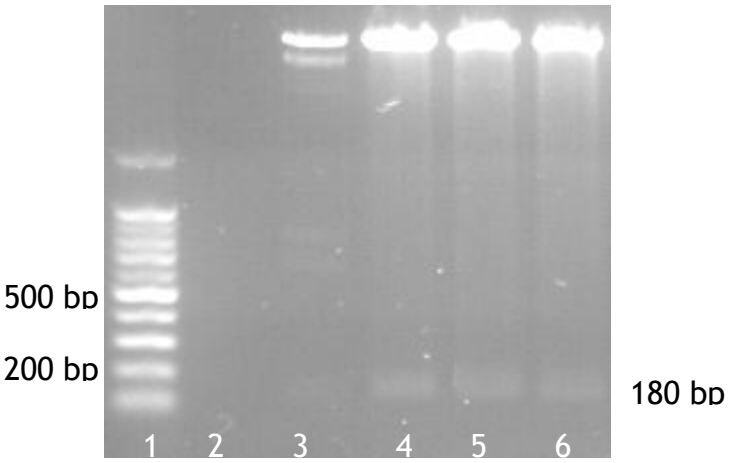

Figure 3-26 Agarose gel electrophoresis of digestion products from candidate clones
Plasmid DNA samples were digested with *Not*I and *Eco*RI and the products (lanes 3 to 6) were separated by electrophoresis. A blank lane (lane 2) separates the samples from a 100 bp DNA ladder (lane 1).

3.3.10.4 Sequencing

To confirm the reading frames were intact, plasmid DNA was sequenced using using the primer DPK9 FR1 (Table 3.1) that anneals to sequences within the scFv reading frame. The data (Figure 3.27, panel A) confirmed that as predicted from earlier experiments, the sequence for pIII originally present in the pIT2 vector had been replaced with that for pVIII, the sequence being flanked by *Not*I and *Eco*RI restriction sites. BLAST analysis (Figure 3.27, panel B) showed the presence of human kappa light chain sequence upstream from pVIII as would be predicted. Differences between the query sequence and database entry in panel B reflect the diversification of the immunoglobulin sequences.

A

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CTGCCGGCAGTCAGAGCATTAGCAGCTATTTAAATTGGTATCAGCAGAAACCAGGGAAAGCCCCTAAGCTCCT
GATCTATAGTGCATCCAGTTTGCAAAGTGGGGTCCCATCAAGGTTCAGTGGCAGTGGATCTGGGACAGATTTC
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AAGAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTG
CAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCG
CAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTT

NotI: GCGGCCGCA
gVIII: GAGGGTGACCCCGCAAAAGCGGCCTTTAACTCCCTGCAAGCCTCAGCGACCGAA
TATATCGGTTATGCGTGGGCGATGGTTGTTGTCATTGTCGGCGCAACTATCGGTATCAAGC
TGTTTAAGAAATTCACCTC
GAAAGCAAGCTGA
EcoRI: AAAGCAAGCTGATAAGAATTCACT
```

B

```
>  gb|DQ184511.1|  Homo sapiens clone ID2E1 immunoglobulin light
chain variable
region mRNA, partial cds
Length=324

 Score =  425 bits (230),  Expect = 6e-116
 Identities = 251/260 (97%), Gaps = 6/260 (2%)
 Strand=Plus/Plus

Query TGCC-GGC-AGTCAGAGCATTAGCAGCTATTTAAATTGGTATCAGCAGAAACCAGGGAAA
      |||| ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct TGCCGGGCAAGTCAGAGCATTAGCAGCTATTTAAATTGGTATCAGCAGAAACCAGGGAAA

Query GCCCCTAAGCTCCTGATCTAT-AGTGCATCC-AGTTTGCAAAGTGGGGTCCCATCAAGGT
      |||||||||||||||||||| | ||||||| | |||||||||||||||||||||||||
Sbjct GCCCCTAAGCTCCTGATCTATTA-TGCATCCTA-TTTGCAAAGTGGGGTCCCATCAAGGT

Query TCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCTGAAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct TCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCTGAAG

Query ATTTTGCAACTTACTACTGTCAACAGTCTTGTACTTCTCCTGATACGTTCGGCCAAGGGA
      ||||||||||||||||||||||||| ||| |||| ||||||||||||||||||||||||
Sbjct ATTTTGCAACTTACTACTGTCAACAGACTTATACTGCTCCTGATACGTTCGGCCAAGGGA

Query CCAAGGTGGAAATCAAACGG
      ||||||||||||||||||||
Sbjct CCAAGGTGGAAATCAAACGG
```

Figure 3-27 Partial nucleotide sequence of the scFV-15-pVIII construct. Recombinant plasmid carrying scFv-15-pVIII fusion was sequenced using DPK9 FR1 primer Nucleotide data are presented in Panel A. Sequence in the blue font shows the *Not*I restriction site, red shows the coding sequence for pVIII and purple shows the *Eco*RI restriction site. Panel B shows BLAST output for sequence upstream of the *Not*I restriction site.

3.3.10.5 Production and characterisation of phage displaying scFv clone 15 as a fusion to pVIII

Bacteria that carried scFv clone 15 in the original pIT2 vector and four modified vectors were grown for the production and characterisation of virus. In the original configuration, the scFv was displayed at the viral surface as a fusion to pIII. Hence at most, each virus would bear 5 copies of the scFv protein at the capsid terminus. In the four modified clones, fusion to pVIII might be expected to present as many as 2700 copies of the scFv protein along the length of the filamentous viral particle. Viral assembly was carried out as described; bacteria were grown in liquid culture, infected with helper phage and viral stocks were precipitated from overnight culture supernatants. Virus were titrated by measuring the conversion of bacteria to ampicillin resistance.

Characterisation was done in ELISA wells coated with the TcdA-MBP fusion or MBP as a control, and about 1x 10^6 cfu for each viral stock. MK13 served as a negative viral control. In Figure 3.28, wells in column 1, rows A, C, E and G were coated with the TcdA-MBP fusion protein and virus displaying the scFv-pVIII fusion were added. Binding was detected with anti-M13 reagents. From the colour apparent in these wells, the functional display of scFv clone 15 and its interaction with the target protein is evident. In column 3, wells in the same rows were coated instead with MBP. The failure of the virus from each stock to adhere to the wells confirmed the specificity of the reactions in column 1 for sequences derived from TcdA. As a positive control, virus displaying scFv clone 15 as a fusion to pIII was tested against the TcdA-MBP fusion (well 5A) and as a further control, KM13 helper phage were tested for reactivity towards the fusion protein target (wells 7A, 7C, 7E and 7G). The data showed without ambiguity

that a TcdA-specific scFv was required linked to either pVIII or pIII for a positive signal in the assay.

Figure 3-28 ELISA characterisation of virus bearing scFv clone 15 as a fusion to pVIII
Wells in columns 1, 7, and 9, rows A, C, E and G, were coated with the TcdA-MBP fusion, whereas the equivalent wells in column 3 were coated with MBP. Well 5A was also coated with the TcdA-MBP fusion protein. Four stocks of virus bearing scFv 15 fused to pVIII were added to wells in columns 1 and 3 wheras KM13 helper phage was added to wells in column 7. Phage displaying scFv 15 as a fusion to pIII was added to 5A. No virus were added to wells in column 9. Attachment of virus to the coated plastic was detected with anti-M13 reagents and TMB substrate.

3.4 Discussion

The aim of this phase of the study was to isolate scFvs from Tomlinson I and J libraries against the purified TcdA-MBP fusion protein, directing the selection strategy towards the clostridial sequences rather than the MBP carrier. Given that the TcdA sequence fused to MBP were small in size yet carried features responsible for receptor-binding, the intention was to attempt to isolate scFvs that might prevent interaction between Toxin A and its receptor. That outcome will be addressed in the next section of the thesis in an evaluation of their ability to protect against native Toxin A *in vitro* and *in vivo*.

Irrespective of the activity of the anti-TcdA scFvs, it was important to assess their capacity to recognise features of native rather than recombinant Toxin A and to attempt to map the epitopes to which they adhered.

3.4.1Panning of Tomlinson libraries

Small molecular weight antibodies can retain antigen-specific affinity and neutralising activities against complex target molecules. For example, Dagan and Eren have reported that scFvs can retain the neutralising activity of full-length parental antibodies (Dagan and Eren, 2003). scFvs antibodies with the ability to detect and neutralise toxins have been isolated in a number of different studies. For example, anti-pertussis toxin scFvs were reported to be able to recognise and neutralise this virulence factor *in vitro* (Williamson and Matthews, 1999) and Chen and colleagues have isolated scFvs against the Protective Antigen of *Bacillus anthracis* toxin and noted that these have the capacity to neutralise the

cytotoxic action of Lethal Toxin *in vitro*. The group reported that the scFvs recognised conformational epitopes at the carboxy-terminus of Protective Antigen (Chen et al., 2006).

In addition, scFv antibodies against *Clostridium botulinum* neurotoxin type A were isolated from immunised and non-immunised sources. Upon epitope mapping, it was found that only a subset of the scFvs were able to neutralise the toxin (Amersdorfer et al., 2002a).

Recently Scott and colleagues isolated more than 50 scFv antibodies specific for the heavy chain carboxy-terminal sub-domain of tetanus toxin. Fifteen scFvs were assessed in a competitive binding to identify non-competing pairs that could then be linked to create Chelating Recombinant Antibodies for assessment of toxin-neutralising activity affinity (Scott et al., 2010).

The Tomlinson I and J libraries represent a rich resource for the isolation of scFvs by phage display. Each contains more than 100 million different scFv fragments cloned into an ampicillin resistant phagemid vector and transformed into TG1 *E. coli* cells. These resources were chosen for the isolation of anti-TcdA scFvs using an MBP fusion protein containing the putative receptor-binding site of TcdA. The goal was to direct selection towards TcdA sequences as a first priority. Before embarking on selection, the libraries were checked for polyreactivity against MBP (the fusion protein carrier), BSA (as an irrelevant purified protein and potential blocking protein) and milk (used as blocker in selection and immunoassay). These studies confirmed that the libraries contained very low numbers of phage that were able to recognise BSA and MBP but some evidence was seen of polyreactivity against the complex protein mixtures present in milk. For this understanding, aliquots of library phage were

pre-incubated with milk and MBP to exclude binding against the blocked surface of the selecting immunotube and the MBP carrier protein. This aimed to direct selection against TcdA sequences carried in the recombinant fusion protein.

The outcome of selection showed that progressive enrichment of phage specific for the fusion protein emerged from the phage display strategy. This was apparent from monoclonal phage ELISA using isolates picked after each round of selection with some flattening off in enrichment from round two to round three. Variations in recoveries of target-specific phage round on round have been documented (de Bruin et al., 1999, Li et al., 2003). Increasing the number of selection cycles from three to five has been done by some researchers but it does not necessarily improve the specificity of the screen or the quality of the binders that emerge. In this study, the selection process provided a reasonable number of phage for analysis and characterisation.

During library construction, some clones arise that lack V_H or V_L components of the scFv reading frame. Bacteria that carry these short inserts can grow more quickly than these with full scFvs and if the antibodies possess affinity for the target, they can emerge from selection. Therefore, genetic characterisation was used to verify the size and integrity of the inserts. Although some clones carried scFv reading frames of exactly the same size as positive control phage, others showed smaller sizes. It has been noted that phage carrying partial inserts can emerge during amplification cycles and come to dominate the output (de Bruin et al., 1999). This is because the expression of recombinant antibodies by bacteria can be toxic and therefore selective pressure can drive the progressive deletion of sequence from scFvs during selection and amplification. Sequencing of clones that carried full length scFv

confirmed the presence of intact reading frames in four clones. In contrast, other clones showed deletion of sequence from the scFv reading frames and some showed stop codons randomly scattered within the sequence of the scFv. Given that the antibodies in the libraries are all constructed on the same heavy and light chain framework sequences, differences would be expected to exist only at those sites in the CDRs that were designated for diversification, though the diversification process for library J (NNK residues at designated positions) has the potential to introduce stop codons. DNA sequence analysis showed that the CDR sequences for the four clones taken forward were unique for which may suggest that they recognise non-overlapping epitopes in the TcdA-MBP target protein. Amino acid differences were located in CDR2 (residues H50, H52, H52a, H53, H55, H56, H58), and CDR3 (H95, H96, H97, H98) for the heavy chain component, and CDR2 (L50, L53) and CDR3 (L91, L92, L93, L94, L96) for the light chain component. Careful scrutiny of the data showed that the VL sequence contained a high frequency of serine and threonine amino acids. Carzaniga and colleagues have reported similar observations (Carzaniga et al., 2002). This may have arisen because of selection pressure imposed by the general nature of the target protein as both serine and threonine share the same physical and chemical properties (neutral-polar/hydrophilic amino acids).

The binding of the scFvs to TcdA sequences in the fusion protein target, their failure to recognise the MBP carrier, and positive results in ELISA using native Toxin A and Toxin B all show that the selection process used in phage display was successful. However, the scFvs failed to recognise the recombinant target protein on Western blots whereas immunoprecipitation was successful, suggesting that their epitopes were susceptible to denaturation through the application of heat, the used of SDS or a combination of these factors. Hence, it

seems likely that the scFvs recognise conformational epitopes on the target recombinant protein. From this observation, attempts were made to map the epitopes.

3.4.2Epitope mapping of anti-Toxin A scFvs

The carboxy-terminal region of Toxin A is thought to form a solenoid-like structure made up from short and long repeats of peptide sequences (Florin and Thelestam, 1983). Each repeat consists of a ß-hairpin and a loop structure and at least some of these features represent the receptor binding site of the toxin (Greco et al., 2006). The sequence conservation in the short and long repeats are thought to play a role in the formation of kinks which are speculated to modulate receptor recognition and cell entry (von Eichel-Streiber et al., 1992, Weis and Drickamer, 1996). Hence, some regions in the carboxy-terminal domain of the toxin may have more importance in receptor recognition than other regions. This was supported by structural analysis (Ho et al., 2005). Therefore, an important goal for the research reported in this thesis was to identify more precisely the regions with Toxin A that are recognised by the scFvs. Potentially, the binding characteristics of these antibodies might have significance with regard to *in vitro* experiments exploring the capacity to inhibit toxin action.

To identify the regions of the putative binding site recognised by the anti-TcdA scFvs, truncated mutants of the target protein were created by site-directed mutagenesis, and the protein products were expressed and purified. The truncated proteins retained MBP as a fusion partner to assist with expression and purification (di Guana et al., 1988). Recognition of the target protein by all four scFvs was lost when the only TcdA sequence present was SR1. However, binding was restored on addition of the LR sequence and was retained by

sequential addition of SR2 and SR3. This suggests that the epitope(s) are located in the LR sequence, at the interface of SR1 and LR, or in the loop connecting these repeat sequences. Interestingly, receptor recognition is mediated to a significant extent by sequences present in the LR motif (Ho et al., 2005).

The next phase of the project explored whether the anti-TcdA scFvs showed any capacity for toxin neutralisation.

Chapter Four Protection

4.1 Introduction

Many studies have shown that antibodies created against bacterial toxins have the capacity to neutralise the activities of the native molecules and, depending on the role of the virulence factor in the pathogenic process, to attenuate bacterial infection. While these antibodies can be triggered by natural infection, vaccination or immunisation with a toxin derivative, passive protection is also possible, the most common, natural examples being the passage of antibodies from the mother to her baby either *in utero* or later during feeding.

These general principles are evident in work with *Clostridium difficile* and monoclonal antibodies (MAbs) and immunoglobulin concentrates are available with activity against *C. difficile* toxins. These agents have been successful *in vitro* and *in vivo* (Lyerly et al., 1991, Corthier et al., 1991, Kelly et al., 1996, van Dissel et al., 2005, Lowy et al., 2010).

Passive immunity induced by intravenous administration of monoclonal antibodies against Toxin A, has been shown to protect experimental animals against *C.difficile* infection. Kelly and colleagues have done similar studies to those initially reported by Lyerly et al 1991. The group immunised Holstein cows as described by Lyerly 1991 and colostrum was collected from the immunised cows from the first six milkings. From this an immunoglobulin concentrate that contained high levels of bovine IgG against Toxins A and B. The researchers then evaluated the protective activity of the bovine IgG in the concentrate, observing that the immunoglobulins showed protective activity against the cytotoxic

effects of TcdA and TcdB when assayed with a human fibroblast cell line. The preparation also blocked the binding of Toxin A to its receptor on enterocytes in the rodent ileum and inhibited enterotoxic effects. Thus, bovine immunoglobulin concentrate neutralised the cytotoxic effects of *C. difficile* toxins *in vitro* and inhibits their enterotoxic effects *in vivo* (Kelly et al., 1996).

Recently Van Dissel and colleagues produced polyclonal antibodies in Holstein-Fresian cows by immunisation with inactivated *C. difficile* toxins and killed whole cells. The researchers collected the milk from immunised cows and prepared a whey protein concentrate that contained polyclonal antibodies against TcdA and TcdB. The immune concentrate contained high levels of secretory IgA antibodies. Protection was demonstrated against the cytotoxic effects of *C. difficile* toxins *in vitro* and *in vivo*. The group also obtained preliminary data in humans showing that the immune concentrate could help prevent relapse of *C. difficile* infection (van Dissel et al., 2005).

In another study Babcock and colleagues used transgenic mice carrying human immunoglobulin genes to isolate human monoclonal antibodies able to neutralise the cytotoxic effects of either TcdA or TcdB. The group tested whether mice injected with various concentrations of purified monoclonals would inhibit the intestinal fluid accumulation provoked by the native toxins (Babcock et al., 2006).

More recently Lowy and colleagues have developed two fully human monoclonal antibodies against *C. difficile* Toxins A and B. The group were able to establish the efficacy of the combined antibodies in a hamster model of *C. difficile* infection and went on to test the antibodies in healthy human volunteers. This was followed by a study of the efficacy of the combined

antibodies in protecting against recurrence of *C. difficile* infection in a group of 200 patients. Patients receiving either metronidazole or vancomycin for symptomatic *C .difficile* infection were infused intravenously with a single dose of both antibodies. The monoclonal antibodies significantly reduced the recurrence of *C. difficile* infection in treated patients when compared to those receiving antibiotics alone (Lowy et al., 2010).

Numerous studies have shown that the biological activities of full length antibodies can be retained in smaller, recombinant constructs. For example, Gould and colleagues have studied the ability of different antibody constructs to protect against West Nile virus infection. Experimental animals were infected intraperitoneally with West Nile virus and injected with doses of rabbit IgG against the pathogen on a schedule beginning 1 day prior to 5 days following the infection. Human IgG1, several scFv constructs and a scFv-Fc fusion protein were also tested. Full length and recombinant antibodies were able to neutralise against the infection. The group commented that the use of phage display libraries provides a productive approach to isolating scFvs to a target of interest (Gould et al., 2005).

4.1.1Aims for the experiments

It is thus clear that antibodies reactive with TcdA and TcdB have the potential to neutralise the effects of these toxins *in vitro* and *in vivo* and that recombinant scFv proteins, at least in other systems, can retain the properties of the original full length immunoglobulins. Having isolated scFvs specific for TcdA, the aims of this phase of the project were to establish if these recombinant antibodies had the capacity to block TcdA activity in a tissue cell assay and in a ligated rat loop challenge system.

4.2 Material and methods

4.2.1 In vitro *protection*

Purified scFvs were tested for the ability to protect against the cytotoxic effects of Toxin A. Earlier experiments confirmed the reactivity of four purified scFvs towards the TcdA sequences carried in an MBP fusion. Hence, a series of experiments were designed to test the ability of the scFvs to protect against Toxin A effects *in vitro* using Vero cell.

4.2.1.1 Biological effects of TcdA

4.2.1.1.1 Examination of the effect of TcdA on Vero cells

TcdA was tested to assess its ability to bind to its receptors on Vero cells and to exert its cytopathic effect on the actin cytoskeleton. Specifically, the action of the toxin is to induce rounding of the target cells. Vero cells were grown in minimal Eagles medium (DMEM medium; BioWhittaker, Belgium) containing 10% (v/v) fetal bovine serum, 1 mM L-glutamine, supplemented with streptomycin-penicillin and fungizone (termed "complete medium"). Freshly trypsinised Vero cells were subcultured into 100 µl of complete medium in flat bottomed 96 well cell culture plates (Nunc, Denmark) at $5x10^3$ cells/well. Cells were allowed to recover for 18 h at 37 ^oC in an atmosphere containing 5% CO_2. The plate was then washed three times with complete growth medium.

Toxin A was the kind gift of Dr Ralf Gerhard, Hannover Medical School, Germany. The TcdA was produced as a full length recombinant protein in *Bacillus megaterium* and purified by nickel affinity chromatography (Burger et al., 2003). The properties of the recombinant protein are highly comparable

with those of TcdA purified from *Clostridium difficile* (Gerhard et al., 2005). The toxin was stored in aliquots at -80 ^{o}C.

TcdA was thawed from storage at -80 ^{0}C and added at 100 ng/well in 100µl of complete growth medium to the Vero cells. Control wells contained only Vero cells and complete medium. The assay was carried out in triplicate. The plate was then incubated at 37 ^{o}C for 5 h in 5% CO_2 and cells were inspected at hourly intervals using an inverted microscope to assess the effect of the native Toxin A. The extent of cell rounding was assessed visually and data collected as the percentage of rounded cells in several fields of view.

4.2.1.1.2 Minimum effective dose of TcdA on Vero cells

Having validated a Vero cell assay of toxin activity, this test was used to determine the minimum dose of Toxin A required to elicit cell rounding. Freshly trypsinised cells were subcultured at $5x10^3$ cells/well in 100 µl of complete medium in flat bottomed 96 well cell culture plates. Cells were then recovered for 18 h as described before. The plate was then washed 3 times with complete medium. Cells were then challenged with ten fold serial dilutions of TcdA starting at 100 ng per well and extending down five dilutions to 1 pg/well. The plate was then incubated as described for 5 h. Cells were inspected for evidence of cytopathic effects (cell rounding) on a regular basis each hour up to 5 h using an inverted microscope. In control wells, Vero cells were cultured in the absence of toxin. The assay was carried out in triplicate.

4.2.1.1.3 Inhibition of TcdA with polyclonal anti-toxin in vitro

These experiments established whether the assay was able to detect the toxin-neutralising activity of a commercial polyclonal anti-Toxin A antibody. As before, Vero cells were subcultured at $5x10^3$ per well and washed. In a separate plate, Toxin A was dispensed at 100 ng/ml and was pre-incubated with serial dilutions of polyclonal anti-Toxin A antibody (List Laboratories) extending from undiluted down to a 500 fold dilution. After two hours of incubation, the mixtures were transferred to the cell culture plate containing the Vero cells and inspected over the course of 3 h. Control wells contained Vero cells without any additional reagents. The experiment was carried out in triplicate.

4.2.1.2 Neutralisation of TcdA with scFvs

Having established the basic parameters of the experimental system and some aspects of the action of Toxin A, experiments were carried out to assess if anti-TcdA scFvs were able to protect Vero cells against the effects of the toxin. Experiments were standardised using the effective dose of the native toxin identified earlier.

4.2.1.2.1 Protective activity of monovalent soluble scFvs against TcdA

a- Ability of soluble scFvs to block the effect of TcdA on Vero cells

Vero cells were prepared as described earlier and Toxin A (100 ng / ml) was pre-incubated in a separate plate with soluble scFvs (100 μg/ml) in a total volume of 100 μl for 2 h at room temperature. Mixtures were then transferred to the cells in the culture plate and incubated at 37 ^{0}C and 5% CO_2 for a total of 5 h. Cells were inspected regularly each hour to assess the extent of cell rounding over the time allowed for the experiment. Given that the toxin dose used triggered rounding of a very high proportion of cells over a short period of time,

protective activity was reported as the percentage of cells that retained their stellate shape during the 5 h of incubation.

b- Minimum scFv concentration required for 50% protection of Vero cells against TcdA challenge

In these experiments, the effective dose of Toxin A was fixed, as was the number of cells in each well ($5x10^3$ cells per well) and the duration of the challenge period (2 h following addition of toxin). To quantify the protective efficacy of each antibody, aliquots of Toxin A were pre-incubated with two fold serial dilutions of each scFvs down to the 5^{th} dilution. Mixtures were then transferred to the cell culture plate, incubated, and inspected over a 2 h period. The test was repeated three times. To guard against experimental bias, plate layouts were unknown to the observer for the second and third tests. Average concentrations, standard errors and the minimum concentration of scFv required for 50% protection were calculated.

4.2.1.2.2 Protective activity of multivalent scFvs

In these experiments phage were used with Toxin A in an attempt to neutralise the action of TcdA. Virus carrying scFv-pIII fusions were compared with those carry many more copies of the scFv at the phage surface (scFv-pVIII fusions). The pVIII constructs were generated just for scFv clone 15. Again, the cytopathic effect of Toxin A was assayed using the rounding of Vero cells *in vitro*.

a- scFv-pIII fusions

Phage were produced from clones carrying scFvs fused to pIII. Vero cells were prepared as described above and fixed numbers of phage for each clone (1×10^6 virus) were pre incubated with Toxin A at 100 ng/ml in total volume of 100 µl/well. Mixtures were then added to the cells in the culture plates and plates

were incubated at 37 ^{0}C and 5% CO_2. Cells were inspected each hour up to 5 h to assess the protective activity of multivalent display of scFv-pIII fusions *versus* TcdA.

b- scFv-pVIII fusion

Similarly, phage were produced from scFv clone 15 clone in which the scFv sequence was fused to pVIII. Vero cells were prepared as mentioned above and similar numbers of phage to those used for scFv-pIII fusions were pre incubated for 2 h with TcdA at 100 ng/ml in total volume of 100 µl/well. The mixture was then added to the cells and culture plate was incubated at 37 ^{0}C and 5% CO_2. Cells were inspected each hour up to 5 h to assess the protective activity of the virus bearing the scFv-pVIII fusion.

4.2.2 In vivo protection

Purified soluble scFv 15 was further examined for its ability to protect against the enterotoxic effects of Toxin A in the rodent lumen. These experiments were carried out in collaboration with Dr M. Lucas, School of Life Sciences, University of Glasgow and adhered to relevant animal welfare legislation.

4.2.2.1 Examination of the effect of TcdA on the intestinal physiology of the rat

To assess the ability of the Toxin A to bind to its receptor on the intestinal epithelium of rats and exert its entertoxic effect, Toxin A was tested in ligated jejunal loops of rats. The rats used for these experiments were 300-410 grams adult Sprague-Dawley males. Rats were anaesthetised and in each animal, a tracheotomy was established to maintain a patent airway. The body of

the animal was maintained at 37 ^{0}C using a heated pad, core temperature being monitored by a rectal thermistor. In each animal, two 10 cm jejunal loops were created and ligated, separated by an interphase loop of 5 cm so that the movement of intestinal contents between the 10 cm loops was prevented. The 10 cm loops were opened carefully from both sides and the intestinal contents were flushed out with isotonic buffer (154 mM saline) to eliminate the debris. Loops were then flushed with air to eradicate the remaining buffer. One of the loops was infused with 1.0 ml of buffer (20 mM ethanolamine, 0.2 M NaCl adjusted to pH 7.5) as control loop to assess the normal jejunal absorption, while the other loop was used to test the activity of Toxin A. Toxin was infused to the test loop at a dose of 10 µg in 1 ml of buffer. The amounts of the infusates injected to both loops were assessed by weighing the syringe before and after injection. The loops were then left undisturbed for 4 h. At the end of this period, the loops were then excised from the animal and weighed before (uncut) and after drainage of the contents with gravity (cut). The drained fluid was also carefully collected and weighed. The length of the excised loops was measured by laying them on the bench gently without stretching. Absorption was calculated for both loops to detect any fluid accumulation in the loop challenged with the toxin when compared with the control loop absorption.

4.2.2.2 Minimum effective dose of native TcdA on the intestinal epithelium cells in rats

Following from these experiments, the minimum effective dose of Toxin A required to achieve a consistent biological effect was determined. To estimate this, the concentration of Toxin A used in the previous experiment was gradually decreased from 10 µg/loop to the least concentration that produced a consistent enterotoxic effect. Experiments were carried out as described but the dose of

Toxin A reduced to 5 and 2.5 µg/loop in separate experiments. The loops were examined exactly in the same way as before. Whilst 5 µg/loop elicited a consistent effect, 2.5 µg/loop was more variable. Hence the dose of Toxin A was increased slightly to 3.5 µg/loop.

4.2.2.3 Protective effect of scFv clone 15 against TcdA

The inhibitory effect of soluble scFv-15 against the toxin was assessed using the minimum effective dose of Toxin A determined earlier. Loop length, injection volume and toxin concentration were increased by half to be 15 cm, 1.5 ml/loop and 4.5 µg/loop respectively. In each animal, two ligated jejunal loops of 15 cm were created with an interphase loop of 5 cm. The 15 cm loops were prepared as described earlier. The first loop was injected with 4.5 µg/loop of Toxin A in 1.5 ml isotonic buffer as control for the experiment. The other loop was used for the test and was injected with 4.5 µg/loop of native Toxin A in 1.5 ml of purified scFv-15. The mixture of TcdA and scFv-15 was pre incubated for 2 h at room temperature to allow interaction. The loops were left undisturbed for 4 h and all measurements were taken exactly as detailed earlier. The experiment was repeated six times. In preparation for these experiments, scFv-15 was initially used as isolated from the purification column. In later experiments, the scFv was dialysed against phosphate buffer to remove the imidazole salt component of the column elution buffer.

4.3 Results

4.3.1 Protective activity of anti-TcdA scFvs in vitro

4.3.1.1 Biological effect of TcdA

Given the recombinant source of the Toxin A, its transport over some distance, and its known instability over time, initial experiments established whether the material was active and whether Vero cells constituted a useful assay system for the toxin and one that could be used to assess the biological activity of the anti-TcdA scFvs isolated in earlier phases of the study.

4.3.1.1.1 Cytotoxic effect of TcdA on Vero cells

To assess the whether TcdA was able to bind to receptors on Vero cells and exert its cytopathic effect, Vero cells were challenged with Toxin A. Intially, cells grown on from an inoculum of $5x10^3$ cells per well were challenged with a fixed dose of 100 ng of TcdA, delivered in 100 µl of complete MEM per well. Cells were inspected every hour to check for evidence of morphological changes. Initially, the Vero cells possessed a characteristic stellate shape (Figure 4.1, panel A). In the first hour after toxin challenge, 98% of the cellular population underwent change to a rounded morphology and 100% were observed to have undergone this change 2 h and 3 h (Figure 4.1, panel B) post challenge. It was thus evident that the TcdA available for the study was able to bind to receptors on Vero cells and exert its cytopathic effect. The minimum effective dose of TcdA required to elicit this effect was investigated in the next set of experiments.

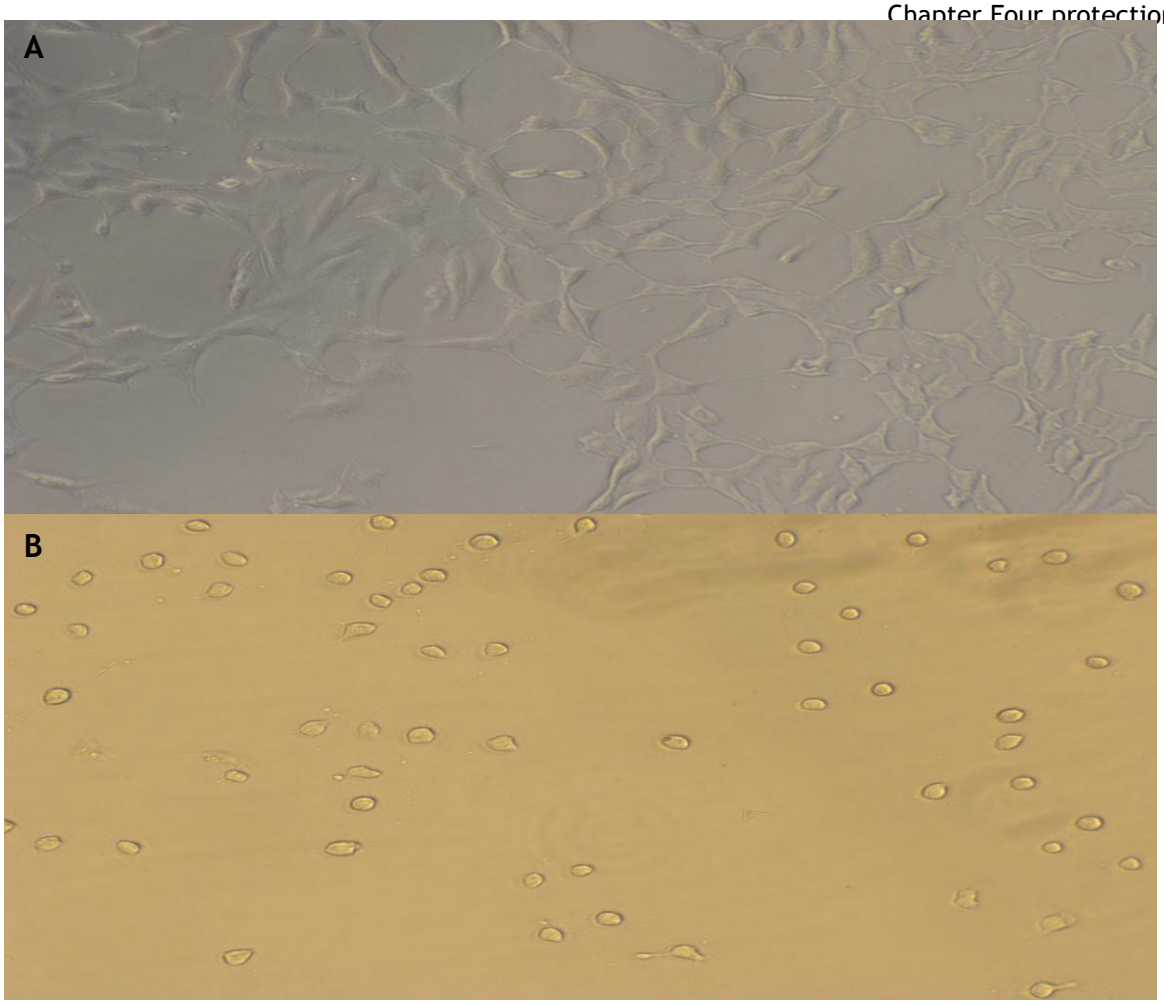


Figure 4-1 Vero cells in culture before and after exposure to TcdA
Panel A shows the normal cell morphology of cells grown for 18 h. Panel B shows the cytopathic effect of Toxin A. The image in Panel B was captured 3 h after exposure of the cells to a dose of 100 ng of toxin.

4.3.1.1.2 Minimum effective dose of TcdA on Vero cells

To determine the minimum dose of TcdA required to elicit cell rounding, Vero cells were plated and challenged with native Toxin A in a series of ten fold serial dilutions starting at 100 ng per well and extending down to 0.001 ng per well. The plate was then incubated and cells were inspected each hour using an inverted microscope. The minimum TcdA concentration that elicited a cell rounding response was 10 ng per well and at this toxin dose, 5-10% of the cell population were rounded after 1 h, about 98% after 2 h and 100% after 3 h of incubation.

4.3.1.1.3 Inhibition of the cytopathic effect of TcdA using polyclonal anti-Tcd A

A commercially-prepared, polyclonal anti-Toxin A antibody was obtained and tested for its ability to inhibit the activity of TcdA upon Vero cells. Native Toxin A was aliquoted at 100 ng/ml and was pre incubated at room temperature with dilutions of the polyclonal anti-Toxin A antibody in 100 fold dilutions ranging from undiluted, down to a 500 fold dilution. After 2 h of incubation, the mixtures were transferred to the wells of a cell culture plate, inoculated earlier with Vero cells at $5x10^3$ cells per well. The plate was then incubated at 37 ^oC for 3 h and the morphology of the cells was inspected each hour. At the final inspection time point, it was found that dilution of the polyclonal antibody to 1:200 was able to provide 100 % protection against cell rounding using a toxin challenge dose of 10 ng per well. Figure 4.2 shows the morphology of Vero cells 3 h after challenge with TcdA that had been pre incubated with polyclonal anti-TcdA antibody (panel B) and control cells that had only received culture medium (panel A). This illustrates the ability of polyclonal anti-TcdA to block the action of the toxin, bearing in mind that the antibody in this experiment was a fully length, bivalent immunoglobulin.

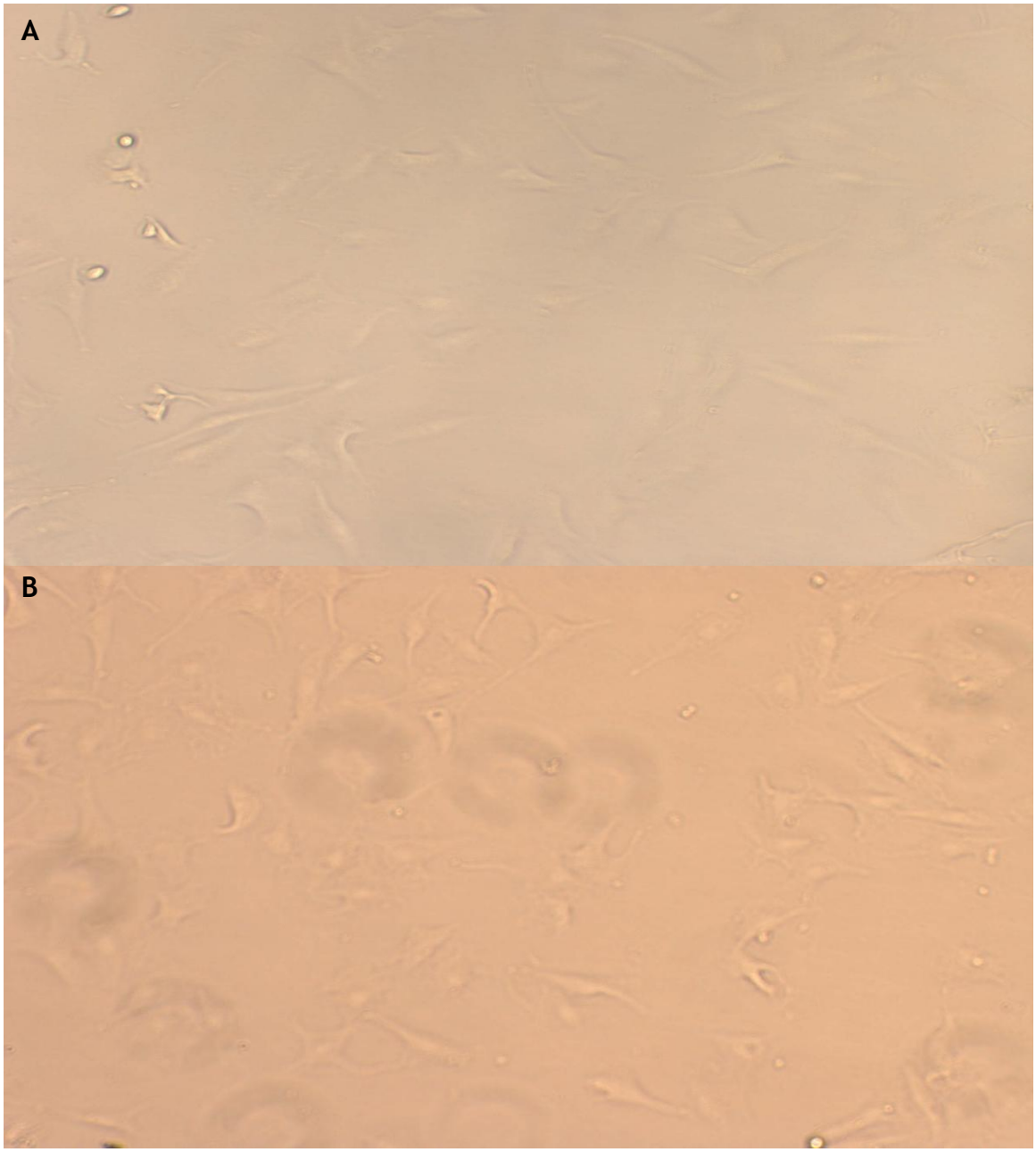


Figure 4-2 Vero cells in culture in the absence and presence of TcdA-antibody mixtures. Panel A shows the appearance of Vero cells grown for 3 h in complete growth medium. Panel B show cells incubated for the same period of time with Toxin A, pre incubated with polyclonal anti-TcdA serum.

4.3.1.2 Neutralisation of TcdA with scFvs

Having established the basic parameters of an *in vitro* assay system, experiments were done to assess whether anti-TcdA scFvs were able to protect cells against the cytopathic effects of the toxin. Experiments were standardised using the effective dose of native Toxin A identified earlier, and seeding concentrations of $5x10^3$ Vero cells per well.

4.3.1.2.1 Protective activity of soluble scFvs (monovalent form) against Native TcdA

a- Ability of soluble scFvs to block the effect of TcdA in vitro

To assess the ability of soluble scFvs to protect cells from the cytopathic effect of Toxin A, cells were prepared as detailed earlier and native toxin was pre incubated with anti-TcdA scFvs in for 2 h at room temperature. Mixtures were then transferred to the cells and incubated at 37 ^oC for 5 h. Cells were inspected each hour to assess the extent of cell rounding during the course of the experiment. Successful protection was observed for the full duration of the post challenge period. Figure 4.4 shows the morphology of cells one hour and five hours post challenge with the scFv-TcdA mixtures. In the upper panel, it can be seen that Vero cells in the fields represented have retained their characteristic stellate morphology despite the presence of mixtures containing a dose of Toxin A known to be capable of triggering rapid cell rounding. In the case of scFv clone 14, some limited cell detachment from the plastic culture plate seemed to have occurred but this cannot be seen for scFv clones 1, 15 or 18: cells appear to have retained adherence to the plate and the morphology characteristic of the Vero cell line. In the second row of the Figure are images captured 5 h after addition of the scFv-TcdA mixtures to Vero cells. Again, there is some evidence that for scFv clone 14, cell detachment and rounding has occurred but it is incomplete and adherent cells were easily observed. For scFv

15 there was more limited evidence of this effect. For clones 1 and 18, the substantial majority of cells appeared to retain their shape and remain adherent to the plastic surface.

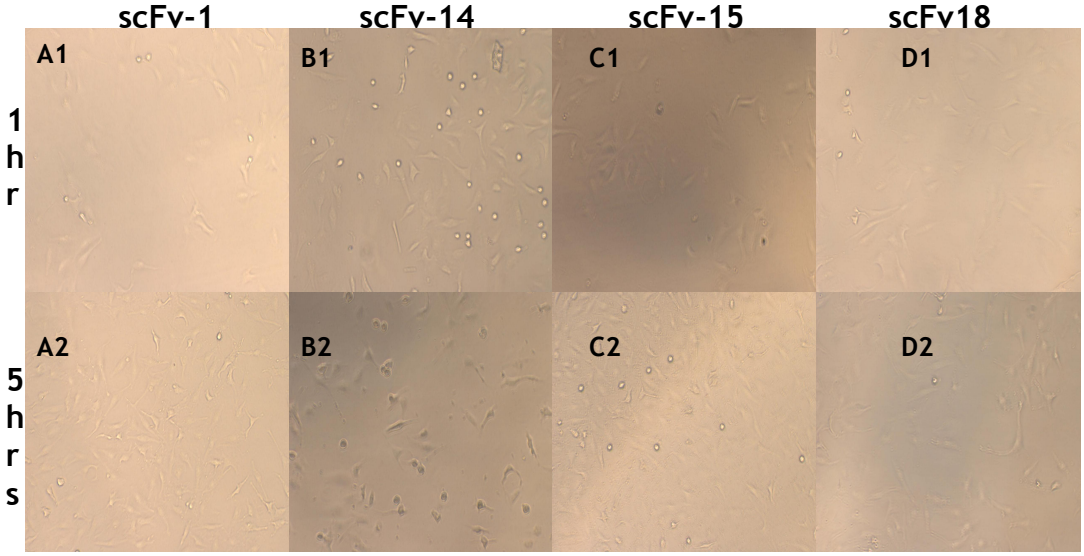
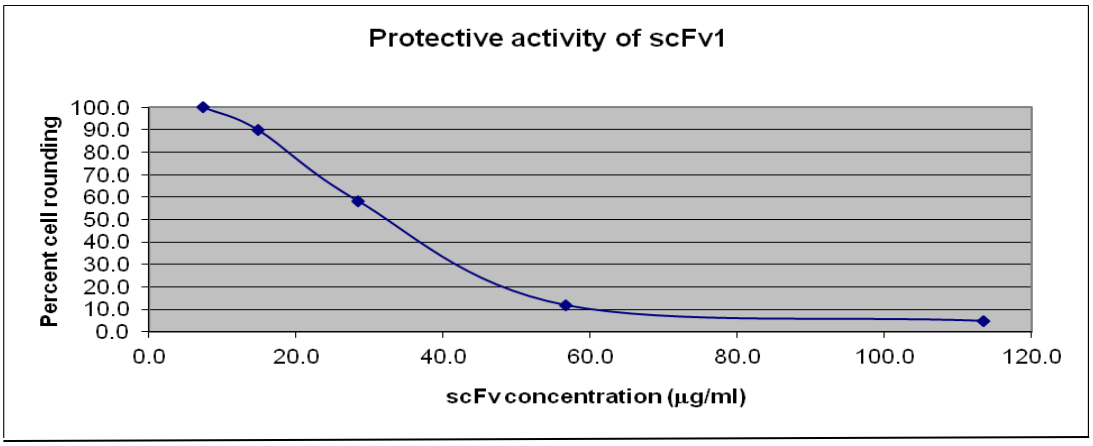
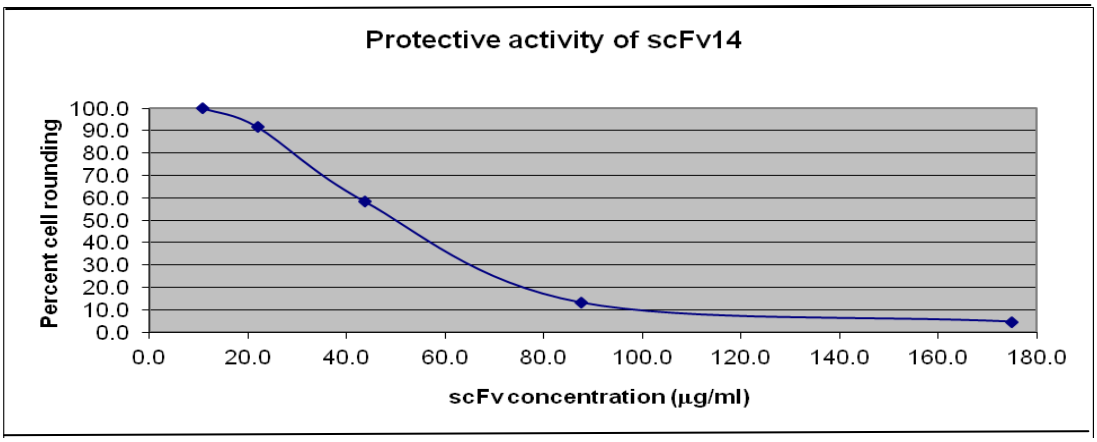


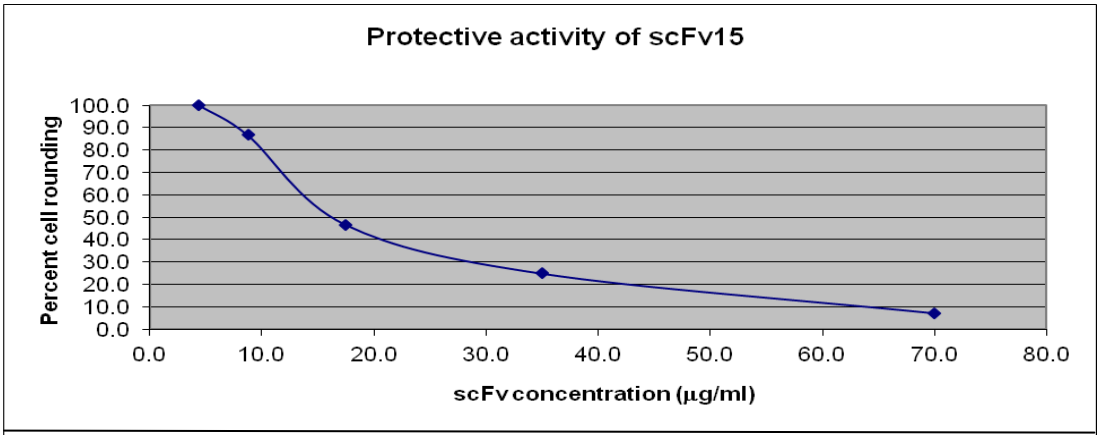
Figure 4-3 Vero cells cultured in the presence of mixtures of anti-TcdA scFvs and Toxin A. The scFvs used in pre incubation with an effective concentration of Toxin A are shown at the top of the Figure. The upper row shows images collected 1 h after addition of the pre incubated mixtures to Vero cells. The lower row shows images collected after 5 h of incubation. All images are available in larger format in Appendix 6.8.

b- Minimum scFvs concentration that elicit 50% protection to Vero cells

To assess the relative potency of the anti-scFvs isolated in this project, an assay was devised to measure the minimum concentration of each scFvs that was able to protect 50% of Vero cells against the cytopathic effects of Toxin A. To do this, each of the four scFvs under investigation were prepared to a known protein concentration and then a series of five 2 fold dilutions were prepared. Each scFv at each concentration was then mixed and incubated with Toxin A. Two hours after this, mixtures were added to Vero cells in culture and a fixed time interval of 2 h was set at the end of which, observations were made of the proportion of cells that had undergone rounding. The experiment was repeated three times and to guard against experimental bias, plate layouts were unknown to the observer for the second and third tests. The data are shown in Figure 4.5. The capacity of each scFv to neutralise the biological activity of Toxin A is evident: at high scFv concentrations, little cell rounding was evident but as the recombinant antibody was progressively diluted, an increasing percentage of Vero cells were seen to have undergone rounding during the 2 h challenge period. For all scFvs, dilution to the lowest protein concentration resulted in rounding of 100% of the Vero cells at the end of the challenge period. However, the shapes of the curves differed somewhat: 50% cell rounding was recorded for scFv 15 at 15 µg/ml; this was apparent for scFv 18 at 18 µg/ml; for scFv 1, an antibody concentration of 35 µg/ml was required; the least potent scFv appeared to be clone 14 for which 53 µg/ml was required to achieve the protective cut-off. It would thus appear that scFv 15 possessed the most potent anti-TcdA activity of the four antibodies isolated.







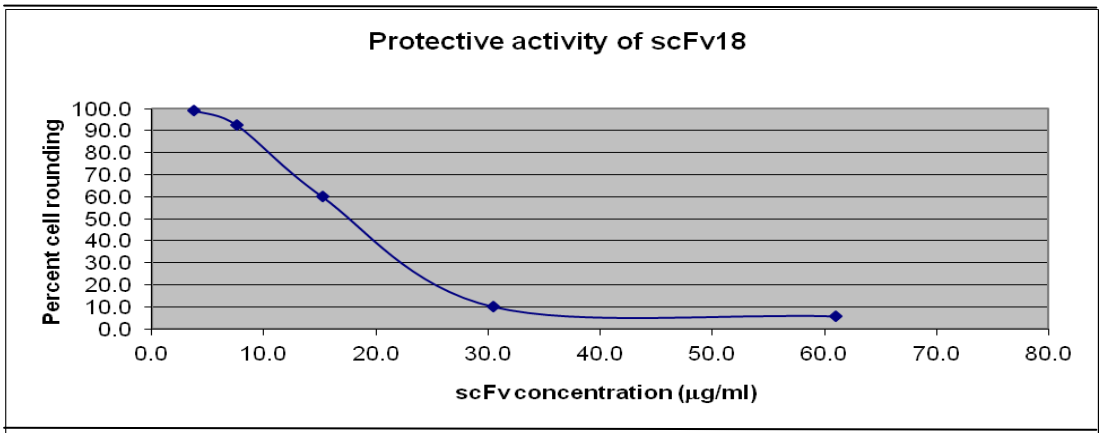


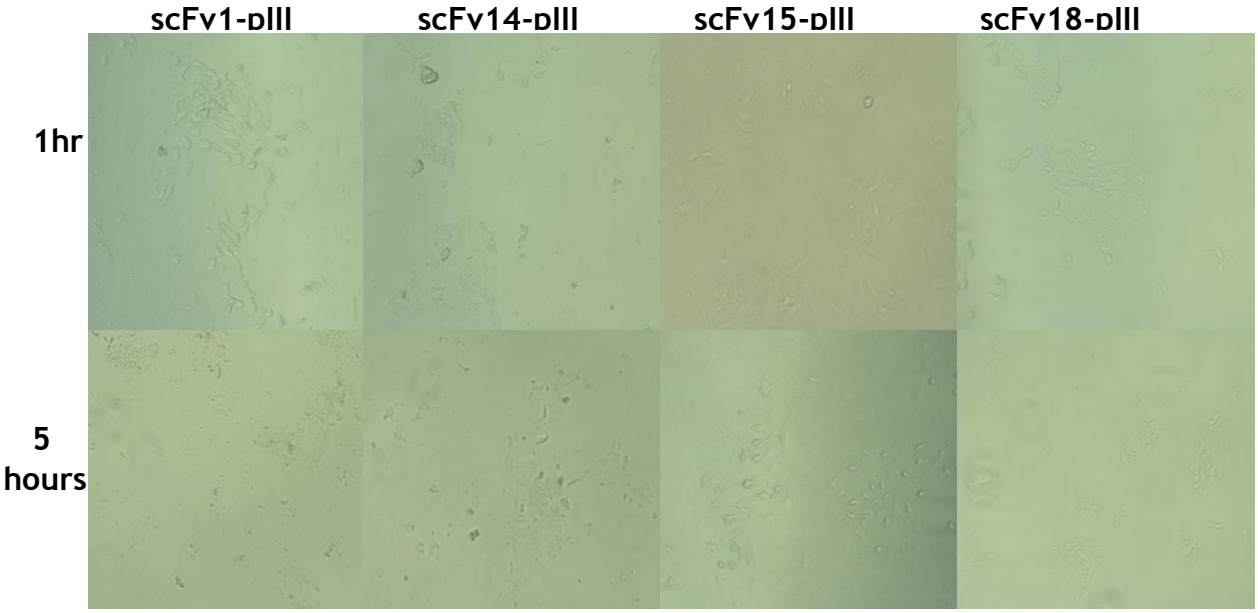
Figure 4-4 Titration of the protective activity of scFvs in Vero cell assay of Toxin A action.

Purified anti-TcdA scFvs were serially diluted, pre incubated with a fixed concentration of Toxin A and added to Vero cells in culture. After 2 h incubation, the proportion of Vero cells that undergone morphological change was assessed visually. Each panel shows data for the designated scFv, the x axis showing the concentration of scFv added to Toxin A (μg/ml) and the y axis the percentage of Vero cells that had rounded at the end of the assay.

4.3.1.2.2 Protective activity of multivalent scFvs

a- Ability of phage carrying scFv-pIII fusions to block the effect of TcdA on Vero cells

To assess the protective activity of multivalent forms of scFvs, phage carrying each scFv as a fusion to phage coat protein pIII were prepared to equal titres, mixed with a fixed dose of TcdA (100 ng/ml) known to be effective in the Vero cell assay and then added to cells that had been cultured from an initial inoculum of 5x 10^3 cells/well as described earlier. Cells were inspected periodically each hour for 5 h post challenge. As shown in Figure 4.6, phage were able to neutralise the cytopathic activity of TcdA during the 5 h that the experiment was conducted with variable degrees of success. Phage carrying scFv clones 1, 14, 15 and 18 as fusions to pIII were all able to neutralise TcdA over the first hour of the incubation, but by 5 h, rounded cells were seen. This is seen most clearly for scFv clone 15. In these experiments 1x10^6 phage were mixed with toxin. At most, each viral particle can carry 5 scFv-pIII fusions per virion and hence the maximum number of scFv proteins present in the experiment was 5 x 10^6 for the least potent soluble scFv shown in the experiments presented in Figure 4.5, complete neutralisation of TcdA might be anticipated at around 100 μg / ml. Given the molecular weight of a scFv is close to 30 kDa, 100 μg represents about 3x10^{-9} moles, or 2x10^{15} scFv proteins. Hence, it is evident from the Figures that the protective activity of the soluble monovalent scFvs was much better than that shown by multivalent scFvs fused to pIII, but there are very substantial differences in the numbers of scFv molecules available in the two assay systems.



Figur 4-5 Vero cells cultured in the presence of mixtures of phage carrying anti-TcdA scFvs and Toxin A.

The scFvs carried by each phage stock are shown at the top of the Figure. The upper row shows images collected 1 h after addition of the pre incubated mixtures of phage and Toxin A to Vero cells. The lower row shows images collected after 5 h of incubation.

b- Ability of phage carrying scFv clone 15 as a pVIII fusion to block the effect of TcdA on Vero cells

In light of the finding that scFv clone 15 appeared to have the highest potency in toxin neutralisation studies (Figure 4.5), this scFv was fused to phage coat protein pVIII to generated virus with high-density, multivalent display of the antibody.These phage were tested for protective activity against Toxin A. The experimental conditions were as described earlier; 1 x 10^6 phage were mixed with Toxin A at 100 ng / ml and then 100 μl of the mix (ie a toxin challenge dose of 10 ng) was added to cultured Vero cells. The morphology of the cells was then checked periodically over a 5 h incubation period. Images taken from the experiment are shown in Figure 4.6. Panel A shows that 1 h after addition of the mixture of toxin and phage, few Vero cells had lost their characteristic stellate morphology. However, 5 h after addition, rounded cells dominate the population and whilst stellate cells can be seen, they are few in number. This is in striking contrast to the behaviour of scFv clone 15 as a pIII fusion or as soluble protein.

Although earlier work showed that the scFv-pVIII was capable of recognising TcdA in ELISA assays, this experiment suggests that folding or display of the antibody is far from optimal.

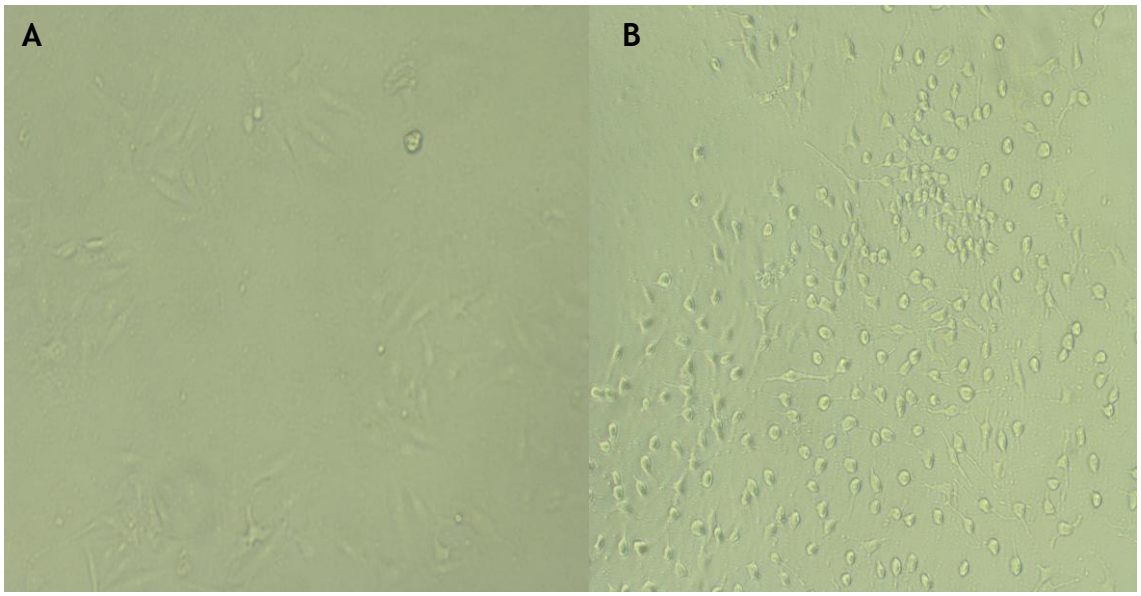


Figure 4-6 Vero cells cultured in the presence of a mixture of Toxin A and phage carrying anti-TcdA scFv clone 15 as a pVIII fusion.

Panel A shows the morphology of Vero cells after one hour after challenge with Toxin A pre-treated with scFv15-pVIII. Panel B shows Vero cells after five hours of exposure.

4.3.2Studies of scFv-mediated protection in vivo

The toxin-neutralising properties of the anti-TcdA scFvs showed sufficient promise to take forward the characterisation with *in vitro* protection experiments. These focussed upon scFv clone 15 and its activities when expressed as a soluble protein. As an experimental system, intestinal loops were created in adult Sprague-Dawley male rats, these serving as a test system to measure the accumulation of fluid in response to Toxin A challenge over a four h incubation period. While other experimental systems (*eg* the hamster) have been developed for studies of *Clostridium difficile* pathogenesis, it was argued that a system generating a simple, measurable output would best meet the aims of the experiments (*ie* to test if scFv 15 could neutralise the effects of Toxin A on intestinal epithelial cells *in vivo*).

4.3.2.1 The effect of TcdA on fluid uptake by the intestine of rats

Initial experiments assessed the ability of Toxin A to bind to its receptor on epithelial cells in the rat intestine and exert its enterotoxic effect. Two ligated jejunal loops were set up in each anaesthetised rat, one to receive Toxin A, the other to serve as a control of intestinal physiology during the following 4 h incubation period. The dose of TcdA used in preliminary experiments was 10 µg per 10 cm loop; this resulted in bulging of the test loop. The loop contained fluid that was red in colour, perhaps as a consequence of the release of blood into the intestinal lumen, and the length of the loop appeared to have increased. In contrast, the control loop seemed unchanged from its appearance at the start of the experiment, it was not distended by fluid accumulation, and it was a little shorter in length than at the time of injection. The volume of fluid recovered from the control loop was zero showing that absorption had taken

place while that from the loop treated with toxin was higher than at the beginning of the test. These values were quantified by comparing volumes that were collected at the end of the experiment with the injected volumes at the start of the experiment, correcting for loop length and time (Table 4.1). These values are expressed in terms of absorption (*ie* fluid uptake rather than secretion).

	Toxin concentration µg /10 cm loop	Fluid absorption µl/cm/h
Control loop	0.0	41.0
Test loop	10.0	-4.1

Table 4-1 Effect of injection of Toxin A on fluid uptake by jejunal epithelial cells *in vivo*.

4.3.2.2 Minimum effective dose of TcdA on fluid uptake by the intestine of rats

Having confirmed that TcdA had a measurable effect on the jejunum of the rat, experiments were designed to establish the minimum dose of TcdA required for a consistent biological effect. To do this, experiments were carried out as described before but using doses of 5, 2.5 and finally 3.0 µg of Toxin A for each 10cm ligated loop. The findings (Table 4.2) showed that injection of 5 µg into each loop elicited a significant effect. Halving this dose to 2.5 µg had a marginal impact on absorption of fluid from the ligated loops when compared to control loops and the biological effect was judged too slight to be reliable. A slight increase to 3.5 µg per loop restored a significant enterotoxic effect (Table 4.2) and net fluid accumulation (*ie* a negative value for absorption) was apparent. In light of these finding the minimum effective dose of Native Toxin A

required to elicit a significant effect on normal fluid absorption in the rat jejunum was determined to be 3.5 µg per loop.

		Toxin concentration µg / 10 cm loop	Fluid absorption µl /cm/h
Control loop	(n=4)	0.0	35.8
Test loop, A	(n=1)	5.0	7.9
Test loop, B	(n=2)	2.5	25.0
Test loop, C	(n=1)	3.5	-5.7

Table 4-2 Effect of injection of graded doses of Toxin A on fluid uptake by jejunal epithelial cells *in vivo*.

4.3.2.3 Protective activity of anti-TcdA scFv clones 15 against Toxin A *in vivo*

In order to assess the protective activity of soluble scFv-15 against TcdA, the toxin dose and the length of the ligated looped was increased by 50% over the values used previous experiments. Hence as control, 4.5 µg of Toxin A was injected in 1.5 ml of buffer into ligated loops of 15 cm in length. For the experimental loops, 4.5 µg was pre incubated with scFv-15 and then the mix was injected in 1.5 ml to loops of 15 cm. These experiments were repeated six times.

In control loops that were injected with Toxin A, the mean increase in weight/cm loop length was 65.9 ±21.4 mg/cm. This value was derived from 6 experiments. When loops were injected with the pre incubated mixture of toxin and scFv 15, the mean increase in loop weight was 20.9 ± 21.5 mg/cm. Again,

this was calculated from 6 experiments though it is worth noting that for 2 of the 6 loops, fluid absorption appeared to be poor.

The pattern of these data is also seen clearly in Table 4.3. The column labelled “Explicit fluid absorption” shows fluid changes normalised by the measured length of the tissue, and the duration of the experiment. Net fluid change in these data were assessed from the volume of fluid injected to each loop and the volume recovered at the end of the experiment. In control loops that were injected only with buffer, fluid absorption was quite evident; over 6 experiments, an average absorption of 35 µl/cm/h was observed. In contrast, there was significant amount of fluid entry into loops injected with Toxin A: an average volumetric change of -16.5 µl/cm/h was recorded. When Toxin A was pre incubated with scFv 15, fluid entry to the ligated loops of rats fell to substantially to about one third that observed in the absence of the scFv (-5.2 ± 5.4 µl/cm/h). In each case (control *versus* Toxin A; unmodified Toxin A *versus* Toxin A pre incubated with scFv 15) differences were significant at $p < 0.001$.

	Explicit fluid absorption µl /cm/h	Implicit fluid uptake µl /cm/h	Weight to length ratio g / cm
Control loops (buffer only)	35.0 ± 1.8	33.5 ± 1.6	0.16 ± 0.01
Toxin A	-16.5 ± 5.4 (p=<0.001)	-19.6 ± 6.0 (p=<0.001)	0.33 ± 0.04 (p=<0.001)
Toxin A plus scFv15	-5.2 ± 5.4 (p=<0.001)	-9.2 ± 5.2 (p=<0.001)	0.3 ± 0.03 (p=<0.01)

Table 4-3 Effect of injection of graded doses of Toxin A on fluid uptake by jejunal epithelial cells *in vivo*.

An alternative method of assessing fluid movement is presented in the column labelled “Implicit fluid uptake”. Here, the fluid present in each loop at the end of the experiment was inferred from the weight of the loop at the end of the experiment *versus* the weight of the tissue after removal of fluid from the lumen of each loop. The pattern and the statistical significance was identical to the observations described earlier: active fluid absorption in loops injected with buffer alone; net fluid accumulation in the presence of Toxin A; a substantial reduction in fluid accumulation when toxin with pre incubated with scFv 15.

An additional interesting feature of the data is presented in the final column of Table 4.3 where the weight of each loop at the end of the experiment was divided by its length. The mean ratio for loops injected only with buffer was 0.16 ± 0.01 g/cm. Effectively, this doubled for loops treated with TcdA (0.33 ± 0.03 g/cm) and pre incubation of Toxin A with scFv 15 reduced this mean by about 10% (0.30 ± 0.03 g/cm). The ratio of wet loop weight to length obscures the action of scFv15 in reducing fluid accumulation in the treated loop. This is because the control loop without toxin absorbed the fluids in the jejunum and the loop will not lengthen. In contrast, fluid secretion increases the volume in the loop treated with toxin plus scFv15 leading to distension of the loop and lengthening. While the net change in fluid accumulation is clearly impacted by the presence of scFv, the length of the loop appeared to be effected less, hence the values in this column of the Table obscure the protective activity of the antibody in reducing fluid accumulation into the loop.

4.4 Discussion

The present management of nosocomial antibiotic-associated diarrhoea arising from infection with *C.difficile* is termination of the offending antibiotic followed by the administration of vancomycin or metronidazole. Antibody treatment has the potential to block the action of toxins and thereby to interfere directly with the course of *C.difficile* pathogenesis. Fast-acting and robust therapeutic effects are a possibility.

Monoclonal antibodies and immunoglobulin concentrates against *C. difficile* toxins have been shown to be successful *in vitro* and *in vivo* (Lyerly et al., 1991, Kelly et al., 1996, Corthier et al., 1991). This study illustrates the protective activity of scFvs isolated against the receptor binding site of TcdA.

4.4.1Protective activity of anti-Toxin A scFvs in vitro

The development of a sensitive and robust Vero cell assay for detecting the activity of Toxin A allowed convenient assessment of the neutralising properties of scFvs antibodies directed against the receptor binding site of TcdA. One uncertainty at the outset of this phase of the project was that the toxin possesses multiple sites for receptor interaction and if a minority of these remained free, there was the potential for receptor interaction, internalisation to the Vero cells and translocation to the cytosol leading to damage to the actin cytoskeleton. A further uncertainty was whether scFvs interacting with key residues in the receptor binding sites might be displaced by receptor engagement with time. Potentially, this might lead to a delay to toxin entry rather than outright blockade of TcdA action.

In the event, protection of Vero cells was apparent and at least for soluble forms of the scFvs, delayed entry to the target cells was not an issue; cells were protected against shape change for 5 h post exposure to complexes of scFv and TcdA. We speculate that this protective action arises from interaction of the scFvs with the repeating peptide motifs required for receptor binding. In turn, this leads to disruption of the binding of Toxin A to the receptor found on the surface of the target cells.

Cell-based assays for the toxins of *C. difficile* are known to be highly specific and very sensitive (Johnson and Gerding, 1998) and are considered to be the "gold standard" for toxin detection. Vero cell lines are believed to be the one of the most sensitive cell lines in this regard (Brazier, 1998, Delmée, 2001) and the changes in morphology that are triggered by the glucosyltransferases of *C. difficile* enable direct assessment of toxin activity in contrast to indirect measures like the agglutination of rabbit erythrocytes (Dingle et al., 2008).

To validate the cytopathic activity of the Toxin A to be used in these experiments, Vero cells were plated out and challenged initially with 100 ng of TcdA. Effects were rapid and complete cell rounding was achieved after 2 h incubation. A minimum effective dose was established at 10 ng per well and under these conditions, complete cell rounding took somewhat longer (3 h).

A polyclonal goat anti-Toxin A antibody was used to set up neutralisation experiments and assess the ability of toxin specific antibody to neutralise the effects of Toxin A. This approach has been employed widely in other studies of interventions to prevent toxin action (Hinkson et al., 2008). From this, further experiments examined the ability of recombinant scFvs to protect Vero cells against TcdA. For these studies, each scFv was pre incubated with the minimum

effective dose of native TcdA for 2 h and then Vero cells were challenged with the mixture for 5 h. Partial protection was observed for all four anti-Toxin A scFvs generated from phage display. Potency of these scFvs was assessed by identifying the concentration able to protect 50% of Vero cells over the course of 2 h of incubation period. This threshold varied from 15 μg/ml (scFv clone 15) through to 53 μg/ml (clone 14). We speculated that polyvalent display of these scFvs at the surface of phage would show still better protection but this did not prove to be the case, indeed fusion of scFv 15 to phage coat protein pVIII was significantly worse in terms of protection than the soluble monovalent form of the same antibody.

4.4.2Protective activity of anti-Toxin A scFvs clone 15 in vivo

These experiments moved to conditions somewhat closer to those likely to exist in the human patient to test whether fluid accumulation triggered by Toxin A in ligated loops of the rat proximal jejunum could be inhibited with scFv 15. This ability to increase fluid entry into the lumen is particularly evident when an adverse osmotic gradient is imposed on the small intestine and most secretion protocols incorporate this. The fluid absorption system in the intestine may be inhibited upon the exposure to enterotoxin. In the case of Toxin A, this might arise through a number of pathways, one being the loss of epithelial integrity as a consequence of cell shape change, thereby compromising normal absorption. There may also be a direct stimulus that triggers enterocytes to secrete fluid, although this secretory concept has been recently challenged (Lucas, 2010) in studies of STa, the small peptide enterotoxin of *E.coli*.

Generally studies to demonstrate fluid entry following *C.difficile* Toxin A challenge are either initiated with hypertonic solutions in the lumen (Johansson

et al., 1997) or by allowing adequate time for loss of cellular integrity to take place, leading to the flow of fluid into the lumen (Triadafilopoulos et al., 1989). In the latter scenario, physical stress forces fluid to leak into the lumen while enterocytes within the loop remain absorptive. Net 'secretion' then becomes apparent if leakage exceeds absorption. Normally, only moderately hypertonic loads of more than 70% milliosmoles are required to draw intestinal fluid into the lumen of the unchallenged jejunum when absorption machinery is functioning normally (Visscher M. B., 1944). The choice of buffers is therefore of substantial importance and in the control experiments described here, the increased luminal osmotic load allowed fluid to be absorbed but only at a rate of around 35 µl/cm/h. This is about 40% less than the normal rate of around 60 µl/cm/h seen for isotonic saline (Lucas et al., 2005).

When *C.difficile* Toxin A was injected in this buffer system, fluid entered the intestinal loops, occasionally distending them. It is normal to refer to fluid entry as 'secretion' implying that enterocytes have been induced to secrete fluid but there is experimental support for the alternative explanation – that intestinal permeability increases – as enhanced mannitol flux into the lumen has been shown (Triadafilopoulos et al., 1989). Loops also become suffused with blood and in experiments to establish an effective dose of Toxin A, discoloration of the loop contents was a frequent observation, associated with elevation of soluble protein in the loop fluid.

Whatever the nature of the underlying process, scFv 15 seemed able to achieve substantial neutralisation of Toxin A activity. Upon exposure to Toxin A in buffer, loops absorption averaged around -16 µl/cm/h. Pre incubation of TcdA with the scFv produced values around -5 µl/cm/h. Interestingly it was noted

that the normal loop wet weight to length ratio was not changed by the presence of antibody. This finding possibly reflects the unsatisfactory nature of any experimental measure of fluid movement that is not based on fluid recovery. Based on the fact that the intestine is highly distensible, any increase in luminal volume will be accompanied by an increase in length as the loop distends. The loop wet weight to length ratio may function adequately as an indicator of toxin challenge when the initial volume added dose not fill up the loops and hence did not distend them; volume is removed during absorption and the intestine cannot be shortened further once all fluid has been removed from the absorbing loop. It is almost certainly more difficult to compare two states of distension using this ratio than it is to compare the distended state. A consequence of this is that antibody efficacy may fail to be recognised using the weight to length ratio but in contrast was definitely detected using both the implicit and explicit methods of estimation by fluid recovery.

Further evidence for the effectiveness of the antibody was that soluble protein and haemoglobin entry into the loops were both reduced when loops were injected with the mix of scFv and Toxin A. Together, the data imply that the effects of TcdA were reduced because antibody neutralised the toxin but did not eliminate its effects completely.

Chapter Five General Discussion

5.1 Overview

The last 40 years have seen a worrying resurgence in a number of important infections and the emergence of new threats to human health. Reasons include the developments of widespread resistance to existing antibiotics, the creation of environments (*eg* hospitals) that facilitate spread, and the evolution of new strains of pathogens with enhanced virulence properties. For the treatment of *C. difficile* and several other bacterial pathogens, alternatives to antibiotics are required with some urgency. Passive immunisation pre or post exposure to the infection is one of the most promising strategies for prophylaxis or therapy.

This project has shown that small, recombinant antibodies – scFvs that can be produced with relative ease in *E. coli* and purified by simple affinity chromatography – have potential against *C. difficile* in this context. The protective properties of these constructs *in vitro* and *in vivo* is attributable to their specificity for features of Toxin A. Selection from the large Tomlinson libraries of synthetically diversified scFvs *via* phage display was directed by use of the sequence from the putative binding site of TcdA, sequences that were fused to MBP to facilitate expression and purification. Formal confirmation that the short clostridial sequence was folded into its native state was not obtained (*eg* the ability of the protein to bind Galα1-3Galß1-4GlcNAc or Galß1-4GlcNac was not tested) but as scFvs selected against the MBP fusion protein were reactive with native Toxin A, is seems reasonable to conclude that it was. Successful recognition of native Toxin A was followed by experiments to test whether the scFvs would protect cells in culture and the intact intestinal

epithelium against the toxin. Given the success of these experiments, future work should examine whether the scFvs can confer protection in an animal system that more closely models infection with *C. difficile* (*eg* the hamster model).

5.2 Future challenges

5.2.1 Epitope mapping

While the reactivity of the scFvs with a series of MBP deletion mutants has provided a degree of localisation for the epitopes, this remains incomplete. The scFvs failed to bind to MBP-SR1, but addition of LR restored reactivity. Given that the scFvs failed to recognise the TcdA-MBP fusion protein in Western blots but immunoprecipitation was successfull, it is likely that the epitopes are conformational. Given this situation, the use of overlapping peptides from the receptor binding domain of TcdA may not be an appropriate way to take forward more precise mapping of the epitopes recognised by these scFvs: alanine scanning would be more appropriate. The scFvs show a strong cross reaction with Toxin B, and alignment of the SR1-LR-SR2-SR3-SR4 sequence from TcdA with that of Toxin B reveals regions of significant similarity, but other areas of divergent sequence. Using this information, hybrids could be generated in which non-identical sequences from SRs or LRs in Toxin A or B are introduced to the TcdA-MBP fusion protein to maintain the overall conformation but modify the primary structure. scFv binding could then be assessed. This approach, combined with alanine scanning mutagenesis, is likely to map the epitopes with greater precision.

5.2.2Overlapping or discrete epitopes, and affinity of binding

Four scFvs were characterised in this study but experiments did not test whether they recognise discrete epitopes within the receptor binding domain of TcdA. The sequences of the scFvs showed that all four were unique with similar but non-identical CDRs. This could infer that they recognise non-identical epitopes but this requires confirmation. This could be established by labelling of the scFvs (*eg* by biotinylation) and then competitive ELISA experiments, testing the binding of the labelled protein to TcdA-MBP in the presence of increasing concentrations of an unlabelled, competing scFv.

Similarly, the affinity of the interaction of each scFv with the TcdA-MBP fusion is unknown. This could be established with BiaCore experiments.

5.2.3Mode of delivery for in vivo *evalution*

Experiments with rat intestinal loops employed a simple approach: pre-incubation of biologically active doses of toxin with each scFv *in vitro* prior to injection to the ligated loops. Whilst the data showed good evidence of toxin neutralisation, the experimental system is significantly different to conditions that would exist in the intestine of an experimental animal or human patient during active infection with *C. difficile*. Each scFv was exposed to Toxin A under ideal conditions whereas the pH, presence of proteases, other proteins *etc* would differ if interaction were to take place *in vivo*.

Similarly, the toxin was delivered to one defined location in the ligated intestinal loop. It is unclear what concentration of scFv would be needed if TcdA was distributed through a larger volume of intestinal lumen, of how effective

neutralisation would be if the intestine was damaged by other products of virulent *C. difficile*.

The best approach to addressing these uncertainties would be to test the scFvs in a hamster model of infection but this represents a significant jump for reasons discussed later. As an intermediate step, scFv could be delivered to ligated intestinal loops created in the rat following initial injection of TcdA. This would establish whether neutralisation *in vivo* was possible in principle.

5.3 Prospects for scFv-mediated immunotherapy against *C. difficile*

The findings from this project show that scFvs isolated by phage display on TcdA-MBP have protective capacity against native Toxin A both *in vitro* and *in vivo*. While many novel immunotherapeutic molecules are under development along with interesting modes of delivery, to date, no monoclonal antibodies have been approved by the licensing authorities for use against bacterial pathogens. While many different molecules have been successfully evaluated in pre-clinical animal models of infection, these *in vivo* models can have significant differences to the situation in humans, particularly due to differences in the pharmacokinetics of the molecules between humans and animal models.

5.3.1 Animal models for CDAD

The anti-TcdA scFvs that been isolated in this study were tested for their ability to protect against native Toxin A both *in vitro* and *in vivo* as detailed earlier. One important milestone in the further development of the reagents would be to study their effects in an animal model of *C.difficile* infection.

One of the best systems is a hamster model. Over 25 years ago, Wilson and colleagues reported a Syrian hamster system in which the normal intestinal microflora was depleted by treatment with clindamycin prior to oral administration of defined numbers of viable spores. The *C. difficile* spores germinated in the intestinal environment (Wilson et al., 1985). More recently, Babcock and colleagues reported the use of a the Syrian hamster model to set up a CDAD model - 140,000 cfu of *C. difficile* spores were administered orogastrically 24 h post intraperitoneal treatment with 10 mg/kg clindamycin. – for testing the ability of full-length human monoclonal antibodies to protect against infection (Babcock et al., 2006).

Another group, Razaq and colleagues, have used *C .difficile* spores to establish CDAD in clindamycin treated Syrian hamsters. Each hamster received a single does of clindamycin orogastrically at 30 mg/kg of body weight on day 0 to establish susceptibility to *C. difficile* infection. Five days later hamsters were challenged by gastric inoculation of very small numbers (100 cfu) of *C. difficile* spores (Razaq et al., 2007).

Protocols for establishing CDAD in the hamster vary. More recently, novel antibiotics have been tested in a hamster model that was established by pre-treatment with a single subcutaneous dose of clindamycin phosphate at 50 mg/kg formulated, followed one day later by oral gavage with a 0.5 ml of *C. difficile* suspension containing 3.2 X 10^7 cfu/ml spores from a clindamycin resistant, toxigenic reference strain of *C. difficile* (Ochsner et al., 2009).

Hence, specific expertise is required to set up the hamster model, aside from the other challenges presented in establishing the potential of anti-TcdA scFvs as immunotherapeutics.

5.3.2 Potential modes of application of anti-TcdA scFvs

This represents a significant area of challenge.

5.3.2.1 Oral treatment

Given that *C. difficile* is an intestinal pathogen, there is logic in attempting to deliver potential immunotherapies by mouth. In a very early report, Lyerly's group raised a conventional mouse monoclonal antibody against *C. difficile* Toxin A using hybridoma methods which was then mixed with Toxin A before delivering to hamsters (Lyerly et al., 1986). The animals were assessed over 72 h. The amount of TcdA used was 0.16 mg/kg body weight. This established the principle that an anti-Toxin A could provide complete protection against a dose of toxin lethal for an experimental animal and that the complexes could be sufficiently stable to survive passage to the intestine (Lyerly et al., 1986).

In a later study, Lyerly and colleagues developed antibodies in cattle and a bovine immunoglobulin G concentrate was generated. This showed protective activity when administered orally, pre-challenge but delaying its delivery until after bacterial challenge was not able to protect (Lyerly et al., 1991). More recently, Van Dissel and colleagues have revisited this approach and shown that a bovine immune whey concentrate containing toxin-specific sIgA and IgG antibodies was effective at preventing *C. difficile*-induced hamster mortality when administered orally before and, crucially, after bacterial challenge. They have reported that 80%-90% of hamsters receiving the immune whey survived. The group have gone on to obtained data from human patients. Sixteen patients with confirmed CDAD, 9 of whom had a history of relapse, received the immune

concentrate three times a day for 2 weeks after the completion of standard antibiotic treatment. Patients were followed up for around 10 months; none of them experienced another episode of *C. difficile* diarrhoea (van Dissel et al., 2005).

The difficulties of using anti-toxin antibodies from animals for systemic delivery to human patients are obvious but these studies show both that oral delivery is practical and that it can be effective against CDAD. Raising potential immunotherapeutics in large animals helps meet some of the challenges of cost but can only result in polyclonal reagents. Antibodies (*eg* scFvs) that can be produced in bacteria might prove economic and also enable the production of monoclonal immunotherapeutics.

5.3.2.2 Intravenous treatment

Passive immunity induced by intravenous administration of monoclonal antibodies against Toxin A has been shown to protect experimental animals against *C.difficile* infection. Corthier and colleagues raised a series of monoclonals against the repeating units located at the carboxy-terminus of TcdA. Intravenous injection 4 days prior to *C. difficile* challenge provided protection against the disease and the injected antibodies were stable, remaining in the circulation for at least 8 days (Corthier et al., 1991).

Recently, Lowy and colleagues have developed two fully human monoclonal antibodies against *C. difficile* Toxins A and B. The group were then able to examine the efficacy of the combined antibodies in a hamster model of *C. difficile* infection before moving to phase 1 trials in healthy human volunteers. In a phase 2 trial, the combined antibodies protected against

recurrence of *C. difficile* infection in a group of 200 patients. Patients with symptomatic *C .difficile* infection who were receiving either metronidazole or vancomycin were infused intravenously with a single dose of both antibodies, each at 10 mg/kg body weight. The addition of monoclonal antibodies to the antibiotic therapy significantly reduced the recurrence of *C. difficile* infection (Lowy et al., 2010).

Hence, monoclonal antibodies also have potential against CDAD when administered systemically, providing they are of human sequence. Phage display libraries of human antibodies are now widely available, as used in the study reported here.

5.3.2.3 Intraperitoneal or subcutaneous treatment

Some useful examples of this treatment modality come from studies of mosquito-borne infection with West Nile virus. There is currently no approved human vaccine or therapy to prevent or to treat this disease but passive immunisation with antibodies specific for the viral envelope protein represents a promising protocol for provide short-term prophylaxis and treatment of West Nile virus infection.

This has been addressed by Gould and colleagues who examined the ability of different antibody constructs to protect against West Nile virus infection in an animal model. Animals were injected intraperitoneally with rabbit IgG against the virus 1 day prior, through to 5 days post intraperitoneal injection with West Nile virus. Human IgG1 or several scFv constructs, along with a scFv-Fc were tested in this system. The antibodies showed viral neutralisation and protection against the infection. The group commented that the use of

phage display libraries is novel and a useful approach to the isolation of high-affinity scFvs to targets of interest (Gould et al., 2005).

In study of *C. difficile*, Babcock and colleagues used transgenic mice carrying human immunoglobulin genes to isolate human monoclonals able to neutralise the cytotoxic effects of either Toxin A or Toxin B. These reagents were injected intraperitoneally to mice to explore their ability to inhibit intestinal fluid accumulation in response to toxin (Babcock et al., 2006).

5.3.2.4 Delivery of multiple scFvs

One notable aspect of the present study is the demonstration that single scFvs have the capacity to protect against Toxin A *in vitro* and *in vivo*, a finding that is surprising given the complexity of the carboxy-terminal domain of Toxin A.

In working with complex targets, a more general finding has been that one scFv from a panel lacks the capacity to protect against the effects of a complex toxin but when several scFvs – an oligoclonal collection – are combined, a protective synergic effect results. One can imagine that each scFv binds to a specific epitope on the target but where redundancy of function exists, multiple epitopes need to be occupied for a protective effect to result.

This is illustrated by findings reported by Nowakowski and colleagues who isolated a panel of scFvs against the botulinum neurotoxins (BoNTs). They discovered that no single scFv was able to neutralise BoNTs but that a combination of three monoclonal antibodies was able to neutralise up to 50% of the lethal doses of BoNT/A. The potency of the oligoclonal panel was primarily

due to a large increase in functional antibody binding affinity (Nowakowski et al., 2002).

More recently, another group has made similar findings in a study of *C. difficile*. Demarest and colleagues developed several murine monoclonal antibodies against the receptor binding domain of TcdA, an interesting parallel with the study reported in this thesis. The panel was screened for neutralisation activity individually or in combination and the authors found that three antibodies delivered together were able to neutralise Toxin A. Each antibody appeared to recognise multiple sites, suggesting that widespread coverage of the receptor binding domain contributed to the potency of neutralisation (Demarest et al., 2010).

This latter finding is strikingly different to the results reported in this thesis. It is possible that some epitopes are naturally immunogenic but that their presence in multiple copies and their functional redundancy creates a way for the toxin to evade the production of neutralising antibodies in the human patient. Perhaps the neutralising epitopes recognised by scFvs from the Tomlinson library are not naturally immunogenic or are concealed in the structure of TcdA such that full length antibodies are unable to gain access for interaction.

The study reported by Hussack and colleagues (Hussack and Tanha, 2010) gathered results similar to those reported in this thesis. Single domain antibodies - small, antigen-binding proteins based upon heavy chain V domains - against the carboxy-terminal domains of TcdA and TcdB were extracted from an immunised library. The reagents recognised a number of common and discrete conformational epitopes. They were tested for protective activity *in vitro*. And

whilst many of the extracted antibodies were able to neutralise the cytopathic effects of Toxin A, this was enhanced when the antibodies were combined.

These studies clearly illustrate the potential of full length or small, recombinant antibodies as alternative therapeutics for CDAD.

5.3.3Alternative delivery systems for scFvs

While studies reported earlier have used the oral or systemic delivery of antibodies to protect against *C. difficile*, this requires purified protein that is often costly to produce. scFvs could be delivered in similar ways (*ie* as an oral or injected therapeutic) but an alternative would be to explore oral delivery of live bacteria that are able to deliver protective scFvs *in situ* in the patient intestine. This could be achieved by fusion to the cell surface proteins of harmless bacteria such as lactobacilli.

Lactobacilli are Gram positive bacteria constituting part of the normal gastrointestinal microflora and are generally considered as safe for human consumption as they have been used in food fermentation and preservation for many centuries. Their ability to colonise and thrive in the gastrointestinal tract has potential for the prophylactic and / or therapeutic delivery of biomolecules. In this context, lactobacilli have been proposed as carrier systems for oral vaccines and as vectors for *in situ* expression of heterologous proteins. Aside from presentation or release of biomolecules by the inoculating dose, the bacteria have the capacity for colonisation and replication in the intestine. This would sustain production of a therapeutic scFv.

Engineered lactobacilli have previously been used to deliver scFvs or antibodies fragments against antigens from various pathogens such as *Streptococcus mutans*, *Porphyromonas gingivalis*, rotavirus, and anthrax edema toxin.

Streptococcus mutans is the major pathogen involved in the development of dental caries. The pathogen produces surface antigen SAI/II as an adhesion factor that binds to salivary pellicles and facilitates the colonisation of bacteria. This is followed by production of a series of surface glucosyltransferases, leading to the later stage of adherence.

SAI/II has been studied as a vaccine candidate to protect against dental caries but in a different approach, the sequence of scFvs against SAI/II were fused to a surface protein of *Lactobacilluis zeae*. The recombinant bacteria were tested for expression of the scFvs and their protective activity in animals. The outcome of the experiment showed decreases in pathogen numbers adhering to the teeth of experimental animals and the development of caries (Krüger et al., 2002).

Anthrax is mediated by a tripartite toxin complex composed of the receptor-binding protective antigen (PA) and the enzymatic subunits lethal factor (LF) and edema factor (EF). PA combines with LF to form the lethal toxin or with EF to form the edema toxin. Recently Andersen and colleagues have constructed an anti-PA scFv that can be expressed in engineered *L .paracasei* as a soluble scFv or anchored to a cell wall protein. The scFv was capable of neutralising toxin activity both *in vitro* and *in vivo*. The ability of the engineered lactobacilli to colonise the gastrointestinal tract may facilitate development of a system for prophylactic as well therapeutic use (Andersen et al., 2011) These

reports show the potential of lactobacilli as vehicles for the delivery of scFvs capable of protecting against bacterial infection. Similar approaches could be used to take forward evaluation of the anti-TcdA scFvs from this study in the hamster model of infection to assess whether replicating lactobacilli carrying anti-TcdA fused to the cell surface or released as soluble products can confer passive immunity against *C. difficile*. While there are many reports of the use of lactobacilli and other probiotic organisms against CDAD, the use of engineered strains for delivery of therapeutic proteins against *C. difficle* has not been reported.

5.4 Conclusions

This study has successfully isolated and characterised a panel of scFv antibodies against features of the carboxy-terminal domain of Toxin A and shown that the reagents possess protective activity against TcdA *in vitro* and *in vivo*.

While other studies have successfully isolated full length or recombinant antibodies with these properties, this study is unique in several respects:

- The target protein used in antibody isolation was designed rationally using information on the structural properties of Toxin A. Many other studies have used the full length toxin, inactivated derivatives or entire domains as immunogens.

- The scFv library was semi-synthetic and was not prepared specially for the project. Where phage display has been used in other studies to isolate antibodies against toxins from *C. difficile*, immunised libraries have been specially prepared. The use of “one pot”

resources like the Tomlinson libraries enables antibodies with potent biological properties to be isolated quickly.

- The recombinant antibodies isolated in this study are of human sequence, a feature that lends promise for human therapeutic use. Other studies have resorted to humanisation to convert antibodies from mice or other animals to minimise immunogenicity in the patient.

While future work could assess protective activity in a hamster model of CDAD using the soluble proteins, it is recognised that this presents challenges. Alternative modes of delivery of the scFvs such as the use of recombinant lactobacilli are worth exploration.

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Appendices

6.1 Preparation of antibiotic solutions

Stock solutions of ampicillin (1g/ml) and kanamycin (0.5g/ml) were dissolved in distilled water. Solutions were filtered by passing them through a filter with 0.2µm pore size. Solutions were stored at -20 ^{o}C.

6.2 Agarose TEA buffer

One gram of agarose powder (Invitrogen) was dissolved in 100 ml of water PBS containing 4 ml of 25x Tris-EDTA-Acetate (TAE; Amresco). Agrose was dissolved by heating in a microwave oven or immersion in a bath of boiling water until the agarose had completely dissolved. Three to 5 µl of SYBR Safe DNA dye (Invitrogen) was added after the solution had cooled and the solution was poured into an electrophoresis tray containing a comb to mold wells for sample loading.

6.3 2xYT medium

Sixteen grams of Tryptone, 10g of yeast extract and 5 g of NaCl were dissolved in one litre deionised water. The broth was then autoclaved at 121 ^{o}C for 15 min.

6.4 TYE medium

This medium was made from 15g of agar, 8g of NaCl, 10g of tryptone and 5 g of yeast extract. The components were dissolved in one litre of deionised water which was then autoclaved at 121 ^{o}C for 15 min.

6.5 0.2% Tween-20 buffer

Two ml of Tween-20 was added to 1 litre PBS and stirred thoroughly until the detergent had completely dispersed. The solution was stored at room temperature.

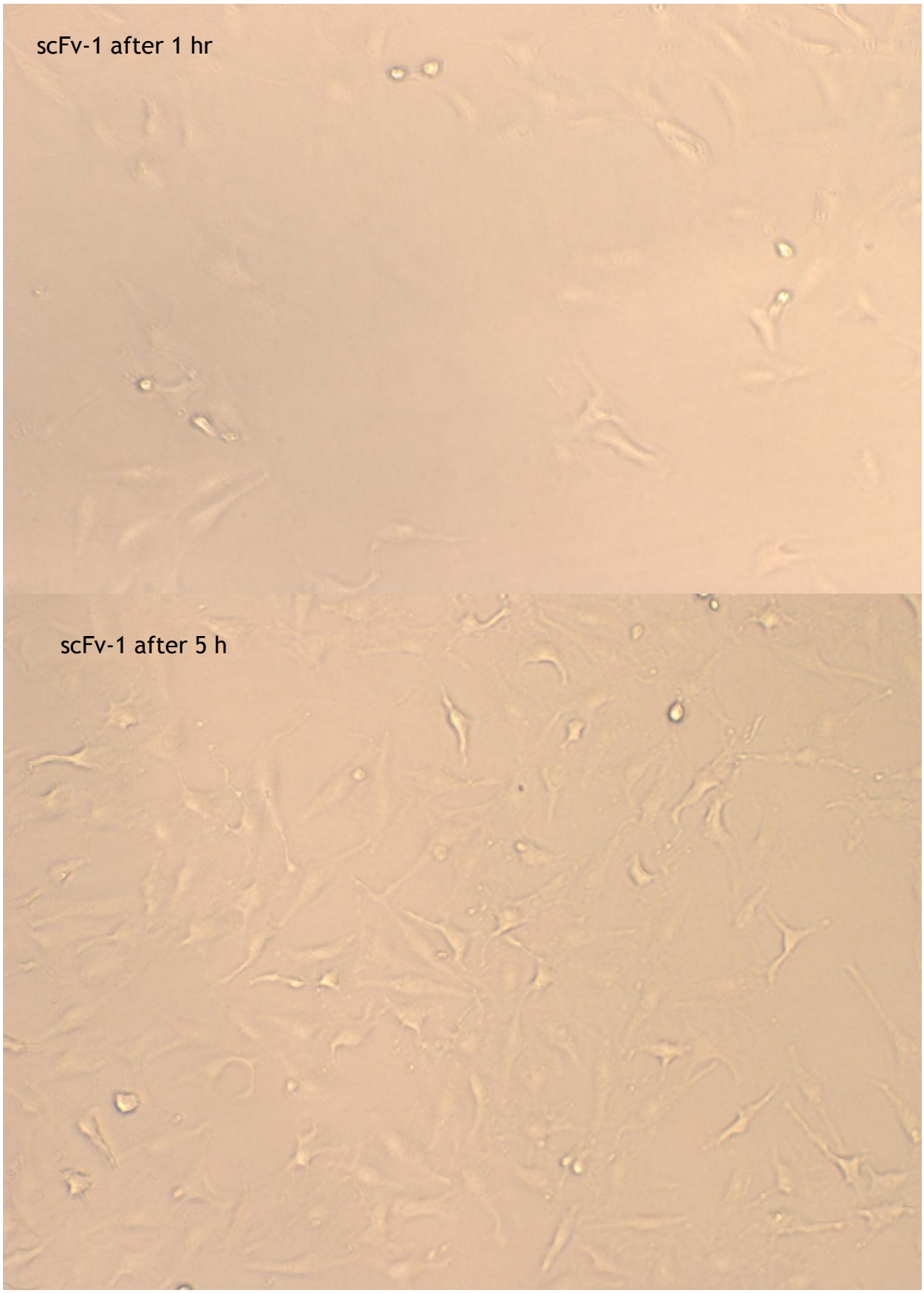
6.6 Blocking buffer

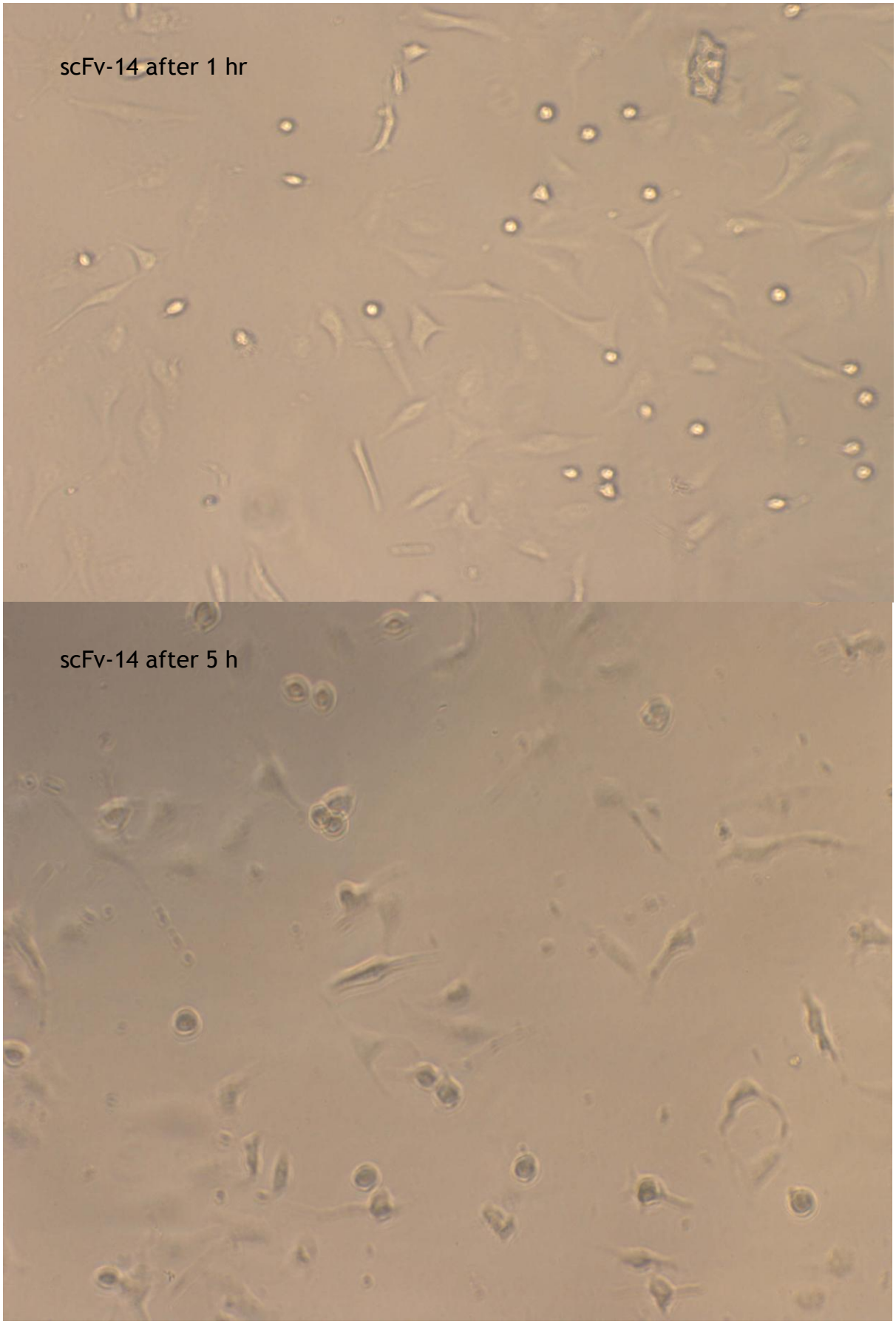
To prepare this, 2g of marvel milk was completely dissolved in 100 ml of PBS and stored at 4 ^{o}C.

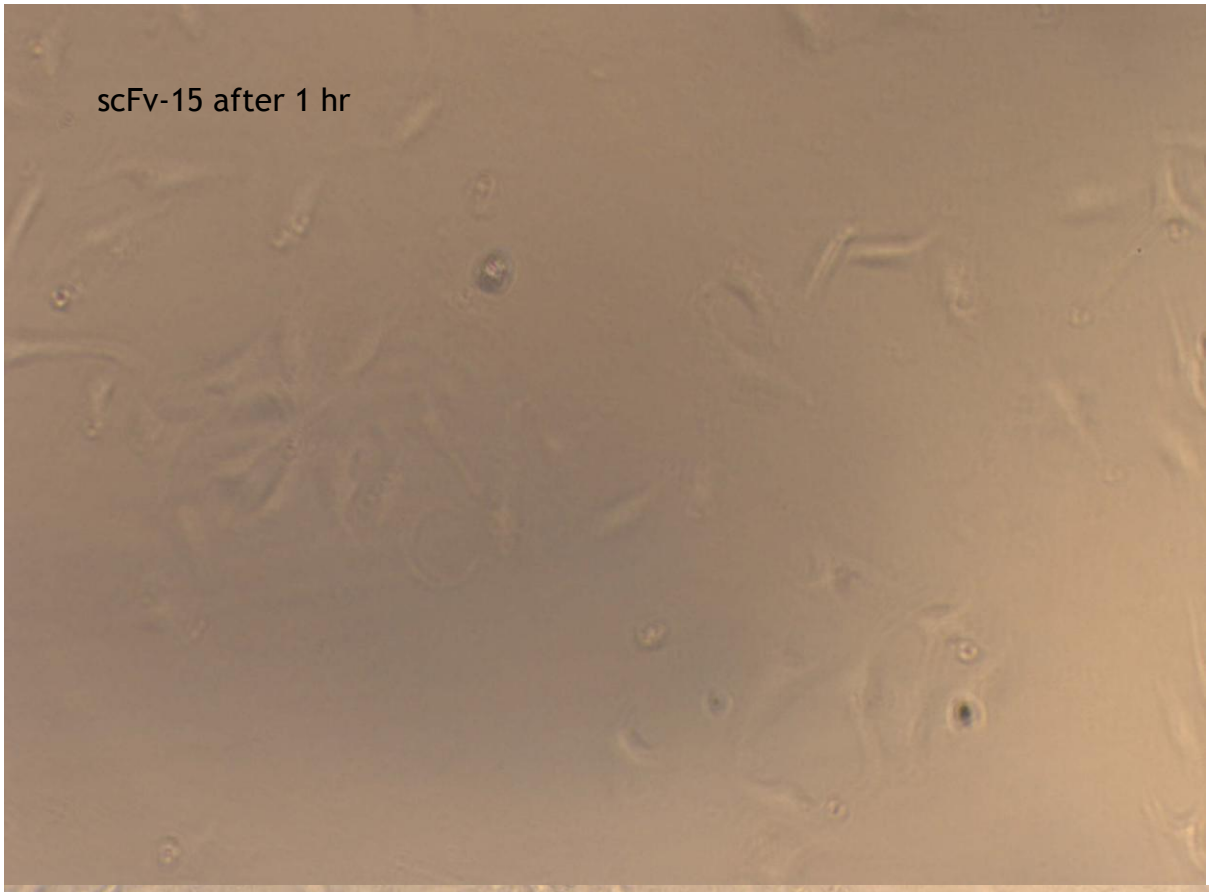
6.7 Electroblotting buffer

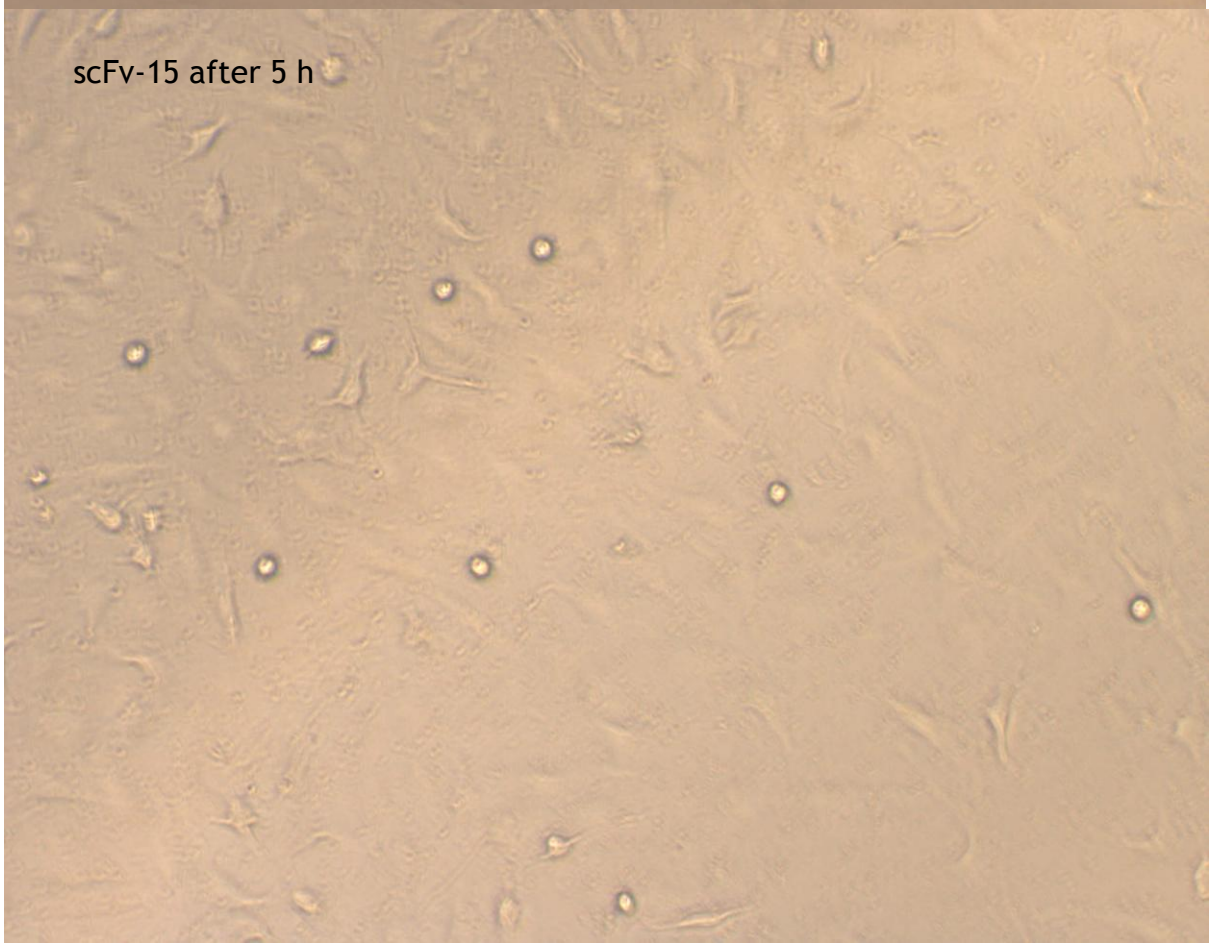
This reagent was made by dissolving 7.2g of Tris base and 33.4g glycine in 2 litres of distilled water. After this, 600 ml of methanol was added and the volume was increased to 3 litres with distilled water.

6.8 Protection activity of the scFvs




scFv-14 after 1 hr
scFv-14 after 5 h


scFv-15 after 1 hr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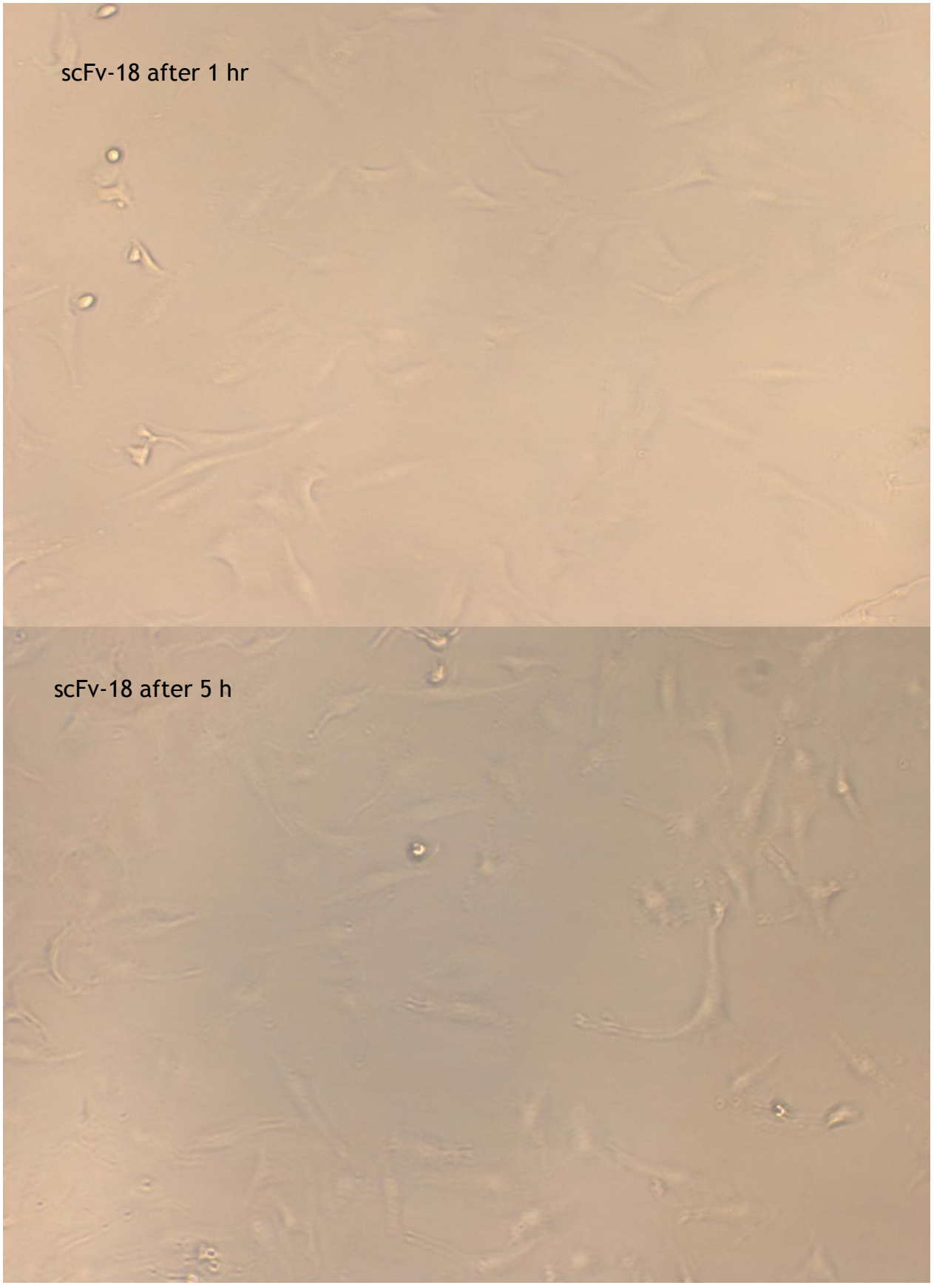
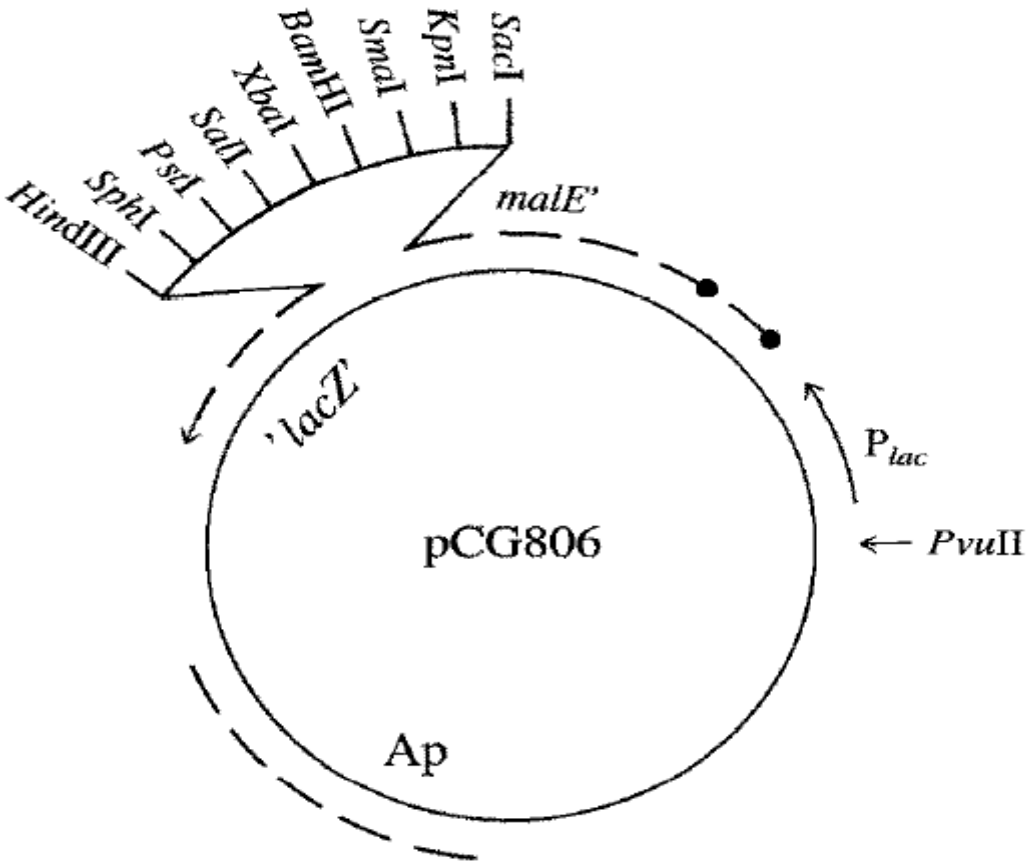

scFv-18 after 1 hr
scFv-18 after 5 h

6.9 pCG 806 vector map



pCG806

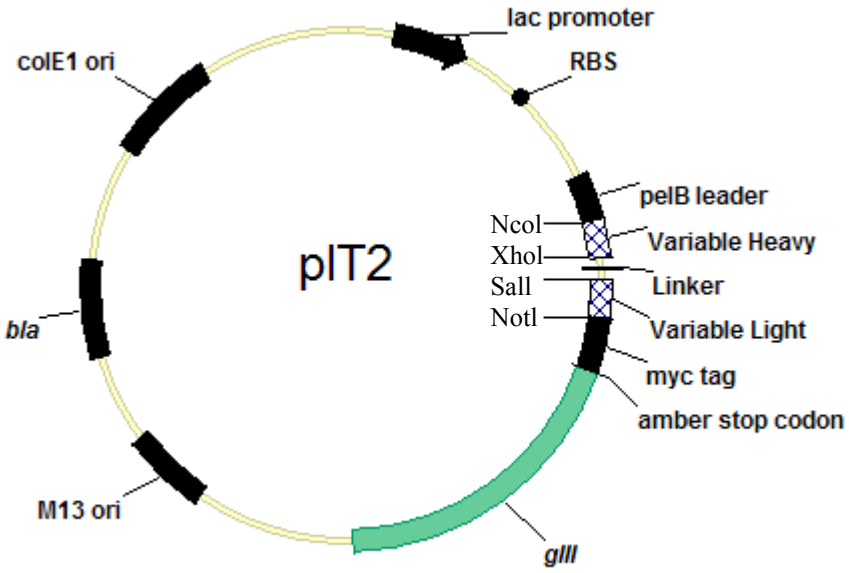
. . . *malE* sequence ⟶ CAG **ACT** AAT TCG AGC TCG GTA CCC

*Sac*I *Kpn*I *Sma*I

GGG GAT CCT CTA GAG TCG ACC TGC AGG CAT GCA AGC TTG ⟶ . . .

*Bam*HI *Xba*I *Sal*I *Pst*I *Sph*I *Hin*dIII *lacZ*

6.10 pIT2


lac promoter
RBS
colE1 ori
pelB leader
NcoI
XhoI
SalI
NotI
Variable Heavy
Linker
Variable Light
myc tag
amber stop codon
pIT2
bla
M13 ori
gIII