The Identification of Therapeutic Targets and Virulence Factors of *Clostridium difficile*

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by

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SUMMARY

Clostridium difficile, a Gram-positive spore forming bacteria, is the leading cause of healthcare-associated diarrhoea in the UK and represents a major healthcare challenge. The vegetative form of the bacterium colonises the gut mucosa and produces two exotoxins, which are responsible for the pathology associated with the bacterium. We thus sort to characterise the host immune response directed against the surface of the bacterium and toxins A and B.

Our studies revealed that the vegetative form of the pathogen is capable of altering its physiology *in vitro* to produce two distinct colony morphotypes. The differences observed in the cell surface, autolytic activity and bile salt sensitivity; suggest that the M2 morphotype may be better equipped to survive in the hostile conditions encountered in the gut. The mechanisms by which these changes are mediated are as yet unclear; however given the characteristics of the morphotypes it may involve one or more phase variable proteins. The identification of immunogenic proteins, including pyruvate-flavodoxin oxidoreductase, an anaerobic metabolism enzyme associated with oxidative stress warrants further investigation. To be effective, a future immuno-therapeutic should target the form of bacteria which is most often encountered during infection.

Toxins A and B remain the primary virulence factors of *C. difficile*, with toxin neutralising antibodies targeting the receptor binding domains conferring protection and reducing recurrent infection. To characterise the antibody response directed against toxins A and B, the cell binding and translocation domains of each toxin were expressed in *Escherichia coli*. To identify immunogenic regions, the native, recombinant and toxoided proteins were subjected to enzymatic digestion with clostripain and probed with toxin neutralising animal sera and sera from *C. difficile* infected patients. The immune sera consistently identified two fragments of 40 and 60 kDa within both toxins A and toxin B, which may contain toxin neutralising epitopes and thus warrant further investigation.

ABBREVIATIONS

AAD	Antibiotic-Associated Diarrhoea
Ab	Antibody
Ag	Antigen
ANOVA	Analysis of Variance
BA	Blood Agar
BAPNA	Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride
BCA	Bicinchoninic Acid
BCR	B Cell Receptor
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
bp	Base Pair
CCFA	Cycloserine-Cefoxitin-Fructose Agar
CDAD	*Clostridium difficile*-Associated Diarrhoea
CDI	*Clostridium difficile* Infection
C.D.M.N	*Clostridium difficile* Moxalactam Norfloxacin
CDT	*Clostridium difficile* Binary Toxin
CHAPS	3-Cholamidopropyl dimethylammonio-propanesulfonic Acid
CROPs	Combined Repetitive Oligopeptides
$CuSO_4$	Copper Sulfate
CWP	Cell Wall Protein
diH_2O	De-ionised Water
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
Fc	Fragment Crystalline
FPLC	Fast Performance Liquid Chromatography

GI	Gastrointestinal
GMSC	Genetic Modification Safety Committee
GPS	Global Proteome Server
His	Histidine
HMW	High Molecular Weight
HPA	Health Protection Agency
HRP	Horse Radish Peroxidase
HSE	Health and Safety Executive
HuMAb	Human Monoclonal Antibody
IEF	Isoelectric Focusing
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukine
IPTG	Isopropyl β-$_D$-thiogalactopyranoside
IVIg	Intravenous Immunoglobulin
kb	Kilobase
kDa	Kilo Daltons
LB	Luria Bertani Broth
LCT	Large Clostridial Toxin
LMW	Low Molecular Weight
mAb	Monoclonal Antibody
MCS	Multiple Cloning Sites
MHC	Major Histocompatibility Complex
MIC	Minimum Inhibitory Concentration
MLST	Multilocus Sequence Typing
MOPS	Morpholinepropanesulfonic Acid Sodium Salt
mRNA	Messenger Ribonucleic Acid
MW	Molecular Weight
NaOH	Sodium Hydroxide

n	Number of Replicates
Ni	Nickle
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PAI	Pathogenicity Island
PaLoc	Pathogenicity Locus
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline+ Tween® 20
PCR	Polymerase Chain Reaction
PCR RT	Polymerase Chain Reaction Ribotype
PERK™	Protein Expression and Rescue Kit
pI	Isoelectric Point
PMC	Pseudomembranous Colitis
PMF	Peptide Mass Fingerprinting
PPY	Proteose Peptone Yeast
PVP	Poly(vinylpyridine)
REC	Research Ethics Committee
R&D	Research and Development
IRAS	Integrated Research Application System
r.p.m.	Revolutions per Minute
SDS	Sodium Dodecyl Sulfate
S-Layer	Surface Layer
SLP	Surface Layer Protein
SSI	Site-Specific Information
σ	Sigma Factor
TBE	Tris-Borate EDTA
TCA	Trichloroacetic Acid
T_C	Cytotoxic T-cell
TcdA	*Clostridium difficile* Toxin A

TcdB	*Clostridium difficile* Toxin B
TcnA	*Clostridium novyi* Alpha Toxin
TcsH	*Clostridium sordellii* Haemorrhagic Toxin
TcsL	*Clostridium sordellii* Lethal Toxin
T_H	T helper
TEM	Transmission Electron Microscopy
TFB	Transformation Buffer
TpeL	*Clostridium perfringens* Toxin
tRNA	Transfer Ribonucleic Acid
SEM	Scanning Electron Microscopy
$V_{H\,(L)}$	Variable Heavy (Light) Chain
VRE	Vancomycin-Resistant *Enterococcus*
v/v	Volume per Volume
w/v	Weight per Volume
1-D	One Dimensional
2-D	Two Dimensional

PUBLICATIONS

During the course of this research the following abstracts were published:

Conference Abstracts:

Siani, H., Groen, H., Maillard, J. Y., Baillie, L. Characterisation of variant *C. difficile* morphotypes – microscopic examination and proteome analysis. 3rd International *C. difficile* Symposium, Bled, Slovenia (09/2010).

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CHAPTER ONE

INTRODUCTION

1.1 Introduction

Clostridium difficile is an anaerobic, Gram-positive, motile rod with sub-terminal spores, responsible for *C. difficile*-associated diarrhoea (CDAD) in humans and animals. Pathogenesis is attributed to the production of two biologically and immunologically distinct toxins; the enterotoxin (toxin A) and the cytotoxin (toxin B) (Voth and Ballard, 2005; Jank *et al.*, 2007; Jank and Aktories, 2008). This introductory chapter provides an overview of *C. difficile*, focusing primarily on the toxins and reviewing the existing and future therapeutic options for *C. difficile* infection (CDI).

1.1.1 The Clostridia

It is estimated that clostridia emerged approximately 2.3 billion years ago, before the 'great oxidation event' (Sheridan *et al.*, 2003; Battistuzzi *et al.*, 2004), with *Clostridium difficile* evolving within the last 1.1–85 million years (He *et al.*, 2010). The genus *Clostridium*, encompasses a wide range of metabolically diverse, obligate anaerobic, spore-forming bacteria (Cato and Stackebrandt, 1989), with genetically diverse genomes, ranging between 22-50% in G+C content (Cato *et al.*, 1986; Lawson *et al.*, 1993). Members of the genus are ubiquitously found inhabiting a wide range of anoxic environments, including the intestinal tracts of insects, humans and animals (Cato *et al.*, 1986). The genus includes fifteen known human and/or animal pathogens (*Clostridium botulinum*, *C. difficile*, *Clostridium novyi*, *Clostridium perfringens*, *Clostridium sordellii* and *Clostridium tetani*) (Popoff and Bouvet, 2013). The organisms themselves are non-pathogenic but are able to exhibit pathogenicity via toxin production.

1.1.2 *Clostridium difficile* Characterisation and History

C. difficile produces irregular, flat to slightly raised colonies ranging between 2-9 mm in diameter with a lobate margin and smooth glossy, greyish-white appearance on blood agar (Hall and O'Toole, 1935; Hafiz and Oakley, 1976). The organism is generally Gram-positive, although older colonies may exhibit Gram stain variability (Brazier and Borriello, 2000). *C. difficile* cells are large, 'drumstick'-like,

with a length of 2-8 μm and width of 0.5 μm (George *et al.*, 1979). The majority of cells are motile with peritrichous flagellae and multiple fimbriae (Hafiz and Oakley, 1976; Borriello *et al.*, 1988). Sub-terminal elongated spores that are slightly wider than the body of the cell are produced under unfavourable growth conditions (Hall and O'Toole, 1935).

CDI presents an array of symptoms, ranging from asymptomatic carriage to pseudomembranous colitis and toxic megacolon (Kelly and LaMont, 1998). Although *C. difficile* was first isolated in 1935 (Hall and O'Toole), the symptoms of pseudomembranous colitis (PMC) were described as early as 1893 (Finney, 1893), thus the bacterium and disease were identified separately, before they were linked together. The antibiotic era saw a rise in the number of reported cases of PMC and in 1952, PMC was recognised as a complication of antimicrobial therapy (Reiner *et al.*, 1952). As it was a major nosocomial pathogen of the time, *Staphylococcus aureus* was initially suspected as the organism responsible for PMC (Altemeier *et al.*, 1963; Hummel *et al.*, 1965), with oral vancomycin quickly becoming the standard treatment of choice (Khan and Hall, 1966). The aetiological role of *S. aureus* in antibiotic-associated diarrhoea (AAD) was not seriously questioned until 1974 (Tedesco *et al.*, 1974), with investigations reporting a high incidence of AAD amongst patients receiving clindamycin treatment.

The presence of a cytotoxin in the faecal exudate of patients was implicated as the causative agent (Larson *et al.*, 1977; Rifkin *et al.*, 1977). As the toxin was neutralised by *C. sordellii* antitoxin, the toxin was believed to be derived from *C. sordellii*, however this organism was rarely isolated from patients with PMC. The 1970s marked the start of the *C. difficile* era resulting in the successful isolation of *C. difficile* from the stools of PMC patients (George *et al.*, 1978; Larson *et al.*, 1980). Whilst investigating antibiotic-associated caecitis in hamsters, in a series of studies Bartlett *et al.* (Bartlett *et al.*, 1977a; Bartlett *et al.*, 1977b; Bartlett *et al.*, 1978; Bartlett *et al.*, 1979) identified vancomycin prolonged survival, this coupled with the earlier work of Khan and Hall (1966) resulted in vancomycin being established as an effective antibiotic of CDI. The latter half of the 1980s, saw *C. difficile* fade into obscurity, as it became perceived as a nuisance disease, manageable by increasingly

effective diagnostics and therapeutics (Bartlett, 1988). However, the turn of the century saw the re-emergence of *C. difficile*, with the prevalence of highly virulent, epidemic strains, associated with increased morbidity, mortality and environmental dissemination.

1.1.3 *Clostridium difficile* Typing and Epidemiology

Early typing methods were initially based on phenotypic characteristics (antibiotic susceptibility, bacteriophage and bacteriocin) which have since been superseded by molecular typing techniques. The most commonly used typing schemes are summarised in Table 1.1.

Table 1.1: Molecular typing methods for *C. difficile*

Method	Summary
Restriction Endonuclease Analysis (REA)	Enzyme restriction (*Hind*III) of total genomic DNA
Toxinotyping	Sequence data of toxin A and toxin B
PCR-Ribotyping (PCR RT)	Amplification of intergenic region between 16S and 23S ribosomal DNA
Multilocus Sequence Typing (MLST)	Sequence analysis of seven housekeeping genes (*aroE*, *ddl*, *dutA*, *tpi*, *recA*, *gmk*, and *sodA*)
Multilocus Variable-Number Tandem Repeat (MLVA)	Number of repeats in a set of VNTR loci is assessed by PCR
Pulsed-Field Gel Electrophoresis (PFGE)	Enzyme restriction of total genomic DNA with rare cutting restriction endonucleases (*Sma*I, *Ksp*I, *SacI*I)
Surface Layer Protein A Gene Sequence Typing (*slpA*ST)	Sequencing of variable region of the surface layer protein A gene (*slpA*)
Amplified Fragment Length Polymorphism (AFLP)	Combination of enzyme restriction and PCR

Table adapted from (Killgore *et al.*, 2008; Cairns *et al.*, 2012)

All of the above methods have been found to be comparable (Killgore *et al.*, 2008), in Europe PCR RT is widely used with over 427 PCR ribotypes identified, to date (Cairns *et al.*, 2012). MLST studies have indicated that *C. difficile* has evolved into six distinct lineages which can be broadly divided by the most important PCR ribotypes (017, 023, 027 and 078) (Knetsch *et al.*, 2013), as summarised in Table 1.2.

Table 1.2: Correlation between sequence type (ST) and PCR ribotype (RT)

Lineage 1	ST-3 (PCR RT001), ST-54 (PCR RT012) and ST-2, ST-14 and ST-49 (all PCR RT014)
Lineage 2	19 STs (PCR RT016, PCR RT036 and PCR RT176, ST-120 (PCR RT006) ST-1 (PCR RT027)
Lineage 3	ST-5 (PCR RT023) ST-22, ST-25 and ST-96 (PCR RT058)
Lineage 4	16 STs ST-37 (PCR RT017)
Lineage 5	ST-11 (PCR RT078, 033, 045, 066, 126 and 193), ST-147 and ST-148
Lineage 6	ST-122 (PCR RT131)

Adapted from (Knetsch *et al.*, 2012)

Voluntary surveillance of *C. difficile* was introduced in 1990 in England and Wales and made mandatory in 2004, for all reported CDI in patients aged 65 and over (CDI is now reported in all patients aged 2 and over). In England and Wales the number of deaths involving *C. difficile* has begun to decrease (Figure 1.1). This is partly attributed to an increase in awareness of CDI, resulting in prompt diagnosis, increased antimicrobial stewardship and improved infection control practices (hand hygiene and hospital cleaning).

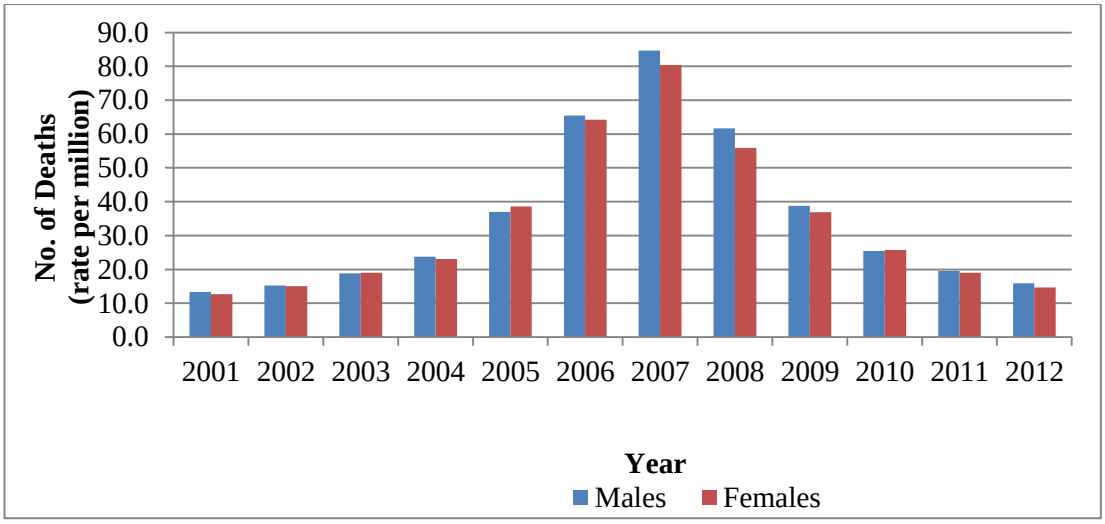

Figure 1.1: Number of deaths involving *C. difficile* in England and Wales.

Data from the Office for National Statistics (2013).

The decline in the three most prevalent PCR ribotypes (027, 106 and 001) in the UK has been offset by an increase in all other ribotypes (Figure 1.2). Recently emerging strains include the toxin A^-B^+ variant strains, which invariably belong to ribotype 017, 053 and 078. Outbreaks due to toxin A^-B^+ strains are increasing globally with strains generally resistant to fluroquinolones and associated with severe CDI. Interestingly, PCR RT078 is predominantly associated with CDI in animal species (pigs, horses, calves) and has also been found in contaminated food, indicating *C. difficile* is a zoonotic disease (Rupnik, 2007) and two-way transmission between humans and animals/food sources occurs (Dawson *et al.*, 2009; He *et al.*, 2012). Although to date, there has been no confirmed case of CDI to be of zoonotic or foodborne origin (Rodriguez-Palacios *et al.*, 2013)

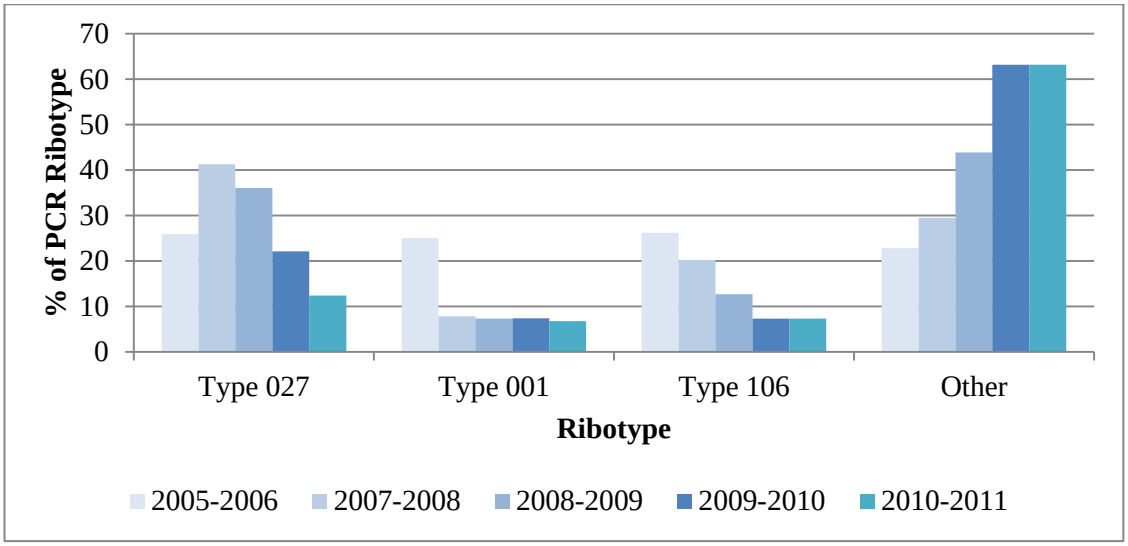

Figure 1.2: Distribution of PCR ribotypes in England and Northern Ireland.
Isolates were typed by the PCR ribotyping method at the Anaerobe Reference Unit or the *C. difficile* Ribotyping Network (CDRN). Adapted from (Brazier *et al.*, 2007; Brazier *et al.*, 2008; Health Protection Agency, 2008, 2009, 2010).

The widespread use of broad spectrum antibiotics facilitated the dissemination of *C. difficile*. In the 1980s and 1990s epidemics were predominantly caused by clindamycin resistant strains (Johnson *et al.*, 1999). More recent epidemic strains

have evolved resistance to fluroquinolones, which has facilitated the global spread of these strains (He *et al.*, 2012).

1.1.4 Risk Factors for *Clostridium difficile* Infection

Disturbance to the microflora, as a consequence of antibiotic exposure is recognised as the primary risk factor for CDI (Kyne *et al.*, 2001a; Drekonja *et al.*, 2011), with the elderly (>65 years) found to be more susceptible (Kim *et al.*, 2010). Other risk factors include proton pump inhibitors and histamine H2-blockers (Dial *et al.*, 2005; Akhtar and Shaheen, 2007; Aseeri *et al.*, 2008; Kim *et al.*, 2010), those with underlying chronic conditions such as solid organ transplant or immunosuppression, (Zilberberg *et al.*, 2010). Increasingly, those previously classified in the low risk group; patients with minimal or no recent exposure to healthcare facilities, peripartum women and children are experiencing CDI (Ananthakrishnan, 2010).

Based on the changing epidemiology and risk factors of CDI, it is likely that *C. difficile* will prevail for the foreseeable future, with the 027 strain replaced by other epidemic strains. Whether these emerging strains represent a new stage in the evolution of *C. difficile* (particularly in regards to transmission, survival and virulence) is yet to be seen.

1.2 *Clostridium difficile* Germination, Colonisation and Toxin Production

There are three main facets to the *C. difficile* lifecycle; the spore, the vegetative cell and toxin production. For CDI to occur the spore must adhere and germinate, the vegetative cells must adhere to the mucosal surface and colonise the intestinal tract followed by toxin production. The following section will summarise aspects related to these mechanisms, highlighting the virulence factors associated with each of these facets.

1.2.1 *Clostridium difficile* Spore Adherence and Germination

Bacterial endospores are of central importance in the transmission, persistence and pathogenesis of *C. difficile*, enabling the organism to survive in both endogenous (within the host) and exogenous environments. *C. difficile* spores are shed into the hospital environment by both symptomatic patients and asymptomatic carriers (Gerding *et al.*, 1995; Muto, 2007; Riggs *et al.*, 2007; Lawley *et al.*, 2009). Transmission is primarily mediated via the hands of patients and healthcare workers via the faecal-oral route (Guerrero *et al.*, 2012).

Initial research in spore adherence to Caco-2 cells (intestinal epithelial cells) has indicated a positive correlation between spore hydrophobicity and adherence, with two spore-surface proteins of ~40-45 kDa implicated in surface hydrophobicity and adherence. Although the *C. difficile* spore receptor(s) is yet to be elucidated, four candidate proteins have been identified, with spore adherence regarded as a key step in pathogenesis (Paredes-Sabja and Sarker, 2012). As the spore is metabolically inactive, for CDI to occur the spore must germinate into a vegetative cell. This is achieved by sensing specific germinants, for example nutrients (amino acids, sugars) or non-nutrient (lysozyme, salts) sources via germinant (Ger) receptors (Setlow, 2003). Analogs of spore specific proteins have been reported in all sequenced *Bacillus* and *Clostridia*, indicating germination proteins are conserved in both species (Howerton *et al.*, 2011). In *C. difficile*, the absence of the GerA, B and K receptor analogs (Paredes *et al.*, 2005; Sebaihia *et al.*, 2006; Sarker and Paredes-Sabja, 2012) indicates the presence of either divergent, uncharacterised receptors and/or germinants (Ramirez *et al.*, 2010; Howerton *et al.*, 2011). Bile salts (sodium taurocholate and sodium cholate) have been reported to trigger germination of *C. difficile* spores in nutrient-rich media (Wilson *et al.*, 1982; Wilson, 1983), with glycine (Sorg and Sonenshein, 2008), l-phenylalanine, l-arginine (Howerton *et al.*, 2011) and histidine (Wheeldon *et al.*, 2011) acting as co-germinants with cholate derivatives. *In vivo*, spores are suspected to interact with bile salts, amino acids and lysozyme (Paredes-Sabja and Sarker, 2011) along the GI tract, wherein multiple interactions stimulate germination. These interactions with host derived factors highlight the ability of *C. difficile* to survive in the human gut. Further to this the ability of the spore to survive within macrophages (72 hours) and persist on surfaces

for prolonged periods (>5 months), highlights the significance of the spore in the pathogenesis, dissemination and transmission of *C. difficile* (Kramer *et al.*, 2006; Paredes-Sabja *et al.*, 2012; Janoir *et al.*, 2013)

1.2.2 *Clostridium difficile* Vegetative Cell Adherence and Colonisation

Fundamental to *C. difficile* colonisation is adherence; the mechanism by which this is achieved is yet to be fully elucidated. Colonisation factors include; capsule (Davies and Borriello, 1990), Cell Wall Protein 66 (Waligora *et al.*, 2001), flagellins, FliC and FliD (Tasteyre *et al.*, 2001a), Fbp68 (Hennequin *et al.*, 2003; Lin *et al.*, 2011), GroEL (Hennequin *et al.*, 2001b) and the S-layer proteins (Slp).

The Gram-positive cell envelope is composed of a single cytoplasmic membrane surrounded by a thick peptidoglycan layer, forming a physical barrier with the environment and providing a scaffold for the attachment of cell wall teichoic acid, capsular polysaccharides or proteins (Scott and Barnett, 2006; Emerson and Fairweather, 2009; Schneewind and Missiakas, 2012; Freudl, 2013). In Gram-positive bacteria, surface proteins can be covalently or non-covalently bound to the peptidoglycan or secondary cell wall (Navarre and Schneewind, 1999). Those which are covalently bound to the cell wall contain a C-terminal sorting signal which is recognised and cleaved by sortase enzymes (sortase-dependent mechanism), allowing for subsequent linkage to the peptidoglycan (Freudl, 2013). The transport of cell wall proteins across the membrane is mediated by the general secretory system (Sec); ATP hydrolysis (SecA ATPase), which allows protein translocation through the aqueous channel (SecYEG), followed by the removal of the signal peptide. In Gram-positive bacteria a second accessory Sec system has been described, SecA2, in *C. difficile* it is this system which is responsible for the transport of S-layer proteins (SlpA, CwpV, Cwp2, Cwp66 and Cwp84) and hence is essential for *C. difficile* viability (Fagan and Fairweather, 2011). This is in contrast to other bacteria where the SecA2 system is largely associated with the transport of virulence and colonization factors.

1.2.2.1 S-Layer Proteins (Slp)

Surface proteins are composed of a LPxTG (Leu-Pro-X-Thr-Gly) motif, a hydrophobic domain and tail of (mostly positive) charged residues (Navarre and Schneewind, 1999). Most *C. difficile* CWPs are non-covalently anchored to the cell wall by an as yet uncharacterized mechanism and hence are not members of the LPxTG protein family (Emerson *et al.*, 2009). LPxTG-like motifs have been identified in *C. difficile* (SPXTG or PPXTG) (Pallen *et al.*, 2001). *C. difficile* is composed of 28 SLP paralogs, each containing a conserved cell wall anchoring domain and a function specifying domain (Sebaihia *et al.*, 2006). The predominant protein in the *C. difficile* S-layer is SlpA, which is post-translationally cleaved by the cysteine protease, Cwp84 (Kirby *et al.*, 2009; Dang *et al.*, 2010), resulting in the formation of a 45 kDa, high molecular weight protein (HMW SLP) and 32 kDa, low molecular weight protein (LMW SLP). The resulting S-layer consists of a thick hexagonally arranged inner S-layer and a squarely arranged outer lattice (Kawata *et al.*, 1984; Cerquetti *et al.*, 2000). The LMW SLP is highly divergent, although a high degree of conservation has been identified in the C-terminal domain, which is necessary for the interaction with the N-terminus of the HMW SLP (Fagan *et al.*, 2009). Although there is some inter-strain variability, SLPs have been detected in all *C. difficile* strains, forming a core component of the bacterium aiding in colonisation and adherence (Karjalainen *et al.*, 2001; Calabi *et al.*, 2002; Cerquetti *et al.*, 2002). During the course of CDI, antibodies to both the HMW and LMW SLPs have been shown (Drudy *et al.*, 2004; Péchiné *et al.*, 2005a; Wright *et al.*, 2008). Whilst the humoral response to the HMW SLP is conserved across strains, the response to the LMW SLP is only similar within the same serogroup (Cerquetti *et al.*, 2000; Calabi *et al.*, 2001). The SLP has also been shown to be a protective factor against the immune response and as such is regarded as a target for therapeutic development.

1.2.2.2 Cell Wall Protein66 (Cwp66)

Cwp66 displays homology to the autolysin CwlB of *Bacillus subtilis*, it has been identified as an immunogenic adhesion and is genetically diverse (Waligora *et al.*, 2001; Péchiné *et al.*, 2005a). Interestingly, *cwp66*, *slpA* and *SecA2* form a 10 kB S-

layer cassette which has co-evolved and through homologous recombination twelve diverse variants are recognised. Such system are comparable to capsular switching in other bacteria, hence S-layer switching would aid in evasion of the host immune system and susceptibility to bacteriophages (Dingle *et al.*, 2013).

1.2.2.3 Cell Wall Protein V (CwpV)

CwpV is a surface expressed phase variable protein, capable of autoproteolysis (Emerson *et al.*, 2009; Reynolds *et al.*, 2011; Dembek *et al.*, 2012). In the OFF orientation, the inverted region between the *cwpV* promoter and open reading frame forms a stem loop terminator preventing full length transcription, this recombination and inversion is mediated by RecV (Emerson *et al.*, 2009). The presence of variable number repeats in the C-terminal of CwpV confers antigenic variability between *C. difficile* strains, with at least five antigenically variable types identified to date, this combined with the proteins ability to promote bacterial aggregation and be expressed in a phase variable manner, suggests CwpV is important for immune evasion and colonization (Reynolds *et al.*, 2011).

1.2.2.4 Collagen Binding Protein A (CbpA)

Binding to host extracellular matrices (ECM), such as fibronectin, fibrinogen, and collagen, is mediated by bacterial surface proteins known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). These proteins contain a surface sorting signal which is recognised by sortase, resulting in the surface localisation of the protein. In *C. difficile* the surface exposed Collagen Binding Protein A (CbpA), has been described as a member of the MSCRAMM family, containing a NVQTG motif at its C-terminus and may well be the substrate for the putative sortase B. CbpA is hypothesised to function as an adhesion facilitating *C. difficile*-host interaction (Tulli *et al.*, 2013).

1.2.2.5 GroEL

GroEL is a heat shock protein which can be both surface located and released extracellularly post-heat shock. Incubation of *C. difficile* cells with GroEL polyclonal antibodies was found to reduce adherence of *C. difficile* to Vero cells (Hennequin *et al.*, 2001a; Hennequin *et al.*, 2001b; Jain *et al.*, 2011), whilst mice

immunised with recombinant GroEL displayed a reduced level of intestinal colonisation, the same was not apparent in the hamster model (Péchiné *et al.*, 2013). The authors suggest this may be due to only a partial inhibition of colonization in the hamster model, which is neither sufficient nor efficient enough to limit the action of toxin A and B. Alternatively the differences observed in the two animal models may be due to experimental differences in the immunization regime and bacterial challenge employed, challenge with $2x10^3$ spores vs. challenge with 10^9 *C. difficile* cells in the hamster and mouse model, respectively.

1.2.2.6 Flagella

In many bacterial pathogens, flagella mediate swimming motility contributes to pathogenesis. In *C. difficile* the flagellin structural monomer (FliC) and the cap protein (FliD) have been reported to aid in biofilm formation and attachment to the intestinal mucus layer and host cells (Tasteyre *et al.*, 2001a; Tasteyre *et al.*, 2001b; Dingle *et al.*, 2011; Đapa *et al.*, 2013). Although in the latter study flagella were not required for virulence in hamsters (Dingle *et al.*, 2011). More recently a study comparing the flagella of 630Δerm and R20291 has identified differences in the production and adherence properties of the two strains, with the latter strain producing a single flagellum and the R20291 flagella mutant displaying reduced adherence *in vitro* as compared to the wildtype (Baban *et al.*, 2013). The generation of serum antibody responses in CDI patients (Péchiné *et al.*, 2005a; Péchiné *et al.*, 2005b) and the variability in flagellar protein glycosylation between strains (Twine *et al.*, 2009) suggests flagella are important virulence factors during the course of CDI, contributing to the evasion of the immune system and virulence. Furthermore, the flagella regulon has been shown to influence the transcription of the PaLoc genes (Aubry *et al.*, 2012; Baban *et al.*, 2013). The role of flagella in colonisation, toxin expression and secretion in *C. difficile* is yet to be determined, and may well display strain-strain variations.

1.2.2.7 Capsule

Capsules have long been regarded as prominent virulence factors aiding in adherence, preventing dehydration, impairing phagocytic uptake into the cells of the

innate immune system and protecting against bacteriophage infection (Foster and Popham, 2002). The major component of capsules is water and is typically composed of polysaccharides of varying structure or amino acid homopolymers (Foster and Popham, 2002). The first indirect demonstration of capsulated *C. difficile* cells was presented by Strelau *et al*., (1989), however the suggestion that a capsule could serve as an antiphagocytic factor in *C. difficile* was proposed two years earlier (Dailey *et al*., 1987). Dailey *et al*., (1987) identified that phagocytosis of *C. difficile* by human polymorphonuclear leukocytes (PMN) (the first line of defence of the innate immune system) was dependant on opsonisation of the bacteria and was independent of the toxins. Confirmation and further characterisation of capsules was provided by Davies and Borriello (1990) who identified two morphologically distinct capsules in different strains of *C. difficile*. This study identified there was no correlation between the presence of capsule and the toxigenicity status of the strain. Later research conducted by Baldassarri *et al*., (1991) identified that glucose enhanced capsule production, with strains more frequently associated with disease able to produce thicker capsules even in the absence of glucose in the media. The role of the capsule in *C. difficile* and its contribution to virulence is yet to be fully investigated.

Undoubtedly the cell surface of *C. difficile* contributes to adherence, colonisation and evasion of the host immune system. The presence/regulation of multiple components (SlpA, CwpV, flagella, capsule) and their precise role in the pathogenesis of this organism is yet to be fully elucidated. Microarray analysis indicates that *C. difficile* alters its cell surface envelope upon contact with Caco-2 cells, resulting in up-regulation of several virulence factors, including capsular proteins and down-regulation of flagellar proteins and Cwp84 (Janvilisri *et al.*, 2010). Although insights have been made in assessing the role of the vegetative cell surface in the pathogenesis of *C. difficile*, it is clear that much is yet to be deciphered.

1.2.3 *Clostridium difficile* Toxin A, Toxin B and Binary Toxin

1.2.3.1 Binary Toxin

In addition to the production of toxins A and B some *C. difficile* strains are capable of producing a binary toxin, CDT (Popoff *et al.*, 1988) which is unrelated to the Large Clostridial Toxins (LCT). CDT binds to lipolysis-stimulated lipoprotein receptor (LSR), irreversibly ADP-ribosylates actin, disrupting the host cell cytoskeleton (Papatheodorou *et al.*, 2011). CDT is capable of inducing a non-haemorrhagic fluid response and thus possesses enterotoxic activity. In a hamster model, animals infected with $A^-B^-CDT^+$ strains became colonized but did not display symptoms of CDI (Geric *et al.*, 2006). More recently, hamsters infected with an isogenic mutant ($A^-B^-CDT^+$), generated using the ClosTron technology, found 38% (3 of 8) of animals died, although these animals displayed atypical symptoms of CDI, the study does indicate a potential role of the binary toxin in *C. difficile* pathogenesis (Kuehne *et al.*, 2014). The destruction of the actin cytoskeleton and induction of microtubule protrusions suggests CDT enhances bacterial adherence and colonization (Schwan *et al.*, 2009).

1.2.3.2 Toxin A

Historically, toxin A has been regarded as the primary virulence factor of *C. difficile*, responsible for the pathophysiological effects of infection. Notably toxin A was viewed as essential for facilitating the exit of toxin B from the gut into the sub-mucosal areas (Lyerly *et al.*, 1988; Du and Alfa, 2004). Toxin A is a potent enterotoxin with slight cytotoxic activity, responsible for the release of inflammatory mediators, including infiltration of neutrophils (Kelly *et al.*, 1994), the production of cytokines (He *et al.*, 2002) and chemokines (Castagliuolo *et al.*, 1998), reactive oxygen intermediates (He *et al.*, 2002) and substance P (Mantyh *et al.*, 1996), the activation of mast cells (Wershil *et al.*, 1998) and sub-mucosal neurons (Hurley and Nguyen, 2002) resulting in fluid accumulation and extensive tissue necrosis.

1.2.3.3 Toxin B

Depending on the cell type, toxin B is a 100–10,000 times more potent than toxin A (Tucker *et al.*, 1990; Chaves-Olarte *et al.*, 1997), is involved in the depolymerisation of filamentous actin, causing destruction in the cell cytoskeleton and cell rounding (Pothoulakis *et al.*, 1986). The isolation of A^-B^+ strains from PMC patients has established that toxin B is capable of causing severe disease and death, even in the absence of toxin A (Limaye *et al.*, 2000; Johnson *et al.*, 2001). Early research indicated toxin B possessed no enterotoxic activity, (Borriello, 1998; Borriello and Wilcox, 1998), it has since been shown toxin B induces fluid secretion and factors associated with enterotoxic and pro-inflammatory action in the human intestine, including epithelial cell damage, release of interlukin-8 (IL-8), neutrophil infiltration and mucosal inflammation (Savidge *et al.*, 2003). The C-terminal region of TcdB has been shown to exhibit cytotoxic effects and in A^-B^+ strains this region is suspected to facilitate access of TcdB to the basolateral surface (Zemljic *et al.*, 2010).

1.2.4 Large Clostridial Cytotoxins

C. difficile toxins A (TcdA) and B (TcdB), the alpha toxin of *C. novyi* (TcnA), the lethal (TcsL) and haemorrhagic toxins (TcsH) of *C. sordellii* and the TpeL toxin of *C. perfringens* are members of the Large Clostridial Cytotoxin (LCT) family (Table 1.3). This is a unique and still emerging group of bacterial toxins that share common sequence similarity, primary structure and enzymatic activity (Table 1.3). Members are recognised by the production of large single chained proteins ranging between 191 to 308 kDa (Rupnik and Just, 2006). A number of LCT variants have been reported to occur in both *C. difficile* and *C. sordellii*, with strains only producing TcdB or TcsL, respectively (Table 1.3). Even in the absence of TcdA and TcsH, both organisms are still able to cause disease. To date, no A^+B^- strain of *C. difficile* has been isolated. With a genetically engineered A^+B^- strain, Lyras *et al.*, (2009) found TcdB was essential for pathogenesis. Since this report, Kuehne *et al.*, (2010) have reported the importance of both TcdA and TcdB in the pathogenesis of CDI. Until the roles of TcdA and TcdB can be unequivocally determined it would be rational to assume, both TcdA and TcdB are required for virulence and pathogenesis.

Table 1.3: Properties of large clostridial toxins and those homologous to LCTs.

Toxin	Organism	Size kDa	CROPS[a]	Location	Substrate	Target GTPases[b]	Homology (%)[c]	Biological Activity [d]	Lethal dose[e]
TcdA	*C. difficile* (VPI 10463)	308	32	Chromosome (PaLoc)	UDP-glucose	Rho, Rac Cdc42, RhoG, TC10, Rap	60	D	50 ng
TcdB	*C. difficile* (VPI 10463)	270	19	Chromosome (PaLoc)	UDP-glucose	Rho, Rac Cdc42, RhoG, TC10	100	D	50 ng
TcsH	*C. sordellii* (VPI 9048)	300	*	*	UDP-glucose	Rho, Rac Cdc42	*	D	75 ng
TcsL	*C. sordellii* (6018)	270	19	Chromosome	UDP-glucose	Ras, Rac, Rap, Ral, (Cdc42)f	75	S	5 ng
TcnA	*C. novyi* (ATCC 19402)	250	14	Phage	UDP-N-acetyl-glucosamine	Rho, Rac Cdc42	27	D	5 – 10 ng
TpeL	*C. perfringens* (Type B and C)	191	*	Plasmid	UDP-glucose UDP-N-acetyl-glucosamine	Ha-Ras, RalA, Rap1B, Rac1	38	D/S	16 µg
$TcdB_{1470}$	*C. difficile*$_{1470}$	270	19	Chromosome (PaLoc)	UDP-glucose	Ras, Rac, Rap, Ral, Cdc42	93	S	50 ng
$TcdB_{8864}$	*C. difficile*$_{8864}$	270	19	Chromosome (PaLoc)	UDP-glucose	Rac, Rap, Ral	85	S	50 ng

[a]Number of Clostridial Repetitive Oligopeptides (CROPs). [b]Target proteins belong to the Ras superfamily of small GTP-binding proteins. [c]Amino acid sequence similarity when compared with that of $TcdB_{10463.}$ [d]Biological activity, D: *C. difficile*-like, S: *C. sordellii*-like. [e]Lethal dose based on intraperitoneal injection of a mouse. [f]Glucosylation of CDC42 is strain specific in *C. sordellii*. UDP-Glucose, glucose-uridine-diphosphate, UDP-N-acetyl-glucosamine, N-acetylglucosamine-diphosphate, * Unknown. Adapted from (Rupnik and Just, 2006; Pruitt and Lacy, 2012).

1.2.5 *Clostridium difficile* Toxin A and B Expression and Secretion

The primary virulence factors of Clostridia are toxins and it is the secreted toxins which are the primary mediators of pathogenesis. Secreted toxins are released into the external media and can act on sites distant from the site of bacterial colonization. This is in contrast to invasive bacteria which survive intracellularly by directly injecting virulence factors into cells, usually by means of a Type III secretion system. Gram-negative bacterial toxins are exported across the inner cytoplasmic membrane, periplasmic space, peptidoglycan and outer membrane whilst in Gram-positive bacteria upon crossing the cytoplasmic membrane the toxin is present in the external media (Popoff and Stiles, 2005). Gram-negative bacteria utilise a range of systems for the secretion of proteins, which are summarised in Table 1.4.

Table 1.4: Toxin secretion in Gram-negative bacteria

	Mechanism	Examples
Type I	3 membrane proteins: ATPase, adaptor protein, TolC form a trimeric structure which forms pore through the outer membrane of Gram-negative bacteria	*E. coli* α-hemolysin leukotoxins hydrolytic enzymes
Type II	Contain N-terminal sequence and use the general secretion pathways to cross inner membrane, cleavage of signal peptide and release of protein into periplasmic space. Protein folding and assembly.	Cholera toxin ExoA Aerolysin Hydrolytic enzymes
Type III	14 proteins that assemble to form a pore through the inner and outer bacterial membrane and eukaryotic membrane. Contact with eukaryotic cell initiates synthesis of Type III secretion proteins and injection of effector proteins.	EPEC/EHEC – EspA,B,D
Type IV	Evolved from conjugation pili to transport DNA or proteins to target cell. 10 proteins that form a central pore connecting the bacterial cytoplasm with the extracellular medium or cytosol of targeted cell.	Pertussis toxin
Type V	N-terminal signal peptide mediates export of the protein across the inner membrane via the general secretion pathway. C-terminus generates pore in the outer membrane, C-terminus is cleaved and protein released into extracellular medium	*Helicobacter pylori* Vacuolating toxin

Modified from Popoff and Stiles (2005).

The precise mechanism by which *C. difficile* secretes toxins A and B is unclear, extracellular proteins generally carry a secretion signal located at either the N or C-terminus or a Tat-signal; neither toxin A or B contain such secretion signals. It was previously believed the toxins were released as a consequence of bacterial lysis, however this does not appear to be true with the extracellular protein proportion accounting for 50% of the total toxin produced in stationary phase cells, whilst <1% of typical cytoplasmic proteins were released (Mukherjee *et al.*, 2002).

In *C. difficile*, TcdA and TcdB are chromosomally located on a 19.6 kb pathogenicity locus (PaLoc) (Hammond and Johnson, 1995; Braun *et al.*, 1996), which is composed of five genes; *tcdA, tcdB, tcdC, tcdR* and *tcdE* (Figure 1.3). In non-toxigenic strains, the integration site of the PaLoc is a highly conserved, 115 bp non-coding region which lies between *cdu1* and *cdd1* (Hammond and Johnson, 1995; Braun *et al.*, 1996). Unique to strain 8864, is an additional ORF, named *tcdF* (Soehn *et al.*, 1998), located at the intergenic region of *tcdA*-8864 and *tcdE*. Amino acid sequence analysis of TcdF has not revealed significant homology with any known proteins and the function of the protein and its role, if any in pathogenesis is yet to be elucidated (Song *et al.*, 1999), Figure 1.3.

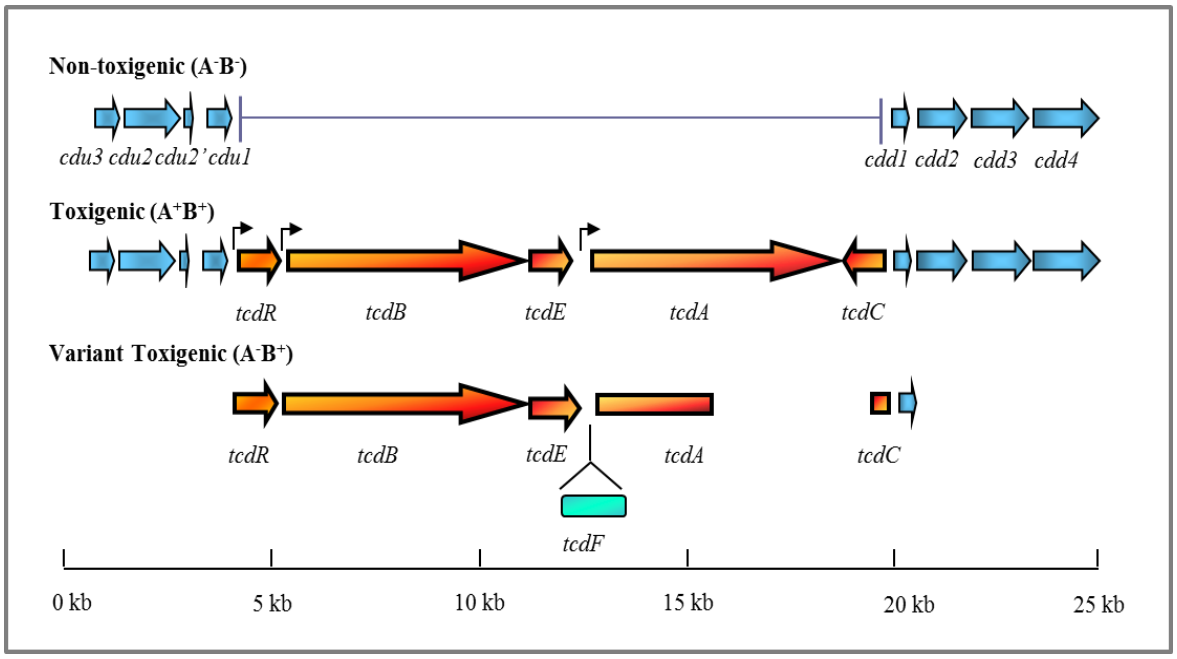

Figure 1.3: Genetic arrangement of the *C. difficile* pathogenicity locus (PaLoc). Schematic diagram of the PaLoc in non-toxigenic (A^-B^-), toxigenic strains (A^+B^+) and variant$_{8864}$ (A^-B^+) strains. In non-toxigenic strains the PaLoc is replaced by 115 bp non-coding sequence. The variant strain 8864 has an insertion (*tcdF*) and truncated *tcdA* and *tcdC*. Black arrows indicate promoters for *tcdR*, *tcdA* and *tcdB*.

Genes *tcdRBEA* form a transcriptional unit, with expression peaking during the late stationary growth phase, suggesting the toxins serve as a means of nutrient scavenging. Transcriptional analysis of the PaLoc has revealed the genes are transcribed in a unified manner in response to a number of environmental stimuli including phase of growth, antibiotics, temperature, nutrient sources and amino acids (Yamakawa *et al.*, 1998; Karlsson *et al.*, 1999; Karlsson *et al.*, 2000; Karlsson *et al.*, 2003). It is suggested toxin production is a cell-density dependent process, with quorum sensing molecules shown to play an important role in transcriptional regulation (Carter *et al.*, 2005; Lee and Song, 2005; Darkoh, 2012).

1.2.5.1 TcdR – Positive Regulator

The *tcdR* gene encodes a 22 kDa protein with a helix-turn-helix DNA motif, homologous with the BotR, TetR and UviA positive regulators of *C. botulinum*,

C. tetani and *C. perfringens*, respectively (Mani and Dupuy, 2001). TcdR is a sigma (σ) factor, directing RNA polymerase to its own promoter and to the promoters of the toxin genes (Moncrief *et al.*, 1997; Mani and Dupuy, 2001; Mani *et al.*, 2002).

1.2.5.2 TcdC – Negative Regulator?

TcdC is a membrane associated protein (Govind *et al.*, 2006) believed to function as an anti-σ factor, negatively regulating *tcdR*-dependent transcription (Dupuy *et al.*, 2008), by preventing the formation of the TcdR-RNA polymerase holoenzyme (Moncrief *et al.*, 1997; Mani and Dupuy, 2001; Matamouros *et al.*, 2007). Deletions and frame shift mutations in *tcdC* have been implicated in contributing to hypervirulence, resulting in increased toxin production and severity of disease (Warny *et al.*, 2005; Freeman *et al.*, 2006; Carter *et al.*, 2011). However, the role of TcdC as a negative regulator of toxin expression is questionable, with studies reporting no correlation between the expression levels of the toxins and TcdC (Merrigan *et al.*, 2010; Vohra and Poxton, 2011; Bakker *et al.*, 2012; Cartman *et al.*, 2012). In the aforementioned studies TcdC is hypothesised to have a modulatory role in regulating toxin expression.

1.2.5.3 TcdE – Holin-Like Protein?

TcdE is predicted to be highly hydrophobic, with three transmembrane domains, a hydrophilic region at the N-terminus and a series of charge rich residues at the C-terminus, indicative of holin proteins (Tan *et al.*, 2001). Sequence analysis and structural similarities between TcdE and a family of bacteriophage holin proteins, suggests TcdE may be involved in holin-mediated cell lysis, facilitating the release of toxins A and B into the extracellular environment (Tan *et al.*, 2001). The role of TcdE is currently under debate with a TcdE-deficient strain displaying no increase in the level of secreted enzyme or delay in toxin release (Olling *et al.*, 2011). These findings are in contrast to the study conducted by Govind and Dupuy (2012), who have proposed TcdE is expressed in sufficient quantities in *C. difficile*, to allow pore formation, facilitating the release of toxins without causing cell lysis. Direct comparisons between the two studies are complicated by differences in growth

media, experimental time point analysis and significantly the parental strain (Govind and Dupuy, 2012)

1.2.5.4 Other Regulators of Toxin Expression

Other regulators of toxin synthesis include; CodY (Dineen *et al.*, 2007; Dineen *et al.*, 2010), Spo0A (Underwood *et al.*, 2009), bacteriophage mediated (Govind *et al.*, 2009; Sekulovic *et al.*, 2011), CcpA (Antunes *et al.*, 2011), SigH (Saujet *et al.*, 2011) and flagella (Aubry *et al.*, 2012; Baban *et al.*, 2013).

1.2.6 *Clostridium difficile* TcdA and TcdB Structure and Uptake

Structural analysis of TcdA and TcdB has revealed a modular structure (Figure 1.4), with amino acid sequence analysis identifying extensive sequence homology (49% identity and 63% similarity) (Pruitt and Lacy, 2012). Due to the structural and functional homologies of the toxins it has been postulated that TcdA and TcdB arose due to a gene duplication event, with subsequent mutation and recombination (von Eichel-Streiber *et al.*, 1992a). The toxins resemble AB-toxins, containing an enzymatic domain and a binding/translocation domain (von Eichel-Streiber *et al.*, 1995; von Eichel-Streiber *et al.*, 1996). The AB model has since been extended to the ABCD model (A, biological activity; B, binding; C, cutting; D, delivery), (Jank and Aktories, 2008), Figure 1.4.

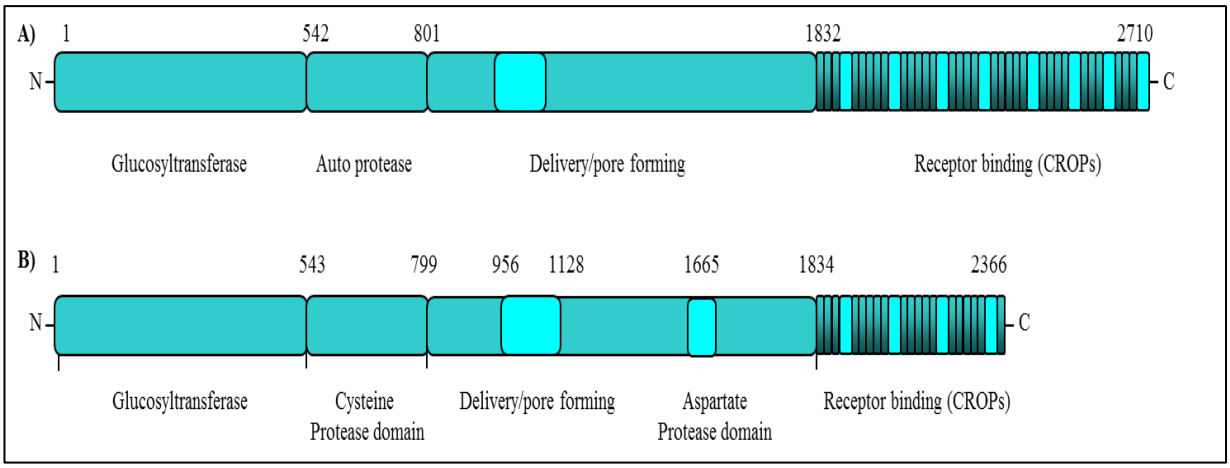

Figure 1.4: Diagrammatic representation of the structure of *C. difficile* toxins.

A) Toxin A and **B)** Toxin B. The glucosyltransferase activity is located at the N-terminus of the protein, the putative transmembrane domain involved in membrane translocation is located in the middle and the C-terminus represents the receptor binding domain, composed of CROPs. Adapted from Jank *et al.*, (2007).

1.2.6.1 C-Terminal

The C-terminus of TcdA and TcdB is composed of multiple repeat regions, termed Combined Repetitive Oligopeptides (CROPs) (Dove *et al.*, 1990; von Eichel-Streiber *et al.*, 1992b). In TcdA, this region accounts for one third of the toxin spanning amino acid residues 1832-2710. The TcdA CROPs are composed of 32 short repeats (20 or 21 amino acids) and seven long repeats (30 amino acids). For TcdB four long repeats (30 amino acids) and 19 short repeats (20 or 21 amino acids) make up the CROPs (Wren, 1991; von Eichel-Streiber *et al.*, 1992a; von Eichel-Streiber *et al.*, 1992b). It is via the CROPs that TcdA and TcdB bind to sugar moieties on the surface of host cells (von Eichel-Streiber *et al.*, 1992a; von Eichel-Streiber *et al.*, 1992b). The C-terminus of TcdA shows homology with ligand binding proteins of Streptococci (Wren, 1991). Crystal structure analysis of 127 and 255 residues at the C-terminus of TcdA (Ho *et al.*, 2005; Greco *et al.*, 2006) has revealed a solenoid-like structure with 7 carbohydrate binding sites (Ho *et al.*, 2005; Greco *et al.*, 2006). Early studies have reported a protein-carbohydrate interaction for TcdA (Krivan *et al.*, 1986; Tucker and Wilkins, 1991), with binding occurring in a multivalent, lectin-like manner (Krivan *et al.*, 1986). The carbohydrate, α-Gal epitope (Galα1-3Galβ1-4GlcNAc) has been shown to bind TcdA, however, this structure is absent in human intestinal receptors (Krivan *et al.*, 1986; Tucker and Wilkins, 1991).

Thus, 3Galβ1-4GlcNAc, which is present in humans is proposed as the receptor for TcdA (Krivan *et al.*, 1986; Tucker and Wilkins, 1991; Jank and Aktories, 2008; Olling *et al.*, 2011). Additionally TcdA has been reported to bind the human blood antigens I (Galb1-14GlcNAcb1-3Galb1-4(Glc)), X (Galb1-4[Fua1–3]GlcNAc) and Y (Galb1-4GlcNAcb1) (Tucker and Wilkins, 1991), human glycosphingolipid (Teneberg *et al.*, 1996) and human gp96 and sucrose-isomaltase (Na *et al.*, 2008).

The receptor for toxin B is yet to be characterised. Its ability to bind to a wide range of cells indicates that the toxin B receptor is ubiquitous. Toxin B's ability to bind to cell lines in a saturated manner and bind more efficiently to some cell lines than toxin A, suggests that the two toxins recognise different receptors (Chaves-Olarte *et al.*, 1997). Studies have also indicated that the toxin B receptor is located on the basolateral side, whilst the toxin A receptor is present on the apical side (Jank *et al.*, 2007). Dingle *et al.*, (2008) assessed the cell binding properties of the repeat regions in TcdA and TcdB, revealing both toxins can withstand structural variations in the carbohydrate binding sites. Microarray screening of fragment TcdA-A2 (amino acid residues 2456-2710) and TcdB-B1 (amino acid residues 2248-2367) against 350 carbohydrate sequences identified a second receptor for TcdA-A2 (Le^a-LacNAc and Sia-Le^a-Le^x); surprisingly no receptor was found for TcdB-B1. Studies have also suggested domains other than the C-terminus of TcdA and TcdB may be important for cell surface binding (Dingle *et al.*, 2008; Gerhard *et al.*, 2013). This notion is supported by the generation of TcdA and TcdB mutants which are devoid of the CROPSs but still retain cytopathicity (Barroso *et al.*, 1994; Genisyuerek *et al.*, 2011; Olling *et al.*, 2011).

1.2.6.2 Endocytosis and Translocation

Upon binding to the host cell receptor(s) the toxins undergo clathrin-mediated endocytosis (Papatheodorou *et al.*, 2010) (Figure 1.5). The acidic nature of the endosome enables the toxin to undergo conformational changes, allowing it to interact with the endosomal membrane, enabling pore formation and the insertion of the hydrophobic toxin domain (Florin and Thelestam, 1986; Qa'Dan *et al.*, 2000; Barth *et al.*, 2001). In TcdB the minimum pore forming region resides between

amino acids 830-990, with the glutamate residues at 970 and 976 crucial for pore formation (Genisyuerek *et al.*, 2011). It is hypothesised that protonation of the acidic glutamate residues occurs at low pH, neutralising the amino acid and allowing for pore formation. Although not conserved in LCTs the presence of other acidic amino acids at these positions suggest a similar mechanism may exist in other LCTs. Differences have been observed in the processing of the *C. difficile* toxins, with TcdA, unlike TcdB, requiring cholesterol for pore-formation (Giesemann *et al.*, 2006). Furthermore, toxins from different strains of *C. difficile* display different processing mechanism, for example TcdB of R20291 (PCR RT027) has been shown to enter cells more quickly, undergoes acid induced conformational changes at a higher pH and is more efficiently auto-processed than TcdB from the prototypical 630 strain (Lanis *et al.*, 2010; Lanis *et al.*, 2012).

1.2.6.3 Autoproteolysis

In the case of toxin B the enzymatic toxic domain is detectable in the cytosol whereas the translocation and binding domains remain in the endosome (Rupnik *et al.*, 2005). Hence, an important step of internalisation is proteolytic cleavage, resulting in the translocation of the enzymatic domain (Pfeifer *et al.*, 2003). The cleavage site for toxin B lies between Leu 543 and Gly 544 (Rupnik *et al.*, 2005). The host cytosolic molecule, inositol hexakisphosphate ($InsP_6$) induces autocatalytic cleavage and is essential for the biological activity of both toxins (Reineke *et al.*, 2007; Egerer *et al.*, 2009), although TcdB is more susceptible to $InsP_6$-induced cleavage than TcdA (Kreimeyer *et al.*, 2011).

Two protease motifs have been identified in toxin B; aspartate protease (D*X*G) and cysteine protease (CPD) (Figure 1.4). The *DXG* motif in toxin B resides at aspartate 1665 and has been identified as the site for protease activity (Reineke *et al.*, 2007). This motif has also been identified in TcdA and TcsL but not in TcnA, even though all the LCTs undergo $InsP_6$-induced auto-catalytic cleavage. Although processing of toxin B can be inhibited with an aspartate inhibitor (Reineke *et al.*, 2007), the D*X*G motif is located nearer to the C-terminal domain (Figure 1.4). This suggests the protease remains in the endosome as opposed to being translocated into the cytosol with the enzymatic region. A second motif that has been identified is a cysteine

protease domain (CPD) (Egerer *et al.*, 2007). The CPD domain has been identified in the RTX (repeats-in-toxin) toxin of *Vibrio cholerae* and is also present in all the LCTs. It is located between the enzymatic and translocation domains (Figure 1.4) and is activated by $InsP_6$ facilitating the release of the enzymatic domain into the target cell. CPD is recognised as essential for toxin processing and represents a target for the development of toxin inhibitors (Puri *et al.*, 2010).

1.2.6.4 Enzymatic Activity

Like all LCTs, toxins A and B are glucosyltransferases which catalyse the transfer of a glucose moiety to Rho and Ras-GTPases (Table 1.1 and Figure 1.5B). GTPases function as molecular switches in a wide range of signalling pathways and are present in all eukaryotic cells (Pruitt and Lacy, 2012). In the cytosol, the glucosyltransferase effector domain of the toxins utilises cellular UDP-glucose to monoglucosylate RhoA, Rac1, and CDC42 (Just *et al.*, 1995a; Just *et al.*, 1995b; Just and Gerhard, 2004). In the inactive, GDP-bound form, the GTPases associate with the guanine nucleotide dissociation inhibitor (GDI) and remain in the cytosol. Activation of GTPases by guanine nucleotide exchange factors (GEF) results in the regulation of a range of host cell functions, including cell proliferation, differentiation and organisation of the actin cytoskeleton (Barth and Aktories, 2005). Hence, the wide ranging impact on downstream signalling and regulatory pathways, following inactivation. Analysis of the structure of the glucosyltransferase domain of TcdA and TcdB has revealed differences in GTPase binding surface and hence substrate specificities (Reinert *et al.*, 2005; Pruitt *et al.*, 2012) of the two toxins. Figure 1.5 summarises the processing of toxins A and B.

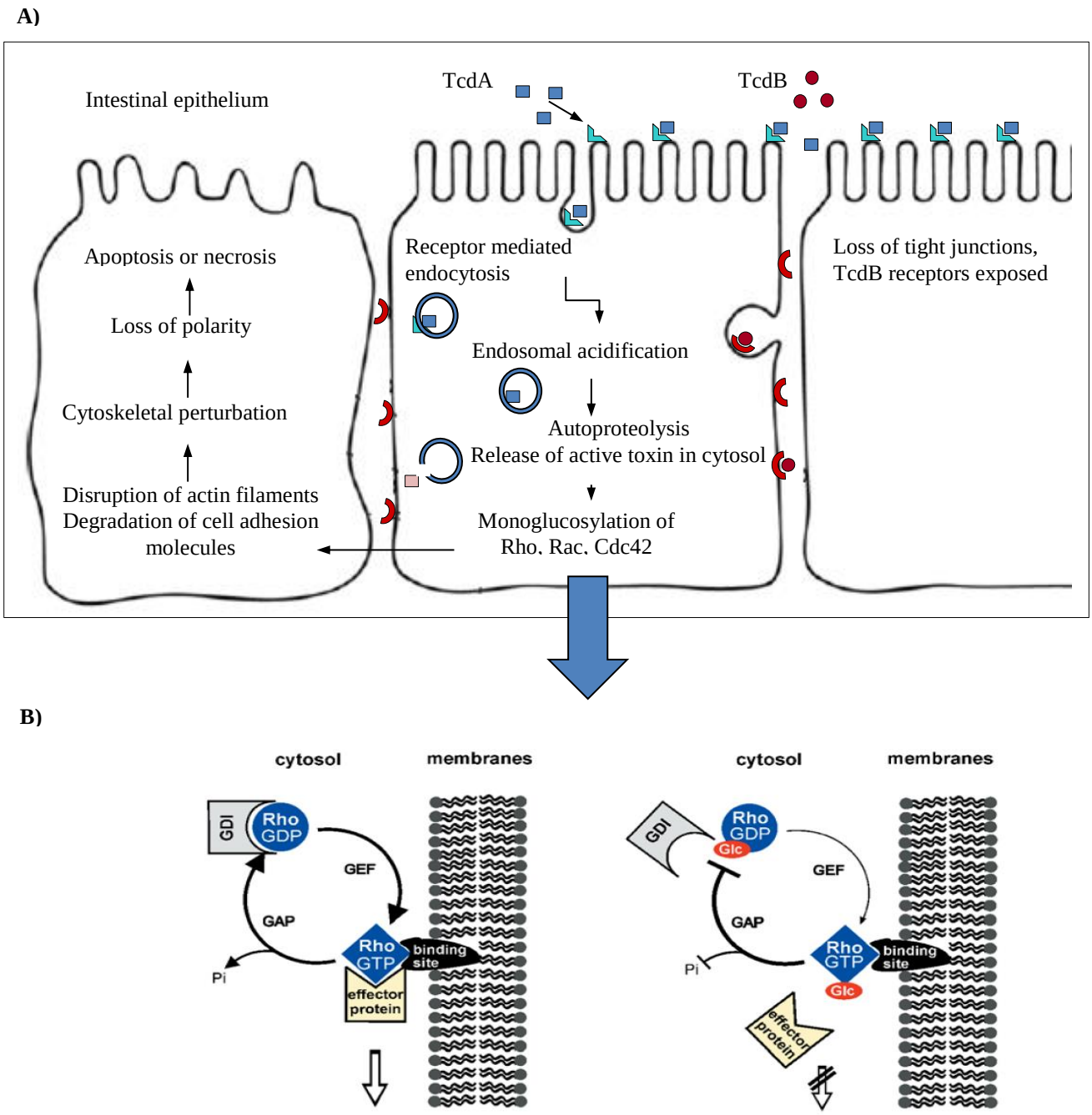

Figure 1.5: Diagrammatic representation of pathogenesis of CDI.

A) Toxin A and B processing. Receptors of TcdA and TcdB are yet to be defined. TcdA receptors are located on the apical surface, the TcdB receptors are hypothesised to be located on the basolateral surface, which are exposed following intestinal damage. **B)** Toxin-mediated monoglucosylation of GTPases. GDI; guanine nucleotide dissociation inhibitor, GAP; GTPase-activating protein, GEF; guanine nucleotide exchange factor, Glc; glucose. Adapted from Keel and Songer (2006) and Just and Gerhard (2004).

1.3 Treatment and Therapy

1.3.1 Antibiotics

In susceptible individuals, CDI develops post-antibiotic treatment as the protective gut flora is compromised. Initial steps in treatment focus on stopping the offending antibiotic. Almost all antibiotics, including metronidazole and vancomycin have been associated with CDI (Saginur *et al.*, 1980; Hecht and Olinger, 1989). These two antibiotics have been the main stay for CDI treatment for over three decades, despite a relapse rate of 15-20%.

1.3.1.1 Vancomycin

Vancomycin binds with high affinity to the d-Ala-d-Ala moiety of the C-terminus of the MurNAc-pentapeptide, thus blocking the addition of muropeptide precursors in the periplasm by transglycosylation and transpeptidation (Courvalin, 2006; Hakenbeck *et al.*, 2010). *C. difficile* is an intraluminal pathogen entirely contained in the colon lumen, hence vancomycin, which is concentrated in the colon is pharmacologically ideal (Bartlett, 2006, 2008) demonstrating a 84-94% cure rate (Tart, 2013). When administered orally vancomycin is not metabolised and has limited absorption, thus reaching levels which are several-fold higher than the minimum inhibitory concentration (MIC) recorded for *C. difficile*. The recommended dosage of oral vancomycin is 125 mg four times daily. Due to its potent antimicrobial activity, the recovery of the indigenous microflora is delayed resulting in a 17% recurrence rate (Louie *et al.*, 2011; Tart, 2013). In an attempt to curb the emergence of vancomycin-resistance Enterococci (VRE), the use of vancomycin in CDI is limited to incidences of treatment failure or severe disease (Tart, 2013).

1.3.1.2 Metronidazole

The recommended metronidazole dose is 500 mg three times daily or 250 mg four times daily for 10 to 14 days. In contrast to vancomycin, metronidazole is highly absorbed from the colon resulting in modest faecal concentration. It is currently unclear why there are an increasing number of treatment failures (22%) and

recurrences (28%) (Musher *et al.*, 2005; Pépin *et al.*, 2005) following metronidazole, given that clinically relevant metronidazole resistant isolates are yet to emerge. Although it should be noted that *in vitro* 3-6% of isolates were found to be metronidazole resistant (Barbut *et al.*, 1999; Pelaez *et al.*, 2008). Whether this is due to the presence of a sub-population of cells with increased resistance is yet to be determined (Moura *et al.*, 2013b). Further to this, two (Louie *et al.*, 2007; Zar *et al.*, 2007) out of three (Bouza *et al.*, 2008) trials have shown metronidazole to be inferior to vancomycin for the treatment of severe CDI. In spite of this metronidazole remains the preferred drug for mild and moderate CDI primarily due to its reduced cost.

1.3.1.3 Fidaxomicin

Fidaxomicin (FDX) administered 200 mg orally twice daily for 10 days, has been shown to be superior to vancomycin in providing sustained clinical response (response without recurrence of disease). The drug inhibits the activity of bacterial RNA polymerase, has a narrow-spectrum of activity against Gram-positive bacteria (Finegold *et al.*, 2004; Hecht *et al.*, 2007), is minimally absorbed from the intestinal tract (Hecht *et al.*, 2007) and inhibits sporulation (Babakhani *et al.*, 2012), thus making it an ideal antibacterial agent for CDI (Ackermann *et al.*, 2004a; Credito and Appelbaum, 2004; Finegold *et al.*, 2004; Hecht *et al.*, 2007; Karlowsky *et al.*, 2008; Shue *et al.*, 2008; Miller, 2010; Louie *et al.*, 2011; Babakhani *et al.*, 2012).

The use of antibiotics to treat CDI appears to be contradictory; by their very nature antibiotics will hinder the re-establishment of the protective microflora and have been implicated in triggering a supershedder (10^8 CFU *C. difficile*/g faeces) state facilitating spore mediated transmission (Lawley *et al.*, 2009). Given that 25% of patients suffer recurrence following initial antimicrobial treatment and that 35-45% of patients with a first recurrence are likely to have a second recurrence, there is a need for new effective therapies (Kelly and LaMont, 2008).

1.3.2 Bacterial Agents

1.3.2.1 Probiotics

Probiotics are live non-pathogenic yeast and bacteria, that resemble the protective microflora of the gut; intended to restore the microbial balance (Pillai and Nelson, 2008). Given that CDI is attributed to the destruction of the protective gut flora, following antibiotic treatment, probiotics such as *Lactobacillus rhamnosus GG, Lactobacillus acidophilus, Lactobacillus plantarum, Bifidobacterium, Bacillus clausii, Sacchromyces boulardii and Sacchromyces cerevisiae* (Dendukuri *et al.*, 2005; McFarland, 2005, 2006; Miller, 2007; Pillai and Nelson, 2008) appear to be a valid and logical means of treatment. In a mouse model the use of a *Bacteroides/Lactobacillus* mixture representing the traditional probiotic bacterial groups failed to suppress the supershedder state and restore recipients' microbiota (Lawley *et al.*, 2009). To date the trials conducted with probiotics for the treatment of CDI have been in addition to standard antibiotic therapy; one trial has lacked controls in antibiotic dose, type and duration of probiotic (McFarland *et al.*, 1994) whilst a second trial has shown a beneficial effect of probiotics in patients treated with a high dose of oral vancomycin (2 g/day) but not in patients treated with metronidazole (1 g/day) or with a lower dose of oral vancomycin (500 mg/day) (Surawicz *et al.*, 2000). The potential benefit of probiotics in the treatment of CDI is inconclusive and requires further research (McFarland, 2009; Miller, 2009).

1.3.2.2 Faecal Infusion

As CDI manifests as a consequence of disruption to the normal intestinal microflora, restoration of said microflora has long been viewed as a viable option for treating CDI, particularly in the case of recurrent infection (Rohlke *et al.*, 2010; Russell *et al.*, 2010; Yoon and Brandt, 2010; Landy *et al.*, 2011; Rohlke and Stollman, 2012; Garborg *et al.*, 2010). As early as 1958, severe antibiotic-associated diarrhoea was treated with faecal infusions from healthy donors (Eiseman *et al.*, 1958). Infusions contain living protective bacteria which are infused into the bowel of the patient via colonoscopy, enema or through a nasogastric tube. This approach although 'unappealing' has been reported to have a 91–100% cure rate (van Nood *et al.*, 2009; Rohlke *et al.*, 2010; Yoon and Brandt, 2010). A small randomised study

investigating the effect of duodenal infusion of donor faeces in patients with recurrent CDI, concluded that infusion with donor faeces was significantly more effective than the use of vancomycin alone or vancomycin with bowel lavage, with cure rates of 81%, 31% and 23% respectively (van Nood *et al.*, 2013). Results from the two phase III randomized trials (FECAL trial and ClinicalTrials.gov, NCT01226992) currently underway, will shed more light on the validity of faecal therapy. Although promising, it is important to note the differences in stool dose (number of infusions and concentration), route of administration and definition of cure/success rate when comparing studies. Given the variability in the composition of donor faecal infusions coupled with the practicalities associated with preparing and screening donor samples, the use of a standardised, defined mixture of bacteria would be beneficial. Such an approach has been utilised in a murine model, in which a mixture of 6 defined bacteria was administered to mice infected with *C. difficile* PCR RT027, resulting in the displacement of the 027 strain and restoration of a healthy microbiota (Lawley *et al.*, 2012).

1.3.2.3 Non-toxigenic *Clostridium difficile* (NTCD)

The use of non-toxigenic *C. difficile* was demonstrated in the early 1980's (Wilson and Sheagren, 1983; Borriello and Barclay, 1985), when it was found to prevent ileocaecitis by toxigenic *C. difficile* in hamsters. A single dose of NTCD spores (10^5 CFU) from naturally colonized humans protected hamsters from challenge with toxigenic *C. difficile* strains (Sambol *et al.*, 2002; Merrigan *et al.*, 2003; Merrigan *et al.*, 2009). Phase I studies have shown NTCD spores to be safe and well tolerated resulting in colonisation of healthy volunteers pre-treated with vancomycin. NTCD spores are currently being studied in a phase II clinical trial to prevent recurrent CDI following treatment (Villano *et al.*, 2012). Recent hamster studies have demonstrated the protection afforded by the administration of NTCD varies between NTCD strains (Nagaro *et al.*, 2013). Furthermore it has recently been demonstrated that the PaLoc can be transferred to non-toxigenic *C. difficile*, resulting in the formation of a toxin producing strain (Brouwer *et al.*, 2013). Given that mixed infections of *C. difficile* occur (Eyre *et al.*, 2012; Eyre *et al.*, 2013), the presence of toxigenic and non-toxigenic *C. difficile* strains within a host could result in the transfer of virulence genes.

1.3.3 Toxin-Binding Agents

Toxin A and B are the causative agents for CDI, responsible for the pathological effects of infection, hence ion-exchange resins, oligosaccharides and various types of polymers which bind the toxins have been developed for treatment.

1.3.3.1 Ion-Exchange Resins

Cholestyramine is a bile acid sequestrant used to prevent diarrhoea in Crohn's disease; its ability to bind vancomycin (McFarland, 2005) and lack of clinical evidence supporting its use in CDI has limited it therapeutic applications (Weiss, 2009). Colestipol was shown to delay death in the hamster model, although a placebo-controlled, randomized trial found the resin to be no better than the placebo, with no difference in faecal excretion of the *C. difficile* toxins (Mogg *et al.*, 1982).

1.3.3.2 Oligosaccharides

SYNSORB is an inert support clay with a synthetic trisaccharide sequence (Galα1-3Galβ1–4GlcNAc) which binds toxin A (Heerze *et al.*, 1994; Castagliuolo *et al.*, 1996). In a placebo-controlled trial no significant difference in recurrence of CDI was observed with SYNSORB (McFarland, 2011). No further trials with SYNSORB were conducted and development was abandoned.

1.3.3.3 Polymers

Tolevamer (GT160-246) is an anionic binder of toxins A and B, it does not alter the gut flora, does not bind vancomycin, and will not result in the development of resistance (Weiss, 2009). An initial phase II trial showed tolevamer to be equivalent to vancomycin in cure and recurrence rates (Louie *et al.*, 2006). In the subsequent phase III trial only 47% of the tolevamer group had diarrhoea resolved by the end of treatment, compared to 81% in the vancomycin group. Based on this, development of tolevamer was terminated, despite only 3% of the tolevamer group suffering recurrence versus 23% and 27% in the vancomycin and metronidazole groups, respectively (Louie *et al.*, 2007; Bouza *et al.*, 2008).

1.3.4 Immunotherapy

Although the introduction of antimicrobials saw an end to serum therapy, antibody-based therapies still provide a viable option for the treatment of infectious diseases. Historically, antibodies target microbial antigens (SLP, polysaccharides) or microbial products (toxins) and have been utilised against a number human of infectious diseases, including; *Bacillus anthracis*, *C. tetani*, *C. botulinum*, *Mycobacterium tuberculosis* and *Streptococcus pneumonia* (Casadevall and Pirofski, 2004). Currently, approved therapeutic antibodies are used for the treatment of cancer and autoimmune disorders; antibodies targeting virulence factors of infectious agents are undergoing clinical trials. The presence of detectable serum IgG and IgA to toxins A and B in 60% of the healthy adult population (Viscidi *et al.*, 1983) has prompted research in the therapeutic activity of Intravenous Immunoglobulin (IVIg) preparations and orally administered anti-*C. difficile* antibodies and vaccines.

1.3.4.1 Intravenous Immunoglobulin (IVIg)

IVIg preparation of pooled human donor serum containing high titres of IgG antitoxin A and B have been administered to patients with severe and recurrent episodes of CDI (Kelly and Kyne, 2011). Thus far, studies have reported conflicting results, with Juang *et al.*, (2007) reporting no beneficial response, Wilcox (2004), O'Horo and Safdar, (2009) and Abougergi *et al.*, (2010), reporting variability between patients and extent of CDI. Whilst Leung *et al.*, (1991), Salcedo *et al.*, (1997) and McPherson *et al.*, (2006) have reported successful treatment with IVIg in children and adults with severe or relapsing CDI. The contradictory results of published studies is attributed to a four-fold variability in antitoxin A and B antibody titres in commercially available IVIg preparations (Grossman *et al.*, 2010). Standardisation of the IVIg preparation and a randomized controlled clinical trial to assess the effectiveness of IVIg for CDI, would determine its validity as a therapeutic for CDI.

1.3.4.2 Anti-*Clostridium difficile* Bovine Immunoglobulin Concentrate (BIC)

Bovine colostrum is a rich source of antibodies composed of IgG (approximately 92%), IgA (7.5%) and IgM (0.5%) (Kelly *et al.*, 1996), BIC is prepared from cows immunized with toxoided *C. difficile* culture filtrate. The oral administration of hyper-immune bovine IgG conferred protection against CDI (Lyerly *et al.*, 1991), blocked toxin A-receptor binding and neutralised the cytotoxicity of toxins A and B in animal models (Kelly *et al.*, 1996). The preparation was also found to survive passage through the human intestinal tract (Kelly *et al.*, 1997; Warny *et al.*, 1999). Human clinical trials are yet to be conducted.

1.3.4.3 Anti-*Clostridium difficile* Bovine Whey Protein

Whey (mature cow milk) is composed of anti-*C. difficile* bovine immunoglobulin secretory IgA, developed from cows immunized with formaldehyde-inactivated whole *C. difficile* cells and toxoid prepared from the *C. difficile* culture filtrate (van Dissel *et al.*, 2005). The preparation (MucoMilk) was found to be safe, well tolerated (van Dissel *et al.*, 2005) and as effective as metronidazole in reducing recurrent CDI in uncontrolled trials (Numan *et al.*, 2007; Mattila *et al.*, 2008). A phase III trial has since been discontinued due to a lack of funding.

1.3.4.4 Monoclonal Antibodies

At present two human monoclonal antibodies directed against toxins A and B have been developed, CDA-1 is directed against toxin A and CDB-1 (formerly MDX-1388) is directed against toxin B (Babcock *et al.*, 2006; Leav *et al.*, 2010; Lowy *et al.*, 2010). A phase II trial revealed a lack of efficacy when CDA-1 alone was administered to CDI patients (Leav *et al.*, 2010). The administration of both CDA-1 and CDB-1, resulted in a reduction in recurrence as compared to standard antibiotic therapy, however a reduction in the severity and duration of CDI was not observed (Lowy *et al.*, 2010). The clinical development of this product has subsequently been put on hold. Despite the cost associated with monoclonal antibodies and the likelihood that protection may be transient, they still represent a

valid therapeutic option for the treatment of recurrent CDI. The above studies emphasise the role of antitoxin antibodies in CDI and lend support to their application as a prophylactic for high risk patients (McFarland, 2005; Kelly and Kyne, 2011).

1.3.4.5 Vaccines

Current research in potential vaccine targets has focused on toxins A and B the S-layer proteins and cell surface polysaccharides, PSI and PSII. In the case of the S-layer proteins, PSI and PSII, research is still in the early stages, in contrast vaccines targeting the *C. difficile* toxins have been in development for the last decade (Torres *et al.*, 1995; Giannasca *et al.*, 1999). Although protection in hamsters has been demonstrated there has been limited studies completed in humans. Hamsters have been found to be protected when administered with either a formalin-inactivated *C. difficile* culture filtrate or toxoid, by nasal, peritoneal, subcutaneous, (Torres *et al.*, 1995) intramuscular, rectal (Giannasca *et al.*, 1999) routes, and intraperitoneal injection (Siddiqui *et al.*, 2012). In mice, transcutaneous immunization with toxoid A induced both IgG and IgA serum antibodies, although the mechanism by which this is achieved is unknown (Ghose *et al.*, 2007). The use of an injectable toxoid vaccine in patients has shown promise in the three patients vaccinated (Sougioultzis *et al.*, 2005). The parenteral administration of a toxoid A and B vaccine, in healthy volunteers has been reported to be safe and immunogenic, capable of eliciting a systematic antibody response relating to increase in serum antitoxin A and B antibodies (Kotloff *et al.*, 2001; Aboudola *et al.*, 2003).

Thus far studies have focussed on either formalin-inactivated culture filtrate or toxoided A and B. Other vaccine approaches include, a DNA vaccine expressing the C-terminal of toxin A (Gardiner *et al.*, 2009) and N-terminal of toxin B (Jin *et al.*, 2013), the oral administration of *Bacillus subtilis* spores expressing a single toxin A C-terminal domain (Permpoonpattana *et al.*, 2011), an injectable fusion protein consisting of the toxin A and B C-terminal domains (Tian *et al.*, 2012), a chimeric protein composed of the C-terminal domain of toxin A and the enzymatic and translocation domains of toxin B (Wang *et al.*, 2012).

Although a vaccine approach appears viable for CDI and is calculated to be cost effective (Lee *et al.*, 2010a), pertinent questions include; the choice of antigen (toxin vs. non-toxin), toxoid vs. recombinant, toxin A vs. toxin B, the route of administration, type of antibody response (mucosal IgA vs. systemic IgG), use of adjuvants, who and when to vaccinate, ability to confer long term protection and efficacy in the at risk population (Gerding, 2012).

1.4 Aims and Objectives

The aims of this research are as follows;

1. Clone and express regions of *C. difficile* toxins A and B in an *E. coli* based expression system.
2. Characterise the immunogenicity of native, toxoided and recombinant toxin A and B using animal and human immune sera, with a view to identify potential targets for therapeutic development.
3. Characterise the variability of vegetative *C. difficile* colonies.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Materials and Methods

Unless otherwise stated all reagents were of molecular biology grade and purchased from Fisher Scientific, UK or Sigma-Aldrich, UK.

2.2 Protein

2.2.1 Protein Precipitation

Prior to protein precipitation 1 M sodium hydroxide (NaOH) was added to the protein samples. Proteins were precipitated by the addition of equal volumes of 10% (w/v) trichloroacetic acid (TCA), incubated on ice for 1 hour and centrifuged at 14,000 *g* for 5 minutes at 4°C (Avanti, JA-14, Beckman Coulter, Inc.). The pellet was washed twice with ice cold acetone and centrifuged as above. The pellet was air dried at 37°C for approximately 5 minutes and re-suspended in collection buffer (8 M urea, 40 mM tris-base, 0.5% (v/v) ampholytes, 1% (w/v) ASB-14) overnight at 4°C.

2.2.2. Protein Quantification – Bicinchoninic Acid Assay (BCA)

A series of bovine serum albumin standards ranging from 0.05-1.0 mg/mL were prepared in either collection buffer or PBS, where required unknown samples were diluted 1/10. All samples were prepared in triplicate. To prepare a BCA working solution 50 parts BCA solution was added to 1 part 4% (w/v) copper sulfate. To each standard or protein sample, 1 mL of BCA working solution was added and the mixture vortexed. Following incubation at 37°C for 30 minutes, the samples were placed on ice and absorbance was read at OD_{562} (Ultraspec 3100*pro*, GE Healthcare). A standard curve was constructed by plotting the mean absorbance at OD_{562} of the standards against the concentration. The final concentration of the sample was corrected for the dilution factor.

2.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For 1D SDS-PAGE, protein samples were analysed using either 4-20% Criterion™ TGX™ Gel, mini-Protean TGX gels, or 5% Criterion™ TGX™ Gel (Bio-Rad, UK). Unless otherwise stated samples were combined with 2x SDS-PAGE buffer (0.09 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS, 0.02% (w/v) bromophenol blue, 0.1 M DTT) and boiled for approximately 7 minutes at 95°C (Techne Dri-Block® DB-3D, Fisher Scientific, UK). For 2D SDS-PAGE samples were analysed using 4-20%, 11 cm IPG Criterion™ TGX™ Gel (Bio-Rad, UK). Electrophoresis was carried out in 1x running buffer (per litre diH_2O, 3.03 g tris base, 14.4 g glycine, 1.0 g SDS) at 150 volts, 30 watts, 400 mA in a mini-Protean Tetra tank or a Criterion™ Cell (Bio-Rad, UK) powered by a GE Healthcare EPS-601 power supply for approximately 1.5 hours. Gels were fixed and double-stained with colloidal Coomassie and silver (Section 2.5).

2.4 2-Dimensional Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (2D SDS-PAGE)

2.4.1 Reduction and Alkylation

Protein samples were reduced and alkylated according to the protocol for the ProteoPrep Reduction and Alkylation Kit (Sigma-Aldrich, UK). The protein sample (pH >7.5) was reduced by adding 2.5 µL tributyl phosphine (TBP) solution per 100 µL of sample and incubated at room temperature for 30 minutes. The sample was alkylated in 3.0 µL of iodiacetamide solution per 100 µL sample and incubated for 1 hour at room temperature. TBP (2.5 µL per 100 µL of sample) was added to the sample and incubated for 15 minutes. The sample was centrifuged at 20,000 *g* for 5 minutes at 20°C (Eppendorf 5417R, UK).

2.4.2 First Dimension Isoelectric Focusing

For cell surface proteome analysis 100 µg of bacterial cell extract was combined with isoelectric focusing buffer (IEF) (7 M urea, 2 M, thio urea, 40 mM tris-base,

0.5% (v/v) ampholytes, 1% (w/v) ASB-14, 0.0002% (w/v) bromophenol blue) in a final volume of 200 µL. For toxin digest analysis 10 µg of protein was combined with 200 µL IEF. ReadyStrip IPG Strips, pH 3–10 nonlinear, 11 cm (Bio-Rad, UK), were rehydrated overnight in a rehydration tray at room temperature overlaid with 2-3 mL mineral oil per strip. IEF was carried out in the Amersham (GE Healthcare) IPGphor 2 at 8,000 volts peak voltage, 50 mA peak current for 30,000 volt-hours.

2.4.3 Second Dimension

Focused strips were equilibrated in buffer (0.375 M Tris-HCl pH 8.8, 6 M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 0.0002% (w/v) bromophenol blue) on a Stuart gyro-rocker (Fisher Scientific, UK) rocker for 20 minutes at 30 r.p.m. The strips were dipped in 1x running buffer overlaid with agarose (125 mg agarose in 25 mL 1x running buffer containing 0.0002% (w/v) bromophenol blue) and electrophoresis completed as above (Section 2.3).

2.5 Staining

2.5.1 Colloidal Coomassie

All solutions were prepared just prior to use and all incubations were carried out on a Stuart gyro-rocker (Fisher Scientific, UK) at 30 r.p.m at room temperature. Following SDS-PAGE, gels were washed in diH_2O for 2 x 5 minutes. Gels were fixed in 100 mL of fixing solution (20% (w/v) trichloroacetic acid (w/v)) overnight; post fixation gels were washed in diH_2O for 3 x 5 minutes. Brilliant Blue G-Colloidal Concentrate (Sigma-Aldrich, UK) was dissolved in 800 mL diH_2O to give a 1x working solution and stored at 4°C. Prior to staining 4 parts working solution was combined with 1 part methanol. Staining was carried out for 24 hours and de-stained with 25% (v/v) methanol overnight at room temperature with constant rocking.

2.5.2 Silver Staining

Following imaging of Coomassie stained gels, gels were silver stained, all solutions were prepared just prior to use. Incubations were carried out at room temperature following the method outlined by Dunn, (2002). The gel was rehydrated by washing in diH_2O for 2 x 20 minutes and soaked in 10% (v/v) glutaraldehyde solution for 30 minutes followed by 3 x 20 minutes washes in diH_2O. Staining was carried out using silver diamine solution, which comprised the following, 73.6 mL diH_2O, 21 mL, 0.36% (w/v) NaOH and 1.4 mL, 35% (v/v) ammonia were mixed, to which 4 mL 20% (w/v) silver nitrate was added drop wise with constant stirring. Gels were stained for 30 minutes followed by 3 x 5 minutes diH_2O washes. The developing solution (2.5 mL, 1% (w/v) citric acid, 0.26 mL, 36% (v/v) formaldehyde in 500 mL diH_2O) was added for 7-10 minutes and terminated by immersing the gel in stopping solution (40% (v/v) ethanol, 10% (v/v) acetic acid).

2.6 Semi-Dry Transfer

Gels were transferred onto nitrocellulose membranes using a Trans-Blot Turbo Midi Nitrocellulose Transfer Packs and a Bio-Rad Trans-Blot® Turbo™ (Bio-Rad, UK). The transfer sandwich was assembled as follows; filter paper, membrane, gel, and filter paper. A blot roller was used to remove air bubbles between the assembled layers. Transfer conditions were 25 volts, 1.0 A for 30 minutes.

2.7 Ferrozine/Ferrocyanide Stain

Ferrozine/Ferrocyanide staining was completed following the method of Patton *et al.*, (1994). Wet membranes were equilibrated for 15 minutes in 2% (v/v) glacial acetic acid at room temperature on a Stuart gyro-rocker. The blot was incubated for 15 minutes in 0.1% (w/v) Poly (vinylpyridine) in 2% (v/v) acetic acid followed by rinsing for 5 minutes in 2% (v/v) acetic acid. Blots were stained with ferrozine stain (0.75 mM ferrozine, 30 mM iron (III) chloride, 5 mM thioglycolic acid in 2% (v/v) acetic acid) for 30 minutes and rinsed with ferrozine stain base (2% (v/v) acetic acid) 5 times for 30 seconds with hand agitation. To achieve greater stain sensitivity, blots were equilibrated in ferricyanide stain base (100 mM sodium acetate, pH 4.0) for

15 minutes at room temperature on a rocker. Followed by staining with ferricyanide stain (100 mM potassium ferrocyanide, 60 mM iron (III) chloride) for 30 minutes and rinsing with ferricyanide stain base 5 times for 30 seconds. The blot was allowed to dry at room temperature for 1 hour. Membranes were de-stained with ferricyanide elution solution (200 mM sodium carbonate, 100 mM ethylenediaminetetraacetic acid, pH 9.6) for 10 minutes.

2.8 Western Blotting

All steps were carried out on a roller mixer (Stuart SR6) at 33 r.p.m., all wash steps were performed in PBST (PBS + 0.1% (v/v) Tween® 20) four times for 5 minutes. Primary and secondary antibodies were diluted in 1% (w/v) dry skimmed milk (Marvel) in PBST. Membranes were washed for 5 minutes with diH_2O, followed by PBST. Membranes were blocked in 5% (w/v) dry skimmed milk in PBST for 1 hour at room temperature and washed in PBST. Membranes were incubated with the primary antibody overnight at 4°C. Following washing the secondary antibody was applied for 1 hour at room temperature and washed.

2.9 Image Capture and Chemiluminescence

Visibly stained gels and membranes were imaged with the Chemi Doc xrs+ system, faint band exposure and analysed using Image Lab™ Software, v.3.0, with a band detection sensitivity of 100. Gels were imaged under white light trans illumination.

All membranes were imaged with SuperSignal West Pico, Dura and Femto; solutions were prepared as per the manufacturer's instructions (Thermo Scientific Pierce, UK). The working solution contained a 1:1 ratio of luminol/enhancer solution and peroxide solution sufficient to cover the membrane. Membranes were incubated for 5 minutes and imaged with a Chemi Doc xrs+ system (Bio-Rad, UK). Membranes were thoroughly washed with PBST prior to the addition of the next detector solution.

2.10. Mass Spectrometry

Mass Spectrometry was performed by Dr. Ian Brewis (CBS Proteomics Facility, Cardiff University) using the method detailed below.

2.10.1 Trypsin Digestion

Spots were manually excised from gels generating 1.5 mm gel plugs. Trypsin digestion was completed by a modified Shevchenko *et al.*, (1996) method. Gel plugs were incubated at 37ºC for 3 hours with 6.25 ng/µL sequencing grade modified trypsin (Promega, UK) in 25 mM ammonium bicarbonate (NH_4HCO_3). Dried peptides were re-suspended in 50% (v/v) acetonitrile in 5 µL of 0.1% (v/v) trifluoroacetic acid for mass spectrometry analysis. An aliquot (0.5 µL) was spotted onto a 384 well MS plate. The samples were allowed to dry and overlaid with α-cyano-4-hydroxycinnamic acid (CHCA, Sigma-Aldrich, UK; 0.5 µL prepared by mixing 5 mg matrix with 1 mL of 50% (v/v) acetonitrile in 0.1% (v/v) TFA).

2.10.2 MALDI TOF Analysis of Trypsin Digested Proteins

Mass spectrometry was performed using a MALDI TOF/TOF mass spectrometer (Applied Biosystems 4800 MALDI TOF/TOF Analyzer; Foster City, CA, USA) with a 200 Hz solid state laser operating at a wavelength of 355 nm (Medzihradszky *et al.*, 2000; Bienvenut *et al.*, 2002; Glückmann *et al.*, 2007; Brennan *et al.*, 2009). MALDI mass spectra and subsequent MS/MS spectra of the 8 most abundant MALDI peaks were obtained following routine calibration. Common trypsin autolysis peaks and matrix ion signals and precursors within 300 resolution of each other were excluded from the selection and the peaks were analysed with the strongest peak first. For positive-ion reflector mode spectra 800 laser shots were averaged (mass range 700-4000 Da; focus mass 2000). In MS/MS positive ion mode 4000 spectra were averaged with 1 kV collision energy (collision gas was air at a pressure of 1.6 x 10-6 Torr) and default calibration.

Combined PMF and MS/MS queries were performed using the MASCOT Database search engine v.2.1 (Matrix Science Ltd, London, UK) (Perkins *et al.*, 1999) embedded into Global Proteome Server (GPS) Explorer software v.3.6 (Applied Biosystems) on the Swiss-Prot database. Searches were restricted to the bacteria domain with trypsin specificity (one missed cleavage allowed), the tolerances set for peptide identification searches at 50 ppm for MS and 0.3 Da for MS/MS. Cysteine modification by iodoacetamide was employed as a fixed modification with methionine oxidation as a variable modification. Search results were evaluated by manual inspection and conclusive identification confirmed if there was high quality tandem MS (good y-ion) data for ≥2 peptides (E value $p < 0.05$ for each peptide; overall $p < 0.0025$) or one peptide (only if E value was $p < 0.0001$).

CHAPTER THREE

PRODUCTION OF RECOMBINANT TOXIN A ($_{R}$TcdA$_{900\text{-}2710}$) AND TOXIN B ($_{R}$TcdB$_{547\text{-}2366}$)

3.1 Introduction

Although the causative role of toxins A and B in CDI has been firmly established, there is much debate as to the contribution of each toxin in pathogenesis and which of the toxins is a primary virulence factor (Lyras *et al.*, 2009; Kuehne *et al.*, 2010). The ability to access and work with pure preparations of each toxin is a key requirement for any researchers attempting to develop new therapeutic agents and diagnostic tools.

The isolation of native *C. difficile* toxins from the culture supernatant of the pathogen has been achieved, but to date this approach has necessitated the use of complex, multi-step processes. One such approach, thyroglobulin affinity chromatography has been successfully used to recover 0.07 mg/mL of pure (single band on an SDS-PAGE gel) toxin A (Krivan and Wilkins, 1987). While yields of toxin B have been as much as tenfold higher (0.765 mg/mL), using ammonium sulfate precipitation, the resulting preparation is frequently associated with multiple contaminated proteins (Pothoulakis *et al.*, 1986). While approaches such as Fast Performance Liquid Chromatography (FPLC), can produce purer proteins of higher yields (0.574 and 0.940 mg/mL for toxin A and B, respectively) (von Eichel-Streiber *et al.*, 1987), there is a requirement for specialist equipment, technical expertise and a high starting culture volume (750 mL). Furthermore any method pertaining to the natural production of toxin A and toxin B in *C. difficile*, will require the decontamination of heat-resistant spores (Donald *et al.*, 2013).

Due to the problems faced in recovering native toxin from *C. difficile*, alternative hosts from which to produce recombinant toxin have been investigated. An approach which has had some success is the use of members of the *Bacillus* spp., such as *Bacillus megaterium*. Burger *et al.* (2003) successfully expressed *tcdA* from a *B. megaterium* system and were able to achieve a yield between 300-500 µg/L of culture. Although the presence of multiple bands in the purified preparation maybe indicative of extensive degradation (as determined by Western blot analysis). More recently, Yang *et al.*,(2008) were able to recover between 5-10 mg/L of recombinant toxin A and B from *B. megatarium* at a purity of 70-80%, as determined by Coomassie

stained SDS-PAGE gels. Purity was further enhanced to >80% using a thyroglobulin column and anion-exchange fractionation.

In this chapter the potential to use *Escherichia coli* as an easy to use recombinant protein expression system was assessed. This Gram-negative bacterium is the most commonly used system for the high level production of heterologous proteins (Makrides, 1996; Terpe, 2006; Fernández and Vega, In Press). The ability of the bacterium to grow rapidly to high cell densities, in inexpensive media coupled with the ease of purification with affinity columns (e.g. Ni-NTA, Glutathione-Sepharose) has added to the popularity of this expression host. Furthermore the availability of the complete genomic sequence and ability to safely produce recombinant protein by large-scale fermentation has insured *E. coli* remains a popular expression host (Rai and Padh, 2001; Terpe, 2006).

Previous attempts to develop *E. coli* based expression systems for these toxins have been hampered by their size, with the gene encoding *tcdA* comprising 8133 nucleotides while that encoding *tcdB* comprising 7098 nucleotides (Dove *et al.*, 1990; Sauerborn and von Eichel-Streiber, 1990; von Eichel-Streiber *et al.*, 1992a). As a consequence, the cloning of each gene required a strategy in which individual fragments of the sequence were inserted into the vector in a step-wise manner which resulted in the eventual reconstruction of the entire gene (Johnson *et al.*, 1990; Phelps *et al.*, 1991). While effective such approaches are time consuming and with the multiple restrictions, ligations and amplification steps can result in sequence errors. More recent attempts to clone full length *tcdB* and *tcdA* using high fidelity Taq polymerase based approaches have proved to be equally problematic. Using the EXPAND™ Long Template PCR cloning system Tang-Feldman *et al.* (2002) observed low cloning efficiency (3/120 clones analyzed), with the positive clones demonstrating variable cytotoxic activity. In a subsequent study using the same approach Ackermann and colleagues (2004b) found that only 50% of their clones showed cytotoxicity (10 clones screened) and that in the majority of cases the insert was lost upon subculture, thus highlighting the unstable nature of *tcdA*.

Indeed attempts to clone and express various regions of TcdA and TcdB in *E. coli* have encountered a range of problems; including the instability of the gene insert, poor protein expression, purification and yield (Craggs, 1999; Zimmer *et al.*, 2002; Letourneur *et al.*, 2003; Castagliuolo *et al.*, 2004; Ho *et al.*, 2005). One possible explanation for these problems is the pronounced difference in the codon usage of the donor gene and that of the host. While *C. difficile* has a genome with a low GC content (30%) (Sebaihia *et al.*, 2006), the GC content of *E. coli* is approximately 50% (Riley *et al.*, 2006).

The genetic code employs 61 codons to represent 20 amino acids and three codons for translation termination (Osawa *et al.*, 1992). Amino acids can be encoded by multiple codons with alternate codons referred to as synonyms or synonymous (Gustafsson *et al.*, 2004). Synonymous codons frequently vary at the third codon position by one nucleotide. A subset of the cell's tRNA is able to recognise each of these codons. This redundancy permits the organism to develop its own codon usage preference which is mirrored by the makeup of the tRNA pool (Osawa *et al.*, 1992; Peden, 1999; Gustafsson *et al.*, 2004). It is suggested this bias allows for improved translational efficiency/accuracy, particularly in the context of highly-expressed genes (Rocha, 2004; Shao *et al.*, 2012). Consequently codon usage plays an important role in determining the level of gene expression, particularly when the gene in question has been derived from a different organism (Kurland, 1987; Kudla *et al.*, 2009; Sharp *et al.*, 2010). In situations where the codon usage of the donor gene differs markedly from that of the expression host it may be necessary to modify either the composition of the host's tRNA pool or the codon usage of the donor gene sequence.

Genetically engineered strains of *E. coli* which over-express rare tRNA's such as BL21-CodonPlus® (http://www.genomics.agilent.com/) and Rosetta™ (http://www.emdmillipore.com/) are commercially available and have been used to express problematic genes (Zdanovsky and Zdanovskaia, 2000; Lu *et al.*, 2009; Lin *et al.*, 2010). Alternatively altering the codon usage of the donor nucleotide sequence has enhanced the expression of clostridial toxin genes by 2-fold for the

tetanus toxin (Makoff *et al.*, 1989) and the botulinum neurotoxins (Zdanovsky and Zdanovskaia, 2000).

In this chapter, the steps taken to develop an *E. coli* based recombinant protein expression system capable of producing the non-toxic translocation and receptor binding domains of TcdA (amino acid residues 900-2710) and TcdB (amino acid residues 547-2366) are described. The receptor binding domain has been shown to be the most antigenic, with toxin neutralising antibodies elicited against this region (Corthier *et al.*, 1991; Kink and Williams, 1998; Genth *et al.*, 2000). Identification of immunogenic regions, particularly within the receptor binding domain would aid in the development of therapeutics.

3.2 Aims and Objectives

The overall aim of this chapter is to express codon optimised regions of *C. difficile* toxin A ($TcdA_{900-2710}$) and toxin B ($TcdB_{547-2366}$) from an *E. coli* based expression system to support further studies.

The experimental objectives are to:

1. To design and construct an *E. coli* based protein expression system capable of expressing recombinant $TcdA_{900-2710}$ and recombinant $TcdB_{547-2366}$.
2. Express and purify sufficient quantities of $rTcdA_{900-2710}$ and $rTcdB_{547-2366}$ from *E. coli* to support future studies.

3.3 Materials and Methods

All plasticware was purchased sterile; DNase, RNase and pyrogen free (Fisher Scientific, UK). Unless otherwise stated all reagents were of molecular biology grade and purchased from Fisher Scientific, UK. Unless otherwise noted, cultures were incubated at 37°C, 300 r.p.m. in an orbital shaker (MaxQ 4450, Thermo Scientific, UK). All centrifugation steps were completed as follows; for 1.5 mL microcentrifuge tubes; Eppendorf 5417R centrifuge, fixed angle rotor F45-30-11, for 50 mL centrifuge tubes, <5,000 *g* Heraeus Primo R, Thermo, UK and for 50 mL centrifuge tubes, >5,000 *g* (Avanti, JA-14, Beckman Coulter, Inc.).

3.3.1 Project Approval

This project was approved for containment Level 2 by Cardiff University Genetic Modification Safety Committee (GMSC) and the Health and Safety Executive (HSE).

3.3.2 Nucleotide Sequences

The nucleotide sequences encoding $TcdA_{900\text{-}2710}$ and $TcdB_{547\text{-}2366}$ were identified within the published genome sequence of *C. difficile* 630 strain (A^+B^+, PCR RT012) (Sebaihia *et al.*, 2006) using NCBI (http://www.ncbi.nlm.nih.gov/) The following software packages were used to analyse the nucleotide and protein sequences of native and codon optimised $TcdA_{900\text{-}2710}$ and $TcdB_{547\text{-}2366}$.

- The Rare Codon Analysis Tool (www.genscript.com).
- EMBOSS isochore (http://www.ebi.ac.uk/Tools/emboss/cpgplot/).
- Graphical Codon Usage Analyser, GCUA (http://gcua.schoedl.de/).

The main features of the sequences encoding each gene are summarised in Table 3.1.

Table 3.1: Summary of recombinant toxin A and B sequence regions.

	Nucleotide	Amino Acid	Gene Length (bp)	MW (kDa)	pI
$TcdA_{900\text{-}2710}$	2701–8101	900–2710	5445	204	6.05
$TcdB_{547\text{-}2366}$	1641–7081	547–2366	5472	206	4.63

3.3.3 Vector Construction

3.3.3.1 Restriction Site Optimisation

To assist in the subsequent cloning of $TcdA_{900\text{-}2710}$ and $TcdB_{547\text{-}2366}$, each sequence was screened for the presence of restriction enzyme sites which could facilitate or hinder cloning. This was achieved using NEBcutter v.2.0 (Vincze *et al.*, 2003). Using this software the nucleotide sequences for *tcdA* and *tcdB* were analysed for the presence of restriction sites recognised by commercially available restriction enzymes.

3.3.3.2 Codon Optimisation

Codon analysis of the native nucleotide sequences of $TcdA_{900\text{-}2710}$ and $TcdB_{547\text{-}2366}$ was performed using the GenScript Rare Codon Analysis Tool (www.genscript.com). Codon optimised versions of each gene region were designed *in silico* in Cardiff to include the *Bam*HI and *Sal*I restriction sites at the 5' and 3' ends of the insert. The newly synthesised sequences were cloned into pQE30 (Qiagen Ltd, UK) by GenScript, Inc., forming pQE30-$TcdA_{900\text{-}2710}$ and pQE30-$TcdB_{547\text{-}2366}$. The pQE plasmids are derived from the pDS56/RBSII and pDS781/RBSII-DHFRS plasmids. DNA sequencing was undertaken by GenScript to confirm the correct orientation and fidelity of the insert. Expression was under the control of an inducible T5 promoter and the gene insert was fused at its N-terminus to a 6x Histidine tag (Figure 3.1).

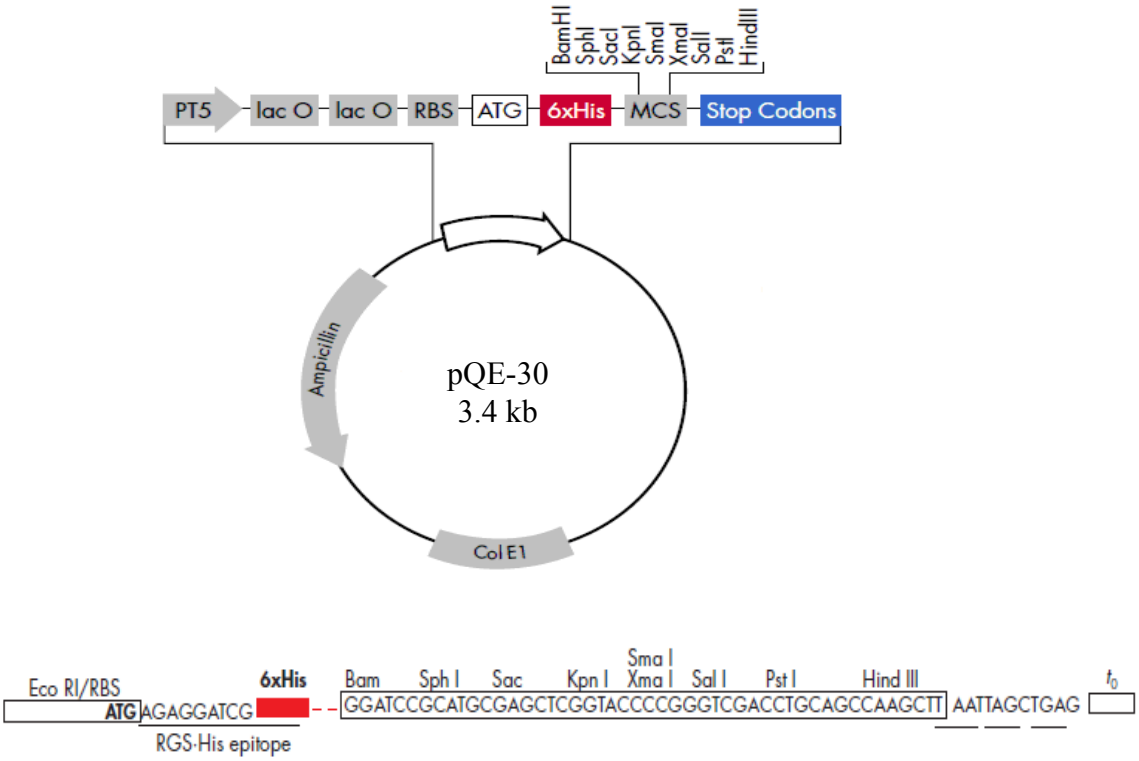

Figure 3.1: A representational map showing the major features of pQE30.

Codon optimised nucleotide sequences of $TcdA_{900\text{-}2710}$, and $TcdB_{547\text{-}2366}$ were cloned into pQE30 at the multiple cloning sites so that a 6xHis affinity tag was inserted at the N-terminus of the protein. PT5: T5 promoter, lac O: lac operator, RBS: ribosome-binding site, ATG: start codon, 6xHis: 6xHis tag sequence, MCS: multiple cloning sites with restriction sites indicated, Stop codon, Col E1: Col E1 origin of replication, Ampicillin: ampicillin resistance gene (Qiagen Ltd, Crawley, UK).

3.3.4 *Escherichia coli* Expression

All strains were stored on PROTECT beads (Fisher Scientific, UK) at -80°C. Strains were routinely cultured at 37°C on LB agar plates and broth supplemented with the appropriate antibiotics (Table 3.2). All three strains are derived from *E. coli* K12. Details of each strain are shown in Table 3.2.

Table 3.2: *Escherichia coli* strain details

E. coli Strain	Phenotype/Genotype	Source	Antibiotic
M15 and SG13009*	F^-, NaI^S, Str^S, Rif^S, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, $RecA^+$, Uvr^+, Lon^+.	Qiagen, Ltd	Kanamycin 25 µg/mL
DH5α™	F- φ80*lac*ZΔM15 Δ(*lac*ZYA-argF)U169, *rec*A1, *end*A1, *hsdR17*(r_k^-, m_k^+), *pho*A supE44 thi^{-1}, *gyr*A96, *rel*A1, λ^-	Invitrogen, Ltd	Naldixic acid 32 µg/mL

*contains pREP4 plasmid confers kanamycin resistance and encodes the *lacI* gene expressing the lac repressor protein.

3.3.5 Preparation of Competent Cells

Competent cells were generated using the method based on Hanahan (1983) and Sambrook *et al.* (1989). A single colony of *E. coli* M15[pREP4], SG13009[pREP4] or DH5α™ was inoculated in 10 mL LB supplemented with the appropriate antibiotic(s) (Table 3.2) and cultured overnight at 37°C in a 50 mL centrifuge tube. From this overnight culture, 5 mL was added to 100 mL of pre-warmed antibiotic supplemented LB in a 250 ml flask, sealed with a foam bung and incubated at 37°C in an orbital shaker until the culture reached an OD_{600} of 0.5 (Ultraspec 3100 *pro*, GE Healthcare). The culture was then cooled on ice for 5 minutes, prior to centrifugation at 4,000 *g* for 5 minutes at 4°C. Cells were gently re-suspended in 30 mL cold transformation buffer (TFB-1: 100mM rubidium chloride, 50 mM manganese chloride, 30 mM potassium acetate, 10 mM calcium chloride, 15% (v/v) glycerol, pH 5.8, sterile-filtered) and placed on ice for a further 90 minutes. Cells were centrifuged as above and gently re-suspended in 4 mL ice-cold TFB-2 buffer: 10 mM MOPS, 10 mM $RbCl_2$, 75 mM $CaCl_2$, 15% (v/v) glycerol, pH 6.8 adjusted with potassium hydroxide, sterile-filtered). Competent

cells were divided into 200 µL aliquots, placed in cold sterile cryovials, flash frozen in liquid nitrogen and stored at -80°C.

3.3.6 Transformation of *Escherichia coli*

Competent cells were thawed on ice and 100 µL of cells were mixed with 10 ng of pQE30-TcdA$_{900-2710}$ or pQE30-TcdB$_{547-2366}$ in 20 µL Tris-EDTA buffer (pH 8.0) (Sigma-Aldrich, UK) in cold sterile 0.5 mL thin wall microcentrifuge tubes and placed on ice for 20 minutes. The suspension was then placed in a 42°C water bath (Fisher Scientific, UK) for 90 seconds. The cells were then added to 600 µL of cold SOC medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) sodium chloride, 250 mM KCl, 2 M $MgCl_2$, plus 2 mL of 1 M filter-sterilised glucose) and incubated for 90 minutes at 37°C in an orbital shaker. Transformation efficiency was checked by transforming competent cells with 10 ng of pQE30 lacking either cloned gene in 20 µL TE buffer (pH 8.0). The culture was diluted with SOC broth and 100 µL was plated onto LB plus appropriate antibiotic plates in duplicate. Negative controls were transformed with 20 µL TE buffer and plated. All plates were incubated overnight at 37°C.

Transformation efficiency was determined using the following calculation:

$$\text{Transformation efficiency (µg)} = \frac{\text{Number of colonies on agar plate}}{\text{Amount of DNA spread on agar plate}}$$

Transformed strains were stored frozen at -80°C on PROTECT beads.

3.3.7 Plasmid Purification

Following transformation plasmids were purified from bacterial cultures using the QIAprep® Spin Miniprep kit as per the manufacturer's instructions (Qiagen Ltd, Crawley, UK). A single colony was inoculated in 5 mL of antibiotic supplemented LB media and incubated overnight at 37°C, 300 r.p.m. in a 50 mL centrifuge tube. Bacterial cells were centrifuged at 5,000 *g* for 10 minutes at 4°C and the cell pellet was re-suspended in 250 µL P1 Buffer containing 200 µg RNaseA.

An equal volume of P2 Buffer was added and the sample gently mixed, followed by the addition of 350 µL of N3 Buffer. Samples were centrifuged at 10,000 *g* for 10 minutes at room temperature. The supernatant was then applied to a QIAprep spin column and centrifuged for 1 minute. The column was then washed with 0.5 mL Buffer PB and centrifuged for 1 minute. PE Buffer (0.75 mL) was added to the column and centrifuged twice. DNA was eluted into a sterile microcentrifuge tube with the addition of 50 µL EB Buffer. The quality and quantity of the plasmid was confirmed by measuring the absorbance at A_{260}/A_{280} (Eppendorf Biophotometer, Cambridge, UK). Purified plasmids were subjected to restriction digest (Section 3.3.8) and agarose gel analysis (Section 3.3.9).

3.3.8 Restriction Digestion

Plasmid extracted from transformed cells was subjected to restriction digestion. In a 0.5 mL thin wall microcentrifuge tube in 2 µL of 10x FastDigest® Green Buffer (Fermentas, UK). FastDigest® *Bam*HI (1 µL/10 Units) and FastDigest® *Sal*I (1 µL/10 Units) (Fermentas, UK) were used to digest 1 µg of pQE30-$TcdA_{900\text{-}2710}$, pQE30-$TcdB_{547\text{-}2366}$ or pQE30. Reactions were incubated in a dry heat block at 37°C (Techne, UK) for 5 minutes.

3.3.9 Agarose Gel Electrophoresis

Agarose gels (1.2% (w/v)) were prepared in 1x TBE (90 mm tris base, 90 mM boric acid, 2 mM EDTA, pH 8.0) using electrophoresis grade agarose. The agarose solution was heated in a microwave oven (Sanyo, EM-S1067, Fisher Scientific, UK) for approximately 5 minutes, the solution was gently swirled every 30 seconds to suspend un-dissolved agarose and prevent super-heating. The agarose was cooled and 0.05 µg/mL of Ethidium bromide was added. Gels were cast on a horizontal perspex plate (7 x 7 cm) at a thickness of 1.0 cm (Bio-Rad, UK). Once set gels were placed in a tank and submerged with 1x TBE. To load samples one part Sample Loading Buffer (Bioline, UK) was combined with 4 parts DNA sample. Electrophoresis was performed at 100 V (constant) in a Bio-Rad Sub-Cell® powered by a Bio-Rad PowerPac™ Basic power supply for approximately 1 hour. Gels were imaged under UV light using the Chemi Doc xrs+ system (Bio-Rad, UK).

3.3.10 Recombinant Protein Expression and Purification

SDS-PAGE gels were completed as detailed in Chapter 2, Section 2.3, stained with colloidal Coomassie (Chapter 2, Section 2.5.1) and silver nitrate (Chapter 2, Section 2.5.2) and imaged using a Chemi Doc xrs+ System (Bio-Rad, UK).

3.3.10.1 Small Scale Expression Trials-Time Course Analysis of Recombinant Protein Expression

A single colony of transformed *E. coli* was inoculated into 5 mL antibiotic supplemented LB and cultured overnight in a 50 mL centrifuge tube. To 100 mL of pre-warmed media 5 mL of the overnight culture was added and the culture was incubated at 37°C until the OD_{600} reached 0.6. Protein expression was induced by the addition of 1 mM Isopropyl-beta-D-thiogalactopyranoside (IPTG: Invitrogen Ltd, UK). A time course of expression and protein solubility was determined by taking 1 mL samples prior to induction and at 0, 1, 2, 3 and 4 hours post-IPTG induction. For all samples the OD_{600} was recorded and samples were centrifuged for 30 minutes at 5,000 *g*, 4°C. To ensure even gel loading of the samples the crude extracts were standardised to 10 OD_{600}/mL (~10^9 CFU/mL) and re-suspended in the appropriate volume of 5x SDS-PAGE buffer using the following calculation;

$$\text{Re-suspension volume (mL)} = \frac{\text{Absorbance at } OD_{600} \times 1 \text{ mL}}{10\ OD_{600}/\text{mL}}$$

For protein solubility studies cell pellets were stored at -20°C until analysis.

3.3.10.2 Recombinant Protein Solubility

To determine if $rTcdA_{900\text{-}27120}$ and $rTcdB_{547\text{-}2366}$ were soluble in the cytoplasm or were aggregated into insoluble cytoplasmic inclusion bodies, protein solubility was assessed as described in The QIAexpressionist™ handbook (Qiagen Ltd, 2003).

3.3.10.2.1 Soluble Expression in the Cytoplasm

The cell pellet was thawed on ice for 20 minutes and re-suspended in native lysis buffer (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) supplemented with 1 mg/mL lysozyme, 10 µL (25 U/µL) Benzonase® Nuclease and Roche Complete EDTA-free protease inhibitor cocktail. The lysate was incubated on ice for 30 minutes and gently inverted every 10 minutes. The cell lysate was sonicated for eight cycles, per bursts of 60 seconds at 14 amplitude microns with a 30 second cooling period on ice (Soniprep 150, MSE, UK). The lysate was then centrifuged at 14,000 *g* for 30 minutes at 4°C. The supernatant containing the soluble cytoplasmic fraction was combined with 2x SDS-PAGE buffer; the insoluble cytoplasmic inclusion body fraction was stored at -20°C for subsequent analysis

3.3.10.2.2 Cytoplasmic Inclusion Bodies

The inclusion body fraction pellet was re-suspended in native lysis buffer, incubated and sonicated as detailed above. Following the sonication cycles, denaturing buffer (native lysis buffer plus 8 M urea, Roche complete EDTA free inhibitor, pH 8.0) was added and the cell lysate incubated at room temperature for 60 minutes with gentle inversion every 20 minutes. The lysate was centrifuged at 10,000 *g* for 20 minutes at room temperature. The supernatant containing the protein extracted from the inclusion bodies was combined with 2x SDS-PAGE buffer.

3.3.10.3 Media Optimisation

To assess the effect of media composition on the efficiency of recombinant protein production, the constructs were expressed in six different media. A single colony of M15[pREP4]-pQE30-$TcdA_{900\text{-}2710}$ or M15[pREP4]-pQE30-$TcdB_{546\text{-}27366}$ was inoculated into 100 mL antibiotic supplemented LB in a 250 mL culture flask. Five mL of the overnight culture was used to inoculate 100 mL of the following media: LB (Fisher Scientific, UK), Superior Broth™, Turbo Broth™, Power Broth™, Glucose M9Y, and Hyper Broth™ (AthenaES®). All media were supplemented with the appropriate antibiotics and prepared as per the manufacturer's instructions, Glucose M9Y, Hyper Broth™ were supplemented with filter-sterilised Glucose Nutrient Mix (20 mL and 50 mL per L, respectively) post-autoclaving. Turbo Broth™ and Power Broth™ were supplemented with 4 mL glycerol (per L)

pre-autoclaving. Time course of expression and protein solubility were determined as above (Section 3.3.10.1 and Section 3.3.10.2).

3.3.10.4 Temperature Optimisation

To further increase the yield of recombinant protein recovered from the soluble fraction, the effect of altering the incubation temperature was investigated. A single colony of transformed M15[pREP4]-pQE-30-$TcdA_{900\text{-}2710}$ was inoculated into 100 mL antibiotic supplemented LB in a 250 mL culture flask. Five mL of the overnight culture was then used to inoculate 100 mL of antibiotic supplemented Superior Broth™. Cultures were grown to an OD_{600} of 0.6, induced with IPTG and transferred to a 16°C (static) incubator. A time course of expression and solubility was determined as detailed in Sections 3.3.10.1 and 3.3.10.2.

3.3.10.5 Biomass and Chaperone Induction

Augmedium™ (AthenaES®) and LB*Booster™ (AthenaES®) were used as additives to induce expression of chaperone proteins and increase biomass production, respectively. Experiments were conducted with the Protein Expression and Rescue Kit (PERK™) following the manufacturer's instructions (Athena Enzyme Systems™). A single colony of transformed M15[pREP4]-pQE30-$TcdA_{900\text{-}2710}$ was inoculated into 10 mL antibiotic supplemented LB in a 50 mL centrifuge tube. Five mL of the overnight culture was then used to inoculate 250 mL antibiotic supplemented Superior Broth™ in a 500 mL culture flask. Cultures were grown to an OD_{600} of 0.6 at 37°C, after which 10 mL aliquots of this culture were dispensed into 50 mL centrifuge tubes. Augmedium™ was added (Table 3.3), and cultures were transferred to either 30°C or 37°C for 20 minutes. IPTG and LB*Booster were added to the cultures as detailed in Table 3.3 and incubated for 3 hours. Samples were processed for solubility as detailed above, Section 3.3.10.2

Table 3.3: PERK™ Optimisation design matrix for rTcdA$_{900\text{-}2710}$.

Culture No.	IPTG (mM)	Temp (°C)	LB*Booster™ (µL)	Augmedium™ (µL)
1	1	37	500	20
2	1	30	500	20
3	0.05	37	500	20
4	1	37	500	200
5	1	30	50	20
6	0.05	37	50	20
7	0.05	37	50	200
8	0.05	30	500	200
9	1	37	50	200
10	1	37	50	20
11	0.05	37	500	200
12	0.05	30	50	200
13	1	30	500	200
14	1	30	50	200
15	0.05	30	50	20
16	0.05	30	500	20

Numbers represent final volume in 10 mL cultures. Stock IPTG, 1 M; Stock LB*Booster™, x20; Stock Augmedium™, x50.

3.3.11 Recombinant Protein Purification

Purification columns (Thermo Scientific, UK) were prepared as follows; a polyethylene porous disc (15 mm diameter, 30 µm pore size) was placed in the bottom of a 10 mL polypropylene column (70 x 15 mm (h x d)). Columns were packed with 1.5 mL of Ni-NTA (Qiagen Ltd, UK) and equilibriated in the appropriate buffer.

3.3.11.1 Purification of rTcdA$_{900\text{-}2710}$

Purification of soluble rTcdA$_{900\text{-}27120}$ was completed as outlined in The QIAexpressionist™ handbook (QIAgen, UK). Ni-NTA slurry was pre-equilibriated in lysis buffer (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole, Roche Complete EDTA-free protease inhibitor cocktail, pH 8.0) and the cleared lysate was applied to the top of the column. The column was washed twice with 4 mL of Wash buffer

(50 mM Na_2HPO_4, 300 mM NaCl, 20 mM imidazole, Roche Complete EDTA-free protease inhibitor cocktail, pH 8.0). The protein was eluted with Elution buffer (50 mM Na_2HPO_4, 300 mM NaCl, 250 mM imidazole, Roche Complete EDTA-free protease inhibitor cocktail, pH 8.0), collecting four 1 mL fractions. The flow through, wash and elution fractions were collected and analysed by SDS-PAGE (Chapter 2, Section 2.3).

To recover $rTcdA_{900\text{-}2712}$ from insoluble inclusion bodies, the purification lysis, wash and elution buffers were supplemented 8 M Urea, pH 8.0. Following application of the cleared bacterial lysate the Ni-NTA column was washed twice with 4 mL of wash buffer (Ni-NTA Wash buffer plus 8 M Urea, Roche Complete EDTA-free protease inhibitor cocktail, pH 8.0). The protein was eluted with Elution buffer (Ni-NTA elution buffer plus 8 M Urea, Roche Complete EDTA-free protease inhibitor cocktail, pH 8.0), collecting four 1 mL fractions. The flow through, wash and elution fractions were collected and analysed by SDS-PAGE (Chapter 2, Section 2.3).

Following IMAC (Immobilized Metal Affinity Chromatography) protein purification of $rTcdA_{900\text{-}27120}$ the eluted fractions were pooled and buffer exchanged (Section 3.3.12). Further purification of the recombinant protein was performed using the method detailed by Krivan and Wilkins (1987) and Kamiya *et al.* (1989). In a 50 mL centrifuge tube, 150 mg of bovine thyroglobulin (Sigma-Aldrich, UK) was dissolved in 30 mL of 0.1 M morpholinepropanesulphonic acid buffer (MOPS) (pH 7.0) and filtered through a 0.22 µm membrane filter. The solution was reacted with 6 mL of activated Affi-Gel® 15 (Bio-Rad, UK) overnight at 4°C on a roller mixer at 33 r.p.m. Any remaining active sites were blocked with 30 mL chilled 0.1 M ethanolamine for 30 minutes at 4°C. Purification columns were prepared as detailed above; the column was washed with pre-warmed 10 mL 0.1 M glycine-sodium hydroxide buffer containing 0.5 M NaCl, pH 10.0 and 0.1 M glycine hydrochloride containing 0.5 M NaCl, pH 2.0. The column was washed with chilled 10 mL TBS (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.0) at 4°C and equilibriated with appropriate elution buffer. The elution fractions were chilled at 4°C and applied to the column at 4°C. The column was then washed twice with 30 mL cold TBS. The

protein was eluted with 30 mL of pre-warmed TBS at 37°C. All fractions were collected and analysed by SDS-PAGE (Chapter 2, Section 2.3).

3.3.11.2 Purification of rTcdB$_{547-2366}$

Purification of soluble rTcdB$_{547-2366}$ was completed as for rTcdA$_{900-27120}$, with the following modification. To maximise recovery of unbound rTcdB$_{547-2366}$ protein lost in the flow through, the purification steps were repeated eleven times.

For rTcdB$_{547-2366}$ recombinant protein recovered from cytoplasmic inclusion bodies was purified as outlined in The QIAexpressionist™ handbook (2003). Cleared lysate was applied to the column. The column was washed twice with 4 mL of 1x Ni-NTA Wash buffer (100 mM Na_2HPO_4, 10 mM Tris-Cl, 8 M Urea, Roche Complete EDTA-free protease inhibitor cocktail, pH 6.3). The protein was eluted Elution buffer D (100 mM Na_2HPO_4, 10 mM Tris-Cl, 8 M Urea, Roche Complete EDTA-free protease inhibitor cocktail, pH 5.9), collecting four 1 mL fractions, followed by elution with Elution buffer E (100 mM Na_2HPO_4, 10 mM Tris-Cl, 8 M Urea, Roche Complete EDTA-free protease inhibitor cocktail, pH 4.5), collecting four 1 mL fractions. The flow through, wash and elution fractions were collected and analysed by SDS-PAGE (Chapter 2, Section 2.3).

3.3.12 Buffer Exchange and Recombinant Protein Concentration

Elution fractions from native purification were buffer exchanged with PBS using a 10 mL Zeba™ Spin DeSalting column (Thermo Scientific, UK). All centrifugations were carried out at 4°C at 1,000 *g* following the manufacturer's instructions. Buffer exchange columns were prepared by centrifugation as follows, removal of storage solution for 2 minutes; two 2 minute washes with PBS; sample application, a 6 minute wash and a 4 minute sample recovery step. Samples were concentrated using a Pierce® Concentrator centrifugal device, with a 150 kDa molecular weight cut off (MWCO) (Thermo Scientific, UK). Samples were centrifuged at 2,000 *g* at room temperature for 5 minutes. Protein concentrations were determined by BCA assay as outlined in Chapter 2, Section 2.2.2.

3.3.13 Detection of rTcdA$_{900-2710}$ and rTcdB$_{547-2366}$ Anti-His Antibodies

The 6x Histidine tag attached to the N-terminus of each recombinant protein can be used to identify the recombinant protein through the use of histidine specific antibodies. Nitrocellulose membranes were prepared as detailed in Chapter 2.0, Section 2.8. Membranes were blocked with 3% (w/v) BSA in PBS-0.1% (v/v) Tween (PBST), washed four times in PBST for 20 minutes and incubated at 4°C overnight on a roller mixer with the antibodies detailed in Table 3.4, diluted to a working concentration of 1:1,000. Following incubation membranes were washed and incubated with goat anti-mouse IgG-HRP-conjugate (Thermo Scientific, UK) diluted 1:10,000 in 5% (w/v) dried skimmed milk (Marvel) for 1 hour. Negative controls included soluble protein from cultures of *E. coli* M15[pREP4], non-induced M15[pREP4]-pQE30-TcdA$_{900-2710}$ and M15[pREP4]-pQE30-TcdB$_{547-2366}$ grown for 3 hours LB supplemented with appropriate antibiotics. Positive control included non-toxic, 6xHis-tagged dihydrofolate reductase (DHFR). Membranes were imaged by chemiluminescence as detailed in Chapter 2.0, Section 2.9.

Table 3.4: Anti-His antibody epitopes.

All antibodies were purchased from Qiagen Ltd, UK, diluted 1:1,000 in 1% (w/v) BSA in PBST.

	RGS-His Ab	**Penta-His Ab**	**Tetra-His Ab**
Epitope	RGSHHHH	HHHHH	HHHH
Detects	Protein expressed from pQE30	Protein with a His tag	Proteins with a His tag

3.4 Results

3.4.1 Codon Optimisation of Gene Sequences Encoding $TcdA_{900\text{-}2710}$ and $TcdB_{547\text{-}2366}$

Expression of clostridial genes in *E. coli* is frequently associated with low protein yields and the subsequent incorporation of the recombinant protein into insoluble inclusion bodies (Craggs, 1999; Letourneur *et al.*, 2003; Davies *et al.*, 2013). A comparison of the overall codon usage of *C. difficile* and *E. coli* is shown in Figure 3.2. In *E. coli* a low usage codon is a codon which displays a lower usage frequency than the smallest value amongst the optimal codons for Met, Trp, Leu, Ile, Val, Ser, Pro, Thr, Ala, Arg, Gly and Gln (Chen and Texada, 2006). The Codon Adaption Index (CAI) enables the analysis of codon bias based on the codon usage in a set of highly expressed genes. For example, in *E. coli* the preferred triplet encoding Proline is CCG (20.9) whereas in *C. difficile* it is CCA (15.4) (Figure 3.2). Analysis of the codon usage patterns of $TcdA_{900\text{-}2710}$ and $TcdB_{547\text{-}2366}$ revealed the presence of a number of codons which are rarely used in *E. coli*, primarily the codons CTA, ATA, AGA, and GGA which encode leucine, isoleucine, arginine and glycine respectively. Given that the codon usage of a gene sequence can affect the efficiency of its translation, codon optimisation of the $TcdA_{900\text{-}2710}$ and $TcdB_{547\text{-}2366}$ sequences was undertaken to maximise expression from *E. coli*. Following codon optimisation, the CAI was increased to 0.87 and 0.88 for $TcdA_{900\text{-}2710}$ and $TcdB_{547\text{-}2366}$, respectively. In addition the GC content of the re-synthesised codon optimised genes was closer to that of *E. coli* as displayed in the GC skew plots (Figures 3.3 and 3.4).

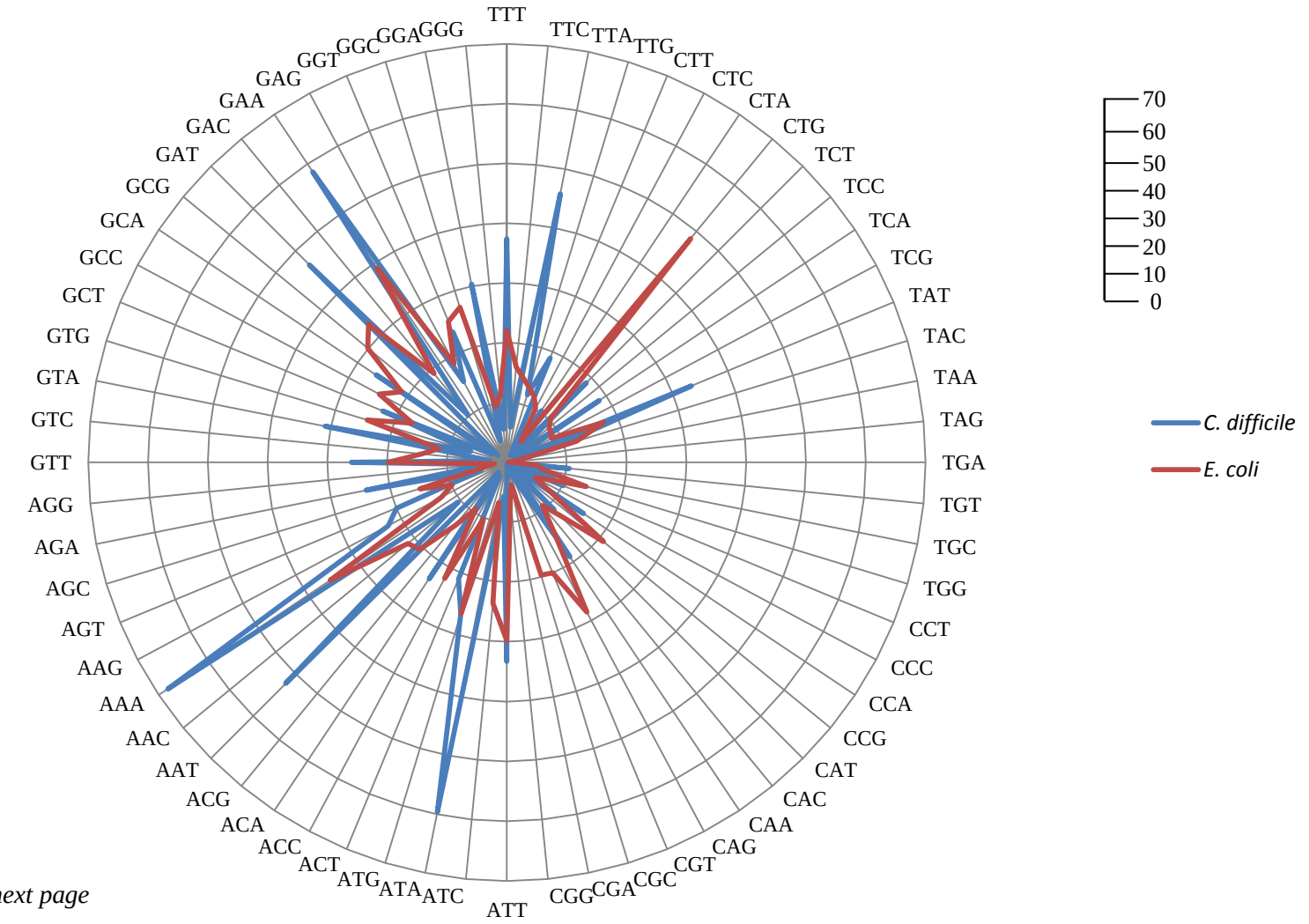

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Figure 3.2: Comparison of codon usage in *E. coli* and *C. difficile*.

E. coli K-12 (blue) and *C. difficile* 630 (red). Codon usage frequency table for *E. coli* is available from (http://www.genscript.com/). Codon usage frequency table for *C. difficile* 630 is available from (http://www.kazusa.or.jp/codon/). Axis value is off-set to the right and represents frequency of occurrence per 1000 codons.

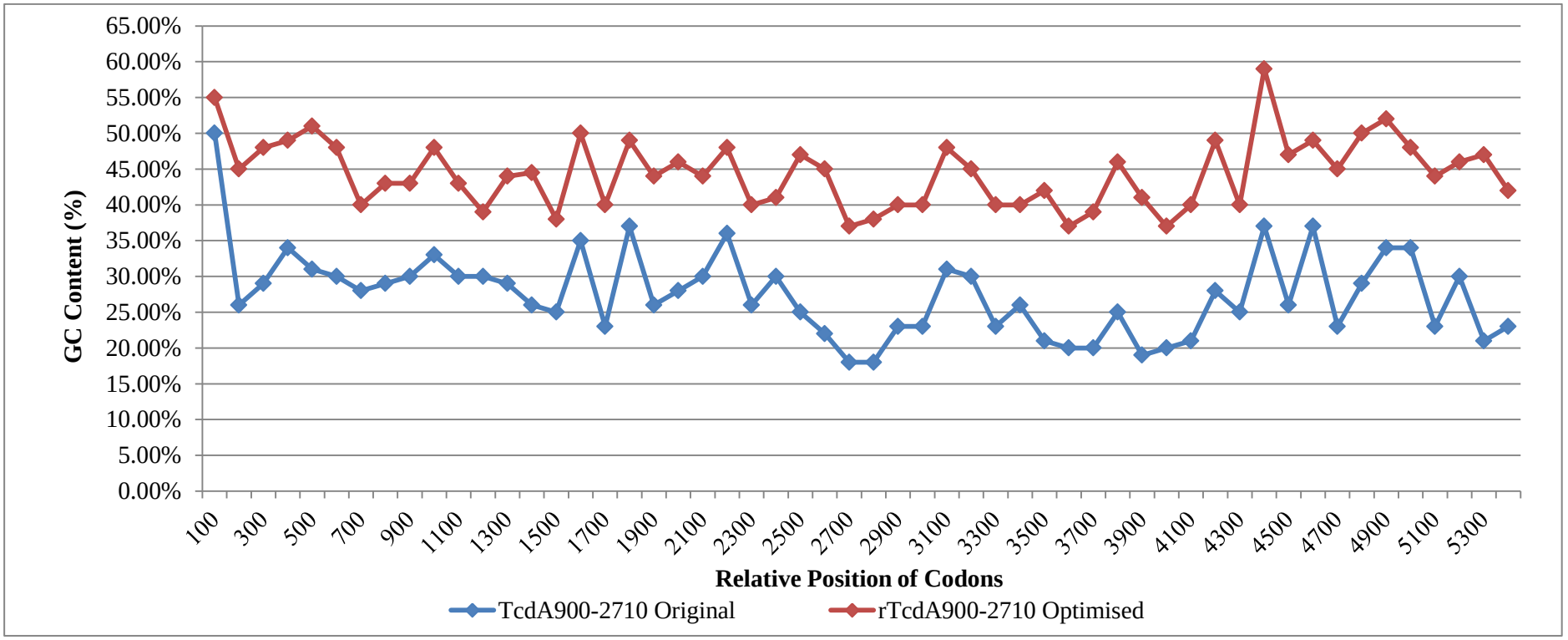


Figure 3.3: A GC content plot for $TcdA_{900\text{-}2710}$ and $rTcdA_{900\text{-}2710}$.

GC percentage was calculated in a 100 bp sliding window using the EMBOSS isochore software. The GC content before and after optimisation was 27.42% and 44.51%, respectively.

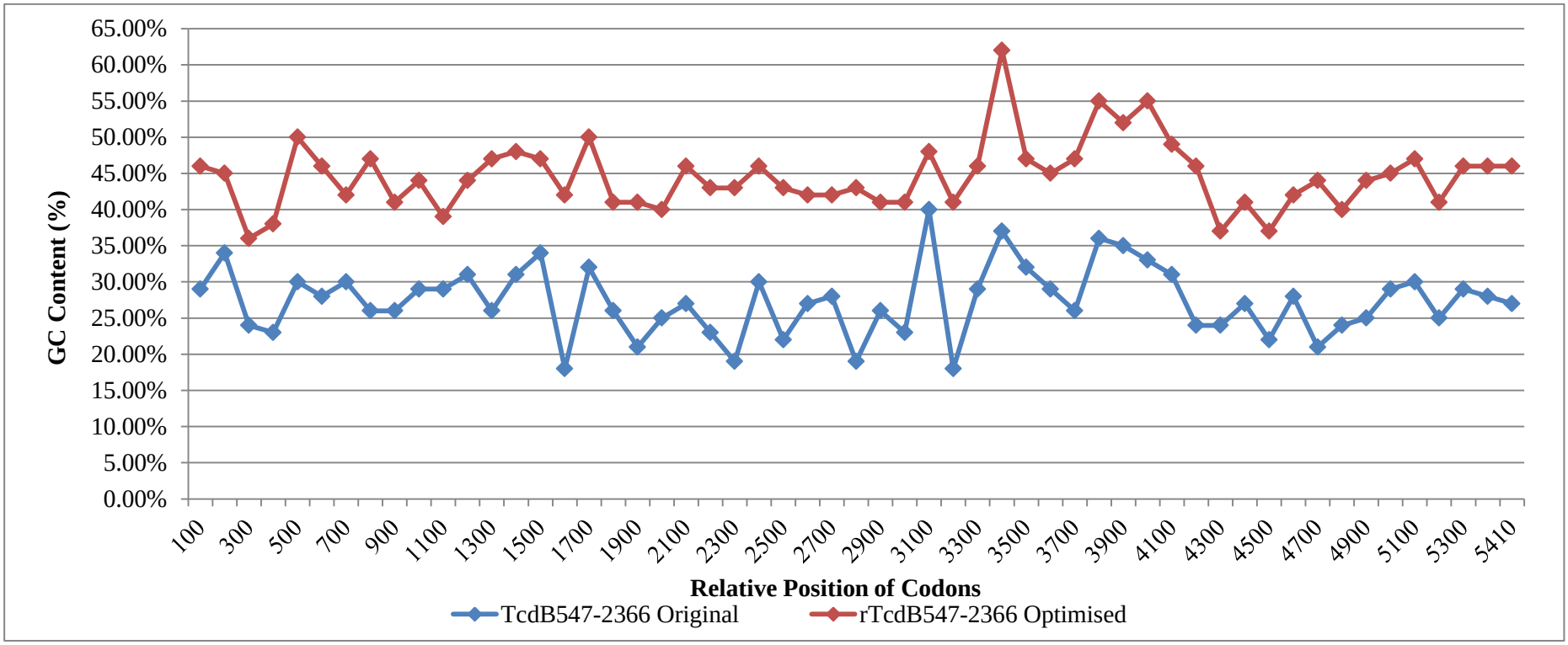


Figure 3.4: A GC content plot for $TcdB_{547-2366}$ and $rTcdB_{547-2366}$.

GC percentage was calculated in a 100 bp sliding window using the EMBOSS isochore software. The GC content before and after optimisation was 27.22% and 44.57%, respectively.

3.4.2 DNA Manipulations

Confirmation of the gene sequences into the pQE30 expression vector was undertaken by GenScript.

3.4.2.1 Transformation

Transformation of *E. coli* expression strains, M15[pREP4] and SG13009[pREP4] with pQE30-TcdA$_{900\text{-}2710}$ and pQE30-TcdB$_{547\text{-}2366}$ was successful but at a lower rate than that seen for the positive control, pQE30 (Table 3.5). In the case of pQE30-TcdA$_{900\text{-}2710}$ only after four attempts was transformation possible. To assess if the transformation efficiency was associated with problems with the donor DNA, the pQE30-TcdA$_{900\text{-}2710}$ plasmid was extracted from SG13009[pREP4] and used to transform DH5α™, a previously non-transformable strain. With this approach a transformation efficiency of 2.25 x 10^2 CFU/µg DNA was obtained.

Table 3.5: Transformation efficiency of rTcdA$_{900\text{-}2710}$ and rTcdB$_{547\text{-}2366}$.

***E. coli* Strain**	**pQE30 (CFU/µg DNA)**	**rTcdA$_{900\text{-}2710}$ (CFU/µg DNA)**	**rTcdB$_{547\text{-}2366}$ (CFU/µg DNA)**
M15 [pREP4]	1.81 x 10^4	9.00 x 10^2	1.73 x 10^3
SG13009 [pREP4]	4.02 x 10^4	1.84 x 10^3	1.55 x 10^3
DH5α™	TNTC	0	ND

All experiments were completed with a negative control of 20 µL of TE buffer and a 10 ng of pQE30 as a positive control. *E. coli* strains were transformed with 10 ng of plasmid DNA. TNTC – Too Numerous To Count at a 1/100 dilution. ND – not done

The incorporation of antibiotics into the culture media enables the selection of positively transformed cells. To confirm that the expression plasmid had been successfully introduced into the *E. coli* host, transformed cells were subjected to alkaline lysis and the plasmids isolated with a purification column. As can be seen from Figure 3.5, digestion with *Bam*H1 and *Sal*1 yielded DNA fragments of the expected sizes of ~5.4 kb for rTcdA$_{900\text{-}2710}$ and rTcdB$_{547\text{-}2366}$. The multiple bands

observed in the undigested pQE30 lane represents the different forms of the plasmid (supercoiled, nicked).

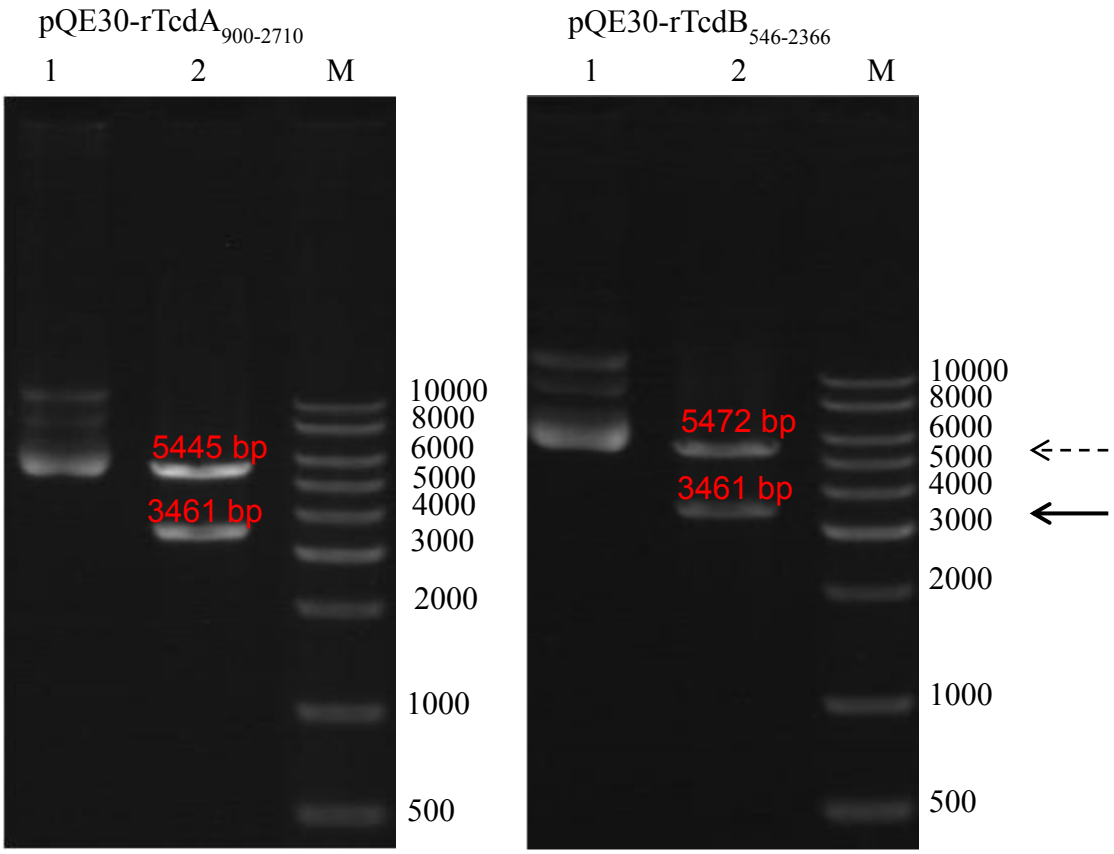


Figure 3.5: Restriction digest analysis.

Lane 1; Undigested pQE30, Lane 2; Digested pQE30A and pQE30B digested with *Bam*HI and *Sal*I. Samples were combined with x5 sample loading buffer, analysed on a 1.2% (w/v) agarose gel and stained with 0.5 µg/mL ethidium bromide. Solid arrow indicates insert and dashed arrow indicates digested pQE30. M; Marker in bp.

3.4.3 Expression and Purification of rTcdA$_{900-2710}$

In an initial series of experiments, the ability of the constructs to produce recombinant protein either as a soluble product (located in the cytoplasm) or as an insoluble product (located in cytoplasmic inclusion bodies) in a range of media was determined. Aliquots were collected pre-IPTG induction and post-IPTG induction

every hour for 4 hours. The optical density was recorded and whole cell crude extracts, soluble and insoluble fractions were analysed by 1D SDS-PAGE.

For the six media analysed no difference in optical density was observed during the course of the experiment (Figure 3.6) suggesting that differences in media composition did not influence the growth rate of M15[pREP4]-pQE30-TcdA$_{900\text{-}2710}$. The positive (6xHis-tagged dihydrofolate reductase (DHFR) and negative (non-induced M15[pREP4]-pQE30-TcdA$_{900\text{-}2710}$) controls were grown in LB media supplemented with appropriate antibiotics.

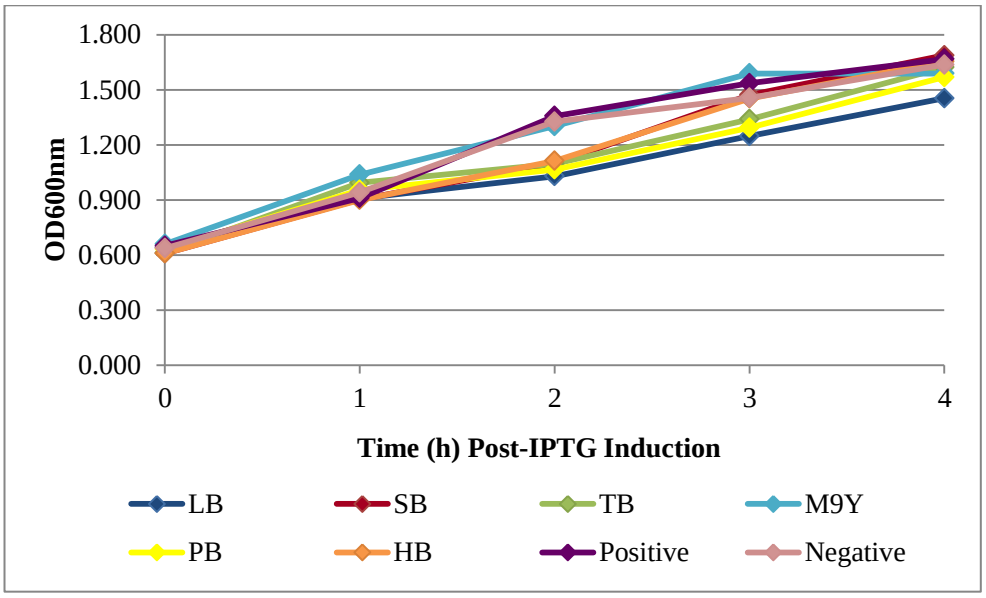


Figure 3.6: Time-course analysis of rTcdA$_{900\text{-}2710}$ expression in a range of media.
The optical density (OD_{600}) pre and post-IPTG induction was recorded. The positive control was non-toxic, 6xHis-tagged dihydrofolate reductase (DHFR) and the negative was non-induced M15[pREP4]-pQE30-TcdA$_{900\text{-}2710}$.

Analysis of the time course of recombinant protein expression by 1D SDS-PAGE (Figure 3.7) found that a band corresponding to the size of rTcdA$_{900\text{-}2710}$ (~205 kDa) was detected in the insoluble fraction of all of the cultures and that production peaked at 3 hours post-IPTG induction. Unusually the recombinant protein band was not detected at 4 hours post-IPTG induction in the insoluble fraction in LB and SB media, it is unclear if this is associated with incomplete lysis. Clearly the band

intensity of rTcdA$_{900\text{-}2710}$ was not comparable to that of the positive control, DHFR, from which protein could be recovered from both the soluble and insoluble fractions.

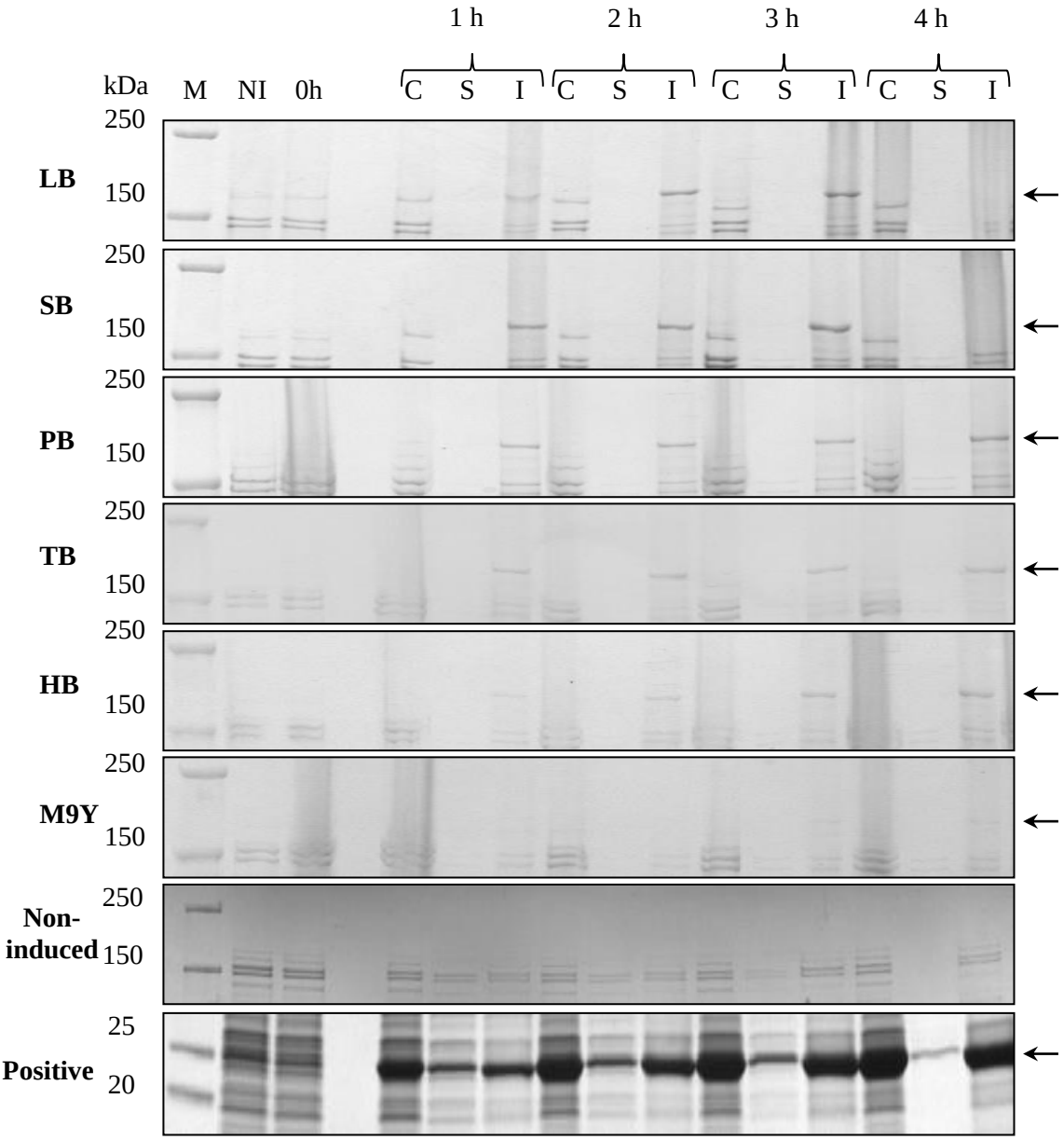


Figure 3.7: 1D SDS-PAGE time-course analysis of rTcdA$_{900\text{-}2710}$ expression in a range of media.

Aliquots were removed pre and post-IPTG induction subjected to lysis, analysed on a 4-20% SDS-PAGE gel and stained with colloidal Coomassie. Controls included pQE30 (positive) and non-induced rTcdA$_{900\text{-}2710}$ (negative). LB; LB Fisher, SB; Superior Broth™, Turbo Broth™, M9Y; Glucose M9Y, PB; Power Broth™, HB; Hyper Broth™, Positive; DHFR, Negative; M15[pREP4], C; crude, S; Soluble, I; Insoluble. Arrows indicate predicted protein size (205 kDa and 26 kDa for rTcdA$_{900\text{-}2710}$ and DHFR, respectively).

To compare the proportion of rTcdA$_{900-2710}$ produced at three hours, samples from all culture media were analysed on a single SDS-PAGE gel and volume intensity analysis conducted. This allowed for direct comparison of the media at a specific time point without introducing gel-to-gel variations in terms of electrophoresis run, staining and de-staining. Gel images were captured with a 12-bit CCD (charge-coupled device) camera and analysed with the in-built software, Image Lab (Bio-Rad, UK) allowing for quantification and comparisons to be made. Using this software an area corresponding to 1.4mm² was drawn around the 205 kDa band. Adjusted volume intensity values were plotted (Figure 3.8) as this took into account the presence of non-data background pixels within each area. Superior Broth™ was identified as the media which generated the highest intensity per 1.4 mm^2 and utilised for subsequent expression of rTcdA$_{900-2710}$.

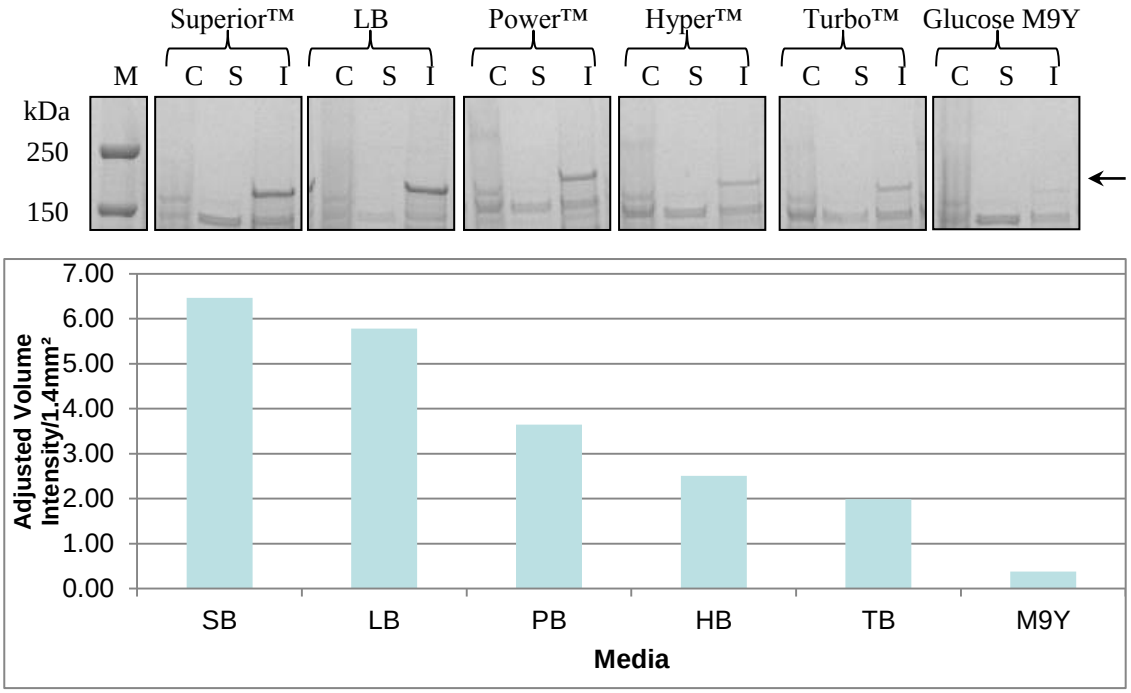


Figure 3.8: Volume intensity analysis of rTcdA$_{900-2710}$ expressed in a range of media. Aliquots 3 hours post-IPTG induction were subjected to lysis, analysed on a 4-20% SDS-PAGE gel and stained with colloidal Coomassie. Gel images were captured with a 12-bit CCD camera and analysed with Image Lab. Adjusted volume intensity values correspond to 1.4 mm² (LB; LB Fisher, SB; Superior Broth™, Turbo Broth™, M9Y; Glucose M9Y, PB; Power Broth™, HB; Hyper Broth™, C; crude, S; Soluble, I; Insoluble. Arrow indicates predicted protein size (205 kDa).

The media optimisation experiment revealed rTcdA$_{900\text{-}271}$ was predominantly located in cytoplasmic inclusion bodies. Inclusion bodies refer to insoluble protein aggregates, which are formed by non-specific hydrophobic interactions between polypeptides (Ventura and Villaverde, 2006). Although the precise physiochemical parameters necessary for inclusion body formation are unknown, a reduction in growth temperature has been reported to reduce inclusion body formation (Schein, 1989; Vera *et al.*, 2007). A reduction in culture temperature to 16°C and the incubation of the culture without shaking (Figure 3.9) produced recombinant protein in both the soluble and insoluble fractions at 2 hours post-IPTG induction. The 205 kDa recombinant band was only detectable in all the soluble fractions upon silver staining.

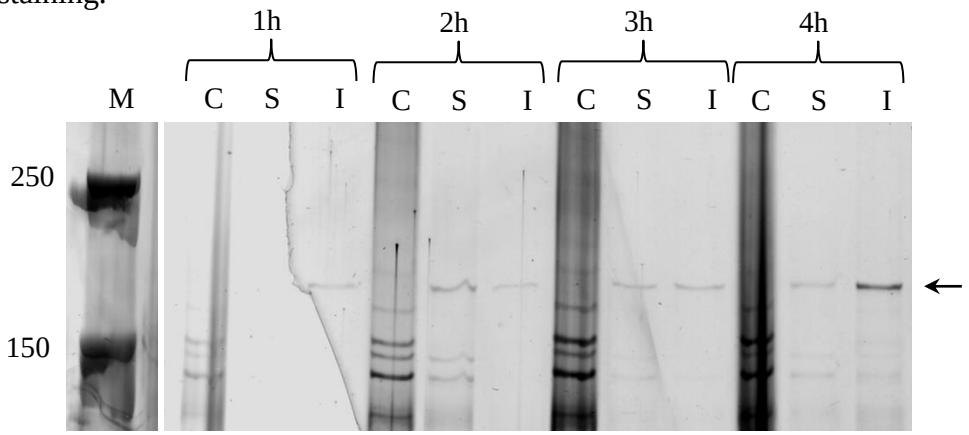


Figure 3.9: Temperature optimisation of rTcdA$_{900\text{-}2710}$.

Culture was grown at 16°C, static, aliquots were removed post-IPTG induction at the times indicated and analysed on a 5% SDS-PAGE gel, double stained with colloidal Coomassie+silver stain. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure. C; crude, S; Soluble, I; Insoluble, NI; Non-induced. Arrow indicates predicted protein size (205 kDa).

The potential yield of recombinant protein attainable is dependent, in part, on achieving a high cell concentration (biomass). For biomass production to be at its maximum, an alternative carbon source to glucose, the most commonly used sugar, must be used, thus LB*Booster™, a fructose based carbon source was assessed. Inclusion body formation is frequently associated with incorrectly folded proteins thus the addition of chaperones may assist in the folding of recombinant proteins and thus enhance recovery from the soluble fraction. Augmedium™ triggers chemical

and oxidative stress responses in *E. coli*, inducing expression of chaperone proteins. Using a commercially available assessment protocol the effect of IPTG, LB*Booster™ and Augmedium were assessed in a series of small scale expression experiments (50 mL culture volume). The crude, soluble and insoluble fractions were analysed (Figure 3.10). From this analysis 1 mM IPTG, x0.1 LB*Booster™ and x1 Augmedium™ incubated at 30°C in Superior Broth was utilised for expression of $rTcdA_{900\text{-}2710}$.

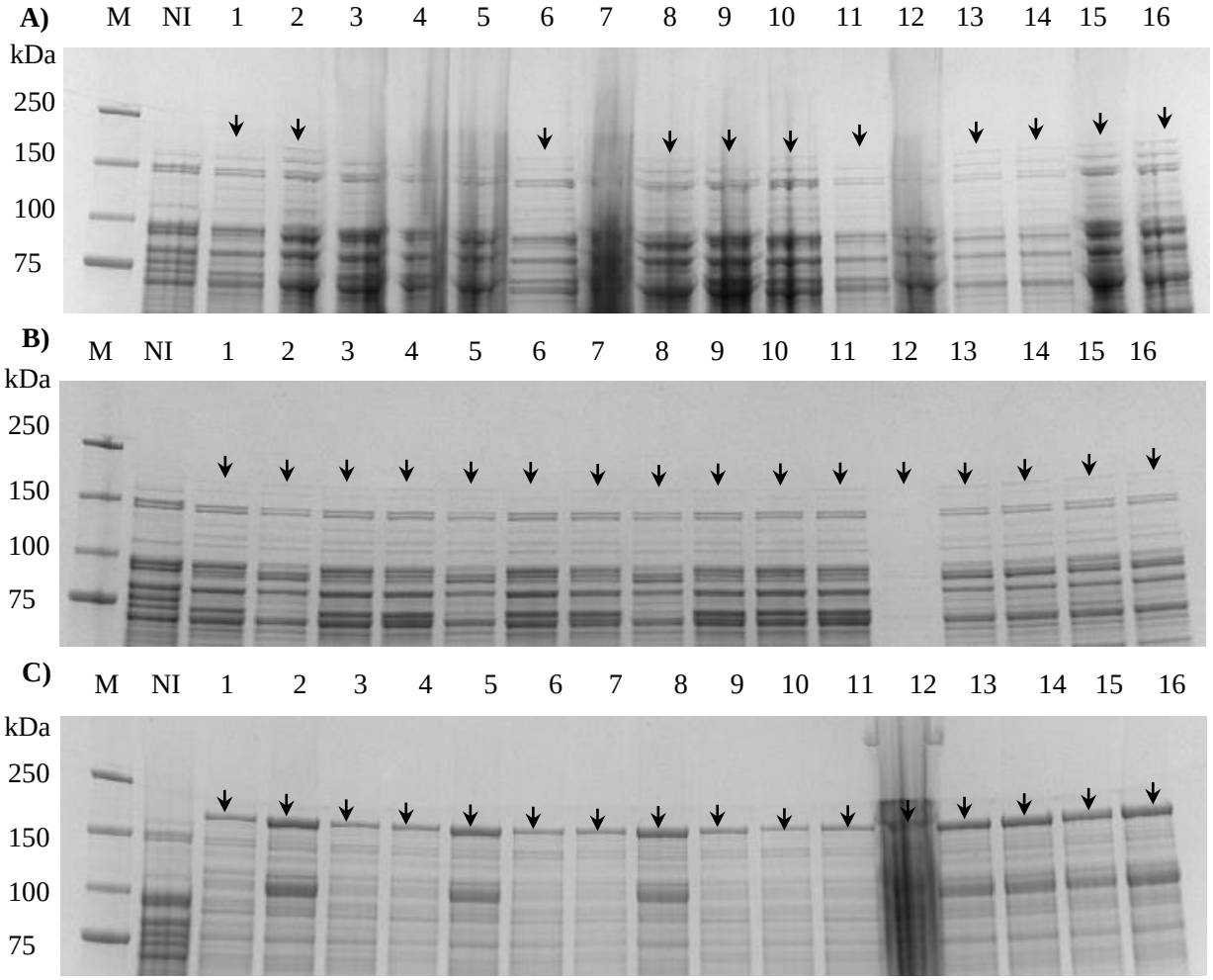


Figure 3.10: Protein Expression and Rescue (PERK™) optimisation of $rTcdA_{900\text{-}2710}$.

A) Crude, **B)** Soluble **C)** Insoluble. IPTG, LB*Booster™, Augmedium™ and Temperature were assessed as detailed in Table 3.3. Aliquots were removed 3 hours after IPTG induction, analysed on a 4–20% SDS-PAGE gel and stained with colloidal Coomassie. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™

Software, v 3.0, with a band detection sensitivity of 100. Arrows indicate predicted protein size (205 kDa).

3.4.3.1 Recovery and Purification of rTcdA$_{900\text{-}2710}$

To maximise the production and recovery of rTcdA$_{900\text{-}271}$, the influence of a range of factors on protein recovery and purification were assessed. Small scale expression experiments were conducted in 100 mL of Superior Broth™ incubated at 37°C for 3 hours. Using this approach a band corresponding to 205 kDa was not visible in the soluble fraction on a Coomassie stained gel. Increasing the culture volume to 1 L did result in a faint band at 205 kDa (Figure 3.11).

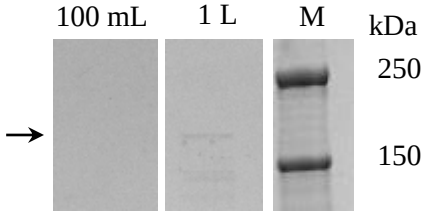


Figure 3.11: Recovery of soluble rTcdA$_{900\text{-}2710}$ based on culture volume.

Cultures were incubated at 37°C; 300 rpm in Superior Broth™, soluble purification was conducted 3 hours post-IPTG induction. The cleared lysate was analysed on a 4–20% SDS-PAGE gel and stained with colloidal Coomassie. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™ Software, v 3.0, with a band detection sensitivity of 100. Arrow indicates predicted protein size (205 kDa).

To further enhance the recovery of the recombinant protein from the bacteria, a lysis approach consisting of a combination of lysozyme and sonication was used to liberate soluble recombinant protein. The insoluble recombinant protein, trapped within inclusion bodies was recovered using a combination of lysozyme, sonication and urea. Based on the visible intensity of the 205 kDa band in the cleared lysate, this new lysis approach increased the amount of recombinant protein recovered from both the soluble and inclusion body fractions (Figure 3.12).

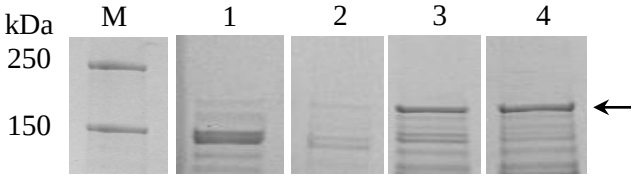


Figure 3.12: Effect of sonication on recovery of rTcdA$_{900\text{-}2710}$.

Lane 1; Soluble fraction, lysozyme, Lane 2; Insoluble fraction, lysozyme and urea, Lane 3; Soluble fraction, lysozyme and sonication, Lane 4; Insoluble fraction, lysozyme, sonication and urea. Aliquots were analysed on a 4–20% SDS-PAGE gel, stained with colloidal Coomassie, imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™ Software, v 3.0, with a band detection sensitivity of 100. Arrow indicates predicted protein size (205 kDa).

Recovery of recombinant protein from the Ni-NTA column requires the disassociation of the 6x His tag, which is located at the N-terminus of the recombinant protein from the Ni ions present in the affinity column. Recovery of His-tagged recombinant protein can be achieved either with an increasing imidazole concentration (100–250 mM) or by reducing the pH (4.5-5.3) gradient. Elution with the pH gradient revealed rTcdA$_{900\text{-}2710}$ was likely to be present as an aggregate, with the protein being eluted at pH 4.5. The lower pH disassociates the protein aggregates allowing the protein to be eluted from the column. Aggregate formation could have further been reduced by the incorporation of reducing agents (dithiothreitol or 2-Mercaptoethanol) in the buffers to disrupt disulphide bonds. In addition to the 205 kDa band corresponding to the recombinant protein, a number of additional proteins (ranging from ~150-20 kDa) were recovered from the elution fractions with both the imidazole and pH gradient (Figure 3.13). These protein bands may represent truncated and/or degraded rTcdA$_{900\text{-}2710}$, or *E. coli* proteins.

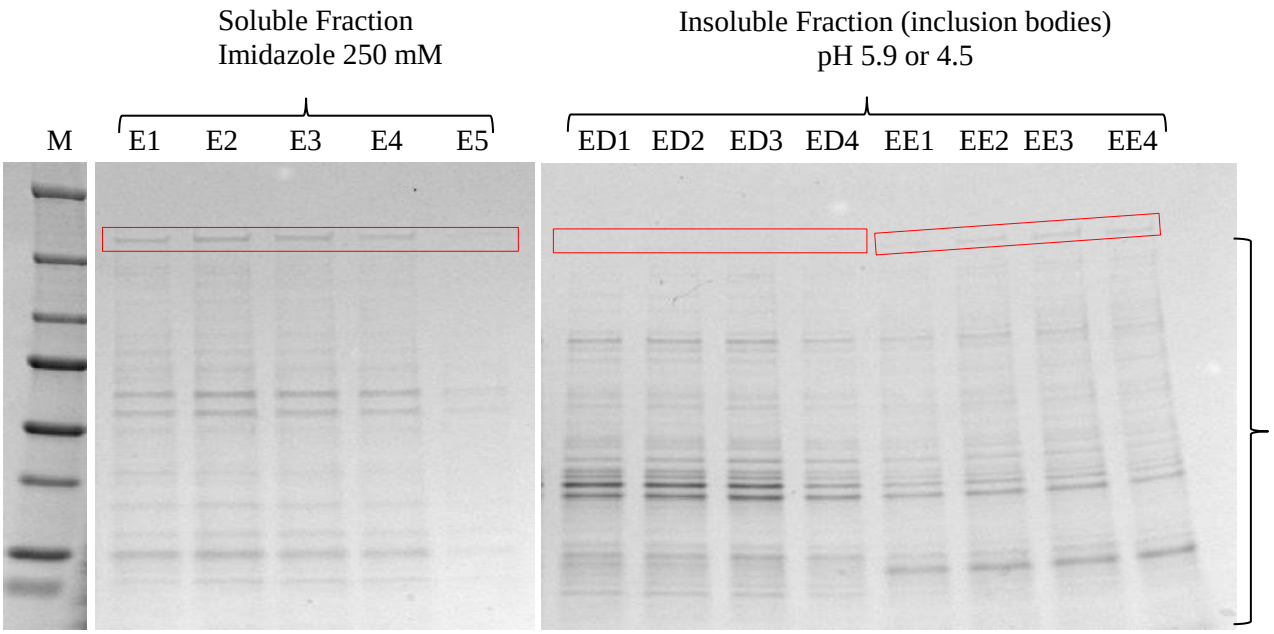


Figure 3.13: Purification of rTcdA$_{900\text{-}2710}$ by imidazole and pH gradient.

Post lysis recombinant protein was recovered from the Ni affinity column by imidazole or pH gradient. E1-E4, Elution by 250 mM imidazole, EE1-EE4; Elution by pH 5.9, ED1-ED4; Elution by pH 4.5, M; molecular weight maker in kDa and analysed on a 4–20% SDS-PAGE gel and stained with colloidal Coomassie. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™ Software, v 3.0, with a band detection sensitivity of 100. Red boxes indicate predicted protein size (205 kDa) and right brace indicates additional proteins present in elution fractions.

Both elution methods gave similar results (Figure 3.13), however the pH gradient was found to be more labour intensive and as a consequence the imidazole gradient was utilised in subsequent purification studies (Figure 3.14).

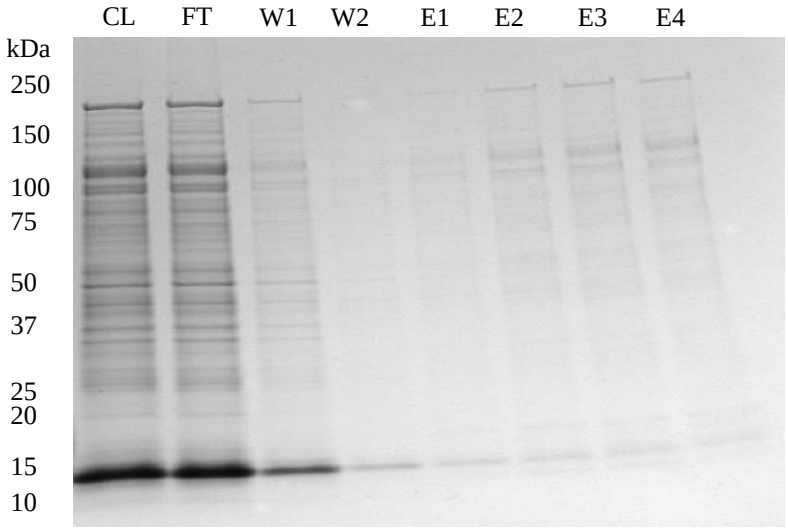


Figure 3.14: Optimized purification of rTcdA$_{900\text{-}2710}$ by imidazole gradient.

Post lysis recombinant protein was recovered from the Ni affinity column by imidazole gradient. CL; Cleared lysate, FT; Flow through, W; Wash, E1-E4, Elution by imidazole. Molecular weight maker in kDa and analysed on a 4–20% SDS-PAGE gel and stained with colloidal Coomassie. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™ Software, v 3.0, with a band detection sensitivity of 100.

To remove the additional bands seen following elution from the Ni-NTA column, a further affinity purification stage, utilising thyroglobulin was employed. This approach exploits the presence of the Galα1-3Galβl-4GlcNAc sugar moiety present in bovine thyroglobulin, which is known to bind the C-terminal of native toxin A (Krivan *et al.*, 1986; Krivan and Wilkins, 1987; Kamiya *et al.*, 1989). As the C-terminal is also present in the recombinant protein expressed herein, purification by thyroglobulin affinity chromatography was feasible. Although this additional step did result in a 50% reduction in the presence of contaminated proteins, based on the pixel intensity of protein bands in the wash fractions and those <200 kDa in elution 1 (E1), multiple bands were still observed following thermal elution (Figure 3.15).

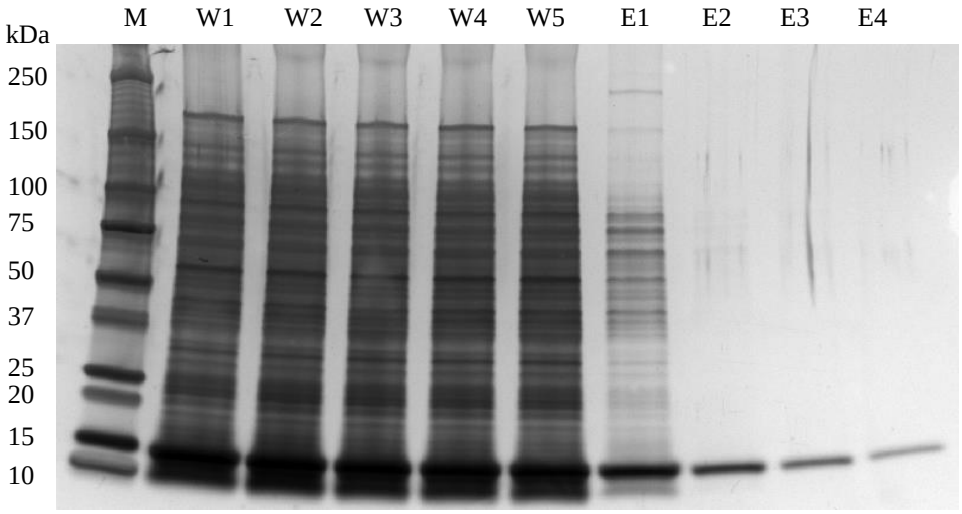


Figure 3.15: Thyroglobulin purification of rTcdA$_{900\text{-}2710}$.

Post Ni-affinity column purification rTcdA$_{900\text{-}2710}$ was subjected to thyroglobulin affinity chromatography. W1-W5; Wash, E1-E4, Elution. Molecular weight maker in kDa and analysed on a 4–20% SDS-PAGE gel and double stained with colloidal Coomassie+silver. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™ Software, v 3.0, with a band detection sensitivity of 100.

As the recombinant protein is a large protein (205 kDa), size exclusion chromatography seemed a logical step to further purify the protein. However, in spite of this, coomassie staining revealed the purified preparations still contained multiple protein bands <150 kDa (Figure 3.16).

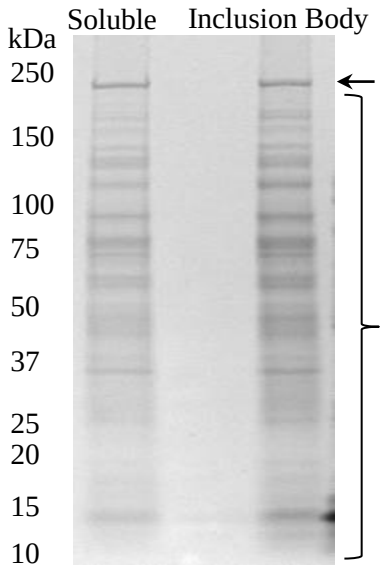


Figure 3.16: Purified rTcdA$_{900\text{-}2710}$ following size exclusion.

Post size exclusion aliquots were analysed on a 4–20% SDS-PAGE gel and stained with colloidal Coomassie. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™ Software, v 3.0, with a band detection sensitivity of 100. Molecular weight maker in kDa. Arrow indicates predicted protein size (205 kDa) and right brace indicates additional proteins present following purification.

In an attempt to determine if the contaminating proteins in the elution preparation represented truncated and/or degraded rTcdA$_{900\text{-}2710}$, or host derived *E. coli* proteins, Western blot analysis was conducted. The commercial availability of antibodies capable of recognising the 6xHis affinity tag incorporated into the N-terminus of the recombinant protein enabled the identification of some of the protein bands. Three commercially available anti-His antibodies (Penta, Tetra and RGS-His) were utilised, the latter antibody is specifically designed to detect the RGS-His$_4$ epitope encoded by the pQE30 expression vector (Figure 3.1).

In order to assess if these bands represent contaminated protein from the *E. coli* host, and may interfere in downstream application, the soluble protein from the non-induced control and M15[pREP4] strain were also assessed (Figure 3.17). Immunoreactive bands could be seen in both controls as indicated by the red boxes, but at a low intensity. This suggests histidine rich sequences or metal binding motifs

maybe present in *E. coli* host proteins (Bolanos-Garcia and Davies, 2006; Robichon *et al.*, 2011). Alternatively as these proteins were also observed in the elution fraction following thyroglobulin purification and size exclusion filtration, they may represent proteins which are bound to the recombinant protein and subsequently resolved as individual proteins following SDS-PAGE (heating and DTT treatment).

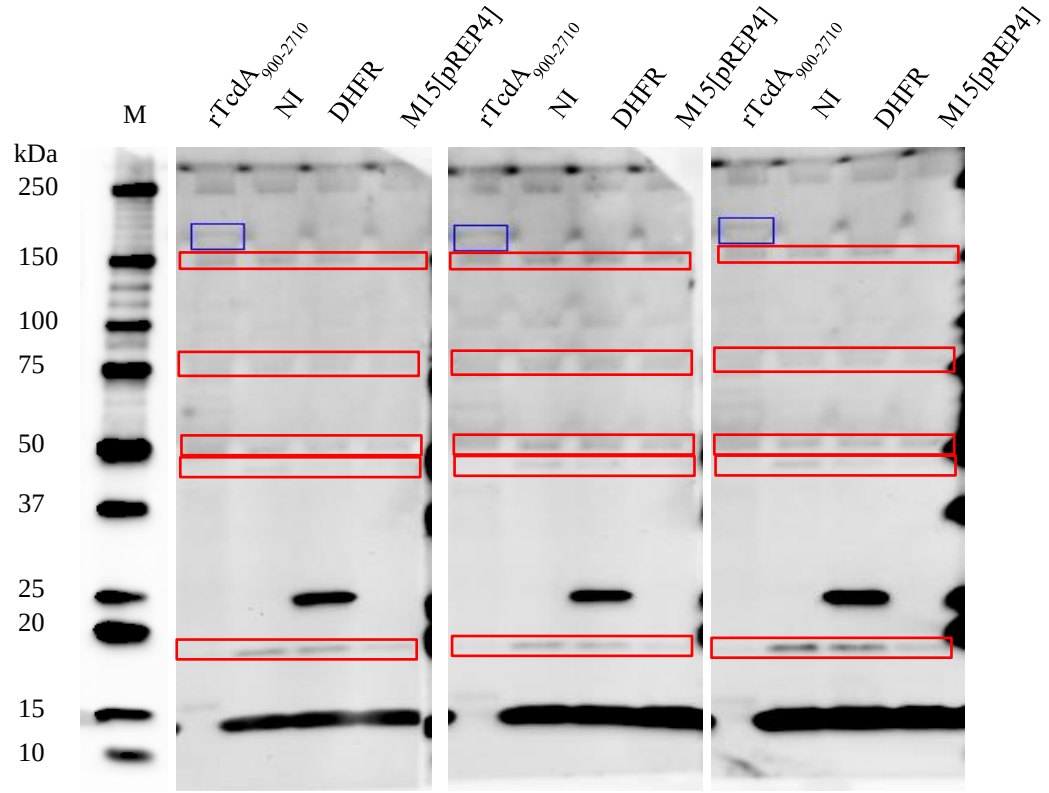


Figure 3.17: Western blot of rTcdA$_{900\text{-}2710}$, non-induced cultures and M15[pREP4].

Membranes were probed with RGS-His (left), Tetra-His (centre) and Pent-His (right), at a 1/1000 dilution and detected with SuperSignal Femto. Soluble fractions from Non-induced cultures (NI) and M15[pREP4] serve as negative controls and soluble DHFR as a positive control. Blots were imaged with the Chemi Doc xrs+ system. Blue box predicted rTcdA$_{900\text{-}2710}$, red boxes possible *E. coli* derived host proteins.

A very faint band at the predicted molecular weight was observed (Figure 3.17, blue box) Repeating of the western blot with a longer lasting ECL reagent (SuperSignal West Dura), showed the presence of rTcdA$_{900\text{-}2710}$ (Figure 3.18).

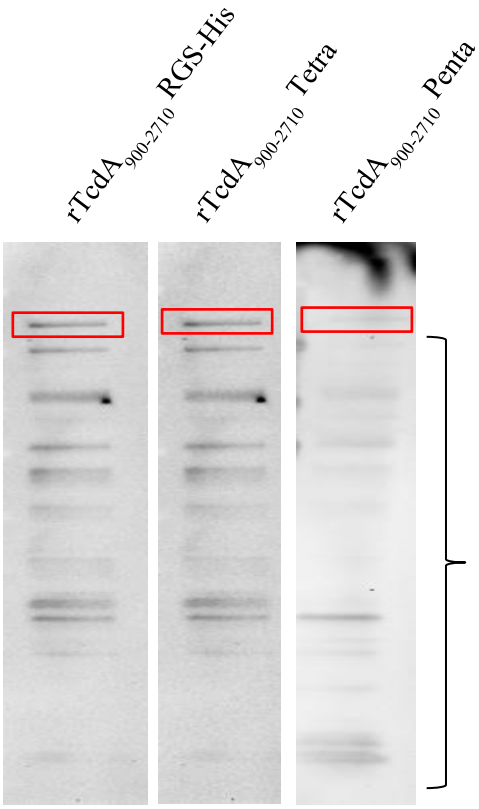


Figure 3.18: Western blot of rTcdA$_{900\text{-}2710}$.

Membranes were probed with RGS-His (left), Tetra-His (centre) and Pent-His (right), at a 1/1000 dilution and detected with SuperSignal Dura. Blots were imaged with the Chemi Doc xrs+ system. Red boxes indicate predicted protein size (205 kDa) and right brace indicates additional proteins detected by anti-His antibodies.

The final yield of rTcdA$_{900\text{-}2710}$ was determined by BCA assay to be 1 mg/L, with 300 µg/L derived from the soluble and 700 µg/L from the insoluble fraction. This yield did include multiple additional proteins, some of which represent degraded rTcdA$_{900\text{-}2710}$ and *E. coli* host proteins.

3.4.4 Expression and Purification of rTcdB$_{547-2366}$

3.4.4.1 Growth Conditions of rTcdB$_{547-2366}$

Expression and purification of rTcdB$_{547-2366}$ was relatively easy when compared to rTcdA$_{900-2710}$. In stark contrast to rTcdA$_{900-2710}$, rTcdB$_{547-2366}$ was recovered predominantly in the soluble fraction, with only a very faint band being detected in the insoluble fraction (Figure 3.19). A faint band could also be observed in the crude fraction of the non-induced control which became more apparent 3 hours post induction. Expression from M15[pREP4] in LB media at 37°C was observed to peak at 3 hour post-IPTG induction.

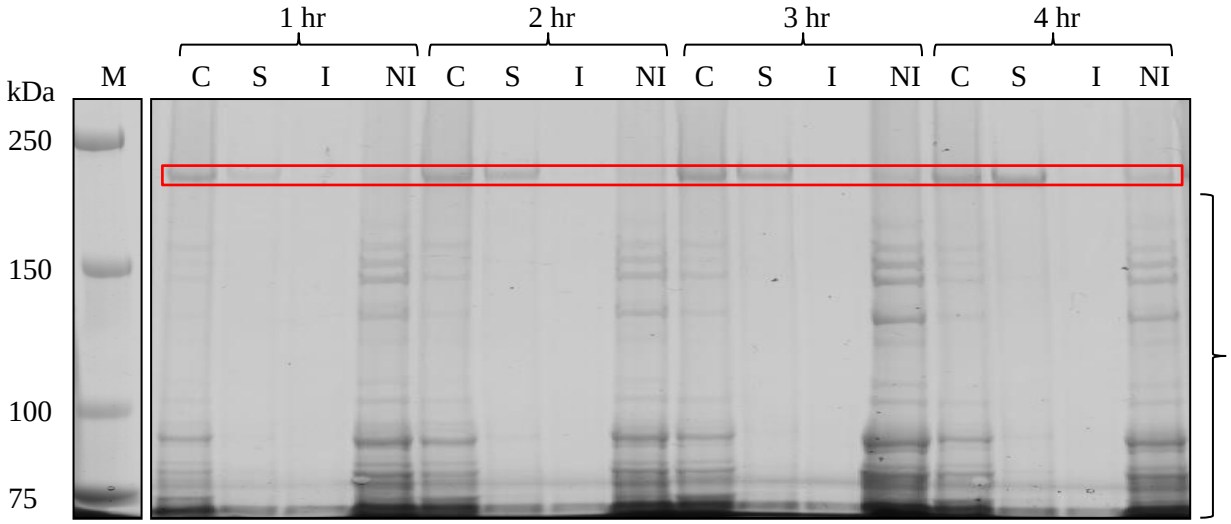


Figure 3.19: Time-course analysis of rTcdB$_{547-2366}$ expression in LB media.

Aliquots were removed post-IPTG induction at the times indicated and analysed on a 5% SDS-PAGE gel and stained with colloidal Coomassie. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure. A non-induced crude sample was analysed as a negative control. Red box indicates predicted protein size (207 kDa) and right brace indicates additional contaminating proteins.

To verify if the composition of the media influenced the level of expression of rTcdB$_{547-2366}$, in a similar manner to rTcdA$_{900-2710}$, the expression of recombinant toxin B from M15[pREP4] was analysed at 3 hours post-IPTG induction (Figure 3.20A). Variations in recombinant protein expression can be observed (Figure 3.20B). As protein was recoverable from the soluble fraction, the insoluble fraction was not analysed by SDS-PAGE, therefore the influence of media recovered from the insoluble fraction was not determined.

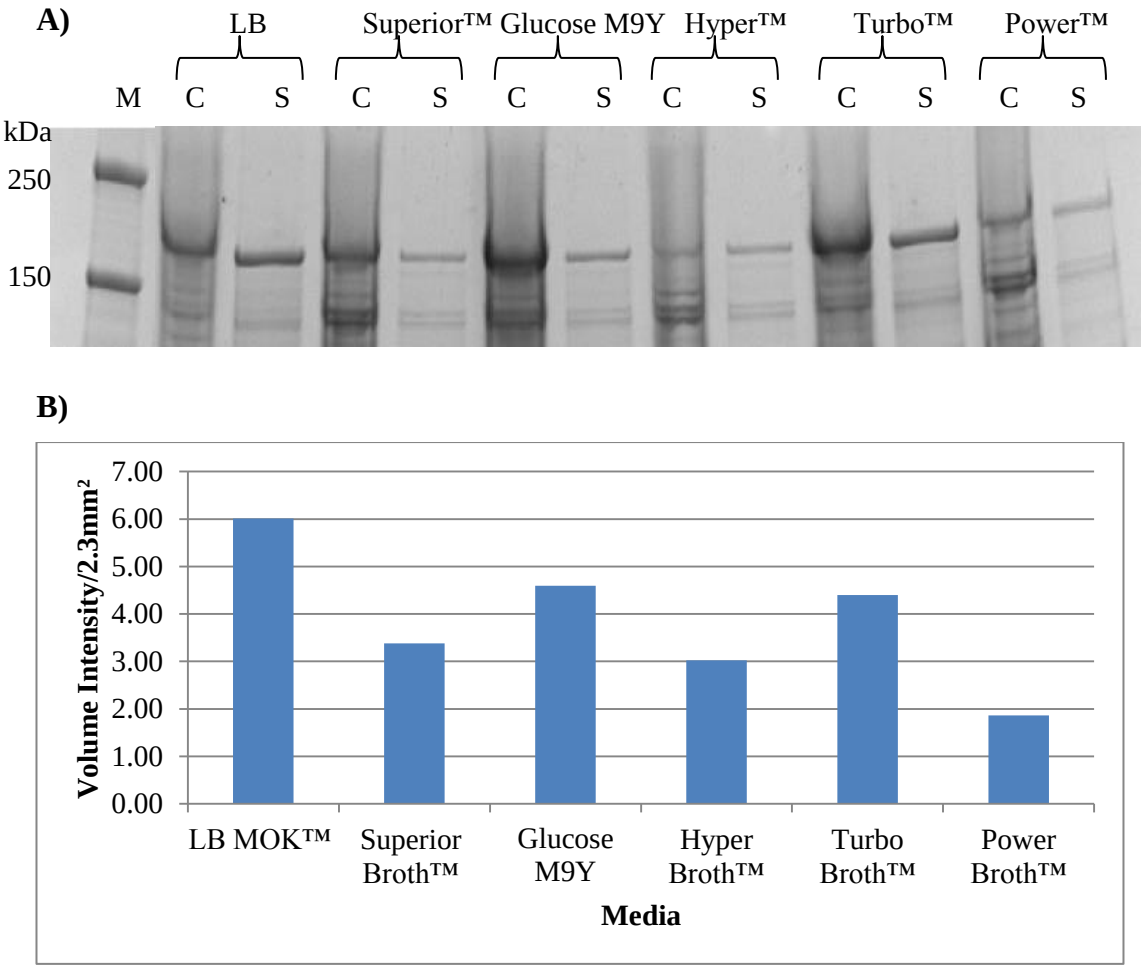


Figure 3.20: Media optimisation of rTcdB$_{547-2366}$ soluble fraction.

A) 1D SDS-PAGE analysis of rTcdB$_{547-2366}$ and **B)** Volume intensity analysis of rTcdB$_{547-2366}$ soluble fraction. Aliquots were removed 3 hour post-IPTG induction and analysed on a 4-20% SDS-PAGE gel, stained with colloidal Coomassie. Gel images were captured with a 12-bit CCD camera and analysed with Image Lab. Adjusted volume intensity values correspond to 2.3 mm². C; crude, S; Soluble.

Based on the results presented above LB media was utilised for large scale expression of rTcdB$_{547-2366}$.

The cleared lysate of both the soluble and insoluble fractions showed a heavily stained band at the expected molecular weight (207 kDa), Figure 3.21. Analysis of the elution fraction by Coomassie stained 1D SDS-PAGE revealed the presence of multiple additional protein bands. Unsurprisingly purification of the inclusion body fraction produced purer protein, with few additional proteins (<200 kDa) detected in the eluted fractions. This is probably due to fewer proteins being present in the inclusion body coupled with more stringent wash and elution steps based on pH.

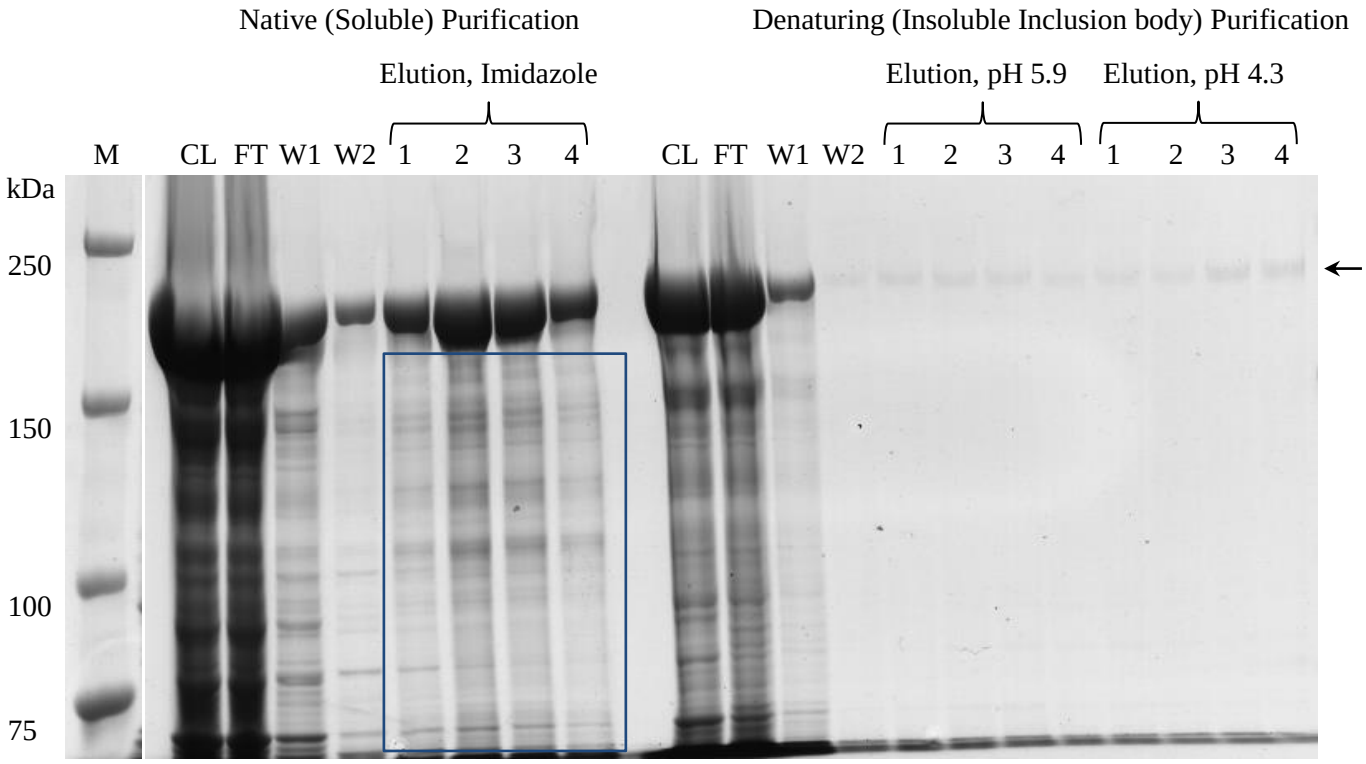


Figure 3.21: rTcdB$_{547-2366}$ purification under native (soluble) and denaturing (insoluble inclusion body) conditions.

Expression was induced with 1 mM IPTG for 3 hour and purified using Ni-NTA agarose. Proteins were analysed on a 5% SDS-PAGE gel and stained with colloidal Coomassie. CL, Cleared lysate; FT, Flow through; W1, Wash 1; W2, Wash 2. Elution of rTcdB$_{547-2366}$ was achieved with 250 mM imidazole, pH 8.0, pH 5.9 or pH 4.5 as indicated above. Arrow indicates predicted protein size (207 kDa) and blue box highlight presence of additional protein bands in elution fractions.

Given the visual intensity of the 207 kDa recombinant protein band in the cleared lysate fraction, the proportion of recombinant protein in the elution fractions recovered from the inclusion bodies appears to be disproportionate. This can partly be attributed to the loss of protein in the flow through and wash steps. It may also be an indication of the protein remaining in the purification column, as it has not been disassociated from the Ni ions. The pI of $rTcdB_{547\text{-}2366}$ is 4.6 which is close to the pH of the elution buffer (pH 4.9). Thus it is possible a significant proportion of protein was not eluted from the column and as such elution by pH is not appropriate for recombinant toxin B, recovered from the inclusion body fraction.

Following the initial binding of the recombinant protein to the Ni-NTA column, a proportion of recombinant protein was lost in the flow through and subsequent wash fractions (Figure 3.21). In order to maximise the recovery of soluble $rTcdB_{547\text{-}2366}$, the flow though was re-applied to the Ni-NTA column and the purification process repeated (Figure 3.22). While analysis of the eluted fractions following this second passage revealed the presence of purer protein, protein continued to be lost in the flow through and wash fractions.

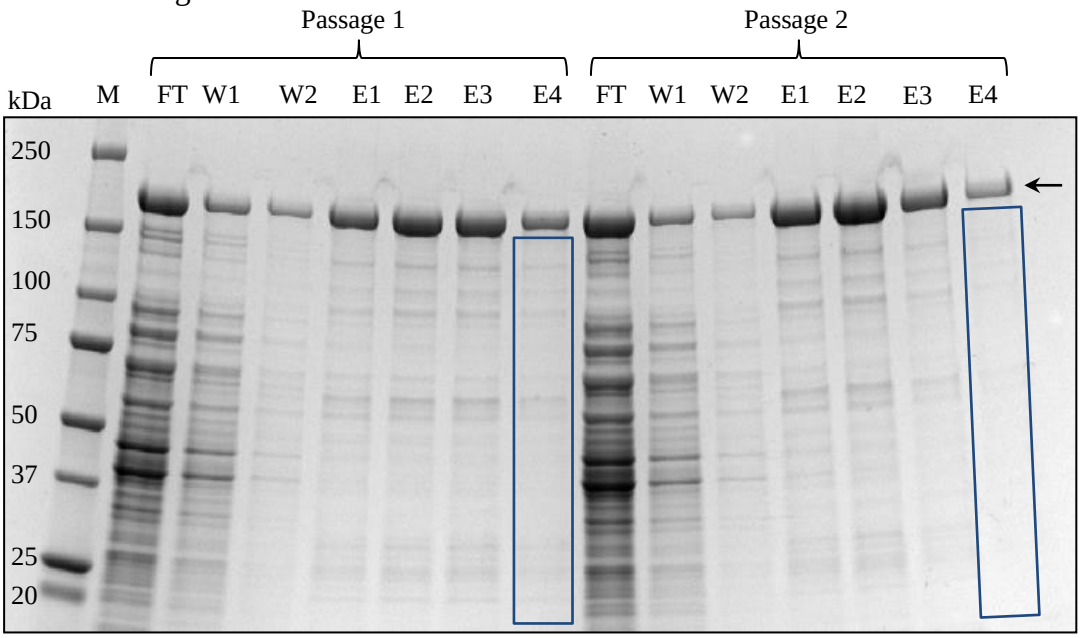


Figure 3.22: Re-application of soluble $rTcdB_{547\text{-}2366}$ flow through.

Purification was completed by re-applying the flow through, through a pre-equilibriated Ni-NTA column. Proteins were analysed on a 4–20% SDS-PAGE gel, stained with colloidal Coomassie and imaged under white light trans illumination, faint band exposure with the Chemi Doc xrs+ system. Arrow indicates predicted protein size (207 kDa) and blue box highlight presence of additional protein bands in elution fractions.

The presence of multiple bands in the elution fractions tends to be associated with non-specific binding interaction, truncated or degraded protein. In the latter case protease mediated degradation of protein was limited by the use of protease inhibitors in the lysis, wash and elution buffers. However proteolytic degradation can occur inside the cell following the production of the soluble recombinant protein, even protease deficient expression strains still have some level of protease activity.

As with $rTcdA_{900\text{-}2710}$ the use of a size exclusion filter did not remove all proteins <150 kDa, which was particularly evident when the purified fractions were concentrated. Western blotting revealed the presence of proteins present between 100-200 kDa which were likely to be truncated and/or degraded recombinant protein as they were absent from the *E. coli* expression strain (M15[pREP4]) and the non-induced negative control (Figure 3.23).

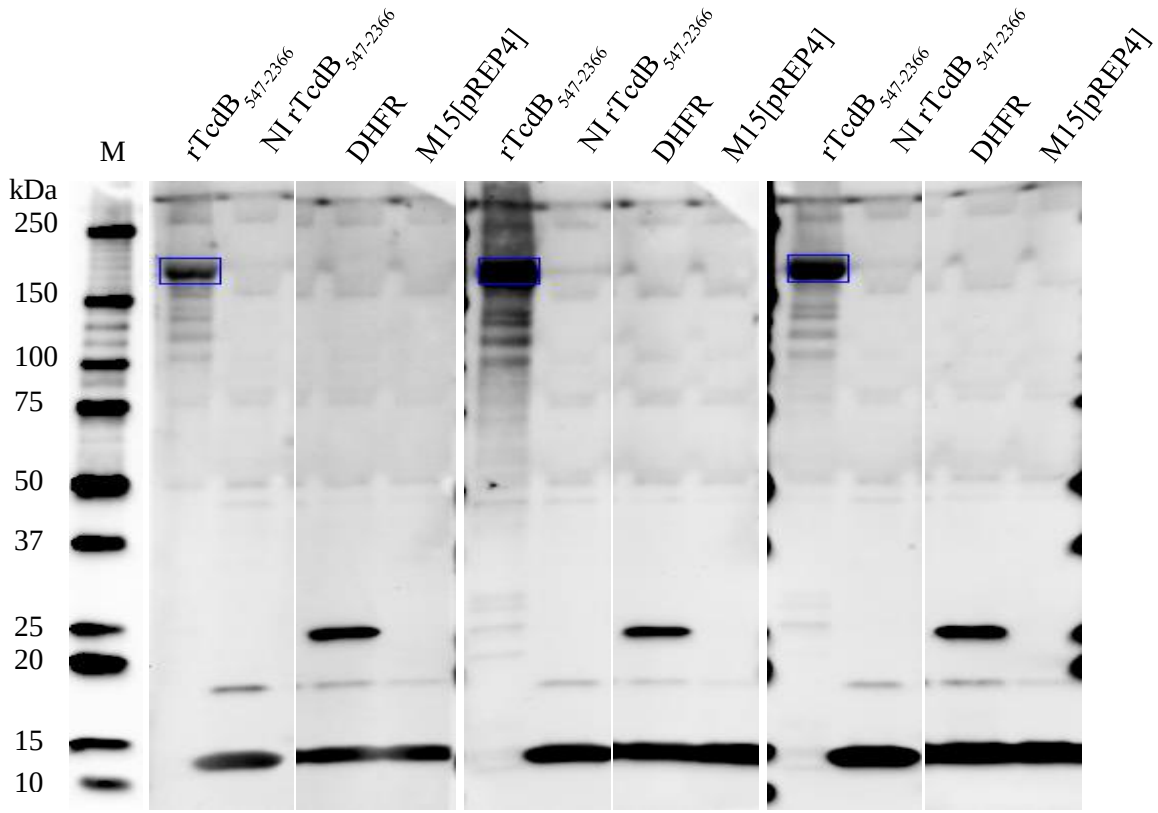


Figure 3.23: Western blot of purified $rTcdB_{547\text{-}2366}$.

A nitrocellulose membrane, probed with RGS-His (left), Tetra-His (centre) and Pent-His (right), at a 1/1000 dilution and detected with SuperSignal Femto. Non-induced cultures (NI) and soluble protein from M14[pREP4] were included as negative controls and soluble DHFR protein as a positive control. Blots were imaged with the Chemi Doc xrs+ system. Blue boxes indicate predicted protein size (207 kDa).

The final yield of rTcdB$_{547-2366}$ from the soluble fraction was 4–6 mg/L. A similar yield was achieved when the recombinant protein was recovered from the insoluble fraction.

3.5 Discussion

3.5.1 Codon Optimisation

The total (soluble and insoluble) purified yields of $rTcdA_{900\text{-}2710}$ and $rTcdB_{547\text{-}2366}$ were ~1 mg/L and ~12 mg/L, respectively. Both purified preparations contained multiple additional protein bands as detected by colloidal Coomassie staining. As non-codon optimised versions of each gene were not expressed during this study, it is not possible to draw any direct conclusions, as to whether codon optimisation has enhanced recombinant protein expression.

In a previous study the yield of non-codon optimised C-terminal domains of TcdA and TcdB in *E. coli* were reported as being 45-89 mg/L and 40 mg/L for TcdA and TcdB respectively (Letourneur *et al.*, 2003). Both the TcdA and TcdB preparations contained multiple additional proteins as detected by Coomassie stained SDS-PAGE. In an early study, Craggs (1999) expressed and purified ten overlapping fragments encompassing the complete *tcdA* gene in *E. coli* BL21 (DE3). Yielding between 5-25 µg/mL (depending on fragment expressed) from different volumes of starting culture, ranging from 10–1000 mL. The same author also attempted to increase the yield of soluble recombinant protein by the inclusion of a plasmid (pDC952) expressing the rare *argU* gene encoding the amino acids AGA/AGG. Unfortunately in the coding sequence of native TcdA the frequency of AGA and AGG are 23 and 0 per 1000 codons, respectively, and had no effect on the yield of recombinant protein produced, possibly due to the presence of other rare codons in the sequence.

A study conducted by Davies *et al.*, (2013) expressed a total of 14 codon optimised fragments from the C-terminal receptor binding domains of TcdA and TcdB, which ranged in size between 14.9–48.1 and 15.9-61.5 kDa, respectively. For the TcdA fragments expressed, 56% (5 out of 9) were found to be located in the insoluble fraction, this is in contrast to 20% of TcdB fragments (1 out of 5). These results are similar to those seen in this study with $rTcdA_{900\text{-}2710}$ expressed predominantly in the insoluble fraction and $rTcdB_{546\text{-}2366}$ easily recoverable from the soluble fraction.

Although the regions expressed in the aforementioned papers are significantly smaller than the >200 kDa fragments presented in this study, the two studies do highlight the problems of expressing TcdA in *E. coli*. Although the expression of such a large protein in a host in which the largest reported protein is 151 kDa (as per the annotated SWISS-2D database) (Hoogland *et al.*, 2004), may account for some of the problems encountered in this study. However the fact that TcdB (207 kDa) was successfully expressed whilst TcdA (205 kDa) was problematic suggests the protein itself is problematic as opposed to the size of the protein.

Codon optimisation is a computer driven process in which the codons of a gene is modified to that seen in genes which are highly expressed in *E. coli* during optimum growth. Given that the introduction and subsequent expression of a foreign protein in *E. coli* does not represent optimum growth, the genes which are highly expressed and hence the optimum codons are likely to be different. Thus the codon optimisation programme utilised in this and indeed in other studies may not be appropriate.

What is evident from this and other studies is expression of the *C. difficile* toxin fragments; particularly TcdA is problematic in *E. coli*. A notion supported by other researchers who have advocated the use of other expression systems such as Baculovirus (Heinrichs *et al.*, 2012) or *Bacillus* spp. (Tang-Feldman *et al.*, 2002; Ackermann *et al.*, 2004b; Wang *et al.*, 2012). The work conducted by Heinrichs and colleagues (2012) indicates, that regardless of the host utilised the resulting recombinant toxin is immunogenic, thus can be utilised as surrogates for therapeutic development.

3.5.2 Transformation Efficiency of $rTcdA_{900\text{-}2710}$ and $rTcdB_{547\text{-}2366}$

The transformation of pQE30-$rTcdA_{900\text{-}2710}$ into various strains of *E. coli* was problematic, with attempts to directly transform DH5α, being unsuccessful, suggesting that the insert was either unstable or toxic to some *E. coli* strains. Similar problems pertaining to the stability of plasmids containing the TcdA

sequence or regions thereof have been reported in the literature (Phelps *et al.*, 1991; Craggs, 1999; Ackermann *et al.*, 2004b). Transformation of M15[pREP4] and SG13009[pREP4] with pQE30-rTcdA$_{900-2710}$ was only achieved following repeated efforts. In order to assess whether the failure to transform consistently was due to problems with the donor DNA, plasmid was extracted from a transformed strain of SG13009[pREP4] and used as donor DNA for a second transformation. This proved successful with DH5α, be it at a very low efficiency (2.25×10^2 CFU/µg DNA). The donor DNA following propagation through an *E. coli* host may have been modified in a manner which enhanced its transformation ability. It has previously been reported that transformation of *B. anthracis* was possible only after the propagation of the plasmid through a *dam* deficient *E. coli* strain (Marrero and Welkos, 1995). Although the reported research does not entirely mirror our findings it does support the notion of propagating plasmids through strains to improve transformation efficiency. Alternatively Tsen *et al.* (2002) and Etchuuya *et al.*, (2011) have suggested that the uptake of plasmid DNA (during the natural transformation process in *E. coli*) is promoted by the presence of cell contents released from *E. coli* lysates, such as DNA-associating molecules. During the cell lysis process it is possible such molecules were released, and in their presence, transformation of DHα became possible.

The transformation problems encountered with TcdA$_{900-2710}$ and TcdB$_{547-2366}$ may be associated with the size of the inserts (5445 and 5471 bp for TcdA$_{900-2710}$ and TcdB$_{547-2366}$, respectively), transformation efficiency has been shown to decrease with an increase in plasmid size (Hanahan, 1983). Although it was not in the remit of this research it would have been interesting to assess the transformation efficiency following electroporation, as electroporation is known to facilitate the uptake of large molecules of DNA (inserts >3 kb) and provides the highest transformation efficiencies (Woodall, 2003).

3.5.3 Expression and Purification of rTcdA$_{900-2710}$

Expression and purification of TcdA in *E. coli* is frequently associated with low yields and the formation of inclusion bodies necessitating the optimisation of

induction conditions and purification procedures (Phelps *et al.*, 1991; Craggs, 1999; Letourneur *et al.*, 2003; Ackermann *et al.*, 2004b). Similarly, our attempts to express and purify $rTcdA_{900\text{-}2710}$ required optimisation at every stage.

Historically, LB media has been used for the cultivation of *E. coli* and expression of recombinant genes from *E. coli* (Aristidou *et al.*, 1999; Broedel Jr *et al.*, 2001). The results from our study are in agreement with those of Broedel Jr *et al*. (2001) who demonstrated that the composition of the growth media effected the accumulation of recombinant proteins. While $rTcdA_{900\text{-}2710}$ was expressed in all of the media assessed during this study, the level achieved varied and maximum expression was seen in Superior Broth™. As this media is proprietary, it was not possible to decipher the composition of the media.

Maximising the yield of recombinant protein is dependent on achieving a high cell concentration (biomass). For biomass production to be at its maximum, an alternative carbon source to glucose - the most commonly used sugar - must be used. During *E. coli* culture, glucose is converted to acidic by-products (such as acetic acid) which inhibit cell growth (limiting biomass production) and have been reported to impede recombinant protein expression (Aristidou *et al.*, 1999). Although not investigated in this study, the effect of non-metabolisable sugars could provide a means of increasing the yield of soluble protein. Bowden and Georgiou (1988) reported that the addition of non-metabolisable sugars (sucrose and raffinose) to the growth media increased the recovery of soluble protein by reducing inclusion body formation and improving folding. The presence of non-metabolisable sugars either increases the osmolarity of the media (Schein, 1989) or the sugars exert a direct stabilising effect on the polypeptides, forming soluble protein and reducing aggregation (Bowden and Georgiou, 1988).

In *E. coli*, Hsp70 (DnaK, DnaJ and GrpE) and Hsp60 (GroEL and GroES) are the major chaperone families involved in protein folding in the cytoplasm. A number of studies have demonstrated that the overproduction of GroESL increases the yield of correctly folded soluble proteins (Goloubinoff *et al.*, 1989; Makrides, 1996), although these observations appear to be protein specific. It is difficult to establish

which, if any, chaperones truly effected protein expression. It is believed *E. coli* has optimised its chaperone content with regards to the expression of host cell proteins (Makrides, 1996). Thus, the chaperones (or the ratio) which are available for the expression of heterologous proteins may not be sufficient and as such would need optimisation.

All other researchers who have experienced difficulties expressing TcdA have performed expression in shake flasks (Phelps *et al.*, 1991; Craggs, 1999; Letourneur *et al.*, 2003; Ackermann *et al.*, 2004b). To date, only one research group (Tian *et al.*, 2012) have reported utilising a fed-batch process for the expression of a recombinant fusion protein, containing the C-terminal regions of TcdA and TcdB, although the authors fail to provide data on the protein yield achieved. Based on the methods provided, it appears soluble protein was extracted, with no reported difficulties encountered in expression or purification (Tian *et al.*, 2012).

The *E. coli* host strain used in this study to express $rTcdA_{900\text{-}2710}$ M15[pREP4] produces a cytoplasmic protease, La, which is known to degrade non-native proteins (Beerens *et al.*, 1963). Thus, it is possible that $rTcdA_{900\text{-}2710}$ has undergone host cell mediated proteolysis. Strategies for minimising proteolysis include use of protease-deficient strains, altering the culture media, temperature, co-expression of chaperones and construction of fusion proteins. The successful transformation of three *E. coli* strains (M15[pREP4], SG13009[pREP4] and DH5α), all of which are Lon^+, limited the assessment if an increase in expression would have occurred with a protease-deficient strain.

More recently Donald and colleagues (2013) have overexpressed full-length genetically inactivated toxin A and B in a non-toxigenic, non-sporogenic *C. difficile* strain. Utilisation of two genetic tools, the ClosTron mutagenesis and *Escherichia coli*–Clostridium plasmid shuttle vector system, enabled inactivation of spore formation (Heap *et al.*, 2007; Underwood *et al.*, 2009) and episomal expression of recombinant proteins (Heap *et al.*, 2009). These recombinant toxins were found to be immunogenic, although they did retain cytotoxicity (Donald *et al.*, 2013). The

advancements in genetic technology and further optimisation of this method may facilitate the safe expression of toxin A, in high yields.

3.5.4 Expression and Purification of rTcdB$_{547\text{-}2366}$

In contrast to the situation with rTcdA$_{900\text{-}2710}$, a high level expression system in which equal quantities of protein were recovered from the soluble and insoluble fractions was developed for rTcdB$_{546\text{-}2366}$. The total average yield of rTcdB$_{547\text{-}2366}$ was 8-12 mg/L of bacterial culture, representing a purity of 80%, as determined by colloidal Coomassie stain. This yield is similar to that reported by Yang *et al*. (2008) (5-10 mg/L), who expressed full length TcdA and TcdB in a *B. megaterium* expression host. However, the reported expression levels are substantially lower than the 40 mg/L (C-terminal TcdB) reported by Letourneur *et al*. (2003). The high yield reported by Letourneur and colleagues is most likely attributed to the size of the insert expressed (71 kDa), which is ~1/3 of the size of the insert expressed herein, generally proteins >60 kDa are difficult to express in *E. coli* (Bell, 2002; Structural Genomics Consortium *et al.*, 2008).

Although the yield and purity of recombinant protein produced in this study was comparable to that reported in the literature, purification of the protein required multiple re-applications through the Ni-NTA column. Analysis of the purification fractions revealed that protein corresponding to the correct molecular weight was being lost in the flow through and wash steps. The presence of protein in the flow through is usually attributed to the protein not binding to the Ni-NTA, either due to protein degradation or inaccessibility of the 6x His tag. The repeated application of the flow through, resulted in further binding of the His tagged protein to the Ni ions on the columns. However as this protein is so large (>200 kDa) it may have rendered the remaining Ni sites inaccessible resulting in the continued loss of recombinant protein in the flow through. Although the re-purification of the protein appears to be tedious, it does provide purer protein through each subsequent passage.

As the strains and vectors used for the expression of $rTcdA_{900\text{-}2710}$ and $rTcdB_{547\text{-}2366}$ are the same, we can conclude that the problems experienced with expression of $rTcdA_{900\text{-}2710}$ was associated with this protein alone.

3.6 Conclusion

This chapter aimed to develop a high yield recombinant expression system for $rTcdA_{900\text{-}2710}$ and $rTcdB_{547\text{-}2366}$. Unsurprisingly, analysis of the sequences identified differences in the codon usage patterns of the original genes. Replacement of these codons was possible through *de novo* synthesis. With regards to $rTcdB_{547\text{-}2366}$, a high yield recombinant expression system was developed; with 8-12 mg/L of protein produced displaying 80% purity following colloidal Coomassie staining. However, this was not the case for $rTcdA_{900\text{-}2710}$, with significant optimisation required at every stage of the process from transformation, expression to purification. In spite of such optimisations, the yield (1 mg/L) and purity (10% colloidal Coomassie) of protein produced $rTcdA_{900\text{-}2710}$ was disappointing. Subsequent analysis of the sequence was unable to definitively identify which, if any, aspects were responsible for the reduced yields obtained. Despite the advancements in recombinant expression technology for *E. coli*, this chapter highlights that the successful expression of genes (particularly large genes) is reliant on a number of factors. Simply altering a presumed key factor may not have any bearing on the expression level obtained. Although there is an array of bioinformatic software available for the analysis of gene sequences, the data generated must be regarded with caution as it may not necessarily reflect experimental outcomes.

CHAPTER FOUR

THE IDENTIFICATION OF IMMUNODOMINANT REGIONS OF TOXIN A AND TOXIN B

4.1 Introduction

Toxins are the primary virulence factors of many clostridial diseases (Introduction, Section 1.2.4, Table 1.1) and the generation of serum antitoxin immune responses following infection has been documented for *C. tetani*, *C. botulinum*, *C. perfringens*, *C. septicum* and *C. difficile* (Johnson, 1997). This anti-toxin response can be used as an indicator of immunity, a measure of the extent of infection in a population or serve as a diagnostic marker. Studies suggest that individuals who succumb to recurrent infections do so as a consequence of being unable to mount a robust toxin neutralising, protective antibody response (Johnson *et al.*, 1992; Kelly *et al.*, 1992; Kelly, 1996; Johnson, 1997; Kyne *et al.*, 2000; Kyne *et al.*, 2001a; Kyne *et al.*, 2001b; Johnson, 2009).

Patients infected with *C. difficile* display an array of symptoms, ranging from asymptomatic carriage, moderate, self-limiting diarrhoea to life-threatening pseudomembranous colitis (Kyne *et al.*, 2001a). It is hypothesised that the range of symptoms is attributed, in part, to the generation (or lack) of an adequate immune response. It is estimated that 1-3% of the healthy adult population is colonised with *C. difficile* (Kelly and LaMont, 1998), with the number increasing to 7-11% for hospital in-patients and 20% following antibiotic usage (McFarland *et al.*, 1989). Despite the relatively low level of colonisation recorded in healthy adults, the serum IgA and IgG antibodies to toxins A and B have been detectable in approximately 60% of the population (Viscidi *et al.*, 1983; Johnson *et al.*, 1992; Kelly *et al.*, 1992; Warny *et al.*, 1994), with the prevalence of antibodies greater after the age of two and falling in the elderly population (Viscidi *et al.*, 1983; Bacon and Fekety Jr, 1994).

The presence of an antitoxin antibody response may be an indicator of prior exposure to toxigenic *C. difficile* in childhood, or may have occurred as a consequence of sub-clinical infection in adults. The importance of an anamnestic toxin immune response has been documented in several studies (Johnson *et al.*, 1992; Warny *et al.*, 1994; Kyne *et al.*, 2000; Loo *et al.*, 2011), with the inability to generate a robust immune response following exposure to *C. difficile* shown to correlate with the

severity and duration of disease and significantly increase the risk of reinfection (Aronsson *et al.*, 1983, 1985; Warny *et al.*, 1994).

As can be seen from Figures 4.1 and 4.2 the *C. difficile* neutralising antibodies generated/reported in the literature predominantly target the C-terminal domains of each toxin. It is hypothesised the antibodies block binding of the toxins to the host receptor, thus preventing toxin uptake. The precise regions (epitopes) recognised by these antibodies can be linear or conformational and are yet to be fully elucidated. Whilst the region of toxin A recognised by PCG-4, has been narrowed down to 44 amino acids, this is not the case for the majority of other neutralising antibodies. The ability to precisely define the regions (epitopes) within toxin A and B which bind toxin neutralising antibodies would aid in the development of therapeutics (vaccines and antibodies) and diagnostics. As part of this project the immunogenicity of the C-terminal and translocation domain of recombinant toxin A and B (as described in Chapter 3), the native toxins and toxoid A were assessed. Regions were identified by probing both the full length and protease digests of each protein with serum from *C. difficile* infected patients and with commercially available toxin neutralising animal serum derived from horse and goat. The latter of which has been derived from the toxoided culture filtrate of a toxin A and B infected *C. difficile* isolate.

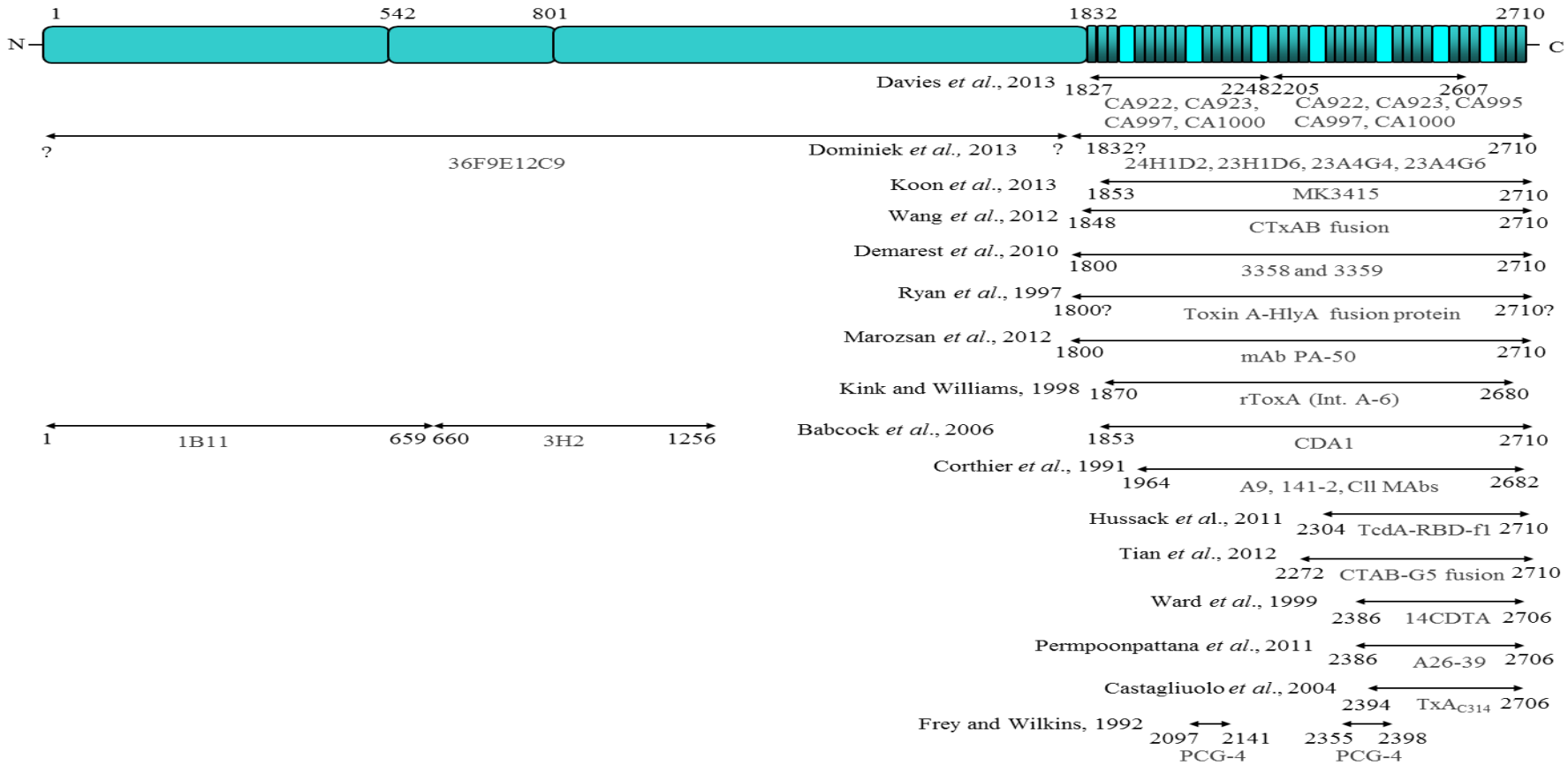


Figure 4.1: Location of TcdA antibodies.

Schematic representation of antibodies currently targeting regions of TcdA. Numbers represent amino acid position based on *C. difficile* 630 toxin A (EMBL CAJ67494.1). ?; amino acid region unknown. CDA1 is also known as MK3415 and MDX006.

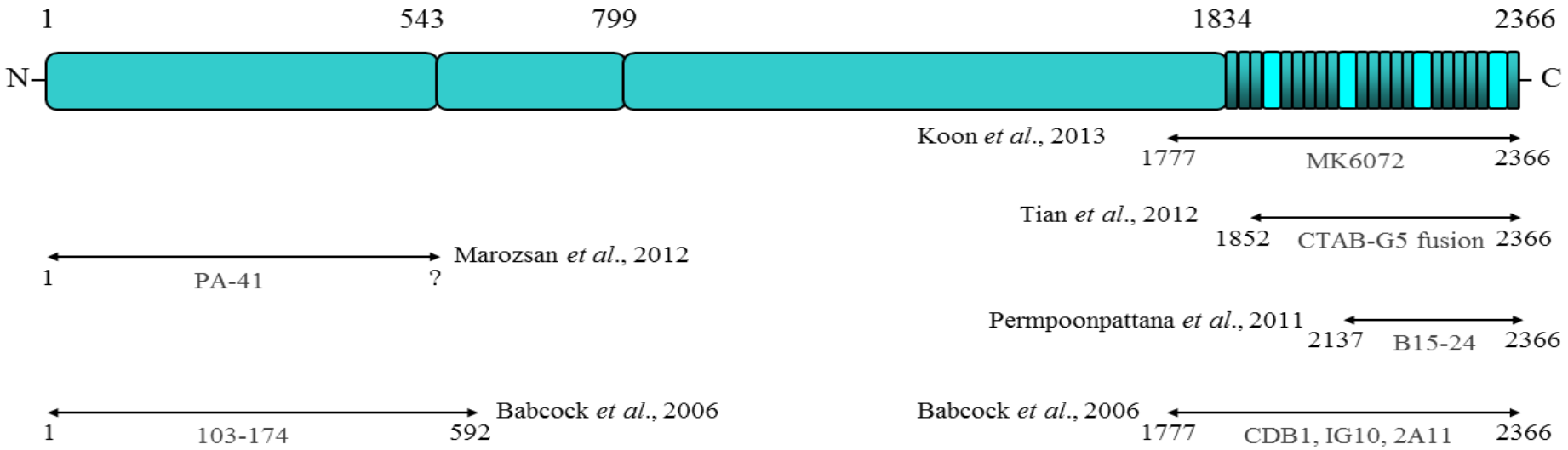


Figure 4.2: Location of TcdB antibodies.

Schematic representation of antibodies currently targeting regions of TcdB. Numbers represent amino acid position based on *C. difficile* 630 toxin B (EMBL CAJ67492.1). ?; amino acid region unknown. CDB1 is also known as MK6072 and MDX1388.

Recombinantly expressed proteins, may be subjected to forms of post-translational processes in their new host, which differ from those they would encounter in *C. difficile* and as a consequence could affect their ability to be recognised by antibodies stimulated in response to infection (Kyne and Kelly, 1998). For this reason the ability of antisera to recognise both recombinant and native toxins were assessed.

In addition, the ability of antisera to recognise formaldehyde inactivated toxins was also investigated. It is highly likely that, in addition to eliminating toxicity, the process has also altered their immunogenicity. Formaldehyde inactivation of toxins, such as those produced by *C. botulinum*, *V. cholera*, *Corynebacterium diptheriae*, *C. tetani* and *C. difficile*, have been employed as a means of generating immunogens which can be used as vaccines or to generate antitoxins (Baxter, 2007; Jones *et al.*, 2008). The loss of toxicity is a consequence of the formation of cross links between amino acids, primarily at lysine residues, altering the structure of the protein (Jones *et al.*, 2008). Given that antibodies recognise linear as well as conformational structures, a by-product of the toxoiding process may be the loss of protective epitopes. Although toxoids are reported to be highly immunogenic in terms of total antibody titres, studies have shown the level of neutralising antibodies stimulated against the toxoid are low as reflected in the level of protection conferred (Fiock, 1962; Libby and Wilkins, 1982; Siegel, 1988; Nencioni *et al.*, 1991). This suggests the toxoiding process alters the confirmation of the antigen such that, certain neutralising epitopes are absent. Given that toxoids are being considered as potential vaccine candidates, this raises issues as to their ability to stimulate a robust protective antibody response (Torres *et al.*, 1995; Sougioultzis *et al.*, 2005; Greenberg *et al.*, 2012; Siddiqui *et al.*, 2012; Anosova *et al.*, 2013).

4.2 Aims and Objectives

The aim of this chapter is to identify immunodominant regions of toxins A and B using toxin neutralising immune sera from animals and sera collected from *C. difficile* infected patients.

The experimental objectives are to:

1. Optimise the enzymatic digestion conditions for native, toxoided and recombinant toxins A and B.
2. Compare the digestion patterns of native, toxoided and recombinantly produced toxin A and toxin B.
3. Develop a Western blot assay to identify immunogenic toxin regions.
4. To collect human immune sera from *C. difficile* infected individuals under an ethically approved protocol.
5. Identify immunogenic toxin fragments recognised by sera from *C. difficile* infected patients and compare the responses to those seen with animal neutralising sera.

4.3 Materials and Methods

Unless otherwise stated, all reagents were of molecular biology grade and were purchased from Fisher Scientific, UK. Clostripain, N-α-Benzoyl-D-arginine *p*-nitroanilide hydrochloride (BAPNA) and 4-Morpholinepropanesulfonic acid sodium salt (MOPS) were purchased from Sigma Aldrich, UK. Native toxin A, toxin B and toxoid A were purchased from List Biological Laboratories, Inc. (California, USA).

4.3.1 Human Sera Immune Study

A research study was designed based on literature searches of peer-reviewed journals and in collaboration with Drs. Robin Howe and Lim Jones, from the University Hospital of Wales. Research Ethics Committee (REC), Research and Development (R&D) and Site-Specific Information (SSI) forms were completed using the Integrated Research Application System (IRAS) (https://www.myresearchproject.org.uk/).

4.3.2 *in silico* Protease Identification

The protein sequences for *C. difficile* 630 toxin A (EMBL CAJ67494.1) and toxin B (EMBL CAJ67492.1) were obtained from Swiss-Prot/TrEMBL database at the ExPASy Molecular Biology Server (http://www.expasy.org). Protease cleavage sites were identified *in silico* using the PeptideCutter software (http://www.expasy.ch/tools/peptidecutter/). The pI and MW of the predicted fragments were estimated with Compute pI/MW Tool (http://www.expasy.ch/tools/pi_tool.html).

4.3.3 Clostripain Activation

Unless otherwise stated, all solutions were prepared in 10 mM MOPS buffer (pH 7.4). Clostripain (226 U/mg) was re-suspended, aliquoted and stored at -20°C. BAPNA was re-suspended in 10 mL DMSO. A working solution of 80 µM was

prepared by diluting in MOPS. Clostripain was activated in MOPS buffer containing 10 mM $CaCl_2$ and 2.5 mM DTT at 25±0.2°C for 3 hours (Techne Dri-Block® DB-3D). Following activation, 1U of clostripain was added to 80 µM BAPNA. The reaction was carried out in cuvettes of 1 cm light-path at room temperature. The formation of *p*-nitoaniline was monitored as an increase in absorbance at OD_{410} for 20 minutes using an automated spectrometer (Helios, Thermo Scientific, UK). All data was recorded in duplicate with a negative control.

4.3.4 Digestion Conditions

$rTcdA_{900-2710,}$ $rTcdB_{547-2366}$, TcdA, TcdB or toxoid A (5 µg) were digested with 1U of activated clostripain. All digestions were carried out at 25±0.2°C (Techne Dri-Block® DB-3D), for 30 minutes, 2, 4, 24, 36 and 48 hours. Digestion was inhibited with Roche Complete mini protease inhibitor cocktail and samples were combined with 2 parts 1x SDS-PAGE buffer. Controls are listed in Table 4.1. Samples were briefly centrifuged (200 *g*, 10 seconds) and stored at -80°C until further use.

Table 4.1: Enzyme digestion controls

Control	Abbreviation
Inactive enzyme	IE
Active enzyme, 3 hours	AE, 3 hours
Active enzyme, 3 hours plus inhibitor	AEI, 3 hours
Active enzyme, 24 hours, 25°C	AE, 24 hours
Active enzyme, 24 hours, 25°C plus inhibitor	AEI, 24 hours
Undigested protein	
Undigested protein, 24 hours 25°C	

1U (4.4 µL) of enzyme was used in each control, 8.8 µL of inhibitor was added were appropriate, all controls were made up to a final volume 38.2 µL with sterile water and combined with 2 parts 1x SDS-PAGE buffer.

4.3.5 1D SDS-PAGE and Western Blot

1D SDS-PAGE gels were completed as detailed in Chapter 2, Section 2.3; Criterion™ TGX™ Precast Gel (4–20%), were used for all digestion profiles. Ten µL of sample was loaded in duplicate. Markers included 4 µL Bio-Rad Precision Protein™ Dual Xtra Standards and Spectra ™ Multicolor High Range Protein Ladder (Thermo Scientific, UK). Gels were double stained as detailed in Chapter 2, Section 2.5. Transfer was completed as detailed in Chapter 2, Section 2.6, membranes stained as detailed in Chapter 2, Section 2.7 and Western blot and chemiluminescence completed as per Chapter 2, Section 2.8 and 2.9 using the antibodies listed in Table 4.2. Western blot controls included active clostripain enzyme and soluble protein extracted and purified from a 3 hour culture of M15[pREP4]. M15[pREP4] was cultured and purified as detailed in Chapter 3.

Table 4.2: Primary and secondary antibodies used for Western blotting in this study.

Antibody	Species	Specificity	Source	Dilution
Goat anti-toxin reagent	Goat[a]	Neutralisation of *C. difficile* toxin	Techlab®, UK	1/25
***C. sordellii* anti-toxin**	Horse[a]	Toxin neutralisation	NIBSC	1/25
***C. difficile* toxin B**	Rabbit[a]	N terminal 60 kDa catalytic fragment TcdB from *E. coli*	Abcam, UK	1/500
***C. difficile* toxin B**	Mouse[b]	Full length TcdB	Abcam, UK	1/110
Anti-toxin A	Goat[a]	Unknown	List Biological Laboratories, Inc.	1/10
Human sera	Human	Unknown	Clinical samples, this study	1/50
Anti-Goat-HRP	Rabbit	Goat IgG (H+L)	Life Technologies, UK	1/10,000
Anti-Mouse-HRP	Goat	Mouse IgG (H+L)	Thermo Scientific Pierce, UK	1/10,000
Anti-Rabbit-HRP	Goat	Rabbit IgG (H+L)	Thermo Scientific Pierce, UK	1/10,000
Anti-Horse-HRP	Rabbit	Horse IgG (H+L)	Abcam, UK	1/10,000
Anti-Human IgG-HRP	Mouse[b]	Human IgG Fc PAN (all subclasses)	Stratech, UK	1/4000
Anti-Human IgA-HRP	Mouse[b]	Human IgA heavy chains (all subclasses)	Life Technologies, UK	1/2000

[a] polyclonal, [b] monoclonal. HRP, Horse Radish Peroxidase. H, heavy chain, L, Light chain. NIBSC, The *National Institute for Biological Standards and Control*.

4.3.6 Silver Stain for Mass Spectrometry

Western blot images captured on the Chemi Doc xrs+ system were aligned and common immunoreactive bands identified. To facilitate mass spectrometric analysis, a compatible silver staining method was employed. Gels were prepared as detailed in Section 4.3.5. For silver staining 250 mL of each solution was prepared just prior to use and all incubations were carried out on a Stuart gyro-rocker (Fisher Scientific, UK) set at 30 r.p.m. at room temperature. Following electrophoresis, gels were immersed for 30 minutes in fixing solution (10% (v/v) acetic acid, 40% (v/v) ethanol) followed by sensitizing solution (30% (v/v) ethanol, 17% (w/v) sodium acetate, 10 mL, and 5% (w/v) sodium thiosulfate for 30 minutes. Gels were washed in diH_2O for three successive 5 minute washes, incubated in 25 mL of 2.5% (w/v) silver nitrate) for 20 minutes, washed twice in diH_2O for 1 minute and then developed (6.25% (w/v) sodium carbonate, 100 µL, 36% (v/v) formaldehyde) for 5-10 minutes. The reaction was stopped with 3.65% (w/v) EDTA and the gel washed three times in diH_2O for 5 minutes. Selected bands were subjected to sequencing following the method detailed in Chapter 2, Section 2.10.

4.4 Results

4.4.1 The Identification of Potential Protease Cleavage Sites in Toxins A and B by *in silico* Analysis

The amino acid sequences of toxins A and B of *C. difficile* strain 630 were obtained from the Swiss-Prot/TrEMBL database at the ExPASy Molecular Biology Server, and were analysed using PeptideCutter software to identify protease recognition sites. Of the 37 chemicals and proteases listed in the programme, 13 were predicted to be unable to digest the toxins. Those capable of digesting the toxins were predicted to generate between 1–1251 fragments. In order to generate toxin digestion patterns which were reproducible and easy to interpret, proteases which yielded greater than 50 digestion fragments were discounted, leaving nine possible enzymes/chemicals (Table 4.3). As can be seen from the table the enzyme clostripain was predicted to yield the greatest number of digestion fragments from both full length and recombinant variants of TcdA and TcdB, and was selected for digestion analysis.

Table 4.3: *in silico* digestion of TcdA, toxoid A, rTcdA$_{900\text{-}2710}$, TcdB and rTcdB$_{547\text{-}2366}$.

Proteases and chemicals predicted to cleave native and recombinant TcdA and TcdB between 1-50 times identified ExPASy PeptideCutter.

Enzyme / Chemical	Specificity	TcdA	rTcdA$_{900\text{-}2710}$	TcdB	rTcdB$_{547\text{-}2366}$
Clostripain[a] and Arg-C proteinase[a]	Hydrolyzes arginine and lysine bonds	51	27	50	32
CNBr[b]	Hydrolyses methionine.	31	18	12	10
Hydroxylamine[b]	Cleaves asparaginyl-glycine bonds	28	27	17	12
BNPS-Skatole[b]	Cleaves tryptophan-containing proteins	27	21	17	12
Iodosobenzoic acid[b]	Cleaves tryptophan	27	21	46	30
NTCB (20-nitro-5-thiocyanobenzoic acid)[b]	Cleaves cysteine	8	6	10	9
Caspase1[a]	A number of different cleavage patterns	6	6	2	3
Proline-endopeptidase [*][a]	Cleaves proline	5	2	5	4
Caspase10[a]	Cleavage sites composed of Leu-Gln-Thr-Asp--Gly	0	0	2	0

Cleavage sites for toxoid A are not reported but are presumed to be the same as those identified for native TcdA. [a] enzyme, [b] chemical. Leu; leucine, Gln; glutamine, Thr; threonine, Asp; asparagine, Gly; glycine.

4.4.2 Proteolytic Digestion of Toxin

On the basis of the *in silico* analysis the protease clostripain was identified as a suitable protease with which to digest both toxins (Tables 4.3). Activation of clostripain was confirmed by the hydrolysis of the substrate BAPNA, resulting in the formation of the coloured product *p*-nitoaniline (Figure 4.3).

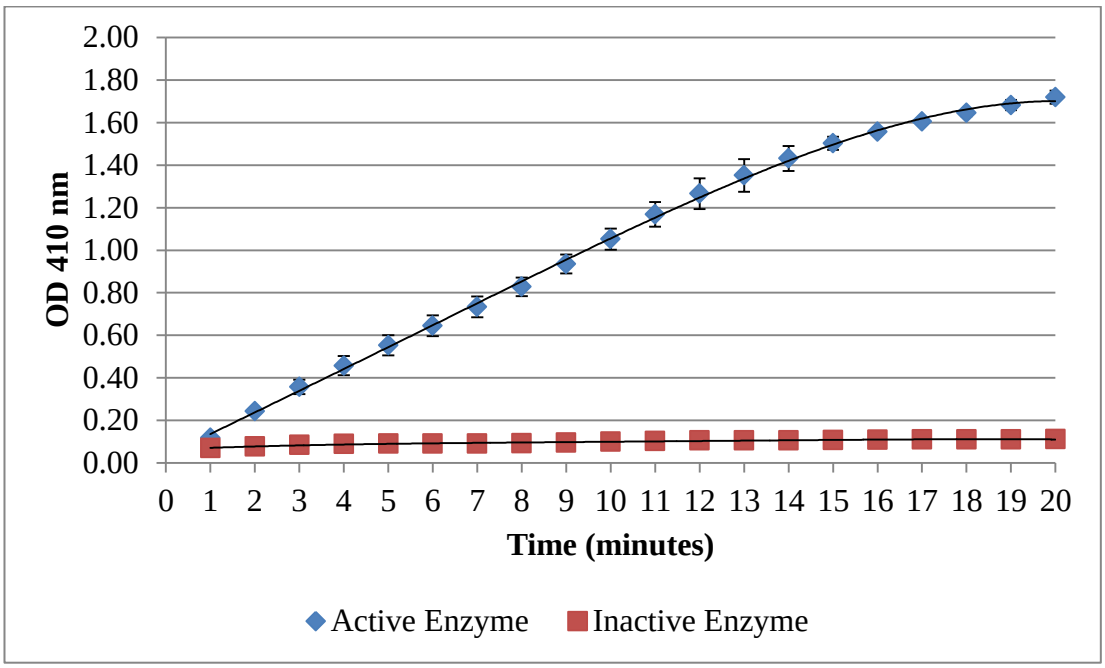


Figure 4.3: Activation of clostripain.

Clostripain (1U) was activated in MOPS buffer containing 10 mM calcium chloride and 2.5 mM dithiothreitol for 3 hours at 25±0.2°C. The hydrolysis of BAPNA, resulting in the formation of *p*-nitoaniline was monitored as an increase in absorbance at OD_{410}. Readings were recorded in duplicate. Negative control included 1U inactive clostripain.

4.4.3 Optimisation of Toxin Digestion

To generate fragments, toxins were subjected to enzymatic digestion with clostripain and a time course analysis conducted (Figures 4.4–4.8). All digestion gels contained the appropriate controls as described in Section 4.3.4; for the sake of clarity, the clostripain digestion controls have only been displayed in Figure 4.4. Due to the limited availability of native toxin A and B and toxoid A, stability analysis could

only be completed once, digestion analysis was completed in duplicate. Analysis of the clostripain control lanes revealed the presence of multiple bands, which as expected were also present in the toxin digestion lanes, making subsequent analysis difficult.

4.4.3.1 Time Course Analysis of Toxin A Digestion

Commercially available native toxin A (List Biological Laboratories, Inc., USA), with a reported high purity was found to contain multiple additional bands (Figure 4.4, Lane 7), these additional bands are most likely to be carried over from the purification process and represent natural toxin breakdown products. These bands were visualised due to the sensitivity of the double staining method employed in this research. The native toxin A from *C. difficile* (Figure 4.4) was found to be unstable when incubated for 24 hours at 25°C in the absence of clostripain (Figure 4.4, Lanes 7, pre-incubation and Lane 8, post incubation), with almost three times as many bands detected post-incubation when analysed using Image Lab™ software, v.3.0, with a band detection sensitivity of 100. Following 24 hours incubation with clostripain the pixel intensity of the band representing full length toxin A (308 kDa) remained unchanged, suggestion that enzyme mediated digestion had not occurred. In an attempt to facilitate clostripain mediated digestion of toxin A, the incubation period was increased from 24 hours to 48 hours. Although a greater number of bands were detected following prolonged incubation/digestion (indicated by red boxes), unfortunately the pixel intensity of the 308 kDa band remained unchanged, suggesting that the additional fragments were either a consequence of the inherent instability of the protein or enzyme mediated degradation of natural toxin breakdown products.

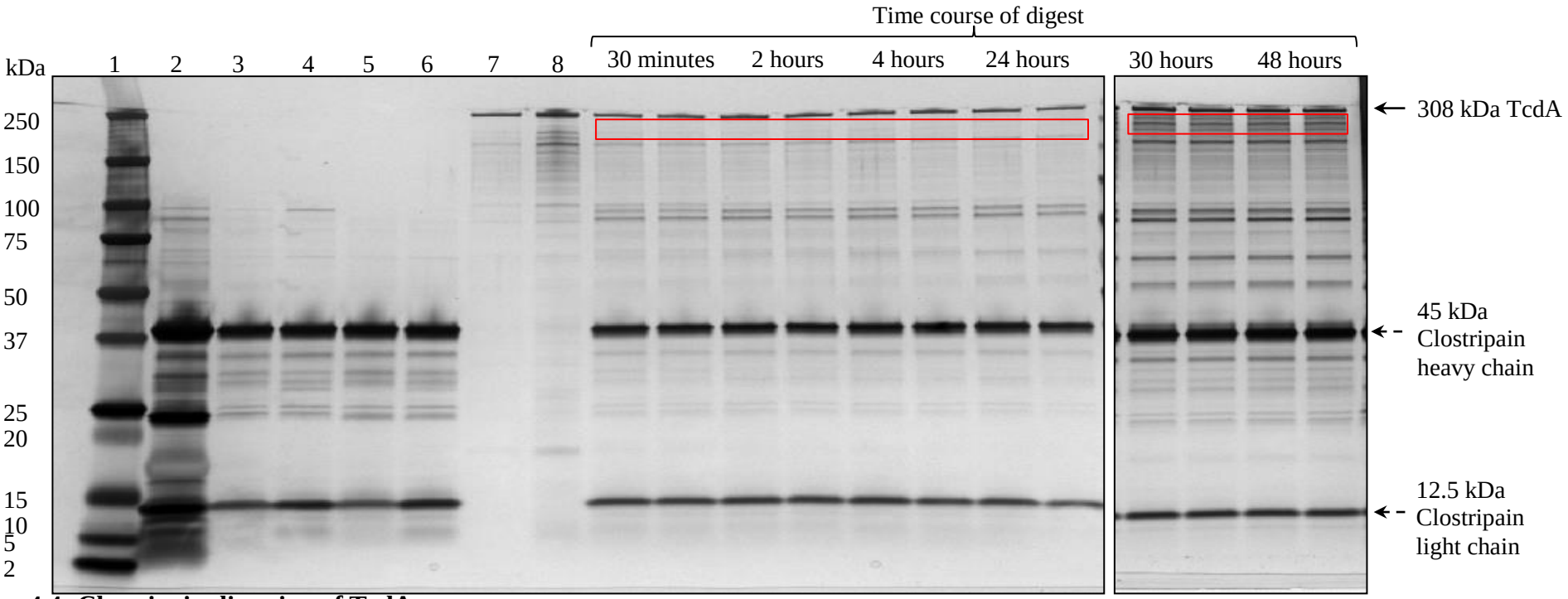


Figure 4.4: Clostripain digestion of TcdA.

Timed digestion of 5 µg TcdA by 1U activated clostripain, analysed on a 4-20% SDS-PAGE double stained by colloidal Coomassie+silver. Controls included, Lane 2, inactive enzyme; Lane 3, active enzyme 3 hours; Lane 4, active enzyme 3 hours+inhibitor; Lane 5, active enzyme 24 hours, 25°C; Lane 6, active enzyme 24 hours, 25°C+inhibitor; Lane 7, undigested TcdA; Lane 8, undigested TcdA 24 hours, 25°C; M, molecular weight marker in kDa. Digestion times are displayed above gel. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™ Software, v.3.0, with a band detection sensitivity of 100. Pre (lane 7) and post incubation (lane 8) of TcdA (n=1), timed digestion of TcdA (n=2). Red boxes, digestion bands.

Due to the problems encountered in obtaining a high yield of rTcdA$_{900-2710}$ (Chapter 3), only a faint band corresponding to the predicted size (205 kDa), was observed in the undigested control (Figure 4.5). Therefore digestion of rTcdA$_{900-2710}$ was not pursued further and undigested rTcdA$_{900-2710}$ was utilised for subsequent Western blotting studies.

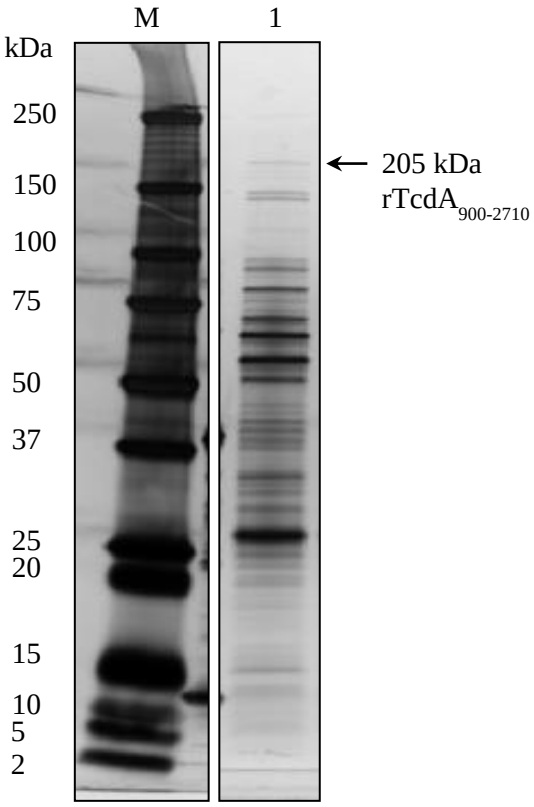


Figure 4.5: Purity of rTcdA$_{900-2710}$.

Analysis of 5 µg rTcdA$_{900-2710}$ analysed on a 4-20% SDS-PAGE double stained by colloidal Coomassie+silver. M, molecular weight marker in kDa. Imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™ Software, v.3.0, with a band detection sensitivity of 100.

As with native toxin A, the toxoid A profile was also found to be unstable, with multiple bands detected following incubation at 25°C for 24 hours in the absence of clostripain (Figure 4.6, Lane 1 and 2). Analysis of the toxoid A profile was complicated by the presence of heavy staining in the high molecular weight region (>180 kDa), which is suspected to be due to protein aggregation following the toxoiding process.

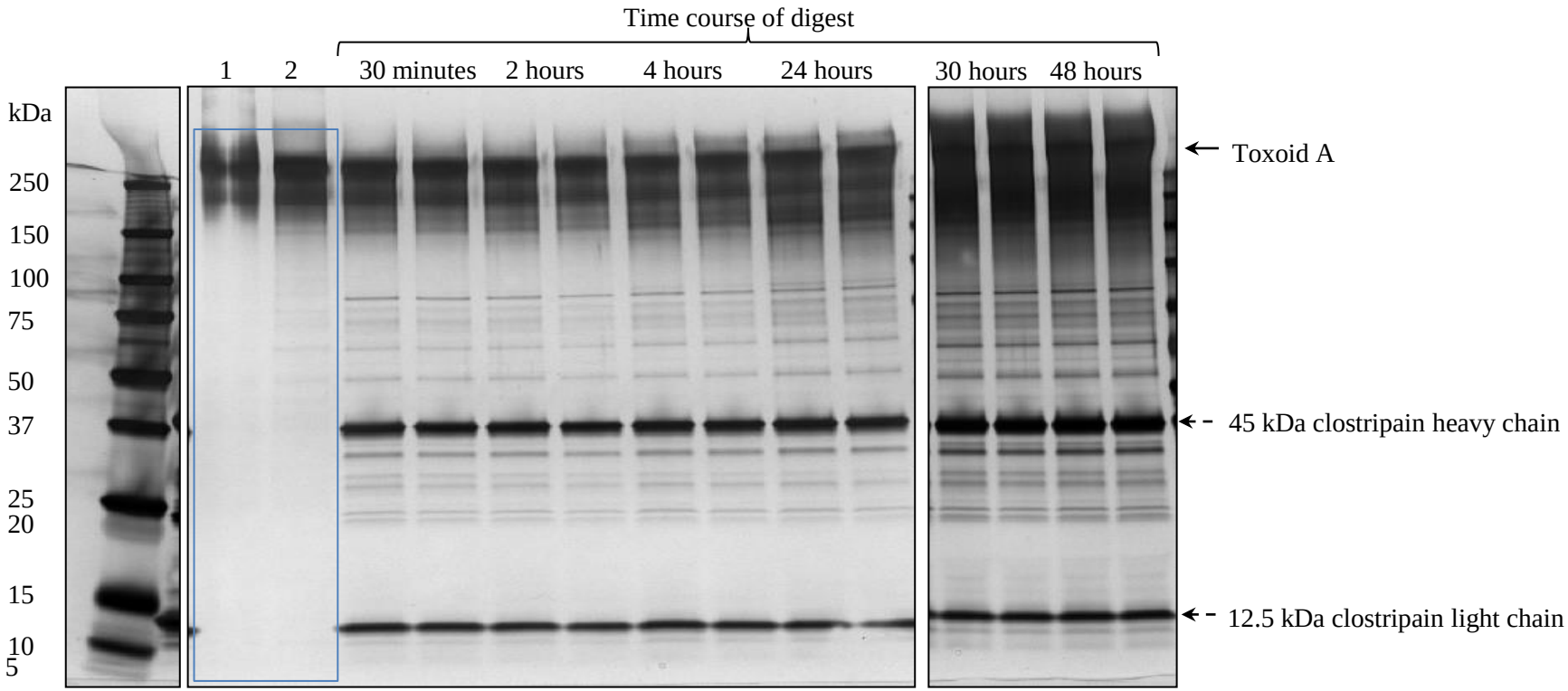


Figure 4.6: Clostripain digestion of toxoid A.

Timed digestion of 5 µg toxoid A by 1U activated clostripain, analysed on a 4-20% SDS-PAGE double stained by colloidal Coomassie+silver. Lane 1, undigested toxoid A; Lane 2, undigested toxoid A incubated for 24 hours, 25°C; M, molecular weight marker in kDa. Digestion times are displayed above gel. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™ Software, v.3.0, with a band detection sensitivity of 100. Pre (lane 1) and post incubation (lane 2) of toxoid A (n=1), timed digestion of toxoid A (n=2). Blue box; stability of toxoid A post 24 hours, 25°C incubation.

From the above results, incubation of TcdA and toxoid A in the absence of clostripain resulted in degradation of the protein. However, complete digestion of TcdA (and toxoid A) with clostripain was not observed. Based on the number of bands resolved a digestion/incubation time of 48 hours was used for Western blot analysis.

4.4.3.2 Time Course Analysis of Toxin B Digestion

As with native toxin A, the toxin B preparation contained multiple protein bands in the pre-incubated, undigested sample (Figure 4.7A, Lane 1). Comparison of native, undigested TcdB pre and post incubation at 25°C for 24 hours using Image Lab™ Software, v.3.0 (band detection sensitivity of 100), revealed the same number of bands (n=29), suggesting that unlike native toxin A, toxin B was stable under these conditions (Figure 4.7). A similar lack of enzyme independent degradation was also observed with $rTcdB_{547\text{-}2366.}$ Both the native and recombinant TcdB were susceptible to clostripain induced digestion, with 30 minutes identified as the optimum incubation period. Prolonged digestion (24 hours) resulted in a 50% reduction in the total number of bands resolved.

For $rTcdB_{547\text{-}2366}$, the gross digestion profiles at 30 minutes appeared to be similar to that of native TcdB, with common bands detected at 100, 70, and 25-37 kDa. A cluster of high molecular weight bands (130-180 kDa) were detected in TcdB digestion but were absent from $rTcdB_{547\text{-}2366}$.

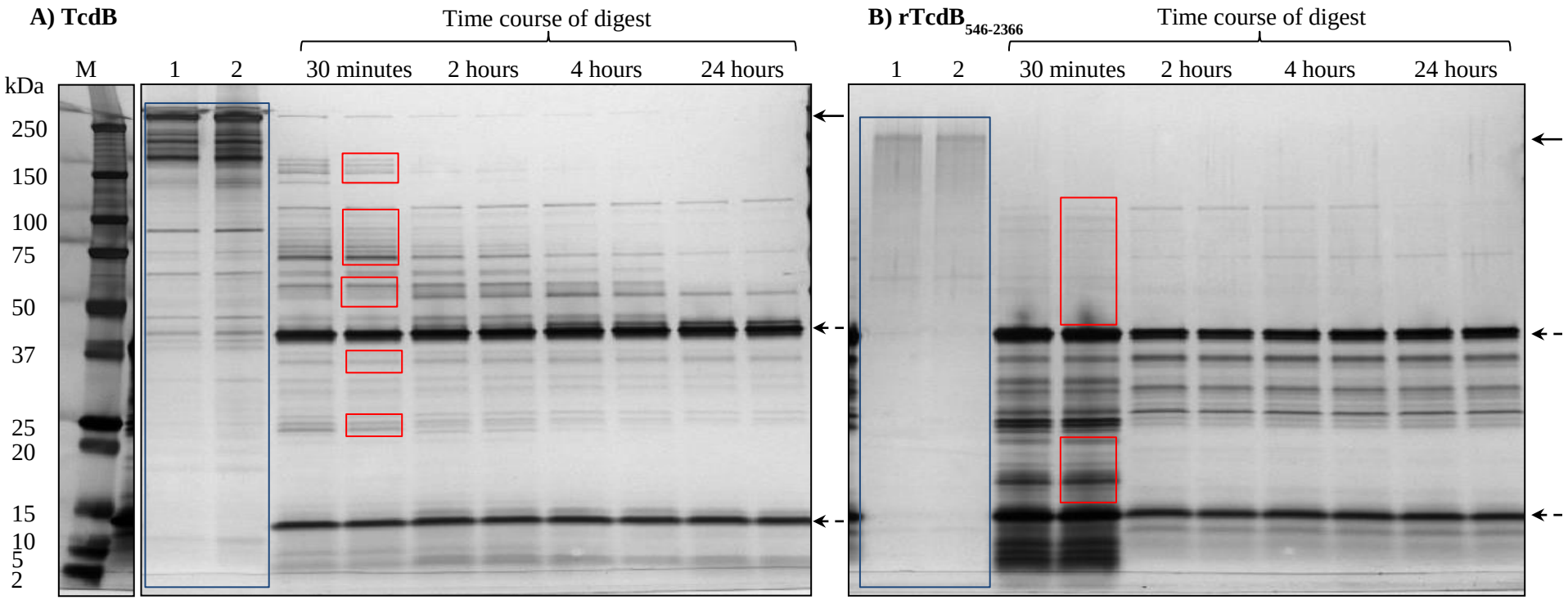


Figure 4.7: Clostripain digestion of TcdB and rTcdB$_{547\text{-}2366}$.

Timed digestion of 5 µg **A)** TcdB and **B)** rTcdB$_{547\text{-}2366}$ by 1U activated clostripain, analysed on a 4–20% SDS-PAGE gel, double stained by colloidal Coomassie+silver. Lane 1, undigested protein, Lane 2, undigested protein incubated for 24 hours at 25°C; M, molecular weight marker in kDa. Digestion times are displayed above gel. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™ Software, v.3.0, with a band detection sensitivity of 100. Pre (lane 1) and post incubation (lane 2) of TcdB and rTcdB (n=1), timed digestion of TcdB and rTcdB (n=2). Blue boxes; stability of TcdB and rTcdB$_{546\text{-}2366}$ post 24 hours, 25°C incubation, Red boxes indicate digestion bands. Solid arrows; 270 and 206 kDa TcdB and rTcdB$_{546\text{-}2366}$, respectively, dashed arrows clostripain heavy and light chain.

The results for the toxin B preparations were in contrast with the results for the toxin A preparations, with native and recombinant toxin B stable pre and post incubation and an optimum digestion time of 30 minutes. From the above data set, the digestions profiles of the various toxin preparations, revealed that the number of observable digested fragments were lower than the number predicted by the PeptideCutter software. Closer inspection of the predicted fragments revealed that 16 and 17 fragments in TcdA and TcdB would have a molecular mass of <2 kDa, thus they may not be resolved on the SDS-PAGE gel. In addition the method employed would be unable to distinguish between multiple bands with the same (or similar) molecular weights (Appendix 1).

4.4.3.3 Toxin Digestion – 2D SDS-PAGE

Whilst a 1D gel is a useful tool with which to determine if the various proteins have undergone proteolytic degradation, it lacks the discrimination provided by a 2D gel based method, which separates fragments on the basis of both pI and size. To further identify clostripain derived protein spots which would also be present in the proteolytic digestion, activated clostripain was incubated at 25°C for 48 hours, treated with a protease inhibitor cocktail and then subjected to 2D SDS-PAGE. As can been seen from Figure 4.8, in addition to the heavy and light chains the clostripain preparation contained multiple protein spots.

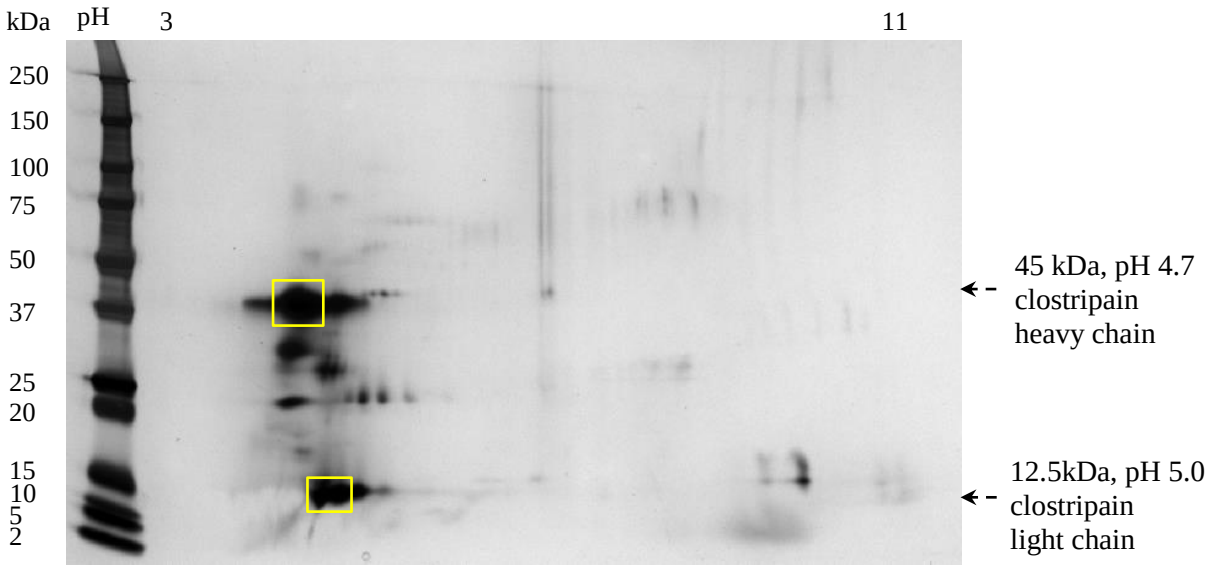


Figure 4.8: 2D SDS-PAGE of clostripain control.

1U of active clostripain was subjected to IEF and resolved on a 4–20% SDS-PAGE gel, double stained with colloidal Coomassie+silver. Molecular weight marker in kDa. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™ Software, v.3.0. Yellow boxes indicate predicted position of the heavy and light chains of clostripain (n=2).

As expected, the clostripain-digested toxin A 2D SDS-PAGE profile (Figure 4.9), revealed the presence of clostripain derived proteins, particularly the heavy and light chains of clostripain (indicated by yellow box). In addition to this the toxin A profile revealed multiple toxin derived proteins which were absent from the clostripain control, these protein spots spanned a pH range of 3-11 and a molecular weight of 2-100 kDa. This analysis emphasised the discriminatory power of 2D SDS-PAGE with multiple proteins of the same molecular weight evident.

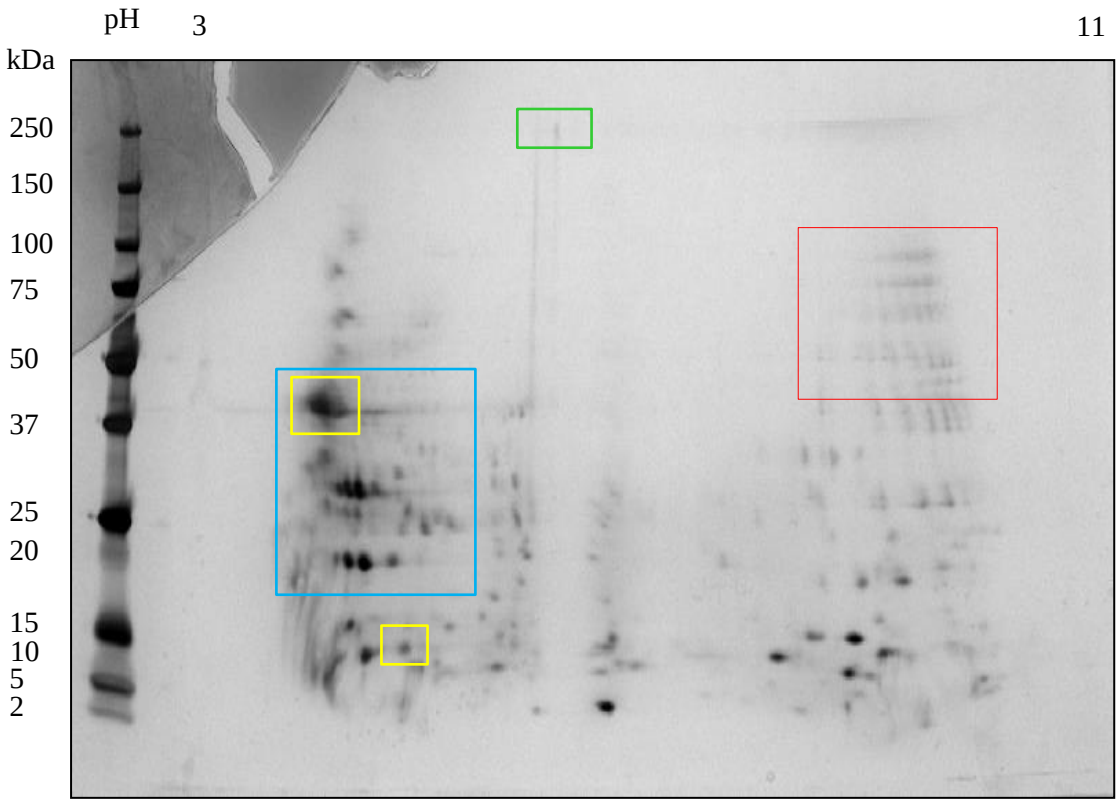


Figure 4.9: 2D SDS-PAGE of clostripain digested TcdA.

10 µg of digested TcdA was subjected to IEF and resolved on a 4–20% SDS-PAGE gel, double stained with colloidal Coomassie+silver. Molecular weight marker in kDa. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™ Software, v.3.0. Red and blue boxes, indicate differences and similarities between TcdA and toxoid A, respectively. Yellow boxes indicate heavy and light chains of clostripain. Green box indicates predicted position of TcdA, 308 kDa, pH 5.5 (n=1).

The 1D toxin A digestion profiles indicated enzyme mediated digestion had not occurred, with a 308 kDa band still visible, it was therefore anticipated that protein spots of this molecular weight, at a pH of 5.5, would also be present in the 2D SDS-PAGE profile. As can be seen from Figure 4.9 this was not the case, it has previously been reported that 2D SDS-PAGE poorly resolves proteins >150 kDa (Mukherjee *et al.*, 2002). This reasoning cannot be applied here as subsequent analysis of toxoid A resolved proteins >300 kDa (Figure 4.10). Although faint spots (green box) can be observed at the indicated molecular weight and pH, it is unclear whether these represent undigested toxin A or are an artefact of staining.

Alternatively the absence of the 308 kDa toxin A protein spot may highlight the instability of the toxin. In order to complete the first dimension of 2D SDS-PAGE, IPG strips were rehydrated overnight at room temperature (20°C) with the digested samples. Thus, toxin A was theoretically incubated for ~60 hours (48 hours for digestion and ~12 hours for rehydration), whether this prolonged incubation was sufficient for complete digestion of toxin A is unclear. As it was not possible to either repeat the 2D SDS-PAGE profile or incubate toxin A for greater than 48 hours, the absence and indeed the stability of toxin A cannot be definitively addressed.

In contrast to the toxin A profile, in the toxoid A digested profile, multiple protein spots were visible at >250 kDa (green box), demonstrating that proteins >150 kDa can be resolved by 2D SDS-PAGE. If indeed the extended incubation condition has resulted in the degradation of toxin A, the presence of toxoid A in the 2D profile suggests the toxoiding process may have conferred stability to the protein (Salnikova *et al.*, 2008), although it is important to note that from the 1D SDS-PAGE (Figure 4.6) profile toxoid A was also found to be display instability under the incubation conditions employed.

As with toxin A, clostripain derived proteins (yellow boxes) were evident in the toxoid A digestion profile (Figure 4.10). The overall spot patterns for clostripain digested toxin A (Figure 4.9) and toxoid A (Figure 4.10) were broadly similar, with protein spots ranging from pH 3-11. A cluster of protein spots at pH 8.5 were absent from the toxoid digest but were present in the native digestion (indicated by the red box in Figure 4.9). Whilst at a pH of 7.0 a number of spots which were absent from the toxin A digest were present in the toxoid digest (indicated by the red box in Figure 4.10). The differences between the digestion patterns of TcdA and toxoid A may be indicative of the toxoiding process, causing structural changes in the toxin which masks proteolytic digestion sites and hence susceptibility to enzyme mediated digestion.

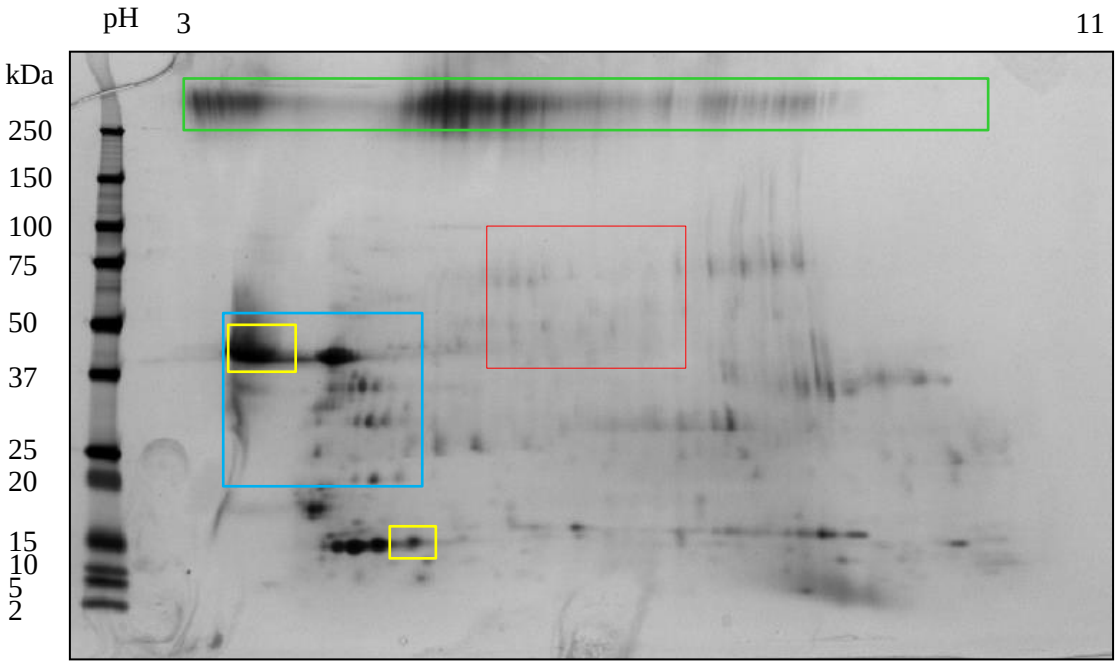


Figure 4.10: 2D SDS-PAGE of clostripain digested toxoid A.

10 µg of digested toxoid A was subjected to IEF and resolved on a 4–20% SDS-PAGE gel, double stained with colloidal Coomassie+silver. Molecular weight marker in kDa. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™ Software, v.3.0. Red and blue boxes indicate differences and similarities between TcdA and toxoid A, respectively. Yellow boxes indicate heavy and light chains of clostripain. (n=1).

From the toxin digested profiles (Figures 4.9 and 4.10) the visual intensity of clostripain heavy and light chains were of lower intensity than that observed in clostripain control (Figure 4.8), it is possible that this is due to staining variation, wherein the clostripain control contains fewer proteins resulting in a greater proportion of Coomassie and silver ions binding to the protein and subsequently being detected. In contrast the toxin digestion profiles were composed of a greater number of proteins at a range of concentrations, hence staining variability may occur.

Due to the previously mentioned problems associated with $rTcdA_{900\text{-}2710}$ purification and subsequent digestion, 2D SDS-PAGE analysis of recombinant toxin A was not pursued.

Comparison of the clostripain digestion profiles of native (Figure 4.11A) and recombinant toxin B (Figure 4.11B) profiles revealed them to be remarkably similar, with the majority of protein spots being acidic in nature. The native profile does appear to contain regions which are absent from the recombinant profile (indicated by red boxes), this is unsurprising given the recombinant protein lacks the N-terminus.

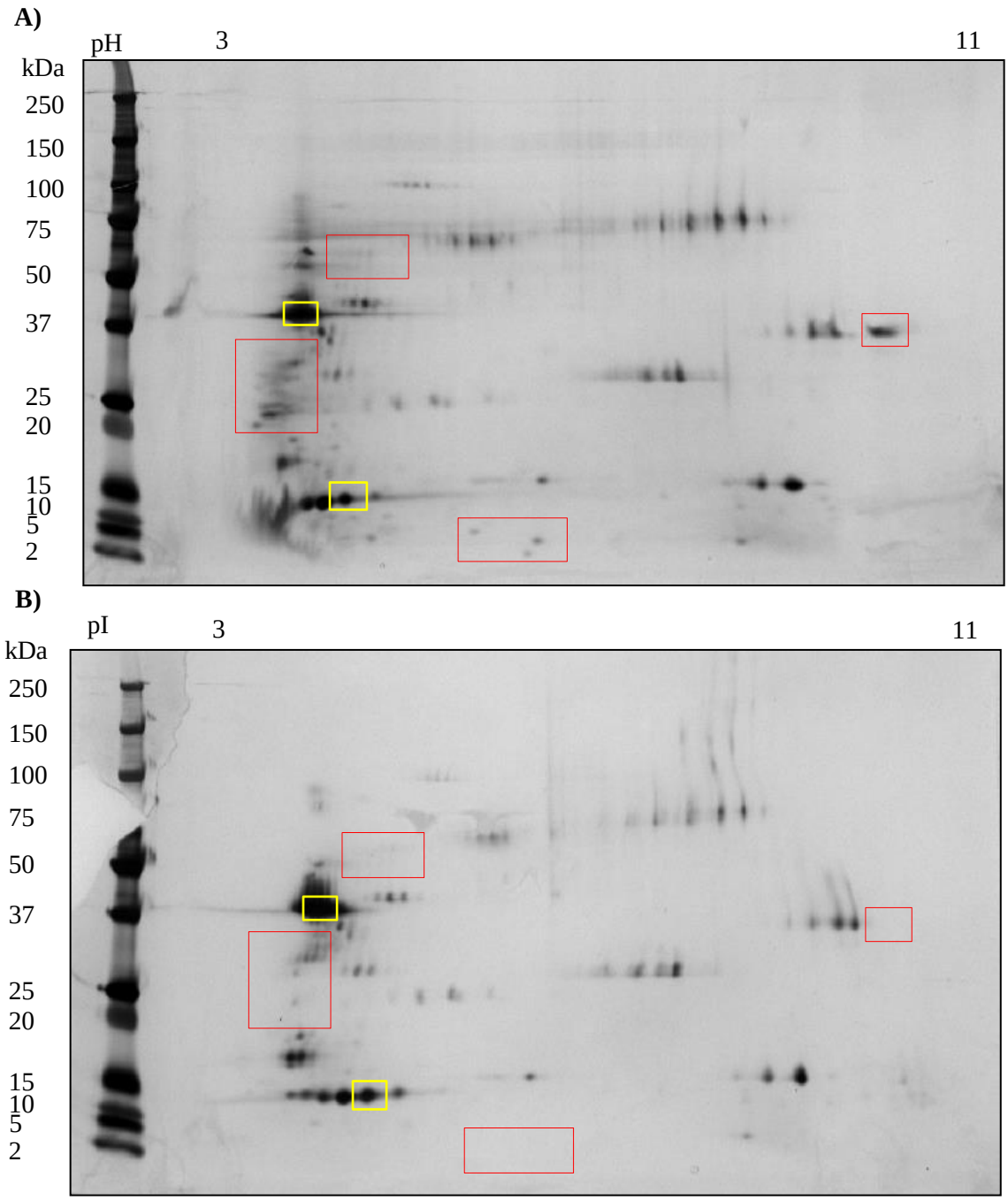


Figure 4.11: 2D SDS-PAGE of clostripain digested TcdB and rTcdB$_{546-2366}$.

A) TcdB and **B)** rTcdB$_{546-2366}$. 10 µg of digested protein was subjected to IEF and resolved on a 4–20% SDS-PAGE gel, double stained with colloidal Coomassie+silver. Molecular weight marker in kDa. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™ Software, v.3.0. Red boxes indicate differences between TcdB and rTcdB$_{546-2366}$. Yellow boxes indicate heavy and light chain of clostripain enzyme. (n=1).

Due to limited availability of the native and toxoided proteins, the concentration of protein resolved by 2D SDS-PAGE was only 10 µg; which is half the manufacturer's recommended concentration (20-50 µg). Hence, it is possible that by using such a low concentration of protein, a complete peptide profile was not obtained. Due to a lack of reagents the repeatability of the 2D SDS-PAGE method could not be verified and as such 2D SDS-PAGE was completed only once

In contrast to the 1D SDS-PAGE approach, the 2D approach provided better discrimination of the peptides in the sample, due to its ability to resolve proteins on the basis of molecular weight and pH. The 2D approach also further highlighted potential stability issues regarding toxin A. It was not possible to utilise the 2D SDS-PAGE approach for subsequent analysis, therefore a 1D approach combined with Western blotting, which has a greater sensitivity than either colloidal Coomassie or silver staining was employed.

4.4.4 Toxin B Immunoblots

4.4.4.1 Probing With Animal Immune Serum

The ability of two commercially available antibodies, a mouse monoclonal (binding site unknown) and a rabbit polyclonal antibody raised against the 60 kDa N-terminal fragment of toxin B, to recognise native and recombinant toxin B was assessed. As can be seen in Figure 4.12A, the mouse monoclonal antibody recognised multiple bands in the digested and undigested TcdB samples. The ability of this murine monoclonal antibody to recognise $rTcdB_{547\text{-}2366}$, suggests that the epitope to which this antibody binds is located within the translocation and receptor binding domain (amino acid residues 547-2366) of toxin B. The binding of the antibody to a fragment of approximately 50 kDa suggests that it might be possible to further define the site recognised by this antibody.

From Figure 4.12B, the polyclonal rabbit antibody bound to multiple fragments in the undigested sample of the native toxin suggesting that the protein had undergone

non-enzymatic degradation, possibly during the purification process. It was somewhat surprising that the bands detected in the digested sample did not correspond to the bands detected in the intact sample; possibly due to the destruction of epitope binding sites following enzymatic digestion.

Given that the polyclonal antibody is directed against the 60 kDa N-terminal region of toxin B, the presence of antibody binding fragments in lanes containing $rTcdB_{547-2366}$, (which is devoid of the catalytic domain) was surprising. Sequence alignment of the 60 kDa N-terminal region and $rTcdB_{547-2366}$ revealed a 5% identity and 10% similarity (calculated using http://www.bioinformatics.org) based on sequence data for *C. difficile* 630. The polyclonal antisera was produced from a recombinant GST-fused 60 kDa protein expressed in *E. coli* (Abcam, UK, personal communication), hence it is possible that the bands observed in the recombinant toxin are due to cross-reacting host derived antibodies. The suppliers (Abcam, UK) of the antisera were unable to provide details on the antibody binding site, but did specify the fragment used to generate the antibody displayed Rho and Rac glycosylation activity (Abcam, UK, personal communication). Hence, the fragment must, at the very least, span from amino acid residues 1–547, in order to encompass the amino acid residues and motifs, which are essential for enzymatic activity (Busch *et al.*, 1998; Busch and Aktories, 2000; Voth and Ballard, 2005; Jank *et al.*, 2007; Jank and Aktories, 2008).

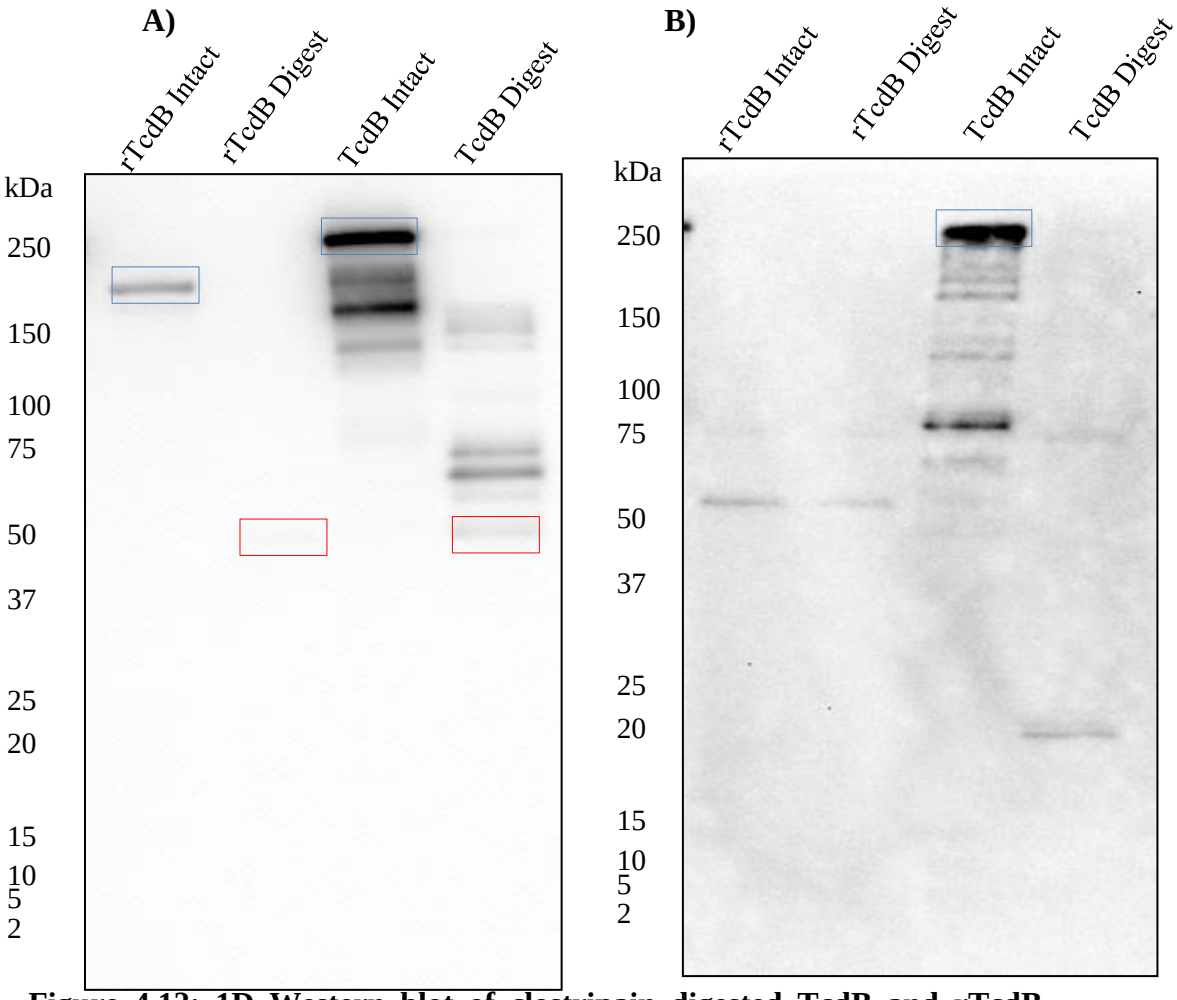


Figure 4.12: 1D Western blot of clostripain digested TcdB and rTcdB$_{547\text{-}2366}$ probed with Ms mAb and Rb pAb.

A) Mouse mAb and **B)** Rabbit pAb. 30 minute digestion of 5 µg of TcdB and rTcdB$_{547\text{-}2366}$ by 1U activated clostripain, analysed on a 4–20% SDS-PAGE gel and transferred to nitrocellulose. Molecular weight marker in kDa. Blue boxes; 207 and 270 kDa rTcdB$_{547\text{-}2366}$ and TcdB, Red boxes; region of commonality.

In the above experiment the ability of two commercially available antibodies to react with native and recombinant toxin B was assessed and there approximate binding sites identified. This approach confirmed the suitability of the recombinant protein as a surrogate for immunogenic studies, with the monoclonal antibody reacting with undigested rTcdB$_{547\text{-}2366}$. The presence of multiple immunogenic bands in the intact native toxin suggests degradation of toxin B, possibly during the purification process. Furthermore to assess the presence of cross-reacting antibodies from the host expression system, nickel purified soluble protein from the *E. coli* expression strain,

M15[pREP4], was used as an additional control in the subsequent neutralising and human sera immunoblots.

4.4.4.2 Probing with Commercial Serum Containing Toxin Neutralising Antibodies

In an attempt to identify toxin protein fragments which contain toxin neutralising antibody binding sites, native and recombinant protein digests were probed with two commercially available toxin neutralising polyclonal antibody preparations; sordellii antitoxin (NIBSC, UK) and goat antitoxin (Techlab®, UK). The sordellii antiserum was prepared from equine immune serum against *C. sordellii* (NIBSC, UK) and the goat antitoxin was prepared from toxoided culture filtrate of *C. difficile* VPI10463. Although, these preparations have been widely used (primarily in cell culture diagnostic assays) (Bartlett *et al.*, 1978; Chang *et al.*, 1978b), the regions of each toxin recognised by each serum has yet to be determined.

Somewhat surprisingly a fragment of ~37 kDa in the clostripain enzyme was recognised by both sera, given its relative size it is likely to represent the heavy chain of the enzyme (Figure 4.13). Sequence alignment of the heavy chain of clostripain, with native and recombinant toxin A and B revealed a low sequence identity of <16% based on sequence data for *C. difficile* 630. Given that *Clostridium* spp. are ubiquitously found it is possible that the animal(s) used to generate the antisera (goat and horse), were previously exposed to *C. histolyticum* and as such possessed anti-clostripain antibodies. Alternatively the toxoided culture filtrate used for the generation of both anti-sera may contain additional proteases, from *C. difficile* and *C. sordellii* which are similar to clostripain. Fortunately on the basis of size comparison this band did not overlap with the common immunogenic fragments seen in any of the toxin preparations.

To verify the bands identified in the recombinant preparations were not host (*E. coli*) derived, soluble protein was extracted from the *E. coli* expression strain,

M15[pREP4], used in Chapter 3. Whilst no cross-reactivity was observed with the goat antitoxin, three bands where identified with the sordellii sera suggesting that this sera contained cross-reacting *E. coli* antibodies.

Probing of the native and recombinant toxin B preparations with the goat and sordellii antisera revealed a broadly similar immuno-profile, with both antisera recognising the intact proteins. Of note is the presence of a 270 kDa band corresponding to intact toxin B in the digestion lane. From the digestion profile of toxin B (Figure 4.7B) enzyme mediated digestion occurred within 30 minutes, however with the more sensitive Western blot approach the presence of a 270 kDa band in the digestion lane suggests complete digestion has not occurred. A cluster of bands ranging from 75-270 kDa was detected by both immune sera in the intact and digested protein. Lower molecular weight common immunogenic bands are indicated by the red boxes in Figure 4.13.

The immuno-profiles for digested rTcdB$_{547\text{-}2366}$ were remarkably similar, with neither antitoxin detecting bands >75 kDa in each digested sample. A group of bands between 30-75 kDa were recognised with both antitoxins, with two particularly dominant bands at 40 and 60 kDa suggesting the possible location of immunodominant epitopes. Although it should be noted the *in silico* analysis did not predict fragments of these molecular weights (Appendix 1).

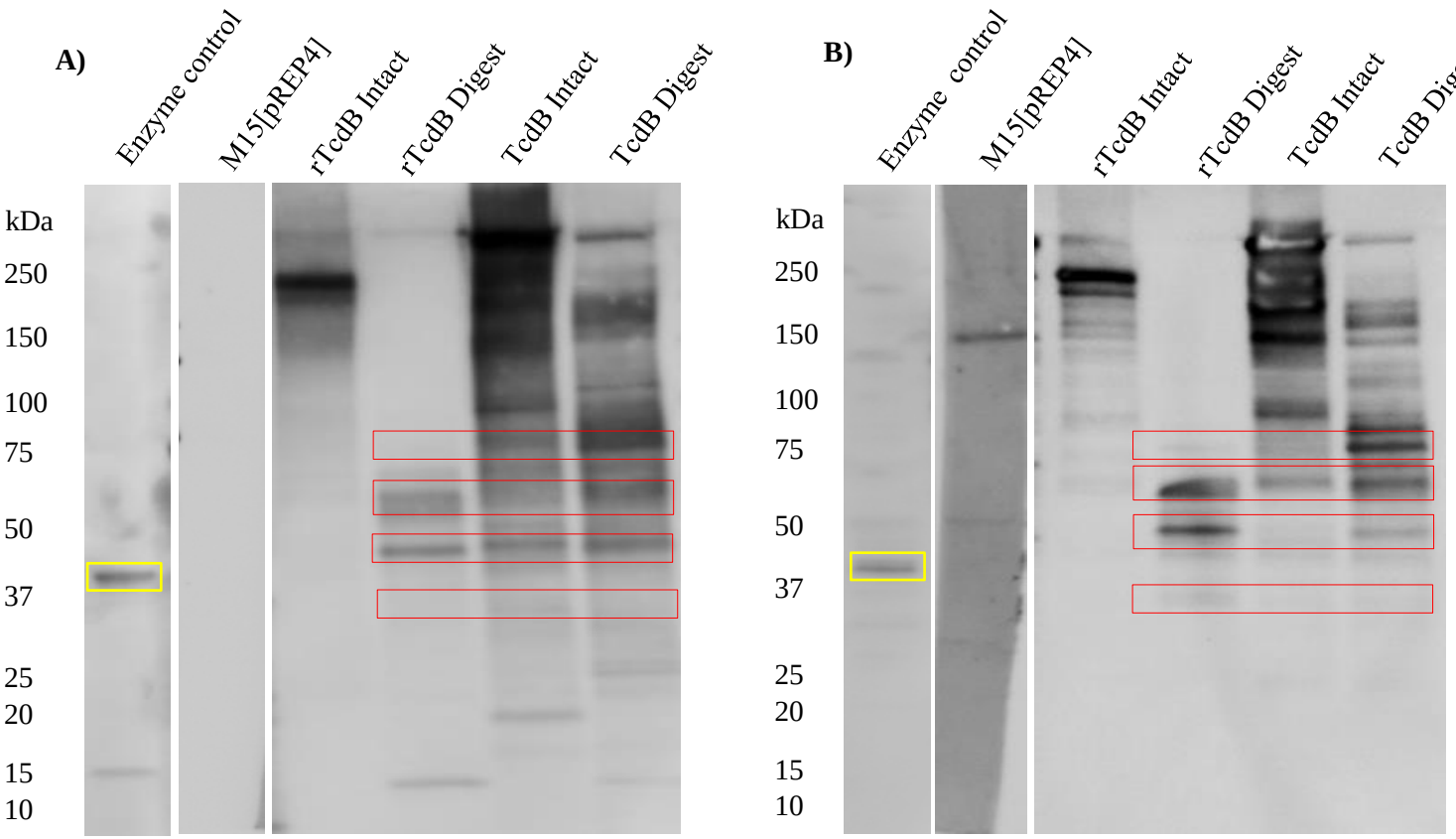


Figure 4.13: 1D Western blot of clostripain digested TcdB and rTcdB$_{547-2366}$ probed with neutralising anti-toxin.

A) Goat anti-toxin **B)** Sordellii anti-toxin. 30 minute digestion of 5 µg of protein with 1U activated clostripain, analysed on a 4-20% SDS-PAGE gel and transferred to nitrocellulose. Clostripain enzyme control, M15[pREP4] Ni purified soluble control. Red boxes indicate commonly recognised fragments. Yellow boxes indicate predicted heavy chain of clostripain enzyme.

In summary both antisera recognised distinct fragments of both native and recombinant toxin B preparations. The fact that there were common bands in the recombinant and native proteins suggests that they share the same/similar epitopes and the recombinant protein can be utilised in the future as a surrogate for native toxin B. The generation of smaller fragments, 30-75 kDa, which were recognised by the antisera, gives the potential to map the location of antibody binding sites, some of which may be neutralising. Given that these sera are used as commercial controls for the majority of cytotoxicity assays, more work needs to be done to determine the basis of their action.

Analysis was complicated by the immunogenicity of the clostripain heavy chain, although the similarity of this protein with the toxin preparations was low, it is evident that cross-reacting antibodies are present in the goat and sordellii sera. The presence of immunogenic bands in the *E. coli* extracted proteins with the sordellii sera further complicated analysis of the recombinant protein. Whether these two results are an indicator of natural exposure to *C. histolyticum* and *E. coli* in the animals used to generate the antisera is unclear.

4.4.5 Toxin A Immunoblots

4.4.5.1 Probing With Animal Immune Serum

As with toxin B, initial efforts focussed on probing blots with non-neutralising animal derived antibodies. It was not possible to determine how the goat polyclonal antibody was produced; the rabbit polyclonal antibody recognises a 60 kDa N-terminal fragment of toxin B. The goat polyclonal antibody recognised a multitude of bands in all toxin A preparations, highlighting particularly the impurity of the commercially available native and toxoided toxin A preparations. As this antibody was found to bind to the 205 kDa recombinant protein, its binding site may well reside within the translocation and C-terminal region. Although the presence of multiple protein bands in the intact recombinant toxin A, confirms the impurity of the recombinant protein, the immunogenic bands may well represent truncated or degraded toxin.

The rabbit pAb, which was raised against the N-terminal catalytic fragment of TcdB (Figure 4.14B), was found to cross react with native and toxoid A preparation. This is not surprising, given that it is in this region that TcdA and TcdB display a 64% homology (von Eichel-Streiber *et al.*, 1990). Although it should be noted the antibody reacted weakly with TcdA and toxoid A. A band of ~20 kDa was observed in all the preparations, indicating the binding site for at least one of the antibodies in this polyclonal preparation is within this region (indicated by the red box).

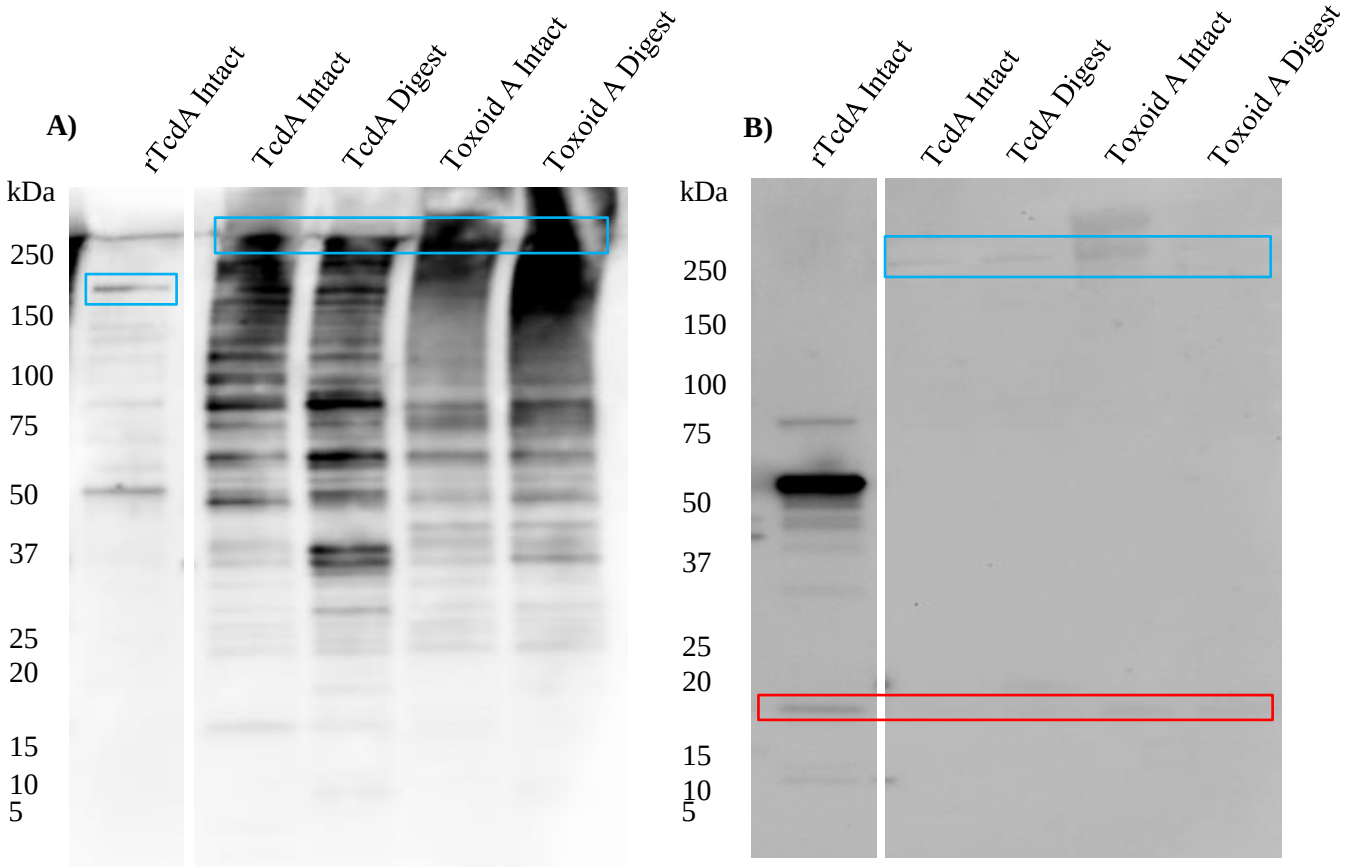


Figure 4.14: 1D Western blot of clostripain digestion of TcdA and toxoid A an intact rTcdA$_{900\text{-}2710}$, probed with Gt pAb and Rb pAb.

A) Goat pAb and **B)** Rabbit pAb. 30 minute digestion of 5 µg protein by 1U activated clostripain, analysed on a 4–20% SDS-PAGE gel and transferred to nitrocellulose. M, Molecular weight marker in kDa. Blue boxes indicate 205 and 308 kDa full length rTcdA$_{900\text{-}2710}$ and TcdA respectively. Red box region of commonality.

4.4.5.2 Probing with Commercial Serum Containing Toxin Neutralising Antibodies

The ability of the two neutralising polyclonal antisera (goat and sordellii) to recognise toxin A preparations is displayed in Figures 4.15A and B, respectively. Both the goat and sordellii antisera reacted with intact $rTcdA_{900\text{-}2710}$, indicating the epitopes (and possible neutralising epitopes) recognised, by at least one of the antibodies in these polyclonal sera resides between amino acid residues 900–2710.

The digestion profiles of native TcdA and toxoid A were similar to their respective intact toxin profiles suggesting enzymatic digestion had not destroyed common epitopes. Although regions of commonality are present between native and toxoided toxin A, multiple immunogenic bands (100-250 kDa) can be observed in the native TcdA profile, which are absent in the toxoid profile. Whether this is a consequence of the toxoiding process, wherein common immunogenic epitopes have been destroyed, or highlights the instability of native toxin A preparations is unclear. In all three toxin preparations (recombinant, native and toxoided) a band of 60 kDa was detected by the goat antisera. Although a band of ~40 kDa is present in the digested native and toxoid lanes, it is unclear whether this is derived from the toxin or the clostripain enzyme.

As with the goat antisera, the sordellii antisera recognised intact recombinant, native and toxoid A. In contrast to the goat antitoxin, the sordellii antisera recognised a limited number of fragments in the native and toxoided profiles. It has not been possible to decipher the exact methodology used to prepare the sordellii antisera, other than it was generated from the culture filtrate of *C. sordellii*. The strain of *C. sordellii* used and hence which toxins and the concentration of these toxins in the preparation is unknown. If the predominant toxin in the preparation was the lethal toxin, which displays a 75% amino acid sequence similarity with TcdB and 41% sequence similarity TcdA (Rupnik and Just, 2006), this would account for the limited immunogenicity observed with toxin A. Although fewer bands were identified with the sordellii serum than the goat serum, when the two blots were overlaid, the bands correlated with those identified with the goat antitoxin, suggesting that both sera recognised a spectrum of similar epitopes.

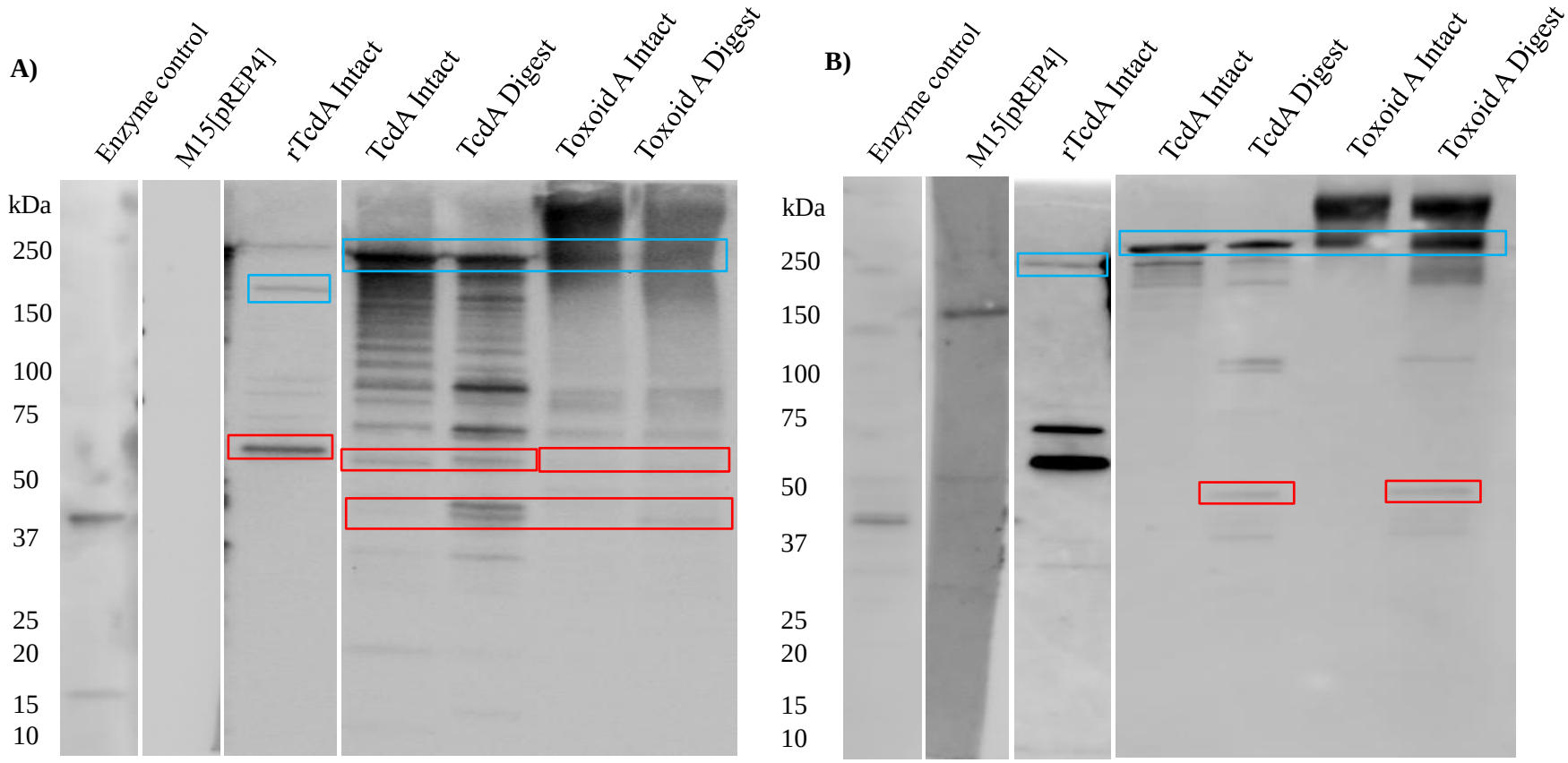


Figure 4.15: Western blot of clostripain digestion of rTcdA$_{900\text{-}2710}$, TcdA and toxoid A.

A) Goat antitoxin and **B)** Sordellii antitoxin. 48 hour digestion of 5 µg of TcdA and toxoid A by 1U activated clostripain, analysed by SDS-PAGE on a 4-20% gel and transferred onto nitrocellulose. Blue boxes indicate 205 and 308 kDa full length rTcdA$_{900\text{-}2710}$ and TcdA, respectively. Red boxes indicate regions of commonality.

In summary both antisera recognised intact recombinant, native and toxoided toxin A preparations indicating that the antisera recognise similar spectrum of epitopes. The identification of 60 and 40 kDa fragments, gives the potential to map the location of antibody binding sites, some of which may be neutralising. The differences observed in the immunogenicity of native toxin A and B with the sordellii antisera may be associated with preparation of the antisera and hence would be related to the homology across the large clostridial cytotoxin family.

4.4.6 Human Sera Immune Study

To characterise the immune response of infected individuals to toxins A and B, blood samples were collected from *C. difficile* infected patients. Data obtained in 2008 for patients admitted to the University Hospital of Wales, Cardiff, revealed that a total of 1185 individuals were diagnosed as *C. difficile* infected, based on an Enzyme-Linked Immunosorbent Assay (Personal communication, Dr Robin Howe). A significant proportion of these patients were in the Internal/General Medicine (40%) and Nephrology/Renal Medicine specialities (11%), with a modal age range of 80-95 years. On the basis of this historical data, these specialties were deemed appropriate for patient recruitment for this study.

The research proposal and study documents (case report form, patient information sheet and consent form) can be found in Appendix 2; the study was designed to recruit 100 patients from the Medical and/or Renal wards, 80 *C. difficile* infected and 20 *C. difficile* negative, control patients. Blood samples were to be collected at infected diagnosis of *C. difficile* and 21 days after infected diagnosis for a single episode of CDI. For recurrent/relapse CDI a total of four serum samples were to be requested and a single sample for *C. difficile* negative control patients. Controls were to be ward, aged and gender matched.

This project was subjected to ethical review by the Dyfed Powys Research Ethics Committee, the Cardiff and Vale University Health Board and the School of

Pharmacy and Pharmaceutical Sciences Ethics Committee and received approval in February 2011, with recruitment commencing in August 2011.

Due to the time taken to obtain ethical approval, it was only possible to collect six serum samples at a single time point, following diagnosis. No negative samples were collected. All serum samples were anonymised and unblinding of clinical data (Table 4.4) occurred following analysis of all experimental data. The average age of the patients was 67 years (range 48 to 89 years). Two of the patients reported a history of CDI and had experienced multiple episodes of infection. The severity of CDI ranged from mild (3 patients) to moderate (2 patients) and severe (1 patient). On average, serum samples were collected 6 days after diagnosis (range 1-15 days).

As part of the current study clinical data from *C. difficile* infected patients was also collected, in other studies similar parameters have been used to assess disease severity and outcome (Walk *et al.*, 2012; Butt *et al.*, 2013; Walker *et al.*, 2013). In these aforementioned studies clinical data from >200 cases of CDI were investigated. Due to the limited number of samples collected in this study, it was not possible to draw any significant conclusions with regards to the clinical data collected.

Table 4.4: *C. difficile* infected patient sera, clinical data.

Sample	Age	Gender	Date of Admission to ward	Date of Infected Stool	Date serum sample obtained	Severity	WBC	Serum Creatine	Albumin	History of *C. difficile*	Major co-morbidities
CD1	89	Male	01/08/2011	01/08/2011	16/08/2011	Mild	11	93	22	None	Rheumatoid arthritis
CD2	43	Female	26/08/2011	31/08/2011	01/09/2011	Mild	12.6	46	24	None	Legionella pneumonia
CD3	68	Female	05/10/2011	06/10/2011	Unknown	Moderate	13.3	49	14	5 previous infecteds	None documented
CD4	81	Male	16/09/2011	30/09/2011	07/10/2011	Mild	8.4	181 (chronic elevation)	15	None	Colon cancer
CD5	75	Male	05/10/2011	17/10/2011	20/10/2011	Moderate	6.8	198 (chronic elevation)	Not recorded	None	Aortic valve replacement, Urostomy
CD6	48	Female	17/10/2011	16/10/2011	20/10/2011	Severe	16	318	Not recorded	4 previous infecteds	Renal transplant

All sera were anonymised until analysis of experimental data.

4.4.7 Probing of Toxin B with *C. difficile* Infected Immune Sera

To identify antibody specific binding sites within toxin B, intact and clostripain digested recombinant and native toxin B were probed with serum from four patients; CD1,CD3,CD5 and CD6. Due to a limited amount of CD2 sera, this sample was omitted from all subsequent analysis. Sera CD4 was found to display a red discolouration, indicating leakage of haemoglobin from the red blood cells due to haemolysis, possibly due to improper separation and was not used in subsequent analysis.

4.4.7.1 Toxin B Specific IgG Response

Surprisingly the sera of two patients (CD3 and CD6) recognised an immunogenic band in the clostripain control, on the basis of size; this is most likely to represent the 42 kDa heavy chain of the enzyme (Figure 4.16). Given the limited number of *C. histolyticum* infections in humans (Health Protection Agency, 2003), the presence of anti-clostripain antibodies is intriguing. Given the numerous antigens the human immune system is exposed to the presence of cross-reacting epitope(s) which are structurally similar to other antigens is feasible. Particularly as the human sera consists of polyclonal antibodies capable of recognising multiple epitopes.

In contrast, a single band was recognised in the *E. coli* protein control by only one serum sample (CD3), suggesting that the majority of the immunogenic bands seen in the recombinant profile preparation represent toxin fragments as opposed to co-purified host derived proteins from the expression system.

Whilst all of the patient sera tested contained IgG antibodies that recognised intact TcdB, only two individuals had antibodies which recognised intact $rTcdB_{547\text{-}2366}$ (CD3 and CD6), suggesting that the majority of the immune response maybe directed towards the N-terminal region of the toxin (Figure 4.16). It has recently been shown, strains of *C. difficile* display antigenically variable C-terminal domains of TcdB

(Lanis *et al.*, 2013). In this study the recombinant protein was produced from *C. difficile* 630 (ribotype 012), therefore if patient sera CD1 and CD5 were infected with, for example *C. difficile* 027, the antibodies generated against toxin B in these individuals may not recognise the recombinant protein.

Sera from CD1 and CD5 displayed a limited ability to recognise digested TcdB; unsurprisingly these two sera also displayed the lowest binding intensity (based on visual observation of blots) for intact TcdB. This suggests that the two sera contain a low concentration of antitoxin B IgG antibodies. Indeed, the variability of toxin-specific antibodies in clinical sera is well documented (Kelly *et al.*, 1992; Kelly, 1996; Kyne *et al.*, 2000, 2001b).

In addition to highlighting the variability in the immune response against the toxin preparations, Figure 4.16 also identified fragments at 80, 60, 40 and 25 kDa, which were consistently identified in both TcdB and $rTcdB_{547\text{-}2366}$ preparations by multiple sera (indicated by red boxes). Thus, indicating the presence of immunodominant regions within the 270 kDa toxin, whether these regions contain neutralising epitopes or can be utilised in diagnostic assays for assessing the immune response generated against the toxins is yet to be determined.

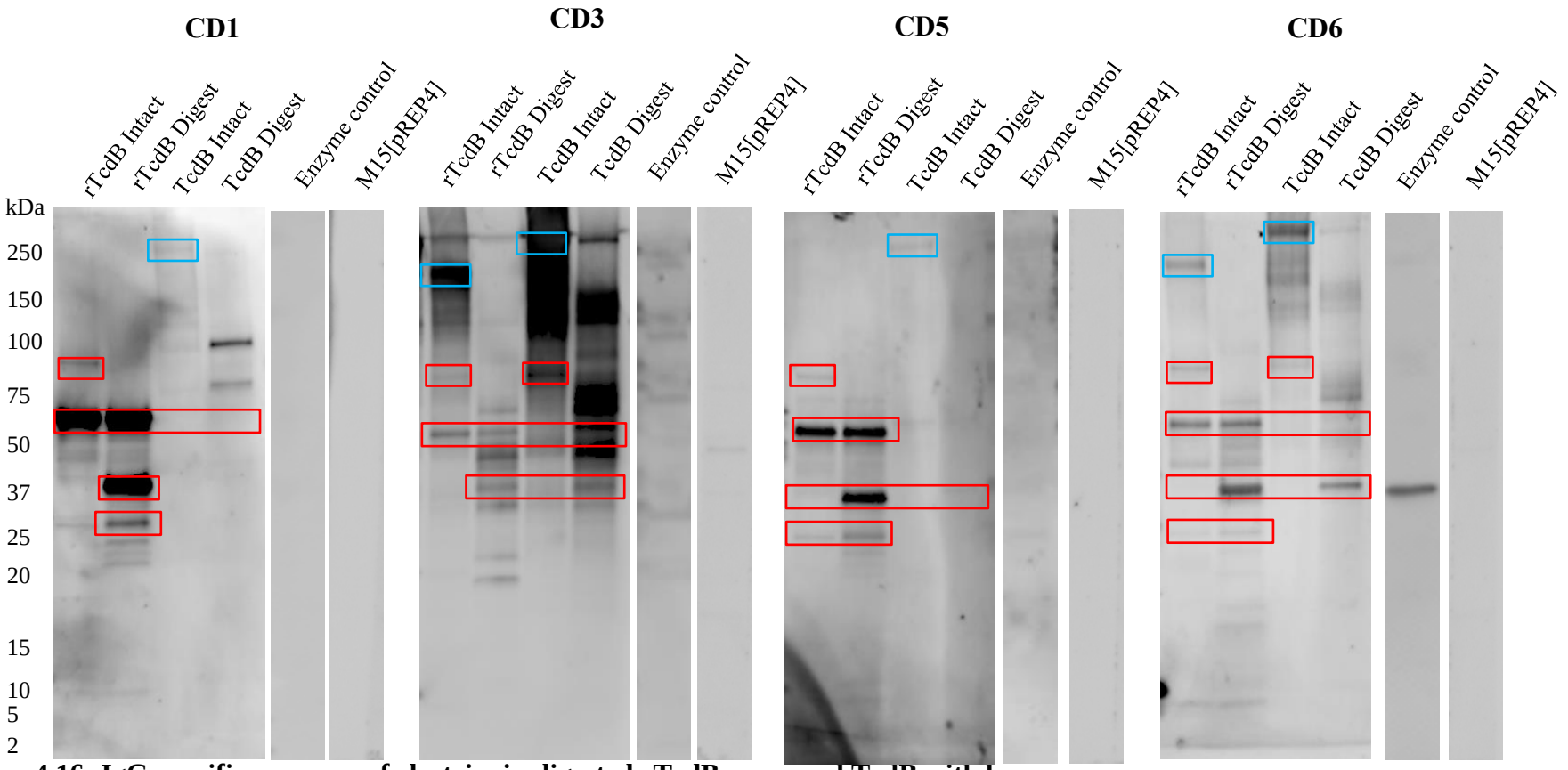


Figure 4.16: IgG specific response of clostripain digested rTcdB$_{547-2366}$ and TcdB with human sera.

30 minute digestion of 5 µg of protein by 1U activated clostripain, analysed by SDS-PAGE on a 4–20% gel and transferred to nitrocellulose and detected with SuperSignal Femto. Molecular weight marker in kDa. Blue boxes indicate 207 and 280 kDa full length rTcdB$_{546-2366}$ and TcdB, respectively. Red boxes indicate commonly recognised fragments.

4.4.7.2 Toxin B IgA Specific Response

From visual observations, the two sera which displayed the strongest IgG responses were also the only sera to display an IgA toxin B specific response (Figure 4.17). For $rTcdB_{547-2366}$, immunoreactive bands were seen between 25-75 kDa, in addition to these bands immunoreactive bands >75kDa were also observed for TcdB (Figure 4.17).

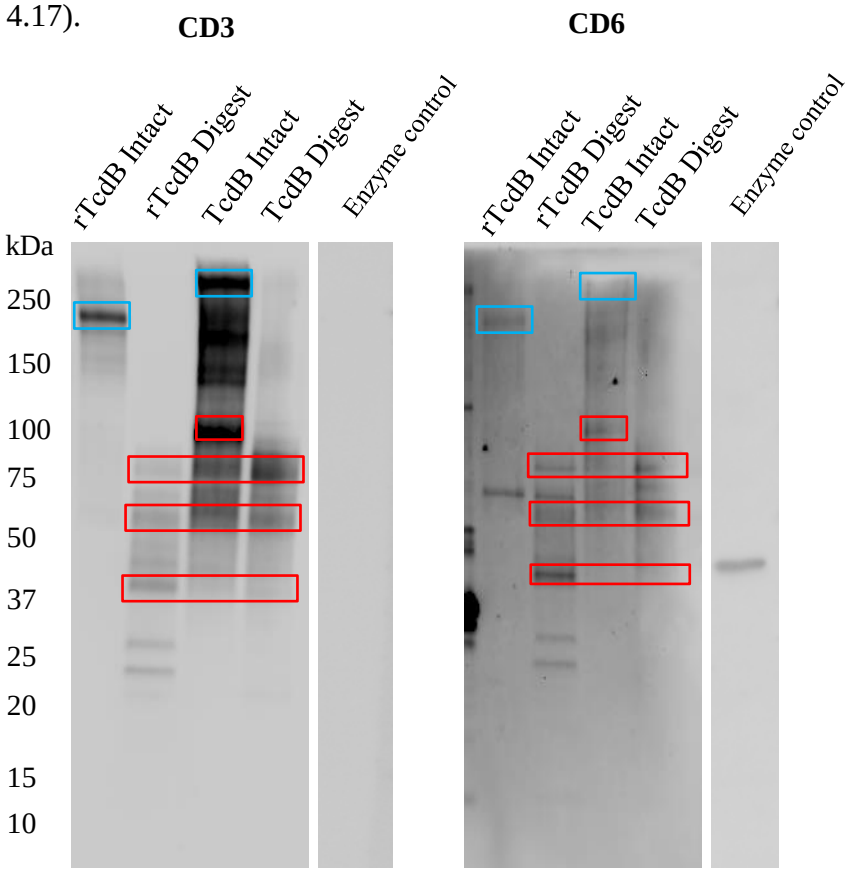


Figure 4.17: IgA specific response of clostripain digested $rTcdB_{547-2366}$ and TcdB with human sera.

30 minute digestion of 5 µg of protein by 1U activated clostripain, analysed by SDS-PAGE on a 4–20% gel and transferred to nitrocellulose and detected with SuperSignal Femto. Molecular weight marker, kDa. Blue boxes indicate 207 and 280 kDa full length $rTcdB_{546-2366}$ and TcdB, respectively. Red boxes indicate commonly recognised fragments.

The human sera recognised multiple immunogenic bands in the toxin B preparations. The cluster of bands (37-75 kDa) identified by the human sera resembled the bands identified by the goat and sordellii antitoxins (Figure 4.13). The cross-reactivity with clostripain and *E. coli* derived host protein(s) further complicated the analysis.

4.4.8 Probing of Toxin A with *C. difficile* Infected Immune Sera

As was the case for toxin B, a wide variation in the toxin A protein fragments which were recognised by immune serum from different patients was observed. The ability of IgG and IgA antibodies from *C. difficile* infected patients to recognise toxin A, toxoid A and rTcdA$_{900\text{-}2710}$ are shown in Figure 4.18 and 4.19, respectively.

4.4.8.1 Toxin A Specific IgG Response

All serum samples contained IgG antibodies which were able to recognise intact TcdA (Figure 4.9, blue boxes). For digested TcdA, the immune response was predominantly centralised in the high molecular weight region (>37 kDa), with sera CD5 and CD6 displaying the weakest IgG response against digested TcdA, both in the number and visual intensity of the fragments identified. All patient sera identified a fragment with an apparent molecular weight of 40 kDa. Regions of 100, 75 and 60 kDa were also found to be immunogenic at varying intensities (Figure 4.9, red boxes).

The overall IgG response to rTcdA$_{900\text{-}2710}$ was found to be consistent across all four patient sera, with a cluster of high intensity bands identified between 30-100 kDa (particularly for CD5 and CD6). An IgG specific response to intact toxoid A was only evident for sera CD1 and CD3. This suggests that the ability of patients to recognise the toxoid will be variable.

Despite the significant amount of variation between the serum samples, these results suggest the presence of common immunogenic regions, amongst all three toxin A preparations. A 40 kDa fragment of toxin A was detected in all of the samples, to a varying degree. Given its close proximity to the predicted molecular weight of the heavy chain of clostripain (42 kDa), it cannot be ruled out that this immunogenic band is enzyme derived. Although its presence in the intact undigested toxin A preparation does suggest that it represents a toxin derived fragment.

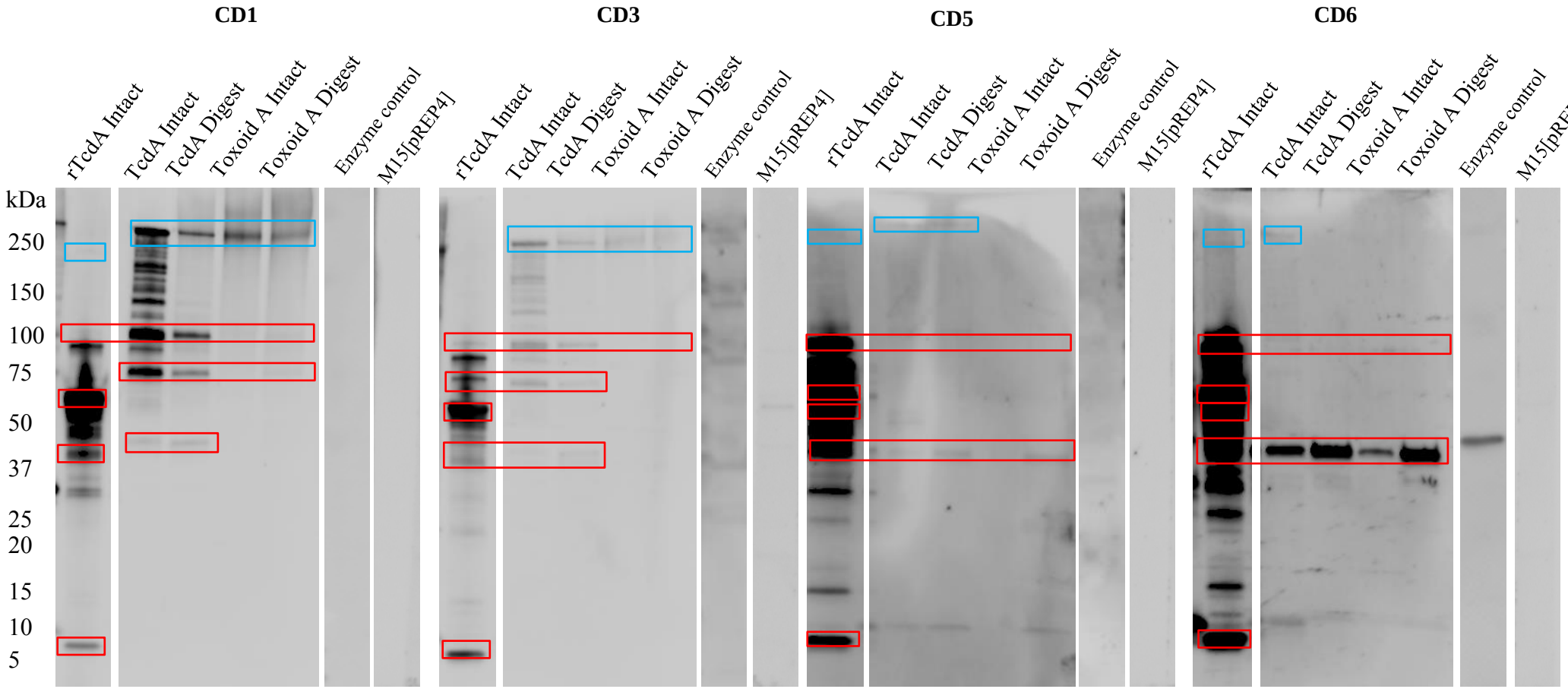


Figure 4.18: IgG specific response of clostripain digested TcdA and toxoid A and intact rTcdA$_{900\text{-}2710}$, probed with human sera.

48 hour digestion of 5 µg of protein by 1U activated clostripain, analysed on a 4–20% SDS-PAGE gel, transferred to nitrocellulose and detected with SuperSignal Femto. Red boxes indicate regions of commonality. Blue boxes indicate 205 and 308 kDa full length rTcdA$_{900\text{-}2710}$ and TcdA, respectively.

4.4.8.2 Toxin A Specific IgA Response

Overall, the pattern of the IgA responses (Figure 4.19) were similar to those seen with IgG response, although there was a reduction in the number of bands detected in the $rTcdA_{900\text{-}2710}$ preparation

Whilst all patient sera contained IgG antibodies which recognised intact TcdA, only two serum samples contained IgA antibodies with specificity for the intact toxin. Only serum CD3 displayed an IgA response against intact toxoid A. As with IgG, a fragment of 40 kDa was consistently observed in each of the toxin A preparations by all of the patient sera. This corresponds with the 40 kDa fragments detected with the goat and sordellii antitoxin (Figure 4.13).

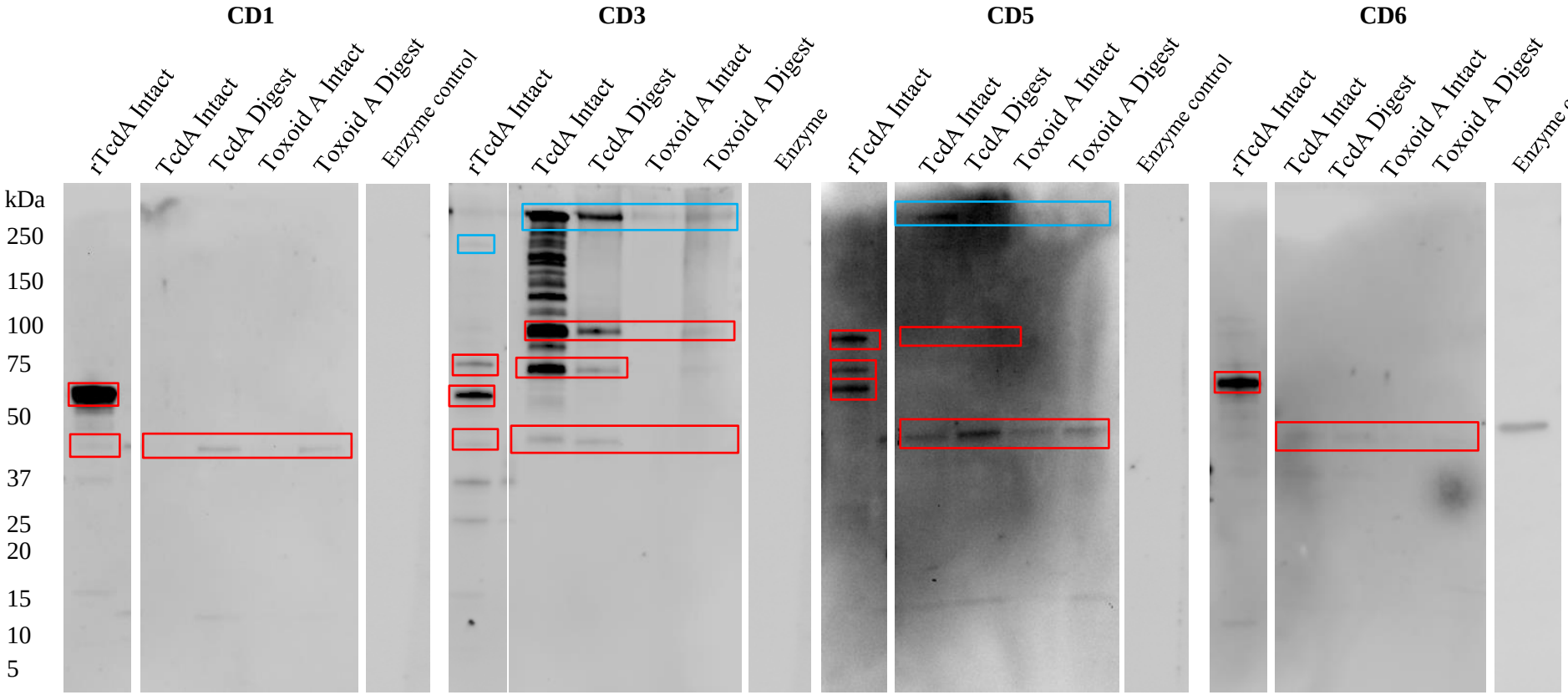


Figure 4.19: IgA specific response of clostripain TcdA and toxoid A and intact rTcdA$_{900\text{-}2710}$, probed.

48 hour digestion of 5 µg of protein by 1U activated clostripain, analysed on a 4–20% SDS-PAGE gel, transferred to nitrocellulose and detected with SuperSignal Femto. Molecular weight marker in kDa. Red boxes regions of commonality. Blue boxes indicate 205 and 308 kDa full length rTcdA$_{900\text{-}2710}$ and TcdA respectively.

4.4.9 Mass Spectrometry

Common immunogenic fragments detected by human immune sera, goat and sordellii neutralising sera were selected for sequencing. All bands were manually selected from silver stained 1D SDS-PAGE gels.

Table 4.5: Bands selected for mass spectrometry.

Toxin preparation	Molecular weight (kDa)
Toxin A	40
	60
	25
rTcdA$_{900-2710}$	40
	60
	25
Toxoid A	40
	60
Toxin B	60
	25
rTcdB$_{547-2366}$	60
	40

Unfortunately it was not possible to identify the immunogenic bands at a sequence level and hence the location of these immunogenic bands could not be determined. Protein identification by mass spectrometry is a multistep process with a number of limitations. Although trypsin is the most commonly used protease for generating peptides for identification, it may not be the optimal protease for all proteins. The generation of sufficient peptides for unambiguous identification is paramount and it may have been beneficial to use a combination of proteases and/or chemicals to facilitate peptide generation. The types of peptides generated may not be reflective of those predicted for trypsin, either due to non-specific or missed cleavage. This may have occurred during peptide preparation or as a consequence of clostripain mediated digestion. The choice of matrix utilised for the identification of peptides will influence ionization, stability and fragmentation of peptides (Lubec and Afjehi-Sadat, 2007). In this study the matrix of choice was α-cyano-4-hydroxycinnamic acid (CHCA), which has been shown to be applicable for a range of peptides (Vestal

and Campbell, 2005). However, if the generated peptides are >2.5 kDa (Jensen *et al.*, 1997), as may be the case if indeed the trypsin digestion has been unsuccessful, then the matrix would need to be optimised. As the bands were selected from 1D SDS-PAGE gels it is also feasible that two or more proteins were simultaneously analysed. In addition to the peptides from the toxins, the presence of the digestion enzymes may have complicated analysis. Although filters were applied to remove these known contaminants from the analysis, it is impossible to eliminate all contaminants. Due to the lack of time and resources (both financial and availability of toxin samples), it was not possible to optimise all of the aforementioned factors and hence it was not possible to attempt sequencing again.

4.5 Discussion

In this chapter we aimed to identify immunogenic fragments of native and recombinant toxins A and B and toxoid A, using animal sera and human sera.

4.5.1 Toxin Digestion

in silico analysis revealed that the proteins could be digested by a number of proteases and chemicals generating upwards of 1000 fragments. With the intended techniques, it was not possible to resolve fragments of this number. Based on its high substrate specificity, preferentially cleaving arginine residues (Mitchell, 1977; Otto and Schirmeister, 1997) and its prediction to yield 51 fragments, clostripain was identified as a suitable enzyme. The number of peptide fragments seen in this study was less than the number predicted by the computer software. Closer analysis of the predicted fragments revealed many of the fragments to be ≤2 kDa, which is below the limit of detection of conventional SDS-PAGE. In addition, some of the fragments were of similar molecular weights, and thus could not be distinguished, using a 1D SDS-PAGE approach. Finally the digestion prediction programme does not take into consideration the conformational structure of the protein. Hence, the predicted number of fragments is likely to be an overestimation of what can be achieved in reality.

Based on the time taken to achieve digestion, 48 hours and 30 minutes, for TcdA and TcdB, respectively, it would appear TcdB is more susceptible to clostripain digestion than TcdA. TcdA has been reported to be resistant to pure trypsin, even at high concentrations (10 mg/mL), whilst TcdB is susceptible (Torres and Lönnroth, 1988; Lyerly *et al.*, 1989). A recent study has utilised trypsin, chymotrypsin, endoproteinases GluC, AspN and LysC separately and in combination to digest both toxin A and B, with digestion conditions of 37°C for 2-24 hours, depending on the enzyme (Moura *et al.*, 2013a). Whether the difference in digestion susceptibility is associated with the grade of protease utilised or the digestion conditions is unclear. If TcdA is resistant to trypsin digestion it may also be resistant to the proteolytic action of numerous enzymes. These observations mirror the natural autoprocessing of TcdA and TcdB, whereby TcdA is more resistant to InsP6-induced cleavage than

TcdB (Kreimeyer *et al.*, 2011; Pruitt and Lacy, 2012), it is intriguing to speculate that an *in vivo* mechanism which (possibly) prevents premature processing of the toxin confers universal resistance to proteolytic degradation. The expression and processing of bacterial toxins is a co-ordinated and controlled approach presumably intended to benefit the bacterium. In the case of *C. difficile* the role of toxin production, to the bacterium, is unclear. Bacterial toxins may be produced to acquire nutrients from the host, thus for *C. difficile* this would allow the bacterium to maintain its vegetative state in an anaerobic environment. Alternatively, the destruction of host cells, aids in the invasiveness and dissemination of the pathogen further into the host tissue. This would appear somewhat contradictory for *C. difficile* as it would result in exposure to aerobic environments resulting in cell death or spore formation. Having said this there are reported cases of extraintestinal *C. difficile* infections, including bacteremia (Libby and Bearman, 2009; Lee *et al.*, 2010b). Given that the majority of *C. difficile* bacteremia are polymicrobial in nature, involving the gut flora (Bacteroides spp., *E. coli*, Enterococci spp.) (Libby and Bearman, 2009), it is intriguing to speculate that the *C. difficile* toxins are expressed to facilitate the translocation and dissemination of other gut microbes, thus reducing the competition within the intestinal niche occupied by *C. difficile* and indirectly enhancing the pathogenicity of multiple gut bacteria.

Recently, Marozsan *et al*., (2012) utilised a proteolysis and Western blot approach to epitope map the location of antitoxin A (PA-50) and antitoxin B (PA-41) monoclonal antibodies. The authors used enterokinase and caspase-1 for the digestion of TcdA and TcdB, respectively. As the authors do not provide coomassie or silver stained gels, comparison of digestion profiles could not be made with the data presented in this thesis. Interestingly the protease, enterokinase, employed by Marozsan and colleagues for the digestion of TcdA, was not predicted to cut the protein when analysed using the PeptideCutter software in this thesis. Thus it may be the case that the fragments identified by Marozsan *et al.*, represent natural degradation products due to the incubation conditions employed (48 hours at 25°C), as opposed to enzymatic digest fragments. In the case of TcdB, Marozsan *et al.*, (2012) digested the toxin for 96 hours at 37°C, this appears to be excessive given the susceptibility of

TcdB to proteolytic digestion as compared to TcdA (results presented in this thesis and Lyerly *et al.*, (1989) and Taylor *et al.*, (1981).

The major limitation of employing an enzymatic digestion approach is the potential immunogenicity of the protease itself, as observed in this study. In order to reduce this effect it may have been feasible to utilise a Fluorescence Difference Gel Electrophoresis (DIGE) approach, whereby the digest enzyme and the toxin sample are labelled with unique fluorescent dyes allowing for differentiation following imaging. This approach would also allow for the recombinant and native toxin preparations to be run simultaneously allowing for easier comparisons. In spite of the difficulties encountered with the enzymatic digestion approach it does appear to be a feasible method for the generation of multiple fragments which can facilitate mapping studies.

4.5.2 Immunoreactive Regions of TcdA and TcdB

It was only possible to make a qualitative assessment on the immunogenic regions of TcdA and TcdB in this study, with attempts at sequencing failing to yield identifiable peptide bands.

The use of recombinant proteins is primarily intended to facilitate the yield and purity of toxin available for investigation (Just and Gerhard, 2004). The immunogenicity of recombinant toxin B, with the monoclonal murine antibody suggests it can be employed as a surrogate for the native toxin B. For toxin A the use of PCG-4 (Frey and Wilkins, 1992), a well characterised murine monoclonal antibody should have been employed. This analysis was not undertaken due to the problems encountered in obtaining a high yield of recombinant toxin A.

Although the neutralising capability of *C. sordellii* antitoxin has been established for over three decades (Rifkin *et al.*, 1977; Chang *et al.*, 1978a; Chang *et al.*, 1978b; Larson *et al.*, 1978; Allo *et al.*, 1979; Popoff, 1987; Martinez and Wilkins, 1988), to the best of our knowledge, no study has attempted to map the regions of TcdA and TcdB which are recognised by this serum. This is surprising given the documented cross-neutralisation of *C. difficile* and *C. sordellii* toxins (Allo *et al.*, 1979; Fernie *et*

al., 1983). The earliest study found to display immunoblot data relating to the cross-reactivity of the lethal toxin of *C. sordellii* and the cytotoxin of *C. difficile* was conducted by Popoff (1987). Although the sera displayed neutralising activity it failed to recognise antigen from *C. difficile* culture supernatant. This may be due to the concentration of antibody used to probe the blot (1/100 compared to 1/25 used in this study) or the low concentration of toxin A and B present in the culture supernatant. As the author does not provide culture conditions for *C. difficile* it was not possible to ascertain the effect of the media used on the production of toxins (Karlsson *et al.*, 1999; Merrigan *et al.*, 2010; Vohra and Poxton, 2011). In 1990, von Eichel-Streiber *et al.*, used *C. sordellii* antiserum to isolate recombinant clones expressing regions of TcdB. Three of the clones were located at the N-terminus and three at the C-terminus of TcdB, suggesting that multiple epitopes are recognised by the sordellii antitoxin and neutralisation may be achieved both by blocking receptor binding (C-terminus) and enzymatic activity (N-terminus). It should be noted that von Eichel-Streiber *et al.* (1990) did not purify the recombinant clones and utilised total cell lysates for Western blot analysis, thus non-specific binding of the polyconal sera with proteins from the *E. coli* expression system cannot be excluded, as found in this thesis (Figure 4.13).

The relatively reduced reactivity observed against TcdA with the sordellii antitoxin, is suspected to be due to the antitoxin being raised against the lethal toxin of *C. sordellii*, which displays a 75% and 41% amino acid sequence similarity with TcdB and TcdA, respectively (Rupnik and Just, 2006), although this has not been definitively determined.

In contrast to the sordellii antitoxin, which was raised against the culture filtrate from *C. sordellii*, the goat serum used in this study was produced from a toxoided culture filtrate of *C. difficile*. The results suggest that the toxoiding process does not appear to affect immunogenicity, at least when compared to native TcdA. However, given that the antitoxin has been generated from a toxoid preparation, this would be expected. If, however the goat antitoxin (like the sordellii antitoxin) predominantly recognises TcdB, it is imperative to assess the effect of toxoiding on the immunoreactivity of toxoid B. Using circular dichroism spectrometry, a recent study

has shown conformational changes in a toxoid B preparation compared to the native toxin (Wang *et al.*, 2012). The authors report the toxoid B preparation displayed lower IgG neutralising antibody response in a mouse model, with highly neutralising epitopes absent in the toxoid preparation, resulting in 70% lethality following challenge with TcdB. This study emphasises the importance of generating a neutralising antibody response, which may not be efficiently generated using a toxoided vaccine.

The utilisation of recombinantly produced regions frequently raises concerns with regards to the level and type of immune response that is likely to be generated, as there is no guarantee that the recombinant protein will produce conformational epitopes which mirror the native protein. Due to limited reagents and time it was only possible to complete the SDS-PAGE and immunoblot profiles once, and although the profiles for the toxin B preparation in this study suggest that similarities exist between the native and recombinant proteins, this would need to be repeated for confirmation.

The use of recombinant toxins in immunisation and neutralisation studies (Lyerly *et al.*, 1990; Kink and Williams, 1998; Ward *et al.*, 1999a; Ward *et al.*, 1999b; Gerhard *et al.*, 2005; Permpoonpattana *et al.*, 2011; Tian *et al.*, 2012), further suggests recombinant proteins would stimulate the appropriate immune response, whilst eliminating problems encountered using a toxoided approach. A chimeric toxin composed of the receptor binding domain of TcdA (amino acid residues 1848-2710) and the glucosyltransferase and translocation domain of TcdB (amino acid residues 1–1851) conferred long-term protection (3 months) in mice against lethal challenge with TcdA and TcdB (Wang *et al.*, 2012).

The location of toxin neutralising epitopes in the receptor binding domain of the toxins (particularly for TcdA) has been demonstrated by a number of researchers (Corthier *et al.*, 1991; Frey and Wilkins, 1992; Kink and Williams, 1998; Babcock *et al.*, 2006; Demarest *et al.*, 2010; Hussack *et al.*, 2011; Marozsan *et al.*, 2012). The presence of multiple epitopes in the receptor binding domain, is likely to be a reflection of multivalent receptor binding sites which are located within this domain

(Ho *et al.*, 2005; Greco *et al.*, 2006). Further evidence of multiple neutralising epitopes within the receptor binding region, has been provided by mapping studies using the toxin neutralising murine monoclonal antibody, PCG-4, which recognises amino acid residues 2097-2141 and 2355–2398 (Lyerly *et al.*, 1985a; Frey and Wilkins, 1992).

The literature suggests that at least one toxin neutralising epitope is located in the N-terminal domain of TcdB. Marozsan *et al.*, (2012) mapped the location of a humanised toxin neutralising monoclonal antibody (PA-41) to a 63 kDa region within the N-terminal domain of TcdB. As the recombinant protein produced in this thesis lacked this domain, potential immunogenic regions within this domain would not be detected. Although this limitation was somewhat circumvented by the incorporation of native toxin B.

CDA1 and CDB1 represent the first human monoclonal antibodies (HuMAbs) specific for TcdA and TcdB used in a human clinical trial for the treatment of recurrent CDAD (Babcock *et al.*, 2006; Lowy *et al.*, 2010). The CDB1 antibody in this study bound to a region within the C-terminal region of toxin B, in contrast to PA-41 which recognised a neutralising epitope at the N-terminus of the toxin (Marozsan *et al.*, 2012). Thus toxin B neutralisation can be achieved by targeting both the enzymatic and receptor binding domains, and when attempting to develop a vaccine, it may be beneficial to target multiple epitopes across various regions of the toxin B molecule.

The variability in the ability of patient sera to recognise individual toxin fragments may be a reflection of a number of factors including the severity of infection and the strain(s) of the pathogen to which the individual has been exposed. In this research, based on visual intensity of immunoblots, sera CD3 and CD6 displayed the strongest toxin specific IgG and IgA response, if this is a reflection of the multiple episodes of CDI experienced by these individuals, it provides further support for the importance of an anamnestic toxin immune response (Johnson *et al.*, 1992; Warny *et al.*, 1994;

Kyne *et al.*, 2000; Loo *et al.*, 2011). For *C. difficile*, sIgA has been suggested to be important for protection (Wada *et al.*, 1980; Kelly *et al.*, 1992; Warny *et al.*, 1994; Johnson *et al.*, 1995; Ward *et al.*, 1999b; Stubbe *et al.*, 2000), thus it was anticipated that in recurrent patients (CD3 and CD6) a IgA response would be evident, this was demonstrated for toxin B (Figure 4.17) but less evident for toxin A (Figure 4.19), with only CD3 recognising intact toxin A. This difference may be a related to the type of epitope (linear or conformational) that is recognised by the sera or the strain responsible for infection.

As part of this study it had been our intension to generate immuno-profiles of individuals with single and recurrent episodes of infection, with the intention to identify differences in the relative quality of the antibody responses of the two patient groups as a means of identify key protective targets. Unfortunately due to the time taken in obtaining ethical approval and subsequently in obtaining serum samples, it was not possible to undertake these studies. We were also unable to determine the *C. difficile* ribotypes/toxinotypes of the strains which had infected the patients from whom we had obtained sera. Given that there are antigenic variations in toxins between strains (Lanis *et al.*, 2013); it is feasible the antibodies present in the patient sera are specific to a particular toxinotype which does not cross-react with the toxins used in this study (VPI 10463 and *C. difficile* 630).

It has recently been shown that the presence of toxin neutralising sIgA antibodies, stimulated by oral immunisation with A26-39 provided a stronger correlation with protection in a hamster model than serum IgG antibody responses (Permpoonpattana *et al.*, 2011). A26-39 (formerly known as 14CDTA) corresponds to amino acid residues 2386-2706 of the C-terminal region of TcdA. This study also showed that serum raised against this region of toxin A cross-reacted with the C-terminal region of toxin B, B15-24 (amino acid residues 2137-2366). Analysis of these regions showed a 29 and 37% sequence identity and similarity, respectively, with conserved regions at sequences YFAPANT, MQ, IGVF, AAT, and YYF. In light of this research the identification of common regions recognised by IgA and IgG (particularly for TcdA) would represent valid targets for future therapeutic developments. For example the 40 and 60 kDa fragment in the toxin A preparations,

identified in this thesis by human (IgG and IgA) (Figure 4.18 and 4.19) and toxin neutralising animal antisera (Figure 4.15) may represent promising candidates. It is feasible that this fragment correlates to the 37 kDa fragment utilised by Permpoonpattana *et al.*,(2011) and would also include one of the PCG-4 epitopes (Frey and Wilkins, 1992). In hindsight probing with the PCG-4 antibody would have been beneficial to further assess the immunogenicity of these regions. The presence of common immunogenic regions in toxin A and B could not be determined in this thesis, due to the failure of protein identification.

One significant limitation of the current study is that the toxin sequences (both native and recombinant) are based on VPI 10463 ($TcdB_{003}$) and *C. difficile* 630 ($TcdB_{012}$), respectively. Whilst the hypervirulent 027 strains, which have been sequenced to date, expressed almost identical TcdA (99% similarity and 98% identity) when compared to that produced by VPI 10463 and *C. difficile* 630, a greater degree of variation in TcdB sequences between these strains was observed (96% similarity, 92% identity) (Lanis *et al.*, 2010; Lanis *et al.*, 2012; Lanis *et al.*, 2013). This variation is most prominent in the C-terminal domain (CTD, 88% sequence identity between $TcdB027_{1651\text{-}2366}$ and $TcdB003_{1651\text{-}2366}$). Given that the recombinant protein produced in this thesis included this region, the immunogenic fragments recognised in this study may not represent all of the immunogenic regions with toxin B homologs produced by 027 strains.

In a recent study the immunogenicity of the C-terminal receptor binding domain (amino acid residues 1651-2366) of toxin B from two different ribotypes of *C. difficile* (ribotype 027 and 003) was assessed (Lanis *et al.*, 2013). Although thirteen overlapping epitopes were recognised, no shared cross-neutralising epitopes were found between the two ribotypes and significantly there was an absence of neutralising epitopes in the C-terminus of $TcdB_{027}$. Given this recent finding the ability of the commercially available neutralising animal sera and indeed the human sera to recognise $TcdB_{027}$, should be under taken.

4.6 Conclusion

This chapter aimed to identify the immunogenic fragments of toxin A and toxin B recognised by toxin neutralising animal sera and human sera from *C. difficile* infected individuals. Utilising a proteolysis based approach; native, recombinant and toxoided proteins were successfully digested and immunogenicity of the resulting fragments identified. Although toxin A and toxoid A were found to display instability under the incubation conditions employed, the proteins were not susceptible to clostripain mediated digestion. This is in contrast with toxin B which was both stable and susceptible to enzyme mediated digestion. Regions of commonality were found between the recombinant and native toxin preparations, which was particularly evident for toxin B, based on 2D SDS-PAGE profiles. In comparison to native toxin A, the toxoid A immunoblot profiles displayed a reduced binding affinity across all antibodies tested.

The two commercially available neutralising animal sera recognised both TcdA and TcdB, with the latter toxin showing greater immunogenicity against the sordellii antisera, possibly due to the sordellii sera being raised against the lethal toxin of *C. sordellii* which displays a greater homology with the *C. difficile* toxin B.

The immunoblot profiles obtained in this study with the human sera varied greatly. Given the limited number of samples analysed and the inherent variability of the immune response to these toxins these preliminary results must be validated to assess repeatability and also with the use of additional serum samples. The presence of a 40 and 60 kDa immunogenic band in the toxin preparations recognised by both neutralising and human sera, warrants further research.

CHAPTER FIVE

THE VARIABILITY OF VEGETATIVE *CLOSTRIDIUM DIFFICILE*

5.1 Introduction

Chapters 3 and 4 have focused on characterising the primary virulence factors (TcdA and TcdB) of *C. difficile*. This chapter examines the vegetative form of *C. difficile* cells, focusing primarily on morphotype variability and their associated surface structures and the contribution they make to the survival of the bacterium in the gut.

5.1.1 Colony Variants

Small colony variants (SCVs) represent a slow growing sub-population of bacteria that are one-tenth the size of wild-type bacterial colonies and display unique phenotypic and pathogenic traits (Proctor *et al.*, 2006). Common traits include slow growth, auxotrophy, antibiotic resistance and ability to revert to wild-type colony (Proctor *et al.*, 2006). They are not restricted to a particular genera or species and have been reported in *Lactobacillus acidophilus* (Kopeloff, 1934), *S. aureus* (Bulger, 1967), *B. subtilis* (Carlisle and Falkinham, 1989), *V. cholerae* (Salles *et al.*, 1976) and *Burkholderia pseudomallei* (Chantratita *et al.*, 2007). It has been suggested that SCVs in these bacteria contribute to disease progression by promoting bacterial persistence and survival (Proctor *et al.*, 2006). The presence of variant colonial morphotypes in *C. difficile* has attracted little or no attention, with the presence of "dwarf" colonies observed by a number of researchers (Brazier and Borriello, 2000). Whether the "dwarf" colonies of *C. difficile* represent SCVs is yet to be determined.

5.1.2 Bile Salts

Despite the relationship between *C. difficile* spores and bile salts (primarily taurocholate) being widely recognised, the interaction between these compounds and vegetative *C. difficile* cells is yet to be fully characterised. Bile is a biological detergent, synthesised from cholesterol and produced by the liver, it is composed of proteins, ions, pigments and bile acids (Merritt and Donaldson, 2009). Cholic acid and chenodeoxycholic acid represent the primary bile acids (Begley *et al.*, 2005; Ridlon *et al.*, 2006), which are conjugated with taurine (to form taurocholate or taurochenodeoxycholate) or glycine (to form glycocholate or glycodeoxycholate).

These secondary bile salts can be deconjugated into the primary bile salts (cholate and chenodeoxycholate) by bile salt hydrolases (BSH) found on the surface of bacterial cells (Ridlon *et al.*, 2006). Intestinal bacteria, such as *Clostridium scindens*, further metabolise the deconjugated primary bile salts, converting cholate into deoxycholate and chenodeoxycholate into lithocholate (Begley *et al.*, 2005; Ridlon *et al.*, 2006). Finally, lithocholate can be metabolised by intestinal bacteria to form the tertiary bile salt ursodeoxycholate (Merritt and Donaldson, 2009), summarised in Figure 5.1.

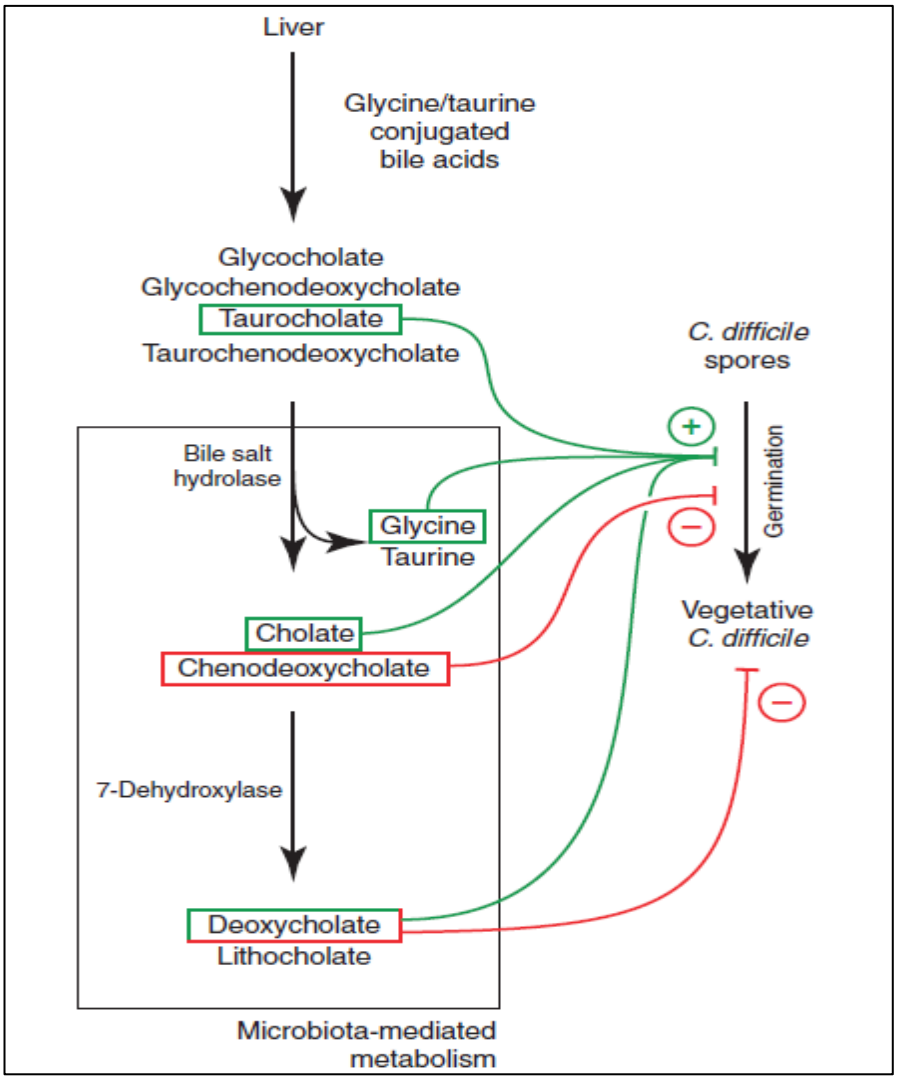


Figure 5.1: Bile acid metabolism and *C. difficile*.

Metabolism of bile salts in the intestine and their positive and negative effects on *C. difficile*. From Britton and Young, (2012).

Understanding the interaction between bile salts (both conjugated and unconjugated as well as primary, secondary and tertiary) and bacteria will facilitate an understanding of how bacteria, particularly *C. difficile*, can survive within the human gut. To date, the relationship of *C. difficile* with bile salts has focussed on the germination of spores in response to taurocholate (Wilson *et al.*, 1982; Wilson, 1983; Sorg and Sonenshein, 2008; Sorg and Sonenshein, 2009, 2010; Heeg *et al.*, 2012). The interaction of *C. difficile* spores and bile salts occurs along the GI tract, resulting in germination (Sorg and Sonenshein, 2008; Wheeldon *et al.*, 2011). Thus far, chenodeoxycholate is the only reported bile component that inhibits the germination of *C. difficile* spores (Sorg and Sonenshein, 2009, 2010) and inhibits the growth of vegetative *C. difficile* cells (Sorg and Sonenshein, 2008) (Figure 5.1), although this relationship appears to be strain specific (Heeg *et al.*, 2012).

Enteric bacteria have developed resistance mechanisms (although poorly understood) to survive the toxic effects of bile. These include efflux pumps (*acr*), Tol genes (*tolQRA*), porin genes (*ompF*) and regulatory genes (Gunn, 2000; Noriega *et al.*, 2004). As *C. difficile* is a gut pathogen, once spores germinate, the vegetative organism is exposed to bile salt concentrations ranging between 0.2-2% (w/v) in the intestines (Gunn, 2000). Thus the organism must possess some mechanism by which it tolerates exposure to bile salts. The mechanism(s) by which this is achieved in *Clostridium* spp. is unknown; analysis of the *C. difficile* genome has identified the presence of a bile acid inducible dehydrogenase gene (CD0065), which converts chenodeoxycholic acid into lithocholic acid (Sebaihia *et al.*, 2006). Orthologs of *bilEA* (CD3215) and *bilEB* (CD3216), which function as bile exclusion systems in *Listeria monocytogenes*, have also been identified in *C. difficile* (Sebaihia *et al.*, 2006).

In *Bacteroides fragilis* (Gram-negative, enteric, anaerobic, commensal), phase variation has been studied in relation to the expression of eight distinct capsular polysaccharides (Chatzidaki-Livanis *et al.*, 2009; Chatzidaki-Livanis *et al.*, 2010). Phase variation is associated with host-pathogen interactions (immune evasion and colonisation) and strain evolution (stress resistant phenotype and protection against foreign DNA and phage) (van der Woude, 2011). *C. difficile* is also capable of phase

variation (Emerson *et al.*, 2009), comparative analysis of three *C. difficile* genomes (630, R202091, CD196) has identified 3 intragenic inversions (Cdi1, Cdi2 and Cdi3) (Stabler *et al.*, 2010) and at least four tyrosine recombinases *recV* (CD1167), CD1222, CD1333 and CD1932, and phage-related recombinases (Stabler *et al.*, 2010). The surface exposed, CwpV is a 160 kDa phase variable protein which is post-translationally cleaved into a 40 kDa cell wall anchoring domain and a 120 kDa surface exposed domain. CwpV is hypothesised to promote aggregation but may also be involved in immune evasion. Intriguingly CwpV mutants display altered colony phenotypes, in these mutants the protein is permanently on (Emerson *et al.*, 2009; Reynolds *et al.*, 2011). Given that both *B. fragilis* and *C. difficile* are intestinal bacteria and phase variation in the former allows evasion and survival (Chatzidaki-Livanis *et al.*, 2010), phase variation in gene regulation of *C. difficile* may also be of equal importance.

The presence of variant colony morphologies in *C. difficile* is an aspect of *C. difficile* microbiology which is yet to be fully investigated. This chapter will address the variability of *C. difficile* at the macroscopic (colony morphotype), microscopic, proteomic and immunological level and attempt to draw some conclusions as to the contribution of these variants to virulence.

5.2 Aims and Objectives

The aims of this chapter are to characterise the vegetative cell surface of *C. difficile*, with a view to identifying components which contribute to virulence and immunogenicity.

The experimental objectives are to:

1. Assess the frequency and stability of variant *C. difficile* morphotypes.
2. Assess the susceptibility of morphotypes to bile salts.
3. Characterise variant *C. difficile* via light microscopy and electron microscopy.
4. Analyse the surface proteome of variant *C. difficile* using 1D and 2D SDS-PAGE
5. Identify immunogenic proteins using immune serum from animals and infected individuals.

5.3 Materials and Methods

All media was prepared in sterile de-ionised water (diH_2O) (inorganics at 25°C, 15 MΩ-cm, Total Organic Carbon <30 ppb) from an Elga PureLab Option-S (Elga, UK). Unless otherwise stated, all reagents were purchased from Fisher Scientific, UK or Sigma-Aldrich, UK.

5.3.1 Bacterial Strains

Twenty-one *C. difficile* isolates were kindly donated by Dr. Jon Brazier of the Anaerobic Reference Unit, Cardiff. NCTC strains were purchased freeze-dried from the Health Protection Agency (Colindale, UK). Details of these strains are noted in Table 5.1.

Table 5.1: *C. difficile* isolates used in this study.

Strain	Ribotype	Patient Sex	Patient Age	Origin
DS1759	001	M	87	Maidstone
DS1747	001	F	82	St James Leeds
R8652	001	*	*	NCTC 11209
DS1750	001	F	88	St James Leeds
DS1813	027	M	80	Hinchingbrooke
DS1801	027	F	64	Leicester
R20291	027	*	*	Stoke Mandeville
DS1807	027	F	31	Salford
R10459	106	*	*	Dudley
DS1798	106	M	94	Poole
DS1787	106	M	83	Leicester
DS1771	106	M	88	Bristol Southmead
DS1742	014	M	27	Bristol Frenchay
DS1748	002	F	83	Leeds
DS1721	005	M	66	Leicester
DS1752	012	F	81	Bradford
DS1723	078	M	71	Leicester
DS1724	020	M	83	Leicester
DS1684	010	F	92	Brighton
DS1665	023	F	92	Bath
NCTC 11204	001	*	*	Sheffield
Unknown	002	*	*	*

*Information not available

5.3.2 Anaerobic Conditions

All work was carried out using a gloveport Bugbox Plus anaerobic workstation (Ruskinn Technology Limited, UK) or an Electrotek AW200SG anaerobic workstation (Electrotek, UK). The anaerobic environment consisted of 85% N_2, 10% CO_2, 5% H_2 and was maintained by a palladium catalyst and circulated by a tangential fan. Disposable anaerobic indicator strips (Becton Dickinson, UK) were replaced daily to confirm a strict anaerobic environment was maintained. The temperature of the work station was set at 37°C and monitored by a temperature probe.

5.3.3 Culture Media

Vegetative *C. difficile* were routinely cultured on BHI agar and BHI broth prepared by following the manufacturer's instructions. *C. difficile* spores were cultured on BHI agar supplemented with 0.1% (w/v) sodium taurocholate. Blood agar plates were prepared with Columbia Blood Agar base (Oxoid Ltd., Basingstoke, UK), supplemented with 5% (v/v) defibrinated horse blood (Oxoid Ltd., Basingstoke, UK) by following the manufacturer's instructions. Cycloserine-Cefoxitin-Fructose Agar (CCFA) plates were purchased from Oxoid Ltd. (Basingstoke, UK). Proteose Peptone Yeast (PPY) extract media was prepared as follows (per litre of diH_2O) (proteose peptone, 40 g; yeast extract, 5 g; agar, 20 g). VL media was composed of (per litre of diH_2O) tryptone, 10 g; NaCl, 5 g; beef extract, 3 g; yeast extract, 5 g; cysteine hydrochloride, 0.4 g, glucose, 2.5 g; agar, 12 g supplemented with 5% (v/v) defibrinated horse blood (Beerens *et al.*, 1963). Media was sterilised for 20 minutes at 121°C (Prestige Medical, UK) and reduced at 37°C for 24 hours in an anaerobic work station prior to use. Cultures were incubated at 37°C for 48 hours in an anaerobic workstation.

5.3.4 Freezer Cultures

PROTECT microbial preservation kit (Technical Service Consultants Ltd, UK) was used for long-term *C. difficile* storage. Multiple colonies from a 48 hour culture

were inoculated in the cryopreservative fluid. The vial was vigorously shaken and the liquid removed using a sterile Pasteur pipette and stored at -80°C. Rescue from these stocks was performed onto blood plates (7% (v/v) defibrin horse blood) supplemented with *Clostridium difficile* Moxalactam Norfloxacin (CDMN) selective supplement (Oxoid, UK).

5.3.5 Spore Production

C. difficile and *C. perfringens* spores were prepared following the method described by Perez *et al.*, (2005). Colonies from a 48 hour culture were inoculated in 25 mL pre-reduced BHI broth and incubated for 5-10 days at 37°C under anaerobic conditions. The culture was centrifuged at 5,000 *g* for 15 minutes at 4°C (Heraeus Primo R, Thermo, UK). The supernatant was discarded and the pellet re-suspended in 2.5 mL chilled sterile deionised water (SDW), to which 10 mL absolute ethanol was added and the suspension was incubated at room temperature for 1 hour. The spore preparation was centrifuged as above for two centrifugation cycles, with SDW washing between cycles. The pellet was re-suspended in 10 mL SDW and heated in a water bath (Fisher Scientific, UK) at 80°C for 10 minutes. The spore suspension was centrifuged and re-suspended in 1 mL SDW, serially diluted from 10^{-1} to 10^{-5} in sterile PBS and 10 µL of the resulting dilutions were spotted onto BHIT agar in triplicate and incubated anaerobically for 48 hours. Spore suspensions were stored at 4°C.

5.3.6 Bacterial Staining

All slides were air dried, stained appropriately and examined under oil immersion at x1000 magnification with an Olympus DX-50 microscope (UK); images were captured on an Olympus DP10 camera (UK).

5.3.6.1 Gram Stain

A single colony was smeared with sterile diH_2O and allowed to air dry; slides were immersed in crystal violet for 1 minute followed by Lugals Iodine for 1 minute.

Slides were de-stained with 95% ethanol for 30 seconds and counter-stained with Safranin (2.5% (w/v)) for 1 minute. Slides were gently rinsed with diH_2O water between each stage.

5.3.6.2 Capsule Stain – Crystal Violet

Singles colonies were air dried onto slides, immersed in double filtered 1% (w/v) crystal violet for 6 minutes and washed with 20% (w/v) copper sulfate.

5.3.7 Proportion of Morphotypes

Spore stocks were prepared as described in 5.3.5, for each culture media spore suspensions were serially diluted in sterile, reduced PBS. From each dilution series 100 µL was spread evenly across the surface of the agar. All dilutions were completed in triplicate. Once dry, the plates were inverted and incubated at 37°C under anaerobic conditions for 48 hours.

5.3.8 RaPID ANA II System

Identification of *C. difficile* was achieved by utilising the RapID ANA II System (Remel, Lenexa). The RapID ANA II panels contain 10 reaction cavities; where cavities 3 through to 10 are bifunctional (Table 5.2). Vegetative cells were produced from spore stocks as described in Section 5.3.7. Spore stock and vegetative cells of the control strain *C. perfringens* 8359 was produced as described in Sections 5.3.5 and 5.3.7. Bacterial cells were suspended in 1 mL of RapID inoculation fluid to achieve a visual turbidity equal to that of a No. 3 McFarland turbidity standard. RapID ANA II panels were inoculated with the test suspension and incubated aerobically at 37°C for 5 hours. Cavities 3 through to 10 were scored; RapID ANA II reagent was added to cavities 3 through to 9 and allowed to react for 1 minute; RapID Spot Indole reagent was added to cavity 10. The resulting microcode was screened against the Electronic RapID Compendium (ERIC).

Table 5.2: Principles and components of the RaPID ANA II reaction panel.

Cavity #	Test Code	Reactive Ingredient	Principle
Before reagent addition			
1	URE	Urea	Hydrolysis of urea produces basic products which raise the pH and change the indicator
2	BLTS	*p*-Nitrophenyl-β,$_D$-disaccharide	
3	αARA	*p*-Nitrophenyl-α,L-arabinoside,	
4	ONPG	*o*-Nitrophenyl-β,$_D$-galactoside	Enzymic hydrolysis of the colourless aryl-substituted glycoside or phosphoester releases yellow *o*- or *p*-nitrophenol
5	αGLU	*p*-Nitrophenyl-α,$_D$-glucoside	
6	βGLU	*p*-Nitrophenyl-β,$_D$-glucoside	
7	αGAL	*p*-Nitrophenyl- α,$_D$-galactoside	
8	αFUC	*p*-Nitrophenyl- α,$_L$-fucoside	
9	NAG	*p*-Nitrophenyl-N-acetyl-β,$_D$-glucosaminide	
10	PO_4	*p*-Nitrophenylphosphate	
After Reagent Addition			
3	LGY	Leucyl-glycine-β-naphthylamide	
4	GLY	Glycine-β-naphthylamide	
5	PRO	Proline-β-naphthylamide	Enzymic hydrolysis of the arylamide substrate releases free β –naphthylamine
6	PAL	Phenylalanine-β-naphthylamide	
7	ARG	Arginine-β-naphthylamide	
8	SER	Serine-β-naphthylamide	
9	PYR	Pyrrolidonyl-β-naphthylamide	
10	IND	Tryptophane	Utilisation of the substrate results in the formation of indole

5.3.9 Transmission Electron Microscopy (TEM) Negative Stain

Variant *C. difficile* colonies were suspended in 1 mL of PBS. A 5 µL drop of sample was placed on a copper grid incubated at room temperature for 5 minutes; excess sample was removed by blotting on filter paper. A 5 µL drop of 2% (w/v) methylamine tungstate was added; incubated for 5 minutes and blotted on filter paper. The grids were dried at room temperature and examined on a transmission electron microscope EM 280 (Phillips, Croydon, UK) at x16,000 magnification. Images were recorded from five randomly selected fields of vision.

5.3.10 Bile Salt Sensitivity

Following 48 hour growth, M1 and M2 colonies were isolated and suspended in sterile diH_20 (Section 5.3.7). Cultures were standardised to an OD_{600} of approximately 1.0. The following solutions were prepared, sodium taurocholate (0.1% (w/v)), chenodeoxycholate (0. 1% (w/v)) and Triton-X (0.05% (w/v)) and their effect on M1 and M2 was assessed spectrophotometrically. This work is based on the study conducted in *C. perfringens* by Camiade *et al.,* (2010). Triton-X-100 can be utilised to determine the general autolytic system of bacteria, as its interaction with lipoteichoic acids form micelles which inhibit autolytic activity in the peptidoglycan. To 900 µL of solution (sodium taurocholate (0.1% (w/v)), chenodeoxycholate (0. 1% (w/v)) and Triton-X (0.05% (w/v)), 100 µL of colony suspension was added. The OD_{600} readings were taken every minute for 30 minutes using an Ultraspec *pro*3100 spectrophotometer (GE Healthcare, UK). All assays were performed from three biological replicates.

5.3.11 Vegetative Cell Surface Protein Extraction

Unless otherwise stated, all centrifugations were completed in an Eppendorf 5417R centrifuge with a F45-30-11 fixed angle rotor (UK).

5.3.11.1 Glycine Extraction

Variant colonies were re-suspended in sterile PBS and extraction was completed as per the method of Wright *et al*., (2005). Cells were centrifuged at 2,000 *g* for 15 minutes at room temperature and washed in PBS twice. Cells were re-suspended in 400 µL of 0.2 M glycine (pH 2.2) containing 200 µL of Complete Mini Protease Inhibitor Cocktail (Roche, UK) and incubated for 30 minutes at room temperature. The suspension was then centrifuged at 17,000 *g* for 10 minutes at room temperature. The supernatant was neutralised with 2 M Tris-HCl and TCA-precipitated as detailed in Chapter 2.0, Section 2.2.1. The resulting pellet was re-suspended in collection buffer (7 M urea, 40 mM Tris-base, 0.5% (v/v) ampholytes, 1% (w/v) ASB-14), the protein concentration determined by BCA assay (Chapter 2.0, Section 2.2.2) and reduced and alkylated (Chapter 2.0, Section 2.1.3). Protein samples were aliquoted and stored at -20°C until subsequent analysis.

5.3.11.2 CHAPS Extraction

Extraction was performed as per the protocol of Hansmeier *et al*., (2006). Variant colonies were re-suspended in 50 mM Tris-HCl (pH 7.2) and centrifuged at 5,000 *g*, 4°C for 10 minutes. The pellet was washed twice with Tris-HCl buffer and re-suspended in 400 µL of Tris-HCl buffer containing 2% (w/v) CHAPS and 200 µL of Complete Mini Protease Inhibitor Cocktail. The preparation was placed on ice for a minimum of 2 hours, followed by centrifugation at 3,500 *g* for 30 minutes at 4°C. The supernatant was TCA-precipitated and the resulting pellet was re-suspended in collection buffer (7 M urea, 40 mM Tris-base, 0.5% (v/v) ampholytes, 1% (w/v) ASB-14), the protein concentration determined by BCA assay (Chapter 2.0, Section 2.2.2) and reduced and alkylated (Chapter 2.0, Section 2.4.1). Protein samples were aliquoted and stored at -20°C until subsequent analysis.

5.3.12 1D SDS-PAGE and Staining

SDS-PAGE gels were completed as detailed in Section 2.3; Criterion™ TGX™ Precast Gel, 4–20% were used. Unless otherwise stated, 10 µg of total protein was loaded. Markers included 4 µL Bio-Rad Precision Plus Protein™ WesternC Standards. Gels were double stained as detailed in Chapter 2.0, Section 2.5 or transferred to nitrocellulose membranes.

5.3.13 Western Blot and Chemiluminescence

Western blot and chemiluminescence was completed as detailed in Chapter 2.0, Sections 2.8 and 2.9 using the antibodies listed in Table 5.3. Rabbit anti-CwpVrptI was kindly provided by Prof. Neil Fairweather (Imperial College, UK).

Table 5.3: Antibodies used in this chapter.

Antibody	Antigen	Dilution	Supplier
Rabbit anti-CwpVrptI	120 kDa repeat sequence	1/5000	Imperial College London, UK
Human Sera[1]	Unknown	1/50	This study
Goat anti-Rabbit-HRP	Rabbit antibodies	1/10000	Thermo Scientific, UK
Mouse Anti-Human IgG-HRP	IgG Fc PAN (all subclasses)	1/4000	Stratech, UK
Mouse Anti-Human IgA-HRP	IgA heavy chain (all subclasses)	1/2000	Life Technologies, UK
Mouse Anti-Human IgM-HRP	Human IgM heavy chain Fc region	1/2000	Life Technologies, UK

[1] Details of *C. difficile* positive patient sera are recorded in Chapter 4, Table 4.5.

5.3.14 2-Dimensional Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2D SDS-PAGE was completed as outlined in Chapter 2.0, Section 2.4 and stained (Chapter 2.0, Section 2.5)

5.3.15 Mass Spectrometry

The 120 kDa immunoreactive band was selected for sequencing following the method in Chapter 2.0, Section 2.10. The band was excised from colloidal Coomassie stained SDS-PAGE gel.

5.4 Results

5.4.1 *Clostridium difficile* Culture

A collection of 22 clinical isolates of *C. difficile* representing a range of PCR ribotypes, including 027, 001 and 106, were cultured from spore stocks on a range of media (BHI, BA, CCFA, PPY and VL). All isolates yielded two distinct colony types (morphotypes): Morphotype 1 (M1) displayed the characteristic large, ground glass, greyish-white irregular colony of *C. difficile* whilst Morphotype 2 (M2) formed a smaller round glossy colony (Figure 5.2). As depicted in Figure 5.2, the M2 colony was often found in close proximity to the M1 colony, making isolation and analysis difficult. While every effort was made to ensure that only M2 variants were sampled it may be possible that small quantities of material from the M1 colonies were also harvested.

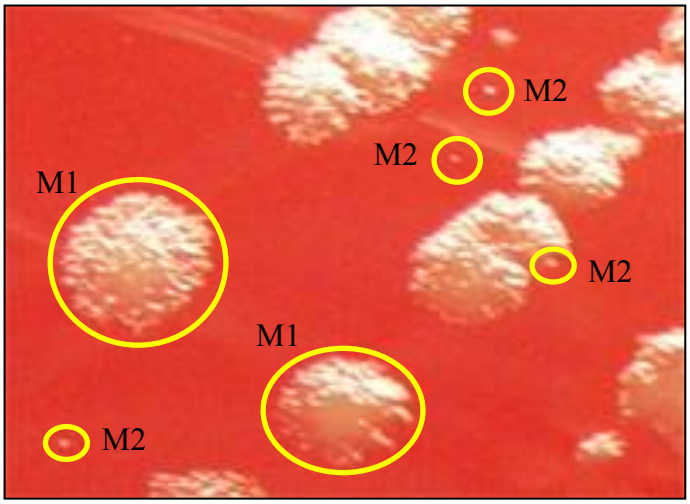


Figure 5.2: Morphotypes of *C. difficile* 11204.

Representative image of *C. difficile* NCTC 11204 (ribotype 001). Cultures were prepared from spore stocks, incubated anaerobically at 37°C for 48 hours on 5% (v/v) Columbia blood agar. (n=3).

Multiple passaging (three times) of the morphotypes onto BHI agar revealed that both the M1 and M2 colonies were unstable, producing cultures of mixed morphotypes. Inoculation of a single M1 or M2 colony into BHI broth for 48 hours resulted in the generation of mixed colonial morphotypes upon sub-culturing onto BHI agar. These results are consistent with those presented by Lynch and colleagues (2013), who were unable to separate *C. difficile* colonies by size, when incubated for

48 hours on Brucella agar supplemented with 10 µg/mL vitamin K, 5 µg/mL haemin, 5% laked sheep blood.

5.4.2 Proportion of Morphotypes

As all 22 strains assessed in this study produced variant colonies, to simplify further analysis NCTC strain 11204 (ribotype 001) was selected and cultured on the following media; BA, CCFA, PPY and VL. As can be seen from Figure 5.3 the frequency of the resulting morphotypes varied depending on the culture media with M1 accounting for between 70-90% of the colonies depending on the culture media. To confirm that the difference in morphotypes was not due to the presence of a contaminant the biochemical profile of each variant was confirmed as being that of *C. difficile* using the RapID ANA II System.

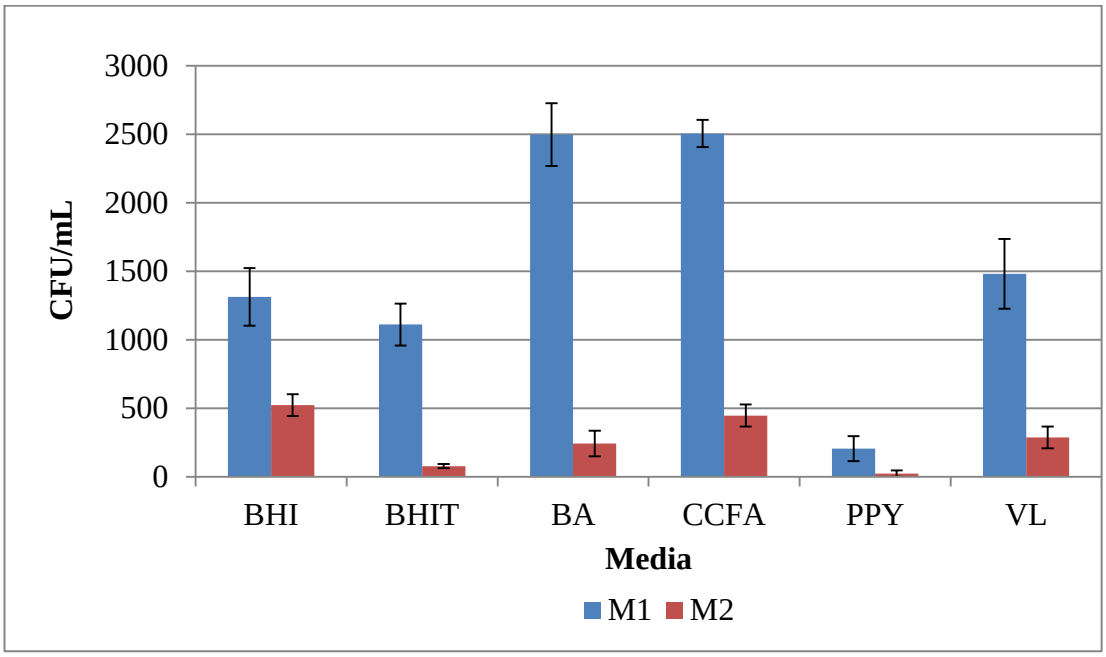


Figure 5.3: Effect of media on proportion of M1 and M2 morphotypes.

Spore stock of *C. difficile* 11204 was serially diluted in PBS with 100 µL spread onto reduced media; cultures were incubated under anaerobic conditions at 37°C for 48 hours. Error bars correspond to standard deviation of three replicates. Statistical analysis was conducted with ANOVA, a statistically significant difference, $p<0.05$, was found in all media tested.

5.4.3 Light and Electron Microscopy of Morphotypes

Light microscopic examination of strain NCTC 11204 grown in BHI broth for 48 hours revealed the presence of multiple Gram positive cells of differing morphologies. Cells can be seen as single individual cells (red arrows) or arranged end to end as short (blue arrows) or long chains of cells (yellow brace) (Figure 5.4).

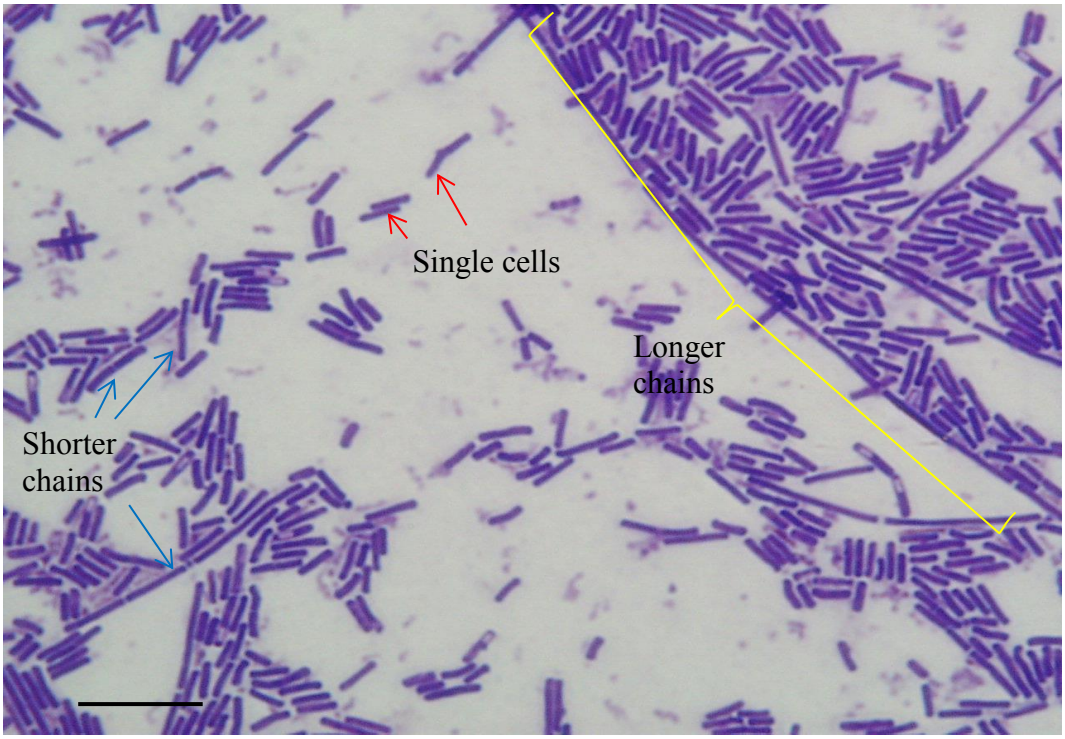


Figure 5.4: Gram stain of *C. difficile* 11204 48 hour broth culture.

Cells were grown in BHI broth for 48 hours, Gram stained and viewed under oil immersion at x1000 magnification with an Olympus DX-50 microscope; images were captured on an Olympus DP10 camera. Bar 10 µm. Red arrows; single cells, Blue arrows; chains comprised of short cells, Yellow brace; Chains comprised of longer cells, Black arrow; spores, (from 5 fields of view).

To assess if the variation in cell morphologies was associated with colony morphology, microscopic examination of material from a M1 colony revealed it to be predominantly comprised of short, encapsulated cells (2.7±0.9 µm in length) (n=5) arranged in chains (Figure 5.5A). In contrast, the M2 colony consisted primarily of filamentous rods (13±0.4 µm in length) (n=5) (Figure 5.5B). Lynch *et al.*, (2013) have recently shown a metronidazole resistant clinical isolate of *C. difficile* to display elongated cell morphology. The length of the bacilli in the wildtype, metronidazole susceptible and resistant isolates were 2.8±0.9 µm, 3.6±2.1µm and 4.0±2.1µm,

respectively. Although the cell length is similar to the cells comprising the M1 colony, they are shorter than those of the filamentous cells reported here.

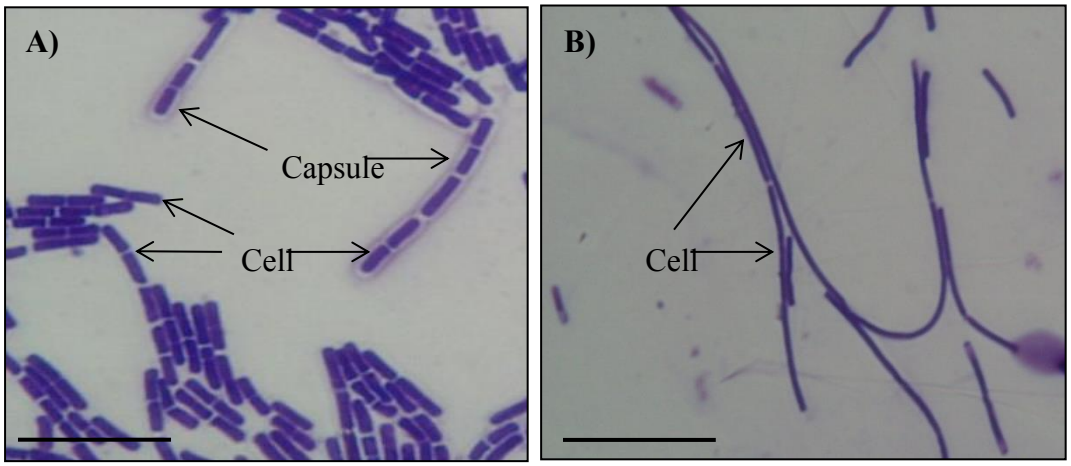


Figure 5.5: Capsule stain of *C. difficile* 11204.

A) M1 and **B)** M2. Cells were Gram stained and viewed under oil immersion at x1000 magnification with an Olympus DX-50 microscope; images were captured on an Olympus DP10 camera. Bar 10 µm. Red arrows; single cells, Blue arrows; chains comprised of short cells, Yellow brace; Chains comprised of longer cells, Black arrow; spores (from 5 fields of view).

Both M1 and M2 colonies contained individual cells. Visual examination suggested that the filamentous cells lacked the presence of a capsular layer; however when viewed using image enhancement tools (brightness and contrast), the presence of a thin capsular layer could be observed.

To further characterise the morphology of the variant cell types of *C. difficile*, samples were negatively stained and imaged by transmission electron microscopy. The images revealed the presence of both filamentous and short cells, but unfortunately, the images were found to be heavily stained making detailed analysis difficult (Figure 5.6). TEM of the M2 colony revealed the presence of all three cell types (individual, chain and filamentous). It was not possible to definitively determine if the short chain represented dividing cells or were two cells arranged end to end in a chain, as seen in the light microscope image. Due to problems associated with staining, detailed cell images could not be obtained.

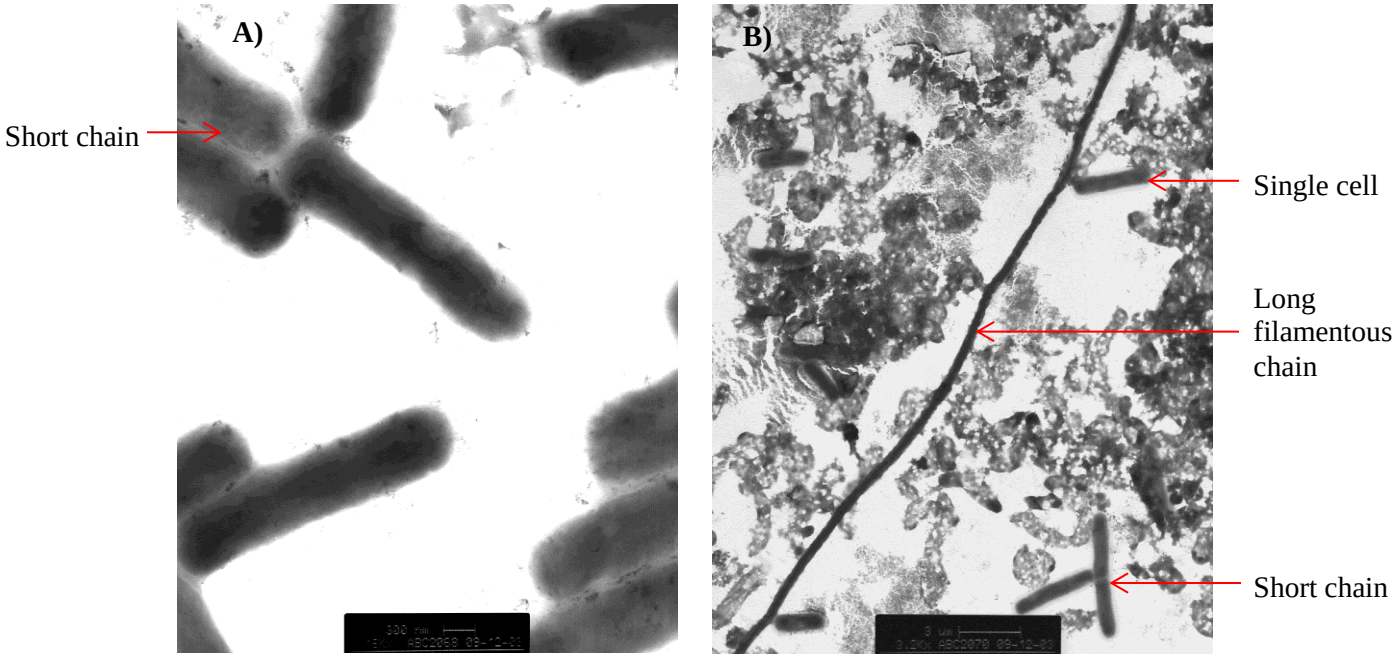


Figure 5.6: Negatively stained TEM of *C. difficile* NCTC 11204.

A) M1 and **B)** M2. Images were negatively stained with 2% (w/v) methylamine tungstate and examined on a transmission electron microscope EM 280. Magnification A)16 Kx and B) 3.2 Kx (from 5 fields of view).

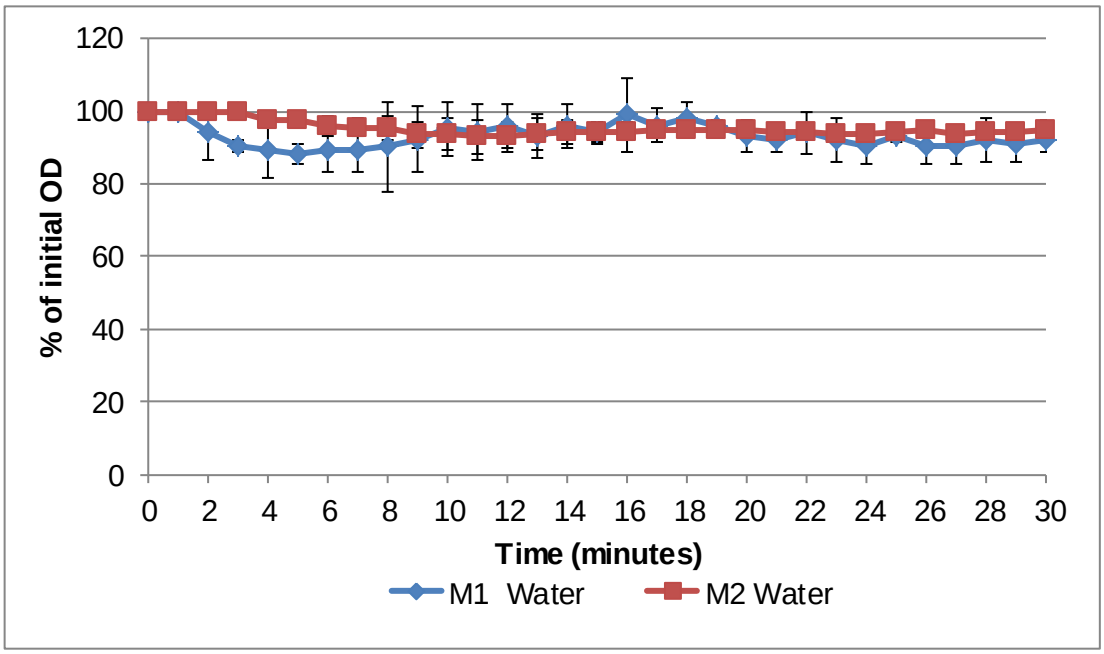
The presence of variant colonies and cells of *C. difficile* raises a number of questions; are they seen *in vivo* and do they display differing properties which contribute to virulence and what purpose, if any, do they serve. The polymorphic nature of *C. difficile* has been reported, with small colony phenotypes (Kirby *et al.*, 2009; Reynolds *et al.*, 2011), filamentous cells (Lynch *et al.*, 2013) and cells arranged in chains (Martin *et al.*, 2013) occurring as a consequence of genetic manipulation. To the best of our knowledge the data presented above is the first to demonstrate such variability in the absence of genetic manipulation. During the course of submitting this thesis, research conducted by Lipovsek *et al*., (2013) has confirmed the presence of multiple cell types of differing lengths within a typical *C. difficile* colony.

5.4.4 Bile Salt Sensitivity

Thus far very few studies have reported the interaction between bile salts and the vegetative cells of *C. difficile*. Triton-X-100 inhibits autolytic activity in the

peptidoglycan by forming micelles with lipoteichoic acid (LTA) (Gutmann *et al.*, 1996; Neuhaus and Baddiley, 2003). The inhibitory effect of LTA is dependent on the net negative charge of the cell surface, which can be reduced by diluting in Triton-X-100 (Gutmann *et al.*, 1996). This method provides an overview of the general bacterial autolytic system in the two morphotypes and can be utilised to assess lysis induced by other detergents, including bile salts. A cuvette based method was employed to determine if the morphotypes varied in their sensitivity to Triton-X-100 and bile salts chenodeoxycholate and taurocholate (Figures 5.7–5.9). Using Triton-X-100 as a model to represent bacterial autolysis, the M1 morphotype lysed significantly more rapidly than M2, (post hoc Tukey's test, $p<0.05$), suggesting that there may be a difference in the autolytic system of the two variants (Figure 5.7A and B).

A)



B)

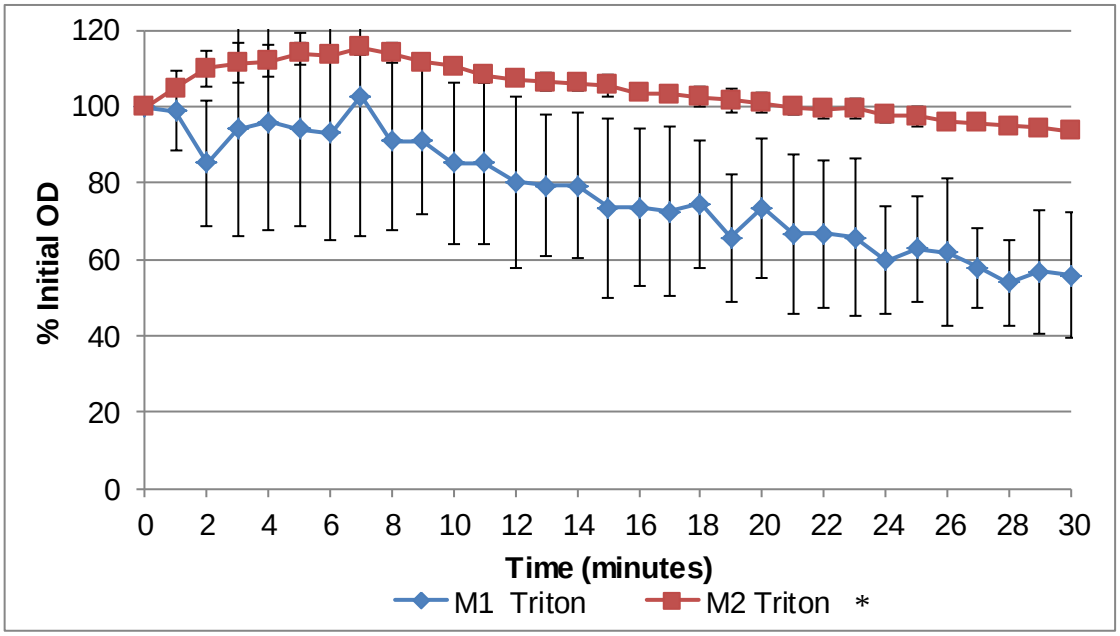


Figure 5.7: Timed Lysis assay of *C. difficile* NCTC 11204 (ribotype 001) M1 and M2 with water and 0.05% (v/v) Trition-X-100.

A) Water and **B)** 0.05% (v/v) Trition-X-100. Autolysis is expressed as percentage reduction of initial OD_{600}. Error bars indicate standard deviations. Statistical analysis was conducted with ANOVA and post hoc Tukey's test. *, indicates statistically significant difference, $p<0.05$. (n=3).

As with Triton-X-100 the M1 variant was found to be significantly more sensitive to the effect of the bile salt, chenodeoxycholate than the M2 variant ($p<0.05$) (Figure 5.8).

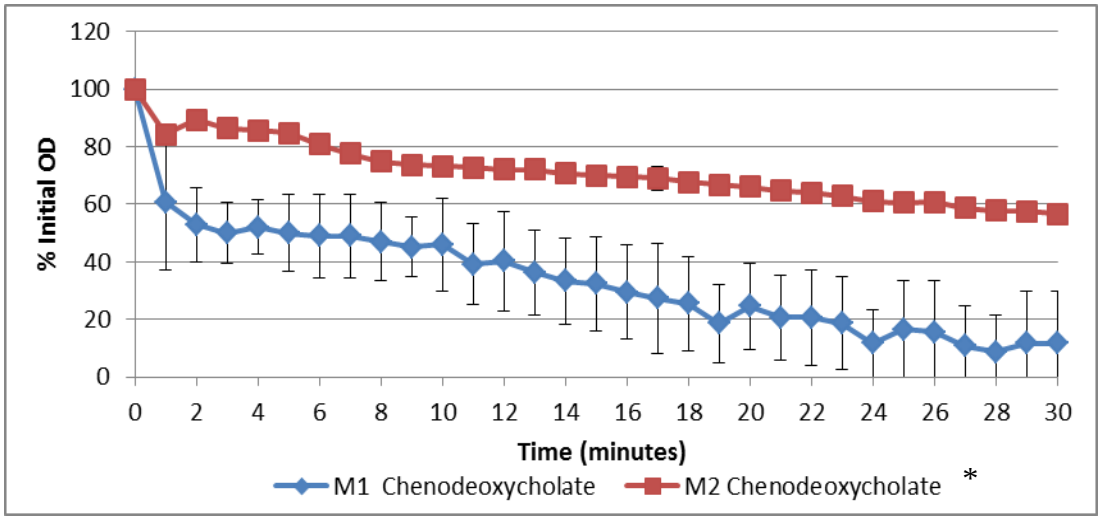


Figure 5.8: Timed lysis assay of *C. difficile* NCTC 11204 (ribotype 001) M1 and M2 with 0.1% (v/v) chenodeoxycholate.

Autolysis is expressed as percentage reduction of initial OD_{600}. Error bars indicate standard deviations. Statistical analysis was conducted with ANOVA and post hoc Tukey's test, *, indicates statistically significant difference, $p<0.05$. (n=3).

In contrast no significant difference in lysis was observed between the morphotypes in the presence of sodium taurocholate ($p>0.05$), Figure 5.9, this is more likely to be a reflection of the need for a greater number of replicates, as the post hoc Tukey's test only compares the difference between means.

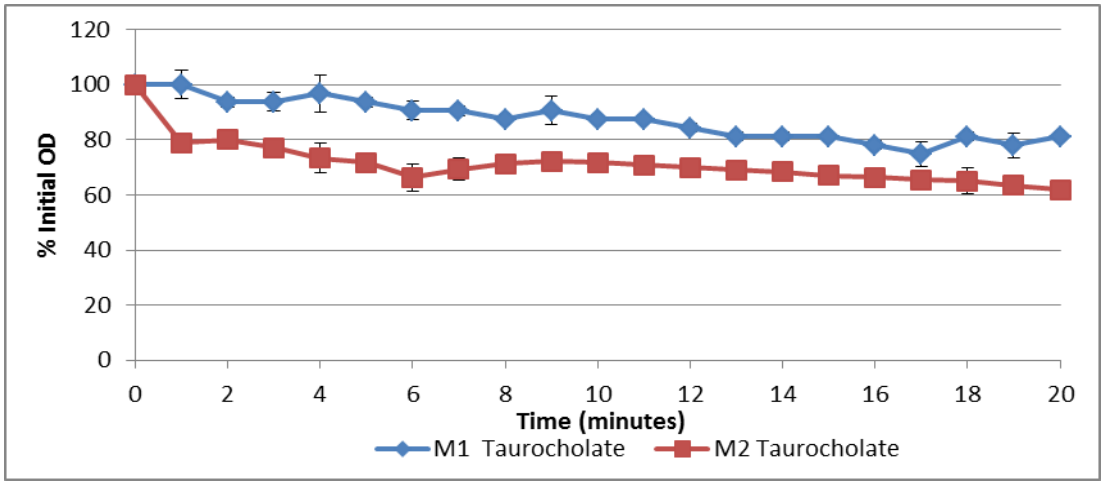


Figure 5.9: Timed lysis assay of *C. difficile* NCTC 11204 (ribotype 001) M1 and M2 with 0.1% (w/v) taurocholate.

0.1% (w/v) taurocholate. Autolysis is expressed as percentage reduction of initial OD_{600}. Error bars indicate standard deviations. Statistical analysis was conducted with ANOVA and post hoc Tukey's test. $p>0.05$. (n=3).

From the results presented above, the M1 variant is significantly more sensitive to lysis in the presence of both Triton-X-100 and chenodeoxycholate than M2. Given that colonial morphology has been associated with virulence in some species of bacteria (*S. aureus* and *Neisseria gonorrhoea*), it is intriguing to speculate the differences observed in lysis susceptibility of M1 and M2 could also be associated with virulence in *C. difficile*. Further studies are required to determine if this is the case and whether the lytic properties of the two morphotypes aid in survival and persistence in the GI tract.

5.4.5 Cell Surface Extraction

To determine if the composition of the cell surface proteins of each morphotype differed, proteins from each morphotype was extracted and subjected to SDS-PAGE. M1 and M2 colonies were harvested from ~200 agar plates and each morphotype pooled to generate sufficient material to support subsequent analysis. The ability of

low pH glycine and CHAPS extraction buffer to extract bacterial proteins was compared using 1D SDS-PAGE (Figure 5.10). Glycine extraction yielded a similar banding pattern to that previously reported in the literature, with two major bands corresponding in size to the HMW (45 kDa) and LMW (36 kDa) S-layer proteins (Wright *et al.*, 2005). The CHAPS extraction method yielded a greater number of protein bands than the glycine extracted method from each morphotype.

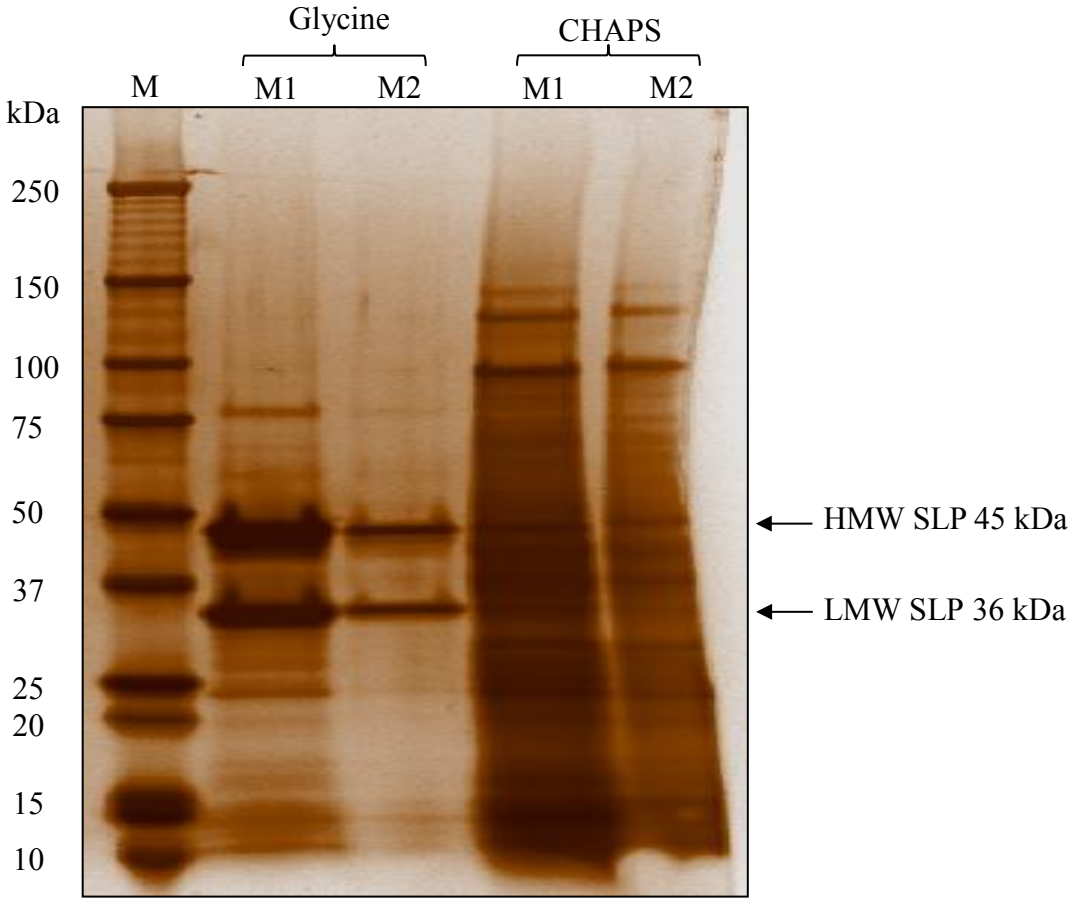


Figure 5.10: Extraction of S-layer proteins from M1 and M2 variants.

Protein extraction was completed as detailed in 5.3.15, 10 µg total protein was analysed on a 4–20% SDS-PAGE gel and stained with colloidal Coomassie. M; molecular weight maker in kDa. HMW SLP, high molecular weight surface layer protein and LMW SLP, low molecular weight surface layer protein.

Subsequent analysis of these cell wall extracts using 2DE showed generally similar protein profiles (Figure 5.11), with the majority of the protein spots located in the

low pH range (3-6). Overall the M1 profile displayed a greater number of protein spots than the M2 profile.

Although the protein concentration was standardised using a BCA assay, staining of the gel revealed unequal protein loading (Figures 5.10 and 5.11). The BCA assay detects the presence of three or more amino acids (particularly cysteine/cystine, tyrosine and tryptophan) in peptides, resulting in the formation of a coloured complex which is proportional to the number of amino acids present in the sample. A visual comparison of the number and intensity of the peptides detected by 1D and 2D SDS-PAGE gels, suggests that contrary to the results obtained with the BCA assay, the concentration of M2 in the loaded samples was lower than that of M1. The reason for this difference is unclear and could be due to some form of interference with the protein sample and the BCA assay. One possible explanation is that the amino acid composition of M1 and M2 may vary, although further studies would need to be undertaken to determine if this is the case. If this is indeed true it may have an impact on the immunoreactivity of the two morphotypes.

Figure 5.11: 2DE of CHAPS and Glycine extracted M1 and M2 variants**.**

A) M1 CHAPS, **B)** M2 CHAPS, **C)** M1 Glycine and **D)** M2 Glycine. Cell surface proteins were resolved by 2D SDS-PAGE and stained with colloidal coomassie. Gels were imaged with the Chemi Doc xrs+ system, under white light trans illumination, faint band exposure and analysed using Image Lab™ Software, v.3.0. (n=2).

5.4.6 1D Western Blot Analysis

Due to the problems mentioned in standardising protein loading, direct comparisons between the immunogenicity of M1 and M2 could not be made. However, given that the gross protein composition of M1 and M2 was found to be broadly similar coupled with the sensitivity of Western blotting; the presence and absence of immunogenic bands may still be assessed.

5.4.6.1 CwpV

CwpV has been identified as a phase variable cell wall protein, expression of which has been shown to affect cellular organisation and hence colony morphology (Emerson *et al.*, 2009; Reynolds *et al.*, 2011). To determine if the M1 and M2 morphotypes differed with regards to CwpV expression, glycine and CHAPS extracts of M1 and M2 were probed with an antibody which recognises CwpVrptI (Figure 5.12). A band of approximately 120 kDa, corresponding in size to CwpV was recognised only in the CHAPS extracts obtained from M1 and M2. This result was surprising given that glycine extracted proteins have previously been shown to contain a 120 kDa immunogenic band detected by anti-CwpVrpt1 (Emerson *et al.*, 2009). The absence of this band in the glycine extract could be due to differences in sample preparation (protein extracted from 48 hour plate culture vs. 24 hour broth culture) or in the concentration of the sample loaded onto the gel. To confirm the validity of the antibody, a positive control in the form of recombinantly expressed CwpV should have been utilised. In addition to the 120 kDa band, eight other bands were detected in the CHAPS extracted M1 and M2 samples. Comparison of the antibody binding profile of the glycine and CHAPS extracts, revealed the presence of a 75 kDa band in all samples; in the case of the glycine extracts this could represent degraded CwpV. Given our problems with standardising the level of protein loading it is not possible to comment with any confidence on the relative intensity of each band in M1 and M2, even though it would appear that a more intense signal was seen for M1.

Figure 5.12: Western blot analysis of glycine and CHAPS extracted M1 and M2 proteins probed with anti-CwpVrptI.

Anti-CwpVrptI (1/5000) probe of glycine and CHAPS extracted protein, resolved on a 4–20% SDS-PAGE gel and transferred to nitrocellulose and detected with SuperSignal Femto. Blots were imaged with the Chemi Doc xrs+ system. 5 µL of Precision Plus Protein™ Western C marker (M).

5.4.6.2 Human Patient Sera

A total of six serum samples were collected from *C. difficile* infected patients who had been treated at the University Hospital of Wales. As discussed in Chapter 4, sera CD2 and CD4 were not used for Western blot studies and will not be referred to in this chapter. Additionally sera CD5 displayed a high level of background staining which made it impossible to identify immunoreactive bands.

Probing of glycine and CHAPS extracts of M1 and M2 with human sera revealed the presence of IgM antibodies which recognised proteins in the CHAPS extracts of both morphotypes (Figure 5.13). A band with an approximate molecular weight of

120 kDa was consistently detected in all patient sera (Figure 5.13). Based on the size of the protein band, this band may represent the CwpV protein which was detected by anti-CwpVrpt1 (Figure 5.12), although further studies are required to determine if these human antibodies are specific for CwpV.

Figure 5.13: Western blot analysis of glycine and CHAPS extracted M1 and M2 proteins, human sera IgM.

Total protein was resolved on a 4–20% SDS-PAGE gel and transferred to nitrocellulose, probed with human sera (1/50) and detected with SuperSignal Femto. Blots were imaged with the Chemi Doc xrs+ system. 5 µL of Precision Plus Protein™ Western C marker (M).

A greater number of immunoreactive bands were observed with IgA than IgM (Figure 5.14), particularly for CD3 and CD6. All sera recognised the 120 kDa band, previously described by the IgM response. In contrast to CD1, CD3 and CD6 recognised multiple bands in the M1 and M2 extracts. Unlike IgM, the IgA response for CD3 and CD6 detected the presence of immunoreactive proteins in the glycine extracted profiles. Given their size the 48 and 36 kDa immunoreactive proteins are

likely to represent the HMW and LMW SLP, respectively, although this would need to be confirmed by additional studies. Four common immunoreactive bands were identified (120, 100, 48, 36 kDa) (Figure 5.14, red boxes), suggesting patients infected with *C. difficile* may recognise common proteins, which in turn could be utilised to support the development of future therapeutics and diagnostics.

Figure 5.14: Western blot analysis of glycine and CHAPS extracted M1 and M2 proteins, Human sera IgA.

Total protein was resolved on a 4-20% SDS-PAGE gel and transferred to nitrocellulose, probed with human sera (1/50) and detected with SuperSignal Femto. Blots were imaged with the Chemi Doc xrs+ system. 5 µL of Precision Plus Protein™ Western C marker (M). Red boxes indicate common immunogenic bands.

Considerably more protein bands were recognised by patient derived IgG (Figure 5.15) than with IgA and IgM, and as before a greater number of immunoreactive bands were detected in the CHAPS extracts than the glycine extracts. Consistent with the IgA and IgM response a band of 120 kDa was identified in all the immune sera. In addition to the 120 kDa band, immunoreactive bands with apparent molecular weights of 100, 36 and 15 kDa were also detected.

Figure 5.15: Western blot analysis of glycine and CHAPS extracted M1 and M2 proteins, Human sera IgG.

Total protein was resolved on a 4-20% SDS-PAGE gel and transferred to nitrocellulose, probed with human sera (1/50) and detected with SuperSignal Femto. Blots were imaged with the Chemi Doc xrs+ system. 5 µL of Precision Plus Protein™ Western C marker (M).

The CHAPS extraction method identified a greater number of bands than the glycine based extraction method. The problems encountered with regards to protein loading made comparisons between immunoreactive profiles of M1 and M2 morphotypes difficult. Consistently an immunoreactive band of 120 kDa was identified with anti-CwpVrptI and human sera from *C. difficile* infected patients, which was frequently absent from the M2 profile.

5.4.7 Mass Spectrometry

Due to time constraints and costs associated with mass spectrometry, only the 120 kDa band was selected for subsequent sequencing. This protein was found to contain one identifiable peptide (LPFIHFFDGFR), the identification of which was accepted based on the e-value (0.00033) being $p<0.0001$. The protein from which this peptide was derived was identified as pyruvate-ferredoxin/flavodoxin oxidoreductase (PFO), Accession Q183B6_CLOD6, CD2682, with an estimated molecular weight of 129 kDa. Following a database search of TrEMBL, this protein was found to be encoded by *nifJ*, which is present in all *C. difficile* genomes, sequenced to date. Figure 5.16 depicts the genetic arrangement of *nifJ* in *C. difficile* 630.

Figure 5.16: Diagrammatic representation of genetic arrangement of *nifJ*.
Image is representative of *C. difficile* 630, created using http://www.xbase.ac.uk/.

PFO is an essential enzyme in anaerobic metabolism, reversibly catalysing the conversion of pyruvate into acetyl-CoA+CO_2+H_2 and reduced ferredoxin resulting in the production of short chain fatty acids (lactate and acetate butyrate), ethanol and butanol (Chabrière *et al.*, 1999; Karlsson *et al.*, 2000; Janoir *et al.*, 2013). During this process the production of CO_2 and H_2 may contribute to maintaining an anaerobic environment (Shimizu *et al.*, 2002). In other intestinal bacteria PFO is associated with an oxidative stress response and bile tolerance.

5.5 Discussion

The presence of variant *C. difficile* cells and colonies raises many questions. What benefit (if any) do these variations provide to the bacterium, and are such variations in bacterial form seen *in vivo*? If so, is there an association between variation, virulence and persistence, particularly in relation to survival in the gut. If one morphotype is favoured over the other in the context of infection, it would make practical sense to ensure that this morphotype is employed when seeking to characterise host specific immune responses.

5.5.1 *Clostridium difficile* Morphotype Characteristics

The polymorphic nature of *C. difficile* has been observed by others, with spontaneously occurring dwarf colonies (Brazier and Borriello, 2000) and small colony phenotypes reported as a consequence of genetic manipulation (Kirby *et al.*, 2009; de la Riva *et al.*, 2011; Reynolds *et al.*, 2011). A recent paper by Lipovsek *et al.*, (2013) has confirmed a number of observations made in this thesis, in that several different cell populations can be seen within an individual *C. difficile* colony. Consistent with the data presented herein, Lipovsek *et al.* identified two colony morphotypes, large irregular colonies and small round colonies, in four strains of *C. difficile* grown on Columbia blood agar plates. Unfortunately the authors failed to provide data on the small colony and therefore direct comparison with the M2 colony cannot be made. This is not surprising given the difficulty encountered during the course of this research in producing sufficient M2 material for analysis.

Interestingly the authors reported differences in colony morphology between 2 and 5 day cultures, with the latter displaying a smoother colony and vegetative cells embedded in an extracellular matrix. The presence of capsular-material in the M1 and M2 colonies in this thesis and its role/contribution to the formation of an extracellular matrix is unclear. In contrast to the Lipovsek *et al.*, a smooth colony with an extracellular capsule layer was observed in 2 day cultures in this thesis, which may be due to strain-to-strain variation. Although it was not possible to quantify the thickness of the capsule layers of M1 and M2, visual analysis of the

light microscope images suggested the short cells of the M1 colony displayed a capsule which extended further from the vegetative cell than the M2 capsule. The M2 capsule surrounding the filamentous cells was found to be closely associated with the vegetative cell surface. Earlier work by Davies and Borriello (1990) had described the presence of two types of capsules in *C. difficile*, a dense layer with or without globular masses and a loose-knit, branching layer. If, the capsules identified herein are indeed morphologically distinct then they would represent the first documented instance of within-strain capsule variability in *C. difficile*. Although this would need to be confirmed by further TEM and SEM studies. Such variability with regards to surface proteins and polysaccharide has been reported in *B. fragilis* (Patrick *et al.*, 1995). *B. fragilis* is an intestinal, anaerobic, Gram-negative organism, capable of producing phase and antigenically distinct capsular structures associated with evasion of the host immune response. van der Woude, (2011) defines phase variation as a heritable, yet reversible regulatory event resulting in a clonal population with a heterogeneous phenotype, which is akin to the observations discussed above for *C. difficile*. Historically, phase variation is related to varied colony morphology due to altered surface proteins, capsule and cell wall composition (van der Woude and Bäumler, 2004), resulting in differences in virulence-associated factors (colonisation and antigenic), as such the morphotypes presented in this work may be indicative of one or more proteins which is capable of phase variation. Capsule variation in *C. difficile* may also be indicative of this. The role of phase variation in the regulation of the cell wall protein, CwpV (Emerson *et al.*, 2009; Reynolds *et al.*, 2011) coupled with the presence of multiple inversions in the *C. difficile* genome (Stabler *et al.*, 2010) suggests that in *C. difficile* (as in other intestinal bacteria) phase variation is important for survival.

The encapsulated chain-like arrangement observed in this study is not unique to *C. difficile*, and has been reported in other *Clostridium* spp. (Betz, 1970; Kuhner *et al.*, 2000). In other bacterial species, bacterial chains rapidly facilitate the transfer of DNA to cells within the chain (Babic *et al.*, 2011) or may serve as a means of resisting infection from bacteriophages, indeed the presence of a capsular layer may well aid in this as well (Betz, 1970; Abedon, 2012). In a recent study of the *agr* locus in *C. difficile*, TEM images of an *agr* mutant revealed the presence of

C. difficile cells arranged in chains, possibly surrounded by an external layer, which mirrors the light microscope images obtained in this thesis (Martin *et al.*, 2013).

Using a Triton-X-100-induced autolysis assay an overview of the general bacterial autolytic system of the M1 and M2 morphotypes was deduced, revealing that the M1 morphotype lysed significantly more rapidly than M2, indicating that the two morphotypes display differences in their autolytic activity. In *B. subtilis* (Margot *et al.*, 1998; Margot *et al.*, 1999; Smith *et al.*, 2000), *C. perfringens* (Camiade *et al.*, 2010) and *Listeria monocytogenes* (Carroll *et al.*, 2003), the formation of long chains is attributed to mutations which effect the activity of peptidoglycan hydrolases (PGHs). A mutant of *C. perfringens* deficient in the expression of the PGH, Acp, grows as a long chain of cells similar to those seen in *C. difficile* (Camiade *et al.*, 2010). While *C. difficile,* contains a homologue to Acp called Acd (59% amino acid similarity), the Acd mutants examined to date revealed no phenotypic differences in chain formation or cell separation (Dhalluin *et al.*, 2005; Peltier *et al.*, 2010). This is possibly due to functional compensation by one or more of the 28 putative autolysins which have been identified in the *C. difficile* genome (Wright, 2006). Autolysins are involved in peptidoglycan turnover, cellular division, sporulation/germination and antibiotic-induced lysis (Peltier *et al.*, 2010), in the latter case the differences observed in autolysis of M1 and M2 may therefore confer the M2 morphotype a competitive advantage *in vivo* upon administration of antibiotics. Furthermore, in the presence of the bile salt, chenodeoxycholate, M1 was more susceptible to lysis than the M2 morphotype, suggesting that the M2 morphotype may be better able to survive in the GI tract.

It should be noted that the lysis assays were performed under aerobic conditions, thus the influence of oxygen related stress cannot be ruled out. Gram stain images taken before and after the bile salt assays did reveal the presence of both spores and vegetative cells, with the latter being predominant. The presence of spores and vegetative cells is not surprising as M1 and M2 colonies were isolated following incubation for 48 hours. Although a viability assay was not performed, *C. difficile*

has been shown to tolerate microaerophilic conditions (Jump *et al.*, 2007; Emerson *et al.*, 2008)

In *C. difficile* genetic analysis of a metronidazole resistant and susceptible strain revealed differences associated with electron transport, including glycerol-3-phosphate dehydrogenase (*glyC*) and PFO (*nifJ)* (Lynch *et al.*, 2013). In addition to affecting the efficiency of metronidazole entry into the cell and subsequent activation, disruptions in electron transport are associated with small colony variants (Lynch *et al.*, 2013). Although Lynch and colleagues did not observe small colonies in their research, it is intriguing to speculate that the similarities observed between their research and that presented in this thesis may indeed be associated with SCVs. The absence of small colonies in the work conducted by Lynch *et al.*, maybe due to difference in the strain or media employed (Brucella agar supplemented with 10 µg/mL vitamin K, 5 µg/mL haemin, 5% laked sheep blood+8 µg/mL metronidazole). In this thesis the sensitivity of M1 and M2 to metronidazole was not assessed but should be investigated in subsequent studies. It is unclear whether our observations are reflective of an adaptive response to culturing in the laboratory or represent an unreported survival mechanism of *C. difficile*. Given the number of factors (including potential virulence factors) affected, a global regulation mechanism may be required.

5.5.2 *Clostridium difficile* Morphotype Proteomics and Immunoreactive Proteins

In support of published data (Wright *et al.*, 2005), glycine extraction revealed the presence of proteins which corresponded in molecular weight to the HMW and LMW SLP. The results presented here indicate that a CHAPS based method is capable of extracting a greater number of cell surface proteins than low pH glycine and as such may give a more complete profile of the surfaceome of *C. difficile.* Although sequencing of the CHAPS extracted bands would aid in identifying how many of these proteins are recognised SLPs. Furthermore, glycine is recognised as a co-germinant for *C. difficile* spores (Sorg and Sonenshein, 2008; Wheeldon *et al.*, 2011), however its interaction with vegetative cells is unreported. Based on the

number of proteins extracted and analysed by 1D SDS-PAGE, the CHAPS based method outperformed the glycine extraction method.

To determine if the morphotypes expressed proteins recognised during the course of human infection, we probed glycine and CHAPS extracted proteins with sera from *C. difficile* infected patients. Due to the problems encountered in standardising protein loading, which may well be attributed to differences in amino acid composition, direct comparison between M1 and M2 extracted proteins could not be made. However, it was noted that the 120 kDa immunogenic band was frequently absent from the M2 extract but present in the M1 extract. Previously, authors have identified immunodominant proteins of *C. difficile* by using a mixture of anti-human IgA, IgM, and IgG HRP conjugated antibodies (Wright *et al.*, 2008). In this study, we probed cell surface extracts of *C. difficile* with antibody conjugates which recognised each subclass individually. Based on the number of the immunoreactive bands detected, IgG displayed the strongest response, followed by IgA and IgM. The immunogenic profiles obtained in this thesis are similar to those obtained in the published literature (Wright *et al.*, 2008). Unfortunately, due to the limited availability of M1 and M2 protein extracts and human patient sera we were unable to perform any further analysis using 2DE Western blots. This would have allowed better resolution and identification of common immunogenic proteins. If the M1 and M2 morphotypes do truly display differences in immunogenicity, this will not only have an impact on the pathogens ability to evade the host immune system but also on the development of therapeutics based on cell surface structures.

Although a number of common proteins were identified following Western blot analysis, due to time restraints and limited financial resources, a single band was selected for sequencing and identified as pyruvate-flavodoxin oxidoreductase (PFO), which is encoded by *nifJ*. The *nifJ* gene is associated with oxidative response and has been identified in other intestinal bacteria, including *Klebsiella pneumoniae*, *B. fragilis* and *Enterococcus faecalis* (Schmitz *et al.*, 2001; Begley *et al.*, 2005), to play a role in bile tolerance. In the latter organism, *nifJ* was one of 45 genes up regulated following bile salt treatment (Flahaut *et al.*, 1996a; Flahaut *et al.*, 1996b; Begley *et al.*, 2005). Whether the low (sub-lethal) concentration of bile salts that

would naturally be present in the most commonly used *C. difficile* culture media, BHI, results in an adaptive response against stresses, is unclear. Currently, it is only possible to speculate if this is occurring in *C. difficile*. Given that intestinal pathogens are exposed to the same environmental stresses, it would be conceivable that they have adapted similar survival mechanisms.

PFO is an essential metabolic enzyme in anaerobic bacteria, in addition to catalysing the conversion of pyruvate into acetyl-CoA+CO_2+H_2 to produce short chain fatty acids (lactate and acetate butyrate), ethanol and butanol, it may also aid in maintaining an anaerobic environment (Chabrière *et al.*, 1999; Karlsson *et al.*, 2000; Janoir *et al.*, 2013). It is intriguing to speculate the role(s) of *nifJ* in *Clostridium* spp., does it confer an advantage in growth and survival by maintaining an anaerobic environment in host tissue (Shimizu *et al.*, 2002), is it involved in bile tolerance (Flahaut *et al.*, 1996a; Flahaut *et al.*, 1996b) or as observed in *Vibrio* does it in some way contribute to virulence by enhancing capsule production and adherence (Pace *et al.*, 1997). Further studies would be required to investigate the relationship between bile salts and morphotypes in *C. difficile*. Furthermore PFO has recently been shown to be the target for the antibiotic, amixicile, which has displayed clinical efficacy against CDI in a mouse model (Warren *et al.*, 2012). PFO has also been shown to be immunogenic in *C. perfringens* (Kulkarni *et al.*, 2007) and *Entamoeba histolytica* (Thammapalerd *et al.*, 1996) and as such may represent a valid therapeutic target.

5.6 Conclusion

This chapter aimed to characterise the differences in variant *C. difficile* colonies. All strains analysed were found to display colonial variance. Two colonies, M1 and M2 were characterised further by microscopy, lysis assay, proteomics and immunoproteome studies.

Colonies of *C. difficile* display differences in autolytic and bile salt sensitivity, with M1 being more susceptible to lysis in the presence of chenodeoxycholate. The identification of a protein (pyruvate-flavodoxin oxidoreductase) associated with oxidative and/or bile salt-related stress warrants further investigation. Given the proteins immunogenicity it may be exploited for therapeutic purposes. The presence of varying cell types within the colonies and the presence of a capsule should also be investigated further.

CHAPTER SIX

GENERAL DISCUSSION

6.1 General Comments

It can be argued that *C. difficile* research has long been neglected, and it is only in the last thirteen years (following a number of major outbreaks) that steps have been taken to decipher the complex nature of this organism and its disease causing ability. Broadly speaking we can categorise *C. difficile* research into three aspects; the vegetative cells, toxins and spores. This thesis has sought to investigate the vegetative cells and toxins A and B of *C. difficile*, focusing on their variability with regards to immunogenicity and possibly virulence.

6.2 Variability of Vegetative *Clostridium difficile*

The vegetative form of the bacteria is associated with toxin production resulting in active disease in the gut. The strain-to-strain variation in growth rate, sporulation, toxin production and surface layer proteins in *C. difficile* is well documented. Thus, it is not surprising that we too have observed differences, intriguingly the aspect of colonial variation adds another dimension to the variability of this organism. In this thesis we have characterised in detail the variant morphotypes (M1 and M2) produced by a single clinical isolate of *C. difficile*. The production of variant morphotypes by twenty-two other clinical isolates indicates that colonial variation is universally present in *C. difficile* isolates.

Examination of the variant colonies revealed morphological differences at a cell structure level, which may impact on adherence, cell lysis, immunogenicity and virulence. Given the relative difference in autolysis and bile salt-induced lysis, it is tempting to speculate that one morphotype maybe better equipped to survive in the hostile environment of the gut. At this stage it is only possible to speculate on the implication(s) of the morphotypes in the context of virulence and pathogenesis.

The presence of a capsular layer in the two morphotypes may well pose a challenge for therapeutics targeting the cell wall, although its presence *in vivo* would need to be confirmed. In the intestinal bacteria, *Bacteroides fragilis*, a phase variable capsule

aids in colonization and host immune evasion (Chatzidaki-Livanis *et al.*, 2010), the presence of CwpV in *C. difficile* demonstrates that this intestinal pathogen is also capable of producing a phase and antigenically variable protein. Based on the visual differences observed in the capsule surrounding the short and long cells of *C. difficile*, it is intriguing to speculate that the capsules are morphologically distinct and as such may well be expressed in a phase variable manner. This thesis has highlighted that there is much to be explored with regards to the vegetative form of *C. difficile*, particularly the interaction of vegetative *C. difficile* with bile salts, given that *C. difficile* is a gut pathogen. Thus far all research has focused on the interaction of *C. difficile* spores and bile salts; given that once the spore germinates it is the vegetative cell which will be exposed to the detergent activity of bile, the organism's ability to survive such conditions must be understood.

Western blotting of vegetative cell extracts of both morphotypes revealed the presence of a common immunogenic band which was recognised by antibodies from animal and human sera. This band was subsequently identified as pyruvate-flavodoxin oxidoreductase (PFO), a protein involved in anaerobic metabolism and associated with an oxidative response in other intestinal pathogens (Flahaut *et al.*, 1996a; Flahaut *et al.*, 1996b; Schmitz *et al.*, 2001; Begley *et al.*, 2005). In *C. perfringens*, PFO is an immunogenic, lytic enzyme released during germination (Ando, 1979), immunization with truncated PFO protected chickens from experimental challenge with virulent *C. perfringens* (Kulkarni *et al.*, 2007). Although the *C. difficile* PFO does not contain a discernible signal sequence it has been identified in the secretome of three *C. difficile* isolates (Boetzkes *et al.*, 2012). Given that this protein has also been shown to mediate immunity to invasive amoebiasis from *E. histolytica* (Thammapalerd *et al.*, 1996), it is intriguing to speculate that these findings may also be mirrored in *C. difficile*. The immunoreactivity of *C. difficile* PFO has been demonstrated in this study, whether this confers to a protective host immune response would need to be investigated. Recently, amixicile, a novel PFO inhibitor, has been shown to display clinical efficacy against CDI in a mouse model (Warren *et al.*, 2012), indicating PFO may well be a promising therapeutic target for antibiotics and immunotherapies. A better

understanding of the form of vegetative *C. difficile* encountered in the gut would aid in the development of new therapeutics and diagnostics.

6.3 Recombinant Expression of Toxin A and B

A high yield *E. coli* based recombinant protein expression system, producing the non-toxic fragments of the C-terminal and translocation domains of toxins A and B was developed. This was undertaken to support the characterisation of immune responses directed against the toxins. Despite the use of identical expression systems (M15[pREP4] and pQE-30) the yield and purity attained for $rTcdA_{900\text{-}2710}$ and $rTcdB_{546\text{-}2366}$ was significantly different. Expression of $rTcdB_{546\text{-}2366}$ yielded 8-12 mg/L of pure protein in contrast to the 0.8–1.0 mg/L for $rTcdA_{900\text{-}2710}$. Difficulties encountered in the expression of non-codon optimised and codon optimised regions of TcdA have been reported by others (Phelps *et al.*, 1991; Craggs, 1999; Letourneur *et al.*, 2003; Ackermann *et al.*, 2004b), suggesting that codon optimisation alone is not sufficient to generate a high yield recombinant expression system for TcdA. Whether the problems encountered with expression are due to the toxic effects of $rTcdA_{900\text{-}2710}$ is unclear. Although the optimisation of expression strain, media and culture conditions, enabled the production of soluble $rTcdA_{900\text{-}2710}$, the yield achieved did not mirror that of $rTcdB_{546\text{-}2366}$. Thus we can only conclude that, although convenient, an *E. coli* based recombinant expression system may not be suitable for the expression of TcdA. In contrast, expression of TcdA with the *B. megatarium* system appears to eliminate the problems encountered with *E. coli* expression (Burger *et al.*, 2003; Yang *et al.*, 2008; Pruitt *et al.*, 2012; Wang *et al.*, 2012). Analysis of gene sequences using bioinformatic software although valuable may not reflect experimental outcomes. Indeed a 'one-rule-fits-all' method cannot be applied for all proteins expressed in *E. coli*.

6.4 Immunogenicity of Toxin A and B

Analysis of the recombinantly expressed toxin B indicated that it is structurally similar to its native counterpart. Comparison between native and recombinant toxin A was complicated by the low yield and purity obtained for the latter protein. Whilst

toxin A was found to be more resistant to enzymatic digestion than toxin B, toxin B demonstrated greater stability following 24 hour incubation at 25°C. Whether these observations have any relevance to the *in vivo* mechanism of cellular intoxication is unclear. During cellular intoxication the toxins undergo a number of conformational changes in response to environmental signals; the ability of TcdA to resist proteolytic digestion may prevent premature proteolysis, whilst the stability of TcdB is reflective of its temporal activity. Toxin A is reported to cause initial damage to the epithelial cells (Krivan *et al.*, 1986; Tucker and Wilkins, 1991), facilitating the subsequent uptake and cytotoxic activity of toxin B (Lyerly *et al.*, 1985b; Bongaerts and Lyerly, 1994). Digestion of the toxins may have been enhanced if exposed to *in vivo* conditions, such as InsP6 or low pH, resulting the conformational changes to the protein and the subsequent exposure of enzymatic digestion sites (Qa'Dan *et al.*, 2000; Barth *et al.*, 2001; Lanis *et al.*, 2010).

The similarities in the 1D and 2D SDS-PAGE and immunogenicity profiles of the recombinant and native proteins obtained indicates that recombinant proteins can be used as surrogates for the development of therapeutics. Although to obtain a true picture, analysis of the complete recombinant toxins would be required. The immunogenic validity of recombinant proteins and there use as surrogates has been demonstrated in a range of expression systems (Heinrichs *et al.*, 2012).

Using animal derived toxin neutralising sera and sera from *C. difficile* infected patients' common immunogenic regions within TcdA and TcdB (40 and 60 kDa) were identified. Attempts to identify these regions by mass spectrometry were unsuccessful. The resistance of toxin A to trypsin digestion (Taylor *et al.*, 1981; Lyerly *et al.*, 1989) may account for the failure to generate meaningful mass spectrometry results. The major limiting factor in peptide identification is protein digestion (van Montfort *et al.*, 2002; Wu *et al.*, 2003; Fischer and Poetsch, 2006), the use of a combination of proteases may have yielded positive results. During the course of this experiment it became apparent that although a proteomic based approach is valuable there are limitations at every stage of the technique, from

peptide preparation (staining, digestion, contamination) to sequence analysis. In hindsight it would have been beneficial to analyse the peptides generated from trypsin digestion of the toxins prior to gel analysis. This approach would have allowed us to ascertain if the toxin was susceptible to trypsin digestion and if so assess the peptides which could be successfully identified. This study successfully identified the major immuno-responsive regions within the 308 kDa (toxin A) and 270 kDa (toxin B) *C. difficile* toxins, with common regions residing within 40 and 60 kDa fragments which may indeed contain toxin neutralising epitopes and are thus worthy of further study.

6.5 Conclusion

The various experiments conducted and observations made during the course of this thesis, highlight the need for a multivalent therapeutic approach to treating CDI, which includes both toxins A and B as well as non-toxic proteins (possibly PFO). Although there is a renewed interest in *C. difficile* research following the 'hypervirulent' outbreaks in 2000, it is evident that some fundamental questions still remain unanswered.

6.6 Future Work

Variability of vegetative *C. difficile*: This work highlights the variability of vegetative *C. difficile* cells, opening many avenues of research. Thus far it has not been possible to stably produce variant colonies and thus samples may contain a small proportion of the other variant. The identification of the trigger for variant colony formation will provide an understanding of the mechanism(s) behind its existence and whether it is replicated *in vivo*. The use of multiple strains is also essential to assess the validity of the results presented herein. To date, transcriptional analysis studies have focussed on the stress response in relation to pH, oxidative shock, sub-inhibitory concentrations of antibiotics and heat (Emerson *et al.*, 2008; Jain *et al.*, 2011; Ternan *et al.*, 2012), it is surprising that no study has assessed the stress response associated with exposure to bile salts. Based on the differences in the autolytic activity of the two morphotypes, it is possible the cell wall chemistry and cell surface composition differs, which may in turn alter the net cell surface charge (Bendinger *et al.*, 1993) and thus effect adherence, as reported in Group A *Streptococcus* (GAS) (Kristian *et al.*, 2005). Although time did not permit us to pursue the differences in adherence properties of the two morphotypes, it is intriguing to speculate that one variant maybe more adherent than the other. Furthermore the differences observed in cell physiology maybe indicative of a difference in the growth phase of the cell which may have an impact on toxin production. The use of the newly developed Cdifftox agar plate assay (CDPA), to assess the presence/absence of toxin production in the two colonies may provide an insight into this (Darkoh *et al.*, 2011), alternatively the use of single-colony whole-genome sequencing would provide a wealth of data as to any differences which exist at a genomic level between the two morphotypes, the use of such techniques has recently been demonstrated with *C. difficile* (Köser *et al.*, 2013). Potential therapeutic targets include PFO and capsule; in the latter case biochemical analysis to assess its composition (carbohydrate or protein capsule) and level of expression should also be conducted. The role of PFO and its impact on other virulence factors can be investigated though mutational analysis using the ClosTron system. The presence of variant cell types within colonies of *C. difficile* is intriguing, and could be further investigated by Fluorescence-Activated Cell Sorting (FACS), which would allow the heterogeneous population of cells to be physically

sorted and subsequently analysed. The presence of multiple cell types of *C. difficile*, suggests like other bacteria (*B. subtilis*) (Lopez *et al.*, 2009), the cells of *C. difficile* may display differences in gene expression, which subsequently effects cell morphology and potentially virulence. Recently studies have utilised bacterial cytological profiling to identify the mechanism of action of antibacterial compounds (Nonejuie *et al.*, 2013). Using this approach *E. coli* was treated with a range of antibiotics at 5x the minimum inhibitory concentration. Post exposure differences in cellular morphology were observed depending on the cellular target of the antibiotic. The presence of single individual cells and long filamentous cells within single colonies cultured under the same conditions suggests the components in the media may be targeting different pathways, resulting in distinct morphological profiles. If this is indeed true, then bacterial cytological profiling by exposing the cells to varying concentrations of the media components, including bile salts could be utilised.

Recombinant expression system: A number of factors could have been assessed to enhance expression of TcdA. Due to the project originally being approved for containment level 2 work, we were restricted in the host strains we could utilise. All expression strains assessed were under the control of the T5 promoter whilst published reports utilised expression systems with a T7 promoter, such as BL21. It would also have been beneficial to assess the use of alternative vectors such as pQE80L which confers higher repression levels. In terms of the culture conditions, a number of factors could have been further investigated (non-metabolisable sugars, chaperones and dissolved oxygen content). The main limitation of this work is the lack of comparison between codon optimised and non-codon optimised proteins. Without this the true effect of codon optimisation on expression yields cannot be determined.

Identifying immunodominant regions of toxin A and toxin B: Sequencing of the immunogenic regions identified by the human sera will enable immunogenic mapping of the toxins and allow for comparisons between toxin A and B, and the native, recombinant and toxoid preparations, which will underpin the development of

therapeutic targets. In order to achieve this, a greater concentration of digested protein must be employed to allow analysis by 2D SDS-PAGE and facilitate sequencing. This would provide clearer resolution of the fragments and reduce the chance of co-purifying multiple protein fragments. Once immunogenic regions have been located, further improvements in resolution can be made by utilising a narrower pI and molecular weight range. The use of peptide gels which allow resolution of peptides between 1–40 kDa could be utilised. Finally the use of multiple strains should also be investigated, with recent studies demonstrating the antigenic variability of toxin B in the current hypervirulent 027 strain (Lanis *et al.*, 2013). Furthermore, it has recently been reported that the toxoiding process alters the conformational structure of toxoid B and hence the presentation of key neutralising epitopes (Wang *et al.*, 2012), the immunogenicity of native and toxoided toxin B should also be a investigated.

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APPENDICES

Appendix 1: Predicted peptide sequences obtained from TcdA and rTcdA$_{900\text{-}2710}$ *in silico* digestion with clostripain.

Of the 51 fragments identified by ExPASy PeptideCutter, 16 had a predicted molecular weight of <2 kDa and would be difficult to visualise on a SDS-PAGE gel and are therefore not displayed. Bold indicates regions in native and recombinant protein.

Resulting Peptide Sequence TcdA	**Peptide Mass (kDa)**
MSLISKEELIKLAYSIR	2
ENEYKTILTNLDEYNKLTTNNNENKYLQLKKLNESIDVFMNKYKTSSR	6
ALSNLKKDILKEVILIKNSNTSPVEKNLHFVWIGGEVSDIALEYIKQWADIN AEYNIKLWYDSEAFLVNTLKKAIVESSTTEALQLLEEEIQNPQFDNMKFYK KR	12
FINYYKSQINKPTVPTIDDIIKSHLVSEYNR	4
ANSLFTEQELLNIYSQELLNR	2
LLALKNFGGVYLDVDMLPGIHSDLFKTISR	3
WEMIKLEAIMKYKKYINNYTSENFDKLDQQLKDNFKLIIESKSEKSEIFSKL ENLNVSDLEIKIAFALGSVINQALISKQGSYLTNLVIEQVKNR	11
YQFLNQHLNPAIESDNNFTDTTKIFHDSLFNSATAENSMFLTKIAPYLQVGF MPEAR	7
STISLSGPGAYASAYYDFINLQENTIEKTLKASDLIEFKFPENNLSQLTEQEI NSLWSFDQASAKYQFEKYVR	8
DYTGGSLSEDNGVDFNKNTALDKNYLLNNKIPSNNVEEAGSKNYVHYIIQ LQGDDISYEATCNLFSKNPKNSIIIQR	9
NMNESAKSYFLSDDGESILELNKYR	3
LKNKEKVKVTFIGHGKDEFNTSEFAR	3

LSVDSLSNEISSFLDTIKLDISPKNVEVNLLGCNMFSYDFNVEETYPGKLLLS IMDKITSTLPDVNKNSITIGANQYEVR	9
KELLAHSGKWINKEEAIMSDLSSKEYIFFDSIDNKLKAKSKNIPGLASISEDI KTLLLDASVSPDTKFILNNLKLNIESSIGDYIYYEKLEPVKNIIHNSIDDLIDE FNLLENVSDELYELKKLNNLDEKYLISFEDISKNNSTYSVR	17
Resulting Peptide Sequence TcdA continued	**Peptide Mass (kDa)**
FINKSNGESVYVETEKEIFSKYSEHITKEISTIKNSIITDVNGNLLDNIQL DHTSQVNTLNAAFFIQSLIDYSSNKDVLNDLSTSVKVQLYAQLFSTGLN TIYDSIQLVNLISNAVNDTINVLPTITEGIPIVSTILDGINLGAAIKELLDE HDPLLKKELEAKVGVLAINMSLSIAATVASIVGIGAEVTIFLLPIAGISA GIPSLVNNELILHDKATSVVNYFNHLSESKKYGPLKTEDDKILVPIDDL VISEIDFNNNSIKLGTCNILAMEGGSGHTVTGNIDHFFSSPSISSHIPSLSI YSAIGIETENLDFSKKIMMLPNAPSR	**36**
VFWWETGAVPGLR	**2**
FYAFFDYAITTLKPVYEDTNIKIKLDKDTR	**4**
NFIMPTITTNEIR	**2**
NKLSYSFDGAGGTYSLLLSSYPISTNINLSKDDLWIFNIDNEVR	**5**
EISIENGTIKKGKLIKDVLSKIDINKNKLIIGNQTIDFSGDIDNKDR	**5**
YIFLTCELDDKISLIIEINLVAKSYSLLLSGDKNYLISNLSNIIEKINTLGL DSKNIAYNYTDESNNKYFGAISKTSQKSIIHYKKDSKNILEFYNDSTLEF NSKDFIAEDINVFMKDDINTITGKYYVDNNTDKSIDFSISLVSKNQVKVN GLYLNESVYSSYLDFVKNSDGHHNTSNFMNLFLDNISFWKLFGFENINF VIDKYFTLVGKTNLGYVEFICDNNKNIDIYFGEWKTSSSKSTIFSGNGR	**29**

NVVVEPIYNPDTGEDISTSLDFSYEPLYGIDR	**4**
YINKVLIAPDLYTSLININTNYYSNEYYPEIIVLNPNTFHKKVNINLDSSS FEYKWSTEGSDFILVR	**8**
YLEESNKKILQKIR	**2**
IKGILSNTQSFNKMSIDFKDIKKLSLGYIMSNFKSFNSENELDR	**5**
DHLGFKIIDNKTYYYDEDSKLVKGLININNSLFYFDPIEFNLVTGWQTI NGKKYYFDINTGAALISYKIINGKHFYFNNDGVMQLGVFKGPDGFEYF APANTQNNNIEGQAIVYQSKFLTLNGKKYYFDNDSKAVTGWR	**16**
IINNEKYYFNPNNAIAAVGLQVIDNNKYYFNPDTAIISKGWQTVNGSR	**5**
YYFDTDTAIAFNGYKTIDGKHFYFDSDCVVKIGVFSTSNGFEYFAPANT YNNNIEGQAIVYQSKFLTLNGKKYYFDNNSKAVTGWQTIDSKKYYFNT NTAEAATGWQTIDGKKYYFNTNTAEAATGWQTIDGKKYYFNTNTAIA STGYTIINGKHFYFNTDGIMQIGVFKGPNGFEYFAPANTDANNIEGQAI LYQNEFLTLNGKKYYFGSDSKAVTGWR	**25**
Resulting Peptide Sequence TcdA continued	**Peptide Mass (kDa)**
IINNKKYYFNPNNAIAAIHLCTINNDKYYFSYDGILQNGYITIER	**5**
NNFYFDANNESKMVTGVFKGPNGFEYFAPANTHNNNIEGQAIVYQNK FLTLNGKKYYFDNDSKAVTGWQTIDGKKYYFNLNTAEAATGWQTID GKKYYFNLNTAEAATGWQTIDGKKYYFNTNTFIASTGYTSINGKHFYF NTDGIMQIGVFKGPNGFEYFAPANTHNNNIEGQAILYQNKFLTLNGKK YYFGSDSKAVTGLR	**23**
TIDGKKYYFNTNTAVAVTGWQTINGKKYYFNTNTSIASTGYTIISGKHF YFNTDGIMQIGVFKGPDGFEYFAPANTDANNIEGQAIR	**10**
FLYLHDNIYYFGNNSKAATGWVTIDGNR	**3**

YYFEPNTAMGANGYKTIDNKNFYFR	**3**
NGLPQIGVFKGSNGFEYFAPANTDANNIEGQAIR	**4**
FLHLLGKIYYFGNNSKAVTGWQTINGKVYYFMPDTAMAAAGGLFEID GVIYFFGVDGVKAPGIYG	**7**

Appendix 1: Resulting peptide sequences for TcdB and rTcdB$_{546\text{-}2366}$ *in silico* digestion with clostripain.

Of the 51 fragments identified by ExPASy PeptideCutter, 17 are predicted to have a molecular weights of <2 kDa and are not displayed. Bold indicates regions in native and recombinant protein.

Resulting Peptide Sequence TcdB	**Peptide Mass (kDa)**
MSLVNR	1

KQLEKMANVR	1
TQEDEYVAILDALEEYHNMSENTVVEKYLKLKDINSLTDIYIDTY KKSGR	6
NKALKKFKEYLVTEVLELKNNNLTPVEKNLHFVWIGGQINDTAIN YINQWKDVNSDYNVNVFYDSNAFLINTLKKTVVESAINDTLESFR	10
MEIIYDKQKNFINYYKAQR	2
EENPELIIDDIVKTYLSNEYSKEIDELNTYIEESLNKITQNSGNDVR	5
NFEEFKNGESFNLYEQELVER	3
ISALKEIGGMYLDVDMLPGIQPDLFESIEKPSSVTVDFWEMTKLEA IMKYKEYIPEYTSEHFDMLDEEVQSSFESVLASKSDKSEIFSSLGD MEASPLEVKIAFNSKGIINQGLISVKDSYCSNLIVKQIENR	15
YKILNNSLNPAISEDNDFNTTTNTFIDSIMAEANADNGR	4
VGFFPDVKTTINLSGPEAYAAAYQDLLMFKEGSMNIHLIEADLR	5
NFEISKTNISQSTEQEMASLWSFDDAR	3
NYFEGSLGEDDNLDFSQNIVVDKEYLLEKISSLAR	**4**
GYIHYIVQLQGDKISYEAACNLFAKTPYDSVLFQKNIEDSEIAY YYNPGDGEIQEIDKYKIPSIISDR	**8**
PKIKLTFIGHGKDEFNTDIFAGFDVDSLSTEIEAAIDLAKEDISP KSIEINLLGCNMFSYSINVEETYPGKLLLKVKDKISELMPSISQ DSIIVSANQYEVR	**11**
ELLDHSGEWINKEESIIKDISSKEYISFNPKENKITVKSKNLPEL STLLQEIR	**6**
NNSNSSDIELEEKVMLTECEINVISNIDTQIVEER	**4**
IEEAKNLTSDSINYIKDEFKLIESISDALCDLKQQNELEDSHFISF EDISETDEGFSIR	**7**

Resulting Peptide Sequence TcdB continued	Peptide Mass (kDa)
FINKETGESIFVETEKTIFSEYANHITEEISKIKGTIFDTVNGKLV KKVNLDTTHEVNTLNAAFFIQSLIEYNSSKESLSNLSVAMKVQ VYAQLFSTGLNTITDAAKVVELVSTALDETIDLLPTLSEGLPIIA TIIDGVSLGAAIKELSETSDPLLR	17
QEIEAKIGIMAVNLTTATTAIITSSLGIASGFSILLVPLAGISAGIP SLVNNELVLR	6
DKATKVVDYFKHVSLVETEGVFTLLDDKIMMPQDDLVISEIDF NNNSIVLGKCEIWR	7
MEGGSGHTVTDDIDHFFSAPSITYR	3
EPHLSIYDVLEVQKEELDLSKDLMVLPNAPNR	4
VFAWETGWTPGLR	2
YFAFIADALITTLKPR	2
SFIVPIITTEYIR	2
EKLSYSFYGSGGTYALSLSQYNMGINIELSESDVWIIDVDNVVR	5
DVTIESDKIKKGDLIEGILSTLSIEENKIILNSHEINFSGEVNGSN GFVSLTFSILEGINAIIEVDLLSKSYKLLISGELKILMLNSNHIQQ KIDYIGFNSELQKNIPYSFVDSEGKENGFINGSTKEGLFVSELPD VVLISKVYMDDSKPSFGYYSNNLKDVKVITKDNVNILTGYYLK DDIKISLSLTLQDEKTIKLNSVHLDESGVAEILKFMNR	24
KGNTNTSDSLMSFLESMNIKSIFVNFLQSNIKFILDANFIISGTTS IGQFEFICDENDNIQPYFIKFNTLETNYTLYVGNR	9
QNMIVEPNYDLDDSGDISSTVINFSQKYLYGIDSCVNKVVISPNI YTDEINITPVYETNNTYPEVIVLDANYINEKINVNINDLSIR	10
YVWSNDGNDFILMSTSEENKVSQVKIR	3
FVNVFKDKTLANKLSFNFSDKQDVPVSEIILSFTPSYYEDGLIG YDLGLVSLYNEKFYINNFGMMVSGLIYINDSLYYFKPPVNNLIT	20

GFVTVGDDKYYFNPINGGAASIGETIIDDKNYYFNQSGVLQTGVFSTEDGFKYFAPANTLDENLEGEAIDFTGKLIIDENIYYFDDNYR	
GAVEWKELDGEMHYFSPETGKAFKGLNQIGDYKYYFNSDGVMQKGFVSINDNKHYFDDSGVMKVGYTEIDGKHFYFAENGEMQIGVFNTEDGFKYFAHHNEDLGNEEGEEISYSGILNFNNKIYYFDDSFTAVVGWKDLEDGSKYYFDEDTAEAYIGLSLINDGQYYFNDDGIMQVGFVTINDKVFYFSDSGIIESGVQNIDDNYFYIDDNGIVQIGVFDTSDGYKYFAPANTVNDNIYGQAVEYSGLVR	**28**
VGEDVYYFGETYTIETGWIYDMENESDKYYFNPETKKACKGINLIDDIKYYFDEKGIMR	**7**
Resulting Peptide Sequence TcdB continued	**Peptide Mass (kDa)**
TGLISFENNNYYFNENGEMQFGYINIEDKMFYFGEDGVMQIGVFNTPDGFKYFAHQNTLDENFEGESINYTGWLDLDEKR	**9**
YYFTDEYIAATGSVIIDGEEYYFDPDTAQLVISE	**4**