Structure – Function Relationships of

Clostridium difficile Toxin A

Joanna K Craggs (BSc. Hons.)

A thesis submitted in partial fulfilment of the requirements of

the Council for the National Academic Awards for

the degree of Doctor of Philosophy

October, 1999

Institute of Infection and Immunity,

University Hospital,

Queens Medical Centre,

Nottingham

****

For my mother Pat

****

SUMMARY

Ten overlapping fragments covering the entire *Clostridium difficile* toxin A gene were cloned and expressed in *Escherichia coli*. Eight fragments (a', a2, b, c, d, e, f and g) represented the first 5.55kb of the gene whereas two fragments (h1 and h2) each spanned the entire C-terminal repeat region of the molecule.

All activities relating to binding to carbohydrates (i.e. cold haemagglutination of rabbit erythrocytes), binding to bovine thyroglobulin and non-specific binding to a murine monoclonal antibody were restricted solely to peptides H1 (amino acids [aa] 1834-2683) and H2 (aa 1832-2683). Peptide H2 alone also displayed the ability to bind to cells and to be internalised into endosome-like compartments within the cells. Taken together with the observation that peptide H2 caused a cytopathic effect on Vero cells which was atypical of the holotoxin, these results may indicate that the repeat region of toxin A stimulates intracellular signalling pathways prior to Rho glucosylation.

Peptide A2 (aa 1-536) glucosylated recombinant RhoA (rRhoA) *in vitro*, whereas peptides A'(aa 1-205), B (aa 542-859), C (aa 114-859), D (aa 869-1330), E (aa 542-1161), G (aa 869-1830) and H2 (aa 1832-2683) did not. The results obtained for peptides A', A2 and C indicate that the first 536 aa encompass the catalytic domain for this activity, that more than the first 205 aa alone are needed for expression of enzymic activity, and that for a peptide to be active it must not lack the first 113 aa. The first 113 aa of the holotoxin are probably essential for the correct folding of the catalytic domain and expression of its activity.

These studies were also the first to locate the toxin A ATP binding site to a peptide spanning aa 542-859 (peptide B) of the holotoxin. Antibody reaction profiles of

antiserum to holotoxin A against toxin A peptides and of antiserum to the peptides against holotoxin A indicate that this region is unexposed in the native state. Also of interest was the observation that the only peptides, which contained the nucleotide-binding site (B and E), lacked the ability to glucosylate rRhoA. Further peptide A2, which possessed glucosyltransferase activity, lacked the nucleotide-binding site. These studies therefore, suggest that a nucleotide-binding site is not required for *in vitro* glucosylation of rRhoA by toxin A, and fail to identify a role for the toxin A nucleotide binding site.

An engineered truncated form of toxin A, consisting of the first 539 aa of the holotoxin (encompassing glucosyltransferase activity) fused to the 852 aa C-terminal peptide H2 (repeat end binding portion) caused a conventional cytopathic effect (CPE), but was 1,400 fold less cytotoxic to Vero cells than the holotoxin. Peptide A2 (aa 1-536) alone had no effect on Vero cells or in rabbit ileal loops suggesting that peptide H2 aided delivery of the glucosyltransferase molecule into cells leading to a CPE. The truncated toxin lacked the nucleotide binding site and the putative membrane-translocating domain (internal hydrophobic region). The reduced activity of the truncated toxin suggests that although not essential for cytotoxic activity, the nucleotide-binding site and the internal hydrophobic region are important for stability and/or efficient translocation of the holotoxin into the cytosol.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council (MRC) and carried out in the Microbial Pathogenicity Research Group, Institute of Infection and Immunity, Queens Medical Centre, Nottingham.

Firstly I would like to thank Professor S. P. Borriello for his constant support, encouragement and patience. He has unknowingly taught me some invaluable lessons about being successful in the scientific world, which I am sure, will stay with me throughout my career.

I would also like to thank all the members of the Institute of Infection and Immunity for providing a friendly and jovial working environment. I would particularly like to thank the other PhD students Vicky Bush and Valia Norte for their moral support and friendship, and Dr Yash Mahida, Dr Paul Dodson and Sue Hyde for many useful and thought provoking discussions on *C. difficile*. I would also like to express my gratitude to Drs Tom Baldwin, Helen Palmer and Alan Cockayne for their excellent technical advice, which has proved invaluable.

My grateful thanks also go to Dr Nicholas Powell for his assistance with the electron microscopy studies, and Dr April Roberts for her collaboration on the Rho glucosylation experiments. For help with the immunohistochemistry studies, I would like to extend my thanks to Shavy Makh.

I would also like to mention with fond memories my late father Malcolm. The knowledge that he would have been proud of me on this day has helped guide me through some of the tougher times. I am extremely grateful to my brother Jonathan and sister Sarah for

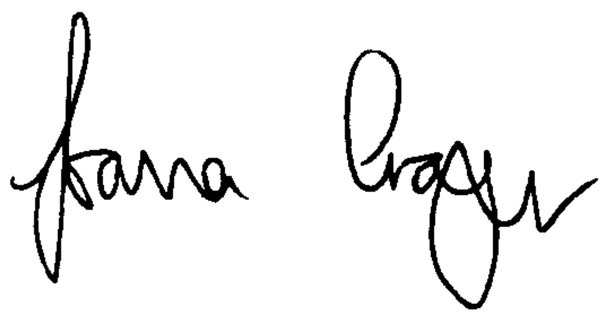
their all round support, both emotional and financial. Thank you both for always believing in me.

A very special mention of thanks must go to my fiancee Damien Wells who has been my strength in times of weakness and whose love and patience has been unceasing. His constant encouragement and support, both practical and emotional, will never be forgotten.

Finally my deepest thanks go to my mother Pat who has always put my life and happiness before her own. I would like to thank her for all the love, kindness and financial support she has provided throughout my student life and for making this day possible. In an attempt to express my gratitude I would like to dedicate this thesis to you!

DECLARATION

I declare that all of the research outlined in this thesis was carried out by myself, with the following exceptions. The rabbit ileal loop studies were performed at Leicester University with the assistance of Professor Julian Ketley. The Rho glucosylation studies were carried out at CAMR Porton Down with the specific assistance of Dr. April Roberts and Mr. Glyn Choules.



Joanna Craggs

LIST OF ABBREVIATIONS

AAC	Antibiotic-associated colitis
AAD	Antibiotic-associated diarrhoea
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CBB	Coomassie brilliant blue
CPE	Cytopathic effect
Da	Dalton
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid disodium salt
ELISA	Enzyme-linked immunosorbent assay
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FPLC	Fast protein liquid chromatography
H/A	Haemagglutination
HT	Haemorrhagic toxin
IEF	Iso-electric focussing
IL	Interleukin
IP3	Inositol triphosphate
IPTG	Isopropyl-β-D-thiogalactosidase
Kb	Kilobase
kDa	Kilodalton
LBA	Luria Bertani agar
LB	Luria Bertani
LBB	Luria Bertani broth
LCTs	Large clostridial cytotoxins
LT	Lethal toxin
Mab	Monoclonal antibody

MBP	Maltose binding protein
MEM	Minimal essential media
Mr	Relative molecular weight
MWT	Molecular weight
ORF	Open reading frame
PaLoc	Pathogenicity locus
PBSA	Phosphate buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point
PMC	Pseudomembranous colitis
PMNs	Polymorphonuclear leukocytes
RBS	Ribosome binding site
RRBCs	Rabbit red blood cells
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SDW	Sterile distilled water
TBS	Tris buffered saline
TNF	Tumour necrosis factor
tRNA	Transfer RNA
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactosidase

TABLE OF CONTENTS

**** **** **** **** ****

SECTION – 2: GENERAL MATERIALS AND METHODS

**** **** **** **** ****

LIST OF FIGURES

Section-5

Section-6

LIST OF TABLES

SECTION-1

INTRODUCTION

SECTION-1

INTRODUCTION

1.1 Pathogenicity

1.2 Toxins

1.3 Clostridia

1.4 Classes of clostridial toxins

1.5 *Clostridium difficile (C. difficile)*

1.5.1 Historical background of *C. difficile* and pseudomembranous colitis (PMC)

1.5.2 *C. difficile* gastrointestinal disease

1.5.3 Animal models

1.5.4 Colonisation resistance

1.6 Factors influencing the outcome of infection with *C. difficile*

1.6.1 Age of the host

1.6.2 Disruption of colonisation resistance

1.6.3 Virulence factors

1.6.3.1 Adherence to host mucosal cells and fimbrae

1.6.3.2 Capsule

1.6.3.3 Enzymes

1.6.3.4 Toxin production

1.6.3.5 Other toxic factors

1.7 Characteristics of toxins A and B

1.7.1 Immunochemical properties

1.7.2 Biological activities

1.7.3 Toxin receptors

1.7.4 Molecular characterisations

1.7.5 Functions of toxins A and B

1.7.5.1 Phosphate and nucleoside binding

1.7.5.2 Interaction with nucleic acid

1.7.5.3 Stimulation of increased inositol triphosphate (IP3) production

1.7.5.4 Carbohydrate binding properties

1.7.5.5 Non-specific binding to monoclonal antibodies (Mabs)

1.7.5.6 Covalent modification of Rho proteins

1.7.5.7 Interaction with cells of the immune system

1.8 Structure-function relationships of the *C. difficile* toxins

1.9 Mechanisms of action of toxins A and B

1.10 Research aims and objectives

SECTION-1

INTRODUCTION

1.1 Pathogenicity

Pathogenicity is the ability to cause disease while a pathogen is any microorganism with that ability. Over 250 bacterial genera are listed in Bergey's Manual of Determinative Bacteriology with only about 10% included as pathogenic species of man or animals. Within a bacterial genus not every species is pathogenic and not every strain of each pathogen is virulent. The percentage of the total microbial biomass capable of causing disease is, therefore, very small (Stephen and Pietrowski, 1986).

To cause a disease a bacterium must be able to attach to and infect broken skin or mucous surfaces before entry into uninfected sites. The organism must be able to proliferate in the host environment then invade and damage the host (Sparling P.F. 1983). The ability to damage the host distinguishes an organism from being pathogenic as opposed to infective. Bacteria adopt two main mechanisms for damaging a host. Firstly, they stimulate self-damaging activities, which lead to hypersensitivity or immunopathological damage in the host. Secondly, some organisms possess the ability to produce toxic substances, which interact directly with specific host cells.

1.2 Toxins

A toxin is a protein or conjugated protein produced by some higher plants, animal, fungi and pathogenic bacteria, that is highly poisonous to other living organisms. Robert Koch, who first described *Vibrio cholerae* as the causative agent of cholera, suggested that cholera was a toxin-mediated disease (Koch R. 1884, cited from van Heyningen W.E. 1970). Later, it was recognised that toxins were also responsible for death from

diphtheria (Roux and Yersin, 1888), tetanus (von Behring and Kitasoto, 1890) and botulism (van Ermengen, 1896) (all cited from Smith H. 1995).

Toxins can be simply divided into two main types, endotoxins and exotoxins. Endotoxins are found in the outer membrane of gram-negative bacteria and are released when cell integrity is disrupted. The endotoxins are antigen complexes of protein, polysaccharide and lipid. It is believed that the protein determines the antigenicity of the toxin, the polysaccharide the immunological specificity and the lipid possibly the toxicity. The endotoxins do not appear to be neutralised by toxin antiserum because antibodies are directed against the non-toxic protein and polysaccharide regions.

Exotoxins are released by bacterial cells into the body tissues enabling bacteria to produce disease without extensive spread or multiplication. Organs and tissue, therefore, can be affected at a distance from the site of infection. Exotoxins are protein toxins and are therefore antigenic. They can be neutralised by toxin antiserum and are considerably more toxic than endotoxins. Some exotoxins exhibit specificity for particular tissues or cell types. For example, neurotoxins (e.g. tetanus toxin) affect cells of the central nervous system while enterotoxins (e.g. cholera toxin) affect cells of the intestinal mucosa often producing severe diarrhoea. Cholera toxin is a cytotonic enterotoxin because it induces a net fluid secretion by interfering with biochemical regulatory mechanisms without causing histological damage. A cytotoxic enterotoxin, however, induces actual damage to intestinal cells as a necessary prelude to fluid secretion (Stephen and Pietrowski, 1986).

Of particular interest in this thesis are Clostridium species which contain one of the most potent collections of toxigenic bacteria in existence.

1.3 Clostridia

Clostridia are widely distributed in nature, present in soil and the intestinal tract of humans and animals. The bacteria are gram-positive, spore forming anaerobic bacilli. The clostridial genus includes organisms that produce organic solvents such as acetone and butanol (e.g. *Clostridium acetobutylicum*). Some convert nitrogen to ammonia by nitrogen fixation (e.g. *Clostridium pasteurianum*) while others are medically important because they are pathogenic to humans and animals. The pathogenic species produce a variety of soluble toxins, some of which are extremely potent. Of more than 85 species of clostridia described in Bergey's Manual of Determinative Bacteriology, at least 20 produce toxins (Lyerly and Allen, 1997).

The important human clostridial diseases include gas gangrene (*C. perfringens, C. novyi, C. septicum, C. sordellii* and *C. histolyticum*), tetanus (*C. tetani*), botulism (*C. botulinum*), pseudomembranous colitis and antibiotic-associated diarrhoea (*C. difficile*), *C. perfringens* type A-induced food poisoning and *C. perfringens* type C necrotising jejunitis (pig-bel). The *C. botulinum* and *C. tetani* neurotoxins are zinc proteases (Schiavo *et al*, 1992a, b, c) and are amongst the most lethal toxins with less than 100ng of either being sufficient to kill an adult (Stephen and Pietrowski, 1986).

In the last twenty years *C. difficile* has been identified as the cause of pseudomembranous colitis (PMC) and antibiotic-associated diarrhoea (George RH *et al*, 1978, Larson *et al* 1978). *C. difficile* is also the most identifiable bacterial cause of nosocomial diarrhoea and is second only to *C. perfringens* as the most common identifiable anaerobe to cause diarrhoea (Borriello, 1995).

1.4 Classes of clostridial toxins

There are currently three characterised classes of clostridial toxins. The first is represented by the clostridial neurotoxins (tetanus and botulinum). These toxins are single polypeptide chains, which are cleaved by an intrinsic protease to produce a heavy and light chain fragment held together by a disulphide bond. Once in the circulation the carboxyl terminal part of the heavy chain fragment binds to the neuronal cells enabling the toxin to be internalised into acidic compartments by receptor mediated endocytosis (RME). The amino terminal 50kDa domain of the heavy chain is then believed to translocate the membrane releasing the catalytic light chain fragment into the cytoplasm where it has its effect. The neurotoxins act to inhibit neurotransmitter release (Schiavo and Montecucco, 1997).

The second, relatively small group of clostridial toxins, is called the 'Binary Toxins'. Examples include the iota toxins of *C. perfringens* and *C.spiroforme* and the *C. botulinum* toxin C2. Binary toxins are composed of two separate and independent protein components, which possess little or no toxicity alone but in combination can cause serious, even fatal effects. The binding component (heavy chain) facilitates uptake of the enzymatic portion (light chain) into the cell. The light chain is an ADP-ribosyltransferase and transfers an ADP-ribosyl group from NAD onto monomeric actin. The modified actin inhibits polymerisation of actin filaments at the barbed end leading to rounding up of cultured mammalian cells. Clostridial binary toxins can also induce fluid accumulation in ligated intestinal loops (Lyerly and Allen, 1997).

The third class is the 'large clostridial cytotoxins'. This group contains cytotoxins of high molecular weight (Mr> 200,000) with an AB^x-type of molecular architecture (^{x} indicating that the molecules are multivalent with respect to receptor binding) which enter cells by

RME (von Eichel-Streiber *et al*, 1996). This group includes toxins A and B of *C.difficile*, alpha toxin of *C. novyi* plus the haemorrhagic (HT) and lethal (LT) toxins of *C. sordellii*. These single polypeptide toxins bind to cells and are internalised by RME. Following endosomal processing and possible activation, the toxins enter the cytoplasm where they have an effect on the cytoskeletal system resulting in cell rounding and eventual cell death (Lyerly and Allen, 1997). The largest of the clostridial cytotoxins is *C. difficile* toxin A. The remainder of this thesis will concentrate on *C. difficile*, its toxins and the role they play in antibiotic associated PMC.

1.5 *C. difficile*

C. difficile is an enterotoxigenic clostridial species which is an anaerobic opportunistic human gut pathogen. The organism is a gram-positive spore former but can stain gram variable in older stationary cultures. It produces two large protein toxins, A (enterotoxin) and B (cytotoxin) which are closely related to the HT and LT toxins of *C. sordellii* (Martinez and Wilkins, 1992). *C. difficile* is the most common identifiable cause of nosocomial diarrhoea and is now established as the aetiological agent of PMC in humans (George *et al* 1978a,b, Larson *et al*, 1978). The spores shed by *C. difficile* infected patients can persist in the environment for long periods of time and care has to be taken by health care workers to avoid transferring them to other patients.

1.5.1 Historical background of *C. difficile* and PMC

One of the earliest descriptions of PMC related to a young woman who presented with pseudomembranous lesions of the intestinal tract and haemorrhagic diarrhoea following surgery (Finney, 1893). *C. difficile* was first identified in the stools of healthy infants and

was originally called *Bacillus difficilis* due to the difficulty in isolating the organism (Hall and O'Toole, 1935). Early experiments on guinea pigs suggested that the organism was toxigenic (Hall and O'Toole, 1935, Snyder, 1937). Observations that *C. difficile* was often isolated from patients who presented with no unusual clinical features led investigators to initially believe that the *C. difficile* toxin, lethal to animals, played no role in clinical disease in humans (Smith and King, 1962). The massive increase in the incidence of PMC in the 1950's was finally associated with antibiotic therapy (Reiner *et al* 1952, Hummel *et al*, 1964, Tedesco *et al,* 1974). At this time PMC was attributed to *Staphylococcus aureus* because the organism was commonly isolated from the stools of diseased experimental animals (Hummel *et al*, 1964).

In 1969 came the first description of *C. difficile* causing intestinal disease in rats (Hammarstrom *et al,* 1969). From this time onwards work to establish the relationship between the lesion, the toxin and the organism evolved rapidly. In 1977 Larson and co-workers identified a cytopathic protein toxin in the stools of patients suffering from PMC. At the same time the observation that the cytotoxic factor present in the caecal contents of diseased animals could be neutralised by polyvalent gas gangrene anti-toxin (Bartlett *et al*, 1977) led to further work which implicated *C. sordellii* as the causative agent of PMC in animals (Rifkin *et al*, 1977). This hypothesis was soon disregarded as it became apparent that *C. sordellii* was not a component of the cecal microbiota of diseased hamsters. *C. difficile* was soon identified as the dominant clostridial species in the caecal contents of diseased hamsters and was shown to produce a cytopathic toxin (Bartlett *et al*, 1977, 1978a) which was neutralised by *C. sordellii* anti-toxin (Bartlett *et al*, 1978b). Evidence that *C. difficile* was also the causative agent of PMC in humans, and that the organism was readily isolated from the faeces of diseased patients was soon to follow

(Bartlett *et al*, 1978c, George R.H *et al*, 1978, George W.L *et al*, 1978, Larson *et al*, 1978).

Until 1980 it was believed that the cytotoxic factor was the only toxin produced by *C. difficile* (Taylor and Bartlett, 1979, Rolfe and Finegold, 1979). Shortly after, however, purification of this cytotoxin resulted in the detection of an additional cytotoxic activity (Humphrey *et al*, 1979, Banno *et al*, 1981, Taylor *et al*, 1981, Sullivan *et al*, 1982) suggesting that *C. difficile* produces at least two distinct toxins. These were initially designated D1 and D2 (Banno *et al*, 1981) but were renamed toxins A (enterotoxin) and B (cytotoxin) respectively due to their different elution patterns on anion-exchange resins (Sullivan *et al*, 1982).

1.5.2 *C. difficile* gastrointestinal disease

C. difficile organisms are present in the normal colonic flora of 2-5% of the general adult population. Thirty to 75% of healthy neonates are also colonised with toxigenic *C. difficile* but are asymptomatic. In an adult the normal bacterial flora in the gut serves as a major barrier against colonisation by pathogens. When the flora is disturbed the host becomes susceptible to colonisation or overgrowth of the pathogen. The prevalence of disease, therefore, is increased when a person is hospitalised or exposed to antibiotics.

C. difficile infection can range from mild diarrhoea to the life threatening condition PMC depending on several factors including the virulence of the infecting strain. In adults *C. difficile* accounts for 15-20% of all cases of antibiotic associated diarrhoea (AAD), 50-70% of all cases of antibiotic associated colitis (AAC) and 90-100% of cases of PMC (Bartlett, 1990). Patients always present with profuse watery or bloody diarrhoea often containing mucus. AAD is defined as diarrhoea associated with recent antibiotic use with

no evidence of colitis. AAC is defined as diarrhoea associated with recent antibiotic use with evidence of non-specific inflammatory reaction but no pseudomembranes. The extreme disease PMC is characterised by the presence of cream to yellow/ green plaques on the colonic mucosa. These are 1-2 mm in diameter in the early stages but increase in size as the disease progresses. Upon closer examination the pseudomembrane consisting of sloughed epithelial cells, fibrin, mucus and polymorphonuclear leukocytes appears to be erupting from the surface of the colonic mucosa (Dodson and Borriello, 1996). The underlying mucosal surface often reveals well-defined lesions and ulcerations consisting of epithelial necrosis and a marked leukocyte infiltration of the lamina propria (Abrams *et al*, 1980, Totten *et al*, 1978).

Antibiotics frequently associated with *C. difficile* diarrhoea include clindamycin, ampicillin and cephalosporins (Trnka and LaMont, 1984, Bartlett, 1984, George *et al*, 1982). However, virtually every antibiotic has been implicated with the disease at some time. The first line of treatment of *C. difficile* disease is to remove the antibiotic. In instances where the symptoms of the disease are more severe, the most common treatment is the oral administration of vancomycin or metranidazole. If treatment of PMC is delayed, life threatening complications can occur such as protein losing enteropathy, toxic megacolon, colonic perforations, severe fluid loss and electrolyte imbalances.

1.5.3 Animal models

C. difficile causes antibiotic–associated pseudomembranous colitis in a number of animal species including hamsters, guinea-pigs, rats and rabbits. The hamster, however, has proved to be the best animal model for the disease. As with PMC in humans, the hamster disease can be initiated with a whole range of antibiotics (Chang *et al*, 1978, Fekety *et al*,

1979, Price *et al*, 1979). The disease in hamsters is localised in the proximal colon and the ileum, whereas PMC in humans occurs primarily in the distal colon. The coverings of inflammatory debris present in the caeca of diseased hamsters, however, are very similar to the pseudomembranes observed in the colon of many PMC patients (Price and Davies, 1977). The hamster became routinely used as a model for studying PMC in the late 1970s. Larson and co-workers isolated cytotoxic *C. difficile* strains from patients with PMC and showed that they induced fatal enterocolitis when inoculated orally into hamsters pre-treated with vancomycin. The organism was re-isolated from the caecal contents of the hamsters and was shown to cause the same cytotoxic effect as the initial inoculum (Larson *et al*, 1978). Another interesting observation is that like human infants, infant hamsters seem to have high numbers of toxigenic *C. difficile* and toxins A and B in their stools whilst remaining asymptomatic for the disease (Griffin *et al*, 1984, Rolfe and Iaconis, 1983).

Other animal models have been used such as clindamycin pre-treated guinea pigs (Rehg, 1980) and lincomycin pre-treated rabbits (Rehg and Pakes, 1982) but they proved less successful than the hamster model. Germ free rats (Czuprynski *et al*, 1983) and mice (Onderdonk *et al*, 1980, Corthier *et al*, 1985, Sugiyama *et al*, 1985) have also been used as animal models for PMC. These animals, however, are considerably more expensive to use than hamsters. Also these gnotobiotic animals are free from other organisms and so do not satisfactorily reflect the *in vivo* situation in humans, whereas the hamster model better approximates to the human conditions.

1.5.4 Colonisation resistance

Colonisation of the gut is important in the development of normal intestinal morphology. The gut flora is extremely complex (i.e. 10^{12} viable organisms/gram of gut content, with 400-500 different species) and acts as a barrier preventing non-resident and other pathogenic bacteria from becoming established (Borriello, 1990). This phenomenon, termed colonisation resistance, is dependent to a large extent on the 'crowding out' effect by the indigenous gut flora. The flora forms an impenetrable layer over the mucosa, and any bacteria that shed from this layer, into the lumen of the tract, multiply and successfully compete with potential pathogens for nutrients. The resident bacteria are also capable of producing antibacterial substances such as colicine (Branche *et al*, 1963), and release short chain fatty-acids which have anti-microbial activity (Meynell, 1963).

Disruption of the normal flora, for example by antibiotic therapy, allows the establishment of opportunistic organisms. Many *in vivo* and *in vitro* studies have shown that disruption of the normal flora is a pre-requisite for *C. difficile* infection of the gut (Rolfe *et al*, 1981, Corthier *et al*, 1985, Barclay and Borriello, 1982, Malamou-Ladas and Tabaqchali, 1982). The role of colonisation resistance in *C. difficile* infection has been demonstrated using faecal emulsion (Borriello and Barclay, 1986, Borriello *et al*, 1988). A study showed that *C. difficile* growth and cytotoxin production were inhibited in faecal emulsions from healthy adults but not in sterilised emulsions. This confirmed the importance of viable bacteria in the inhibitory system (Barclay and Borriello, 1986). Caecal emulsions from the hamster, pre-treated with clindamycin or ampicillin, were also found to support *C. difficile* growth and cytotoxin production, whereas caecal emulsions from untreated animals did not (Borriello *et al*, 1988a).

Attempts have been made to reconstitute colonisation resistance to *C. difficile* infection in patient's following antibiotic therapy. Faecal enemas have been used to successfully

reintroduce a complete gut flora (Schwan *et al*, 1984). Micro-organisms, known to have an inhibitory effect on *C. difficile* growth, e.g. human source strain *Lactobacillus* GG (Gorbach *et al*, 1987) and *Sacchromyces boulardii* (Elmer *et al*, 1989), have also been used to treat relapsing *C. difficile* colitis. The protective effects of *S. boulardii* on *C. difficile*-induced inflammatory diarrhea in humans have been shown to be due, in part, to proteolytic digestion of toxins A and B by a secreted protease (Castagliuolo *et al*, 1999). Feeding *S. boulardii* to mice during oral immunisation with *C. difficile* toxin A, has also been shown to stimulate a 19-fold increase in specific intestinal IgA anti-toxin A levels (Qamar *et al*, 1999). Non-toxigenic avirulent strains of *C. difficile* have been shown to be protective against subsequent colonisation with toxigenic virulent strains of *C. difficile* (Borriello and Barclay, 1985, Seal *et al*, 1987).

All of these models were successful in demonstrating the importance of the normal gut flora and certain probiotics as barriers to colonisation by *C. difficile*.

1.6 Factors influencing the outcome of infection with *C. difficile*

1.6.1 Age of the host

Antibiotic-associated susceptibility to *C. difficile* infection appears to increase with age. The elderly who receive antibiotic therapy in a hospital setting comprise the population at highest risk. It has been suggested that age-related changes in the gut flora of the elderly (Ellis-Pegler *et al*, 1975, Yamagishi *et al*, 1976) cause a reduction in the amount of disruption of colonisation resistance required for *C. difficile* to colonise (Borriello and Barclay, 1986). It has also been shown that toxin A causes an increased chemotactic response of leukocytes, in elderly subjects compared to young subjects, explaining the

more severe inflammatory response seen in the elderly with *C. difficile* disease (Triadafilopoulos *et al*, 1991).

Another interesting aspect of PMC is that new-borns and young infants often harbour *C. difficile* and its toxins in their intestines without developing disease (Donta and Mayers, 1982). This is also true in the hamster model (Borriello *et al* 1985). This can be explained to some extent by the fact that neonates have a developing and incomplete intestinal flora that is unable to exclude *C. difficile* (Borriello, 1990). It is possible that infants lack the receptors for toxin A in their intestines, or that the receptors exist in an immature form that are not recognised by toxin A (Lyerly *et al*, 1988). Another possible explanation is that the mucin layer covering the receptors on infant intestinal cells is much thicker than in adults thus preventing toxin A from binding (Lyerly *et al*, 1988).

1.6.2 Disruption of colonisation resistance

Most antibiotics, but particularly clindamycin, ampicillin and cephalosporins, kill or suppress the normal flora to such an extent that *C. difficile* is free to colonise and cause toxinogenic diarrhoea. Other procedures that may disrupt the normal gut flora and predispose patients to *C. difficile* infection include the use of medications that decrease intestinal motility (Trinka and LaMont, 1984), gastrointestinal tract surgery (McFarland *et al*, 1991) and cancer chemotherapy treatment (Fainstein *et al*, 1981).

1.6.3 Virulence factors

The outcome of infection with *C. difficile* is dependent primarily on the virulence potential of the infecting strain and on the extent to which the virulence determinants are

allowed to be expressed. Many virulence factors or potential factors have now been identified for *C. difficile*.

1.6.3.1 Adherence to host mucosal cells and fimbrae

C. difficile is regularly isolated from areas of the gut that are continually washed by intestinal fluids, suggesting that the bacterium produces specific factors that mediate adherence to host cells. *C. difficile* has been found in association with the intestinal mucosa of man (Borriello, 1979) and hamsters (Borriello *et al*, 1988b). Highly virulent toxigenic strains of *C. difficile* have been found to adhere and colonise much more efficiently than poorly virulent toxigenic or avirulent non-toxigenic strains (Borriello *et al*, 1988b). It has been suggested that the toxins damage the host cells exposing masked receptor sites for *C. difficile* or that they affect the ability of the gut cells to produce mucosal secretions (Laboisse, 1995).

Trefoil peptides and mucin glycoproteins have been shown to protect T84 monolayers from *C. difficile* toxin A injury *in vitro* (Kindon *et al*, 1995). It has been suggested that cells of the intestinal mucosa must be poorly covered by the visoelastic gel produced by trefoil peptides and mucin glycoproteins to enable virulent toxigenic *C. difficile* strains to adhere to and colonise the gastrointestinal tract.

Heat shocking of *C. difficile* at 60°C appears to greatly enhance its ability to adhere to CaCo-2 and HT29-MTX cells (Eveillard *et al*, 1993). The adhesion factors involved are believed to be two surface proteins, which, although scarcely expressed by *C. difficile*, become over-expressed following induction by antibiotic therapy, or some other stimuli, allowing the bacteria to adhere to the mucus (Eveillard *et al*, 1993). One of these

adhesins is believed to be a lectin because glucose and galactose partially block attachment of *C. difficile* to tissue culture cells (Karjalainen *et al*, 1994).

Typing of *C. difficile* strains has proved successful in revealing a consistent correlation between certain protein types and virulent strains (Pantosti, *et al*, 1988, Tabaqchali, 1990). A 36 kDa protein has been identified (Pantosti *et al*, 1988) and purified (Cerquetti *et al*, 1992) and appears to be specifically expressed by highly virulent toxigenic *C. difficile* strains isolated from hospitalised patients with PMC. Its role in *C. difficile* virulence remains unclear, although it has been suggested that it may be a promoter for cell adhesion (Mastrantonio *et al*, 1995).

Some strains of *C. difficile* have been shown to possess fimbrae that are 4-9nm in diameter and which originate from the pole of the cell (Borriello *et al*, 1988c). To date, no direct correlation has been made between the presence of the fimbrae and the ability of *C. difficile* to adhere to the gut mucosa of hamsters or to cause disease. The role of the fimbrae in *C. difficile* disease, therefore, remains unclear.

1.6.3.2 Capsule

For many bacteria the presence of a capsule is an important virulence factor that protects against phagocytosis (Duerden *et al*, 1994). *C. difficile* appears to be resistant to phagocytosis by polymorphonuclear leukocytes (PMNs) even though there is a great infiltration of PMNs during disease. It was concluded, therefore, that there must be an anti-phagocytic factor, such as a capsule, present on the surface of the bacterium. In support of this hypothesis, recent studies have demonstrated that some strains of *C. difficile* produce capsule-like material *in vitro* (Davies and Borriello, 1990, Baldassari *et al*, 1991) that has apparent phenotypic similarities to the small capsule associated with

Bacteroides fragilis (Borriello *et al*, 1990). Although these studies were unable to demonstrate a correlation between possession of a capsule and virulence status of the *C. difficile* strain *in vitro*, it remains a possibility that the possession of a capsule by *C. difficile* confers resistance to phagocytosis.

1.6.3.3 Enzymes

C. difficile produces a variety of hydrolytic and proteolytic enzymes (Seddon *et al*, 1990, Seddon and Borriello, 1992, Hafiz, 1974). Different *C. difficile* strains have been shown to be positive for hyaluronidase, chondroitin-4-sulphatase, collagenase, heparinase and general protease activity (Seddon *et al*, 1990). Toxigenic *C. difficile* strains produce more collagenase than non-toxigenic strains and the most virulent strains in the hamster model, are the most proteolytic (Karjalainen *et al*, 1995).

Hydrolytic and proteolytic enzymes, as well as permitting tissue degradation of the intestinal wall, break down host polymers into smaller components, thus providing the bacteria with energy sources. For example, *C. difficile* requires N-acetylglucosamine but lacks the enzyme necessary for cleaving this monosaccharide from oligosaccharide side chains (Wilson and Perini, 1988). To compensate, *C. difficile* is able to produce hyaluronidase and obtain N-acetylglucosamine from hyaluronic acid.

1.6.3.4 Toxin production

At least five toxic factors have been described for *C. difficile* but only two of these, toxins A and B (which possess an overall homology of 69% at the amino acid level) have been implicated in disease and studied in detail. The molecules are single polypeptides of 303kDa (toxin A) and 269kDa (toxin B), making toxin A the largest protein known to

date (Lyerly and Allen, 1997). In order to differentiate between the two toxins, toxin A is described as an enterotoxin and toxin B as a cytotoxin. This description is not strictly correct because both toxins are potent cytotoxins, although toxin B is at least 1000 times more cytotoxic for most cell lines. Unlike toxin A, toxin B does not appear to be able to affect the undisturbed mucosa of the hamster gut (Lyerly *et al*, 1985a, Mitchell *et al*, 1986, Triadafilapoulous *et al*, 1987). It has been suggested that toxin B acts synergistically with toxin A, which is required for initial tissue damage and escape of toxin B from the gut (Lyerly *et al*, 1985a).

A cytotoxigenic strain of *C. difficile* has been isolated that fails to haemagglutinate rabbit red blood cells, or to react in toxin A specific enzyme immunoassays (Haslam *et al*, 1986). This strain, 8864, was initially believed to be toxin A negative, toxin B positive. The interest shown in this isolate was due to the prior assumption that all strains were either non-toxigenic or produced both toxins (Lyerly *et al*, 1988). Interestingly, this strain was shown to cause haemorrhage and diarrhoea in hamsters (Borriello *et al*, 1992), initially thought to be due to the effects of toxin B or an additional enterotoxic factor. Further characterisation revealed that strain 8864 produced a modified form of toxin B (Borriello *et al*, 1992, Torres, 1991), and that the lack of the toxin A protein was not due to a silent copy of the gene, but to the absence of the 3' end of the gene (Borriello *et al*, 1992). It was later shown that the front portion of the toxin A gene was present in strain 8864 (Lyerly *et al*, 1992) but was not being expressed due to rearrangements in the pathogenicity locus resulting in altered transcription (Soehn *et al*, 1998). It was concluded that the effects seen in the hamster model were due to the altered activity of the modified toxin B gene. Both toxins A and B will be discussed in more detail in later sections.

1.6.3.5 Other toxic factors

Several investigators have described an additional non-haemorrhagic enterotoxic activity expressed by *C. difficile* (Banno *et al*, 1984, Mitchell *et al*, 1987, Guiliano *et al*, 1988, Torres and Lonnroth, 1989). In 1989 Torres and co-workers described an enterotoxin designated toxin C which caused a clear haemorrhagic fluid secretion similar to that caused by cholera toxin. It was concluded that toxin C was antigenically and biologically distinct from toxins A and B.

A motility – altering factor has also been described in culture filtrates of *C. difficile* which is believed to cause altered motor activity in the intestine, but does not cause tissue damage or fluid secretion (Justus *et al*, 1982).

In an isolated study, one *C. difficile* strain (CD196) out of fifteen isolated from patients with PMC produced a 43 kDa actin-specific ADP-ribosyltransferase (Popoff *et al*, 1988) homologous to the enzymatic component of other clostridial binary toxins (Popoff and Boquet, 1988). It has recently been reported that this strain produces both the enzymatic and binding components forming a complete binary toxin which is cytotoxic for cells (Perelle *et al*, 1997). This toxin is unlikely to be an essential virulence factor for *C. difficile* because it is not produced by all pathogenic strains.

The production of toxins A and B by *C. difficile* is thought to be the most important virulence factor because, irrespective of the presence or absence of any other factors, non-toxigenic strains are avirulent in the hamster model of the disease (Borriello, 1990, Fluit *et al*, 1991,).

1.7 Characteristics of toxins A and B

One of the most unusual features of both toxins is their extremely large size. There has been much controversy over the actual molecular weights and sub-unit composition of the toxins. Past studies have estimated native toxin A to have a Mr of between 400,000 and 600,000 and native toxin B to have a Mr of between 360,000 and 500,000. Under denaturing conditions both toxins were found to have a Mr in excess of 250,000 and did not dissociate into subunits (Banno *et al*, 1984, Lyerly *et al*, 1986a). Other investigators have reported sizes of 50 kDa (Pothoulakis *et al*, 1986, Rihn *et al*, 1988, Meador and Tweten, 1988) and 43 kDa (Torres and Lonnroth, 1988a) for toxin B. In an isolated study, toxin A was reported to have an Mr of 52,000 and a sub-unit composition of A_1 (Mr 41,500) and A_2 (Mr 16,000) (Rihn *et al*, 1984).

Others have observed minor protein bands following SDS-PAGE of toxins A and B which were smaller than the major toxin band and which reacted with toxin specific antibodies. It was suggested that the toxin exists as aggregates of small sub-units that were dissociated under denaturing conditions (Lyerly *et al*, 1986b).

The molecular masses of 303,103 Da for toxin A and 269,696 Da for toxin B were finally deduced from the published DNA sequence data (Dove *et al*, 1990, Barroso *et al*, 1990). These sizes are in agreement with other estimations (Banno *et al*, 1984, Lyerly *et al*, 1986a) but contradict others (Pothoulakis *et al*, 1986, Rihn *et al*, 1984, 1988). The native Mr values of 600 kDa (toxin A) and 500 kDa (toxin B) suggest that both toxins exist as dimers, either in the native state, or as a result of purification procedures. There is evidence to suggest that toxin A is produced as a 'pro-toxin' form that lacks haemagglutinating activity but retains cytotoxicity and is activated within the bacterial

cell to the fully active form. Pro- toxin A has been shown to contain twice as many α-helices as toxin A (Borriello *et al*, 1990, Kamiya and Borriello, 1992).

The coding DNA of both toxin genes is extremely AT rich (toxin A gene has an AT content of 73.1 mol%). Both proteins contain high concentrations of the amino acids glycine, aspartic acid, and glutamic acid and low concentrations of histidine and methionine (Banno *et al*, 1984, Lyerly *et al*, 1986a,b). Both toxins appear to be acidic molecules with pI values reported as 5.31 for toxin A (Sauerborn and von Eichel-Streiber, 1990) and 4.24 for toxin B (Barroso *et al*, 1990).

Toxins A and B are susceptible to a variety of proteases and extremes of pH and temperature, with toxin B being more susceptible and less stable than toxin A (Lyerly *et al*, 1986a, Banno *et al*, 1984, Sullivan *et al*, 1982, Taylor *et al*, 1981). Proteases shown to inactivate both toxins include amylase, pronase and chymotrypsin (Rolfe and Finegold, 1979) whilst trypsin inactivates only toxin B (Rolfe and Finegold, 1979, Lyerly *et al*, 1989a, Torres and Lonnroth, 1988b). Both toxins are inactivated by oxidising agents such as H_2O_2 and O_2 but can be protected by the addition of a reducing agent such as dithiothreitol. The toxins are not sensitive to reducing agents or sulphydryl-inactivating agents, suggesting that these groups are not involved in binding or enzymatic action of the toxins (Lyerly *et al*, 1986a).

1.7.1 Immunochemical properties

Studies have shown that toxins A and B are immunologically distinct molecules which do not cross react significantly in immunoassays (Lyerly *et al*, 1983, Laughan *et al*, 1984). Polyclonal toxin A antibodies do not neutralise cytotoxic activity of toxin B and toxin B polyclonal antibodies do not neutralise cytotoxic or enterotoxic activity of toxin A (Libby

and Wilkins, 1982, Taylor *et al*, 1981, Banno *et al*, 1984). Interestingly, the *C. sordellii* HT cross reacts antigenically and is highly related genetically and biologically to toxin A. Similarly, the *C. sordelli* LT is similar in biological activity and antigenic structure to toxin B (Martinez and Wilkins, 1992). Although highly related neither toxin A nor B cross-react antigenically with *C. novyi* α-toxin (Hofmann *et al*, 1995). Toxin A has, however, been shown to be antigenically cross-reactive with the glucan binding protein from *Streptococcus mutans* (Wren *et al*, 1991).

Some mouse monoclonal antibodies (Mabs) have been isolated that cross-react with both toxins A and B (Lyerly *et al*, 1986b, von Eichel-Streiber *et al*, 1987, Rothman *et al*, 1988) initially suggesting that the toxins share some immunodeterminants. It was later discovered that these cross-reacting antibodies were able to bind to the toxins in a non-immune fashion with non-specific binding being greater to toxin A than B (Lyerly *et al*, 1989b). Several specific Mabs have also been raised to toxin A but only two of these appear to block any biological activity of the toxin. The first, PCG-4 (Lyerly *et al*, 1985b) neutralises the enterotoxic and haemagglutinating activities of toxin A but not the cytotoxic activity. As the PCG-4 Mab precipitates toxin A, it was concluded that it was recognising a repeating unit in the molecule (Lyerly *et al*, 1986b). It was later confirmed that this antibody recognises epitopes in amino acid residues 2,097 through 2,141 and 2,355 through 2,398, both of which are within the C-terminal repeat region (Frey and Wilkins, 1992). As the PCG-4 Mab blocks toxin A induced haemagglutination of rabbit red blood cells, it was concluded that enterotoxicity was prevented by blocking binding of the toxin to its receptor on the target cell (Frey and Wilkins, 1992). The failure of the antibody to neutralise cytotoxicity suggests that the enterotoxic and cytotoxic activities of toxin A are distinct (Lyerly *et al*, 1986b).

In contrast, the second Mab 37B5 (Kamiya *et al*, 1991) was shown to neutralise the enterotoxic activity of toxin A but not the haemagglutinating or cytotoxic activities. It was concluded that the antibody was blocking an active site for enterotoxicity not associated with receptor binding (Kamiya *et al*, 1991). The binding site for this antibody in the toxin A molecule has not yet been identified.

In toxin A the C-terminal region is immunodominant (Frey and Wilkins, 1992). Pre-treatment with PCG-4 reduces the binding of polyclonal toxin A antiserum to native toxin A by about 75%, suggesting that the PCG-4 binding sites are particularly immunodominant (Lyerly and Wilkins, 1995). In contrast, immunodominant epitopes are located along the entire length of the toxin B molecule (Sauerborn *et al*, 1994). Some of these epitopes in the C-terminal repeat region also elicit protective antibodies (Torres and Monath, 1996).

1.7.2 Biological activity

Although toxins A and B are extremely homologous at the amino acid level, they differ in their biological activities. Unlike toxin B, toxin A is referred to as a potent enterotoxin (Mitchell *et al*, 1986) because in the rabbit ligated intestinal loop model (Rifkin *et al*, 1978a) toxin A causes the accumulation of proteinaceous, haemorrhagic fluid (Banno *et al*, 1984). Toxin A differs from classically studied enterotoxins (e.g. cholera toxin, which produces a clear rice-water fluid in the loop assay and so is often referred to as a 'histotoxin') by causing extensive tissue damage which results in an increased permeability of the gut mucosa and eventual vessel leakage (Mitchell *et al*, 1986, Lima *et al*, 1988). Toxin B, however, appears to lack enterotoxic activity in rabbit intestinal loops but it does cause some haemorrhaging.

A study has shown that when toxin A was given intragastrically to hamsters it caused haemorrhagic fluid secretions, severe inflammation and death, whereas intragastric administration of toxin B had no effect on the hamster caecum or small intestine. When toxin B was administered with sub-lethal amounts of toxin A, however, the animals died (Lyerly *et al*, 1985a). This suggests that toxins A and B act synergistically with the tissue damage caused by toxin A facilitating action of toxin B. The inability of toxin B to affect intestinal cells may be due to the lack of a receptor for toxin B on these cell types.

The effects of toxins A and B on human colonic epithelium in Ussing chambers has also been studied (Riegler *et al*, 1995). The results of this *in vitro* assay suggested that the human colon is approximately ten times more sensitive to the damaging effects of toxin B than A. Although this is the first observation of toxin B having activity on undisturbed intestinal mucosa, it is possible that manipulation during surgery may have damaged the tissue enabling toxin B access to the deeper layers of the colonic mucosa. Both toxins A and B are lethal when injected intraperitoneally or subcutaneously in mice, hamsters, rats and rhesus monkeys (Arnon *et al*, 1984, Banno *et al*, 1984, Lyerly *et al*, 1986a, Sullivan *et al*, 1982, Taylor *et al*, 1981).

Toxin B is generally 1000 fold more cytotoxic than toxin A for most cell lines (Sullivan *et al* 1982). Results have indicated that cell lines which express high levels of the trisaccharide residue Galα1-3Galβ1-4 GlcNAc are 100 to 1000 times more sensitive to toxin A than other cell lines (Tucker and Wilkins, 1989). The morphological effect elicited by both toxins has been termed 'actinomorphic' (Chang *et al*, 1978) and is characterised by cell rounding (Chang *et al*, 1979, Donta *et al*, 1982). Approximately 3-200 ng/ml of toxin A is required to cause complete cell rounding as opposed to only 1.5-5 pg/ml of toxin B (McFarland, 1995). A variety of mammalian cells are affected by toxins

A and B (Pothoulakis *et al*, 1986, Donta *et al*, 1982, Wedel *et al*, 1983, Fiorentini and Thelestam, 1991, Sullivan *et al*, 1982, Borriello and Welch, 1984) suggesting that the receptors involved in the intoxication process are ubiquitous.

It is assumed that the toxins affect the microfilament system of the cell because early investigators observed disorganisation of the actin filaments in toxin infected cells (Pothoulakis *et al*, 1986, Thelestam and Bronnegard, 1980). Both toxins have been shown to cause accumulation of F-actin and α-actin in an irregularly organised form opposite to the nucleus of the cell. Other components such as filamen and vinculin also showed abnormal distribution. The effect on the cytoskeleton causes the cell to retract and round, accompanied by marginalisation of the nucleus which is localised at one pole of the cell (Ahlgren *et al*, 1983, Fiorentini *et al*, 1990, Aktories and Just, 1995). Further data has shown that toxin A causes surface blebbing with apoptosis-like cell death and that these changes are achieved without any measurable effect on the cellular calcium homeostasis (Fiorentini *et al*, 1993). Due to the change in F-actin organisation, the toxins were suspected to affect Rho proteins, the small GTPase proteins involved in regulation of the microfilament system. It has been shown that toxins A and B monoglucosylate Rho proteins using UDP-glucose as a substrate (Dillon *et al*, 1995, Just *et al*, 1995b,c). This modification inactivates Rho and its ability to induce polymerisation of actin filaments therefore causing cell retraction.

Toxin A is also described as a lectin due to its ability to haemagglutinate rabbit red blood cells (rRBCs) via the high numbers of branched Galα1-3Galβ1-4 GlcNAc trisaccharide residues present on the surface of the erythrocyte (Krivan *et al*, 1986). This activity is greater at 4°C than at 37°C. Sequencing of the toxin A gene revealed a series of repeats at the carboxy-terminus of the molecule (Dove *et al*, 1990) which are thought to be

responsible for the lectin properties of toxin A (Price *et al*, 1987). Bovine thyroglobulin also contains high numbers of the Galα1-3Galβ1-4 GlcNAc residues and has been shown to bind toxin A at 4°C (Krivan *et al*, 1986). Thermal thyroglobulin affinity chromatography (Krivan and Wilkins, 1987, Kamiya *et al*, 1988), followed by anion-exchange and fast protein liquid chromatography (Kamiya *et al*, 1989) is now effectively used to purify toxin A to homogeneity. Toxin B lacks the ability to agglutinate rRBCs or bind bovine thyroglobulin.

1.7.3 Toxin receptors

The repeat region at the carboxy terminus of toxin A has long been identified as the receptor binding portion of the molecule (Price *et al*, 1987, Dove *et al*, 1990). Binding of toxin A to rabbit erythrocytes and hamster brush border membranes at 4°C, and to a lesser extent at physiological temperatures, led to the identification of the trisaccharide residue Galα1-3Galβ1-4 GlcNAc as a possible receptor for toxin A (Krivan *et al*, 1986). Many observations support this theory. Firstly, it was shown that pretreatment of hamster brush borders with α-galactosidase (which cleaves terminal α-galactose residues), or with the lectin from *Bandeirea simplificolia*, reduces binding to toxin A (Krivan *et al*, 1986). Secondly, toxin A is 1000 times more cytotoxic for the mouse carcinoma F9 cell line which expresses high levels of the Galα1-3Galβ1-4 GlcNAc trisaccharide on its cell surface than other cell lines (Tucker and Wilkins, 1989). Finally, a further study has shown that a human anti-galactose antibody (which recognises Galα1-3Galβ1-4 GlcNAc) not only inhibits binding of toxin A to its receptor in the rat colon, but also mimics the effects of the toxin by causing fluid secretion and increased vascular permeability (Pothoulakis *et al*, 1996a). An earlier study reported that the membrane receptor for toxin

A in rabbit ileum is a galactose and N-acetyl glucosamine containing glycoprotein that appears to be coupled to a G-protein. The report concluded that binding of toxin A to its receptor stimulates a G-protein dependant signal transduction pathway which activates the cell prior to internalisation of the toxin or expression of the enzymatic activity (Pothoulakis *et al*, 1991).

More recently, the rabbit small intestine brush border membrane enzyme, sucrase/isomaltase, has been identified as a receptor for toxin A (Pothoulakis *et al*, 1996b). Chinese hamster ovary cells transfected with sucrase/isomaltase cDNA showed increased intracellular calcium release in response to both holotoxin and recombinant repeat end peptide. Anti- sucrase/isomaltase IgG also inhibited toxin A induced secretion in rabbit ileum suggesting that binding alone stimulates signal transduction pathways across the membrane (Pothoulakis *et al*, 1996b).

Although the Galα1-3Galβ1-4 GlcNAc trisaccharide appears to be a receptor for toxin A in hamsters, it cannot be the receptor in the human intestine because the α-galactosyltransferase required for its formation is absent in humans (Thall *et al*, Galili *et al*, 1988). Current work on the toxins has therefore concentrated on identifying a receptor for toxin A in human intestinal epithelium. Three additional blood group carbohydrate antigens, designated Lewis X, Y and I, were identified as possible intestinal epithelial cell receptors for toxin A (Tucker and Wilkins, 1991). Their data indicates that toxin A binds the X and I antigens only at 4°C, but binds the Y antigen with greater affinity at both 4°C and 37°C. A common feature of these three carbohydrates is that they contain a Galβ1-4 GlcNAc residue (type 2 core) with a branch either on, or adjacent to, the core. It has been suggested that this branch holds the carbohydrate in a conformation that favours binding to toxin A (Tucker and Wilkins, 1991). It has been shown that N-acetylglucosamine

inhibits binding of toxin A to the Lewis Y antigen suggesting that the disaccharide Galβ1-4 GlcNAc is the minimum structure required for binding to toxin A (Tucker and Wilkins, 1991). Interestingly, human neonates appear to be resistant to toxin A damage although they express the Y antigen on their intestinal epithelium (Kapadia *et al*, 1981, Karlsson and Larson, 1981). A possible explanation is that the receptor coupled G-protein system has not been fully developed in the neonates.

The direct binding of toxin A to human intestinal epithelial cells has been demonstrated recently (Smith *et al*, 1997). Pre-treatment of the cells with β-galactosidase (which cleaves terminal β-galactose residues) reduced toxin A binding, supporting the idea that the Lewis X, Y and I antigens may be receptors for toxin A on human intestinal epithelial cells. As α-galactosidase treatment also reduced toxin A binding, however, it has been suggested that more than one receptor type may be involved (Smith *et al*, 1997).

Cell receptors for toxin B have not yet been fully characterised. Recent results however, have shown that oligosaccharides containing sialic acid and n-acetyl glucosamine mediate toxin B binding and biological effects in human colonic mucosa (Castagliuolo *et al* 1998a). The toxin receptor(s) involved in the cytotoxic activity are likely to be fairly ubiquitous molecules because both toxins are active against a wide variety of cell types.

1.7.4 Molecular characterisation

Prior to sequencing of the toxin A and B genes, several investigators isolated fragments of the toxins from *C. difficile* genomic libraries using polyclonal anti-toxin sera. A 0.3kb DNA fragment of the toxin A gene was isolated and found to hybridise to a 4.5 kb *Pst*I generated fragment of *C. difficile* DNA (Muldrow *et al*, 1987) An immunoreactive toxin A peptide coded for by a 4.7kb *Pst*I digested DNA fragment was also isolated from a

genomic library (Price *et al*, 1987). This DNA fragment was believed to code for a non-toxic binding portion of toxin A because it haemagglutinated rabbit erythrocytes but lacked cytotoxic and enterotoxic activity (Price *et al*, 1987). Further, a 14.3Kb DNA fragment was isolated from a λEMBL3 *C. difficile* genomic library using polyclonal toxin A antiserum. This fragment encoded a 235 kDa protein that haemagglutinated rabbit erythrocytes and caused a cytotoxic effect on tissue culture cells, properties which are characteristic of native toxin A (Wren *et al* 1987).

DNA sequencing has revealed that the coding region of the toxin A gene is 8.133Kb in length (Dove *et al*, 1990, Sauerborn and von Eichel Streiber, 1990). This encodes a single chain protein of 308 kDa (2, 710 amino acids). One third of the gene (2, 499bp) at the 3' end, consists of 38 contiguous repeating units which have been classified into two main groups dependant on length and sequence similarities (Dove *et al*, 1990). The 3' end of the toxin B gene lies 1,350bp upstream of the toxin A translational start with the two genes being separated by a small ORF encoding a 16-19 kDa polypeptide (Dove *et al*, 1990). The coding region of the toxin B gene was shown to be 7.098 kb in length encoding a single chain protein of 269.696 kDa (2,366 amino acids) (Barroso *et al* 1990, Johnson *et al*, 1990). Toxin B also possesses repeating units at the carboxyl terminus but it is unclear whether these function as the binding portion of the molecule.

Other proteins that cross-react antigenically with toxin A have been reported to contain C-terminal repeat sequences including four glycosyltransferases and a glucan binding protein from *Streptococcus mutans* (Wren, 1991, Wren *et al*, 1991, von Eichel-Streiber and Sauerborn, 1990).

The amino acid sequences of the toxin A and B genes show 49% identity and 63% similarity, suggesting that the toxins arose by duplication. The greatest homology (64%)

appears between the N-terminal two-thirds of the molecules (von Eichel-Streiber *et al*, 1992). *C. difficile* toxin B has also been shown to be 75.7% identical and 90.3% similar to the *C. sordellii* LT at the amino acid level. Similar homologies are expected between *C. sordellii* HT and *C. difficile* toxin A (Green *et al*, 1995). *C. difficile* toxins A and B, *C. sordellii* LT and HT, and the *C. novyi* α-toxin, all contain repeat sequences at the C-terminal end of their molecules. These toxins have been classified as large clostridial cytotoxins (LCT's) (Bette *et al*, 1991) with reference to their high molecular weights, their close structural and functional relationships and their similar *in vivo* and *in vitro* biological activities. Based on acquired knowledge and sequence similarities, a structural and functional domain map was postulated for both toxins (Barrosso *et al*, 1994) (see figure 1.1). Although no direct evidence was presented, the N-terminus of the toxins was believed to encode the cytotoxic activity. A 50 amino acid hydrophobic region near the centre of the toxin was tentatively assumed to be involved in membrane translocation while the repetitive C-terminal domain was known to be involved in binding to glycoconjugates (Barroso *et al*, 1994). The development of a series of toxin B mutants also highlighted the importance of four conserved cysteine residues (Cys_{597}, Cys_{700}, Cys_{1169}, Cys_{1623}) and a putative nucleotide binding site (aa's 651-683) for full expression of biological activity (Barroso *et al*, 1994).

A sequence comparison between toxin B and a variant form (ToxB-1470), which is far less potent to endothelial cells, revealed that the majority of the changes in the variant sequence resided between amino acids 1-868 (von Eichel-Streiber *et al*, 1995). This provides further evidence to suggest that the N-terminal third of the toxin B gene encodes the cytotoxic activity.

Using PCR, the *C. difficile* toxigenic element was found to be chromosomal, 19.6Kb in length, and comprising of five ORFs including the toxin A and B genes. Non-toxigenic strains were shown to lack this entire element but possess a unique 127 bp DNA fragment in its place (Hammond and Johnson, 1995). The toxigenic element has also been described as a pathogenicity locus (PaLoc) because its very existence is a prerequisite for the pathogenicity of a strain (Braun *et al*, 1996). The locus is a distinct genetic element integrated at a single site (running in one direction) in the *C. difficile* genome. The five ORFs were named TcdA- TcdE inclusive, with TcdA and TcdB being the original toxin A and B genes. TcdA, TcdB, TcdD and TcdE were shown to be transcribed in the same direction whilst TcdC is transcribed in the opposite direction (see figure 1.2) (Braun *et al*, 1996).

Transcriptional analysis of the TcdA- TcdE genes showed that they are all transcribed (Hundsberger *et al*, 1997). Interestingly, in the early exponential phase, high levels of TcdC and low levels of TcdA, B, D and E transcripts were detected, but in the stationary phase this was reversed. It has been suggested that TcdC may have a negative effect on transcription of the other genes (Hundsberger *et al*, 1997). TcdD contains a helix-turn-helix motif typical of DNA binding proteins (Hundsberger *et al*, 1997) and is believed to be a positive regulator that activates expression of *C.difficile* toxins A and B (Moncrief *et al*, 1997). Eight additional ORFs have been identified outside of the PaLoc, which also appear to be present in non-toxigenic strains (Braun *et al*, 1996) (see figure 1.2). The function of these small ORFs still remains unclear.

1.7.5 Functions of toxins A and B

1.7.5.1 Phosphate and nucleoside binding

Studies have indicated that toxin B has a phosphate binding site(s) which, when occupied, delays cytotoxicity but does not prevent it. Toxin B also binds nucleoside triphosphates, nuclear, di-, tri-, and tetraphosphates, inorganic polyphosphates and polyphosphorylated sugars (Florin and Thelestam, 1984). The polyphosphate-binding site in toxin B shares characteristics with the site in the diptheria toxin. Both bind all nucleoside triphosphates as well as di-and tetrphosphates, but bind ATP with a greater affinity than other nucleotides. Also, binding of ATP to both molecules caused a dose dependant inhibition of the cytopathic effect in the 1-5mM range (Florin and Thelestam, 1984). In one study, approximately 7-20% of a toxin B preparation was shown to bind ATP (Florin and Thelestam, 1984). ATP was protective to cells even upon addition 10 minutes after the toxin B binding step, suggesting that the ATP binding site and the receptor binding site are separate in this molecule. It has been suggested that the ATP binding site may be involved in the interaction of toxin B with the cell shortly after the binding step (Florin and Thelestam, 1984). A putative ATP binding site has been located in toxin B between amino acids 651-683 and shows high levels of homology with conserved ATP binding sites (Barroso *et al*, 1994).

More recently, the change in intrinsic fluorescence of tryptophan residues has been used to demonstrate that toxin A also binds nucleotides and tetraphosphates. The binding affinity was greatest for tetraphosphates, adenosine triphosphate then guanosine triphosphate respectively (Lobban and Borriello, 1992). Both toxins A and B bind to ATP-agarose columns although a greater proportion of toxin A (35%) than toxin B (20%) appeared to be retained on the column (Lobban and Borriello, 1992, Florin and Thelestam, 1984). The phosphate-binding site in toxin A appears to be analogous to the

'P' site of fragment B of diptheria toxin (Lory and Collier, 1980, Boquet and Duflot, 1981).

Toxin A also binds to NAD^+ suggesting that it contains a nucleoside-binding site (Lobban and Borriello, 1992). In diptheria toxin, the phosphate and nucleoside binding sites are distinct but appear to come together in the tertiary structure of the molecule to contribute to the nucleotide-binding site (Boquet and Duflot, 1981). It is not known if this is also true of toxin A or if toxin B has a nucleoside-binding site. The function of the phosphate binding site and, for toxin A at least the nucleoside binding site, are unknown for both toxins A and B.

1.7.5.2 Interaction with nucleic acid

Several investigators have reported that both DNA and RNA co-purify with toxin B but not toxin A (Meador and Tweten, 1988, Bisseret *et al*, 1989, Borriello, 1991). The DNA fragments recovered ranged from 20MDa (Meador and Tweten, 1988) to 20 nucleotides (Bisseret *et al*, 1989). Contradictory observations were made by these investigators. The first authors found that the presence of nucleic acid decreased toxin B induced cytotoxicity, while the latter implied that removal of the nucleic acids with DNAase had a detrimental effect on toxin B induced cytotoxicity. Further investigation (in agreement with Bisseret and co-workers) found that treatment of toxin B with DNAase reduced cytotoxic activity but treatment with RNAase had no effect (Borriello *et al*, 1991). However, it was also shown that the effect of the DNAase was due to contamination with proteases (Borriello *et al*, 1991), DNAase treated with protease inhibitors no longer caused a reduction in toxin B cytotoxicity. Purification of the DNAase showed the presence of a protease which was active against toxin B but not toxin A, and which was

sensitive to the effects of the serine protease inhibitor PMSF. It would appear, therefore, that the reduced cytotoxic activity observed by Bisseret and co-workers was due to a contaminating serine protease.

The relevance of the interaction of toxin B with nucleic acid, if any, is unknown. It is unlikely that toxin B binds directly to host cell DNA switching off an essential gene involved in cytoskeletal control, as Vero DNA does not inhibit toxin B induced cytotoxicity for Vero cells (Borriello, 1991).

1.7.5.3 Stimulation of increased inositol triphosphate (IP3) production

Binding of toxins A and B to cells has been shown to subvert host phosphatidyl inositol signal transduction mechanisms. A study revealed that the toxins caused a net stimulation of increased IP3 production of 25.5% in duodenal membrane preparations (Smith *et al*, 1995). Incubation of toxin A with membrane preparations for 10 minutes at 37°C increased the accumulation of IP3 by 42%, whereas incubation of toxin B in the same conditions reduced the accumulation of IP3 by 16.5%. The stimulation of IP3 was greater at 4°C than 37°C, which is consistent with the known thermal binding characteristics of toxin A to its receptor (Krivan *et al*, 1986, Smith *et al*, 1995). Stimulation of IP3 production may be involved in the toxin A induced secretory response.

1.7.5.4 Carbohydrate binding properties

The observation that toxin A haemagglutinates rabbit erythrocytes at 4°C led to the identification of a trisaccharide residue Galα1–3Galβ1-4GlcNAc, as a receptor for toxin A. This trisaccharide is present on hamster brush border membranes, bovine thyroglobulin and rabbit erythrocytes (Krivan *et al*, 1986, Clark *et al*, 1987), but is not

present on human cells. Human tissues, however, express the Galβ1-4GlcNAc structure, e.g. Lewis X, Y, and I blood group antigens, which also bind toxin A (Tucker and Wilkins, 1991). The Galβ1-4GlcNAc disaccharide appears to be the minimum structure required for binding, and probably forms a receptor for toxin A in human intestinal tissue. The toxin B receptor is also believed to be carbohydrate based but no particular structure has yet been identified (Thelestam and Bronnegard, 1980).

1.7.5.5 Non-specific binding to monoclonal antibodies (Mabs)

Both toxins A and B bind non-specifically to many murine Mabs raised against antigens other than toxin A, although this is a feature more of toxin A than B (Lyerly *et al*, 1989b). None of the cross-reacting Mabs neutralised biological activity of either toxin leading investigators to conclude that the antibody binding does not occur via a true immune response (Lyerly *et al*, 1989b). Toxin A also appears to bind to human paraproteins in a manner believed to be non-specific (Borriello, 1991).

Many other bacterial proteins have been shown to bind non-immunologically to antibodies e.g. Protein A from *Staphylococcus aureus* (Forsgren and Sjoquest, 1966). These proteins, along with several others from gram-negative organisms, appear to bind non-specifically to the Fc domain of the antibody molecule (Forsgren and Grubb, 1979, Labbe and Grenier, 1995, Yarnall *et al*, 1988). Recently, however, it has been shown that non-specific binding of *C. difficile* toxin A to an IgG3λ murine Mab was mediated through the Fab domain of the immunoglobulin (Cooke and Borriello, 1998). This represents the first observation of a bacterial antigen binding to the Fab component of antibodies.

Pre-treatment of the Fab fragment with α-galactosidase reduced binding to toxin A suggesting that the non-specific binding was mediated through a carbohydrate moiety on the Fab domain (Cooke and Borriello, 1998). The Lewis X antigen, also present on the secretory component of immunoglobulins, is unlikely to be involved in the non-specific interaction because it was unable to compete with the antibody for binding to toxin A (Cooke and Borriello, 1998). The region of the toxin A molecule responsible for binding to the Fab region of Mabs has not yet been determined. It has been suggested that the ability of toxin A to bind to carbohydrate residues on the Fab fragment of secretory IgA antibodies may promote interaction with mucus (Cooke and Borriello, 1998).

1.7.5.6 Covalent modification of Rho proteins

Several clostridial species produce toxins that disrupt the actin cytoskeleton by ADP-ribosylation of either actin, such as the C2-toxin of *C. botulinum* (Aktories *et al*, 1986), or the small GTPase Rho, such as *C. botulinum* exoenzyme C3 (Aktories *et al*, 1987, Chardin *et al*, 1989). Early observations showed that micro-injection of *C. difficile* toxins A and B into cells caused disruption of the actin cytoskeleton suggesting that the target for the toxins was cytosolic (Meuller *et al*, 1992). Due to changes in F-actin organisation induced by *C. difficile* toxins A and B, Rho proteins were also suspected to be cellular targets for these proteins. For many years, however, investigators failed to demonstrate that the *C. difficile* toxins possessed ADP-ribosyltransferase activity (Florin and Thelestam, 1991, Popoff *et al*, 1988).

Finally it was shown that despite their different *in vivo* biological activities, both toxins A (Just *et al*, 1995a) and B (Just *et al*, 1994) covalently modify Rho proteins in such a way that subsequent ADP-ribosylation by exoenzyme C3 was prevented. Also shown was that

the modification was dependent on an unknown heat stable cytosolic factor with a molecular mass between 500 and 3,000 Da (Just *et al*, 1995a). Further investigation revealed that the molecular mass of toxin A modified recombinant Rho was 162 Da higher than unmodified Rho. As this difference corresponded to the molecular mass of a hexose, but the estimated size of the required cytosolic factor was >500 Da, the authors tested a number of sugar nucleotides for their ability to act as a co-factor for toxin A (Just *et al*, 1995b). It was finally shown that toxins A and B monoglucosylate Rho at the amino acid position Thr-37 using UDP-glucose as the substrate (Just *et al*, 1995b and 1995c). It was further shown that the three subtypes of small G-proteins within the Rho family (Rho A, Rac1 and Cdc 42) were all substrates for the toxins but that other subtypes of the Ras superfamily (H-Ras, Rab 5 and Arf 1) were not (Just *et al*, 1995b).

The observation that a mutant cell line, which produces low levels of UDP-Glucose was resistant to both *C. difficile* toxin B and to *C. sordellii* LT suggested that both toxins require UDP-glucose as a co-factor (Chaves-Olarte *et al*, 1996). It was later confirmed that the *C. sordellii* LT is also a glucosyltransferase which uses UDP-glucose as a co-substrate, but that this toxin modifies Rac and Ras proteins but not Rho or Cdc 42 proteins (Just *et al*, 1996). The *C. novyi* α-toxin, however, is an N-acetyl-glucosaminyltransferase that utilises UDP-N-acetyl-glucosamine as a co-substrate to modify Rho subfamily proteins (Rho, Rac, Cdc 42, RhoG) (Selzer *et al*, 1996). All of the large clostridial cytotoxins appear to modify the threonine in the effector region (Thr-37 in Rho, Thr-35 in Rac) of the GTP-binding protein (Just *et al*, 1995c, 1996, Selzer *et al*, 1996). The interaction of *C. difficile* toxins A and B with Rho proteins will be discussed in more detail in Section 6.

The Rho GTPases form a subgroup of the Ras superfamily of low molecular mass GTP-binding proteins comprising RhoA, RhoB, RhoC, Rac1, Rac2, Cdc 42 and RhoG. They are primarily involved in the organisation of the actin cytoskeleton but have also been associated with protein phosphorylation, phospholipid synthesis, formation of protein-protein complexes and regulation of transcription factors (Machesky and Hall, 1996). Rho has been shown to control the formation of stress fibres and focal adhesins as well as being involved in growth regulation. Rac is involved in lamellipodia ruffling and Cdc 42 induces filapodia (Machesky and Hall, 1996).

Cellular actin rapidly turns over between the monomeric (G-actin) and the polymeric (F-actin) forms. The turnover rate is controlled by two classes of actin-binding proteins called sequestering proteins and capping proteins (Symons, 1996). Sequestering proteins bind to G-actin and inhibit actin polymerisation whereas capping proteins inhibit polymerisation by binding to the barbed (fast polymerising) ends of filaments. Activated Rho GTPases are believed to be involved in the mechanism of filament uncapping thus allowing further actin polymerisation (Hartwig *et al*, 1995). Rho GTPases function as molecular switches shuttling between the active GTP-bound state and the inactive GDP-bound state. The cycling between the two states of Rho is controlled by three additional factors, GTPase activating proteins, GTPase exchange factors and GDP dissociation inhibitors (Symons, 1996).

Figure 1.3 illustrates the transition between the active and inactive states of Rho and the conformational changes that allow modification by *C. difficile* toxins. Thr-$_{37}$ is part of the switch region involved in the transition between the active and inactive states of the GTP-binding proteins. In the inactive state the OH groups of Thr-$_{37}$ of Rho is made accessible for modification by *C. difficile* toxins A and B (von Eichel-Streiber *et al*, 1996). The

toxins glucosylate Rho at Thr-$_{37}$, disrupting the normal switch mechanism rendering the molecule permanently inactive. This leads to a breakdown of the actin filament network of cells. A consequence of the depolymerisation of F-actin to G-actin is that the tight junctions between cells is destroyed (von Eichel-Streiber *et al*, 1996).

Toxins A and B have become powerful tools for delineating the signal transduction pathways mediated by the small GTP-binding proteins. They have been used to study Rho protein-dependent phospholipase D (PLD) activity in human embryonic kidney cells (Schmidt *et al*, 1996) and in human promyelocytic leukaemia HL60 cells (Ohguchi *et al*, 1996). Inactivation of Rho by toxins A and B caused a marked inhibition of membrane associated PLD activity. PLD hydrolyses membrane phospholipids but has also been shown to act as a mediator of actin reorganisation downstream of Rho proteins (Steed *et al*, 1996). Blockage of PLD activation may, therefore, lead to cytoskeletal rearrangements. It has been suggested that *C. difficile* toxins may mediate their cytotoxic and enterotoxic effects, in part, by inhibiting the Rho-PLD pathway (Ohguchi *et al*, 1996).

1.7.5.7 Interaction with cells of the immune system

Toxins A and B are potent activators of human monocytes, with toxin A also being a potent chemoattractant of granulocytes (Pothoulakis *et al*, 1988, Flegel *et al*, 1991, Linevsky *et al*, 1997). Toxin A has a direct effect on mast cells, stimulating them to release small amounts of TNF-α. Longer exposure to toxin A however, affects the release of inflammatory mediators from mast cells, possibly due to the alteration of the cytoskeleton and induction of apoptosis (Calderon *et al*, 1998). Toxin B has been shown to stimulate monocytes to produce the inflammatory products interleukin-1 (IL-1), IL-6

and tumour necrosis factor (TNF) which contribute to epithelial damage, mucus release and membrane permeability. Toxin B is extremely toxic to monocytes and eventually eliminates them thus preventing phagocytosis of bacteria (Flegel *et al*, 1991). Toxin A causes a rapid dose dependent increase in the concentration of free cytosolic calcium in human granulocytes which stimulates a chemotactic response by granulocytes to toxin A (Pothoulakis *et al*, 1988). The increased cytosolic calcium is thought to initiate an infiltration of the intestinal lamina propria with granulocytes, which are activated to release inflammatory mediators and cause epithelial damage. Toxin A also stimulates the release of macrophage inflammatory protein-2 (MIP-2) from macrophages and intestinal epithelial cells *in vitro* (Castagliuolo *et al*, 1996). MIP-2 has chemotactic properties on polymorphonuclear cells (PMNs). By blocking the effects of MIP-2 in the rat ileal loop model with MIP-2 antiserum, investigators were able to inhibit toxin A induced inflammatory diarrhoea (Castagliuolo *et al*, 1996).

More recently came the demonstration that toxin A induces the release of neutrophil chemotactic factors, including TNFα and IL-1B, from rat peritoneal macrophages (Rocha *et al*, 1997). Toxin A stimulated macrophages have also been shown to release an additional factor capable of inducing intestinal secretions *in vitro* (Rocha *et al*, 1998). The release of this intestinal secretory factor (ISF) was significantly reduced by platelet activating factor (PAF), TNFα inhibitors, cyclo-oxygenase inhibitors and pertussis toxin. Both monoclonal anti-IL-1B and recombinant human IL-1 receptor agonist completely blocked the activity of the ISF. The investigators determined that cyclo-oxygenase products, PAF and TNFα are involved in the release of the ISF. From their data they concluded that the ISF was IL-1B and that its regulation was dependent on the activation of a G-protein (Rocha *et al*, 1998).

A recent study has shown that T84 and HT29 cells produce IL-8 in response to toxin A exposure (Mahida *et al*, 1996). In this study, toxin A induced epithelial cell rounding, detachment and apoptosis in human colonic biopsy specimens. Others have reported that IL-8 is induced in intestinal epithelial cell lines in response to cytokines such as IL-1 and TNFα (Eckmann *et al*, 1993). Loss of adherence between intestinal epithelial cells and of cells to the extracellular matrix component of the basement membrane has also been shown to induce apoptosis (Bates *et al*, 1994, Ruoslahti and Reed, 1994). It was therefore concluded that intestinal epithelial cell IL-8 production and apoptosis most likely occurred as a consequence of cell injury and detachment rather than as a direct effect of the toxin (Mahida *et al*, 1996). As IL-8 is a potent chemoattractant of PMNs, however, it is likely that its production is essential for the induction of the intestinal inflammation seen in PMC. The effect of toxin A on lamina propria T-cells, macrophages, and eosinophils was also studied (Mahida *et al*, 1998). None of these cells require adherence-dependent signalling from components of the extracellular matrix for survival and therefore any induction of apoptosis would most likely be due to a direct effect of the toxin on the cells. The results showed that toxin A induced an early rapid loss of colonic lamina propria macrophages followed by apoptotic death of T-cells and eosinophils (Mahida *et al*, 1998). Toxin A also induced the release of TNFα from lamina propria cells, however, neutralisation of this cytokine did not influence toxin induced apoptosis of T-lymphocytes. The investigators were able to demonstrate toxin A induced apoptosis of purified T-lymphocytes implying that toxin A induces cell death following a direct interaction with the cells (Mahida *et al*, 1998). Toxin A also increased the proportion of T-cells expressing the IL-2 receptor (CD25) suggesting that toxin A induces activation of T-cells prior to their death by apoptosis. These studies demonstrate that *C. difficile* toxin

A is capable of suppressing human colonic mucosal immune responses by inducing early loss of macrophages followed by T-cell apoptosis. It is possible that toxin A induces cell death by apoptosis by inactivating the cytosolic Rho GTPases (Mahida *et al*, 1998).

C. difficile toxin B has also been shown to potently activate monocytes to release IL-8 (Linevsky *et al*, 1997) and to induce apoptosis of intestinal cells (Fiorentini *et al*, 1998).

1.8 Structure-function relationships of the *C. difficile* toxins

When the research for this PhD was initiated, the only structure–function relationship that had been made for either toxin A or B was the observation that toxin A binds to carbohydrate residues via repeating sub-units at the C-terminal end of its molecule.

The trisaccharide residue Galα1-3Galβ1-4GlcNac was identified as a receptor for toxin A on the rabbit erythrocyte (Krivan *et al*, 1986). There are 38 contiguous repeating units at the C-terminal end of toxin A, which were identified as a binding site for this trisaccharide (Price *et al*, 1987). Although this trisaccharide residue is not expressed on human cells, it led to the identification of the Lewis X, Y and I antigens as possible receptors for toxin A on intestinal cells (Tucker and Wilkins, 1991). A putative ATP binding site had also been located in the toxin B molecule between amino acids 651-683 (Barroso *et al*, 1994) although no direct evidence has been provided to confirm this location.

1.9 Mechanisms of action of toxins A and B

Due to their high molecular weight and typical cytopathic action on cells in culture, toxins A and B have been classified as 'large clostridial cytotoxins' (LCTs). The LCTs

have an N-terminal catalytic domain and a C-terminal receptor binding domain composed of repeating units (von Eichel-Streiber *et al*, 1996). The advantage of encoding several binding epitopes within one chain is that it allows the toxins to interact with more than one cell receptor thus increasing the affinity of the toxins for the cells.

The C-terminal repeat regions of toxins A and B are the least homologous (von Eichel-Streiber *et al*, 1992) probably suggesting that they use different receptor structures for their cellular uptake. The fact that toxin B is not a potent enterotoxin may be explained by the lack of a receptor for the toxin on intestinal cells.

Following receptor binding, the toxins are internalised by receptor mediated endocytosis (Henriques *et al*, 1987 Florin and Thelestam, 1983) via coated pits (Kushnaryov and Sedmak, 1989). Cells pre-treated with compounds that raise the pH of endosomes and lysosomes were reported to be protected from the cytopathic effect (CPE) suggesting that the toxins require passage through an acidic compartment for activation (Henriques *et al*, 1987, Florin and Thelestam, 1983). In addition, intracellular processing of the toxin appears to be required in post endosomal compartments. The nature of the putative enzymatic processing has not yet been clarified although it has been suggested that activation of the toxins can only take place following partial unfolding of the proteins at low pH (Henriques *et al*, 1987).

Experiments have shown that LCTs are active upon microinjection into the cytosol (Meuller *et al*, 1992). Induction of the CPE by microinjection, however, requires several fold higher amounts of the toxins than intoxication by the normal route. It is reasonable to suggest that the required processing conditions for the toxins are also present in the cytosol, although the natural route via endosomal compartments appears to provide a more efficient activation of the toxins. All LCTs share a central hydrophobic region

which is believed to be involved in membrane translocation (Barroso *et al*, 1994, Hofmann *et al*, 1995, von Eichel-Streiber *et al*, 1992). Following activation, this putative transmembrane-spanning region is thought to allow the toxins access into the cytosol where they have their effect.

Once inside the cytosol the *C. difficile* toxins glucosylate GTP-binding proteins of the Ras-related Rho subfamily using UDP-glucose as a substrate (Just *et al*, 1995a,b,c). This leads to actin depolymerisation, general cytoskeletal breakdown and cell rounding. The rounding of the cells, especially at the perijunctional ring, increases tight junction permeability between the cells, allowing for the leakage of fluids and soluble components into the colonic lumen. Another hypothetical model was suggested following the observation that toxin A stimulates intracellular calcium release which also alters epithelial permeability (Pothoulakis *et al*, 1988). This model states that binding of toxin A to its intestinal cell receptor stimulates transmembrane signals that result in the release of a mediator on the basolateral side of the epithelium. This mediator, of unknown nature, is thought to activate neurons that trigger fluid secretion and immune cells prior to Rho glucosylation (Castagliuolo *et al*, 1994). In this model, toxin B is unable to stimulate these transmembrane signalling events and thus unable to trigger fluid secretion.

Following toxin A induced damage to the intestinal epithelium, both toxins access deeper cell layers of the gut where the damage continues. Interaction of toxins A and B with cells of the immune system (See Section 1.7.5.7) results in the release of inflammatory mediators which cause additional cellular damage and increased permeability of the lamina propria.

1.10 Research aims and objectives

When this research was initiated a very limited amount of information was available on the structure-function relationships of either toxin A or B. In order to control *C. difficile* associated disease, it is essential to understand the mechanism of action of these toxins and the part they play in the disease process. The aim of this PhD is to increase the understanding of the structure-function relationship of toxin A, the most important virulence factor of *C. difficile*.

It is proposed to PCR amplify, clone and express overlapping DNA fragments covering the entire toxin A molecule. The recombinant toxin A peptides will be purified then used to raise polyclonal monospecific antisera in rabbits in order to provide important information of the tertiary structure of toxin A. In addition, the peptides will be used to localise a full range of biological activities of toxin A, including glucosyltransferase activity, nucleotide binding, haemagglutination, cytotoxicity, non-specific binding to monoclonal antibodies and receptor ligand binding. The identification of regions of the enterotoxin molecule that are important for toxicity will be essential for the future development of preventative therapy.

The overlapping peptides covering the entire toxin A molecule will also prove to be an invaluable and unique tool for future research into many other aspects of *C. difficile* disease.

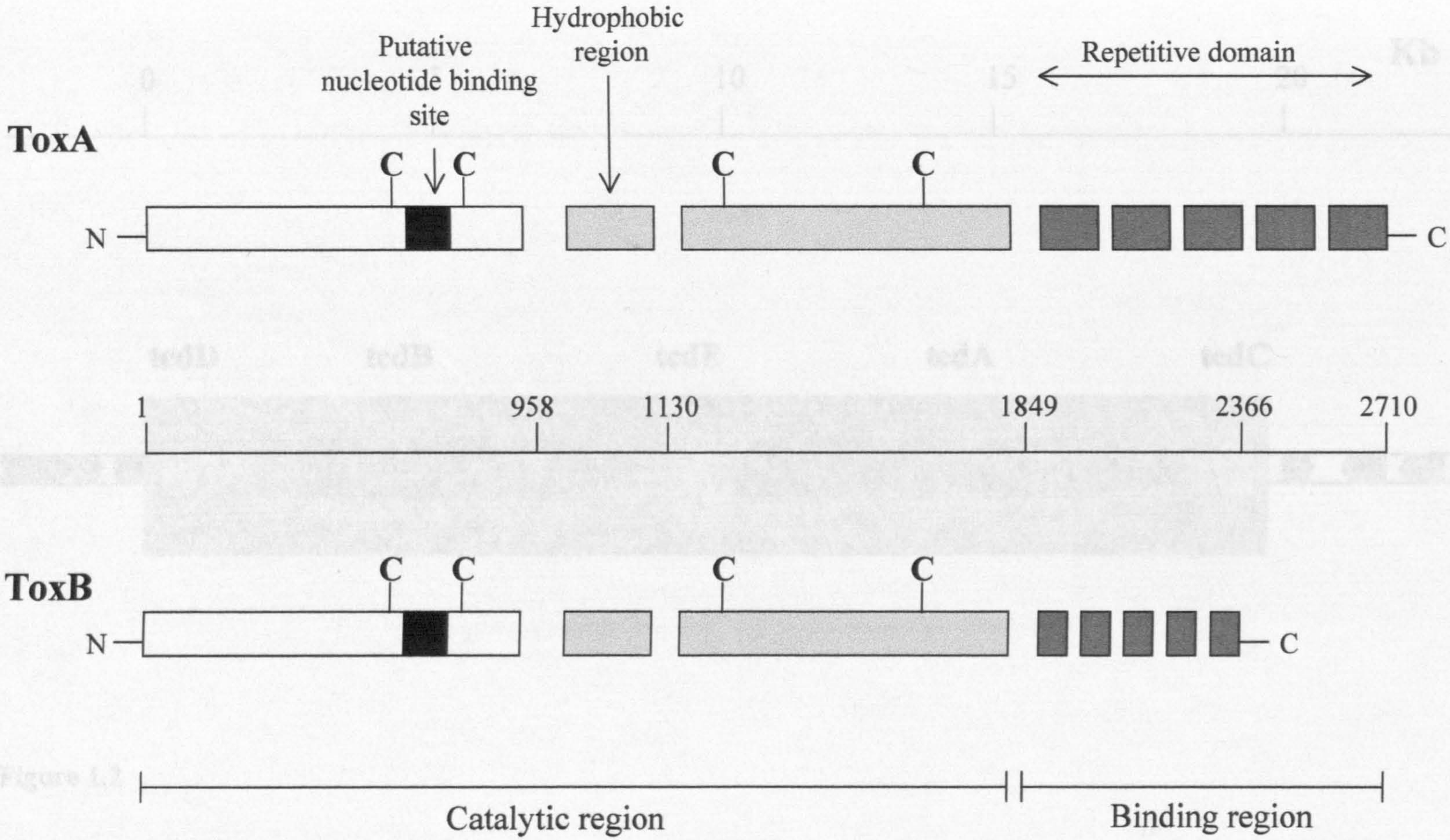


Figure 1.1

Schematic model depicting the domain structure of the *Clostridium difficile* toxins showing the conserved cysteine residues **(C)**, the putative nucleotide binding site (for toxin B) and the hydrophobic region. Taken and modified from Thelestam *et al*, 1997.

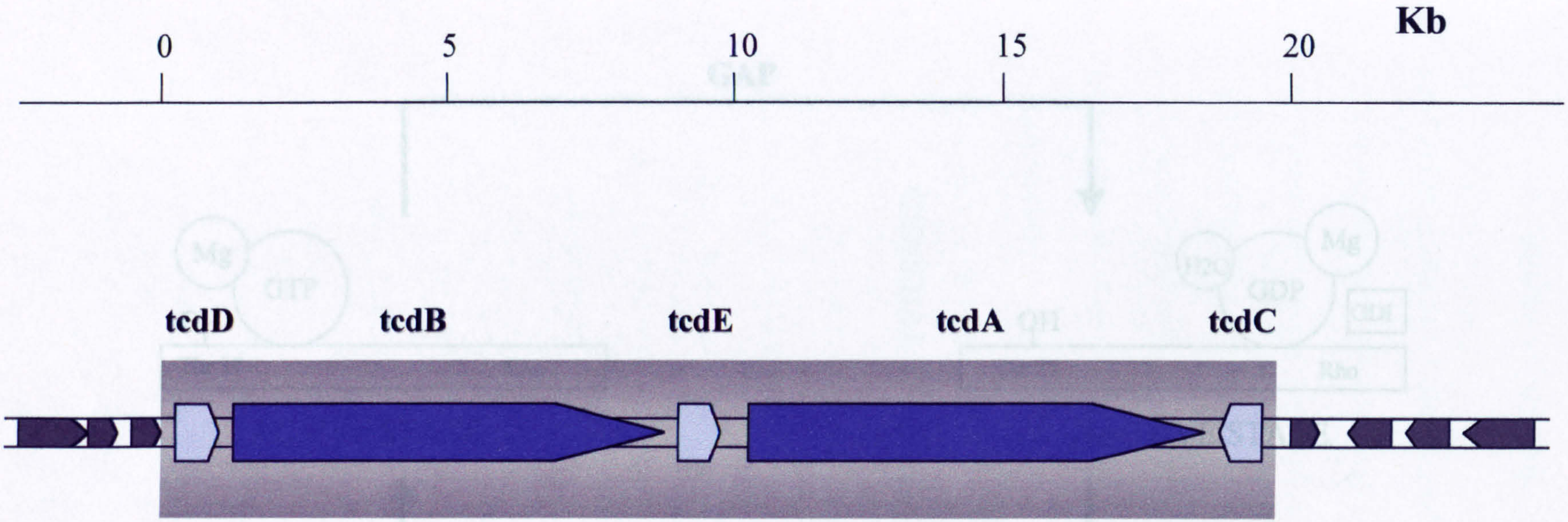


Figure 1.2

Map of the pathogenicity locus (PaLoc) of *C. difficile* VPI10463 taken and modified from Hundsberger *et al*, 1997). Filled arrows indicate open reading frames (ORFs), the direction of the arrows showing the direction of gene transcription. Parts belonging to the *C. difficile* PaLoc are underlaid in grey. The filled black arrows indicate ORFs outside of the PaLoc. These ORFs are also present in non-toxinogenic strains and their functions are not yet fully understood (Braun *et al*, 1996).

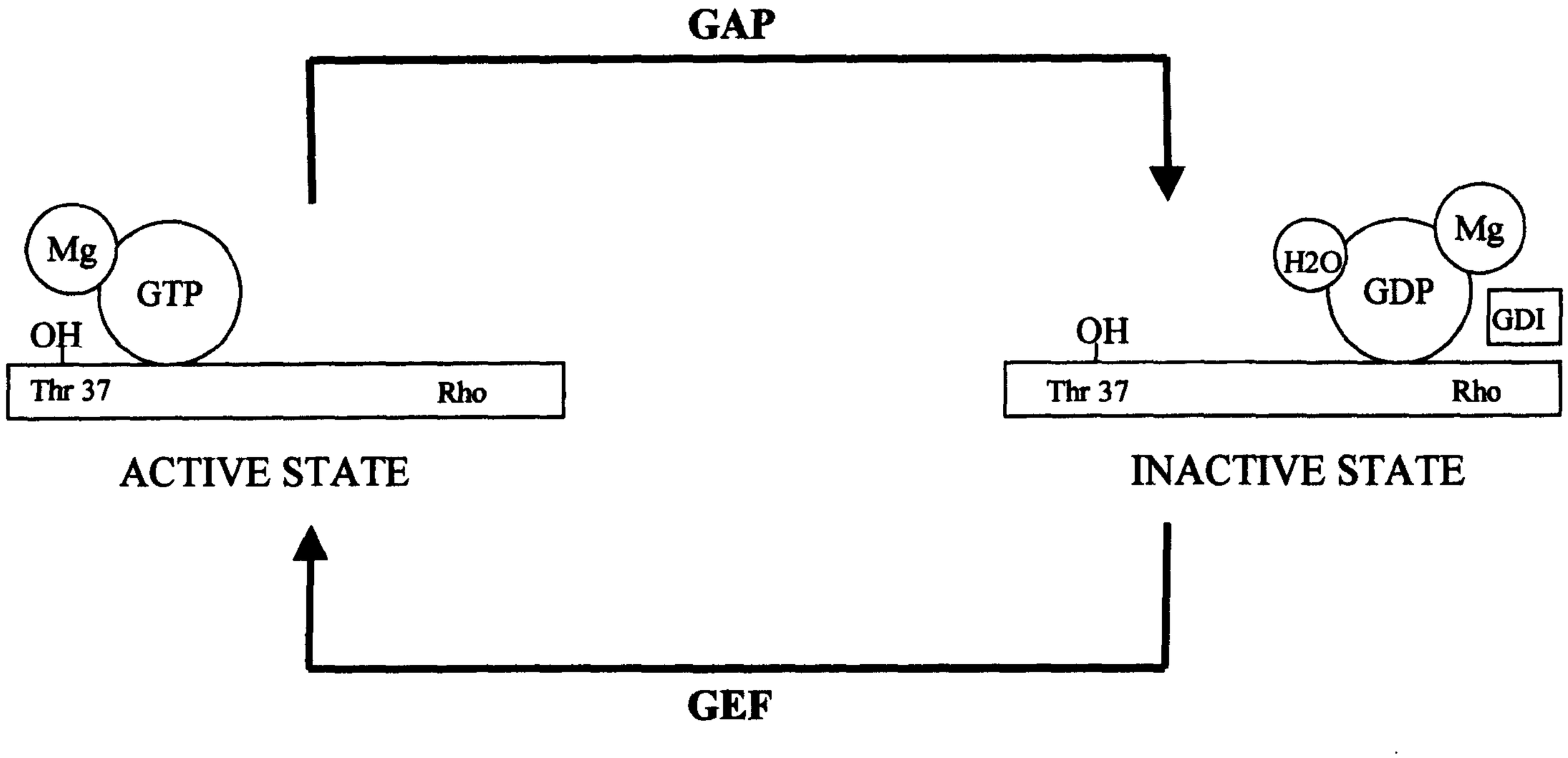


Figure 1.3

Transition between active and inactive states and conformational changes of Rho. In the active state Rho is bound to GTP. GTPase activating proteins (GAP) catalyse the hydrolysis of GTP to GDP, inactivating Rho. GDP dissociation inhibitor (GDI) confers solubility on inactive Rho. GTPase exchange factors (GEF) catalyse the GDP-GTP exchange, returning Rho to its active state. In the inactive state the OH side group of the Thr 37 is made accessible for modification by CDTxA and B. (Taken and modified from Aktories and Just, 1995 and Olson, 1996.

SECTION-2

GENERAL MATERIALS AND METHODS

SECTION – 2

GENERAL MATERIALS AND METHODS

2.1 Bacterial strains, plasmids and growth conditions

2.1.1 Source and culture of *Escherichia coli* strains

2.1.2 Source and culture of *Clostridium difficile* strains

2.1.3 Plasmids and antibiotic resistance

2.1.4 Preparation of calcium chloride competent *E. coli* cells

2.1.5 Preparation of electrocompetent *E. coli* cells

2.2 DNA Manipulations

2.2.1 *C. difficile* chromosomal DNA extractions

2.2.1.1 Calculation of DNA concentration

2.2.1.2 Calculation of DNA purity

2.2.2 Isolation of plasmid DNA

2.2.2.1 Small scale preparation

2.2.2.2 Large scale preparation

2.2.3 Agarose gel electrophoresis

2.2.4 Purification of DNA from agarose gels using spinbind columns

2.2.5 Ethanol precipitation of DNA

2.2.6 Restriction digestion of DNA

2.2.7 DNA ligation

2.2.8 Transformation of calcium chloride competent *E. coli* cells

2.2.9 Transformation of electrocompetent *E. coli*

2.3 PCR amplification of regions of the toxin A gene

2.4 Cloning of PCR products using the Original TA Cloning Kit

2.9.1.2 Micro assay procedure

2.9.2 Estimation of the concentration of a protein within a mixed population

2.10 Optimisation of expression of toxin A peptides

2.10.1 Varying the IPTG concentration and length of induction time

2.10.2 Varying the host *E. coli* strain

2.11 N-terminal sequencing of proteins

2.12 Electro-elution of proteins from SDS-PAGE

2.13 Determination of the pI value of proteins by Isoelectric focusing (IEF)

2.14 Ion-exchange chromatography

2.15 Enzyme-linked immunosorbent assay (ELISA)

Section - 2

General Materials and Methods

2.1 Bacterial strains, plasmids and growth conditions.

2.1.1 Source and culture of *Escherichia coli* strains.

The genotypes and sources of *E. coli* strains used in this study were BL21 (DE3) [F^- ompT $hsdS_B(r_B^-m_B^-)$ gal dcm(DE3)] (Novagen Inc), JM109 [endA1, recA1, gyrA96, thi, hsdR17, (r_K^-,m_K^+), relA1, supE44, λ^-, Δ(lac-proAB), [F',traD36,proAB,lacI^{q}ZΔM15]] (Stratagene) and TB1 [*ara*Δ(*lac proAB*) *rpsL* (ϕ80 *lacZ*ΔM15) *hsdR*] (New England Biolabs).

The *E. coli* strains stored at -70^{0}C in Luria-Bertani broth (LBB) plus 30% glycerol were inoculated onto Luria-Bertani agar (LBA) plates (containing antibiotics where necessary) and incubated at 37^{0}C overnight to isolate single colonies. Overnight cultures of the bacterial strains were produced by inoculating a single bacterial colony into 10 mls of LBB (plus antibiotics where necessary) and incubating for 15 hrs at 37^{0}C aerobically with shaking (250 rpm).

2.1.2 Source and culture of *Clostridium difficile* strains.

The virulent *C. difficile* strains used in this study were clinical isolates from the stools of patients who presented with *C. difficile* infection. The avirulent strain was a clinical isolate from a patient who presented with diarrhoea due to *Shigella sonnei.* The strains used included strain VPI 10463 [highely virulent and toxigenic] (Krivan *et al*, 1986), M-1 [avirulent and non-toxigenic] (Borriello *et al*, 1987) and 8864 [toxin B positive, toxin A negative, poorly virulent] (Haslam *et al*, 1986; Torres, 1991; Borriello *et al*, 1992).

The *C. difficile* strains stored in Robertsons Cooked Meat Medium (Southern Group Laboratories, U.K) were inoculated onto blood agar plates and incubated anaerobically (MK3 Anaerobic Work Station; Don Whitley Scientific Ltd, England) for 2 days at 37^0C to isolate single colonies.

To produce overnight cultures of the *C. difficile* strains, a single bacterial colony was inoculated into 10 mls of Modified Brain Heart Infusion (MBHI) broth (3.7% BHI, 0.5% yeast extract, 0.05% cysteine hydrochloride and 0.03% sodium formaldehyde sulphoxylate, pH 7.3) and incubated anaerobically at 37^0C for 2 days.

2.1.3 Plasmids and antibiotic resistance.

The T-tailing vector pCRII (Invitrogen) confers on *E. coli* strains resistance to ampicillin ($100\mu gml^{-1}$). The expression vectors pMalc2, pMalp2 (New England Biolabs) pET3a and pET3d (Novagen) confers on *E. coli* strains resistance to ampicillin ($100\mu gml^{-1}$). The expression vector pET30a (Novagen) confers on *E. coli* strains resistance to kanamycin ($30\mu gml^{-1}$).

E. coli transformants containing these plasmids were grown as described in section 2.1.1 with the exception that the relevant antibiotic described above was included in the LBB and LBA media.

2.1.4 Preparation of calcium chloride competent *E. coli* cells.

Calcium chloride ($CaCl_2$) competent JM109 and XL2-Blue cells were purchased from Stratagene Ltd while BL21 DE3 cells were obtained from Novagen Inc. $CaCl_2$ competent TB1 cells were purchased from New England Biolabs Ltd. Cells were also prepared 'in house' by the method described by Ausubel *et al*, 1992.

A single colony of *E. coli* BL21 DE3, TB1 or JM109 was inoculated into 50 mls of LBB and grown overnight at 37°C with shaking. Four mls of the overnight culture were inoculated into 400mls of LBB in a 2 litre flask and grown at 37°C, with shaking (250 rpm), to an OD_{600} of 0.4. The culture was aliquoted into eight 50ml pre-chilled polypropylene tubes and incubated on ice for 5-10 mins. The cells were gently pelleted at 1,600 xg for 7 mins at 4^0C, then each was resuspended in 10 mls of ice-cold calcium chloride ($CaCl_2$) solution (60mM $CaCl_2$, 15% glycerol, 10mM piperazine N, N'-bis 2-ethane-sulphonic acid [PIPES]). The cells were pelleted at 1,100 xg for 5 mins at 4^0C, then each resuspended in 10 mls of fresh ice-cold $CaCl_2$ solution. The cells were incubated on ice for 30 mins and then centrifuged at 1,100 xg for 5 mins at 4^0C. Each pellet was resuspended in 2 mls of ice-cold $CaCl_2$ solution. Aliquots (250µl) of $CaCl_2$ competent cells were placed into pre-chilled eppendorf tubes and stored at -70°C.

2.1.5 Preparation of electrocompetent *E. coli* cells.

Electrocompetent *E. coli* BL21 DE3 cells were purchased from Novagen Inc., USA. Electrocompetent *E. coli* BL21 DE3 or JM109 cells were also prepared using the method described by Ausubel *et al*, 1992.

A single colony of *E. coli* cells was inoculated into 5 mls of LBB and grown overnight at 37^0C with shaking, then 2.5 mls of the overnight culture were inoculated into 500 mls of LBB and grown at 37^0C with shaking to an OD_{600} of 0.5-0.6.

The cells were chilled in an ice-water bath for 10-15 mins, then centrifuged at 5,000 xg for 20 mins at 4^0C. The cells were resuspended gently in 5 mls of ice-cold sterile distilled water (SDW) before adding a further 500 mls of ice-cold SDW. The cells were mixed and centrifuged at 5,000 xg for 20 mins at 4^0C. Again the pellet was resuspended in ice-cold

SDW, mixed and centrifuged as described above. The supernatant was gently poured off and the pellet was resuspended by swirling in the remaining liquid. The cells were mixed with 40 mls of ice-cold 10% glycerol and then centrifuged at 5,000 xg for 20 mins at 4^{0}C. The cells were resuspended in an equal volume of ice-cold 10% glycerol and 100µl aliquots were placed into pre-chilled eppendorf tubes then stored at -70^{0}C.

2.2 DNA Manipulations.

2.2.1 *C. difficile* chromosomal DNA extractions.

Toxigenic *C. difficile* VPI 10463 was grown in dialysis tubing suspended in Brain Heart Infusion Broth (BHIB) (see section 2.1.2). The culture (≈ 100mls) was centrifuged at 10,179×g for 1 hr at 4°C (High Speed 18 Refrigerator Centrifuge; MSE). The cell pellet was washed with 20 mls of SDW and the cells harvested at 9,000×g for 10 mins. The pellet was resuspended in 15 mls of 1M sucrose and 15 mls of lysozyme solution (10 mg/ml in SDW) then incubated at 37°C for 1 hr.

Following centrifugation at 9,000×g for 5 mins, the pellet was resuspended in 7.5 mls 50mM Tris-HCl pH 8.0, 2.5mls 0.25 M EDTA pH 8.0 and 600 µl pronase (20 mg/ml) then incubated at 37°C for 30 mins.

The cells were lysed by firstly incubating with 2.5 mls N- lauroylsarcosine for 20 mins at 37°C, then with 2 mls 10% SDS for 10 mins at 65°C. RNA was removed by incubating with 12.5 mls SDW and 250µl of 10 mg/ml RNase for 30 mins at 37°C. The DNA preparation was purified by adding an equal volume of phenol: chloroform: IAA (6:4:1), (Sigma) mixing gently and then centrifuged at 9,000 ×g for 5 mins. The upper layer was removed to a clean tube and extracted twice more with phenol/chloroform/IAA as described. To remove contaminating phenol two successive chloroform extractions were

performed. In each case the upper layer was mixed with an equal volume of chloroform and then centrifuged at 9,000 ×g for 5 mins. To precipitate the DNA, the upper layer was gently mixed with an equal volume of isopropanol and 0.05 volumes of 7.5M NH_4Ac. The DNA was spooled out of solution using a glass rod and placed in a clean eppendorf tube. The DNA was washed in 5× 1 ml of 80% ethanol, then air dried and resuspended in 500µl of SDW.

2.2.1.1 Calculation of DNA concentration:

To calculate DNA concentration, 5µl of DNA was mixed thoroughly with 495µl of SDW then transferred to a 0.5 cm UV glass cuvette. Spectrophotometer readings were taken at 260 nm and 280 nm using 500µl of SDW in an identical cuvette as a blank reference. The following equation was used to calculate the concentration of double stranded DNA:

A_{260} value × 2* × 100** × 50*** = DNA concentration (µg/ml)

* Pathlength: cuvettes are 0.5 ml thick but the equation relies on 1 cm cuvettes.

** Dilution factor of DNA.

*** When A_{260} = 1, the double stranded DNA concentration is 50µg/ml or the single stranded DNA concentration is 37µg/ml. The 50*** value is replaced with 37 for single stranded DNA.

2.2.1.2 Calculation of DNA purity:

For pure DNA:

$$\frac{A_{260}}{A_{280}} = 1.9$$

A value lower than this suggests contamination with phenol or protein and requires further purification.

2.2.2 Isolation of plasmid DNA.

2.2.2.1 Small scale preparation:

Plasmid DNA (1-5μg) was isolated from an overnight bacterial culture using the Wizard™ Minipreps DNA Purification System (Promega), as outlined by manufacturers instructions. The Wizard™ DNA Purification protocol is based on a modified alkaline lysis procedure. Briefly, 3ml of an overnight bacterial culture was harvested by centrifugation at 11,600xg for 2 min. Bacterial pellets were resuspended in 0.2ml of cell resuspension buffer (50mM Tris-HCl, pH 7.5, 10mM EDTA, 100μg/ml RNase A), and then lysed by the addition of 0.2ml of cell lysis buffer (0.2M NaOH, 1% SDS). The cell lysate was neutralised by the addition of 0.2ml of cell neutralisation buffer (1.32M Potassium acetate, pH 4.8), and the precipitate that formed was pelleted by centrifugation at 11,600xg for 5 min. 1ml of Wizard Minipreps DNA Purification Resin was added to the supernatant before the Resin/DNA mix was passed through a Wizard purification column. The DNA bound to the column was washed by the addition of 2ml of column wash solution (200mM NaCl, 20mM Tris-HCl, pH 7.5, 5mM EDTA, 55% ethanol), followed by centrifugation at 11,600xg for 30 sec. Plasmid DNA was eluted from the column by the addition of 50μl of SDW (heated to 65^0C), followed by centrifugation at 11,600xg for 30 sec. Plasmid DNA stocks were stored at 4^0C.

2.2.2.2 Large scale preparation:

Plasmid DNA (100-500μg) was isolated from an overnight bacterial culture using the Qiagen Maxi Plasmid Purification Kit (Qiagen Inc.), as outlined by the manufacturers instructions. The Qiagen plasmid purification protocols are based on a modified alkaline lysis procedure.

Briefly, 100ml of an overnight culture containing high copy number plasmid, or 500ml of a culture containing low copy number plasmid was harvested at 6000xg for 15min at 4^0C. The bacterial pellet was resuspended in 10ml of buffer P1 (50mM Tris-HCl, pH 8.0; 10mM EDTA; 100μg/ml RNase A), and then lysed by the addition of 10ml of buffer P2 (200mM NaOH; 1% SDS). The cell lysate was neutralised by the addition of 10ml of ice cold buffer P3 (3.0M Potassium acetate, pH 5.5), and the precipitate that formed was pelleted by centrifugation at 10,000 xg for 30min. A Qiagen-tip 500 was equilibrated by applying 10ml of buffer QBT (750mM NaCl; 50mM MOPS,pH 7.0; 15% ethanol; 0.15% Triton X-100) and allowing the column to empty by gravity. The centrifugation supernatant was allowed to drip through the purification column, and the plasmid DNA that bound to the membrane was washed with 2x 30ml of buffer QC (1.0M NaCl; 50mM MOPs, pH 7.0; 15% ethanol). Plasmid DNA was eluted from the purification column by the addition of 15ml of buffer QF (1.25M NaCl; 50mM Tris-HCl, pH 8.5; 15% ethanol), and was then precipitated by the addition of 0.7 volumes of isopropanol followed by centrifugation at 10,000 xg for 30min. DNA pellets were washed with 70% ethanol, air dried and then resuspended in 200μl of SDW. Plasmid DNA stocks were stored at 4^0C.

2.2.3 Agarose gel electrophoresis

DNA was analysed by agarose gel electrophoresis as outlined by Maniatis *et al*, 1982. Two horizontal agarose gel electrophoresis systems were used throughout this project. The Anachem mini gel tank (model H2-SET) was used to run gels with dimensions 10cm x 5.8cm x 0.5cm, and the Northumbria Biologicals Ltd. System (model SPH20100) was used to run gels with dimensions 15cm x 13cm x 1cm.

Unless otherwise stated, electrophoresis grade agarose (Boehringer Mannheim) was dissolved in 1 x TBE buffer (90mM Tris Base, 90mM Boric acid, 2mM EDTA) to a final concentration of 0.8%. Linear DNA has an efficient range of separation that is related to the percentage of agarose in the gel (see table 2.1). DNA samples were diluted with 0.16 volumes of 6 x bromophenol blue loading buffer (0.25% bromophenol blue, 40% (w/v) sucrose in water) before being applied to the wells of the agarose gel. A 1Kb DNA ladder (Boehringer Mannheim) was included on all gels for DNA size estimations. Gels were resolved in 1 x TBE buffer containing 0.5μg/ml ethidium bromide at 80 Volts for 1-3hr. Following electrophoresis, DNA fragments were visualised by Ultra-violet illumination (Transilluminator model TM40, Ultra Violet Products Inc).

2.2.4 Purification of DNA from agarose gels using spinbind columns.

DNA was purified from agarose gels using the 'Spinbind® DNA Recovery System' (FMC Bioproducts, Denmark). A brief outline of the methodology is provided below.

Following agarose gel electrophoresis (see section 2.2.3) the DNA fragment of interest was excised from the gel minimising the amount of agarose. The agarose was removed from the slice by centrifugation through glass beads (Sigma) at 11,600×g for 10 mins. The supernatant containing the DNA was collected, mixed with 1.5 volumes of sodium iodide (NaI) solution (reagent A), applied to a Spinbind® unit, and then incubated for 1 min to

allow the solution to wet the MPS® membrane uniformly. The unit was centrifuged at 11,600×g for 10secs then the supernatant was reapplied to the unit and spun for a further 10secs. The supernatant was discarded and the unit transferred to a clean eppendorf tube. The DNA was washed by applying 100µl of ice-cold ethanol wash buffer (Reagent E) to the Spinbind® unit and spinning at 11,600×g for 10secs. The buffer was discarded and the wash stage repeated twice to remove NaI. The empty unit was centrifuged at 11,600×g for 30secs to remove any remaining ethanol wash buffer, and subsequently transferred to a clean eppendorf. The DNA was eluted from the unit by applying 50µl SDW followed by centrifugation for 10secs at 11,600×g. The purified DNA was analysed by agarose gel electrophoresis (see section 2.2.3).

2.2.5 Ethanol precipitation of DNA.

Two volumes of 100% ethanol and 0.1% volume of 3M sodium acetate pH 5.2 were added to the DNA solution, mixed and incubated at –20^{0}C for 1hr. The mixture was centrifuged at 11,600xg for 30mins and the supernatant was removed. The DNA pellet was washed with 0.5ml of 70% ethanol and recentrifuged at 11,600xg for 10mins. The supernatant was removed and the DNA pellet was air dried then resuspended in 10-200µl of SDW (volume dependant on the amount of DNA expected).

2.2.6 Restriction digestion of DNA.

DNA was digested with DNA restriction enzymes as outlined by the manufacturers instructions (Boehringer Mannheim).

Approximately 5µg (1-10µl) of DNA were digested with 1µl of the relevant restriction enzyme (4-10 Units; Boehringer Mannheim) and 2µl of its respective 10 x buffer

(Boehringer Mannheim) in a 20µl reaction volume made up with SDW. Digests were incubated at 37^0C or 30^0C (as outlined by manufacturers instructions) for 2 hrs. Restriction digest profiles were analysed by agarose gel electrophoresis (see section 2.2.3). Where it was necessary to perform a double digest, the DNA was precipitated using ethanol (see section 2.2.5) following the first digestion before proceeding with the second digest.

2.2.7 DNA Ligation.

The compatible 'sticky ends' of the digested vector and insert DNA were ligated in a 1:1 or 1:3 ratio of vector to insert. To calculate a 1:1 molar ratio of vector ends to insert ends, the following equation was used:

$$\text{Xng DNA insert} = \frac{(\text{Ybp DNA insert}) \quad (50\text{ng vector})}{(\text{size in bp of vector})}$$

Ligations were performed in a 20µl volume reaction containing Xµl digested insert, Yµl digested vector, 2µl 10 x ligase buffer (Gibco BRL), 1µl T4 DNA ligase (4U/µl, Gibco BRL), and were made up to 20µl with SDW. The reactions were incubated at 4^0C overnight.

2.2.8 Transformation of calcium chloride competent *E. coli* cells

Approximately 50ng of plasmid DNA or 10µl of a ligation reaction (see section 2.2.7) were incubated with 100µl of ice-cold $CaCl_2$ competent *E. coli* cells (see section 2.1.4) on ice for 30mins. The cells were heat shocked in a 42^0C water bath for 2mins then quickly placed on ice for a further 2mins. Nine hundred µl of LBB (pre-warmed to 37^0C) was

added and each transformation reaction was incubated at 37^{0}C with shaking (225rpm) for 1hr. The cells were pelleted by centrifugation at 11,600xg for 30secs then resuspended in 200µl of LBB. The cell suspension was spread on the surface of LBA plates containing the appropriate antibiotics and selective reagents (outlined in the relevant sections). The plates were incubated at 37^{0}C for 16hrs then stored at 4^{0}C.

2.2.9 Transformation of electrocompetent *E.coli* cells

Approximately 10ng of plasmid DNA or 5µl of a ligation reaction (see section 2.2.7) were dialysed to remove salt prior to electroporation. The samples were placed on a filter (vs 0.025µ ; Millipore) and floated on the surface of SDW for 30mins at RT. One hundred µl of electrocompetent *E. coli* cells (see section 2.1.5) were thawed on ice then incubated with the dialysed DNA sample for 2mins on ice. The cells were added to a Gene Pulser™ cuvette (0.2cm Electrode Gap; Biorad) which was subsequently placed in the Gene Pulser™ (Biorad). The electroporator was set at a resistance of 200 Ohms then 2.5Volts were passed through the cuvette. The transformation reaction was diluted immediately with 900µl of LBB then incubated at 37^{0}C with shaking (250rpm) for 1hr. The cells were pelleted by centrifugation at 11,600xg for 30secs then resuspended in 300µl of LBB. The cells were spread on the surface of LBA plates containing the appropriate antibiotics and selective reagents (outlined in the relevant sections) then incubated at 37^{0}C for 16hrs.

2.3 PCR amplification of regions of the toxin A gene.

Fragments of the toxin A gene were amplified from *C. difficile* strain VPI 10463 chromosomal DNA (see section 2.2.1) on a Techne Thermocycler (PHC-1) by the polymerase chain reaction (PCR). Oligonucleotide primers were designed based on the

published toxin A sequence data (Dove *et al.* 1990; see appendix 1). Forward primers read 5' to 3' on the positive strand of the DNA helix, and reverse primers read 5' to 3' on the negative complementary DNA strand. Restriction endonuclease recognition sites were engineered into the primers for efficient cloning into plasmid vectors.

One hundred ng of chromosomal DNA was amplified in a 100µl volume reaction containing 10mM Tris-HCl (pH. 8.3), 50mM KCl, 2mM $MgCl_2$, 0.01% gelatin, 0.005% Tween-20, 0.2mM dNTP's (Pharmacia) with Taq DNA polymerase (5U, Boehringer Mannheim U.K) and 1µg of each primer (outlined in the relevant sections). Controls containing only one of the two primers, and a control lacking template DNA were included in each amplification reaction. Each reaction mixture was overlayed with 50µl of mineral oil (Sigma). Various thermocycling conditions (outlined in the relevant sections) were used to denature, anneal and extend the template DNA for a total of 35 cycles, with a final extension cycle of 72^0C for 10 min. Amplified DNA was analysed by agarose gel electrophoresis (see section 2.2.3), and stored at 4^0C.

2.4 Cloning of PCR products using the Original TA Cloning Kit.

PCR products were first cloned into a T-tailing vector as outlined by the manufacturers instructions (Invitrogen). A brief outline of the procedures is given below.

2.4.1 Ligation to the pCR11 plasmid vector.

A 1:1 or 1:3 ratio of vector DNA to PCR product DNA was used for ligation reactions (see section 2.2.7) which were performed in a 10µl volume reaction containing 5µl SDW (TA1), 1µl 10 x ligation buffer (TA2), 2µl pCR11 vector (TA3, 25ng/µl), 1µl of purified

PCR product (Xng), and 1µl of T4 DNA ligase (TA5). The ligation reactions were incubated at 4^0C for a minimum of 4hr but usually overnight.

2.4.2 Transformation of constructs into Inv α F' competent cells.

One vial of One Shot Inv α F' competent cells per ligation reaction was defrosted on ice. Two µl of 0.5M β-mercaptoethanol (TA11) followed by 1µl of the ligation reaction was added to the competent cells, which were mixed by gentle stirring. The cells were incubated on ice for 30 min, heat shocked at 42^0C for 30 sec then immediately cooled on ice for 2 min. The transformed bacteria were supplemented with 450µl of the supplied SOC medium and allowed to grow with shaking (225 rpm) at 37^0C for 1hr. One hundred µl of each reaction was spread onto the surface of an LBA plate containing 100µgml^{-1} of ampicillin (LB Amp) that had been pre-spread with 50µl of 20mgml^{-1} 5-bromo-4-chloro-3-indoyl-β-D-galactosidase (X-gal) 1hr previously. The plates were inverted and incubated at 37^0C overnight.

White colonies indicated transformation of recombinant plasmids. Ten such colonies were selected and the plasmids isolated using the Qiagen Plasmid Mini kit (see section 2.2.2.1). Each plasmid was digested with the relevant restriction endonucleases (see section 2.2.6) to excise the cloned DNA fragment. The DNA profiles were examined by agarose gel electrophoresis (see section 2.2.3) to identify clones with the correct size insert. Positive clones were stored in glycerol at -70^0C (see section 2.1.1).

2.5 Automated DNA sequencing

A detailed account of the methodology can be found in the ABI PRISM Dye Termination Cycle Sequencing Ready Reaction Kit protocol (Perkin Elmer). A brief outline is provided below.

Recombinant plasmids were purified (see section 2.2.2.2) and resolved on a 0.8% agarose gel (see section 2.2.3) with a DNA mass ladder (Gibco) to establish the plasmid DNA concentration. The plasmid insert DNA was sequenced in the DNA sequencing laboratory, Queens Medical Centre, Nottingham. The plasmid was used as a template in a thermocycling reaction to incorporate a fluorescent dye termination. The plasmid was precipitated, washed in ethanol and dried under vacuum (Speed Vac Plus SC21OA; Saivant). The dried plasmid was resuspended in formamide EDTA and resolved on a sequencing gel (Applied Biosystems 373A DNA Sequencer) for 14hrs before signal analysis.

2.6 Toxin A production.

Highly purified toxin A was obtained from crude culture filtrate using bovine-thyroglobulin affinity chromatography followed by two step anion-exchange chromatography on Q Sepharose Fast Flow (QSFF) and Mono Q columns. Cytotoxicity and haemagglutination (H/A) assays were used to test fractions collected at each stage of purification, such that fractions containing toxin A (positive in both assays) could be identified and pooled for further purification. Polyacrylamide gel electrophoresis (see section 2.7.1) was used to check the homogeneity of the final product eluted off the Mono Q column. Full details are given below.

2.6.1 Growth of *C difficile* in dialysis bags.

Toxigenic *C. difficile* VPI 10463 was grown in dialysis tubing (size 2, 14.3mm; Medicell International Ltd., England), suspended in BHI broth (BHI [3.7%], yeast extract [0.5%], cysteine hydrochloride [0.05%] and sodium formaldehyde sulphoxylate [0.03%], pH 7.3) and incubated anaerobically at 37^{0}C for 3 d. Dialysis tubing was cut to length and secured with string to the tubing in a rubber bung, so that the tubing was able to hang freely in an 11 side-arm flask containing BHI broth. A small amount of sterile phosphate buffered saline (PBSA) was added to the tubing before the bung was wrapped in foil and autoclaved at 121^{0}C for 15 mins. Following autoclaving, 50 mls of PBSA was added to the dialysis tubing and the system incubated aerobically at 37^{0}C overnight, to allow equilibration of micronutrients between the dialysis bag contents and the BHI broth. The following day, the dialysis tubing was inoculated with 1 ml of an 18 h 10 ml *C difficile* VPI 10463 suspension, followed by enough sterile PBSA to make the tubing taut. The top of the flask was covered with autoclaved foil and the system was incubated anaerobically at 37^{0}C for 3 d. The contents of the dialysis tubing were harvested and centrifuged at 10179 x g at 4^{0}C for 1 h (High Speed 18 Refrigerator Centrifuge; MSE). The culture supernatant containing the toxin A protein was filtered (0.45 μm; Acrodisc) and stored at 4^{0}C until purification could be carried out as outlined below.

2.6.2 Thyroglobulin affinity chromatography.

The method used was that of Krivan and Wilkins (1987). Two hundred and fifty mg of bovine thyroglobulin (Sigma Chemical Co, St Louis, USA) was dissolved in 50mls of 0.1 M MOPS buffer. The thyroglobulin solution was then reacted with 10mls of activated affinity gel 15 (Bio-Rad Laboratories), overnight at 4°C on a shaker. Any remaining active sites on the gel were then blocked by treatment with 100 μl of 0.1M ethanolamine

for 3mins at 4°C. The coupled beads were packed into a glass column (Pharmacia, Uppsala, Sweden: column C10/10, 10 x 100 mm) and washed at 37°C, firstly with 20 column volumes of a basic buffer (0.1 M glycine 0.5 M NaCl pH 10) then 20 column volumes of an acidic buffer (0.1 M glycine, 0.5 M NaCl pH 2.0). These stringent washes ensured that free thyroglobulin did not remain ionically bound to the gel. Finally the gel was equilibrated with 20 column volumes of TBS (0.05 M Tris buffer containing 0.15 M NaCl pH 7.0) at 4°C. Approximately 100 mls of crude *C. difficile* culture filtrate (see section 2.6.1) was applied to the column at 4°C. The column was washed with 100mls of TBS at 4°C to remove all proteins other than toxin A, which remained bound to the column. The column was then warmed to 37°C for 30 mins before toxin A was eluted by washing with 50mls of TBS at 37°C. The thermal eluate was collected in 5ml fractions, and each fraction was monitored for absorbency at 280nm, for cytotoxicity (see section 2.6.5) and H/A (see section 2.6.4).

2.6.3 Anion-exchange chromatography.

Two types of anion-exchange chromatography were used as part of a Fast Protein Liquid Chromatography (FPLC) apparatus to analyse the toxins of *C. difficile* (as described in Borriello *et al,* 1987)

The thyroglobulin eluted fractions containing toxin A were pooled and filtered (0.2μm; Acrodisc). The sample was first applied to a QSFF column (Pharmacia) via a 10ml super - loop (Pharmacia) and then eluted by a 0 - 1.0M sodium chloride gradient in 20mM Tris-HCl, pH. 7.5. For each fraction collected, the absorbance at 280nm, H/A (section 2.6.4) and cytotoxicity (section 2.6.5) were measured. Following QSFF chromatography, the fractions containing toxin A were desalted by dialysis against 20mM Tris-HCl, pH 7.5,

overnight at 4°C. The toxin A sample was then further purified by Mono Q chromatography. The sample was applied to, and eluted from the Mono Q column (Pharmacia) in the same way as for QSFF chromatography, with the exception that a plateau was added to the salt gradient just before the toxin A elution point to remove a known closely eluting contaminant (Kamiya *et al,* 1988).

2.6.4 Haemagglutination assay.

This assay is based on a method described by Krivan *et al* (1986) with the slight modification that a 1% rabbit red blood cell (RRBC) suspension was used (Kamiya *et al*, 1988), instead of a 2.5% suspension. Two-fold serial dilutions of the samples (50µl) were prepared with Tris Buffered Saline (isotonic buffer) in the wells of a v-bottom microtitration plate (Sero-well). Fifty µl of a 1% RRBC suspension was added to each well and left to incubate for at least 3 h at 4°C. H/A titres were expressed as the reciprocal of the highest dilution giving a positive H/A when viewed by eye.

2.6.5 Cytotoxicity assay.

This assay makes use of African Green Monkey Kidney (Vero) cells, as described by Kamiya *et al* (1988). Vero cells were grown in culture flasks in Medium 199 (Sigma, St Louis, USA) containing 5% newborn calf serum, 1% penicillin and 1% glutamine (Growth medium). When confluent, the cells were recovered from the flask by incubating with 3 mls of trypsin / EDTA solution (Sigma U.K.) at 37^{0}C for 2mins. The cells were loosened from the flask by gentle tapping and were resuspended in 20mls of growth medium. Two hundred µl aliquots of the cell suspension were added to each well of a 96 well sterile microtitre plate (Falcon). The cells were incubated overnight at 37^{0}C to reach confluency

before the medium was replaced with 100µl of maintenance medium (Medium 199 containing 2% newborn calf serum, 1% penicillin and 1% glutamine). Ten-fold serial dilutions of the toxin A samples were prepared in PBSA, and 100µl of each sample was added to the Vero cells in the microtitre plate. Following an incubation of 24 h at 37^0C, cells were monitored for a cytopathic effect (CPE), commonly seen as cell rounding. The cytotoxicity titre (cytotoxic unit [CU]/100 µl) was expressed as the highest dilution that induced more than 50% CPE after an incubation of 24 h.

2.7 Protein Manipulations.

2.7.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Protein samples were analysed by separating on linear SDS-PAGE gels using the method of Laemmli (1970). The larger the size of the protein to be analysed, the higher the percentage of acrylamide that was included in the gel.

Polyacrylamide gels were poured using the solutions and volumes outlined in table 2.2. The Protean II electrophoresis system (Biorad) was assembled as outlined by manufacturers instructions and used to run gels with dimensions 20 cm x 16 cm. The Protean II mini electrophoresis system (Biorad), on the other hand, was used to run gels with dimensions 7cm x 9cm.

The separating gel was poured then overlaid with a small layer of propan-2-ol to hasten the setting time. The propan-2-ol was washed from the surface of the separating gel with SDW before the stacking gel was applied. A comb (Biorad) was inserted into the stacking gel prior to its setting to form wells for protein sample loading. Once the stacking gel was set, the comb was removed and the gel was placed into the Protean electrophoresis tank containing three litres of running buffer (25mM Tris base, 250 mM glycine pH 8.3, 0.1%

SDS). Protein samples were mixed with an equal volume of 2 x SDS loading buffer (100mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 0.2% bromophenol blue, 5% β-mercaptoethanol), heated to 100^0C for 5 mins then loaded into the wells in the stacking gel. Ten µl of either high or low molecular weight markers (Pharmacia) were also included on the gel to allow protein size estimations to be made. Protein samples were resolved on large gels at a constant current of 35 mA for 4 hrs or 8 mA for 16 hrs. Small gels were run at 200 Volts for 40 mins.

Protein profiles were visualised by incubating in coomassie blue solution (60mls SDW, 30 mls propan-2-ol, 10 mls glacial acetic acid, 0.5g coomassie brilliant blue [Biorad]) for 1 hr with shaking then in destain solution (SDW: propan-2-ol : glacial acetic acid in a 6:3:1 ratio) for 4 x 2 hrs. Alternatively the protein profiles on the gel were transferred to nitrocellulose by western blotting (see section 2.7.3).

2.7.2 Silver staining of SDS-PAGE gels.

Protein samples were resolved on an 8% SDS-PAGE gel (see section 2.7.1). The apparatus was dismantled and the gel placed in fixative (30% ethanol, 10% acetic acid) for 30mins at RT. The gel was transferred to sensitiser solution (0.28M NaAc, 30% ethanol, 0.1% sodium thiosulphate, 0.5% glutaraldehyde) for 1hr at RT then washed for 4 x 15mins in SDW. The gel was placed in 200mls of stain solution (0.1% $AgNO_3$, 0.005% formaldehyde) for 1hr and the proteins visualised by incubating in developing solution (2.5% Na_2CO_3, 0.016% formaldehyde) for 30mins at RT. The gel was rinsed in SDW to remove excess stain and the reaction stopped by placing in 10% acetic acid.

2.7.3 Western blotting and immunological detection.

The SDS-PAGE gel (see section 2.7.1) was incubated in transfer buffer (39mM glycine, 48mM Tris base pH 8.3) for 2mins with shaking to remove SDS. One sheet of nitrocellulose (Schleider and Schull BA85, 0.45μm), two sheets of 3MM blotting paper (Whatmann) and two transfer pads (Biorad) were pre-soaked in transfer buffer for 5mins. The sheet of nitrocellulose was placed on top of the gel and together they were sandwiched between the sheets of 3MM blotting paper. A transfer pad was placed either side and the sandwich was clamped then placed in the western transfer tank containing 3l of transfer buffer. One Biorad Transblot system was used for blotting gels with dimensions 20 cm x 16 cm at a constant current of 220mA for 2hrs. A second Biorad Transblot system was used for blotting gels with dimensions 7cm x 9cm and was also set at 220mA for 2 hrs.

Following protein transfer from the SDS-PAGE gel to the nitrocellulose, the apparatus was dismantled and the nitrocellulose placed protein side up in a plastic container. The proteins were stained red with Ponceau stain (Sigma) for 30s and the bands corresponding to the molecular weight markers were drawn onto the nitrocellulose. The ponceau stain was removed from the blot by rinsing in tap water. The western blot was blocked by incubating in 1% gelatin/PBSA/0.05% Tween 20 for 1 hr at RT with shaking. The nitrocellulose was washed briefly in PBSA/0.05% Tween 20 (PBST) and was then incubated with a primary antiserum to the protein of interest (diluted in 0.1% gelatin/PBST) for 2hrs at RT with shaking. The details of specific antisera and their corresponding dilution factors are provided in the appropriate sections.

The filter was washed in PBST for 5 x 5mins at RT with shaking. The protein of interest was localised by incubating with a 1:1000 dilution of an alkaline phosphatase conjugated whole molecule IgG anti-rabbit or anti-mouse conjugate (Sigma) for 1 hr at RT with shaking. The blot was washed for 5 x 5mins with PBST and then for 5mins with SDW.

The immune complex was detected by developing with BCIP/NBT alkaline phosphatase substrate solution (Sigma). Once the colour had developed, the reaction was stopped by rinsing in tap water and the nitrocellulose was air dried.

2.7.4 Dot blotting of proteins.

Two µl of each test solution was spotted onto a small strip of nitrocellulose (Schleicher and Schuell, 0.2µm), and allowed to air dry. Blots were blocked with 1% gelatin (Biorad) in PBSA for 1hr at RT, and then incubated with 3mls of primary antibody (diluted 1:1000 or 1:10000 in 0.1% gelatin / PBSA) for 2hr at RT. The blots were washed five times in PBSA / 0.05% Tween 20 (Sigma) and then incubated with 3ml of a 1:1000 dilution of either goat anti-rabbit alkaline phosphatase conjugate (Sigma) or goat anti-mouse alkaline phosphatase conjugate (Sigma) for 1hr at RT. Blots were washed five times with PBSA/Tween 20 and binding was visualised by incubating with 3ml of BCIP/NBT substrate solution (Sigma).

2.8 Expression and purification of recombinant peptides.

2.8.1 Induction of expression of recombinant fusion protein.

Cells containing the fusion plasmid were incubated overnight in LBB containing ampicillin (100µg/ml) at 37^0C with shaking. LBB (9.9ml) containing ampicillin (100µg/ml) was inoculated with 0.1ml of the overnight culture and the cells were incubated at 37^0C with good aeration to $2x10^8$ cells/ml ($O.D._{600}$ of approx. 0.5). One ml of cells was removed, pelleted at 11,600 x g for 2min and resuspended in 100µl of 2 x SDS-PAGE sample buffer (see section 2.7.1). This sample represented the uninduced control cells. Expression was induced in the remaining culture by the addition of isopropyl-β-D-thiogalactosidase

(IPTG) to the final concentration of 0.3mM (for pMal vectors), 0.4mM (for pET3d vectors) or 0.1mM (for pET30a vectors), with continued incubation at 37^{0}C for 2hrs or 3hrs, for pMal^{TM} vectors (New England Biolabs) or pET vectors (Novagen) respectively. Five hundred µl of cells was removed, pelleted at 11,600 x g for 2min and resuspended in 100µl 2 x SDS-PAGE sample buffer. This sample represented the induced cells. The uninduced and induced cells were boiled for 5min and the proteins resolved by 8% SDS-PAGE (section 2.7.1). The proteins were transferred to nitrocellulose by western blotting (section 2.7.3), and the presence of recombinant fusion protein was determined by immunological detection (see section 2.7.3).

2.8.2 Purification of maltose binding protein (MBP) fusion products.

The MBP fusion products were purified by amylose affinity chromatography as outlined by the manufacturers instructions (New England Biolabs).

One litre of LBB containing ampicillin (100µg/ml) was inoculated with 10ml of an overnight culture of bacterial cells (section 2.1.1) containing the MBP fusion plasmid. The cells were incubated at 37^{0}C with good aeration to 2 x 10^8 cells/ml ($O.D._{600}$ of approx. 0.5). Expression was induced by the addition of IPTG to the final concentration of 0.3mM and the cells were incubated for a further 2hr. The bacteria were harvested by centrifugation at 4,000 x g for 20min, resuspended in 50mls amylose column buffer (20mM Tris-HCl, 200mM sodium chloride [NaCl], 1mM EDTA) and incubated at -20^{0}C overnight. The cells were thawed in cold water and disrupted by 15sec bursts of sonication (Soniprep 150; MSE) at an amplitude of 8 microns, in an ice-water bath. Cell debris and insoluble proteins were pelleted by centrifugation at 9,000 x g for 30min and discarded.

The supernatant (crude extract) was diluted 1:5 with amylose column buffer and stored at 4^0C before being loaded onto the amylose column.

Amylose resin (New England Biolabs) was poured into a 2.5 x 10cm column (Biorad) and allowed to settle by gravity flow. The resin was washed with 8 column volumes of amylose column buffer before the diluted crude extract was applied at a flow rate of approximately 1ml / min. The resin was washed with 10-12 column volumes of amylose column buffer, before being sealed and stored at 4^0C overnight. The fusion protein was eluted from the resin with amylose column buffer containing 10mM maltose, and was collected in 10 fractions of 3ml each. The three fractions containing the highest concentration of fusion product were pooled, diluted in 50mls amylose column buffer then passed through an amylose column a second time. Each eluted fraction was analysed for the presence of fusion protein by 8% SDS-PAGE (section 2.7.1).

2.8.3 Cleavage of the MBP fusion products with factor Xa.

The MBP was cleaved from fusion products by treatment with Factor Xa as outlined by the manufacturers instructions (New England Biolabs).

Factor Xa cleavage was carried out at a w/w ratio of 1% the amount of fusion protein. The reaction mixture was incubated for 3hr to overnight at 4^0C. Protein profiles were analysed by SDS-PAGE (section 2.7.1). Depending on the particular fusion protein, the amount of Factor Xa was adjusted within the range of 0.1-5.0% to get an acceptable rate of cleavage.

2.8.4 Determination of the solubility of recombinant toxin A peptides.

One hundred ml cultures of bacterial cells containing a plasmid with a toxin A DNA insert and a plasmid lacking a DNA insert were incubated at 37^0C with shaking to an OD_{600} of

0.5. Half of each culture was removed and the cells pelleted at 5,000xg for 20mins at 4^0C (uninduced cells). Expression of recombinant protein was induced in the remaining culture by the addition of IPTG (for concentrations see section 2.8.1) with continued incubation at 37^0C. The uninduced cell pellets were resuspended in 10mls of PBSA then sonicated in an ice-water bath for bursts of 15secs at an amplitude of 10 microns. The insoluble proteins and cellular debris were pelleted from the sonicate at 10,000xg for 20mins at 4^0C. The 10ml supernatants (uninduced soluble fractions) were stored at 4^0C until further analysis. The pellets were resuspended with the aid of sonication into 10mls of PBSA (uninduced insoluble fractions) then stored at 4^0C until further analysis.

Following the 2 or 3hr incubation of the second half of each culture, the induced cells were pelleted, sonicated and processed as already described for the uninduced samples to produce induced soluble and induced insoluble fractions.

Each fraction was mixed 1:1 with 2x SDS-PAGE sample buffer and resolved on two 8% SDS-PAGE gels (see section 2.7.1). One gel was stained with coomassie blue (see section 2.7.1) and the remaining gel was western blotted (see section 2.7.3). The peptide of interest was detected on the blot using the method outlined in section 2.7.3. The primary antibodies used are outlined in the relevant sections.

2.8.5 Purification of proteins from inclusion bodies present within *E. coli* cells.

Based on a method for purifying proteins from inclusion bodies present within *E. coli* cells (Ausubel, F.M. *et al*, 1992).

Briefly, 1L of LBB containing 100μg/ml of ampicillin was inoculated with 10ml of an overnight culture of bacterial cells (section 2.1.1) containing the recombinant plasmid. The cells were incubated at 37^0C with good aeration to 2 x 10^8 cells/ml ($O.D._{600}$ of approx.

0.5). Expression was induced by the addition of IPTG to the final concentration of 0.4mM and the cells were incubated for a further 3hr. The cells were pelleted by centrifugation at 5,655 x g for 10min and resuspended in 10ml of HEMGN buffer (100mM KCl, 25mM HEPES pH 7.6, 0.1mM EDTA pH 8.0, 12.5mM $MgCl_2$, 10% glycerol, 0.1% Nonidet P-40) containing protease inhibitors (1mM dithriothreitol, 1μg/ml leupeptin, 1μg/ml pepstatin, 0.1mM PMSF and 0.1mM sodium meta-bisulphite). Cells were disrupted by incubating at 4^0C for 30min with 500μg/ml of lysozyme (Sigma), followed by two 15sec bursts of sonication (Soniprep 150; MSE) at an amplitude of 8 microns. The inclusion bodies were pelleted at 27,000 x g for 15min, and then solubilised by rotating for 30min in 10ml of HEMGN buffer containing 4M guanidine HCl (Sigma) at 4^0C. Following centrifugation at 27,000 x g for 30min the supernatant was dialysed for periods of 4hr to overnight against 1L of HEMGN buffer containing 1M guanidine HCl, 2 x 1L of HEMGN buffer alone and against 2 x 5L of Tris-buffered saline pH.7 (TBS). Samples were collected prior to guanidine hydrochloride treatment and following final dialysis. The samples were resolved on an 8% SDS-PAGE gel (see section 2.7.1) and transferred to nitrocellulose by western blotting (see section 2.7.3). The antibodies used in detection are outlined in the relevant sections.

2.9 Protein concentration determinations.

2.9.1 Coomassie® plus protein assay reagent kit.

Unknown protein concentration determinations were made by directly comparing an unknown absorbance value to the absorbance values of a set of albumin standards of known concentrations using the Coomassie® Plus Protein Assay Reagent Kit (Pierce,

U.K). Two procedures were adopted dependant on the approximate protein concentration expected:

2.9.1.1 Standard assay procedure.

The standard assay was used for determining protein concentrations in the range of 75µgml^{-1} to 1,500µgml^{-1}. A known protein concentration series was produced by diluting the stock albumin standard (Pierce, U.K) in the same diluent as the protein sample whose concentration was to be determined. The standard data points selected were 50, 100, 250, 400, 500, 1,000 and 1,500µgml^{-1}. Ten µl of each dilute standard, of the unknown protein sample and of the sample diluent (to be used as a blank reference) were placed, in duplicate, into the wells of a microtitre plate (EIA; Biorad). Two hundred and ninety µl of Protein Assay Reagent (Pierce, U.K) was applied to each sample and mixed by gentle tapping. The absorbance values at 595nm of the protein/dye mixtures were recorded immediately. The absorbance at 595nm of the blank was subtracted from each standard or unknown protein sample absorbance. A standard curve was prepared by plotting the average net absorbance at 595nm for each dilute albumin standard. The standard curve was used to determine the protein concentration for each unknown protein sample.

2.9.1.2 Micro assay procedure.

The micro assay was used for determining protein concentrations in the range 1 to 25µgml^{-1}. This assay was performed as outlined in section 2.9.1.1 with the exception that the standard albumin data points were 1, 5, 10, 15 and 25µgml^{-1}. Each sample was mixed with Protein Assay Reagent (Pierce, U.K) in a 1:1 ratio before reading the absorbance values at 595nm.

2.9.2 Estimation of the concentration of a protein within a mixed population.

A stock albumin solution (Pierce, U.K) was diluted to 1μgμl^{-1} and 100ngμl^{-1} in 2x SDS-PAGE sample buffer (see section 2.7.1). One hundred ng to 1μg, in 100ng incremental rises, plus 2μg and 3μg of albumin were added to the wells of a 10% SDS-PAGE gel (see section 2.7.1). Five μg to 50μg, in 5μg incremental rises of albumin, were added to the wells of a second 10% SDS-PAGE gel. An aliquot of the sample containing the protein of interest was included on each gel, as was an aliquot of high molecular weight markers (Pharmacia).

The two gels were resolved at a constant current of 70mA for 3-4hrs. The gels were stained in coomassie blue to visualise the protein bands (see section 2.7.1). Protein concentrations were estimated by visually comparing the band intensity of the protein of interest to the band intensities of the albumin standards.

2.10 Optimisation of expression of recombinant peptides.

2.10.1 Varying the IPTG concentration and the length of induction time.

To optimise the expression of toxin A peptides, the method outlined in section 2.8.1 was adapted in the following ways.

Aliquots of the recombinant toxin A bacterial culture were grown to an OD_{600} of 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0. Expression was induced in each of the aliquots by the addition of IPTG to final concentrations of 0.1mM, 0.2mM, 0.3mM and 0.4mM with continued incubation at 37°C. For each different sample, expression was stopped after 30mins, 60mins, 90mins, 120mins, 150mins and 180mins by pelleting then resuspending in 2 ×

SDS sample buffer. Each sample was run on an 8% SDS-PAGE gel, stained with coomassie blue and western blotted (see section 2.7.1 and 2.7.3).

2.10.2 Varying the host *E. coli* strain.

Each recombinant plasmid containing a toxin A insert was transformed into electrocompetent BL21 DE3, JM109 and TB1 *E. coli* cells (see section 2.1.5) by electroporation (see section 2.2.9).

Each clone was incubated at 37^0C before expression of the toxin A peptide was induced (see section 2.8.1). Prior to addition of IPTG, 1ml of the cells was removed, pelleted at 11,600 xg for 2mins then resuspended in 0.5ml PBSA (uninduced cells). After incubation with IPTG 1ml of cells was removed, pelleted and resuspended in 0.5ml PBSA (induced cells). Both samples were sonicated in an ice-water bath for bursts of 15secs at amplitude of 8 microns. Two µl of the uninduced and induced samples were spotted onto nitrocellulose (Schleicher and Schull BA85, 0.45µm). The sonicated induced sample was pelleted at 11,600 xg for 2mins and 2µl of the supernatant (induced supernatant fraction) was spotted onto the nitrocellulose. The nitrocellulose was left to air dry and the dot blot was performed (see section 2.7.4). Primary antibodies used are outlined in the relevant sections.

2.11 N-Terminal sequencing of proteins.

The toxin A peptides were cleaved where necessary from the MBP fusion using factor Xa at 4^0C overnight as described above (see section 2.8.3). The protein mixture was resolved on a 10% SDS-PAGE mini gel (see section 2.7.1) then incubated for 5mins in 10mM CAPS (3-[cyclohexylamino]-1-propanesulphonic acid) buffer pH 11. The proteins were

transferred in CAPS buffer onto Hybond PVDF membrane (Amersham International) by western blotting (see section 2.7.3). The PVDF membrane blot was rinsed in SDW and the proteins stained for a few seconds in amido black solution (0.1% napthol black in 5% acetic acid). Excess stain was removed by rinsing in SDW and the protein band of interest was excised from the membrane using a sterile scalpel blade. The protein on the membrane strip was sequenced 'in house' (Dept. of Biochemistry, Queens Medical Centre).

2.12 Electro-elution of proteins from SDS-PAGE gels.

Approximately 500µg of the MBP fusion product purified by amylose affinity chromatography (see section 2.8.2) was cleaved with factor Xa at 4^0C overnight (see section 2.8.3).

The protein mixture was resolved on a 10% SDS-PAGE mini gel alongside a sample of low molecular weight markers (see section 2.7.1). The gel was fixed in 20% methanol for 15 mins. The proteins were stained in 20% methanol, 0.1% coomassie blue for 15mins then destained in 20% methanol for 15mins. Using a sterile scalpel blade and a clean surface, the protein band corresponding to the toxin A peptide of interest was cut from the gel and placed in 3mls of elution buffer (50mM Tris, 50mM glycine, 0.1% SDS) for 15mins.

A small circular disc (14 KDa cut off; Biorad) was softened by incubating in elution buffer at 65^0C for 1 hr. The electroelution tank, glass capillary tube and filter holder (Biorad) were cleaned thoroughly in SDW then equilibrated in elution buffer for 10mins. The electroelution equipment was assembled making sure not to trap air bubbles between the fritt and the membrane. One litre of elution buffer was poured into the assembled tank and the buffer was kept circulating using a magnetic stirrer.

The protein/gel slice was given extra weight by dissolving 1g of sucrose powder into the buffer. The entire sample including the gel slice was loaded into the glass capillary in the electroelution tank. The protein sample was eluted at a constant current of 10mA (per tube to be eluted) for 16 hrs. To dislodge proteins stuck to the membrane, the direction of the current was reversed for 10secs. The equipment was dismantled and the protein-containing fraction under the fritt was pipetted into an eppendorf. The eluted protein sample was analysed by 10% SDS-PAGE (see section 2.7.1).

2.13 Determination of the pI value of proteins by isoelectric focusing (IEF).

The Phastsystem equipment (Pharmacia) was used to determine the pI value of proteins.

Two Broad Range IEF gels (Phastgel pH 3-9; Pharmacia) were pre-run for 75 Vhrs to equilibrate. Two µl droplets of each protein sample were placed in the wells formed in a strip of Parafilm (Alpha Laboratories Ltd.; England). Two µl of Broad Range IEF Markers (Pharmacia) were placed into one of the wells to allow pI determinations to be made. An eight well comb (Pharmacia) was dipped into the protein samples and the comb then gently lowered to touch the surface of the centre of the gel. The same procedure was repeated to produce an identical second gel. The separation and control unit was set to run the gels for a further 425 Vhrs.

At the end of the programme one of the gels was transferred to the development unit. Tubes attached to the equipment (1-9) were submerged into the following buffers respectively: - 20% trichloroacetic acid (100mls); 50% ethanol, 10% glacial acetic acid (500mls); 10% ethanol, 5% glacial acetic acid (500mls); 8.3% glutaraldehyde (100mls); 0.5% silver nitrate (100mls); 12.5% sodium carbonate (40mls), SDW (160mls), formaldehyde (100µl); 5% glacial acetic acid (100mls); 10% glacial acetic acid, 5%

glycerol (100mls); SDW (1,000mls). The chamber was set for developing a silver stain gel and the pre-set programme allowed to proceed.

The second gel was western blotted onto nitrocellulose. The gel loading assembly was removed from the equipment and was replaced with the development unit. Six pieces of 3MM paper (Whatmann), one piece of cellulose acetate (Whatmann) and one piece of nitrocellulose (Schleicher and Schull BA85; 0.45μm) were cut to the size of the gel and pre-soaked in transfer buffer (39mM glycine, 48mM Tris pH 8.3, 5% methanol). Three of the sheets of 3MM and the nitrocellulose were placed onto the western blot platform. Using a cheese wire the gel was loosened from its plastic backing, transferred onto the pre-soaked sheet of cellulose acetate and placed cellulose acetate side down on top of the nitrocellulose on the blotting platform. The three remaining sheets of 3MM were placed on top of the gel. Transfer buffer was applied to the electrode and the equipment was run for 5 Vhrs. The blot was then developed (see section 2.7.3) to locate the protein of interest.

2.14 Ion-exchange chromatography.

When performing ion-exchange chromatography it is important to consider the pI of the protein of interest, the charge of the purification column and the pH of the ion-exchange buffer. A generalised outline of the methodology is provided below.

The protein sample was dialysed (Slide-A-Lyzer Cassette, 10Kda cut off; Pierce) against 5l of 20mM Tris pH 7.5 (for anion-exchange chromatography) or 5L of 50mM phosphate buffer (25mM disodium hydrogen phosphate, 25mM potassium dihydrogen phosphate) pH 7.5 (for cation-exchange chromatography) overnight at 4^0C. The sample was recovered, filtered (0.2μm filters; Acrodisc) and applied to the ion-exchange column via a 10 ml super-loop (Pharmacia). A positively charged anion-exchange column (Mono Q;

Pharmacia) was used for purifying negatively charged proteins and a negatively charged cation-exchange column (Mono-S; Pharmacia) was used for purifying positively charged proteins. The proteins were eluted with a 0-1.0M sodium chloride gradient (made up in the relevant ion-exchange buffer). For each fraction collected, the absorbance at 280nm was determined. Fractions containing protein were analysed by SDS-PAGE (see section 2.7.1).

2.15 Enzyme-linked immunosorbent assay (ELISA).

Protein samples were diluted in 0.05M carbonate buffer pH 9.6 to the final concentration of 5 or 10$\mu g ml^{-1}$. The wells of a 96 well Enzyme Immunoassay plate (EIA; Biorad) were coated in multiples of six with 50µl of the protein samples and incubated at 4^0C overnight. The protein samples were removed and the wells blocked with 200µl of blocking buffer (1% gelatin/PBSA/0.05% Tween-20 [Sigma]) for 1hr at RT. Fifty µl of primary antibody (specific details outlined in the relevant sections) diluted in 0.1% gelatin/PBSA/0.05% Tween-20 (diluent) were applied to three of the six wells coated with each protein sample. The remaining three wells for each sample were coated with 50µl of diluent (conjugate control samples) and all of the wells were incubated for 2 hrs at RT. The plate was washed with 4 x 200µl of PBSA/Tween-20 (PBST) and the wells incubated with 50µl of a 1:1,000 dilution of either goat anti-mouse or goat anti-rabbit (specified in the relevant sections) alkaline phosphatase conjugate (Sigma) for 1 hr at RT. The plate was washed with 4 x 200µl of PBST then 2 x 200µl of SDW before the reaction was developed by applying 50µl of p-nitrophenyl phosphate substrate solution (pNpp; Sigma) to each well. When sufficient colour had developed the absorbency at a wavelength of 405nm was determined using an ELISA plate reader (Labsystems iEMS Reader MF).

Amount of Agarose in Gel (%[w/v])	Efficient range of Separation of Linear DNA Molecules (kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

Table 2.1.

Range of separation of DNA in gels containing different amounts of agarose (taken from Maniatis *et al,* 1982).

Reagents	Percentage Separating Gel 5%	8%	10%	11%	12%	Stacking Gel
Acrylamide (National Diagnostics)	7.5 ml	12 ml	15 ml	16.5 ml	18 ml	1.3 ml
Separating ** Buffer	11.25 ml	11.25 ml	11.25 ml	11.25 ml	11.25 ml	
Stacking Buffer *						2.5 ml
Sterile Water	26.25 ml	21.75 ml	18.75 ml	16.25 ml	15.75 ml	6.1 ml
10% Ammonium persulphate	150µl	150µl	150µl	150µl	150µl	100µl
TEMED*	100µl	100µl	100µl	100µl	100µl	50µl

** 3M Tris-HCl pH 8.8, 0.4% SDS

* 0.5M Tris-HCl pH 6.8, 0.4% SDS

Table 2.2

Reagent volumes required for the preparation of SDS-PAGE gels.

SECTION – 3

PCR AMPLIFICATION, CLONING, EXPRESSION AND PURIFICATION OF THE TOXIN A CONSTRUCTS

SECTION – 3

PCR Amplification, Cloning, Expression and Purification of the Toxin A Constructs.

3.1 SUMMARY

3.2 INTRODUCTION

3.3 MATERIALS AND METHODS

3.3.1 Polymerase chain reaction (PCR) amplification of regions of the toxin A gene

3.3.1.1 PCR amplification of fragment **a1**

3.3.1.2 PCR amplification of fragment **a2**

3.3.1.3 PCR amplification of fragment **b**

3.3.1.4 PCR amplification of fragment **c**

3.3.1.5 PCR amplification of fragment **d**

3.3.1.6 PCR amplification of fragment **e**

3.3.1.7 PCR amplification of fragment **f**

3.3.1.8 PCR amplification of fragment **g**

3.3.1.9 PCR amplification of fragment **h1**

3.3.1.10 PCR amplification of fragment **h2**

3.3.2 Cloning of toxin A PCR products into a T-tailing vector

3.3.3 Sub-cloning of toxin A fragments into expression vectors

3.3.3.1 Sub-cloning of the N-terminal toxin A fragments

3.3.3.2 Sub-cloning of the C-terminal toxin A fragments

3.3.4 DNA sequencing of the cloned toxin A fragments

3.3.5 Induction of expression of recombinant toxin A peptides

3.3.6 Optimisation of expression of toxin A peptides

3.3.6.1 Transforming with rare tRNA encoding plasmids

3.3.6.1.1 Introductory comment

3.3.6.1.2 Materials and methods

3.3.6.2 Varying the host *E. coli* strain

3.3.6.3 Varying the IPTG concentration and length of induction time

3.3.7 Purification and cleavage of recombinant toxin A peptides

3.3.7.1 Purification of MBP fusion peptides

3.3.7.2 Thyroglobulin affinity chromatography

3.3.7.3 Concentration of toxin A peptide H1 by evaporation

3.3.7.4 Factor Xa cleavage of toxin A MBP fusion products

3.3.7.5 Purification of proteins from inclusion bodies present within *E. coli* cells

3.3.7.6 Effect on the formation of inclusion bodies by inducing the expression of peptide H1 at 30^0C or 37^0C

3.3.8 Concentration determinations of the recombinant toxin A peptides

3.3.8.1 Concentration of toxin A peptide / MBP fusion products

3.3.8.2 Concentration of peptide H2 purified from inclusion bodies

3.3.9 Further purification of toxin A peptide H2

3.3.9.1 Introductory comment

3.3.9.2 Thyroglobulin slurry

3.3.9.3 Isoelectric focusing of peptide H2

3.3.9.4 Ion-exchange chromatography

3.3.9.4.1 Introductory comment

3.3.9.4.2 Methodology

3.3.10 Further purification of toxin A peptide B

3.3.10.1 Introductory comment

3.3.10.2 Electro-elution from an SDS-PAGE gel

3.3.10.3 Isoelectric focusing of toxin A peptide B

3.3.10.4 Anion-exchange chromatography

3.3.10.5 Direct cleavage of the peptide B fusion product on an amylose column

3.3.10.6 N-terminal sequencing of the toxin A peptides

3.4 RESULTS

3.4.1 Introductory comment

3.4.2 PCR amplification of the overlapping toxin A fragments

3.4.3 T-tailing of the toxin A fragment PCR products

3.4.4 Sub-cloning into expression vectors

3.4.4.1 N-terminal fragments

3.4.4.2 C-terminal fragments

3.4.5 DNA sequencing from the expression vectors

3.4.6 Induction of expression of toxin A peptides

3.4.6.1 N-terminal peptides

3.4.6.2 C-terminal peptides

3.4.7 Optimisation of expression of toxin A peptides

3.4.7.1 Transforming with rare tRNA encoding plasmids

3.4.7.2 Varying the host *E. coli* strain

3.4.7.3 Varying IPTG concentration and induction time

3.4.8 Purification and cleavage of N-terminal toxin A peptides

3.4.9 Determination of the solubility of the peptide F fusion product

3.4.10 Further purification of toxin A peptide B

3.4.10.1 Introductory comment

3.4.10.2 Electro-elution from an SDS-PAGE gel

3.4.10.3 Iso-electric focusing of toxin A peptide B

3.4.10.4 Anion exchange chromatography

3.4.10.5 Cleavage of the peptide B fusion product on an amylose column

3.4.10.6 N-terminal sequencing of the toxin A peptides

3.4.11 Purification and cleavage of toxin A peptide H1

3.4.11.1 Thyroglobulin affinity chromatography

3.4.11.2 Concentration of peptide H1 by evaporation

3.4.11.3 Determination of the solubility of peptide H1

3.4.11.4 Effect on the formation of inclusion bodies by inducing expression of peptide H1 at 30^0C or 37^0C.

3.4.11.5 Factor Xa cleavage of the peptide H1 fusion product

3.4.12 Purification of toxin A peptide H2

3.4.12.1 Introductory comment

3.4.12.2 Thyroglobulin affinity chromatography

3.4.12.3 Purification of peptide H2 from inclusion bodies

3.4.13 Further purification of toxin A peptide H2

3.4.13.1 Thyroglobulin slurry

3.4.13.2 Iso-electric focusing (IEF) of peptide H2

3.4.13.3 Ion-exchange chromatography

3.5 DISCUSSION

Section - 3

PCR Amplification, Cloning, Expression and Purification of the Toxin A Constructs

3.1 SUMMARY

To study the structure-function relationships of *Clostridium difficile* toxin A it was necessary to produce overlapping peptides spanning the entire toxin A molecule. This section was successful in the PCR amplification of eight N-terminal (a1, a2, b, c, d, e, f and g) and two C-terminal (h1 and h2) overlapping fragments covering the entire toxin A gene. Both fragments h1 and h2 spanned the complete C-terminal repeat end-binding portion of the toxin. Fragments a1, a2, b, c, d, e, f, g and h1 were cloned into the pMal vectors and were shown to be 'in frame' with the *mal E* gene encoding the N-terminal maltose binding protein (MBP) fusion. The C-terminal repeat end fragment, h2, was cloned into the *NcoI* site of the pET 3d vector which bypasses the T7 purification tag.

All of the toxin A fragments, except a1, were successfully expressed in *E. coli* and named peptides A2-H2 respectively. With the exception of peptide E, the size of each recombinant peptide compared to the size estimated from the length of the insert DNA. Peptide E was estimated to be 125.85 kDa but was truncated at 108 kDa. Peptides A2, B, C, D, E, F, G and H1 were purified by amylose affinity chromatography. The purified peptides B, D, E and G were cleaved to completion with 1% factor Xa, whereas peptides A2, C and F were only partially cleaved. Peptide H1 was resistant to factor Xa cleavage and was subsequently replaced by unfused peptide H2 for studies involving the C-terminal repeat region of toxin A. The expression of peptide H2 within inclusion bodies in the *E. coli* cell made purification by thyroglobulin affinity chromatography difficult.

The peptide was successfully purified from inclusion bodies, however, and was found to retain biological activity.

This section was successful in the expression and purification of nine overlapping toxin A peptides. Of these, seven N-terminal peptides (A2, B, C, D, E, F and G) and one C-terminal peptide (H2) were studied throughout the remainder of this thesis. These eight overlapping peptides covered the entire toxin A protein. For a more detailed description of the location of these peptides in the toxin A molecule see appendices 4 and 5.

3.2 INTRODUCTION

Many functions have been attributed to both toxins A and B and have been discussed in full in section 1. Very few structure-function relationships, however, have been made for either toxin, particularly toxin A. For eventual vaccine development, it is essential to understand the way in which these toxins are contributing to *C. difficile* disease. This is where understanding the relationships between the structure and function of toxin A is important.

There are many approaches to studying the structure-function relationships of toxin A. It is possible to cleave the toxin into smaller peptides using proteases such as trypsin or chymotrypsin. These proteases would cleave toxin A into more than thirty peptides by cleaving at arginine or lysine residues (Lyerly, D.M and Wilkins, T.D, 1988). It is also possible to disrupt regions of the toxin A molecule by site-directed mutagenesis then look for a loss of function of the mutated toxin. The method adopted in this thesis for structure-function analysis however was to PCR amplify and clone overlapping fragments of the toxin A gene. These fragments would be expressed in the pMal vectors and purified from *E. coli* making use of the MBP fused to the N-terminus of the

recombinant peptide. This fusion would later be removed by factor Xa cleavage. As the entire toxin A gene has been sequenced (Dove *et al*, 1990), this approach would make it possible to control the exact boundaries of each toxin A peptide. The limitation of these studies is that it cannot be guaranteed that the recombinant peptides will fold in the same way as their complementary regions in the native toxin A molecule. It is also possible that cloning the gene in small sections may result in the disruption of an active site causing the loss of a function. In a given assay, therefore, a positive result is a good indication that a particular peptide is responsible for a function, however a negative result does not rule out the involvement of that region of the toxin in the function in question.

The aim of this section was to produce overlapping toxin A peptides representing the entire toxin A molecule. It was hoped that each peptide would later be assessed for its ability to contribute to particular functions displayed by the native toxin.

3.3 MATERIALS AND METHODS

3.3.1 Polymerase chain reaction (PCR) amplification of regions of the toxin A gene.

Throughout the next few sections, regions of the PCR primers in bold represent an engineered restriction endonuclease site, whereas underlined regions of the PCR primers represent an engineered translational stop codon. For all fragments amplified, 10µl of the product was analysed by 0.8% agarose gel electrophoresis (see section 2.2.3).

3.3.1.1 PCR amplification of fragment **a1**.

The forward primer 5' TATTGCTCTT**GGATCC**ATAAAACAA 3' (1) and the reverse primer 5' CAGAAAGAG**GTCGAC**CAGTCTAATC 3' (2) (see appendix 2) were used in a PCR reaction (see section 2.3) to amplify a 1.306 Kb fragment (a1) of the toxin A gene covering base pairs (bps) 324 - 1,630. The thermocycling conditions were denaturation at 96^0C for 1min, annealing at 57^0C for 1min and extension at 72^0C for 2mins.

3.3.1.2 PCR amplification of fragment **a2**.

Due to the failure of expression of peptide A1 (see section 3.4.6.1), this region of the toxin A gene was reamplified using a different forward primer to produce fragment a2.

The forward primer 5' **GGATCC**ATGTCTTTAATATCTAAAGAAG 3' (3) and the reverse primer (2) (see appendix 2) were used in a PCR reaction (see section 2.3) to amplify a 1.630 Kb DNA fragment (a2) of the toxin A gene covering bps 1 - 1,630. The thermocycling conditions were denaturation at 96^0C for 1min, annealing at 51^0C for 1 min and extension at 72^0C for 2mins.

3.3.1.3 PCR amplification of fragment **b**.

The forward primer 5' TATACTGGT**GGATCC**CTTTCTGAAG 3' (4) and the reverse primer 5' CATTTTCAA**GTCGAC**TGAACTAATC 3' (5) (see appendix 2) were used in a PCR reaction (see section 2.3) to amplify a 0.99Kb DNA fragment (b) of the toxin A gene covering bps 1,609 - 2,599. The thermocycling conditions were denaturation at 97^0C for1 min, annealing at 50^0C for1 min and extension at 72^0C for 1.5mins.

3.3.1.4 PCR amplification of fragment **c**.

The forward primer (1) and the reverse primer (5) (see appendix 2) were used in a PCR reaction (see section 2.3) to amplify a 2.275Kb DNA fragment (c) of the toxin A gene covering bps 324 - 2,599. The thermocycling conditions were denaturation at 97^0C for 1 min, annealing at 50^0C for 1min and extension at 72^0C for 3mins.

3.3.1.5 PCR amplification of fragment **d**.

The forward primer 5' ACTTGAAAAT**GGATCC**GATGAATTA 3' (6) and the reverse primer 5' TTTATATTC**GTCGAC**ATTGGCTATG 3' (7) (see appendix 2) were used in a PCR reaction (see section 2.3) to amplify a 1.424Kb DNA fragment (d) of the toxin A gene covering bps 2,589 - 4,013. The thermocycling conditions were denaturation at 96^0C for 1min, annealing at 54^0C for 1min and extension at 72^0C for 2mins.

3.3.1.6 PCR amplification of fragment **e**.

The forward primer (4) and the reverse primer (7) (see appendix 2) were used in a PCR reaction (see section 2.3) to amplify a 2.404Kb DNA fragment (e) of the toxin A gene

covering bps 1,609 - 4,013. The thermocycling conditions were denaturation at 97^{0}C for 1min, annealing at 52^{0}C for 1min and extension at 72^{0}C for 3mins.

3.3.1.7 PCR amplification of fragment **f**.

The forward primer 5' GGAGCAGGA**GGATCC**TACTCTTTAT 3' (8) and the reverse primer 5' TATATTGATT**AAGCTT**TAAACTAAT 3' (9) (see appendix 2) were used in a PCR reaction (see section 2.3) to amplify a 1.553Kb DNA fragment (f) of the toxin A gene covering bps 3,955 - 5,508. The thermocycling conditions were denaturation at 97^{0}C for 1min, annealing at 44^{0}C for 1min and extension at 72^{0}C for 2mins.

3.3.1.8 PCR amplification of fragment **g**.

The forward primer (6) and the reverse primer (9) (see appendix 2) were used in a PCR reaction (see section 2.3) to amplify a 2.919Kb DNA fragment (g) of the toxin A gene covering bps 2,589 - 5,508. The thermocycling conditions were denaturation at 96^{0}C for 1min, annealing at 44^{0}C for 1min and extension at 72^{0}C for 3.5mins.

3.3.1.9 PCR amplification of fragment **h1**.

The forward primer 5' AATTAGTTAA**AGGATCC**ATCAATAT 3' (10) and the reverse primer 5' CTCGAAAA**GTCGACC**AGCTTAAGCC 3' (11) (see appendix 2) were used in a PCR reaction (see section 2.3) to amplify a 2.587Kb DNA fragment (h1) of the toxin A gene covering bps 5,483 - 8,070. The thermocycling conditions were denaturation at 96^{0}C for 1min, annealing at 51^{0}C for 1min and extension at 72^{0}C for 3mins. Ten μl of the amplified product was analysed by 0.8% agarose gel electrophoresis (see section 2.2.3).

3.3.1.10 PCR amplification of fragment **h2**.

Due to failure of Factor Xa cleavage of peptide H1 (see section 3.4.11.5), fragment h2 was amplified for cloning into an alternative expression vector. The forward primer 5' **CCATGGGATTAATCAATATAAATAATTCA** 3' (12) and the reverse primer (11) (see appendix 2) were used in a PCR reaction (see section 2.3) to amplify a 2.576Kb DNA fragment (h2) of the toxin A gene covering bps 5,495 - 8,070. The thermocycling conditions were denaturation at 96^0C for 1min, annealing at 50^0C for 1min and extension at 72^0C for 3mins.

3.3.2 Cloning of toxin A PCR products into a T-tailing vector.

The PCR products amplified in section 3.3.1 were purified from agarose gels (see section 2.2.4), ligated into the pCRII T-tailing vector (Invitrogen; see section 2.4.1) then transformed into Inv αF' competent cells (Invitrogen; see section 2.4.2). Recombinant plasmid DNA was isolated (see section 2.2.2.1) from selected white colonies, and the purity checked by agarose gel electrophoresis (see section 2.2.3).

Plasmids thought to contain toxin A fragments a1, a2, b, c, d, e and h1 were digested with the restriction enzymes *Bam*HI and *Sal*I (see section 2.2.6) and the DNA profiles were analysed by 0.8% agarose gel electrophoresis (see section 2.2.3) to identify clones with the correct sized inserts. Similarly plasmids thought to contain toxin A fragments f and g were analysed following digestion with *Bam*HI and *Hind*III (see section 2.2.6) whereas the plasmid thought to contain fragment h2 was analysed following digestion with *Nco*I (see section 2.2.6). Digestion of a plasmid containing an inverted h2 insert would produce two bands of 1.586 Kb and 5.177Kb, whereas a correct orientation would produce two bands of 4.154 Kb and 2.609 Kb. Overnight cultures (see section 2.1.1) of the host cells

containing plasmids with the desired inserts were mixed 1:1 with 60% sterile glycerol and stored at -70^0C.

3.3.3 Sub-cloning of toxin A fragments into expression vectors.

3.3.3.1 Sub-cloning of the N-terminal toxin A fragments.

The pCRII plasmids containing toxin A fragments a1, a2, b, c, d, e, f and g were isolated from their host *E. coli* cells (see section 2.2.2.2).

Plasmids containing fragments a1, a2, b, c, d and e were digested at 37^0C for 2 hrs with the restriction enzymes *Bam*HI and *Sal*I (see section 2.2.6) and the DNA profiles were analysed by 0.8% agarose gel electrophoresis (see section 2.2.3). The 1.281Kb, 1.616Kb, 0.967Kb, 2.25Kb, 1.4Kb and 2.382Kb DNA fragments corresponding to toxin A inserts a1, a2, b, c, d and e respectively, were purified from the agarose gels (see section 2.2.4).

The pMal^{TM}- p2 and pMal^{TM}- c2 MBP fusion vectors (New England Biolabs Ltd. U.K; see appendix 3) were digested with *Bam*HI and *Sal*I (see section 2.2.6), analysed by 0.8% agarose gel electrophoresis (see section 2.2.3) then purified from the agarose gel (see section 2.2.4). The purified *Bam*HI, *Sal*I digested toxin A fragments were ligated (see section 2.2.7) into the *Bam*HI, *Sal*I digested pMal-p2 and pMal-c2 expression vectors.

The pCRII plasmids containing toxin A fragments f and g were treated as described with the following exceptions. The 1.528Kb and 2.893Kb DNA fragments corresponding to toxin A inserts f and g were excised from the pCRII vector by digestion, at 37^0C for 2 hrs, with the restriction enzymes *Bam*HI and *Hind*III (see section 2.2.6). These DNA inserts were ligated (see section 2.2.7) into the *Bam*HI, *Hind*III digested pMal-p2 and pMal-c2 expression vectors (New England Biolabs Ltd. U.K).

The ligation reactions containing fragments a1, b and f were transformed (see section 2.2.8) into $CaCl_2$ competent TB1 *E. coli* host cells (New England Biolabs Ltd.; see section 2.1.1). Ligation reactions containing fragments a2, c, d, e and g were transformed (see section 2.2.8) into $CaCl_2$ competent JM109 *E. coli* host cells (see sections 2.1.1 and 2.1.4). Two hundred µl aliquots of the transformation reactions were spread onto the surface of LB agar plates containing $100\mu gml^{-1}$ of ampicillin (LB Amp). Following incubation at 37^0C overnight, colonies were selected and restreaked onto both a master LB Amp plate and an LB Amp plate containing $80\mu gml^{-1}$ X-gal and 0.1mM IPTG. Following incubation at 37^0C for 8-16 hrs the lac phenotype of each clone was determined on the X-gal plate. White colonies were recovered from the master plate and the recombinant plasmid DNA was isolated (see section 2.2.2.1). Plasmids containing toxin A inserts were identified by digesting with the restriction enzymes (see section 2.2.6) *Bam*HI and *Sal*I (fragments a1, a2, b, c, d and e) or *Bam*HI and *Hind*III (fragments f and g) then analysing the DNA profiles on 0.8% agarose gels (see section 2.2.3).

Toxin A fragment a2 was also sub-cloned as a *Bam*HI fragment into a *Bam*HI digested (see section 2.2.6) pET3a vector (Novagen Inc.). The recombinant plasmid was transformed (see section 2.2.8) into $CaCl_2$ competent BL21 DE3 cells (see section 2.1.4), plated onto LB Amp plates then incubated at 37^0C overnight. Plasmid DNA was isolated from ten colonies (see section 2.2.2.1). Digesting with *Bam*HI (see section 2.2.6) then analysing the DNA profiles by 0.8% agarose gel electrophoresis (see section 2.2.3) identified plasmids with an insert. Overnight cultures (see section 2.1.1) of the cells containing plasmids with the desired inserts were mixed 1:1 with 60% sterile glycerol and stored at -70^0C.

3.3.3.2 Sub-cloning of the C-terminal toxin A fragments.

The pCRII plasmid containing toxin A fragment h1 was isolated using the method outlined in section 3.3.2. The recombinant plasmid and the pMal-p2 expression vector were digested at 37^0C for 2 hrs with the restriction enzymes *Bam*HI and *Sal*I (see section 2.2.6) before analysing the DNA profiles by 0.8% agarose gel electrophoresis (see section 2.2.3). The 2.562Kb and 6.721Kb DNA fragments, corresponding to the *Bam*HI, *Sal*I digested toxin A fragment h1 and pMal-p2 vector respectively, were purified from the agarose gel as outlined in section 2.2.4. The two DNA fragments were ligated together (see section 2.2.7) then transformed (see section 2.2.8) into $CaCl_2$ competent TB1 host cells (New England Biolabs; see section 2.1.4). Two hundred µl aliquots of the transformation reaction were spread onto the surface of LB Amp plates and incubated at 37^0C overnight. Colonies were selected and re-streaked onto a master LB Amp plate and a LB Amp plate containing 80µgml^{-1} X-gal and 0.1mM IPTG. Following incubation at 37^0C for 8-16 hrs the lac phenotype of each clone was determined on the X-gal plate. White colonies were recovered from the master plate and the recombinant plasmid isolated (see section 2.2.2.2). A plasmid containing toxin A fragment h1 was identified by digesting with the restriction enzymes *Bam*HI and *Sal*I (see section 2.2.6) then analysing 10µl of the DNA profile by 0.8% agarose gel electrophoresis (see section 2.2.3).

Due to insufficient factor Xa cleavage of the peptide H1 fusion protein (see section 3.4.11.5), fragment h was re-amplified using a second set of PCR primers to produce fragment h2 (see section 3.3.1.10). Following cloning of fragment h2 into the pCRII T-tailing vector (see section 3.3.2), the recombinant plasmid containing an inverted insert was isolated (section 2.2.2.2). The pCRII plasmid containing fragment h2 and the pET3d

expression vector (Novagen Inc; see appendix 3) were digested with the restriction enzymes *Nco*I and *Bam*HI (see section 2.2.6) at 37^{0}C for 2 hrs. The DNA profiles were analysed by 0.8% agarose gel electrophoresis (see section 2.2.3). The 2.568Kb and 4.5Kb DNA fragments, corresponding to the *Nco*I, *Bam*HI digested fragment h2 and pET3d vector respectively, were purified from the agarose gel (see section 2.2.4). The two DNA fragments were ligated (see section 2.2.7) then transformed (see section 2.2.9) into electrocompetent *E. coli* BL21 DE3 cells (see sections 2.1.1 and 2.1.5). Two hundred µl aliquots of the transformation reaction were plated onto LB Amp plates and incubated at 37^{0}C overnight. Plasmid DNA was isolated from ten selected colonies as outlined in section 2.2.2.1. Plasmids with the h2 insert were identified by digesting with the restriction enzymes *Nco*I and *Bam*HI (see section 2.2.6) then analysing the DNA profiles by 0.8% agarose gel electrophoresis (see section 2.2.3).

Overnight cultures (see section 2.1.1) of host cells containing a plasmid with either fragment h1 or h2 were mixed 1:1 with 60% sterile glycerol and stored at –70^{0}C.

3.3.4 DNA sequencing of the cloned toxin A fragments.

The toxin A DNA fragments cloned in sections 3.3.3.1 and 3.3.3.2 were sequenced (see section 2.5). DNA fragments cloned into the pMal^{TM} vectors were sequenced using the forward Mal E primer (New England Biolabs) and the reverse M13 primer (New England Biolabs). Toxin A fragments cloned into pET^{TM} vectors (Novagen Inc.) were sequenced using the forward primer 5' CGACTCACTATAGGGAGA 3' and the reverse primer 5' TAGTTATTGCTCAGCGGT 3'. The additional primers 5' ATGTCATTATCTATAGCTGC 3' (eInt1; bps 3217-3236) and 5'

AATAATTCGATAAAACTAGG 3'(eInt2; bps 3480-3499) were also used to sequence further into the toxin A fragment e insert.

The sequence profiles obtained were compared to the known toxin A sequence data (Dove *et al*, 1990).

3.3.5 Induction of expression of recombinant toxin A peptides.

IPTG was used to induce expression of the toxin A peptides in their host *E. coli* strains (see section 2.8.1) with the same strains containing plasmids without inserts included as negative controls. The protein profiles were analysed by 8% SDS-PAGE (see section 2.7.1) and western blotting (see section 2.7.3). Western blots containing potential toxin A peptides A1, A2, B, C, D, E, F and G (resulting from the induction of plasmids containing toxin A fragments a1, a2, b, c, d, e, f and g respectively) were probed with a 1:10,000 dilution of anti-MBP antiserum (New England Biolabs) followed by a 1:1,000 dilution of goat anti-mouse alkaline phosphatase conjugate (Sigma). Western blots containing toxin A peptides H1 and H2 (resulting from the induction of plasmids containing toxin A fragments h1 and h2 respectively) were probed with 3μgml^{-1} of the monoclonal antibody PCG-4 followed by a 1:1,000 dilution of goat anti-mouse alkaline phosphatase conjugate (Sigma).

3.3.6 Optimisation of expression of toxin A peptides.

3.3.6.1 Transforming with rare tRNA encoding plasmids.

3.3.6.1.1 Introductory comment.

For the successful expression of recombinant genes it is essential that the codon usage of the foreign protein is compatible with that of the host strain. A characteristic feature of

clostridial DNA is its low G and C base content (Hill *et al*, 1966), which in the coding region of the *C. difficile* toxin A gene is only 26.9 mol% (Dove *et al*, 1990). The codon usage of clostridial genes is therefore, biased towards codons in which A and U predominate. Certain codons (e.g. AGA for arginine) preferentially used in *C. difficile* genes are extremely rare in *E. coli* gene expression (see appendix 6). Problems with premature termination of translation of these AT rich recombinant peptides can occur due to the low levels of the cognate tRNAs that recognise these rare codons in *E. coli*.

A pBR322 based plasmid carrying the genes encoding the tRNAs that recognise the AGA and AGG arginine codons was made available from SmithKline Beecham. By introducing this plasmid, and thus these rare tRNAs, into the host *E. coli* cell it was hoped that the expression of the toxin A peptides would be improved.

3.3.6.1.2 Materials and methods.

In an attempt to improve the expression of toxin A peptides A2, C, D, E and H1 from the pMalp2 vector and H2 from the pET3d vector, each was expressed in the presence of the plasmid pDC952. This plasmid contains the *argU* gene encoding the rare tRNAs for the AGA/AGG codons. Dr. Shatzman from SmithKline Beecham Pharmaceuticals kindly supplied the pBR322 based plasmid pDC952.

The recombinant plasmids containing toxin A inserts and the pDC952 plasmid were isolated from their host *E. coli* strains (see section 2.2.2.1) and the DNA concentrations were determined (see section 2.2.1.1).

For each toxin A fragment, electrocompetent *E. coli* BL21 DE3 (see section 2.1.5) were transformed by electroporation (see section 2.2.9) with 100ng of the recombinant toxin A plasmid plus 100ng of pDC952 or 100ng of the toxin A plasmid only. Transformants

containing both plasmids were selected for on LBA plates containing 100μgml^{-1} of ampicillin and 35μgml^{-1} of chloramphenicol. Transformants containing the recombinant toxin A plasmids were selected for on LBA plates containing only ampicillin.

Expression of the toxin A peptide from each clone was induced as described in section 2.8.1, with the exception that clones containing plasmid pDC952 were grown in the presence of ampicillin and 35μgml^{-1} of chloramphenicol. Levels of expression of the toxin A peptides with and without the presence of the rare arginine encoding tRNAs were compared by 8% SDS-PAGE (see section 2.7.1) and western blotting (see section 2.7.3). Blots containing peptides A2, C, D and E were probed with a 1:10,000 dilution of anti-MBP antiserum (New England Biolabs) followed by a 1:1,000 dilution of goat anti-rabbit alkaline phosphatase conjugate (Sigma). Blots containing peptides H1 and H2 were probed with 3μgml^{-1} of the monoclonal antibody PCG-4 followed by a 1:1,000 dilution of goat anti-mouse alkaline phosphatase conjugate (Sigma).

3.3.6.2 Varying the host *E. coli* strain.

In an attempt to increase the expression of toxin A peptides A, C, E, H1 and H2, each was transformed into different *E. coli* hosts (see section 2.10.2).

Blots containing peptides A, C and E were probed with a 1:10,000 dilution of anti-MBP antiserum (New England Biolabs) followed by a 1:1,000 dilution of goat anti-rabbit alkaline phosphatase conjugate (Sigma). Blots containing peptides H1 and H2 were probed with 3μgml^{-1} of the monoclonal antibody PCG-4 followed by a 1:1,000 dilution of goat anti-mouse alkaline phosphatase conjugate (Sigma).

3.3.6.3 Varying the IPTG concentration and length of induction time.

In an attempt to increase the expression of toxin A peptides A, C, H1 and H2, the IPTG concentration and length of induction time were varied for each peptide (see section 2.10.1). Samples were resolved on 8% SDS-PAGE gels (see section 2.7.1) and western blotted (see section 2.7.3).

Blots containing peptides A and C were probed with a 1:10,000 dilution of anti-MBP antiserum (New England Biolabs) followed by a 1:1,000 dilution of goat anti-rabbit alkaline phosphatase conjugate (Sigma). Blots containing peptides H1 and H2 were probed with $3\mu gml^{-1}$ of the monoclonal antibody PCG-4 followed by a 1:1,000 dilution of goat anti-mouse alkaline phosphatase conjugate (Sigma).

3.3.7 Purification and cleavage of recombinant toxin A peptides.

3.3.7.1 Purification of MBP fusion peptides.

Recombinant toxin A fusion peptides A2, B, C, D, E, F, G and H1 were purified by amylose affinity chromatography (see section 2.8.2) and the concentrations of the peptides in the 3ml eluted fractions determined (see section 2.9.1.1). The fractions collected were resolved on an 8% SDS-PAGE gel (see section 2.7.1) and western blotted (see section 2.7.3). Blots were probed with a 1:10,000 dilution of anti-MBP antiserum (New England Biolabs) followed by a 1:1,000 dilution of goat anti-rabbit alkaline phosphatase conjugate (Sigma). Bacterial cells containing the pMal vector without an insert were treated in the same way and the MBP purified was used as a negative control in all experiments.

3.3.7.2 Thyroglobulin affinity chromatography.

Recombinant toxin A peptides H1 and H2 were purified by thyroglobulin affinity chromatography (see section 2.6.2) with the following modifications. One litre of induced cells containing toxin A peptide H1 or H2 was prepared (see section 2.8.2). Expression of peptide H1 was induced by the addition of IPTG to the final concentration of 0.3mM whereas peptide H2 was induced with 0.4mM IPTG. The bacteria were harvested by centrifugation at 4,000 xg for 20mins, re-suspended in 50mls of ice-cold TBS then re-centrifuged as above. The pellet was re-suspended in 50mls of ice-cold TBS then stored at -20^0C overnight. The cells were thawed in cold water then disrupted by sonication (see section 2.8.2). The sonicate containing toxin A peptide H1 or H2 was then applied to, and eluted from the thyroglobulin column (see section 2.6.2). The concentration of protein in the eluted fractions was determined (see section 2.9.1.2) along with the ability of the protein to haemagglutinate rabbit red blood cells (see section 2.6.4). The protein in the fractions collected was resolved by 8% SDS-PAGE (see section 2.7.1) and transferred to nitrocellulose by western blotting (see section 2.7.3). The blots were probed with 3μgml^{-1} of the monoclonal antibody PCG-4 followed by a 1:1,000 dilution of goat anti-mouse alkaline phosphatase conjugate (Sigma).

3.3.7.3 Concentration of toxin A peptide H1 by evaporation.

Toxin A peptide H1 was purified by thyroglobulin affinity chromatography (see section 3.3.7.2). Two 5ml fractions containing peptide H1 were pooled and a 100µl aliquot was removed for further analysis. The remaining 10mls of the protein sample was concentrated by bringing the volume down from 10mls to 1ml by evaporation at 60^0C (Speed Vac Plus SC21OA; Saivant).

The ability of the protein to haemagglutinate rabbit red blood cells, prior to and after evaporation, was determined (see section 2.6.4). Two µl of each sample was spotted onto nitrocellulose for dot blot analysis (see section 2.7.4) and subsequently probed with 3µgml^{-1} of the monoclonal antibody PCG-4 followed by a 1:1,000 dilution of goat anti-mouse alkaline phosphatase conjugate (Sigma).

3.3.7.4 Factor Xa cleavage of toxin A / MBP fusion products.

Toxin A fusion peptides B, D, E, F, and G were cleaved with 1% factor Xa whereas toxin A fusion peptides A, C and H1 were cleaved with both 1% and 2% factor Xa (see section 2.8.3). The cleaved and uncleaved products were resolved in adjacent lanes on 8% SDS-PAGE gels (see section 2.7.1). One gel containing peptides A, B, C, D, E, F and G was stained with coomassie blue (see section 2.7.1) whereas one gel containing peptide H1 was silver stained (see section 2.7.2). A second gel containing peptides A, B, C, D, E, F and G was western blotted (see section 2.7.3) and probed with a 1:10,000 dilution of anti-MBP antiserum (New England Biolabs) followed by a 1:1,000 dilution of goat anti-rabbit alkaline phosphatase conjugate (Sigma). A second gel containing peptide H1 was western blotted and probed with 3µgml^{-1} of the monoclonal antibody PCG-4 followed by a 1:1,000 dilution of goat anti-mouse alkaline phosphatase conjugate (Sigma).

3.3.7.5 Purification of proteins from inclusion bodies present within *E. coli* cells.

The method outlined in section 2.8.4 was used to determine the solubility of toxin A peptides F, H1 and H2.

The method outlined in section 2.8.5 was then used to purify peptides F, H1 and H2 from inclusion bodies present within *E. coli* cells. Forty µl samples, of the soluble sonicate

prior to and the solubilised pellet after guanidine hydrochloride treatment, were resolved on an 8% SDS-PAGE gel (see section 2.7.1) and western blotted (see section 2.7.3). Blots containing peptide F were probed with a 1:10,000 dilution of anti-MBP antiserum (New England Biolabs) followed by a 1:1,000 dilution of goat anti-rabbit alkaline phosphatase conjugate (Sigma). Blots containing peptides H1 and H2 were probed with $3\mu gml^{-1}$ of the monoclonal antibody PCG-4 followed by a 1:1,000 dilution of goat anti-mouse alkaline phosphatase conjugate (Sigma). Bacterial cells containing the expression vector without an insert were also treated as described and the *E. coli* proteins purified were used as a negative control in all experiments.

3.3.7.6 Effect on the formation of inclusion bodies by inducing the expression of peptide H1 at 30^0C or 37^0C.

An attempt was made to prevent toxin A peptide H1 being placed into inclusion bodies within the *E. coli* host cell. BL21 DE3 cells containing pMal-p2 with the fragment h1 insert were treated as described in section 2.8.4 with the exception that one aliquot was incubated and induced at 37^0C whilst a second aliquot was incubated and induced at 30^0C. Induced soluble and insoluble fractions from each temperature were resolved on 8% SDS-PAGE gels (see section 2.7.1) and western blotted (see section 2.7.3). The blot was probed with $3\mu gml^{-1}$ of the monoclonal antibody PCG-4 followed by a 1:1,000 dilution of goat anti-mouse alkaline phosphatase conjugate (Sigma).

3.3.8 Concentration determinations of the recombinant toxin A peptides.

3.3.8.1 Concentration of toxin A peptide / MBP fusion products.

The concentration of the full-length peptide A2, B, C, D, E, F, G and H1 / MBP fusion products were determined (see sections 2.9.1.1 and 2.9.1.2. The toxin A peptide fusion products were cleaved with factor Xa (see section 3.3.7.4). Fifty µl of the cleavage reaction was resolved on an 8% SDS-PAGE gel (see section 2.7.1) alongside 50µl of the uncleaved product and the gel was stained with coomassie blue (see section 2.7.1). The concentration of the toxin A peptide present within the cleavage mixture was estimated as a percentage of the calculated concentration of total fusion protein.

3.3.8.2 Concentration of peptide H2 purified from inclusion bodies.

The concentration of full-length peptide H2 was determined (see section 2.9.2). Forty µl of the peptide H2 protein preparation purified in section 3.3.7.5 was resolved on an 8% SDS-PAGE gel (see section 2.7.1) alongside the albumin standards of known concentration. The gel was stained with coomassie blue and the concentration of full-length peptide H2 was estimated by visual comparison to the albumin standards.

3.3.9 Further purification of toxin A peptide H2.

3.3.9.1 Introductory comment.

When toxin A peptide H2 was isolated from inclusion bodies (see section 3.3.7.5), a few *E. coli* proteins were co-purified. The following procedures were performed in an attempt to further purify peptide H2.

3.3.9.2 Thyroglobulin slurry.

A 1ml sample of the peptide H2 preparation (see section 3.3.7.5) was dialysed (Slide-A-Lyzer Cassette, 10,000Da cut off; Pierce) against 2 x 1 litre of TBS. A 0.5ml fraction of

the dialysed protein sample was incubated with 2mls of activated bovine thyroglobulin (see section 2.6.2) on a rotator at 4^0C overnight. The excess protein sample was removed and stored at 4^0C (unbound protein). The thyroglobulin was washed at 4^0C with 6 x 2mls of TBS with intermittent centrifugation steps at 6,500 xg for 1 min (wash).

Bound peptide H2 was eluted from the thyroglobulin by applying 0.5ml of TBS (pre-incubated at 37^0C) and incubating rotating at 37^0C for 2.5 hrs. The slurry was centrifuged at 6,500 xg for 30 secs and the supernatant containing peptide H2 was aspirated (thyroglobulin eluted protein).

Aliquots of the unbound protein, the wash and the thyroglobulin eluted protein were tested for their abilities to haemagglutinate rabbit red blood cells (see section 2.6.4) and were compared to the H/A titre of the original peptide H2 preparation.

3.3.9.3 Isoelectric focusing (IEF) of peptide H2.

The isoelectric point (pI value) of peptide H2 was determined by applying a 1µl sample of the peptide (see section 3.3.7.5) to the centre of two Broad Range IEF gels (Biorad). The gels were processed (see section 2.13) and the resulting western blot was probed with 3µgml^{-1} of the monoclonal antibody PCG-4 followed by a 1:1,000 dilution of a goat anti-mouse alkaline phosphatase conjugate (Sigma) (see section 2.7.3).

3.3.9.4 Ion-exchange chromatography.

3.3.9.4.1 Introductory comment.

Separation in ion-exchange chromatography depends upon the reversible adsorption of charged solute molecules to an immobilised ion-exchange group of opposite charge.

Separation is obtained since different substances have different degrees of interaction with the ion exchanger due to differences in their charges and charge densities.

Positively charged exchangers (e.g. Mono Q columns) have negatively charged counter ions (anions) available for exchange and so are termed anion exchangers. Proteins with a net negative charge (i.e. a low pI value) are therefore, preferentially purified on these positively charged exchangers. Negatively charged exchangers (e.g. Mono S columns) on the other hand, have positively charged counter ions (cations) and are termed cation exchangers. Proteins with a net positive charge (i.e. a high pI value) are therefore, more easily purified by cation exchange chromatography. When the pH of the buffer system is the same as the pI of a protein, the protein has no net charge (amphoteric molecule) and will not bind to either column. In these circumstances it is necessary to alter the pH of the buffer to give the protein a net charge. At a pH below its isoelectric point (pI) a protein has a net positive charge and will adsorb to cation exchangers. When the pH is above the pI therefore, a protein has a net negative charge and will adsorb to anion exchangers.

3.3.9.4.2 Methodology.

Taking the pI value of peptide H2 to be approximately 9.0 (see section 3.4.13.2), attempts were made to purify peptide H2 by cation-exchange chromatography (see section 2.14). In separate runs the buffer systems applied were 50mM phosphate buffer pH 7.5 and 6.0.

Taking the pI value of peptide H2 to be approximately 6.5 (see section 3.4.13.2), attempts were made to purify peptide H2 by anion-exchange chromatography (see section 2.14). In separate runs the buffers systems applied were 20mM Tris-HCl pH 7.5 and 8.5. After each run the samples eluted with the 0-1M salt gradient were screened for the presence of

peptide H2 using the H/A assay (see section 2.6.4). Fractions showing a positive H/A titre were analysed on silver stained 8% SDS-PAGE gels (see section 2.7.2).

3.3.10 Further purification of Toxin A peptide B.

3.3.10.1 Introductory comment.

Following factor Xa cleavage of the maltose binding protein (MBP) fusion products (see section 3.3.7.4), the toxin A peptide preparations were contaminated with the 43 kDa MBP. The following procedures were undertaken to remove the MBP from the toxin A peptide preparations. The toxin A peptide B fusion protein has been used as an example.

3.3.10.2 Electro-elution from a SDS-PAGE gel.

Approximately 500µg of the toxin A peptide B / MBP fusion product was cleaved with factor Xa at 4^0C overnight (see section 3.3.7.4). Ten µl of the protein mixture were resolved on a 10% SDS-PAGE mini gel (see section 2.7.1) alongside an aliquot of low molecular weight (LWT) markers (Pharmacia). The 35 kDa protein corresponding to toxin A peptide B was electro-eluted from the gel (see section 2.12). A 40µl aliquot of the eluted peptide was analysed by 10% SDS-PAGE (see section 2.7.1) and the remainder was stored at -20^0C.

3.3.10.3 Isoelectric focusing (IEF) of toxin A peptide B.

The toxin A peptide B / MBP fusion product was cleaved using factor Xa (see section 3.3.7.4) and an attempt was made to determine the pI value of peptide B (see section 2.13). One µl samples of the cleavage mixture and of MBP alone were applied to the centre of the Broad Range IEF gel (Biorad) (see section 2.13). The protein band in the

cleavage mixture that did not correspond to the MBP was taken to be the pI value of toxin A peptide B.

3.3.10.4 Anion Exchange chromatography.

One ml of a 1mgml^{-1} solution of the toxin A peptide B fusion product was cleaved with 1% factor Xa (see section 3.3.7.4). To separate peptide B from the MBP, the cleaved mixture was applied to a Mono-Q anion exchange column (Pharmacia) (see section 2.14). In separate runs the buffer systems used were 20mM Tris-HCl pH 6.0, 7.5 and 8.0. After each run, 40µl aliquots of the samples eluted with the salt gradient that corresponded to a protein absorbance peak on the printout were analysed by 10% SDS-PAGE (see section 2.7.1).

3.3.10.5 Direct cleavage of the peptide B fusion product on the amylose column.

The toxin A peptide B / MBP fusion product produced from one litre of induced cells was applied to an amylose affinity column (see section 2.8.2). The column was washed with 200mls of column buffer (20mM Tris-HCl, 200mM NaCl, 1mM EDTA), saturated with 12mls of column buffer containing 150µg of factor Xa, then sealed and incubated at 4^{0}C overnight. Toxin A peptide B was eluted in 20 x 1ml fractions of column buffer. The bound MBP was eluted in 9 x 1ml fractions of column buffer containing 10mM maltose. Forty µl aliquots of the eluted fractions were analysed for protein content by 8% SDS-PAGE (see section 2.7.1) and western blotting (see section 2.7.3). Blots were probed with a 1:10,000 dilution of anti-MBP antiserum (New England Biolabs) followed by a 1:1,000 dilution of a goat anti-rabbit alkaline phosphatase conjugate (Sigma).

3.3.10.6 N-terminal sequencing of the toxin A peptides.

One $mgml^{-1}$ solutions of the toxin A peptide B, D and E fusion products, purified by amylose affinity chromatography (see section 3.3.7.1), were cleaved with 1% factor Xa (see section 3.3.7.4). Each cleaved peptide was applied in 10µl aliquots across the wells of a 10% SDS-PAGE mini-gel (see section 2.7.1). The protein bands were transferred onto Hybond PVDF membrane and stained with amido black (see section 2.11). Membrane strips containing the 35 kDa, 49 kDa and 65 kDa proteins, corresponding to peptides B, D and E respectively, were excised from the PVDF membrane and air-dried. The N-terminal sequences of the proteins on the strips were determined (see section 2.11) and were compared to the known toxin A amino acid sequence data (Dove *et al,* 1990).

3.4 RESULTS

3.4.1 Introductory comment

Due to the large number of toxin A fragments that were cloned, expressed and purified, it was impractical to include the controls for every experiment in the figures although they were included at the time the experiments were performed. The figures for toxin A fragment b, however, show all the controls and have been included as a representation.

3.4.2 PCR amplification of the overlapping toxin A fragments

Using the primers and thermocycling conditions outlined in section 3.3.1, eight N-terminal (a1, a2, b, c, d, e, f and g) and two C-terminal (h1 and h2) fragments of the toxin A gene were successfully amplified using the polymerase chain reaction (PCR).

A PCR reaction using primers 4 and 5 (see appendix 2) at a concentration of 10ng/µl was successful in amplifying a 0.99 Kb DNA product (bps 1,609-2,599 of the toxin A gene; see appendix 1) corresponding to toxin A fragment b (see figure 3.1, lane 2). At a concentration of 5ng/µl, however, the primers failed to amplify this product (figure 3.1 lane 3). The reactions containing either only primer 4 or primer 5 showed no amplification suggesting that the 0.99Kb fragment was the result of an amplification using both primers (figure 3.1 lanes 4 and 5). When VPI template DNA was omitted from the reaction there was no amplification of a DNA fragment (figure 3.1 lane 6).

Primers 1 and 2 (see appendix 2) were successfully used to amplify a 1.306Kb DNA product (bps 324-1,630 of the toxin A gene; see appendix 1) corresponding to toxin A fragment a1 (figure 3.2 lanes 1 and 2).

Due to failure of expression of toxin A fragment a1 (see section 3.4.6.1), primers 3 and 2 (see appendix 2) were used to successfully amplify a 1.630Kb DNA product (bps 1-1,630

of the toxin A gene; see appendix 1) corresponding to toxin A fragment a2 (figure 3.2 lanes 3 and 4).

A PCR reaction using primers 1 and 5 (see appendix 2) resulted in the amplification of a 2.275Kb DNA product (bps 324-2,599 of the toxin A gene; see appendix 1) corresponding to toxin A fragment c (see figure 3.2 lanes 5 and 6).

Primers 6 and 7 (see appendix 2) were successfully used to amplify a 1.424Kb DNA product (bps 2,589-4,013 of the toxin A gene; see appendix 1) corresponding to toxin A fragment d (figure 3.3 lanes 1 and 2).

A PCR reaction containing primers 4 and 7 (see appendix 2) was successful in amplifying a 2.404Kb DNA product (bps 1,609-4,013 of the toxin A gene; see appendix 1) corresponding to toxin A fragment e (figure 3.3 lanes 3 and 4).

Primers 8 and 9 (see appendix 2) were successfully used to amplify a 1.553Kb DNA product (bps 3,955- 5,508 of the toxin A gene; see appendix 1) corresponding to toxin A fragment f (figure 3.3 lanes 5 and 6). The 2.919Kb product corresponding to toxin A fragment g (bps 2,589-5,508 of the toxin A gene; see appendix 1) was successfully amplified (figure 3.3 lanes 7 and 8) using primers 6 and 9 (see appendix 2).

The 2.587Kb DNA product corresponding to the C-terminal toxin A fragment h1 (bps 5,483-8,070 of the toxin A gene; see appendix 1) was successfully amplified (figure 3.3 lanes 9 and 10) using primers 10 and 11 (see appendix 2). Due to low levels of expression of fragment h1 (see section 3.4.6.2) and poor factor Xa cleavage of the resultant peptide (see section 3.4.11.5) this region of the toxin A gene was reamplified using primers 12 and 11 (see appendix 2). The resultant 2.576Kb DNA product (bps 5,495-8,070 of the toxin A gene; see appendix 1) corresponded to toxin A fragment h2 (figure 3.3 lanes 11 and 12).

A summary of the amplified and subsequently cloned toxin A fragments can be seen in figure 3.4.

3.4.3 T-tailing of the toxin A fragment PCR products

Each of the PCR products amplified in section 3.4.2 was cloned successfully into the pCRII T-tailing vector as outlined in section 3.3.2. (results not shown).

3.4.4 Sub-cloning into expression vectors

The toxin A fragments cloned into the T-tailing vector (see section 3.4.3) were removed and sub-cloned into expression vectors (see sections 3.3.3.1 and 3.3.3.2).

3.4.4.1 N-terminal fragments

Toxin A fragments b and f were successfully sub-cloned into the pMal-p2 expression vector. Digestion of fragment b clones with *Bam*HI and *Sal*I produced a 0.967Kb fragment corresponding to fragment b and a 6.721Kb fragment corresponding to the linear vector (figure 3.5). Digestion of fragment f clones with *Bam*HI and *Hind*III produced the vector band plus a 1.528Kb fragment corresponding to fragment f (figure 3.7 lanes 3 and 4).

Toxin A fragments a1, a2, c, d, e and g were sub-cloned into both the pMal-p2 and pMalc2 vectors although only the results for pMal-c2 are shown. Successful cloning of the 1.281Kb (figure 3.6 lanes 1 and 2), 1.616Kb (Figure 3.6, lanes 4 and 5), 2.25Kb (Figure 3.6, lanes 1 and 3), 1.4Kb (Figure 3.7, lanes 1 and 2) and 2.382Kb (Figure 3.6, lanes 4 and 6) DNA bands corresponding to fragments a1, a2, c, d and e respectively was

verified by digestion with *Bam*HI and *Sal*I. In each case a linear vector band of 6.646Kb was present.

Digestion of fragment g clones with *Bam*HI and *Hind*III produced the vector band and a 2.893Kb band corresponding to toxin A fragment g (see Figure 3.6, lanes 7 and 8). Toxin A fragment a2 was also successfully sub-cloned as a *Bam*HI fragment from the pCRII vector into the pET3a expression vector. Digestion of positive clones with *Bam*HI produced a 4.5Kb linear vector band and a 1.616Kb band corresponding to toxin A fragment a1 (results not shown).

3.4.4.2 C-terminal fragments

Toxin A fragment h1 was successfully sub-cloned into the pMal-p2 expression vector. Digestion with *Bam*HI and *Sal*I produced a 6.721Kb vector band and a 2.562Kb band corresponding to fragment h1 (Figure 3.6, lanes 9 and 10). Toxin A fragment h2 was sub-cloned as an *Nco*I, *Bam*HI fragment from the pCRII vector into the pET3d expression vector. Digestion of positive clones with *Nco*I and *Bam*HI produced a 4.5Kb vector band and a 2.568Kb band corresponding to fragment h2 (see Figure 3.7, lanes 5 and 6).

3.4.5. DNA Sequencing from the expression vectors

The cloned toxin A fragments were sequenced as outlined in section 3.3.4. In all cases the sequence data obtained was compared to the known toxin A sequence data (Dove *et al*, 1990) and was shown to be the correct fragment (data not shown). The data showed the engineered restriction sites in both primers and the engineered stop codon in the reverse primer. Each cloned fragment was shown to be in the correct reading frame for over-expression from the vector's own promotor.

3.4.6 Induction and expression of toxin A peptides

Expression was induced from the pMal and pET vectors (see appendix 3) using IPTG. In the pMal vectors the cloned DNA was inserted downstream from the *malE* gene which encodes a 43 kDa maltose binding protein. IPTG induction resulted in the expression of a toxin A peptide / MBP fusion product. In the pET3a vector the cloned DNA was inserted downstream from an 11 amino acid T7 tag peptide.

The size of the recombinant peptide was estimated by taking 1Kb of cloned DNA to be equivalent to a peptide of 35 kDa. Peptides expressed from the pMal vectors were then expected to be 43 kDa larger than the estimated size due to the N-terminal MBP fusion.

3.4.6.1 N-terminal peptides

IPTG induction of fragment b in the pMalp2 vector resulted in high levels of expression of a MBP fusion product of approximately 80 kDa which was clearly visible on a coomassie gel (see Figure 3.8, lane 6). This compared well to the estimated size of peptide B of 76.85 kDa. The peptide B fusion product reacted with anti-MBP antiserum on a western blot (see Figure 3.8, lane 7) and was clearly larger than the control product obtained by inducing expression from the pMalp2 vector in the absence of an insert (see Figure 3.8, lane 9).

Induction of expression of fragment a1 from the pMalp2 vector failed to produce a MBP fusion product (see Figure 3.9, lanes 1-6). This was also the case when inducing expression from the pMalc2 vector (results not shown).

Due to the failed expression of fragment a1 , fragment a2 was amplified and cloned (see sections 3.4.2, 3.4.3 and 3.4.4.1) into both the pMalp2 and pMalc2 vectors. Induction of

expression of fragment a2 from the pMalp2 vector again failed to produce a fusion product (results not shown). IPTG induction from the pMalc2 vector, however, did result in the expression of a fusion product of approximately 100 kDa which was visible on a coomassie gel (see Figure 3.9, lanes 7 and 8). This compared well to the estimated size of A2 of 99.28 kDa. Western blot analysis revealed that as well as the full length product there was high levels of expression of a series of truncated products the most predominant of which were approximately 65 kDa in size (see Figure 3.9, lanes 9 and 10). As the first 43 kDa of this product is the MBP it would appear that there is some termination of translation approximately 22 kDa into peptide A2. In an attempt to overcome this problem, fragment a2 was cloned into the pET3a expression vector (see section 3.4.4.1). Unfortunately, induction of expression of fragment a2 from this vector failed to produce a fusion product (results not shown).

IPTG induction of fragment c from the pMal-p2 vector resulted in very low levels of expression of fusion product (peptide C). The product was smaller than the estimated 121.33 kDa and many truncations were observed (results not shown). In the pMal-c2 vector the number of truncations was reduced and a product of the correct size was observed. The levels of expression of peptide C was still low but was detectable on a western blot and a coomassie gel (see Figure 3.10, lanes 4 and 9).

Induction of expression of fragments d and e from the pMal-p2 vector failed to produce fusion products (results not shown). Induction from the pMal-c2 vector, however, produced high levels of expression of the peptide D/MBP fusion product of approximately 90 kDa which was visible on both a coomassie gel and a western blot (see Figure 3.10, lanes 3 and 8). This compared to the estimated size of peptide D of 91.51 kDa. Induction of expression of fragment e from the pMalc2 vector produced a product

of approximately 108 kDa which was visible on both a coomassie gel and a western blot (see Figure 3.10, lanes 5 and 10). This protein was smaller than the estimated size of peptide E of 125.85 kDa. It would appear that translation was being terminated 65 kDa into peptide E. This may have been due to a change in the reading frame of the DNA between the region 1641 bp to 2237 bp generating a stop codon. The additional sequencing primers outlined in section 3.3.4 were used to sequence this region of the insert DNA. The eInt1 primer was used to sequence the region 1641 bp to 1991 bp in the fragment e insert. The eInt2 primer was used to sequence the region 1938 bp to 2237 bp in the fragment e insert. These results proved that there was no change in the reading frame of the DNA in the region covering 57 to 70 kDa of the peptide E product (results not shown).

IPTG induction of fragment f from the pMal-p2 vector resulted in high levels of expression of a MBP fusion product of approximately 95kDa which was clearly visible on an SDS-PAGE gel (figure 3.11 lanes 1-3) This compared well to the estimated size of peptide F of 96.24kDa. On the western blot there appears to be some smearing of the antibody reaction possibly due to the protein not entering the separating gel efficiently (figure 3.11 lane 5).

Induction of expression of fragment g from the pMal-p2 vector resulted in very low levels of expression of a fusion protein of approximately 145kDa (results not shown). Expressing from the pMal-c2 vector increased the amount of fusion protein produced. The size of the recombinant peptide compared well to the estimated size of peptide G of 144.01 kDa. Levels of expression were still low but the protein was clearly visible on both a coomassie gel (figure 3.11 lanes 7-9) and a western blot (figure 3.11 lanes 10-12).

3.4.6.2 C-terminal peptides

IPTG induction of fragment h1 in the pMal-p2 vector resulted in low levels of expression of a MBP fusion product of approximately 130 kDa. This compared to the estimated size of peptide H1 of 132.67 kDa. The protein reacted on a western blot with PCG-4 (see Figure 3.12, lanes 3 and 4) but due to low levels of expression the peptide was barely visible on a coomassie gel (see Figure 3.12, lanes 1 and 2). Further, this protein cleaved insufficiently with factor Xa (see section 3.4.11.5) and was, therefore, re-amplified as fragment h2 for cloning into an alternative vector (see sections 3.4.2, 3.4.3 and 3.4.4.2). By cloning the repeat region of toxin A into the *Nco*I site of the pET3d vector (see appendix 3) it was possible to bypass the T7 tag and produce an unfused recombinant peptide. Due to low levels of expression the peptide was not clearly visible on a coomassie gel (see Figure 3.13, lane 4). A peptide of approximately 90 kDa did react with PCG-4 on a western blot (see Figure 3.13, lane 10). This compared to the estimated size of peptide H2 of 89.46 kDa. *E. coli* cells containing pET3d without an insert were also induced with IPTG but showed no reaction with the PCG-4 monoclonal antibody (see Figure 3.13, lanes 11 and 12).

3.4.7 Optimisation of expression of toxin A peptides

3.4.7.1 Transforming with rare tRNA encoding plasmids

In an attempt to improve the expression of toxin A fragments a2, c, d, e and h1 from the pMal-p2 vector, and h2 from the pET3d vector, each was induced in the presence of a plasmid encoding the rare tRNAs for the AGA / AGG codons (see section 3.3.6.1). The results showed that no more peptide was expressed in the presence of these tRNAs than

when the pDC952 plasmid was excluded from the reaction. The results for peptides A2, C, D and E are shown in Figure 3.14.

3.4.7.2 Varying the host *E. coli* strain

Toxin A peptides A2, C, E, H1 and H2 were transformed into different *E. coli* strains in an attempt to improve the levels of expression (see section 3.3.6.2). Levels of expression of peptides A2, C and E were unaffected by varying the *E. coli* strain (results not shown). The level of expression of peptide H1, however, was greater in the *E. coli* strain BL21 DE3 (see Figure 3.15, lanes 4a to 4c) than in the *E. coli* strains TB1 (see Figure 3.15, lanes 2a to 2c) or JM109 (see Figure 3.15, lanes 6a to 6c). The concentration of peptide H1 in the induced sonicated whole cell preparation (figure 3.15, lane 4b), was far greater than in the sonicated supernatant fraction suggesting that a high percentage of the peptide was being lost in the insoluble fraction. When the pMal-p2 plasmid lacking the insert was induced in the different cell lines, the resultant proteins did not react with the PCG-4 monoclonal antibody (see Figure 3.15, lanes 3, 5 and 7). The results of peptide H2 were as shown for peptide H1. For future work the yields of peptides H1 and H2 were maximised by inducing and purifying the peptides from the *E. coli* strain BL21 DE3.

3.4.7.3 Varying IPTG concentration and induction time

The experiment outlined in section 3.3.6.3 was used in an attempt to increase the expression of peptides A2, C, H1 and H2. For each peptide the level of expression was constant when sampling at an $O.D_{600}$ of 0.4 or above, and an IPTG concentration of 0.3 mM or above. Below these values the levels of expression was reduced (results not

shown). By using the IPTG concentrations and length of induction times outlined in section 2.8.1, the level of expression for each peptide was therefore maximised.

3.4.8 Purification and cleavage of N-terminal toxin A peptides

The toxin A peptide fusion products were purified by amylose affinity chromatography as outlined in section 3.3.7.1. The concentration of protein in each fraction was determined as outlined in section 2.9.1.1.

The protein concentration in the 3ml aliquot containing the highest concentration of the peptide A2 fusion product was typically in the range of 400 – 500 $\mu g/ml^{-1}$. Factor Xa, at a concentration of 1%, was insufficient for complete cleavage of the 99.28 kDa peptide A2 fusion product, although the amount of full length peptide was significantly reduced (see Figure 3.16, lanes 3 to 4 and Figure 3.17, lanes 3 to 4). Cleavage with 2% and 3% factor Xa was more successful but was still incomplete (results not shown).

The highest protein concentration for the peptide B fusion product was typically in the range of 1.0 – 1.5 mg/ml^{-1}. One percent factor Xa successfully cleaved the 76.85 kDa peptide B fusion product resulting in the 43 kDa MBP and the 33.85 kDa toxin A peptide B product (see Figure 3.16, lanes 5 and 6). On a western blot the 33.85 kDa peptide B did not react with anti-MBP antiserum (see Figure 3.16, lane 6 and Figure 3.17, lane 6).

The protein concentration in the aliquot containing the highest concentration of the peptide C fusion product was typically in the range of 400-500 μgml^{-1}. Factor Xa failed to cleave the 121.33 kDa peptide C fusion product into the 43 kDa MBP and the 78.33 kDa peptide C product (figure 3.16 lanes 7-8 and figure 3.17 lanes 7-8).

The typical concentrations of the purified peptide D and E fusion products were 0.8 – 1.0 $mgml^{-1}$ and 1.0 – 1.5 $mgml^{-1}$ respectively. One percent factor Xa successfully cleaved the

91.51 kDa peptide D fusion product into the 43 kDa MBP and the 48.41 kDa peptide D product (see figure 3.16 lanes 12 and 13). On a western blot the 49 kDa peptide D did not react with the anti-MBP antiserum (see figure 3.16 lane 13 and 3.17 lane 13). One percent factor Xa cleaved the 108 kDa peptide E fusion product into the 43 kDa MBP and the 65 kDa truncated peptide E (see figure 3.16 lanes 9 and 10). Again on a western blot the 65 kDa truncated peptide E did not react with the anti-MBP antiserum (see figure 3.16 lane 10 and 3.17 lane 10). After an overnight incubation with factor Xa there did appear to be some uncleaved full-length protein in the two samples (D and E), but increasing the incubation time to 24 hrs completed the cleavage.

The typical concentration of the purified peptide G fusion product was 300 – 400 $\mu g ml^{-1}$. One percent factor Xa cleaved the 144.01 kDa peptide G fusion product into the 43 kDa MBP and the 101.01 kDa peptide G product (see figure 3.16 lanes 14 and 15). On a western blot, although the anti-MBP antiserum reacted with a small amount of uncleaved peptide, the 101.01 kDa peptide G did not react (see figure 3.16 and 3.17 lanes 14-15).

The average concentration of the purified peptide F fusion product was 200 – 300 $\mu g ml^{-1}$ (see figure 3.18). This concentration appeared to be low and did not reflect the over-expression seen in figure 3.11 A and B. Factor Xa failed to cleave the 96.24 kDa fusion product into the 43 kDa MBP and the 53.24 kDa peptide F product (see figure 3.18 lanes 7, 15, 16 and 24).

A summary of the expressed and purified toxin A peptides can be seen in appendices 4 and 5.

3.4.9 Determination of the solubility of the peptide F fusion product

As the concentration of the peptide F fusion product purified by amylose affinity chromatography was lower than expected, the method outlined in section 3.3.7.5 was used to determine the solubility of the peptide. Only a small percentage of the peptide was purified by amylose affinity chromatography (see figure 3.19 lanes 3 and 8). Following purification from inclusion bodies only a small percentage of the original peptide was located in the soluble fraction (see figure 3.19 lanes 4 and 9). At the end of this procedure it is expected that approximately 50% of a soluble recombinant protein will remain in solution whilst the remainder forms a precipitate. In the case of the toxin A peptide F fusion product the majority of the peptide precipitated following renaturation (see figure 3.19 lanes 5 and 10). These results implied that the peptide F fusion product was highly insoluble.

3.4.10 Further purification of toxin A peptide B

3.4.10.1 Introductory comment

The following results were obtained when trying to remove the MBP from the toxin A peptide B / MBP cleavage mixture. Peptide B has been included as an example although attempts were also made to further purify other toxin A peptides.

3.4.10.2 Electro-elution from an SDS-PAGE gel.

Following factor Xa cleavage the 33.85 kDa toxin A peptide B (see figure 3.20 lane 3) was successfully eluted from a SDS-PAGE gel (see figure 3.20 lane 5). There was no trace of the 43 kDa MBP (seen in figure 3.20 lane 4) in the electro-eluted sample. After one freeze / thaw reaction the eluted peptide B showed a degree of degradation on an

SDS-PAGE gel, even with the addition of the protease inhibitors PMSF and pepstatin (results not shown).

3.4.10.3 Iso-electric focusing of toxin A peptide B.

Iso-electric focusing was used to determine the pI value of peptide B as outlined in section 3.3.10.3. The results were inconclusive, as only one band was visible on the gel from the cleavage mixture running at a pI value of 4. Unfortunately this band also lined up with the control band corresponding to the MBP alone (results not shown).

3.4.10.4 Anion exchange chromatography

Although the pI determination for peptide B was inconclusive (see section 3.4.10.3) an attempt was made to purify the peptide by anion exchange chromatography. The buffer systems attempted for purification were 20mM Tris-HCl pH 7.5, pH 6.0 and pH 8.0. Prior to injection onto the column both peptide B and the MBP were present in the sample. An aliquot of each eluted fraction that corresponded to a protein absorbance peak on the printout was resolved on the SDS-PAGE gel. In each case where protein was present it corresponded to the 43 kDa MBP and not peptide B (results not shown). In summary anion-exchange chromatography failed to purify peptide B from the cleavage mixture.

3.4.10.5 Cleavage of the peptide B fusion product on an amylose column

The method outlined in section 3.3.10.5 was used to purify peptide B. Following factor Xa cleavage of the fusion product whilst bound to the column, it was hoped that peptide B would be released leaving the MBP still attached to the column. Each of the eleven

fractions eluted with amylose column buffer (see figure 3.21, lanes 4-13) contained both the MBP and peptide B. Both of these proteins were clearly visible in the original cleavage mixture (see figure 3.21, lane 14). Some of the later fractions also contained the uncleaved 76.85 kDa product (see figure 3.21 lanes 11-13). The fractions eluted with 10mM maltose were also mixed containing the MBP, the uncleaved fusion product and peptide B (see figure 3.21 lane 15).

3.4.10.6 N-terminal sequencing of the toxin A peptides

Following factor Xa cleavage, the 33.85 kDa peptide band corresponding to toxin A peptide B was sequenced (see section 3.3.10.6). The first ten amino acids of the peptide were ISEFGSLSED. The 'ISEF' motif corresponds to the last four amino acids of the MBP to one side of the factor Xa cleavage site. The 'GSLSED' motif was as expected and corresponds to the first six amino acids of peptide B (amino acids 540-545 of the toxin A protein). The sequence data was 100% accurate.

The sequence data obtained for the 65 kDa peptide E was also ISEFGSLSED because the initiation sites for expression of both peptides B and E are at the same point in the toxin A gene.

The sequence data obtained for the 49 kDa peptide D was ISEFGSDELY. The expected sequence data for this region of the toxin A protein was GSDELK giving an accuracy of 83.3%.

3.4.11 Purification and cleavage of toxin A peptide H1

3.4.11.1 Thyroglobulin affinity chromatography

Toxin A peptide H1 was purified by thyroglobulin affinity chromatography as outlined in section 3.3.7.2. A 40µl aliquot of the fraction containing the highest concentration of peptide H1 was just visible on a coomassie gel (see figure 3.22 lane 5). The fusion peptide reacted strongly on a western blot with the monoclonal antibody PCG-4 (see figure 3.22 lanes 7-9) whereas the MBP alone did not (see figure 3.22 lane 11). The full-length product was approximately 130 kDa, which compared to the estimated size of the fusion product of 132.67 kDa. The PCG-4 monoclonal antibody also reacted with a large number of breakdown products of peptide H1, because the antibody is directed against a repeated unit, which is found at intervals throughout the C-terminal region of toxin A.

Three millilitre aliquots of peptide H1 at a concentration of $10\mu gml^{-1}$ were routinely purified by thyroglobulin affinity chromatography. A similar result was obtained when peptide H1 was purified by amylose affinity chromatography (result not shown).

A summary of peptide H1 can be found in appendices 4 and 5.

3.4.11.2 Concentration of peptide H1 by evaporation

Prior to concentration, peptide H1 was at a concentration of $10\mu gml^{-1}$, had a H/A titre of 1:32 and reacted strongly with PCG-4 on a dot blot. Following evaporation the peptide concentration had increased to $25\mu gml^{-1}$ but the H/A titre had dropped to 1:8 and the peptide reacted more weakly with PCG-4 (results not shown). This implied that heating to 60^0C had reduced both the biological activity and antigenicity of peptide H1.

3.4.11.3 Determination of the solubility of peptide H1

As the yield of peptide H1 purified from the soluble fraction was poor (see section 3.4.11.1), it was assumed that a large proportion of the peptide was being lost in the

insoluble fraction. The method outlined in section 2.8.4 was, therefore, used to determine the solubility status of peptide H1.

The results showed that only a small percentage of the total peptide was soluble and available for purification by amylose affinity chromatography (see figure 3.23 lanes 2 and 5). The majority of the peptide was insoluble (see figure 3.23 lanes 3 and 4) and was being lost prior to purification by affinity chromatography. The method outlined in section 3.3.7.5 showed that peptide H1 was being placed into inclusion bodies within the *E. coli* cell (result not shown).

3.4.11.4 Effect on the formation of inclusion bodies by inducing expression of peptide H1 at 30^0C or 37^0C.

Inducing the expression of recombinant peptides at 30^0C as opposed to 37^0C has been shown to reduce the formation of inclusion bodies within *E. coli* cells (Ausubel *et al*, 1992). The method outlined in section 3.3.7.6 was used to determine whether or not reducing the temperature would increase the proportion of soluble peptide H1. The results showed that lowering the temperature to 30^0C only reduced the overall expression of peptide H1 but did not effect the ratio of soluble: insoluble protein (result not shown).

3.4.11.5 Factor Xa cleavage of the peptide H1 fusion product

The peptide H1 fusion product was treated with both 1% and 2% factor Xa in an attempt to remove the MBP (see section 3.3.7.4). Neither concentration was successful in cleaving the full-length fusion protein (see figure 3.24 lanes 4 and 9) into the 43 kDa MBP and the 89.67 kDa peptide H1 (see figure 3.24 lanes 5 and 10, 6 and 11).

3.4.12 Purification of toxin A peptide H2

3.4.12.1 Introductory comment

Due to poor levels of expression (see section 3.4.11.1), insufficient factor Xa cleavage (see section 3.4.11.5) and insolubility of fusion peptide H1, it was decided to re-clone the C-terminal repeat region of toxin A into a vector that would express an unfused peptide (see section 3.4.6.2). This would overcome the problem of removing a fusion protein whilst still allowing purification by thyroglobulin affinity chromatography. The resultant peptide was named H2.

3.4.12.2 Thyroglobulin affinity chromatography

Toxin A peptide H2 was purified by thyroglobulin affinity chromatography as outlined in section 3.3.7.2. The purified recombinant peptide was not visible on a coomassie gel but did react with the PCG-4 monoclonal antibody on a western blot (results not shown). The size of the full-length peptide was approximately 90 kDa, which compared to the estimated size of 89.46 kDa. The typical concentration of a batch of peptide H2 purified by thyroglobulin affinity chromatography was 5.5μgml^{-1}. The H/A titre corresponding to this concentration was only 1:8. A summary of peptide H2 can be found in appendices 4 and 5.

3.4.12.3 Purification of peptide H2 from inclusion bodies

Due to the poor recovery of peptide H2 by thyroglobulin affinity chromatography, it was assumed that peptide H2, like H1 (see section 3.4.11.3), was being placed into inclusion bodies within the host cell. The method outlined in section 3.3.7.5 was, therefore, used to purify peptide H2 from inclusion bodies. A small proportion of the recombinant peptide

was present in the soluble sonicate fraction (see figure 3.25, lanes 5 and 11) but the majority was found in the insoluble fraction (see figure 3.25, lanes 6 and 12). Following solubilisation of the inclusion bodies and renaturation of the proteins, peptide H2 still reacted on a western blot with the PCG-4 monoclonal antibody (see figure 3.26 lane 8). Although some *E. coli* proteins were isolated during the purification procedure, the full length peptide H2 was clearly visible on a coomassie gel running at 89.46 kDa (see figure 3.26 lane 4). The negative control *E. coli* proteins showed a similar banding pattern to that of the peptide H2 preparation, but did not react with the PCG-4 monoclonal antibody on a western blot (see figure 3.26 lanes 3 and 7).

This purification procedure typically yielded 25mls of recombinant peptide H2. When compared to a series of BSA standards on a coomassie gel it was estimated that 40µl of the preparation contained 1µg of full length peptide H2 (see figure 3.27 lanes 11 and 14). The total concentration of peptide H2 was, therefore, 25µgml^{-1}.

3.4.13 Further purification of toxin A peptide H2

3.4.13.1 Thyroglobulin slurry

Bovine thyroglobulin was used as outlined in section 3.3.9.2 to remove unwanted *E. coli* proteins from the peptide H2 preparation purified in section 3.4.12.3. Prior to thyroglobulin purification, peptide H2 was visible on a coomassie gel running at 89.46 kDa and gave a H/A titre of 1:128. Following thyroglobulin purification, peptide H2 was barely visible on a coomassie gel and the H/A titre had dropped to 1:8 (results not shown).

The peptide H2 preparation isolated in section 3.4.12.3 was also further purified by thyroglobulin affinity chromatography as outlined in section 3.3.7.2. Again, an

unsatisfactory amount of peptide H2 was recovered with a low H/A titre (results not shown).

3.4.13.2 Isoelectric focusing (IEF) of toxin A peptide H2

Isoelectric focusing was used to determine the pI value of peptide H2 (see section 3.3.9.3). The presence of the contaminating *E. coli* proteins made it difficult to determine which band corresponded to peptide H2 on a silver stain gel (see figure 3.28 lane 2). The toxin A band lined up with the markers at a pI value of approximately 5.5 (see figure 3.28 lane 4). The IEF gel was western blotted and probed with the PCG-4 monoclonal antibody. Two main bands from the peptide H2 preparation reacted with antibody. These protein bands lined up with the markers at pI values of approximately 6.5 and 9.0 (see figure 3.28 lane 5). It is possible that the band at a pI value of 6.5 was due to residual protein in the loading well, because the sample was loaded in the centre of the gel. From these results it was difficult to conclude whether the pI value of peptide H2 was 6.5 or 9.0.

3.4.13.3 Ion-exchange chromatography

The method outlined in section 3.3.9.4.2 was used to purify peptide H2 by both anion and cation-exchange chromatography. Taking the pI value of peptide H2 to be 6.5 (see section 3.4.13.2) it was decided to use a positively charged anion-exchange column for purification. Prior to application to the column, the peptide H2 preparation purified in section 3.4.12.3 gave a H/A titre of 1:128 (see figure 3.29 lanes 3 and 11). Using a 20mM Tris-HCl pH 8.5 buffer system, the fractions eluted that corresponded to protein absorbance peaks on the printout gave H/A titres between 1:2 and 1:4 (see figure 3.29

lanes 12-16). Each fraction, therefore, contained a small amount of peptide H2. On a silver stain gel however, it was evident that the purification was unsatisfactory because other proteins were still present in the peptide H2 fractions (see figure 3.29 lanes 4-8). A similar result was obtained using a buffer system at a pH of 7.5 (results not shown).

Taking the pI value of peptide H2 to be 9.0 (see section 3.4.13.2), a negatively charged cation-exchange column was selected for purification. None of the buffer systems outlined in section 3.3.9.4.2 resulted in the satisfactory purification of peptide H2. In each case the H/A titres of the eluted fractions were low and the samples were still contaminated with other proteins (results not shown).

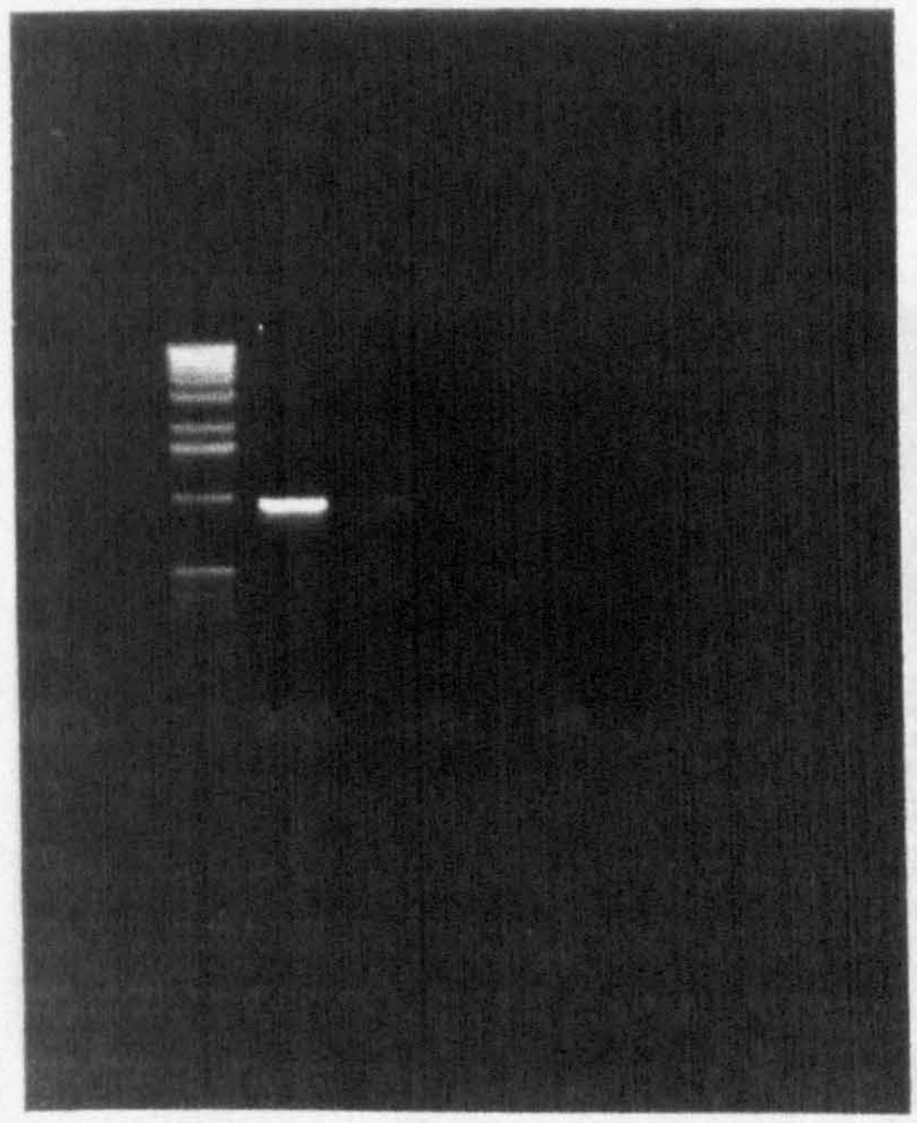


Figure 3.1

0.8% agarose gel showing the PCR amplification of toxin A fragment b from *C. difficile* VPI 10463 chromosomal DNA using primers 4 and 5.

Lane 1, 1Kb ladder; lane 2, 10ng/μl of both primers; lane 3, 5ng/μl of both primers; lane 4, 10ng/μl of primer 5 only; lane 5, 10ng/μl of primer 4 only; lane 6, no template DNA.

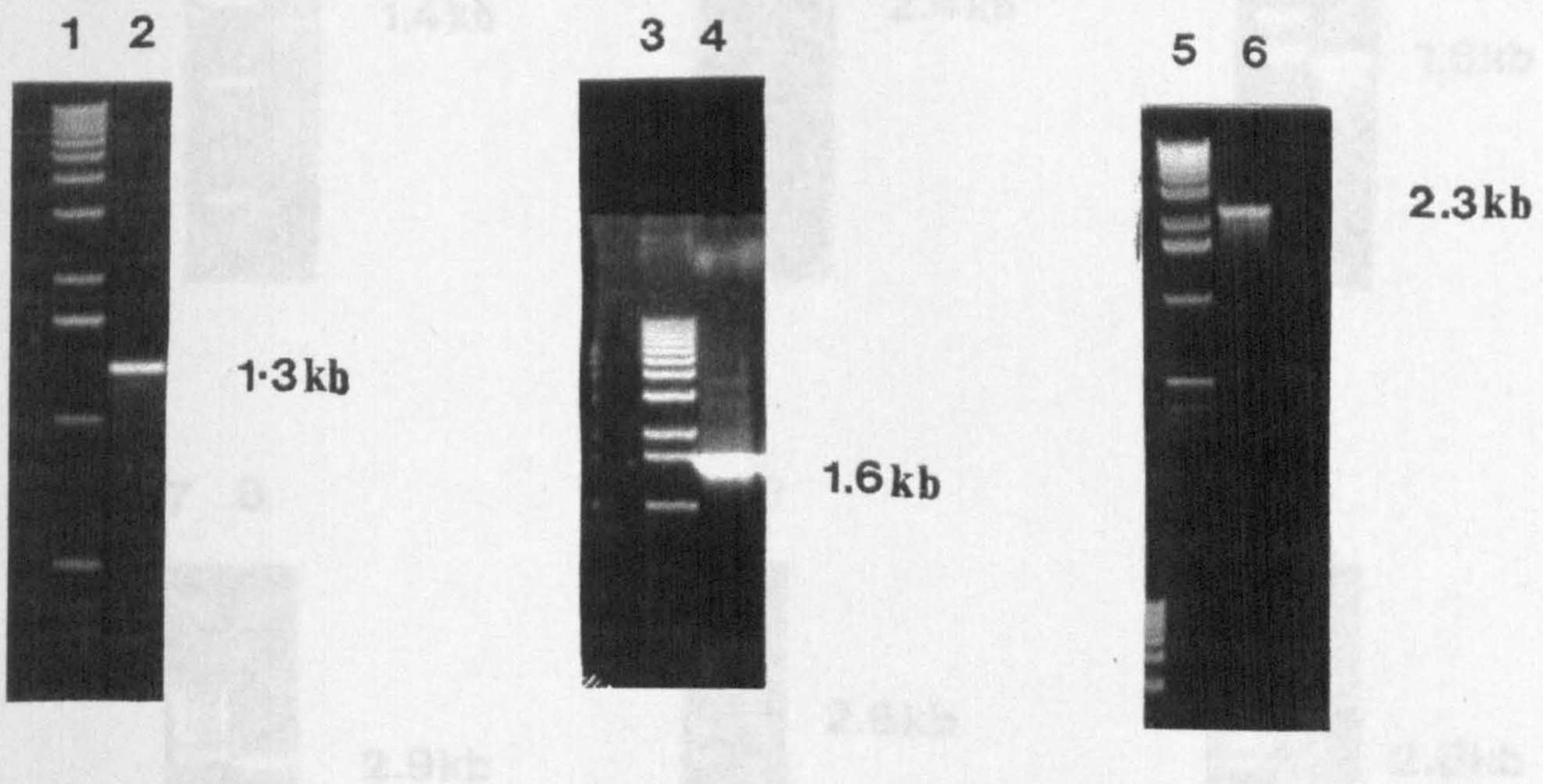


Figure 3.2

0.8% agarose gels showing toxin A fragment PCR products amplified from *C. difficile* VPI 10463 chromosomal DNA.

Lanes 1, 3 and 5, 1Kb ladder; lane 2, fragment a1; lane 4, fragment a2; lane 6, fragment c.

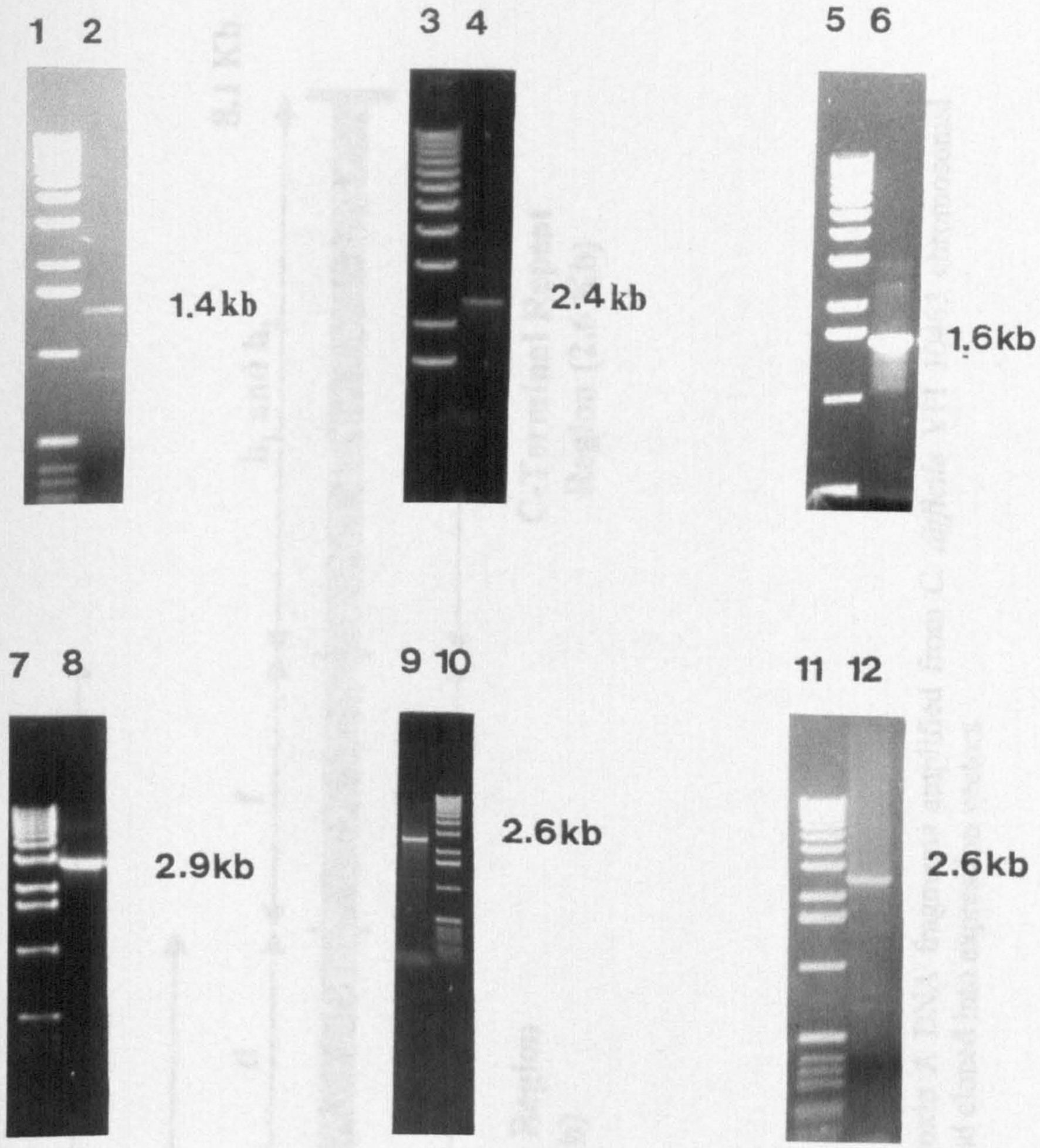


Figure 3.3

0.8% agarose gels showing toxin A fragment PCR products amplified from *C. difficile* VPI 10463 chromosomal DNA.

Lanes 1, 3, 5, 7, 10 and 11, 1Kb ladder; lane 2, fragment d; lane 4, fragment e; lane 6, fragment f; lane 8, fragment g; lane 9, fragment h1; lane 12, fragment h2.

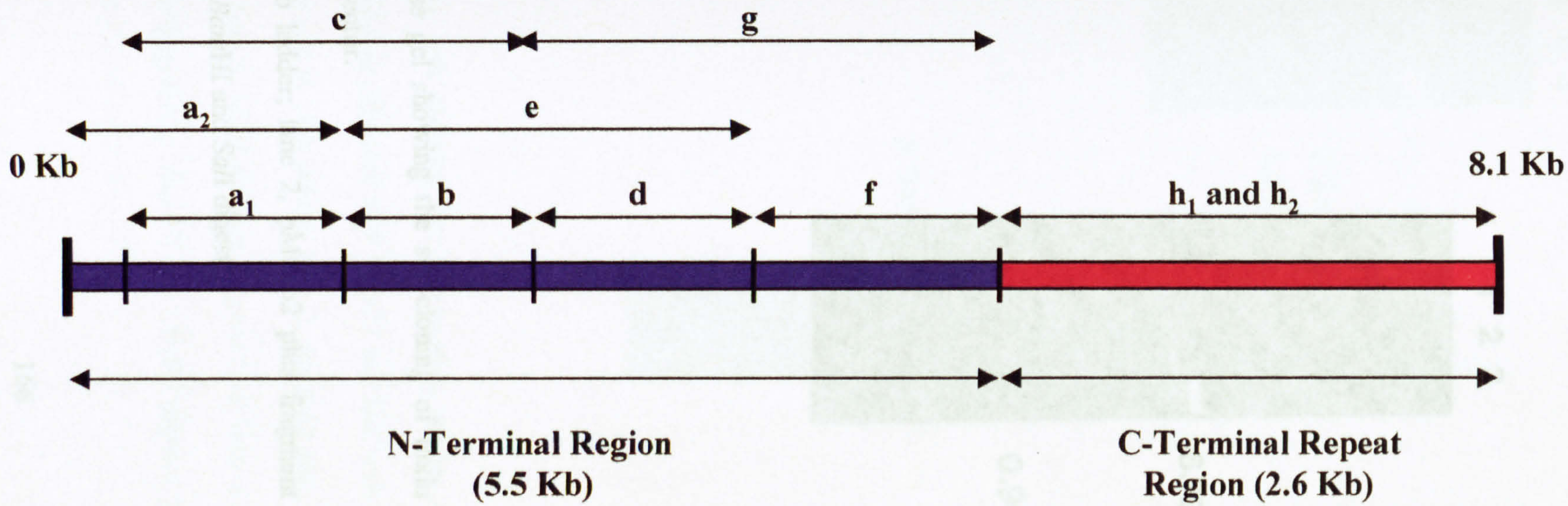


Figure 3.4

A schematic diagram summarising the toxin A DNA fragments amplified from *C. difficile* VPI 10463 chromosomal DNA by the polymerase chain reaction and cloned into expression vectors.

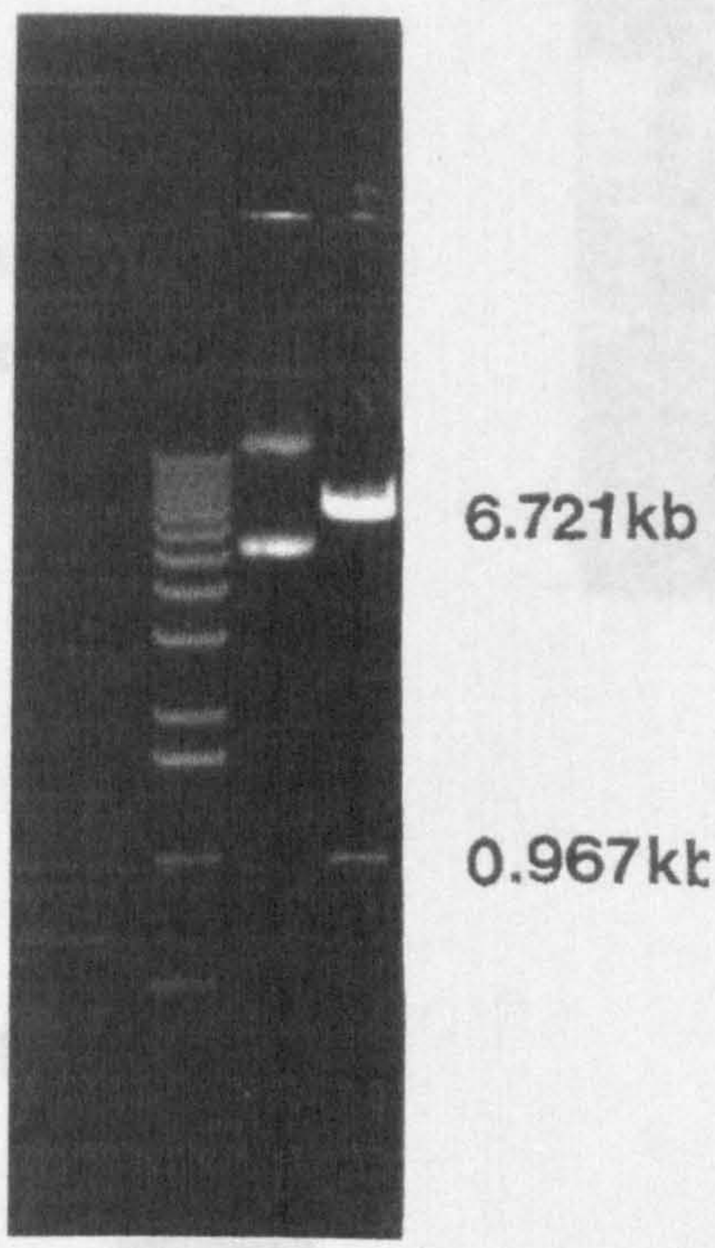


Figure 3.5

0.8% agarose gel showing the sub-cloning of toxin A fragment b into the pMal-p2 expression vector.

Lane 1, 1Kb ladder; lane 2, pMal-p2 plus fragment b (uncut); lane 3, pMal-p2 plus fragment b (*Bam*HI and *Sal*I digest).

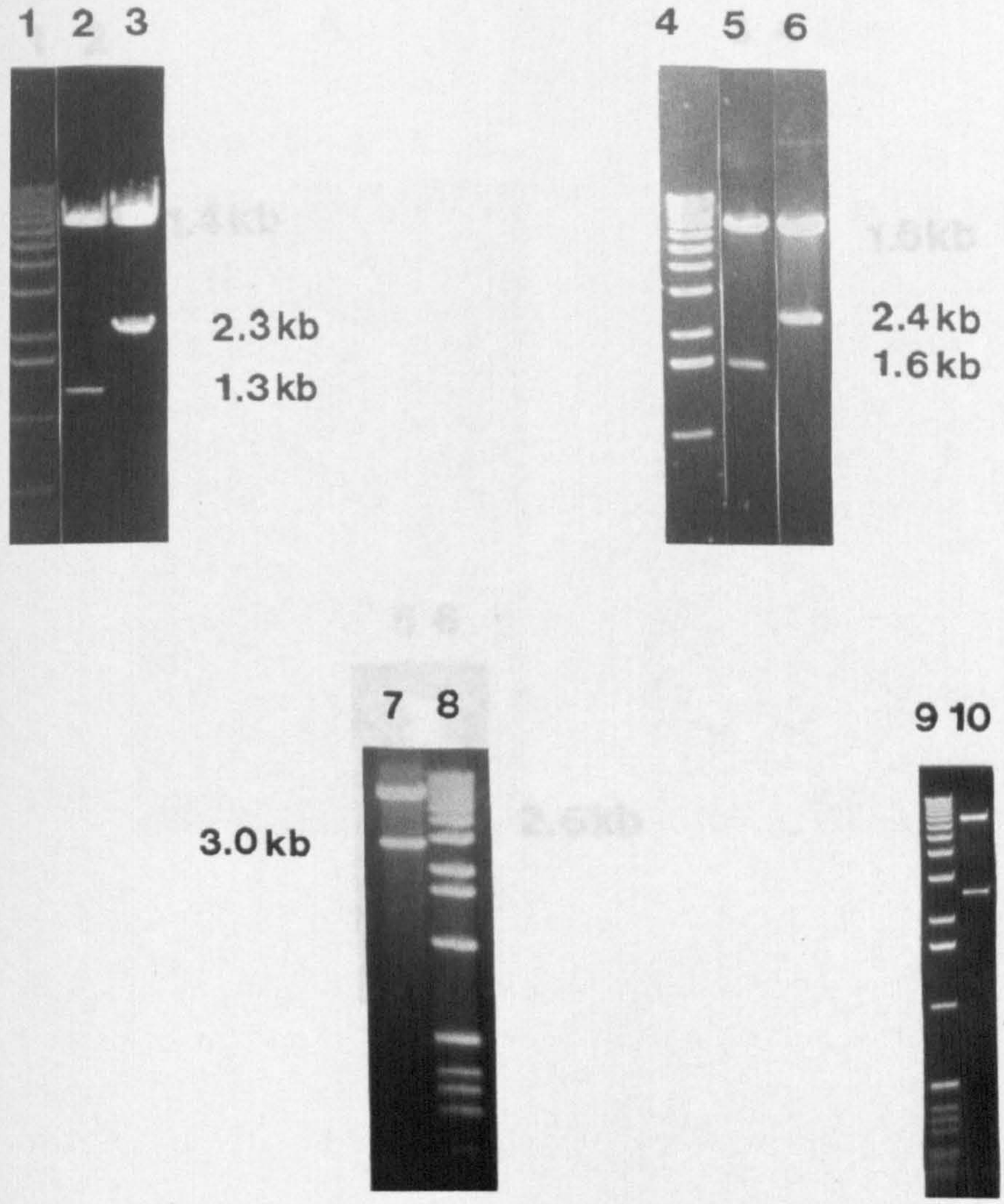


Figure 3.6

0.8% agarose gels showing the sub-cloning of the toxin A fragments into the pMal expression vectors (digested with *Bam*HI and *Sal*I unless otherwise stated).

Lanes 1, 4, 8 and 9, 1Kb ladder; lane 2, pMal-c2 plus fragment a1; lane 3, pMal-c2 plus fragment c; lane 5, pMal-c2 plus fragment a2; lane 6, pMal-c2 plus fragment e; lane 7, pMal-c2 plus fragment g (*Bam*HI and *Hind*III digest); lane 10, pMal-p2 plus fragment h1.

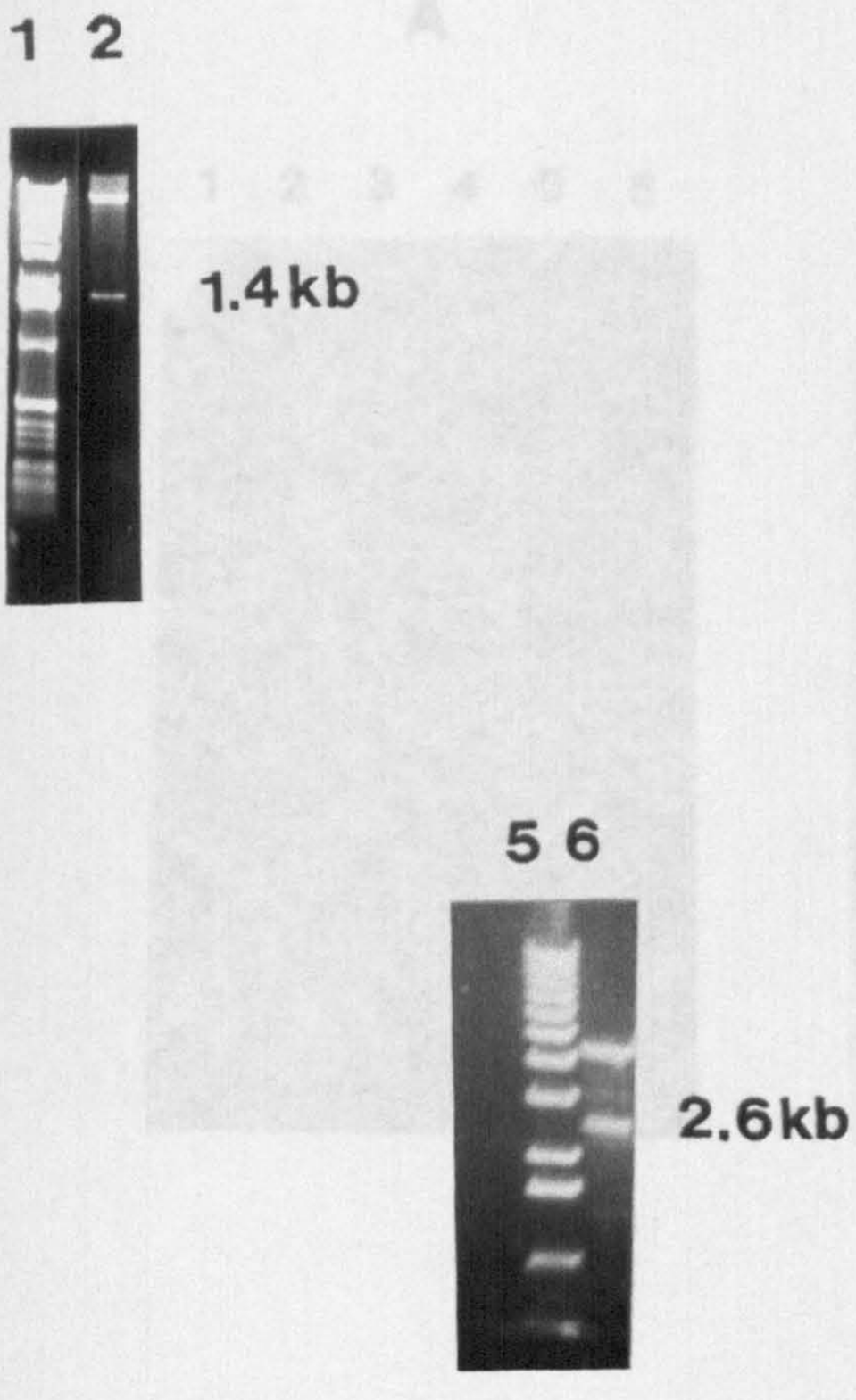


Figure 3.7

0.8% agarose gels showing the sub-cloning of toxin A fragments into the pMal or pET vectors.

Lanes 1, 3 and 5, 1Kb ladder; lane 2, pMal-c2 plus fragment d (*Bam*HI and *Sal*I digest); lane 4, pMal-p2 plus fragment f (*Bam*HI and *Hind*III digest); lane 6, pET3d plus fragment h2 (*Bam*HI and *Nco*I digest).

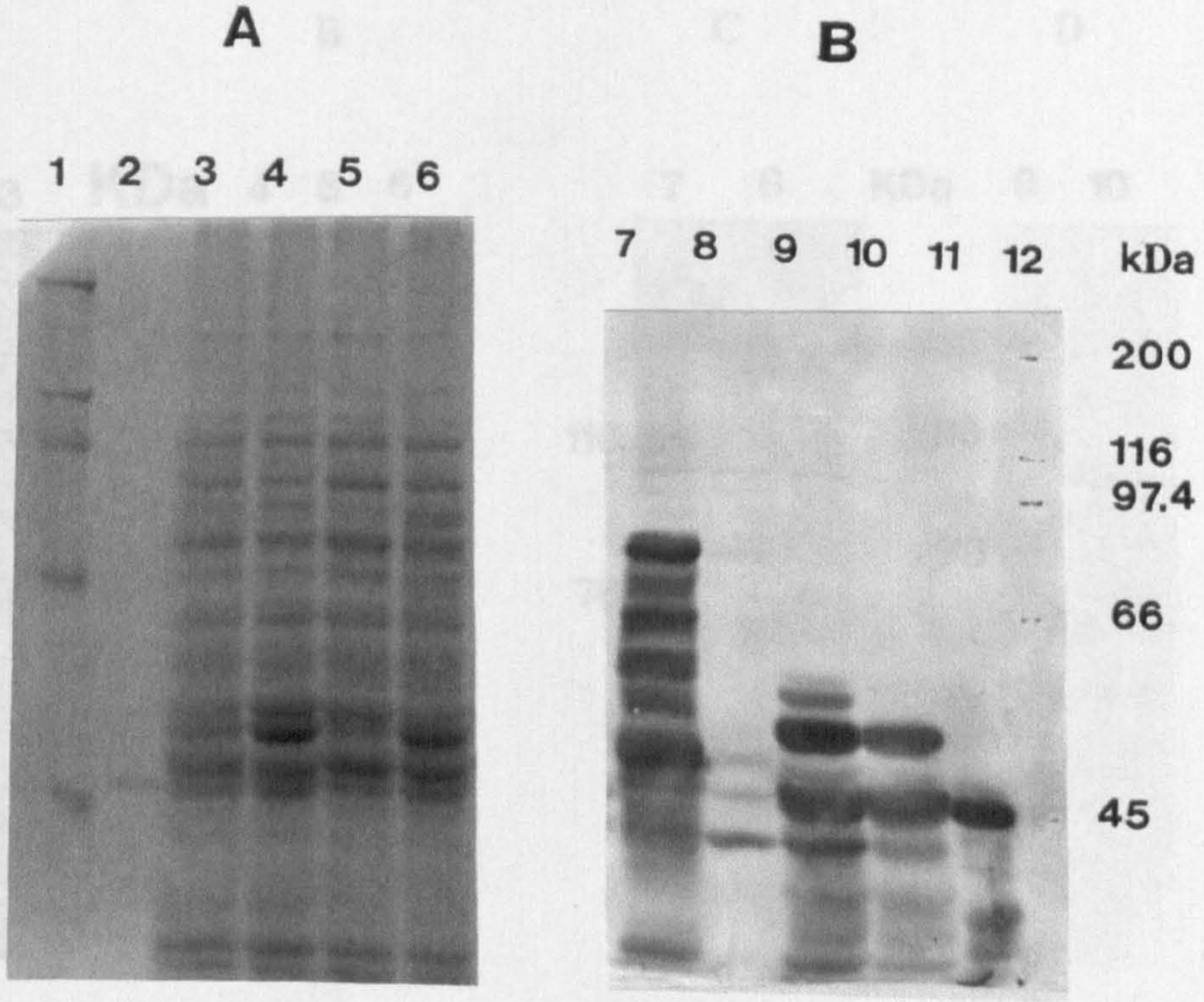


Figure 3.8

8% SDS-PAGE gel (A) and respective western blot (B) probed with MBP antiserum showing the induction of expression of toxin A peptide B.

Lanes 1 and 12, high molecular weight (MWT) markers; lanes 2 and 11, MBP; lanes 3 and 10, uninduced TB1 cells containing pMal-p2 only; lanes 4 and 9, induced TB1 cells containing pMal-p2 only; lanes 5 and 8, uninduced TB1 cells containing pMal-p2 plus fragment b; lanes 6 and 7, induced TB1 cells containing pMal-p2 plus fragment b.

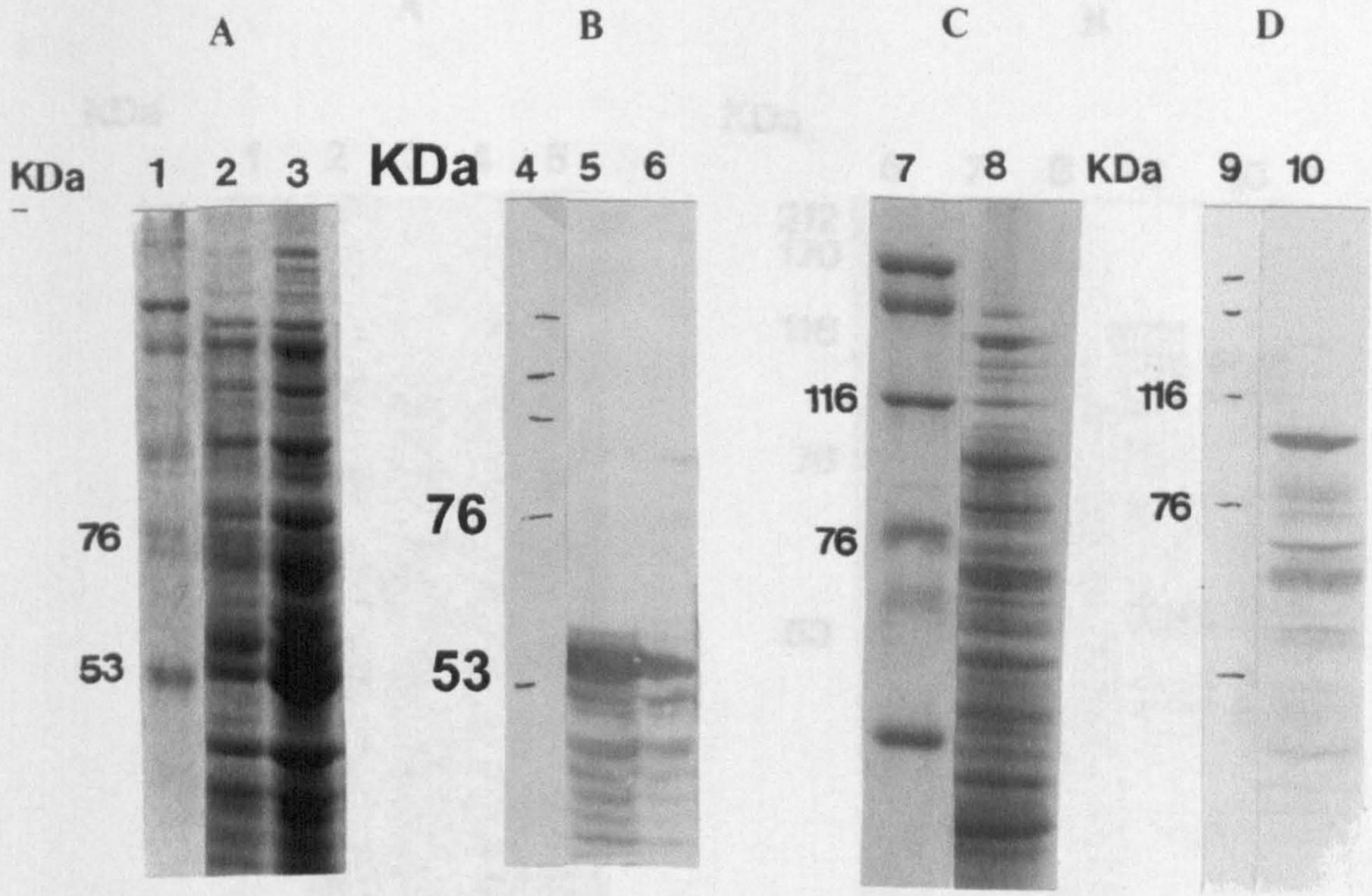


Figure 3.9

8% SDS-PAGE gels and their respective western blots probed with MBP antiserum showing induction of expression of toxin A peptides.

Coomassie blue stain (A) and western blot (B) of lanes 1 and 4, high MWT markers; lanes 2 and 5, induced TB1 cells containing pMal-p2; lanes 3 and 6, induced TB1 cells containing pMal-p2 plus fragment a1.

Coomassie blue stain (C) and western blot (D) of lanes 7 and 9, high MWT markers; lanes 8 and 10, induced JM109 cells containing pMal-c2 plus fragment a2.

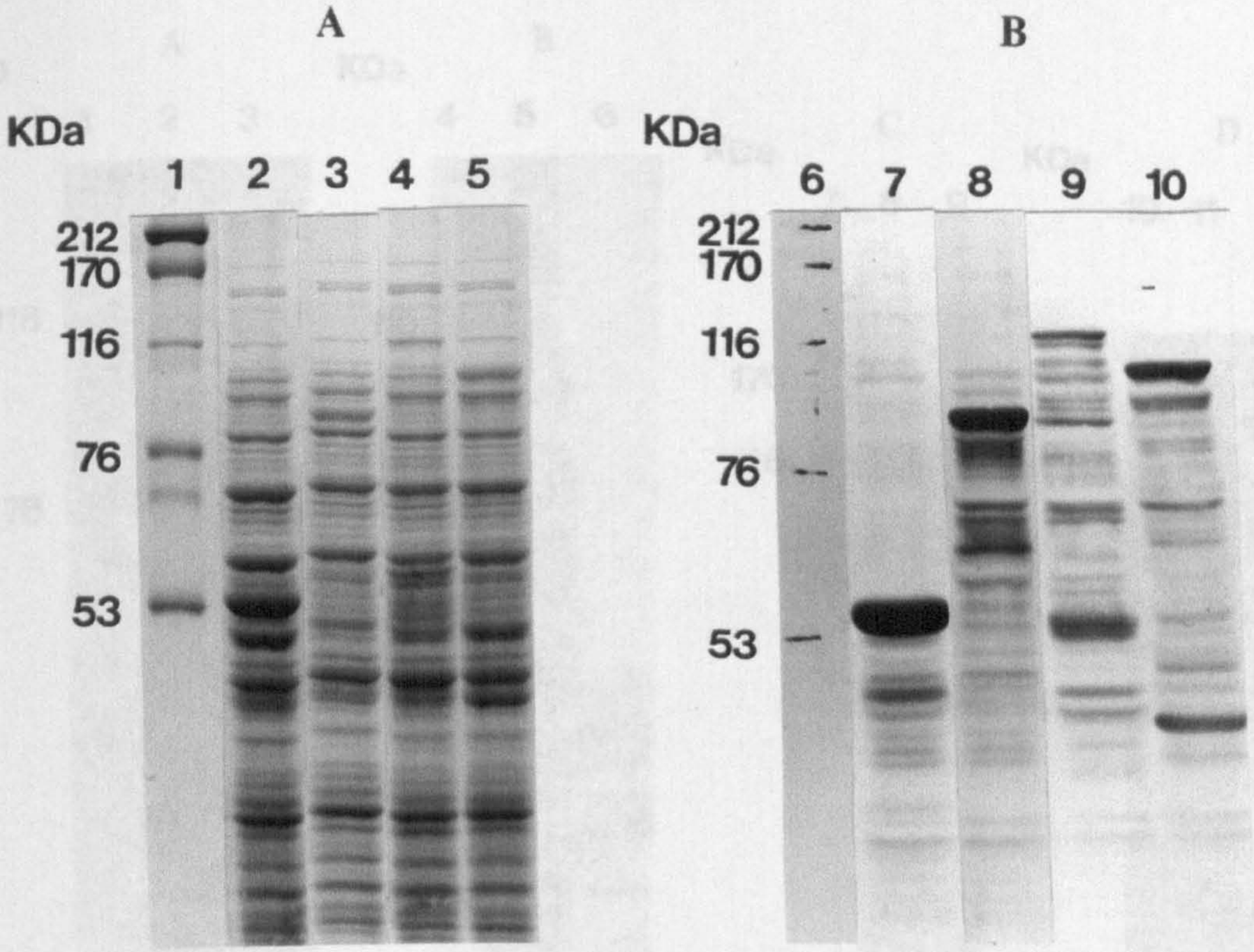


Figure 3.10

8% SDS-PAGE gel stained with coomassie blue (A) and respective western blot (B) probed with MBP antiserum showing the induction of expression of toxin A peptides. Lanes 1 and 6, high MWT markers; lanes 2 and 7, induced JM109 cells containing pMal-c2; lanes 3 and 8, induced JM109 cells containing pMal-c2 plus fragment d; lanes 4 and 9, induced JM109 cells containing pMal-c2 plus fragment c; lanes 5 and 10, induced JM109 cells containing pMal-c2 plus fragment e.

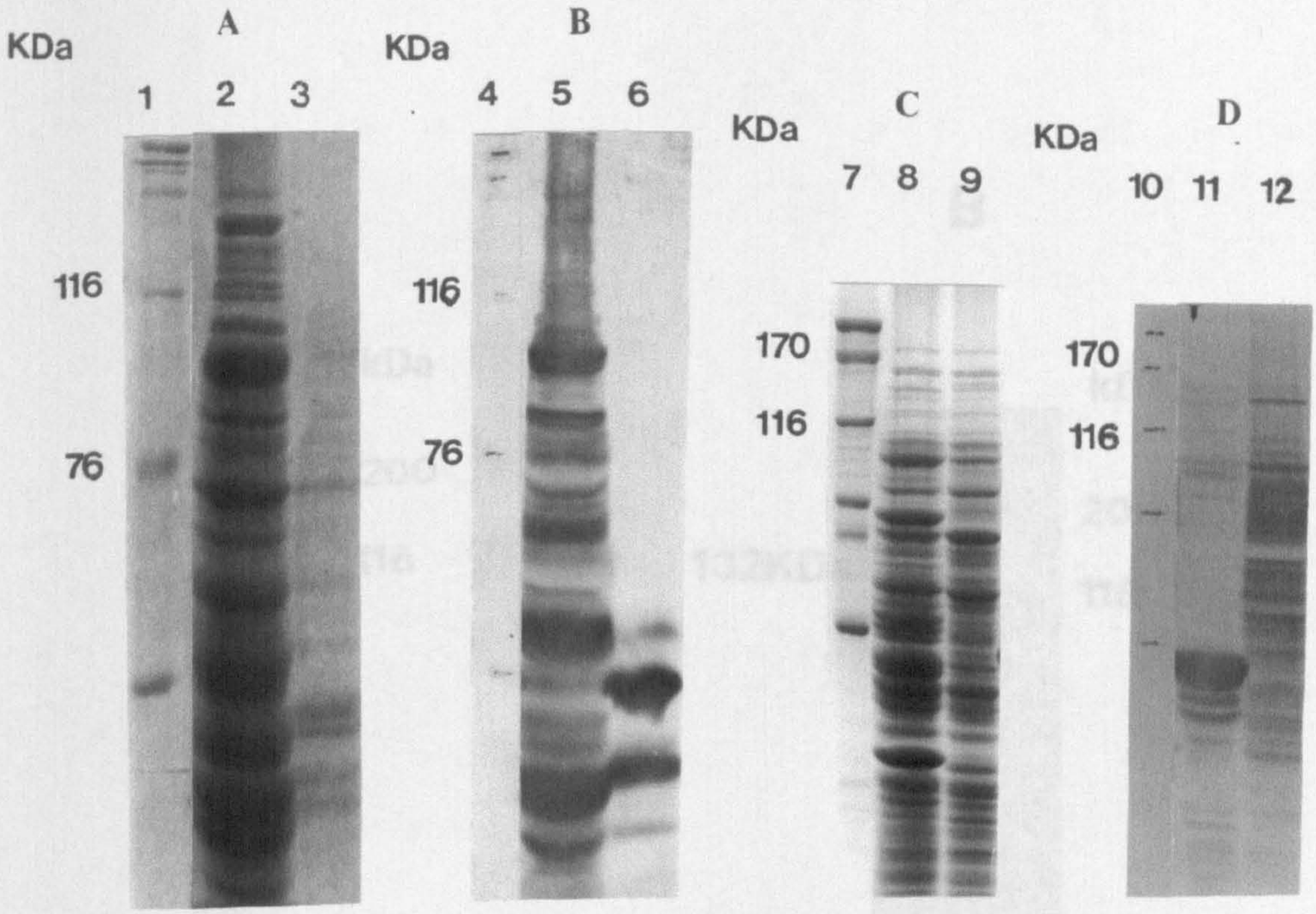


Figure 3.11

8% SDS-PAGE gels and their respective western blots probed with MBP antiserum showing the induction of expression of toxin A peptides.

Coomassie blue stain (A) and western blot (B) of lanes 1 and 4, high MWT markers; lanes 2 and 5, induced TB1 cells containing pMal-p2 plus fragment f; lanes 3 and 6, induced TB1 cells containing pMal-p2 only.

Coomassie blue stain (C) and western blot (D) of lanes 7 and 10, high MWT markers; lanes 8 and 11, induced JM109 cells containing pMal-c2 only; lanes 9 and 12, induced JM109 cells containing pMal-c2 plus fragment g.

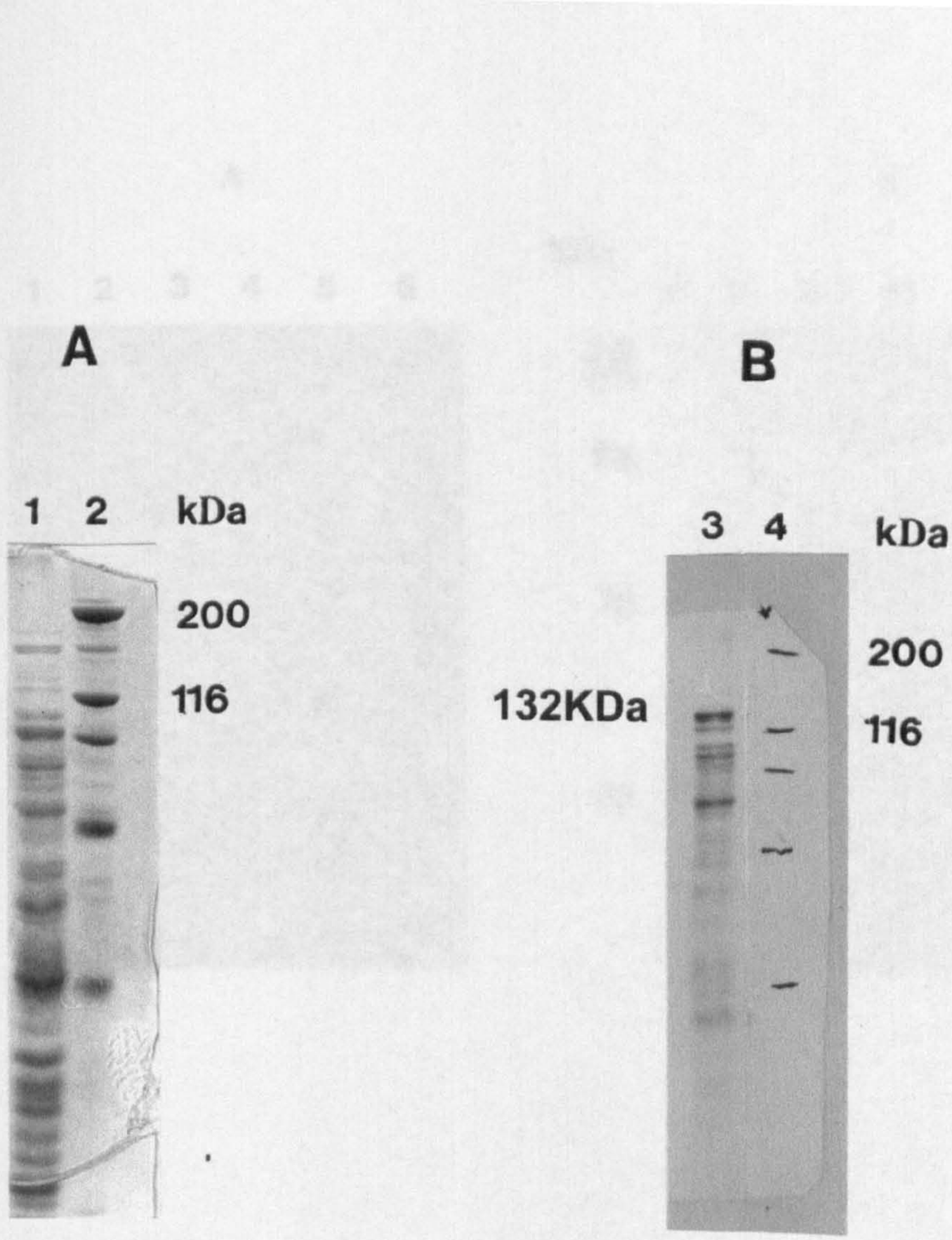


Figure 3.12

Coomassie blue stain (A) and western blot (B) of lanes 1 and 3, induced BL21 DE3 cells containing pMal-p2 plus fragment h1; lanes 2 and 4, high MWT markers.

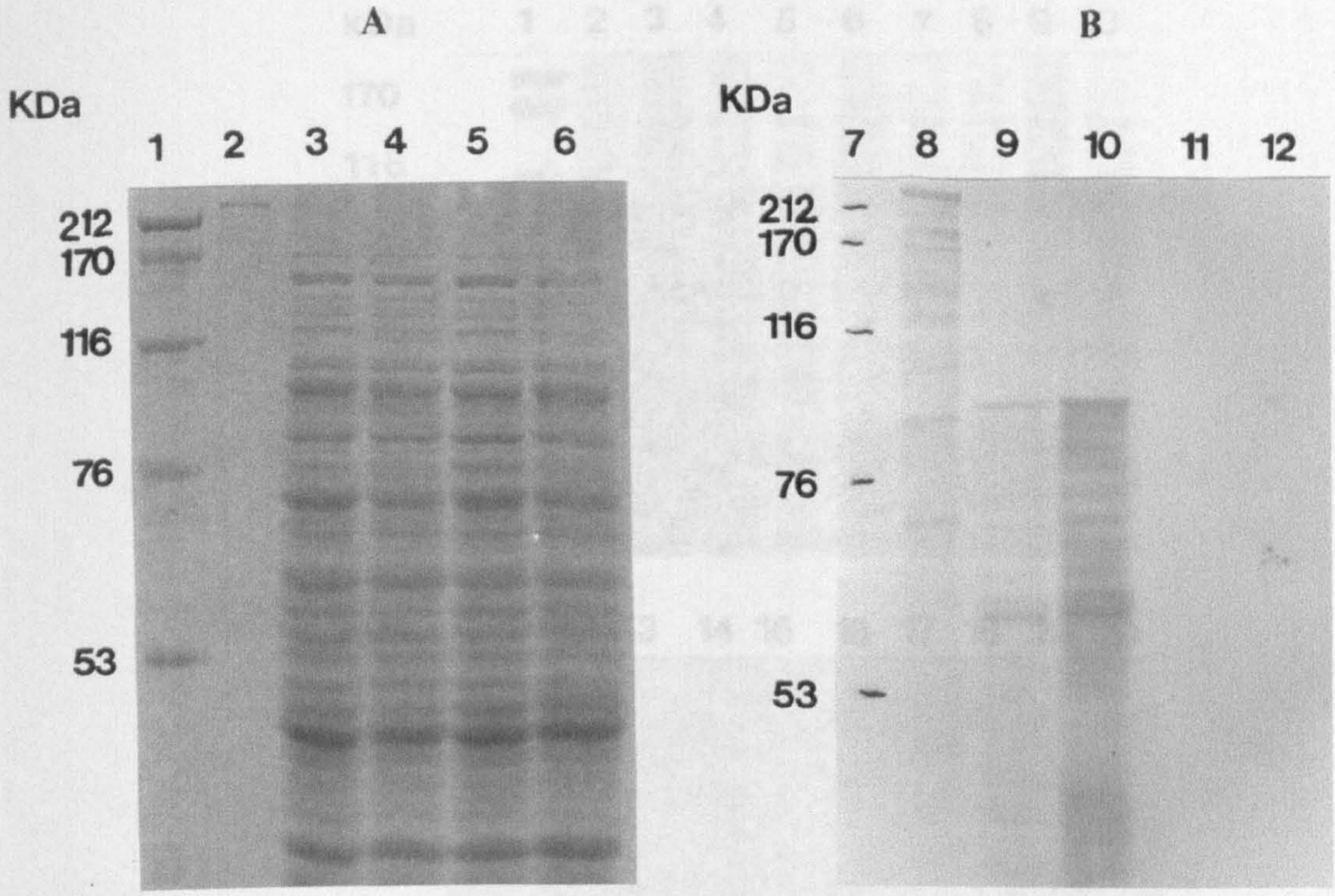


Figure 3.13

8% SDS-PAGE gel (A) and its respective western blot (B) probed with the monoclonal antibody PCG-4 showing the induction of expression of toxin A peptide H2.

Lanes 1 and 7, high MWT markers; lanes 2 and 8, toxin A; lanes 3 and 9, uninduced BL21 DE3 cells containing pET3d plus fragment h2; lanes 4 and 10, induced BL21 DE3 cells containing pET3d plus fragment h2; lanes 5 and 11, uninduced BL21 DE3 cells containing pET3d only; lanes 6 and 12, induced BL21 DE3 cells containing pET3d only.

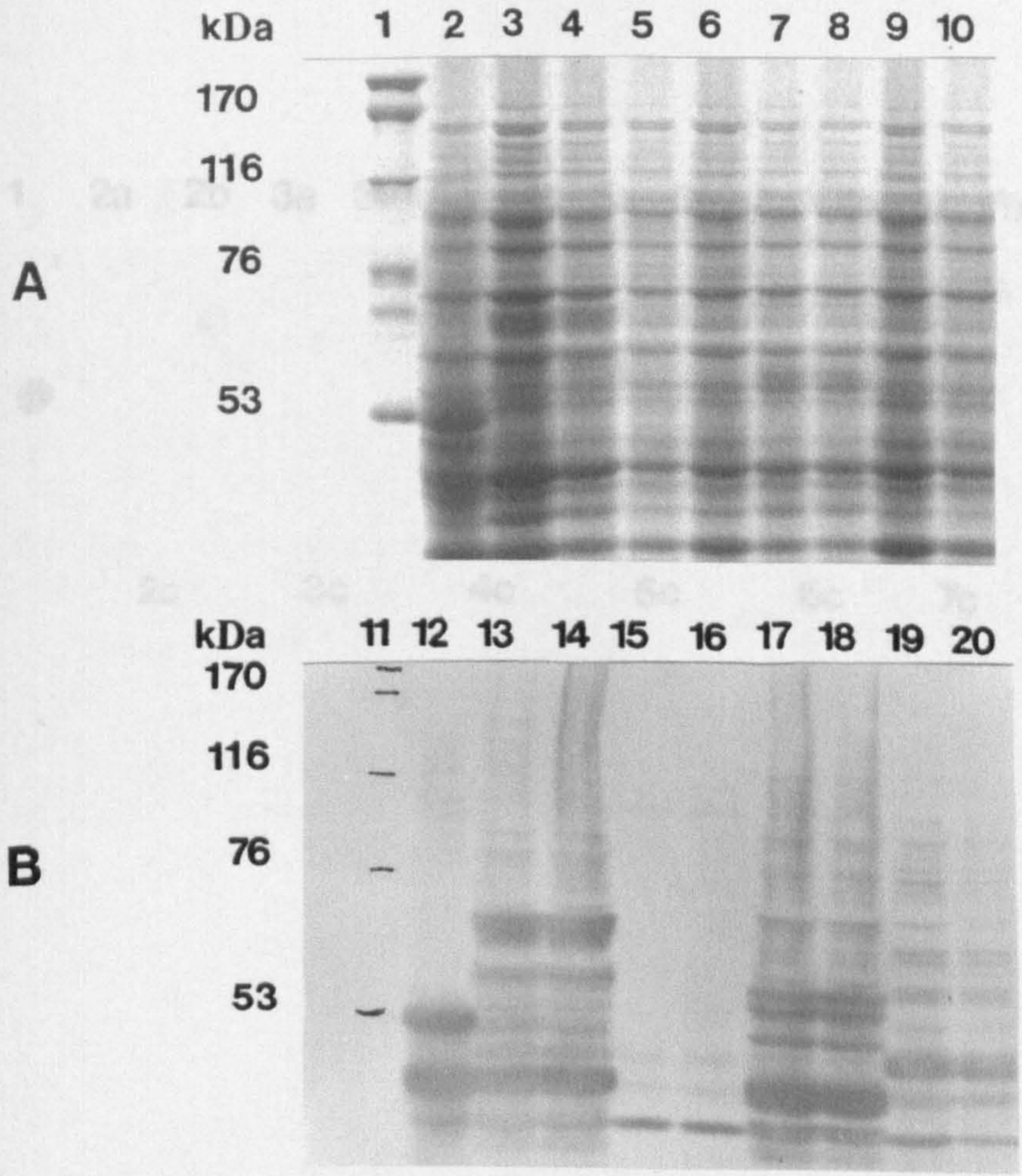


Figure 3.14

8% SDS-PAGE gel and its respective western blot of *E. coli* BL21 DE3 cells showing the induced expression of toxin A fragments in the presence of plasmid pDC952 encoding rare tRNA's.

(A) Coomassie blue stain of lane 1, high MWT markers; lane 2, pMal-p2 only; lane 3, pMal-p2 plus fragment a2; lane 4, as lane 3 containing plasmid pDC952; lane 5, pMal-p2 plus fragment d; lane 6, as lane 5 containing plasmid pDC952; lane 7, pMal-p2 plus fragment c; lane 8, as lane 7 containing plasmid pDC952; lane 9, pMal-p2 plus fragment e; lane 10, as lane 9 containing plasmid pDC952.

(B) western blot of (A) probed with MBP antiserum; Lanes 11-20 as lanes 1-10.

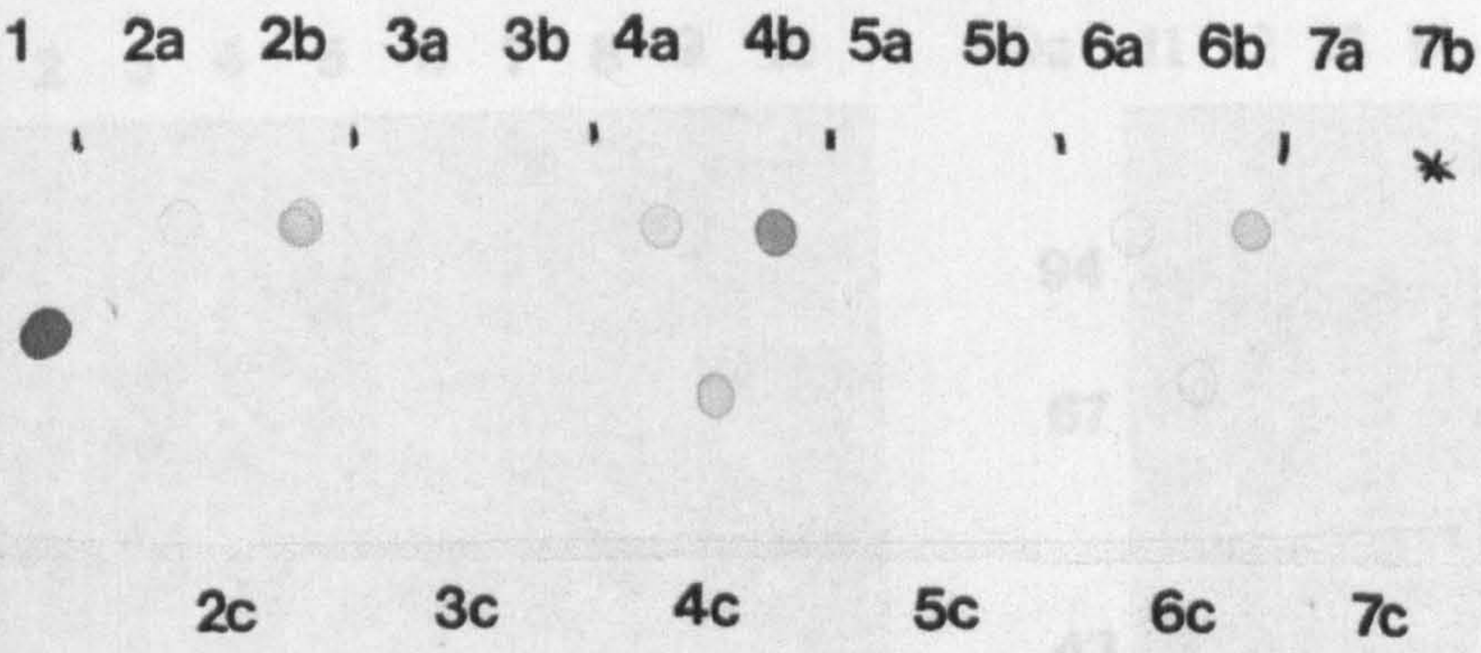


Figure 3.15

Optimisation of expression of toxin A peptide H1 by transforming into different *E. coli* cell lines.

Lane 1, toxin A; lane 2a, uninduced pMal-p2 plus fragment h1 in *E. coli* TB1; lane 2b, as lane 2a but induced; lane 2c, as 2b but supernatant fraction only; lane 3a, uninduced pMal-p2 only in *E. coli* TB1; lane 3b, as lane 3a but induced; lane 3c, as lane 3b but supernatant fraction only; lanes 4a-4c, as lanes 2a-2c but in *E. coli* BL21 DE3; lanes 5a-5c, as lanes 3a-3c but in *E. coli* BL21 DE3; lanes 6a-6c, as lanes 2a-2c but in *E. coli* JM109; lanes 7a-7c, as lanes 3a-3c but in *E. coli* JM109.

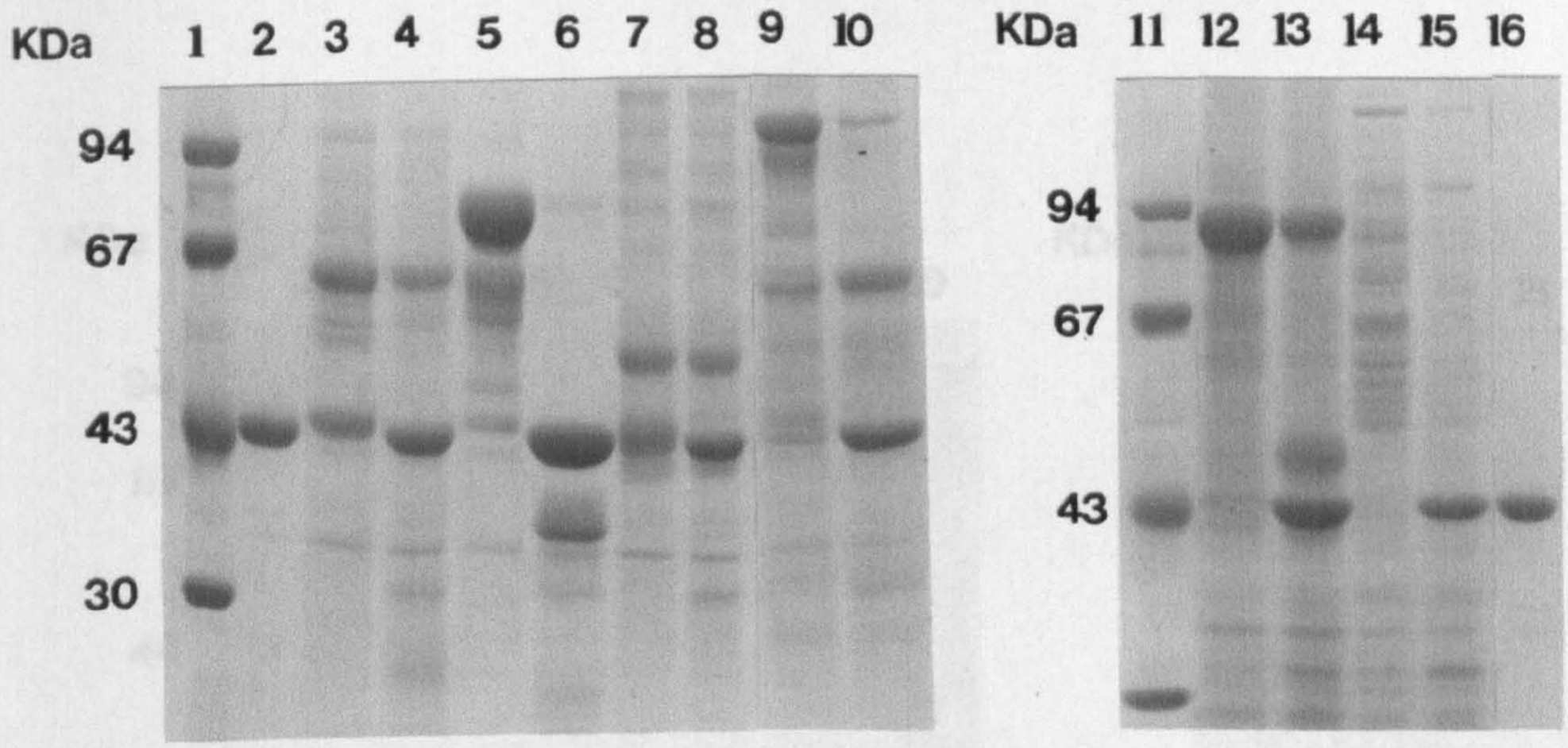


Figure 3.16

8% SDS-PAGE gels showing the amylose affinity purification and factor Xa cleavage (1%) of the toxin A peptide fusion products.

Lanes 1 and 11, Low MWT markers; lanes 2 and 16, MBP; lane 3, uncleaved fusion peptide A2; lane 4, cleaved fusion peptide A2; lane 5, uncleaved fusion peptide B; lane 6, cleaved fusion peptide B; lane 7, uncleaved fusion peptide C; lane 8, cleaved fusion peptide C; lane 9, uncleaved fusion peptide E; lane 10, cleaved fusion peptide E; lane 12, uncleaved fusion peptide D; lane 13, cleaved fusion peptide D; lane 14, uncleaved fusion peptide G; lane 15, cleaved fusion peptide G.

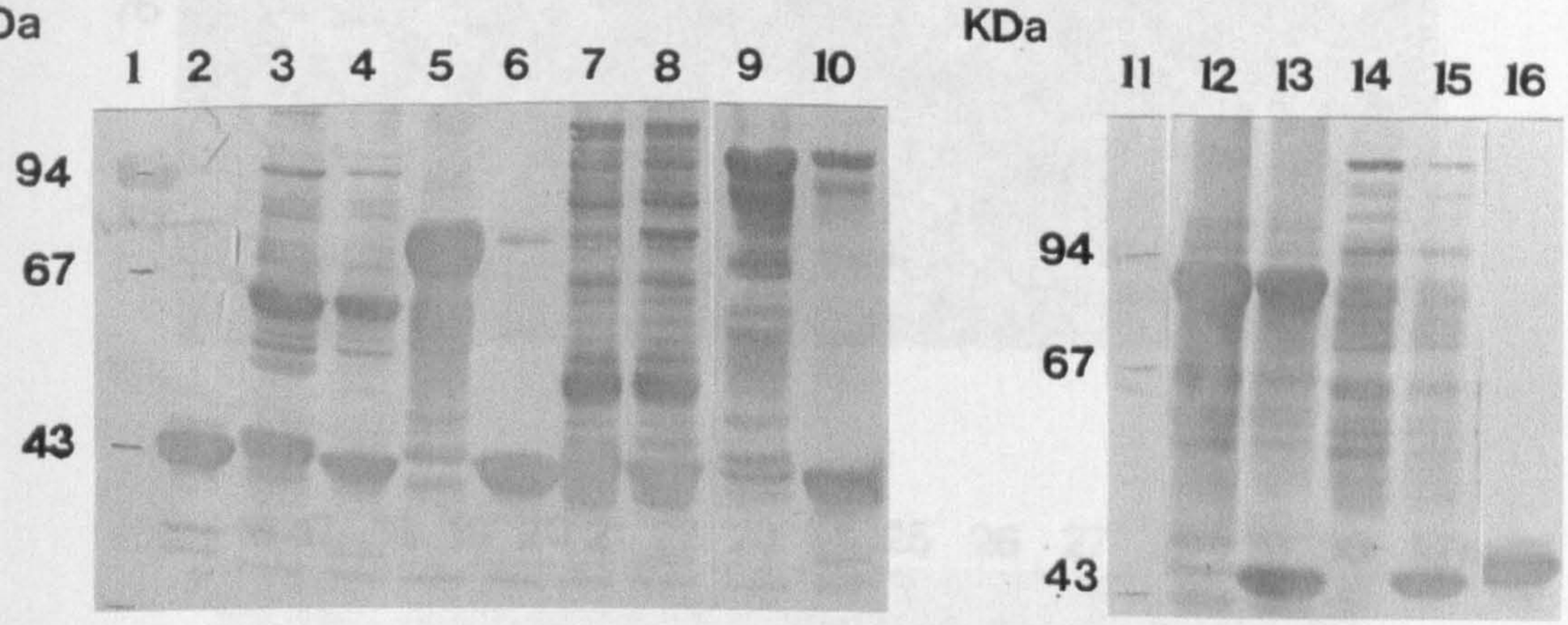


Figure 3.17

Western blots of the gels shown in figure 3.16 probed with MBP antiserum.

Lanes 1-16 as figure 3.16 lanes 1-16.

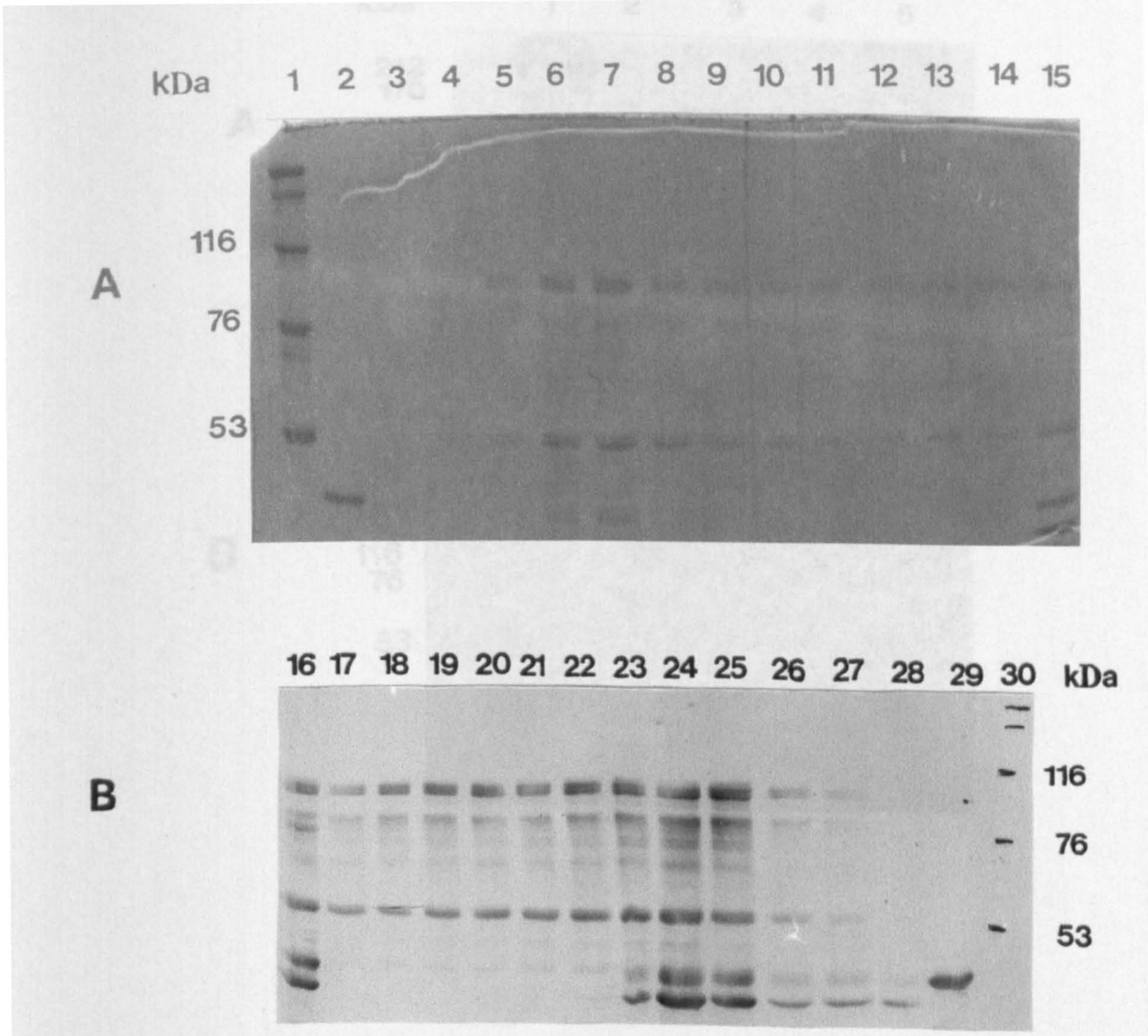


Figure 3.18

8% SDS-PAGE gel (A) and western blot (B) probed with MBP antiserum showing amylose affinity purification and factor Xa cleavage of the peptide F fusion product.

Lanes 1 and 30, High MWT markers; lanes 2 and 29, MBP; lanes 3-14, uncleaved fusion peptide F (fractions 1-12); lane 15, cleaved fusion peptide (fraction 5); lane 16, as lane 15; lanes 28-17, as lanes 3-14.

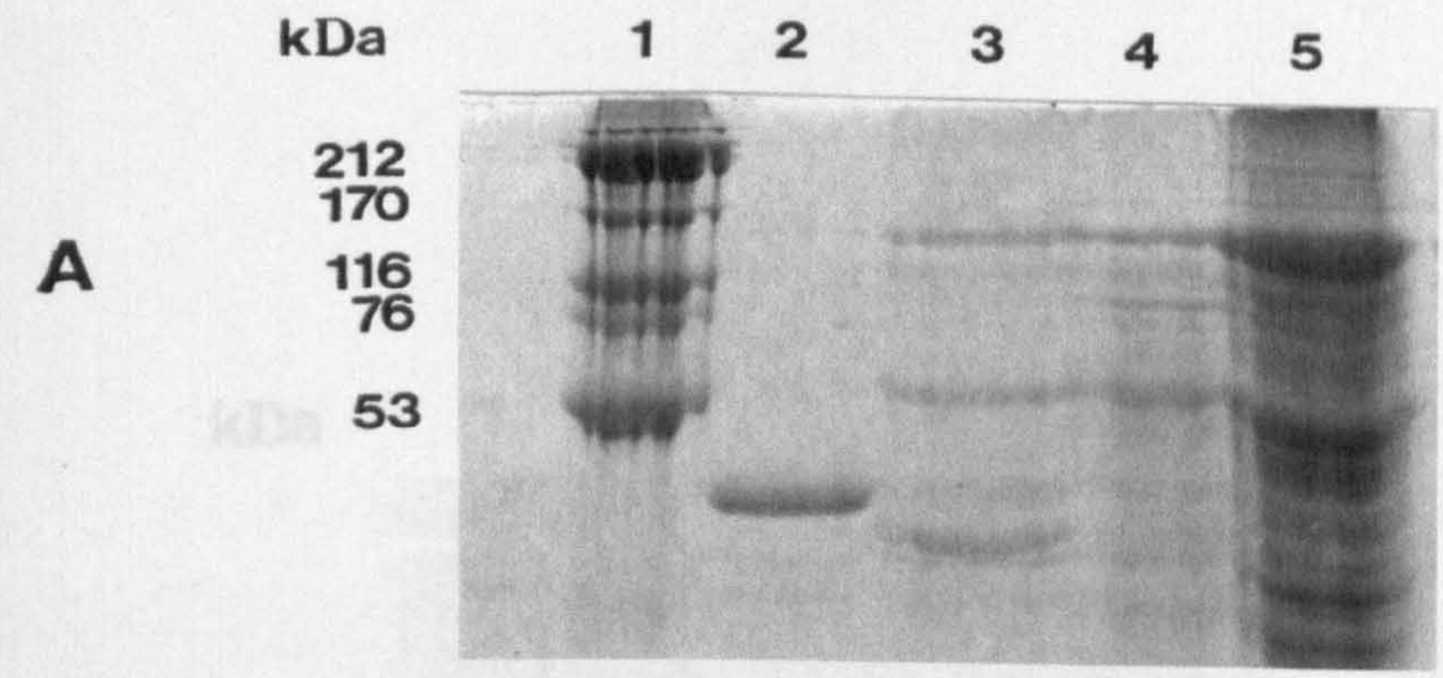


Figure 3.19

10% SDS-PAGE gel (A) and western blot (B) probed with MBP antiserum to determine the solubility of the toxin A peptide F fusion product.

Lanes 1 and 6, High MWT markers; lanes 2 and 7, MBP; lanes 3 and 8, peptide F fusion product purified by amylose affinity chromatography; lanes 4 and 9, peptide F fusion product purified from inclusion bodies (supernatant fraction); lanes 5 and 10, as lanes 4 and 9 (pellet fraction).

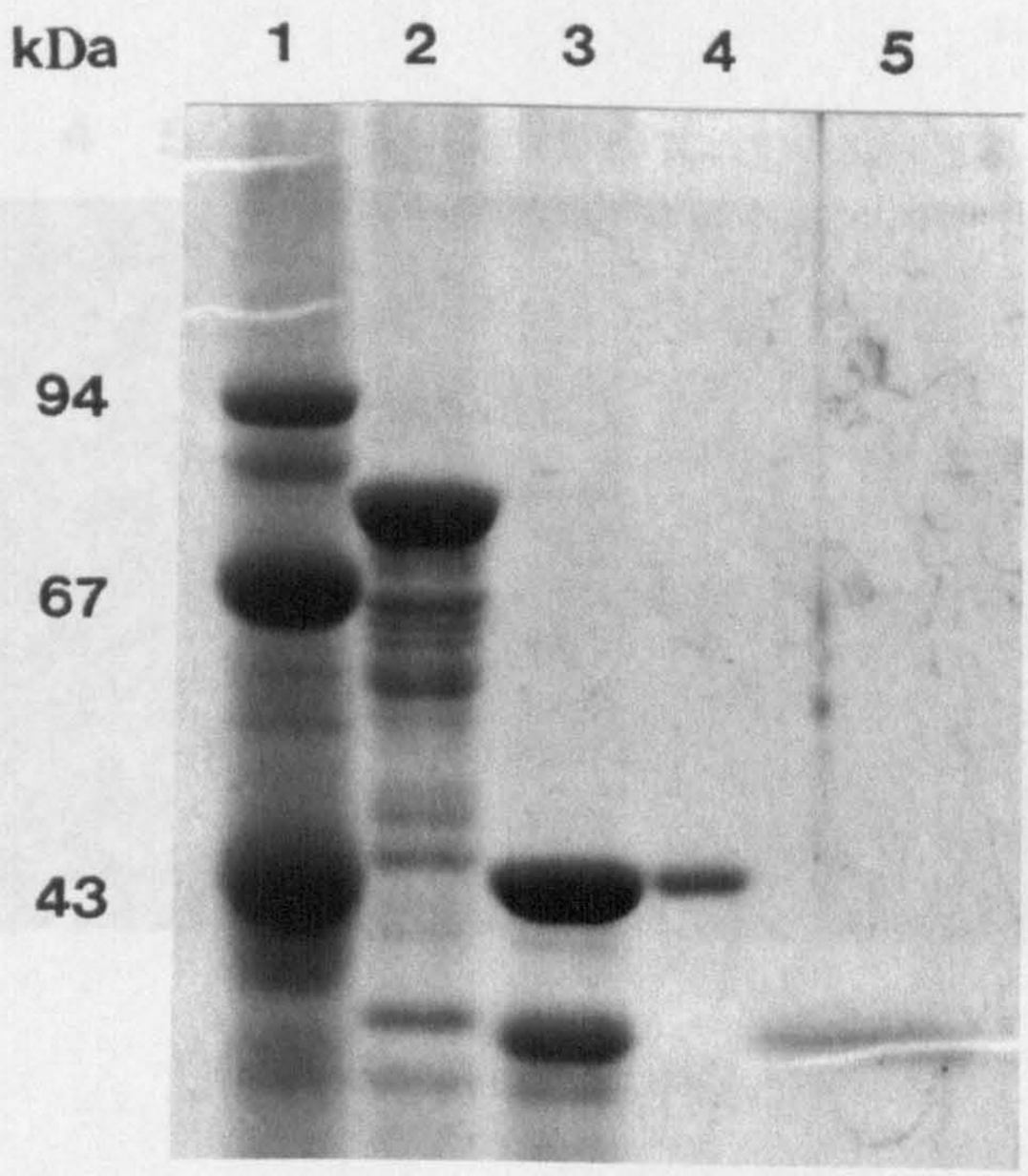


Figure 3.20

8% SDS-PAGE gel showing electro-eluted toxin A peptide B.

Lane 1, Low MWT markers; lane 2, uncleaved toxin A peptide B fusion product; lane 3, cleaved toxin A peptide B fusion product; lane 4, MBP; lane 5, electro-eluted toxin A peptide B.

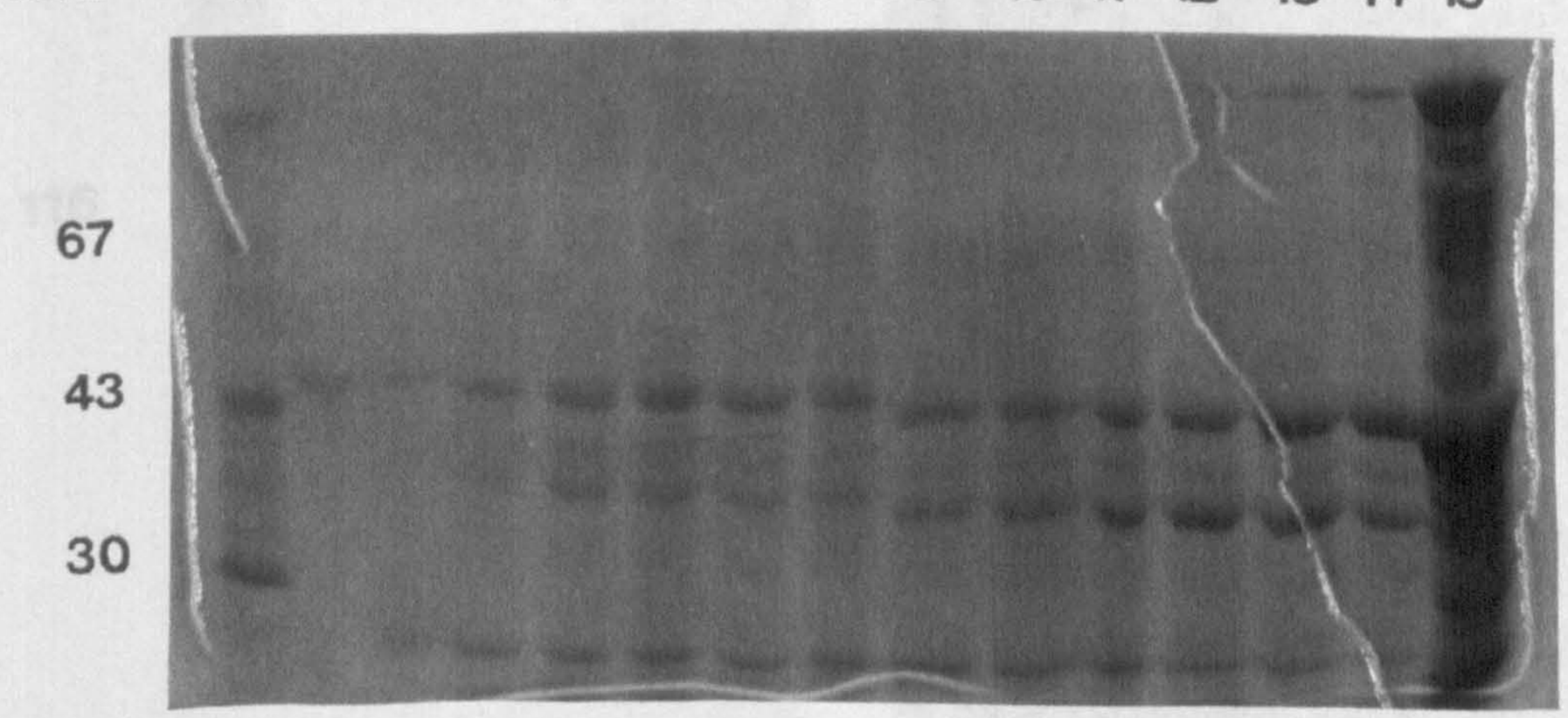


Figure 3.21

8% SDS-PAGE gel showing the fractions eluted following factor Xa cleavage of the toxin A peptide B fusion product on an amylose affinity column.

Lane 1, Low MWT markers; lane 2, MBP; lanes 3-13, amylose column buffer wash fractions (1-11) following overnight cleavage; lane 14, cleaved toxin A peptide B fusion product; lane 15, 10mM maltose eluted fraction following wash.

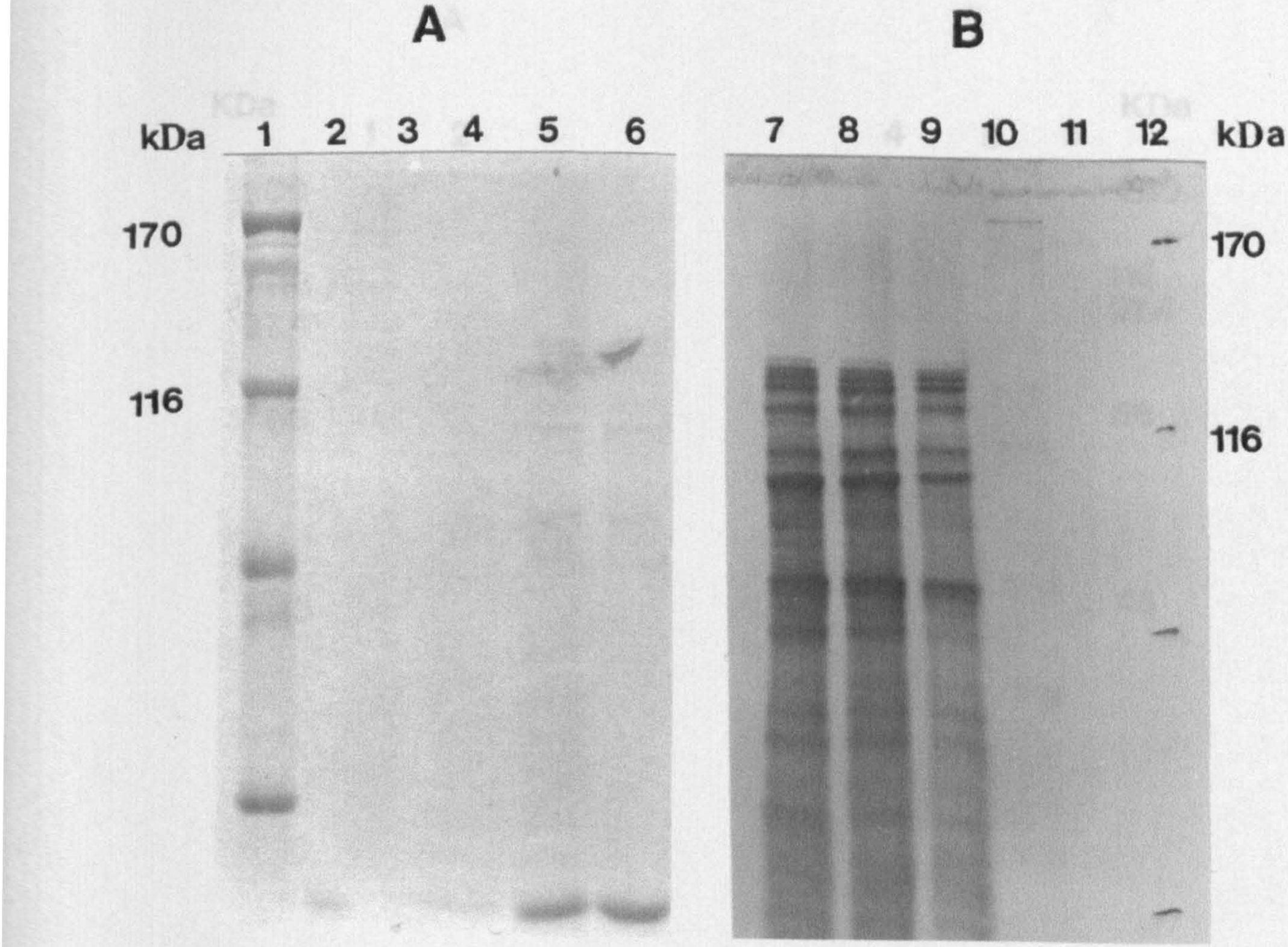


Figure 3.22

8% SDS-PAGE gel (A) and subsequent western blot (B) probed with the monoclonal antibody PCG-4 showing the peptide H1 fusion product purified by thyroglobulin affinity chromatography.

Lanes 1 and 12, High MWT markers; lanes 2 and 11, MBP; lanes 3 and 10, toxin A; lanes 4 and 9, purified peptide H1 fusion product (fraction 4); lanes 5 and 8, as lanes 4 and 9 (fraction 5); lanes 6 and 7, as lanes 4 and 9 (fraction 6).

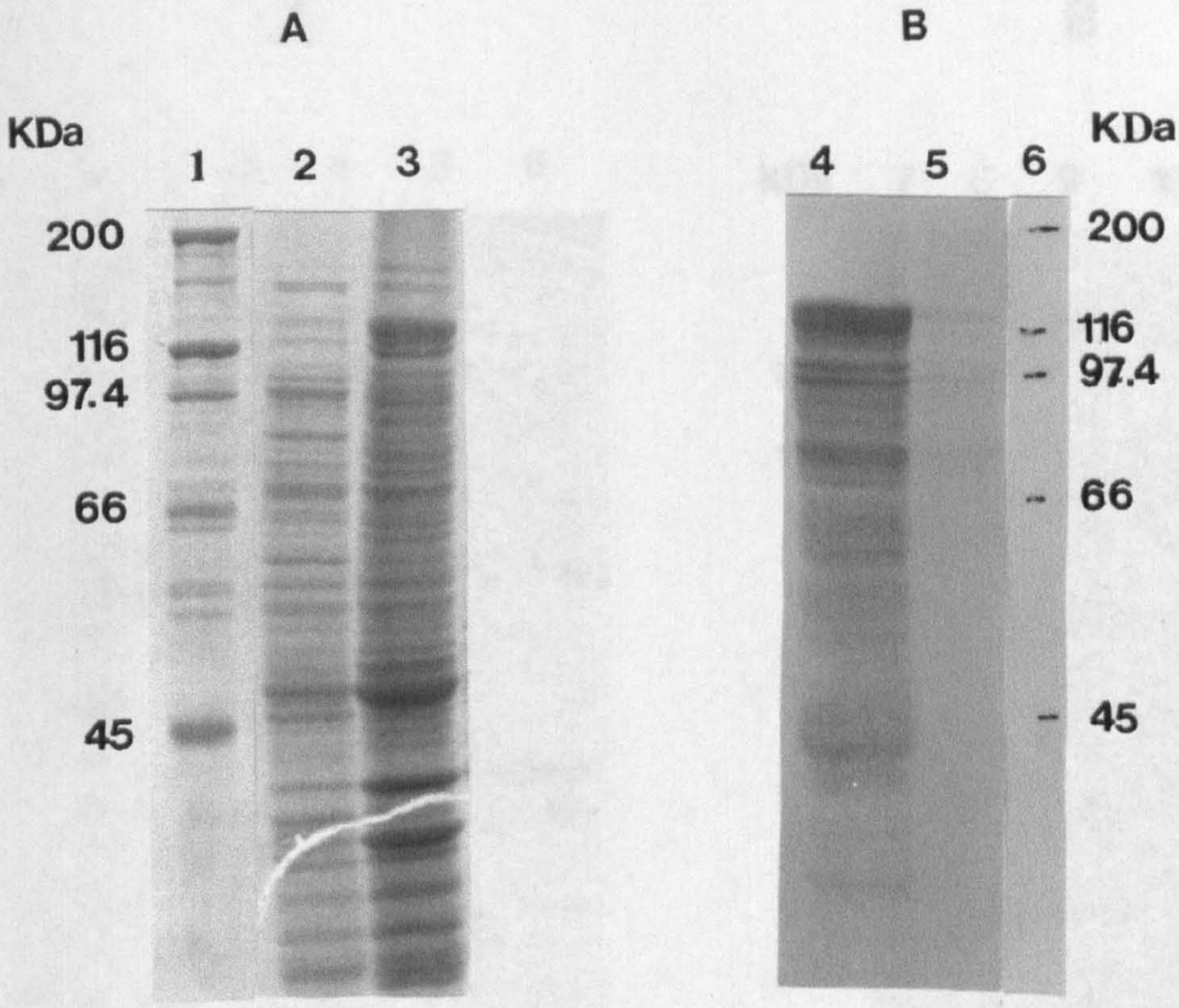


Figure 3.23

8% SDS-PAGE gel (A) and subsequent western blot (B) probed with the monoclonal antibody PCG-4 to determine the solubility of the toxin A peptide H1 fusion product. Lanes 1 and 6, High MWT markers; lanes 2 and 5, induced BL21 DE3 cells containing pMal-p2 plus toxin A fragment h1 (supernatant fraction); lanes 3 and 4, as lanes 2 and 5 (pellet fraction).

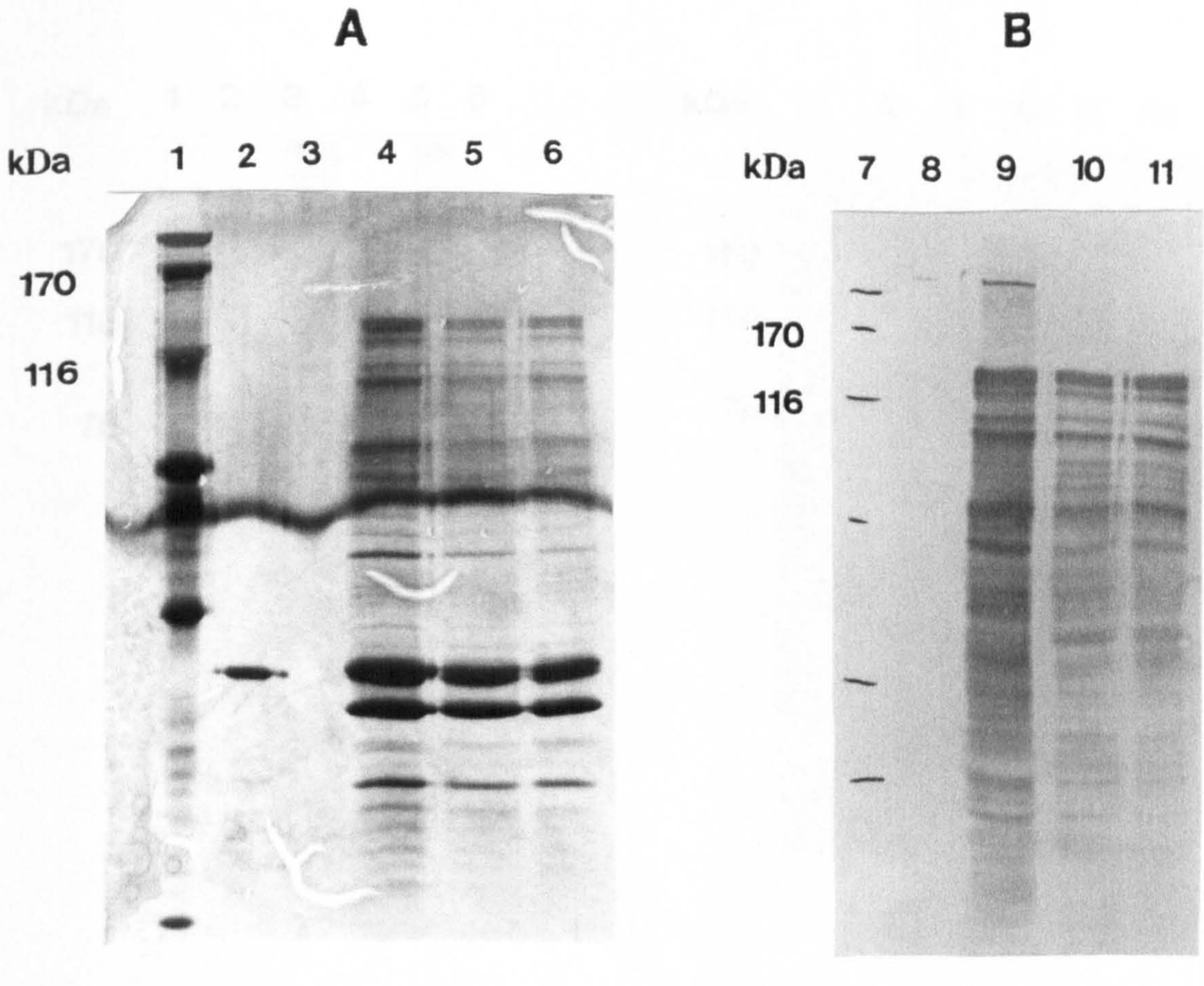


Figure 3.24

8% silver stained SDS-PAGE gel (A) and western blot (B) probed with the monoclonal antibody PCG-4 showing the factor Xa cleavage of the peptide H1 fusion product purified by amylose affinity chromatography.

Lanes 1 and 7, High MWT markers; lanes 2 and 8, MBP; lanes 4 and 9, uncleaved peptide H1 fusion product; lanes 5 and 10, peptide H1 fusion product cleaved with 1% factor Xa; lanes 6 and 11, as lanes 5 and 10 cleaved with 2% factor Xa.

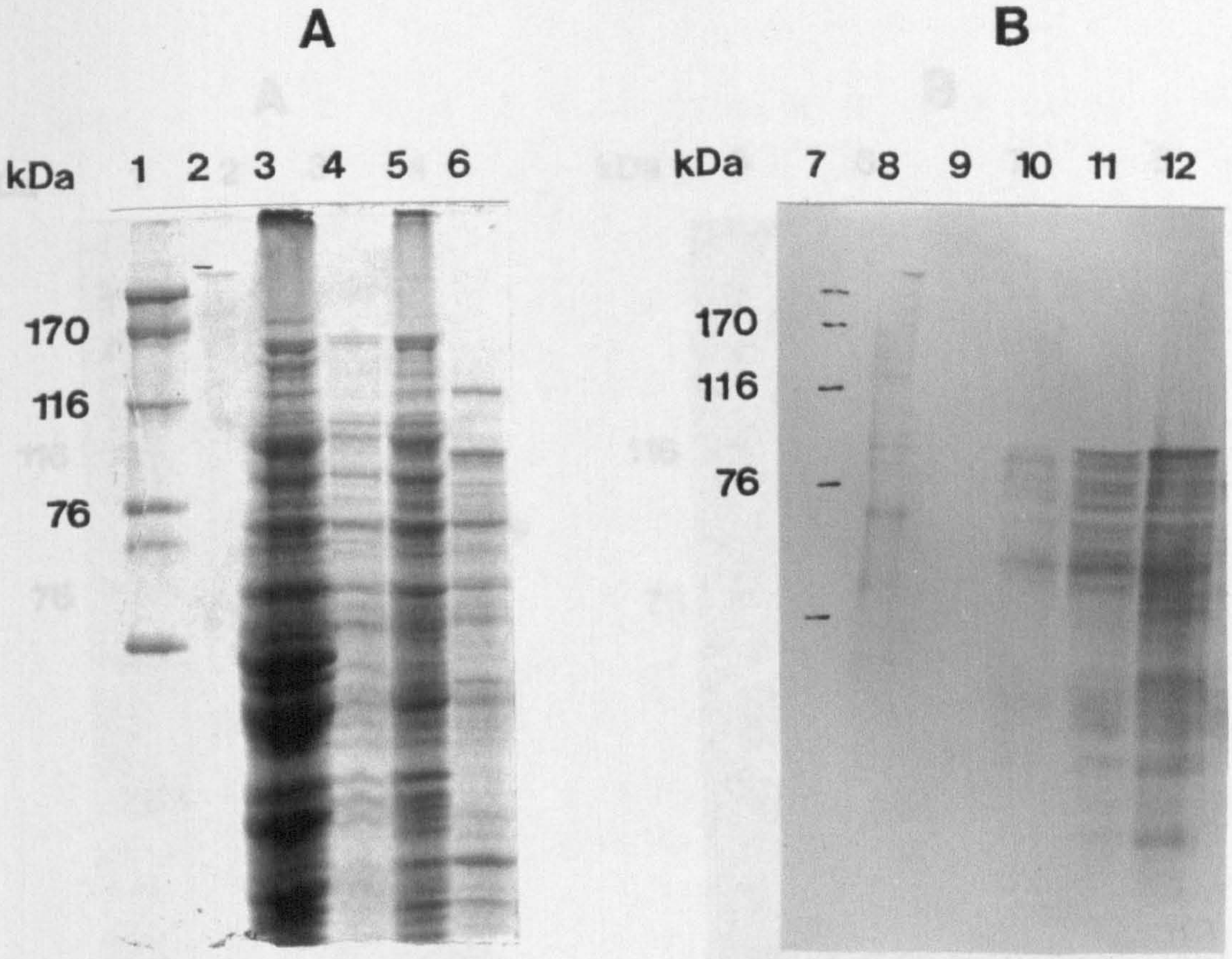


Figure 3.25

8% SDS-PAGE gel (A) and western blot (B) probed with the monoclonal antibody PCG-4 showing the location of peptide H2 at different stages of purification from inclusion bodies within BL21 DE3 cells.

Lanes 1 and 7, High MWT markers; lane 2 and 8, toxin A; lanes 3 and 9, induced whole cells containing the pET3d plasmid only; lanes 4 and 10, induced cells containing pET3d plus toxin A fragment h2 (supernatant fraction following PBS wash); lanes 5 and 11, as lanes 4 and 10, (soluble sonicate supernatant); lanes 6 and 12, as lanes 4 and 10 (insoluble sonicate fraction following solubilisation with guanidine hydrochloride).

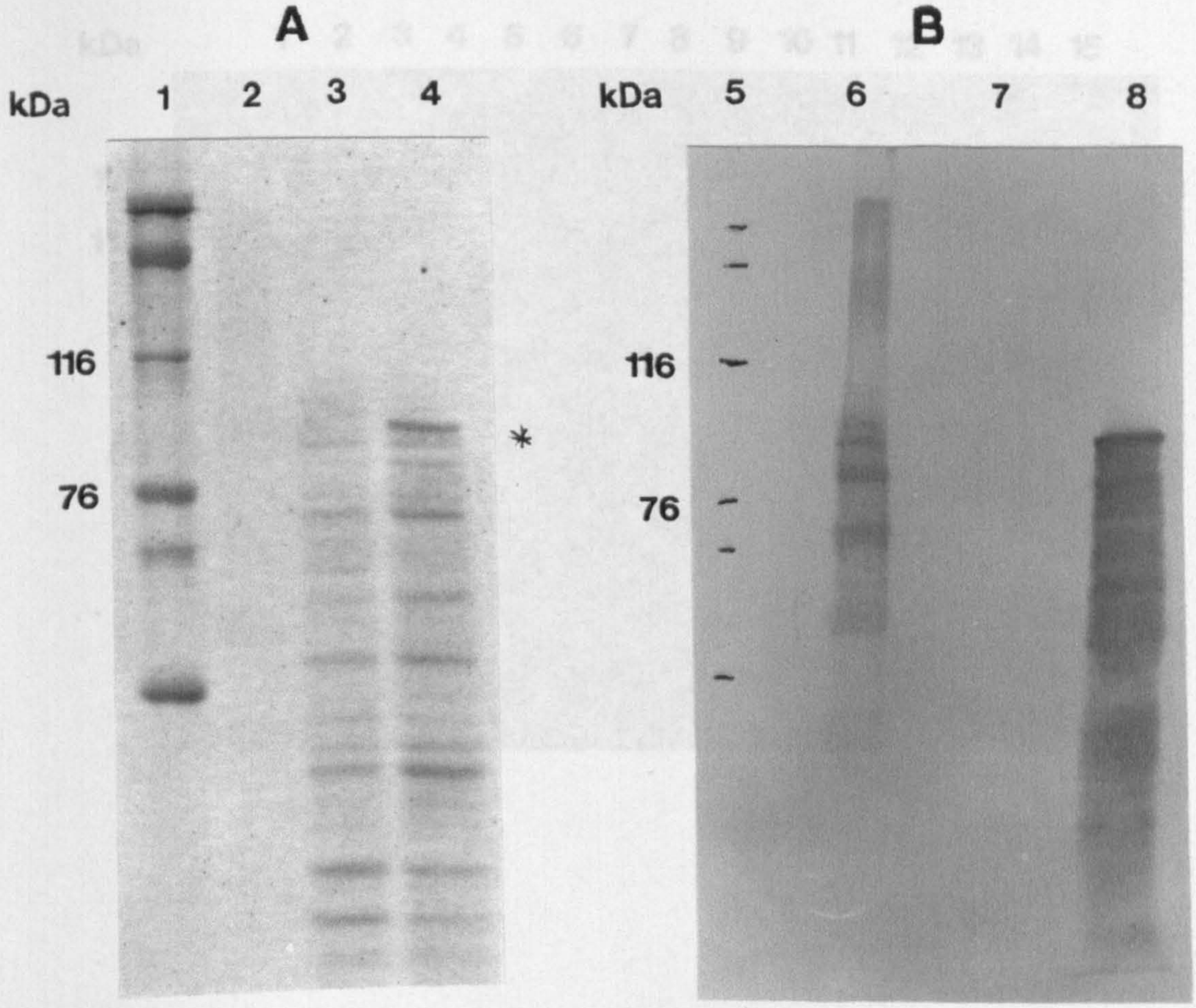


Figure 3.26

8% SDS-PAGE gel (A) and western blot (B) probed with monoclonal antibody PCG-4 showing toxin A peptide H2 and its respective *E. coli* negative control purified from insoluble inclusion bodies within the *E. coli* BL21 DE3 cells.

Lanes 1 and 5, High MWT markers; lanes 2 and 6, toxin A; lanes 3 and 7, *E. coli* negative control purified from BL21 DE3 cells containing plasmid pET3d only; lanes 4 and 8, toxin A peptide H2 (*) purified from BL21 DE3 cells containing pET3d plus toxin A fragment h2.

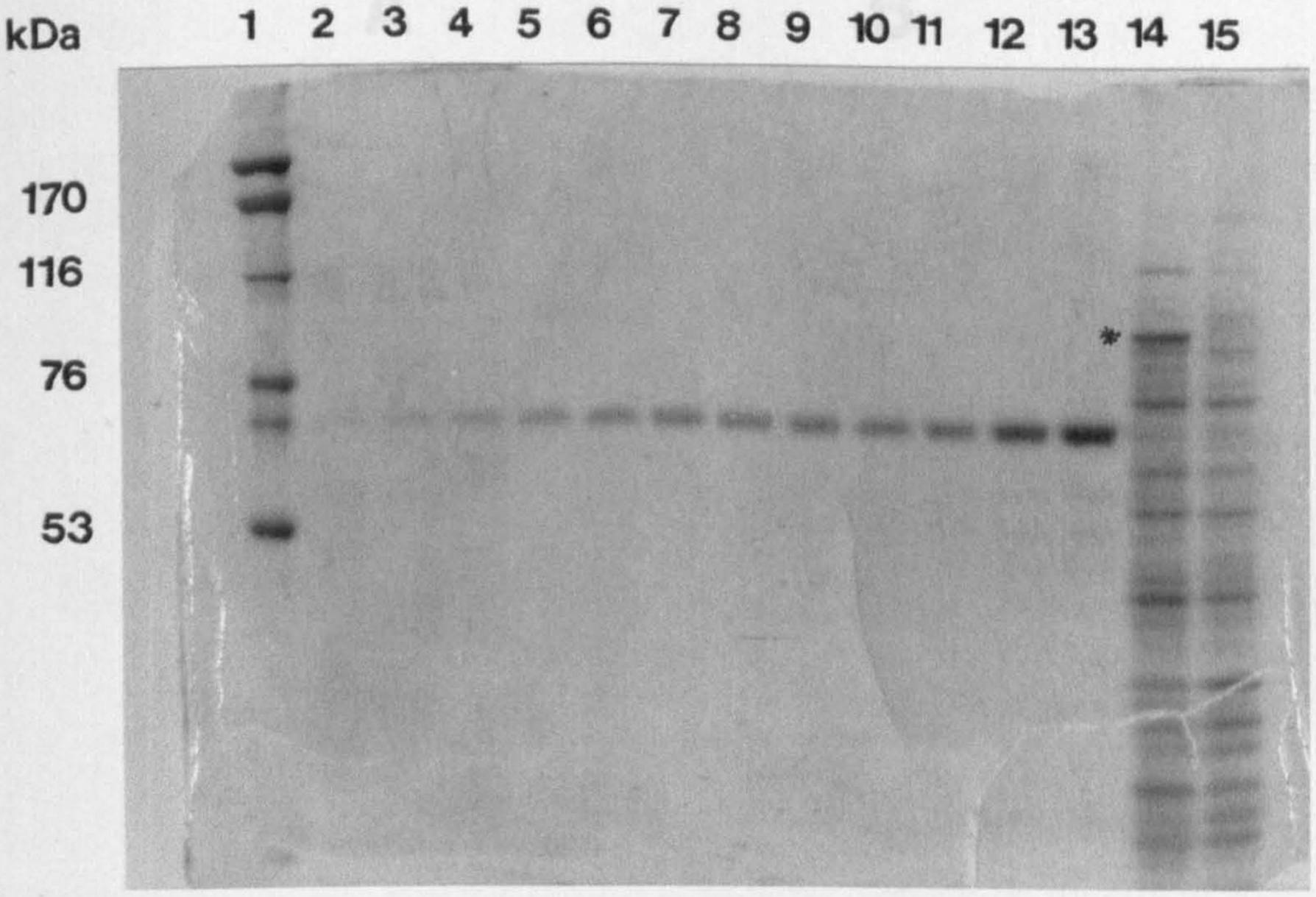


Figure 3.27

8% SDS-PAGE gel showing Bovine Serum Albumin (BSA) concentration standards for the estimation of the concentration of peptide H2 in the protein mixture purified from inclusion bodies.

Lane 1, High MWT markers; lane 2, 100ng BSA; lane 3, 200ng BSA; lane 4, 300ng BSA; lane 5, 400ng BSA; lane 6, 500ng BSA; lane 7, 600ng BSA; lane 8, 700ng BSA; lane 9, 800ng BSA; lane 10, 900ng BSA; lane 11, 1μg BSA; lane 12, 2μg BSA; lane 13, 3μg BSA; lane 14, full length toxin A peptide H2 (*) in protein mixture purified from inclusion bodies; lane 15, *E. coli* negative control proteins purified from inclusion bodies.

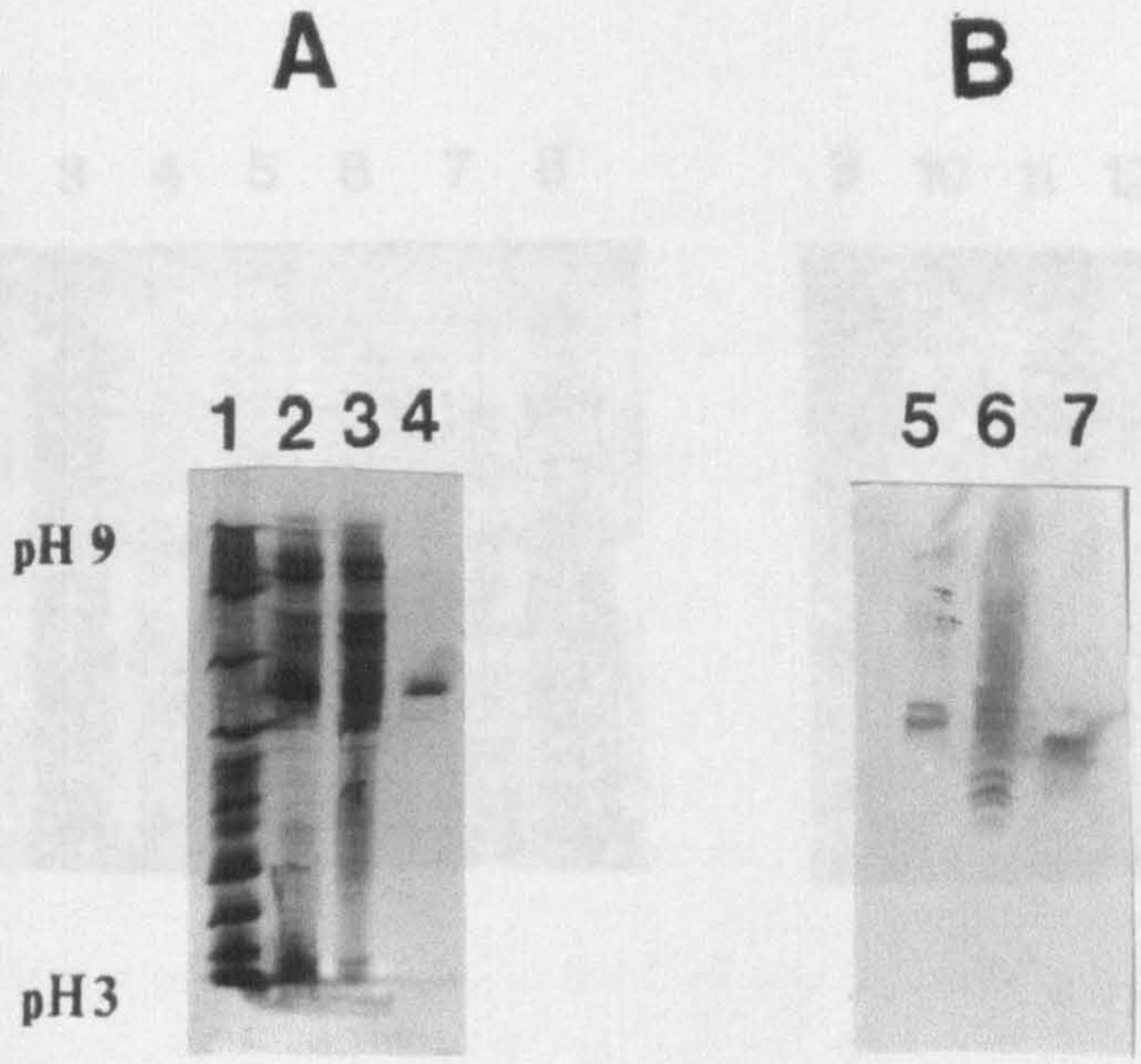


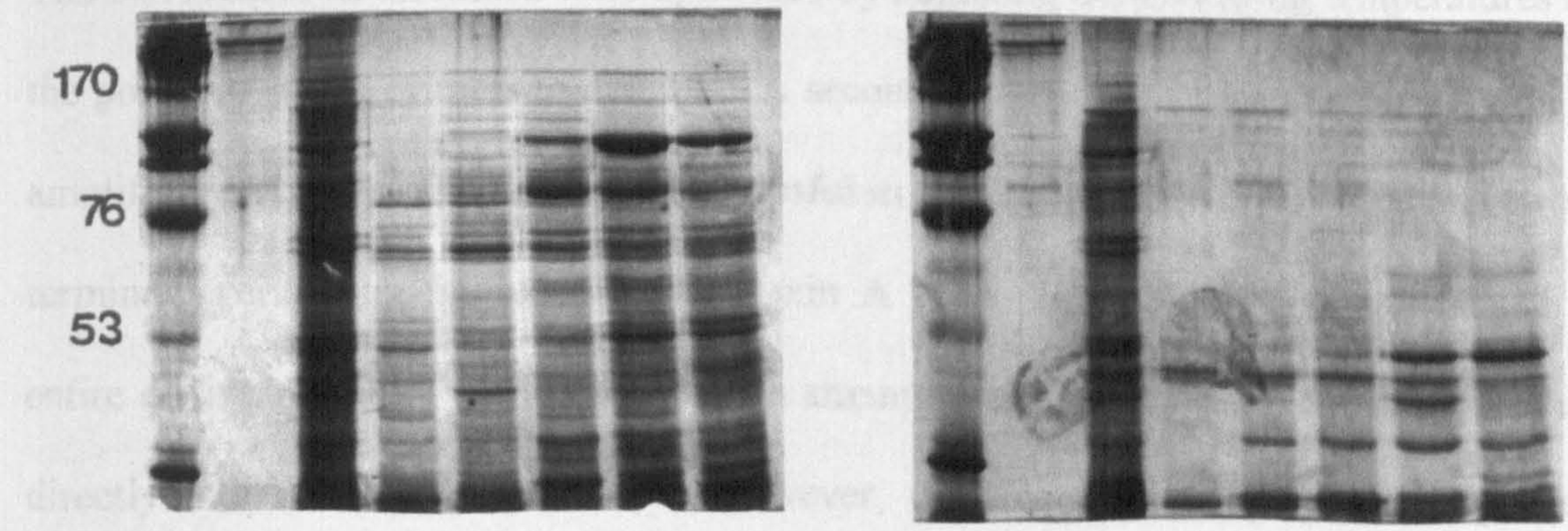
Figure 3.28

Determination of the pI values of the toxin A peptides H1 and H2 by isoelectric focusing.

A) Silver stained pH. 3-9 Broad Range Phastgel of lane 1, broad range markers; lane 2, toxin A peptide H2 purified from inclusion bodies; lane 3, toxin A peptide H1 purified from inclusion bodies; lane 4, toxin A.

B) Western blot of (A) probed with the monoclonal antibody PCG-4. Lanes 5-7, as lanes 2-4.

A



B

Lane	3	4	5	6	7	8	11	12	13	14	15	16
H/A titre	1:128	1:4	1:2	1:2	1:2	1:2	1:128	1:2	1:2	1:2	1:2	1:2

Figure 3.29

A) 10% silver stained gels showing the fractions eluted after the peptide H2 / *E. coli* protein mixture was applied to a Mono Q anion-exchange column at pH. 8.5.

Lanes 1 and 9, High MWT markers; lanes 2 and 10, toxin A; lanes 3 and 11, peptide H2 preparation purified from inclusion bodies; lane 4, eluted fraction 12; lanes 5-8, eluted fractions 20-23; lanes 12-16, eluted fractions 24-28.

B) A table to show the H/A titres of the fractions (shown in A) eluted from the anion-exchange column.

3.5 DISCUSSION

The PCR reactions discussed were optimised by adjusting the annealing temperatures and the positions of the primers to avoid DNA secondary structure loops interfering with the amplification cycles. The study was successful in amplifying eight N-terminal and two C-terminal overlapping fragments of the toxin A gene. These fragments represented the entire coding region of the toxin gene. An attempt was made to clone the PCR products directly into the expression vectors, however, the close proximity of the engineered restriction endonuclease sites to the ends of the primers made this task difficult. To overcome this problem the PCR products were first cloned into a T-tailing vector by taking advantage of the poly A tail, which is added by *Taq* polymerase. Once in the expression vectors, the toxin A fragments were sequenced to confirm the relative position of each fragment in the toxin A gene. The sequence data also confirmed that each fragment was in the correct reading frame for expression from each host plasmid.

Of the ten toxin A fragments cloned, nine resulted in the expression of a recombinant fusion protein upon IPTG induction. The tenth, toxin A fragment a1, would not express in either the pMal-p2 or pMal-c2 vectors. It has been suggested that the second codon in a gene could affect its expression by as much as 15-fold. Codons such as AAA, AAU and ACC in the second position after the initiation codon have been shown to enhance expression in *E. coli* (Komine, Y *et al*, 1990). Although this is an important consideration, recombinant peptides expressed from the pMal vectors have an N-terminal MBP fusion and this protein alone is expressed in high levels in *E. coli*.

A new forward PCR primer was designed 335bp upstream from the primer used to produce fragment a1 in the hope of amplifying the first 1.6Kb of the toxin A gene. The PCR product amplified (fragment a2) was successfully expressed in *E. coli* from the

pMal-c2 vector. A large proportion of the expressed protein (A2) however, was truncated. In an attempt to overcome this problem, fragment a2 was also cloned into the pET3a vector. This vector allows expression of a peptide with a six amino acid histidine fusion on its N-terminus. Unfortunately, expression from this vector failed to produce a recombinant peptide. These results suggested that the presence of a large N-terminal fusion such as the MBP, as opposed to a small fusion, was favourable and facilitated translation to proceed into the toxin A fragment.

The toxin A fragments a2, c, d, e and g expressed fusion products from the pMal-c2 vector but not the pMal-p2 vector. In the pMal-p2 vector the signal sequence of the *mal E* gene is intact, which potentially allows fusion proteins to be exported to the periplasm. The lack of expression of these fragments from the pMal-p2 vector suggested that they were not exported to the periplasm, possibly due to secondary structure that is incompatible with passage through the membrane. These peptides were more stable from the pMal-c2 vector, which allows cytoplasmic expression. Peptides B, F and H1 were satisfactorily expressed from the pMal-p2 vector. As passage of a protein through the membrane allows disulphide bond formation, it cannot be assumed that the peptides expressed in the cytoplasm (A2, C, D, E and G) were folded correctly. Indeed it is unlikely that all of the peptides produced '*in vitro*' were folded in the same way as their corresponding regions in the native holotoxin. With the exception of peptide E, the sizes of the expressed toxin A peptides matched the estimated sizes. Peptide E was approximately 108 kDa as opposed to the estimated 125 kDa. DNA sequencing failed to reveal a premature stop codon or a change in the reading frame of the cloned fragment e DNA. Although the reason for the premature termination of translation was unknown, N-

terminal sequencing confirmed that the correct peptide (albeit shorter) was being expressed.

The levels of expression in *E. coli* varied between peptides. Generally expression was better for peptides B, D, E and F than for peptides A2, C, G and H. Where expression was poor, there appeared to be a large number of truncated products that reacted with the antiserum as well as the full length peptide. Other workers have also encountered difficulties with expression of *C. difficile* toxin A in *E. coli* (von Eichel-Streiber *et al*, 1989; Johnson *et al*, 1988) both at the level of transcription / translation and product stability. Similarly problems have also been encountered with expression of tetanus and botulinum toxin genes (Eisel *et al*, 1986; Fairweather *et al*, 1986).

There are several possible explanations for the poor expression of *C. difficile* toxin A in *E. coli*. Firstly, a characteristic feature of clostridial DNA is its low G and C base content (Hill L.R, 1966). The coding region of the *C. difficile* toxin A gene has an extremely low G+C content of 26.9 mol% (Dove *et al*, 1990). When expressing A+T rich genes, such as toxin A (A+T content of 73.1 mol%) in *E. coli* there can be a problem with reinitiation of translation and / or transcription giving rise to the expression of additional smaller peptides. *E. coli* promoter sequences (comprising the –10 and –35 TATA boxes) are also characteristically A+T rich (Young *et al*, 1989), therefore it is likely that regions within the toxin A coding sequence may resemble additional *E. coli* promoter sequences. In this situation, *E. coli* RNA polymerase could bind to these additional sites and transcribe shorter mRNAs. These shorter mRNAs would be more abundant because they would be transcribed more quickly, and the RNA polymerase transcribing them would stall other RNA polymerase running behind them. The shorter mRNAs would not be translated however, unless there was a recognised ribosome-binding site (RBS) the correct distance

downstream from the pseudo promoter. Even with translation occurring there would only be a 1:3 chance that the DNA would be in the correct reading frame. Finally any peptides produced by these means would not contain a MBP fusion and would, therefore, not react with the anti-MBP antiserum.

Like the promoter sequences, some *E. coli* RBSs are also A+T rich (Young *et al*, 1989). It is also possible, therefore, that the A+T rich toxin A DNA contains internal sequences that resemble *E. coli* RBSs (Shine-Dalgarno sequences). If present, the ribosome would bind to these additional sites and reinitiate translation resulting in the production of shorter peptides. The efficiency of ribosome binding is dependent on the spacing between the Shine-Dalgarno sequence and the translational start codon. The optimal spacing between these sites in *E. coli* is 7 +/- 2 nucleotides (Young *et al*, 1989). Successful translation of these truncated peptides would therefore be dependent on the presence of start codons (most frequently AUG) 7 +/- 2 nucleotides downstream from the pseudo RBSs. Should this coincidence occur there would be a 1:3 chance that the peptides would be expressed in the correct reading frame. Again, the peptides that were successfully expressed would not be fused to the MBP and would not react with the anti-MBP antiserum. It is unlikely therefore, that the two processes already discussed were the main contributors to the poor expression of the toxin A peptides, because a large number of the truncated products reacted with the anti-MBP antiserum on western blots.

It is much more likely that the problem was with premature termination of translation rather than re-initiation of transcription / translation. The poor expression of clostridial genes in *E. coli* is most likely due to incompatible codon usage. The inefficient production of tetanus toxin in *E. coli* has been attributed to conflicting codon usage (Eisel *et al*, 1986). The extremely low G+C content of clostridial DNA has a marked effect on

codon usage, which is strongly biased towards codons in which A and U predominate. The biased codon usage is most common for amino acids with six synonymous codons (see appendix 6). It is evident from the codon usage table that the amino acid arginine is of particular concern. The codons AGA and AGG are used for an arginine amino acid in *C. difficile* genes at frequencies of 75.7% and 7.4% respectively, and in *E. coli* genes at frequencies of 4.5% and 2.7% respectively. Unfortunately these two codons are the rarest in global gene expression in *E. coli* (Chet *et al,* 1994). On average only 2.5 AGA codons and 1.5 AGG codons exist in every 1,000 codons in the *E. coli* chromosome (taken from Netscape site: http://www.dna.affrc.go.jp/~nakamura/). Genes such as toxin A that contain a significant proportion of these rare codons may cause translational problems in expression systems in *E. coli*. The problems would result from a combination of high levels of expression coupled with low levels of the cognate tRNAs that recognise these rare codons (Rosenberg *et al,* 1993. During translation where a cognate tRNA is not readily available, the acceptor region on the ribosome is left vacant with nothing to transfer to the growing polypeptide chain. This stalling or idling of the ribosome causes premature chain termination. Toxin A peptides produced by this process would be truncated within the toxin A coding sequence and would therefore contain the MBP and react with anti-MBP antiserum.

Expression of toxin A fragment a2 in *E. coli* resulted in the expression of several additional truncated products, the most predominant of which were in the range of 61-68 kDa. These truncated species also reacted with the anti-MBP antiserum (see figure 3.9). It was calculated that these premature terminations occurred approximately 18-25 kDa into the peptide. This region of the toxin A gene contains seven AGA or AGG codons representing arginine amino acids (see appendix 7). It is likely, therefore, that the lack of

the cognate tRNAs for these codons was responsible for the poor expression of peptide A2. In an attempt to resolve this problem the toxin A peptides were expressed in the presence of a pBR322 plasmid containing the tRNA genes for recognition of the AGA/AGG codons (plasmid pDC952 supplied by SmithKline Beecham Pharmaceuticals). A potential problem with this experiment was that like the pDC952 plasmid, the pMal vectors are also based on pBR322. As both plasmids have a colE1 origin of replication it was considered unlikely that both would function to full efficiency within the same cell. It was hoped however, that although the overall levels of expression may be lower, the proportion of full-length protein would be increased. Unfortunately, the results showed that there was no improvement in expression with these tRNAs possibly due to the fact that *C. difficile* also shows a strong preference towards other codons (e.g. UUA for leucine).

Other possible contributory reasons for the presence of additional bands in the purified peptide preparations may include denaturation of the products on SDS-PAGE gels or host proteases recognising and cleaving the foreign proteins.

Expression of peptides H1 and H2 were improved by re-transforming into the host strain *E.coli* BL21 DE3 every 2-3 months. This strain lacks the *lon* protease and the *ompT* outer membrane protease and so reduces proteolytic degradation within the host cell (Furlong *et al*, 1992). It is possible that expression could have been further improved in a different host-vector system such as the baculovirus system, but unfortunately time did not allow for these studies. Having considered all of the potential problems in expressing *C. difficile* genes in *E. coli* however, it is both surprising and satisfying that peptides representing the entire toxin A molecule were finally produced.

All of the peptides containing MBP were successfully purified by amylose affinity chromatography. It became evident however, that peptide F was highly insoluble and was resistant to factor Xa cleavage. As this region of the toxin A molecule was also covered in peptide G, peptide F was omitted from several studies. Peptide C was purified at a late stage in this thesis and so was included in some, but not all, of the following functional assays.

As with peptide F, peptide H1 was also resistant to factor Xa cleavage. Other workers have reported that when screening a *C. difficile* genomic library with anti-toxin A antiserum, only clones containing the C-terminal repeat region of toxin A (equivalent to peptides H1 and H2) were detected (von Eichel-Streiber *et al* 1989, Muldrow *et al* 1987, Dove *et al* 1990 and Wren *et al* 1987). This may suggest that the C-terminal repeat region of toxin A is wrapped around the rest of the molecule in the native state. It is possible therefore, that peptide H1 was wrapping itself around the MBP in the fusion product making the factor Xa site inaccessible to cleavage. Peptide H2, covering the same region of the toxin as peptide H1, was expressed without a fusion tag and therefore did not require cleavage. This peptide was used in all functional studies in place of peptide H1.

Unfortunately attempts to further purify the toxin A peptides were unsatisfactory. Thyroglobulin affinity chromatography did result in low levels of purified peptide H2, but these levels were too low to allow for the necessary dilution factors required for analysis. Despite the problems of purification it was decided to proceed with the planned functional assays with appropriate *E. coli* negative controls. Toxin A peptides A2, B, D, E, G and H2 covering the entire toxin A molecule were included in all of the functional assays. These peptides were cleaved where necessary and included in the experiments alongside the relevant controls outlined in section 3.3.7.1 and 3.3.7.5. It became apparent

that some of the peptides were more stable at 4^0C as MBP fusion products than following factor Xa cleavage. It may be that the peptides were more soluble or less susceptible to degradation as MBP fusions. The fusion proteins were, therefore, routinely cleaved the night before inclusion in an experiment.

A summary of the purified toxin A peptides can be found in appendices 4 and 5.

SECTION – 4

IMMUNOLOGICAL PROPERTIES OF HOLOTOXIN A AND THE TOXIN A CONSTRUCTS

SECTION – 4

Immunological Properties of Holotoxin A and the Toxin A Constructs

4.1 SUMMARY

4.2 INTRODUCTION

4.3 MATERIALS AND METHODS

4.3.1 Production of polyclonal monospecific antisera to toxin A and toxin A peptides

4.3.2 Cross-reactivity of polyclonal monospecific toxin A antiserum

4.3.2.1 Toxin A and crude culture filtrate from *C. difficile* strains VPI 10463 and M-1

4.3.2.2 Toxin A peptides

4.3.2.2.1 By SDS-PAGE and western blotting

4.3.2.2.2 By ELISA analysis

4.3.3 Cross-reactivity of the polyclonal monospecific anti-peptide A2, B, D, E, G and H2 antisera

4.3.3.1 With toxin A and crude culture filtrates from strains VPI 10463 and M-1

4.3.3.2 With holotoxin A

4.3.3.3 With the respective toxin A peptides

4.3.3.3.1 By ELISA analysis

4.3.3.3.2 By SDS-PAGE and western blot

4.3.4 Reactivity of the monoclonal antibodies (Mabs) PCG-4 and 37B5 with the toxin A peptides

4.3.4.1 By SDS-PAGE and western blot

Section 4

Immunological Properties of Holotoxin A and the Toxin A Constructs

4.1 SUMMARY

Monospecific polyclonal antisera were raised in rabbits to formalised holotoxin A and to the peptides A2, B, D, E, G and H2. All of the toxin A peptides were antigenic eliciting a specific antibody response. The polyclonal antiserum to formalised holotoxin A also recognised peptides A2, C, D, G and H2 but not peptides B, F or truncated E. Polyclonal monospecific antisera to peptides A2, D, G and H2 recognised formalised holotoxin A, whereas antisera to peptides B and truncated E did not. All of the antisera recognised non-formalised holotoxin A and the crude culture filtrate from the toxigenic *C. difficile* strain VPI 10463. None of the antisera to peptides A2, B, D, E, and G, representing the N-terminal part of toxin A, bound non-specifically to the C-terminal peptide H2. These results suggest that peptides B, F and truncated E are unexposed in the native toxin molecule.

The monoclonal antibodies (Mabs) PCG-4 and 37B5 bound to the repeat end peptide, H2, but not to the N-terminal peptides A2, B, C, D, E, F and G. These results suggest that both Mabs block toxin A induced enterotoxicity by blocking binding of the holotoxin to its target cell.

The C-terminal 'repeat end' peptide, H2, bound non-specifically to the murine Mab IgG3λ, whereas the N-terminal peptides A2, E and G did not. The binding was significantly reduced by prior treatment of the antibody with α-galactosidase suggesting that the C-terminal repeat region of toxin A binds non-specifically to Mabs via galactose residues on the Fab domain of the molecule.

4.2 INTRODUCTION

The tertiary structure of the *C. difficile* toxin A molecule has yet to be solved by X-ray crystallography. Results to date have shown that only clones that express part, or all, of the C-terminal repeat region of toxin A are isolated from *C. difficile* genomic libraries by screening with polyclonal antiserum to toxin A (Price *et al* 1987, von Eichel-Streiber *et al* 1989, Muldrow *et al* 1987, Wren *et al* 1987). These results suggest that either the N-terminal portion of toxin A is not antigenic or that it is hidden inside the molecule with the C-terminal repeat region wrapped around it and surface exposed.

Many Mabs have been raised to toxin A (von Eichel-streiber *et al* 1987, Rothman *et al* 1988, Lyerly *et al* 1985b, Kamiya *et al* 1991) but only PCG-4 (Lyerly *et al*, 1985b) and 37B5 (Kamiya *et al*, 1991) have been shown to neutralise biological activity of toxin A. The Mab PCG-4 has been reported to neutralise toxin A induced H/A and enterotoxicity but not cytotoxic activity (Lyerly *et al*, 1986b). The binding site for this Mab has been localised to the repeat region of toxin A between amino acid residues 2,097 through 2,141 and 2,355 through 2,389. It was concluded that PCG-4 was neutralising enterotoxicity by blocking binding of the toxin to its target cell (Frey *et al*, 1992). The Mab 37B5 blocks toxin A induced enterotoxicity but not H/A and the binding site for this antibody has not yet been identified. As this antibody did not neutralise H/A it was concluded that it was not preventing binding of the toxin to its target cell but was blocking an additional active site for enterotoxicity in the N-terminus of the molecule (Kamiya *et al*, 1991). These predictions make this antibody invaluable and very interesting to study. As these two Mabs neutralise biological activity of toxin A it is believed that they react with the toxin by a true immune reaction.

Other Mabs raised to toxin A have been shown to cross-react with both toxins A and B (von Eichel-Streiber *et al* 1987, Rothman *et al* 1988, Kamiya *et al* 1991, Lyerly *et al* 1985b, Lyerly *et al* 1986b). Although it has been suggested that these cross-reacting antibodies are recognising an epitope that is shared between toxin A and B, it has been shown that both toxins react non-specifically with murine monoclonal antibodies (IgM, IgG, and IgA) (Lyerly *et al*, 1989). It was suggested, therefore, that these cross-reacting Mabs were binding non-specifically to the toxins and not by a true immune reaction. It has recently been shown that toxin A binds non-specifically to the Fab domain of IgG Mabs (Cooke *et al*, 1998). It is important to have an effective screening assay for detection of these non-specific Mabs to prevent misleading results.

The initial aim of this section was to raise polyclonal monospecific antisera to holotoxin A and to the toxin A peptides. These antisera would be used to generate reaction profiles of antiserum to holotoxin A against toxin A peptides and of antiserum to the peptides against holotoxin A. The results would be used to predict which regions of holotoxin A are surface exposed in the molecule's native state. Another aim of this section was to locate an active site for enterotoxicity by pinpointing the binding site for the 37B5 Mab in the toxin A molecule. Finally, an attempt would be made to determine which region of the toxin A molecule is responsible for binding non-specifically to the Fab of an IgG3λ murine Mab.

4.3 MATERIALS AND METHODS

4.3.1 Production of polyclonal monospecific antisera to toxin A and toxin A peptides.

Toxin A peptides A2, B, D, E, G and H2 (100μgml^{-1}) were used to raise polyclonal monospecific antisera in rabbits. The peptides were purified by amylose affinity chromatography as outlined in section 2.8.2. Prior to injection, peptides A2, B, D, E and G were cleaved from the maltose binding protein by incubating in a final concentration of 10μgml^{-1} of Factor Xa (New England Biolabs) at 4^{0}C overnight. Toxoid was prepared by incubating toxin A (100μgml^{-1}) in a final concentration of 0.4% formaldehyde for 36hr at 37^{0}C. Prior to injection a 10ml pre-bleed blood sample was taken from seven 5Kg New Zealand White female rabbits (Shrubacre Rabbits, Ltd.). Six of these rabbits were given 4 x 0.25ml subcutaneous injections of 1:1 v/v toxin A peptide with Freund Complete Adjuvant (FCA). The seventh rabbit was given 4 x 0.25ml subcutaneous injections of 1:1 v/v toxoid with FCA. Two weeks later fresh antigen was prepared and mixed 1:1 v/v with Freund Incomplete Adjuvant (FIA) and injected as already described. The rabbits were boosted fortnightly with antigen: FIA for a further two injections, after which they were terminated and bled. The blood was left to clot at room temperature and the serum decanted off. Each polyclonal monospecific antiserum was adsorbed against whole *E. coli* cells four times as follows. Three millilitres of an overnight culture of *E. coli* cells were pelleted at 13, 000×g for 2min and the supernatant removed. The pellet was resuspended in 1ml of antiserum and the mixture incubated, rotating overnight, at room temperature. The adsorbed antiserum was retained following centrifugation at 13,000×g for 2min. Following the final adsorption the antiserum was aliquoted and stored at -20°C.

The titre of each antiserum was determined using an ELISA based assay (see section 2.15). Each protein used to raise antiserum was diluted to a final concentration of 5µg/ml and applied to 32 wells of an ELISA plate. Each antiserum was diluted two-fold from a 1:5 dilution down to a 1:163,840 dilution. Each dilution of the relevant antiserum was applied in duplicate to the wells containing the corresponding antigen. The remaining 16 wells coated with each antigen were incubated with the same dilutions of the relevant pre-bleed serum. The binding of each antiserum to each antigen was detected using a 1:1000 dilution of goat anti-rabbit alkaline phosphatase conjugate. The absorbance (405nm) for the pre-bleed serum was deducted from the corresponding absorbance, using the peptide antiserum, and the difference subsequently plotted on a graph.

4.3.2 Cross-reactivity of polyclonal monospecific toxin A antiserum.

4.3.2.1 With toxin A and crude culture filtrates of *C. difficile* strains VPI 10463 and M-1.

Dot blot analysis (see section 2.7.4) was used to determine the binding of the polyclonal monospecific toxin A antiserum (see section 4.3.1) to toxin A and crude culture filtrates of VPI 10463 and M-1. *C. difficile* VPI 10463 is highly virulent and produces both toxins A and B. The M-1 strain, however, is avirulent and does not produce either toxin. In duplicate, toxin A (20µg/ml) was spotted onto nitrocellulose as a positive control along with the crude filtrates of the test strains VPI and M-1. One set was incubated with 1:1000 dilution of toxin A antiserum while the other was incubated with 1:1000 dilution of pre-immune serum. Both blots were then incubated with 1:1000 dilution of goat anti-rabbit alkaline phosphatase conjugate.

4.3.2.2 With toxin A peptides.

4.3.2.2.1 By SDS-PAGE and western blotting.

The toxin A peptides were cleaved where necessary with factor Xa (see section 2.8.3). Toxin A peptides A2, B, D, E, F, G and H2 were resolved on three 8% SDS-PAGE gels (see section 2.7.1). Two of the gels were western blotted (see section 2.7.3) whilst the third was stained with coomassie blue (see section 2.7.1). One of the western blots was probed with a 1:1000 dilution of the polyclonal monospecific toxin A antiserum whilst the second was probed with a 1:1000 dilution of pre-immune serum. Both blots were then incubated with a 1:1000 dilution of goat anti-rabbit alkaline phosphatase conjugate before being developed.

4.3.2.2.2 By ELISA analysis.

ELISA analysis (see section 2.15) was used to determine the binding of the toxin A antiserum (see section 4.3.1) to the native toxin A peptides. Toxin A peptides were cleaved where necessary with factor Xa (see section 2.8.3). Each peptide (A2, B, C, D, E, F, G and H2) was diluted to a final concentration of 10µg/ml and applied to six replica wells of the ELISA plate. Toxin A and MBP were included on the plate as the positive and negative controls respectively. Three of the six wells coated with each sample were incubated with a 1:1000 dilution of toxin A antiserum with the remaining three incubated with a 1:1000 dilution of pre-immune serum. The wells were then treated with a 1:1000 dilution of goat anti-rabbit alkaline phosphatase conjugate before being developed. The mean absorbances at 405nm in the pre-immune wells were deducted from the corresponding absorbances for the anti-toxin A treated wells and the differences plotted on a graph.

4.3.3 Cross-reactivity of the polyclonal monospecific anti-peptide A2, B, D, E, G and H2 antisera.

4.3.3.1 With toxin A and crude culture filtrates of VPI and M-1.

The cross-reactivity of the peptide antisera (see section 4.3.1) with toxin A, VPI and M-1 were determined by dot blot analysis (see section 4.3.2.1). The peptide used to raise an antiserum was included as a positive control on the dot blots treated with that antiserum. Each nitrocellulose square also contained holotoxin A, VPI and M-1 culture filtrates. The first of each pair of blots was treated with a 1:1000 dilution of the relevant polyclonal monospecific peptide antiserum and the second with a 1:1000 dilution of the corresponding pre-immune serum. The remainder of the method has been previously described (see section 4.3.2.1).

4.3.3.2 With holotoxin A.

An ELISA assay (see section 2.15) was used to determine cross-reactivity of the polyclonal monospecific peptide antisera (see section 4.3.1) with formaldehyde treated and untreated toxin A.

Toxin A was treated with 0.4% formaldehyde solution for 36 hrs at 37^0C, diluted to $10\mu gml^{-1}$ then used to coat an ELISA plate. A second plate was coated with $10\mu gml^{-1}$ of untreated toxin A. Each peptide antiserum and pre-immune serum was diluted 1:1,000 and applied to three replica wells on both plates. Toxin A and MBP antisera were also included as positive and negative controls respectively. All wells were treated with a 1:1,000 dilution of goat anti-rabbit alkaline phosphatase conjugate prior to being developed. The triplicate mean absorbance for each pre-immune serum was deducted from

the mean absorbance for the corresponding peptide antiserum and the differences were plotted on a graph.

An ELISA plate was also coated with 10μgml^{-1} of peptide H2 and processed as already described to determine the level of non-specific binding of the repeat region of toxin A to the different polyclonal monospecific peptide antisera.

4.3.3.3 With the respective toxin A peptides.

4.3.3.3.1 By ELISA analysis

ELISA analysis (see section 2.15) was used to determine the binding of each peptide antiserum (see section 4.3.1) to its native toxin A peptide. The toxin A peptides were cleaved with factor Xa (see section 2.8.3). The peptides (A2, B, D, E, G and H2) were diluted to 10μgml^{-1} and each applied to six replica wells across the plate. In each case, three of the six wells were incubated with a 1:1,000 dilution of the relevant peptide antiserum, whereas, the other three wells were incubated with a 1:1,000 dilution of the corresponding pre-immune serum i.e. wells containing peptide A2 were incubated with the peptide A2 antiserum. All of the wells were then treated with a 1:1,000 dilution of goat anti-rabbit alkaline phosphatase conjugate. The mean absorbance obtained with the pre-immune serum was subtracted from the mean absorbance obtained with the corresponding peptide antiserum and the results were plotted on a graph.

4.3.3.3.2 By SDS-PAGE and western blot

This method was adopted to determine the cross reactivity of each peptide antiserum with its own antigen. The toxin A peptides were cleaved where necessary with factor Xa (see section 2.8.3). One microgram of each toxin A peptide were applied to the wells of one

8% SDS-PAGE gel (see section 2.7.1). On a further two 8% SDS-PAGE gels 1μg of each peptide were applied in duplicate to two adjacent wells. The three gels were resolved and the first was stained in coomassie blue. The other two gels were western blotted and stained in ponceau solution (see section 2.7.3). The different lanes were labelled and the nitrocellulose was cut into strips. In each case, one of the two duplicate strips was incubated with a 1:1,000 dilution of the relevant peptide antiserum (see section 4.3.1), whereas, the second strip was incubated with 1 1:1,000 dilution of the corresponding pre-immune serum. The strip containing peptide C (which contains part of the A2 fragment) was probed with peptide A2 antiserum and the strip containing peptide F (the C-terminal portion of peptide G) was probed with anti- peptide G antiserum. All of the strips were then treated with a 1:1,000 dilution of goat anti-rabbit alkaline phosphatase conjugate before development. The bands on the western blots were compared to the protein profiles on the coomassie blue stained gel.

4.3.4 Cross-reactivity of the Mabs PCG-4 and 37B5 with the toxin A peptides.

4.3.4.1 By SDS-PAGE and western blot

The toxin A peptides were cleaved where necessary with factor Xa (see section 2.8.3). On duplicate 8% SDS-PAGE gels, 1μg of each toxin A peptide (A2, B, D, E, F, G and H2) was applied to separate wells and the gels were resolved (see section 2.7.1). Toxin A and MBP were included on both gels as positive and negative controls respectively. One of the gels was stained in coomassie blue for reference and the second was western blotted (see section 2.7.3). The blot was probed with a $2\mu g ml^{-1}$ of the Mab 37B5 followed by a 1:1,000 dilution of goat anti-mouse alkaline phosphatase conjugate. This experiment was

repeated as described above with the exception that the western blot was probed with $2\mu g ml^{-1}$ of the Mab PCG-4.

4.3.4.2 By ELISA analysis

ELISA analysis was used to determine the cross-reactivity of the Mabs PCG-4 and 37B5 with the native toxin A peptides as described in section 2.15 with the following exceptions.

The toxin A peptides were cleaved where necessary with factor Xa (see section 2.8.3). The peptides were then diluted to $10\mu g ml^{-1}$ and each applied to six wells on two duplicate ELISA plates. MBP and the *E. coli* protein sample were included as negative controls whilst toxin A was included as a positive control. On one ELISA plate, three of the replica wells for each peptide were incubated with $2\mu g ml^{-1}$ of the Mab PCG-4. On the second plate, three of the replica wells were incubated with $2\mu g ml^{-1}$ of the Mab 37B5. On both plates, the remaining three replica wells for each peptide were incubated with antibody diluent only (conjugate control). Each well was treated with a 1:1,000 dilution of goat anti-mouse alkaline phosphatase conjugate prior to development. In each case, the mean absorbance obtained at 405nm for the conjugate control was deducted from the corresponding mean absorbance for the wells incubated with Mab. The differences in absorbances were plotted on two separate graphs.

4.3.5 ELISA determination of non-specific binding of the MAb IgG3λ to the toxin A peptides.

This assay was based on a method described by Cooke *et al*, 1998.

Where necessary toxin A peptides were cleaved with Factor Xa to remove the MBP (see section 2.8.3). The wells of a Maxisorp C96 microtitre plate (Life Technologies Ltd., Paisley, U.K) were coated, in triplicate, with toxin A and toxin A peptides (diluted in 0.05M carbonate buffer pH 9.6) at 4^0C overnight. Toxin A was coated at 10μgml^{-1} but the concentrations of the toxin A peptide fusions were adjusted to account for their relative size when compared to toxin A (peptide A2; 3.3μgml^{-1}, peptide E; 3.6μgml^{-1}, peptide G; 4.8μgml^{-1}, MBP; 1.4μgml^{-1}, peptide H2; 3.0μgml^{-1} and the *E. coli* sample; 3.0μgml^{-1}). Therefore for every one molecule of holotoxin A in the assay there was one molecule of a toxin A peptide fusion product. Wells coated with 1.4μgml^{-1} MBP acted as a negative control for peptides A2, E and G, whilst wells coated with the *E. coli* protein sample were included as a negative control for peptide H2. Wells coated with toxin A acted as a positive control for non-specific binding to the Mab IgG3λ (Sigma). The plate was washed three times with PBS containing 0.05% Tween-20 (PBST) and then blocked with 2% Bovine Serum Albumin (BSA; Sigma) in PBST for 1hr at room temperature. Fifty μl of the IgG3λ Mab (40μgml^{-1}) was added to each well and incubated for 2hr at 37^0C. The plate was washed three times with PBST, and 50μl goat anti-mouse immunoglobulin G-alkaline phosphatase conjugate (Sigma) in 1% BSA-PBST added to each well. After an incubation of 1hr at 37^0C the plate was washed three times with PBST and twice with sterile distilled water. Fifty μl of pNpp alkaline phosphate substrate solution (Sigma) was added to each well and the absorbance at 405nm was monitored hourly for 3hr. The highest A_{405} obtained with wells that lacked primary antibody was taken as the background value. The absorbance values were plotted on a graph.

4.3.6 Non-specific binding of the MAb IgG3λ to toxin A and to peptide H2 following treatment of the antibody with α-galactosidase.

The α-galactosidase treatment of the Mabs was based on a method described by Cooke *et al*, 1998.

To determine whether or not α-galactosidase could reduce the binding of the Mab IgG3λ to toxin A or peptide H2, the ELISA procedure (section 4.3.5) was adopted with the following exceptions.

One third of a Maxisorp C96 microtitre plate (Life Technologies) was coated with 5μgml^{-1} of toxin A, one third with 5μgml^{-1} of peptide H2 and the final third with 5μgml^{-1}of the *E. coli* negative control sample at 4^{0}C overnight.

Four test antibody preparations were prepared. In the first test the Mab IgG3λ was diluted in 1% Bovine Serum Albumin (BSA)/PBSA/0.05% Tween-20 pH 6.0 (diluent) to a final concentration of 40μgml^{-1} (Mab preparation) and then pre-incubated with an equal volume of 30μgml^{-1} α-galactosidase (Sigma) solution (diluted in diluent) for 1 hr at 22^{0}C. In the second test the Mab preparation outlined in test 1 was pre-incubated as above but with an equal volume of diluent to determine the level of non-specific binding of the untreated Mab to the proteins. In the third test the diluent was pre-incubated as above with an equal volume of the α-galactosidase solution (30μgml^{-1}) to determine the direct effect, if any, of the α-galactosidase on the proteins. The fourth test involved the pre-incubation of diluent only and acted as a conjugate control.

After blocking the wells in 2% BSA/PBS/0.05% Tween-20, each protein sample on the plate was incubated, in triplicate, with 50μl of each of the four test antibody preparations for 2 hrs at 37^{0}C. Following this step, the ELISA proceeded as outlined in section 4.3.5.

This method was repeated using 2μgml^{-1} of the specific Mab to toxin A, PCG-4, in place of the non-specific Mab IgG3λ. The absorbance values obtained with untreated antibody were plotted on a graph alongside the absorbance values obtained with α-galactosidase treated antibody.

4.3.7 Statistical analysis of ELISA derived data.

In each case, the mean absorbance value of the conjugate control wells or the pre-immune serum wells was compared to the mean absorbance of the test sample in a two sample T-test (Minitab). Where $p<0.05$ we reject H0 ($\mu 0=\mu 1$) with 95% confidence limits. The alternative hypothesis H1 was therefore accepted ($\mu 0\neq\mu 1$). If $p>0.05$ we accept H0 ($\mu 0=\mu 1$) with 95% confidence limits. This statistical analysis was carried out on all ELISA data.

4.4 RESULTS

Statistical analyses stated in this section were performed as outlined in section 4.3.7.

4.4.1 Titres of the polyclonal monospecific toxin A and peptide antisera

Rabbit polyclonal monospecific antisera was raised to holotoxin A and to the toxin A peptides A2, B, D, E, G and H2 (see section 4.3.1). To determine an effective working dilution, each antiserum was diluted and directed against the antigen that it had been raised against in an ELISA based assay. The absorbance values obtained were plotted against the dilution factors on line graphs. All of the antisera recognised homologous antigens, suggesting that each toxin A peptide was antigenic. At a dilution of 1:40,960 each antiserum still detected its antigen, however, at this low dilution the line graphs tailed off. It was decided therefore, to use each antiserum at a dilution of 1:1,000 – 1:2,000 because this dilution represented a much more sensitive area on the line graphs (results not shown).

4.4.2 Cross-reactivity of toxin A antiserum

4.4.2.1 With crude culture filtrate from strains VPI 10463 and M-1

On the dot blots a 1:1,000 dilution of the polyclonal toxin A antiserum recognised the toxin A positive control as well as the crude culture filtrate from strain VPI 10463 (see figure 4.1, lanes A1 and A2). This strain is highly virulent and produces both toxin A and B, which would have been present in the filtrate. The antiserum did not however, recognise the crude culture filtrate from the avirulent, non-toxigenic strain M-1 (figure 4.1, lane A3). There was a weak non-specific interaction of the conjugate with toxin A but this was not seen with VPI or M-1 (figure 4.1, lanes C1, C2 and C3).

4.4.2.2 With the toxin A peptides

On a western blot the polyclonal toxin A antiserum recognised peptide H2 (figure 4.2 lanes 9 and 19) and the cleaved toxin A peptides A2, D and G (see figure 4.2 lanes 3 and 13, 5 and 15, 8 and 18 respectively). The reaction with peptide H2 was far stronger than with peptides A2, D and G which, only reacted weakly. The antiserum did not recognise peptides B, E and F (see figure 4.2 lanes 4 and 14, 6 and 16, 7 and 17 respectively). Although an attempt was made to load equal concentrations of each full- length peptide, the proportion of each preparation represented by truncated products or MBP varied greatly. It would appear, however, that the reaction of the antiserum with peptides A2, D and G was specific and not due to a background binding caused by overloading of the peptides on the gel. This was shown by peptides B (35 kDa; figure 4.2 lane 4) and E (65 kDa; figure 4.2 lane 6) which clearly did not react with the antiserum although there was more of these two peptides on the gel than the two reacting peptides A2 (99 kDa; figure 4.2 lane 3) and G (105 kDa; figure 4.2 lane 8). The pre-immune serum did not react with any proteins on the western blot (result not shown).

Cross-reactivity of the toxin A antiserum with the native peptides was also studied by ELISA analysis (see section 4.3.2.2.2). The results were plotted on a graph (see figure 4.3). Again the antiserum was shown to bind significantly to peptides A2 ($p=0.0009$), D ($p=0.0009$), G ($p<0.0001$) and H2 ($p=0.0001$). Peptide C, which was not screened in the western blot, was also shown to bind significantly ($p=0.0034$) to the toxin A antiserum. Binding of the polyclonal toxin A antiserum to peptides B, E and F was significant with 95% confidence limits but not with 90% confidence limits (in each case $p>0.01$). For all three peptides the mean absorbance obtained with toxin A antiserum was less than 1.2 x greater than the mean absorbance obtained with the corresponding pre-immune serum.

MBP and the *E. coli* negative control sample did not bind significantly to polyclonal toxin A antiserum (p=0.33 and 0.42 respectively).

4.4.3 Cross-reactivity of the polyclonal peptide antisera

4.4.3.1 With toxin A and crude culture filtrates from strains VPI10463 and M-1

Toxin A peptides A2, B, D, E, G and H2 were injected into rabbits to produce polyclonal monospecific antisera (see section 4.3.1). Dot blot analysis was used to determine the cross-reactivity of the different antisera with toxin A and crude culture filtrates of *C. difficile* strains VPI 10463 and M-1 (see section 4.3.3.1). Each antiserum reacted most strongly with the homologous immunogen, followed by toxin A then filtrate from strain VPI 10463. The weak reaction with VPI was probably due to the low levels of toxin A in the filtrate. None of the antisera reacted with the filtrate from the non-toxigenic strain M-1 (see figure 4.4 blots 1, 3, 5, 7, 9 and 11). The pre-immune sera did not cross-react (or did so weakly) with the peptides, toxin A, VPI or M-1 suggesting that the results obtained with the peptide antisera were specific (see figure 4.4 blots 2, 4, 6, 8, 10 and 12). These results suggest that all of the peptide specific antisera recognise domains in native toxin A.

4.4.3.2 With holotoxin A by ELISA and western blot

An ELISA assay was used to demonstrate the affinity of the polyclonal peptide antisera to toxin A and the results were plotted on a graph (see figure 4.5). All of the peptide antisera bound significantly to holotoxin A (in each case $p<0.05$). The MBP antiserum however, did not bind significantly to toxin A ($p=0.12$). These results did not correlate with those obtained when toxin A antiserum was directed against the peptides (see

section 4.4.2.2) where only peptides A2, D, G and H2 were recognised. This ELISA was repeated therefore, using peptide H2 as the ligand instead of holotoxin A to determine whether or not the peptide antisera were binding non-specifically to the repeat end-binding portion of the holotoxin. Polyclonal peptide H2 antiserum bound significantly to peptide H2 with a p-value of <0.0001. Anti-peptide B, D and E antisera did not bind significantly (in each case p>0.05). Anti-peptide A2 and G antisera bound significantly at the 5% level but not at the 10% level with p-values of 0.012 and 0.042 respectively. In both of the latter cases however, the level of binding of the antisera to peptide H2 was less than 1.5 x the level of binding of the pre-immune sera to peptide H2 (results not shown).

These small differences did not account for the discrepancies seen between figures 4.3 and 4.5. It was concluded that formaldehyde treatment of toxin A prior to rabbit immunisation had fixed the toxin in a certain configuration, perhaps its native state, only allowing antibodies to be raised to exposed epitopes. The ELISA was again repeated to determine whether or not the peptide antisera were able to cross-react with formalised toxin A. The results obtained were similar to those obtained in figure 4.3 and were plotted on a graph (see figure 4.6). The antisera to peptides A2, D, G and H2 bound significantly to formalised holotoxin A with p-values of 0.0072, 0.0007, 0.0013 and 0.0061 respectively. There was no significant binding of anti-peptide B (p=0.082), anti-peptide E (p=0.15) or anti-MBP (p=0.62) antisera to formalised toxin A.

4.4.3.3 With their respective immunogens

An ELISA assay was used to determine the ability of each peptide antiserum to bind to its respective native peptide and the results were plotted on a graph (see figure 4.7). Each

polyclonal peptide antiserum bound significantly to homologous immunogen (in each case $p<0.05$). As there was a possibility that these reactions were due to cross-reactivity of only the MBP with the antisera, the cleaved peptides were also probed on western blots (see section 4.3.3.3.2). Each cleaved peptide was probed with its respective peptide antiserum and pre-immune serum (see figures 4.8 and 4.9). The peptide A2 antiserum had a high affinity for binding to the full-length peptides A2 (99 kDa) and C (123 kDa) (see figure 4.8, lanes 5a and 8, lanes 3a and 10). There was a weak background binding of each pre-immune serum to the peptides (see figure 4.8, lanes 5b and 3b). As peptides A2 and C did not cleave to completion it was difficult to determine whether this binding was due to antibodies directed against MBP, peptide A2 or both.

The polyclonal peptide B antiserum reacted with both the 43 kDa MBP and the 35 kDa toxin A peptide B (see figure 4.8, lanes 4a and 9). The pre-immune serum reacted weakly with the MBP but not with peptide B (see figure 4.8, lane 4b).

Unfortunately there was some uncleaved peptide D in the preparation loaded onto the gel, which masked the western blot results to some extent. Close observation does reveal however, that the polyclonal peptide D antiserum recognised both the MBP and the 49 kDa peptide D (see figure 4.9, lanes 3 and 7a). Although there was a small amount of background binding of the pre-immune serum to the fusion product there was no reaction with the 49 kDa peptide D (see figure 4.9, lane 7b).

The polyclonal peptide E antiserum reacted with the 43 kDa MBP and the 65 kDa band corresponding to peptide E (see figure 4.8, lanes 2a and 11). This fusion product did not react with the pre-immune serum (see figure 4.8, lane 2b).

The polyclonal peptide G antiserum reacted with both peptide F and peptide G (see figure 4.8, lanes 1a and 12 and figure 4.9, lanes 2 and 6a). Peptide F was resistant to cleavage

and so it was difficult to determine whether the binding was due to antibodies recognising peptide F, MBP or both. The antiserum was clearly binding to the cleaved 105 kDa band corresponding to peptide G however (see figure 4.9, lane 6a). These proteins did not react significantly with the pre-immune serum (see figure 4.8, lane 1b and figure 4.9, lane 6b). Finally, peptide H2 (89.46 kDa) had a high affinity for binding to the polyclonal peptide H2 antiserum (see figure 4.9, lanes 4 and 8a). There was also a significant amount of non-specific binding of the pre-immune serum to this peptide (see figure 4.9, lane 8b).

4.4.4 Cross-reactivity of the toxin A peptides with the Mabs PCG-4 and 37B5.

The 37B5 Mab reacted only with toxin A (see figure 4.10, lanes 3 and 12) and peptide H2 (see figure 4.10, lanes 9 and 18) on a western blot. The antibody did not cross-react with the MBP (see figure 4.10, lanes 2 and 11) or with the toxin A peptides A2, B, D, E and F (see figure 4.10, lanes 4 and 13, 5 and 14, 6 and 15, 7 and 16, 8 and 17 respectively). The same result was obtained when the peptides were probed with the Mab PCG-4 (results not shown).

These results were confirmed by screening the native peptides in ELISA assays. Again, holotoxin A and peptide H2 were bound significantly by the 37B5 Mab (p-values of 0.0065 and 0.0027 respectively) and PCG-4 Mab (p-values of 0.0028 and 0.0042 respectively)(see figure 4.11 and 4.12). The *E. coli* and MBP negative controls and the peptides A2, B, C, D, E and G did not react with either Mab in the ELISA assays (in each case $p>0.05$).

4.4.5 Non-specific binding of the toxin A peptides with the murine Mab IgG3λ.

An ELISA assay was used to determine the region of the toxin A molecule responsible for non-specifically binding to Mabs (see section 4.3.5). Only holotoxin A and peptides A2, E, G and H2 were examined. These peptides represent the entire toxin A molecule. The antibody only bound significantly to holotoxin A and peptide H2 (see figure 4.13) with p-values of 0.004 and 0.0001 respectively. Binding of the antibody to the other protein samples was not significant at the 10% level (in each case $p > 0.01$).

A second set of ELISA assays was used to determine whether or not peptide H2 binds non-specifically to the antibody via a galactose residue. The non-specific binding was significantly reduced by prior treatment of the monoclonal antibody with α-galactosidase (see figure 4.14), with p-values of 0.0001 and 0.0005 for toxin A and peptide H2 respectively. With 90% confidence limits, α-galactosidase treatment had no effect on the binding of the *E. coli* negative control sample to the Mab. The ELISA was repeated using the specific Mab PCG-4 as a negative control. Prior treatment of the PCG-4 antibody with α-galactosidase failed to significantly reduce binding to holotoxin A or peptide H2 with p-values of 0.12 and 0.34 respectively (results not shown).

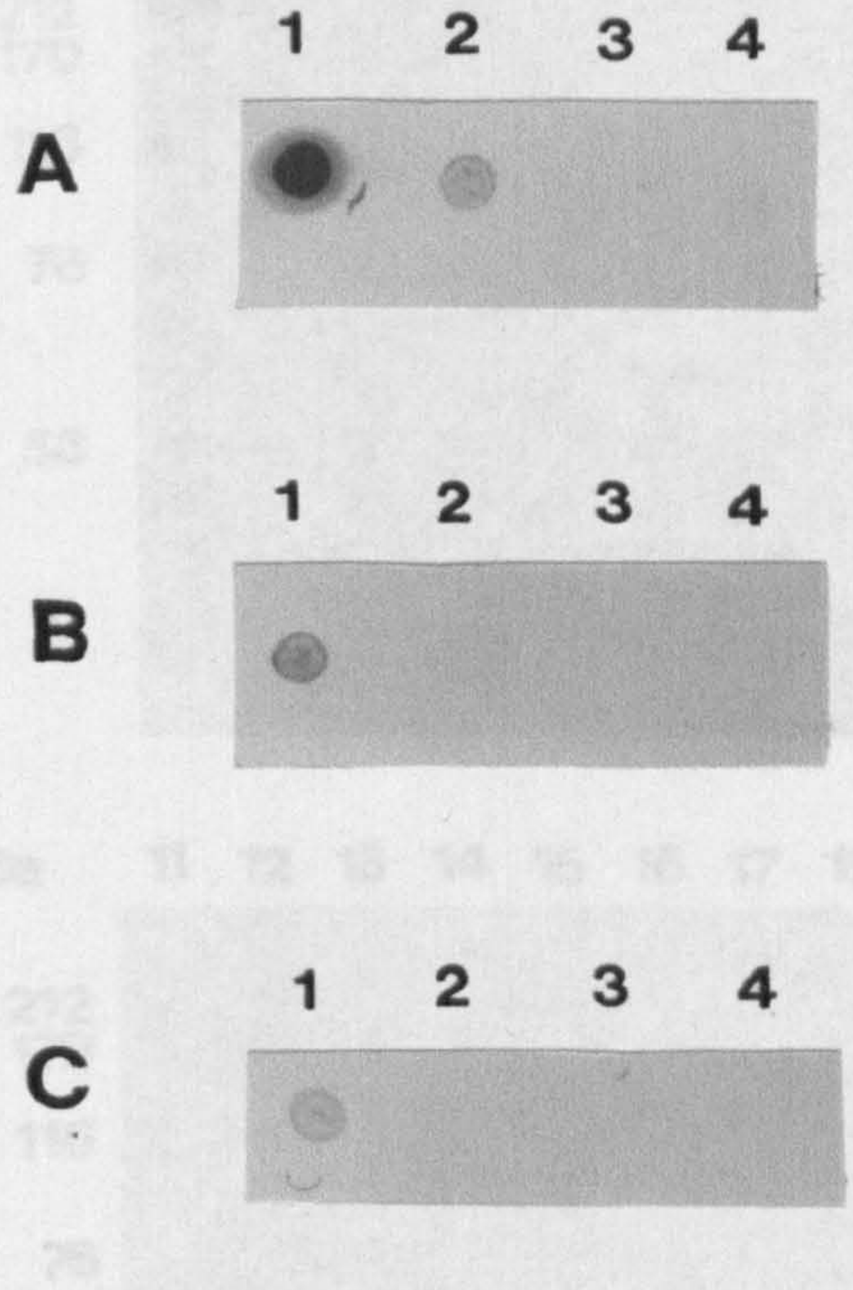


Figure 4.1

Dot blots showing the cross-reactivity of the polyclonal monospecific toxin A antiserum with toxin A and the crude culture filtrates from strains VPI 10463 and M-1.

Samples 1, toxin A; 2, strain VPI 10463; 3, strain M-1; 4, PBSA. Dot blots were probed with A, polyclonal monospecific toxin A antiserum followed by anti-rabbit alkaline phosphatase conjugate; B, pre-immune rabbit serum followed by anti-rabbit alkaline phosphatase conjugate; C, anti-rabbit alkaline phosphatase conjugate only.

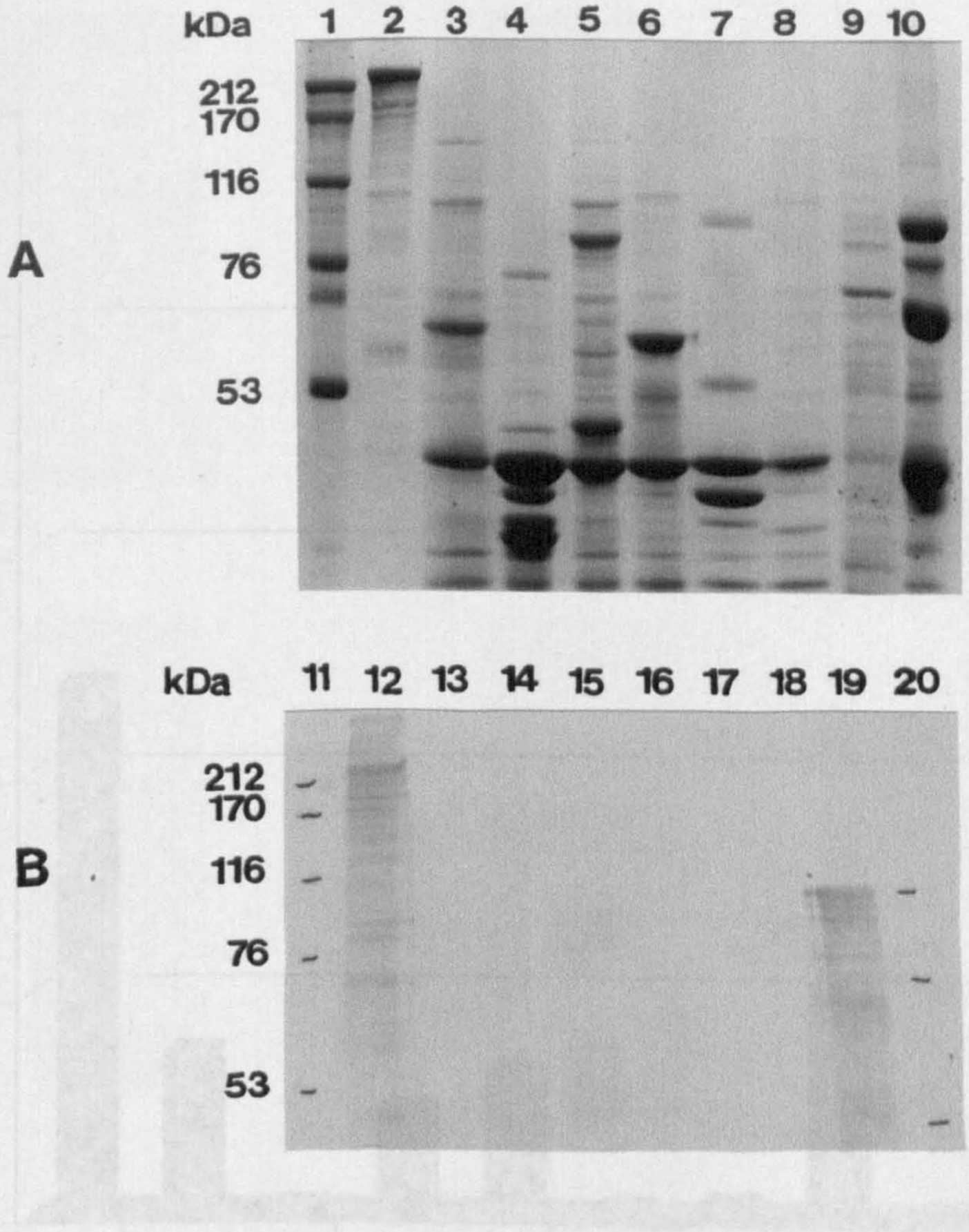


Figure 4.2

8% SDS-PAGE gel (A) and western blot (B) showing the cleaved toxin A peptides probed with the polyclonal monospecific toxin A antiserum.

Lanes 1 and 11, high MWT markers; lanes 2 and 12, toxin A; lanes 3 and 13, peptide A2; lanes 4 and 14, peptide B; lanes 5and 15, peptide D; lanes 6 and 16, peptide E; lanes 7 and 17, peptide F; lanes 8 and 18, peptide G; lanes 9 and 19, peptide H2; lanes 10 and 20, low MWT markers.

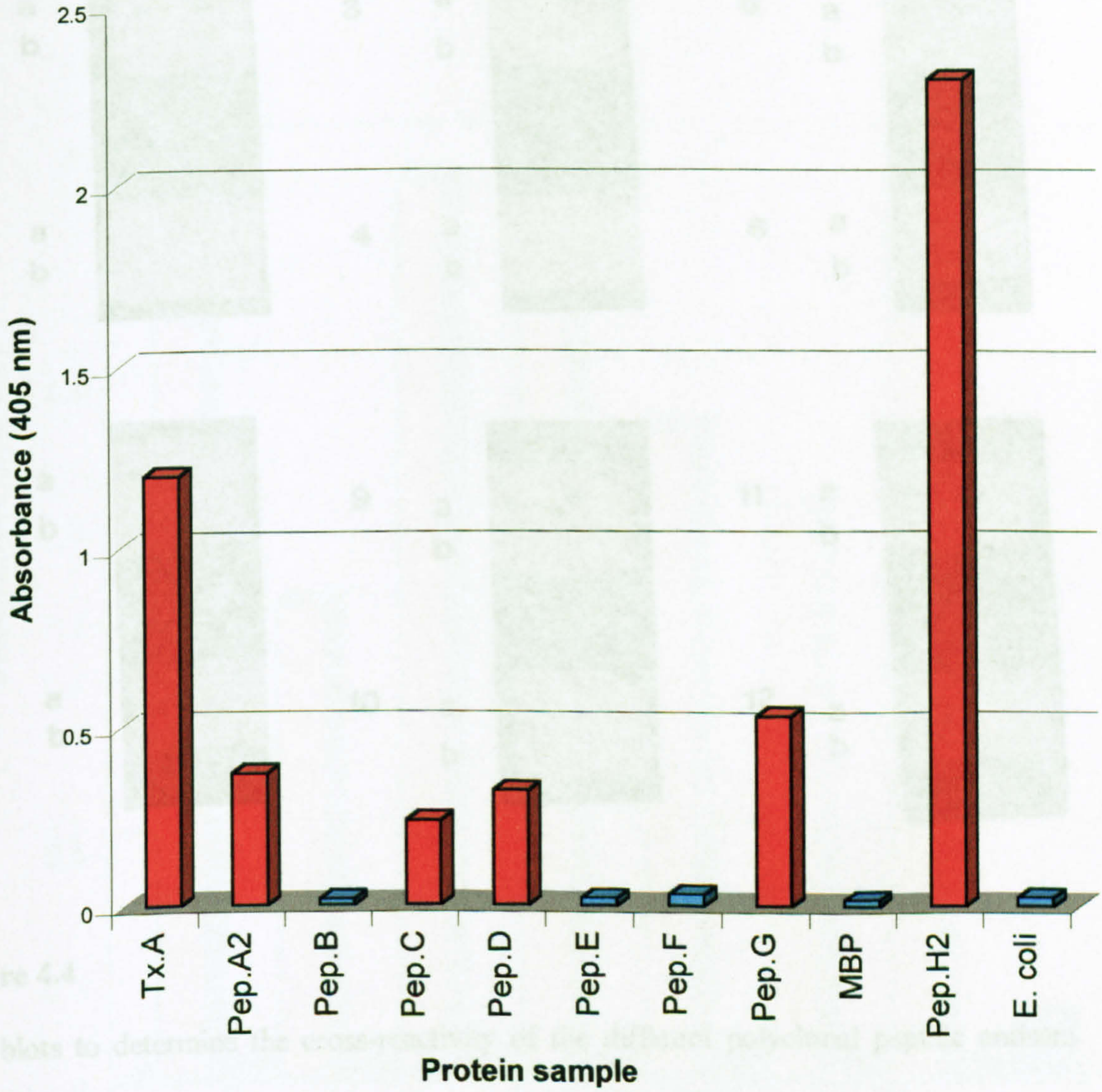


Figure 4.3
A graph to show the ELISA values obtained when the cleaved toxin A peptides were probed with the polyclonal monospecific anti-toxin A antiserum. Peptides shown in red bound significantly to the anti-toxin A antiserum.

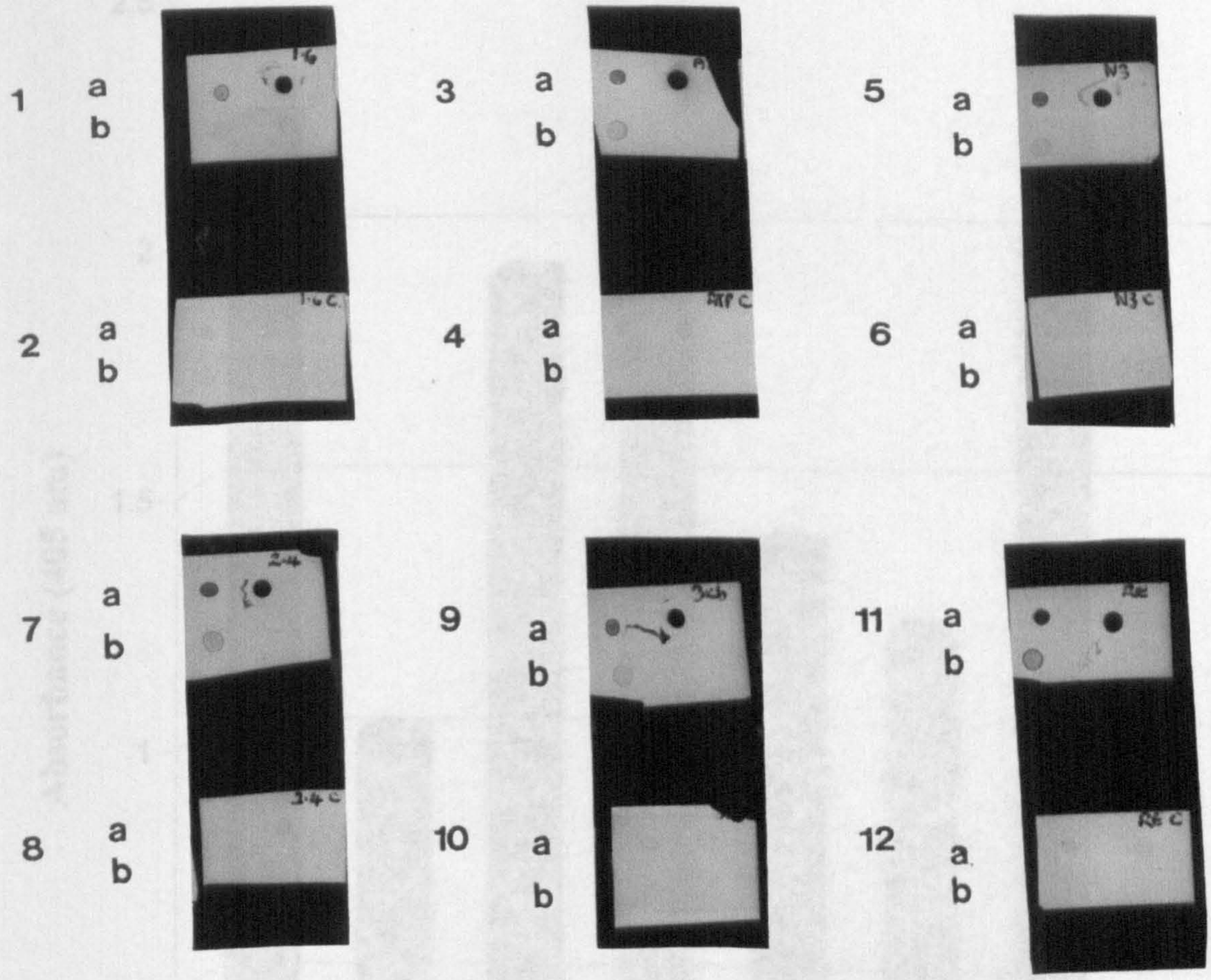


Figure 4.4

Dot blots to determine the cross-reactivity of the different polyclonal peptide antisera with toxin A and with crude culture filtrates from strains VPI 10463 and M-1.

Blots were probed with 1, peptide A2 antiserum; 3, peptide B antiserum; 5, peptide D antiserum; 7, peptide E antiserum; 9, peptide G antiserum; 11, peptide H2 antiserum; 2, 4, 6, 8, 10 and 12, rabbit pre-immune sera. Samples on each blot were a, holotoxin A and the peptide specific to the antiserum (positive control); b, culture filtrate from strains VPI 10463 and M-1.

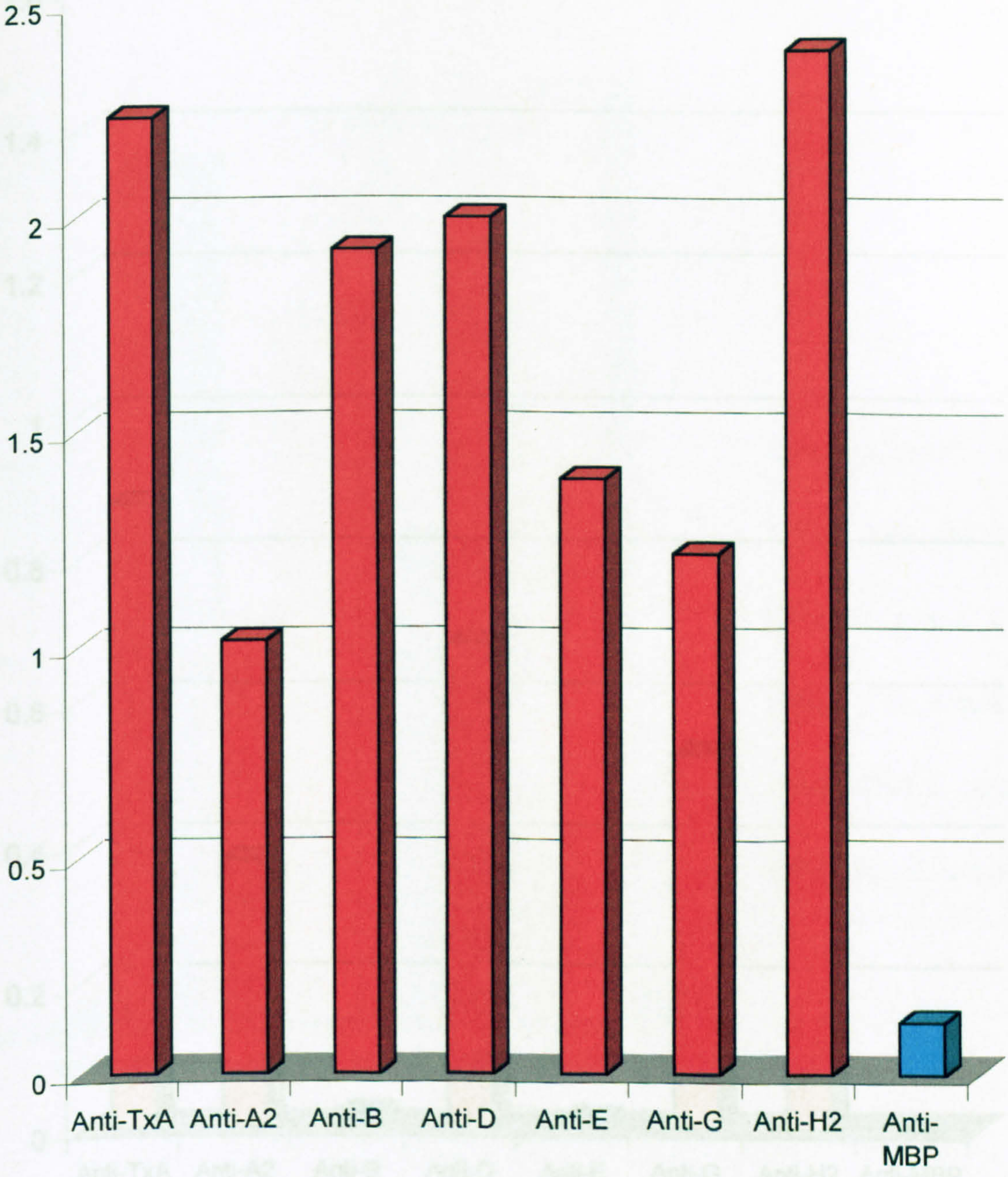


Figure 4.5

A graph to show binding of the different polyclonal monospecific anti-peptide antisera to wells containing untreated holotoxin A in an ELISA assay. The antisera shown in red bound significantly to native holotoxin A.

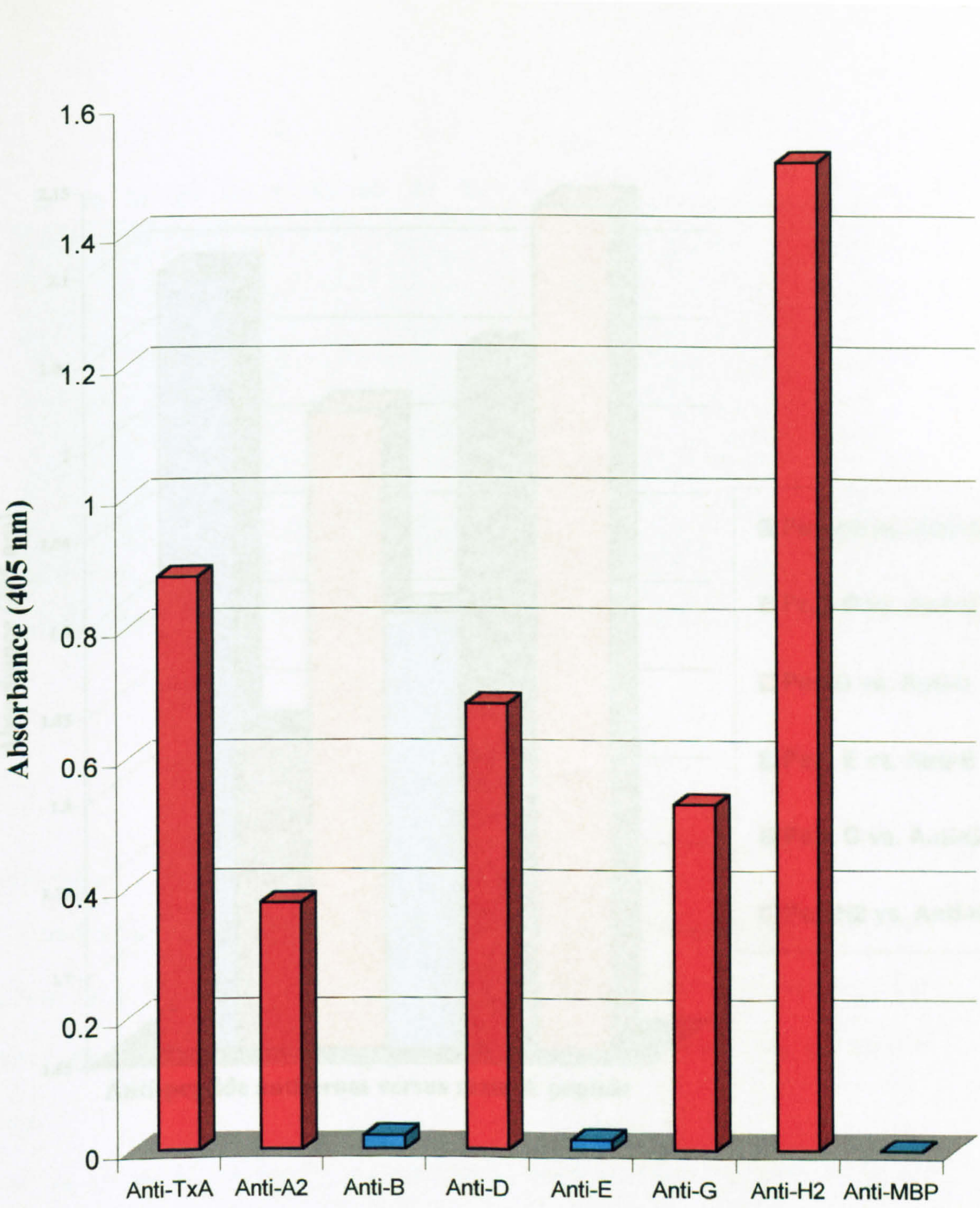


Figure 4.6
A graph to show binding of the different polyclonal monospecific anti-peptide antisera to wells containing formalised holotoxin A in an ELISA assay. The antisera shown in red bound significantly to formaldehyde treated toxin A.

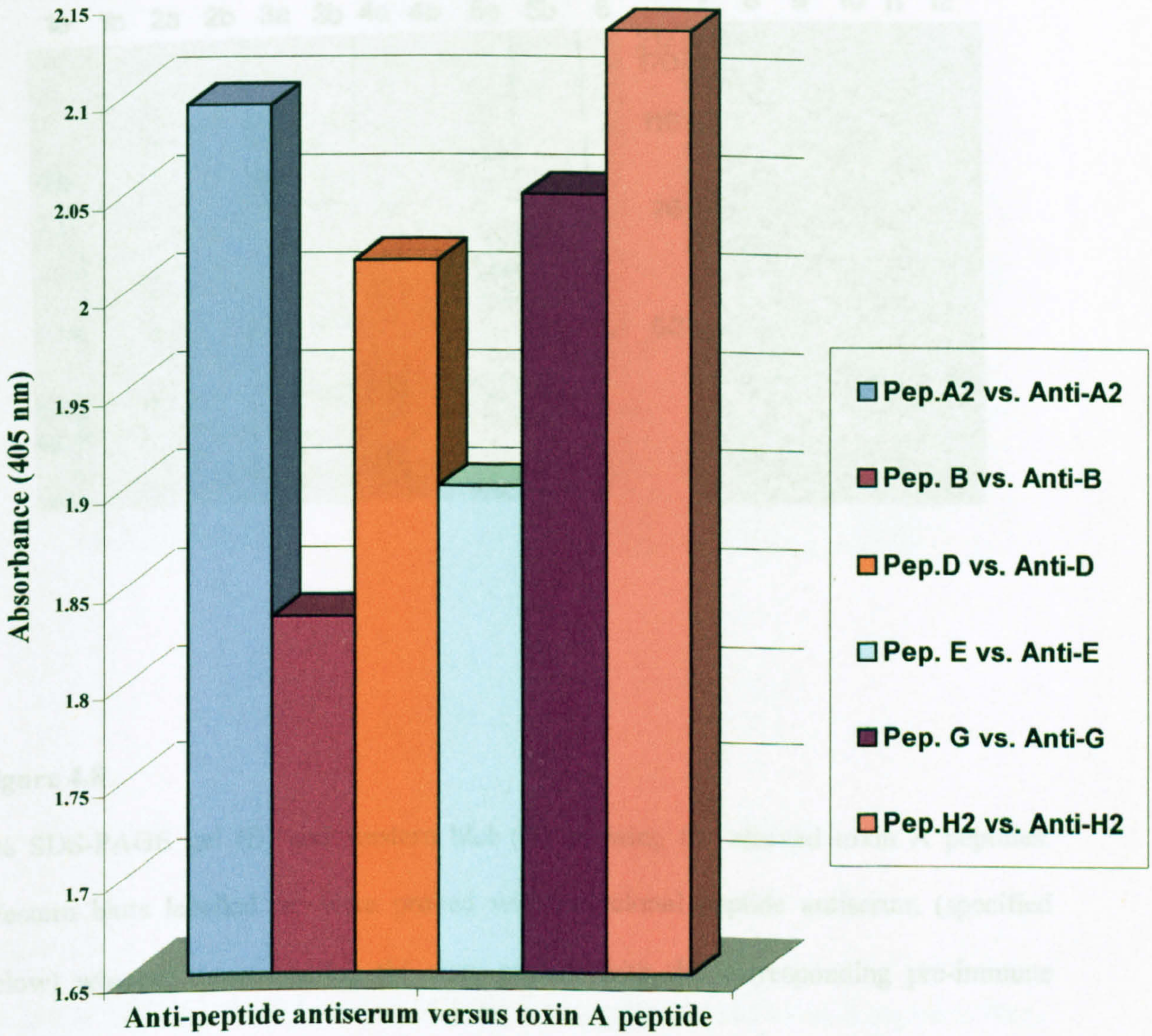


Figure 4.7
A graph to show binding of each native toxin A peptide to its respective polyclonal monospecific anti-peptide antiserum in an ELISA assay. Each binding was statistically significant.

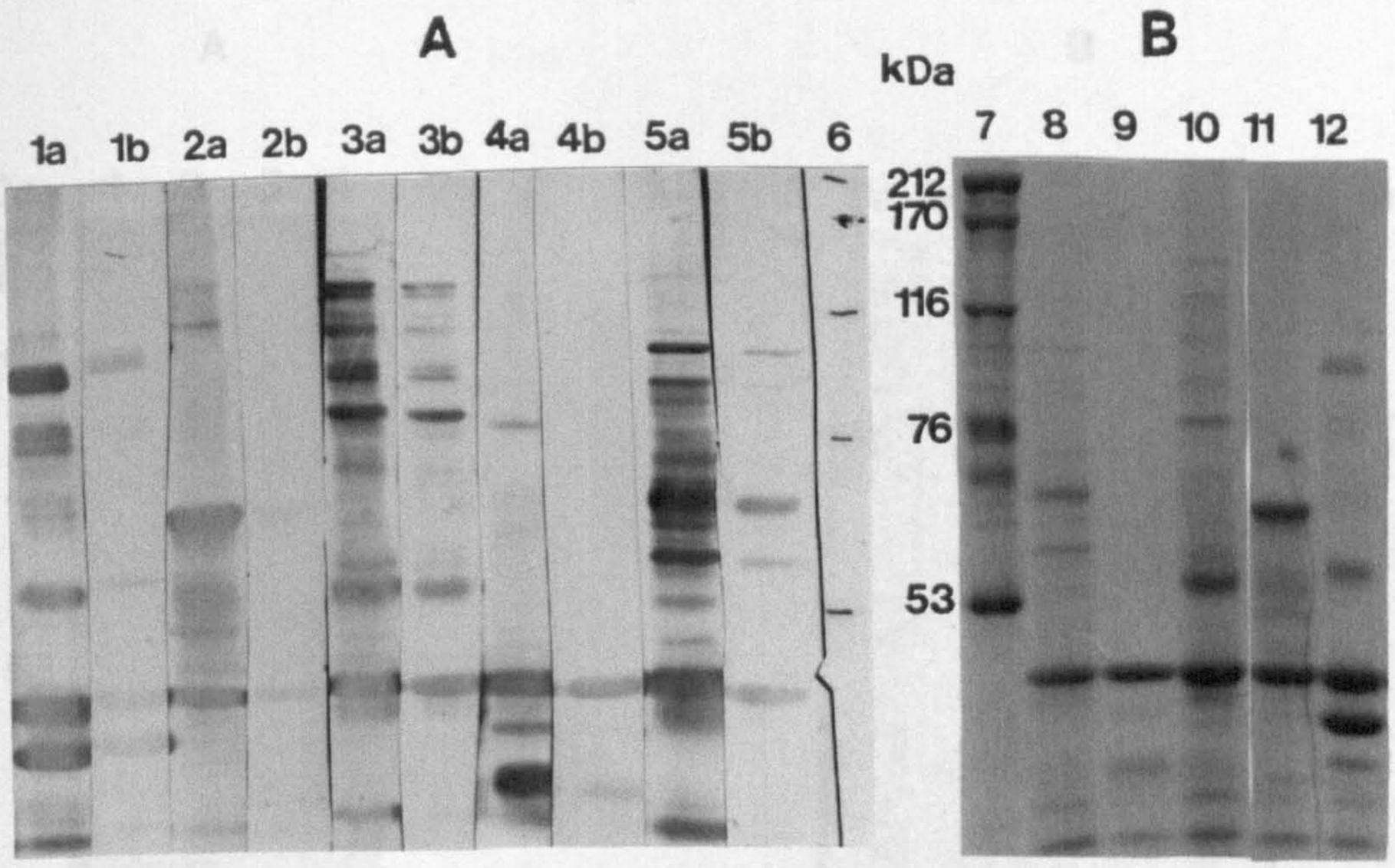


Figure 4.8

8% SDS-PAGE gel (B) and western blot (A) showing the cleaved toxin A peptides. Western blots labelled 'a' were probed with polyclonal peptide antiserum (specified below) whereas those labelled 'b' were probed with the corresponding pre-immune serum.

Lanes 1a, 1b and 12, peptide F (1a probed with peptide G antiserum); lanes 2a, 2b and 11, peptide E (2a probed with peptide E antiserum); lanes 3a, 3b and 10, peptide C (3a probed with peptide A2 antiserum); lanes 4a, 4b and 9, peptide B (4a probed with peptide B antiserum); lanes 5a, 5b and 8, peptide A2 (5a probed with peptide A2 antiserum); lanes 6 and 7, high MWT markers.

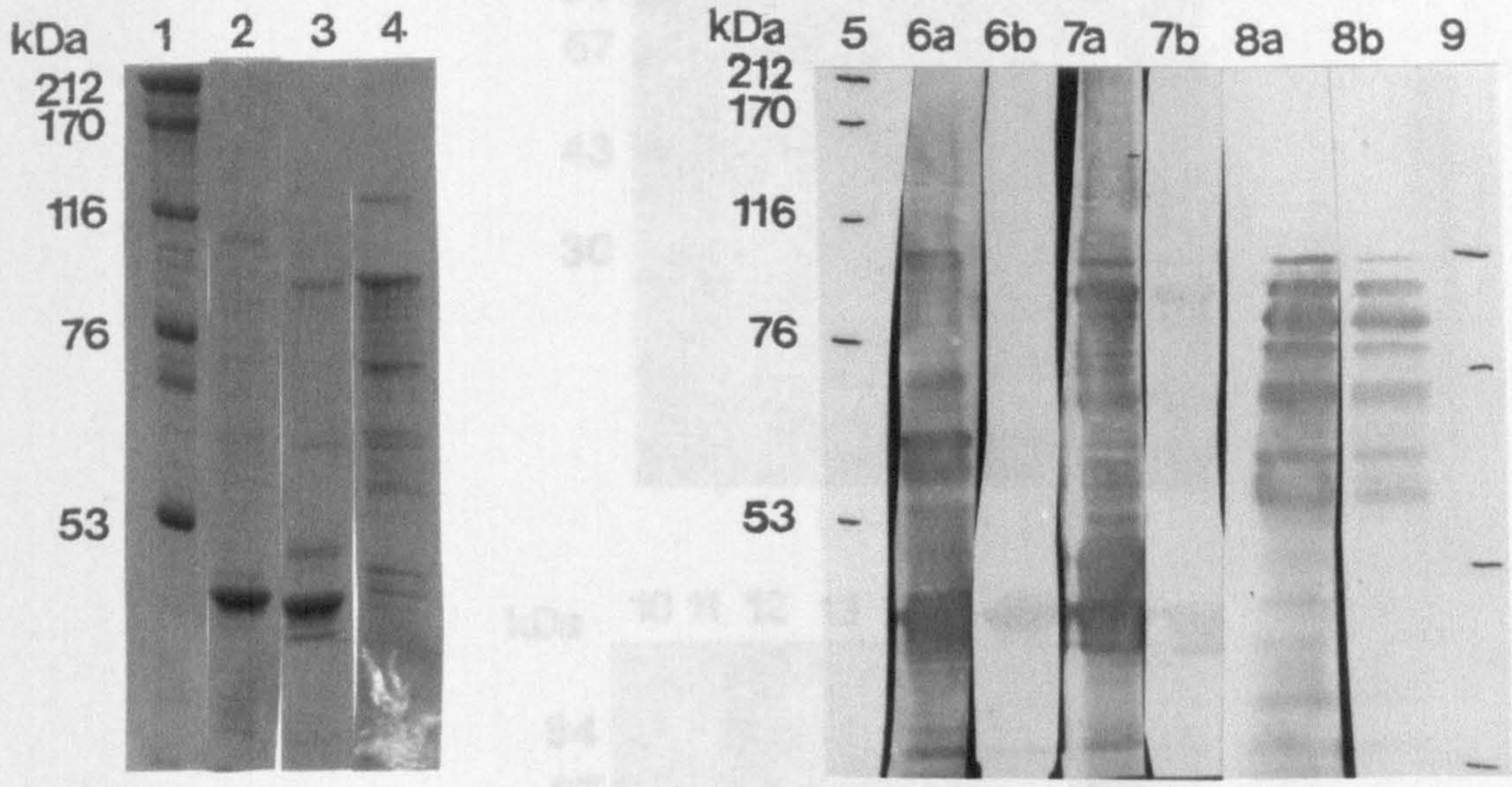


Figure 4.9

8% SDS-PAGE gel (A) and western blot (B) showing the cleaved toxin A peptides. Blots labelled 'a' were probed with polyclonal peptide antiserum (specified below) whereas those labelled 'b' were probed with the corresponding pre-immune rabbit serum.

Lanes 1 and 5, high MWT markers; lanes 2, 6a and 6b, peptide G (6a probed with peptide G antiserum); lanes 3, 7a and 7b, peptide D (7a probed with peptide D antiserum); lanes 4, 8a and 8b, peptide H2 (8a probed with peptide H2 antiserum); lane 9, low MWT markers.

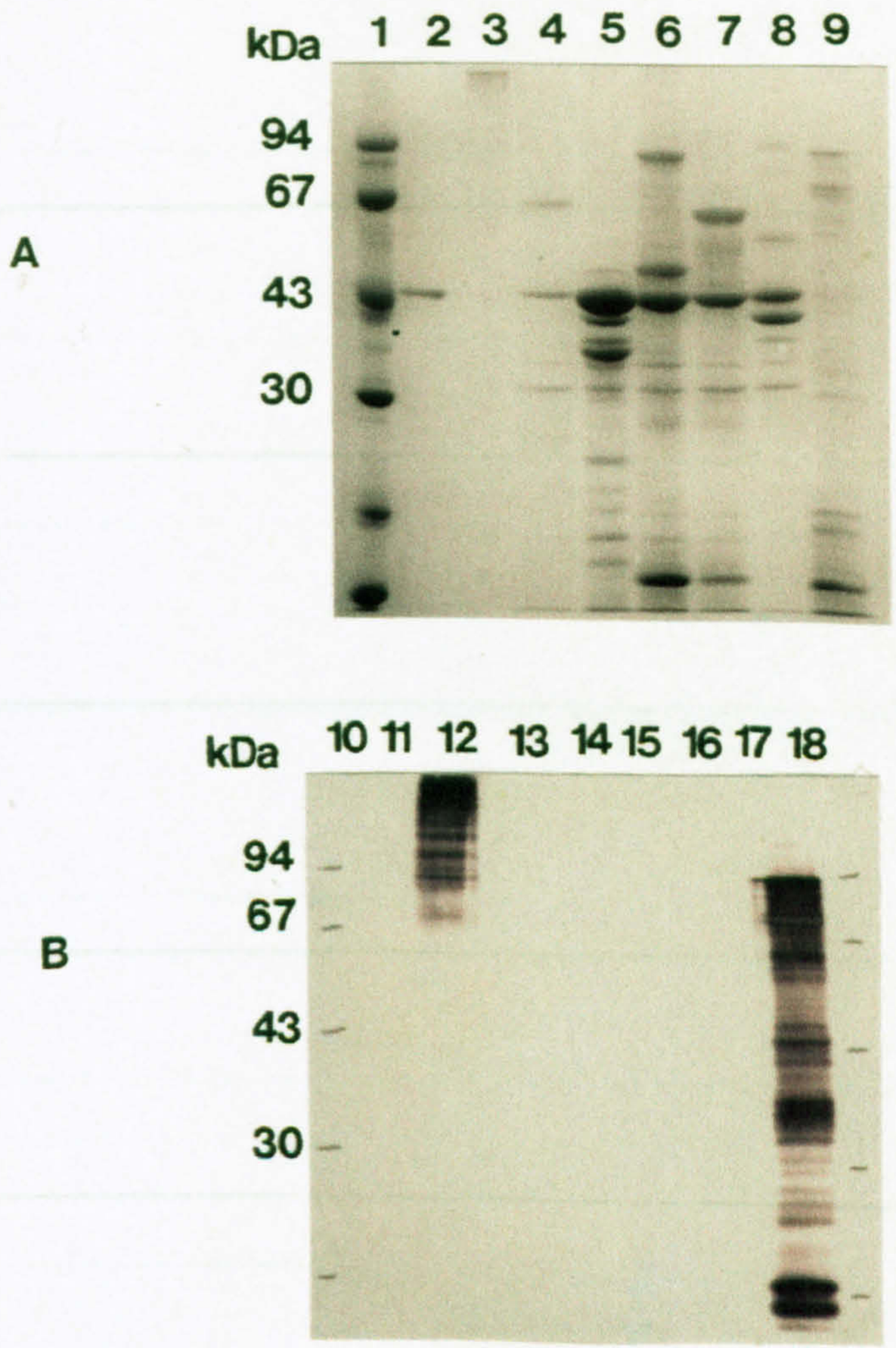


Figure 4.10

8% SDS-PAGE gel (A) and western blot (B) showing the cleaved toxin A peptides probed with the monoclonal antibody 357B.

Lanes 1 and 10, low MWT markers; lanes 2 and 11, MBP; lanes 3 and 12, toxin A; lanes 4 and 13, peptide A2; lanes 5 and 14, peptide B; lanes 6 and 15, peptide D; lanes 7 and 16, peptide E; lanes 8 and 17, peptide F; lanes 9 and 18, peptide H2.

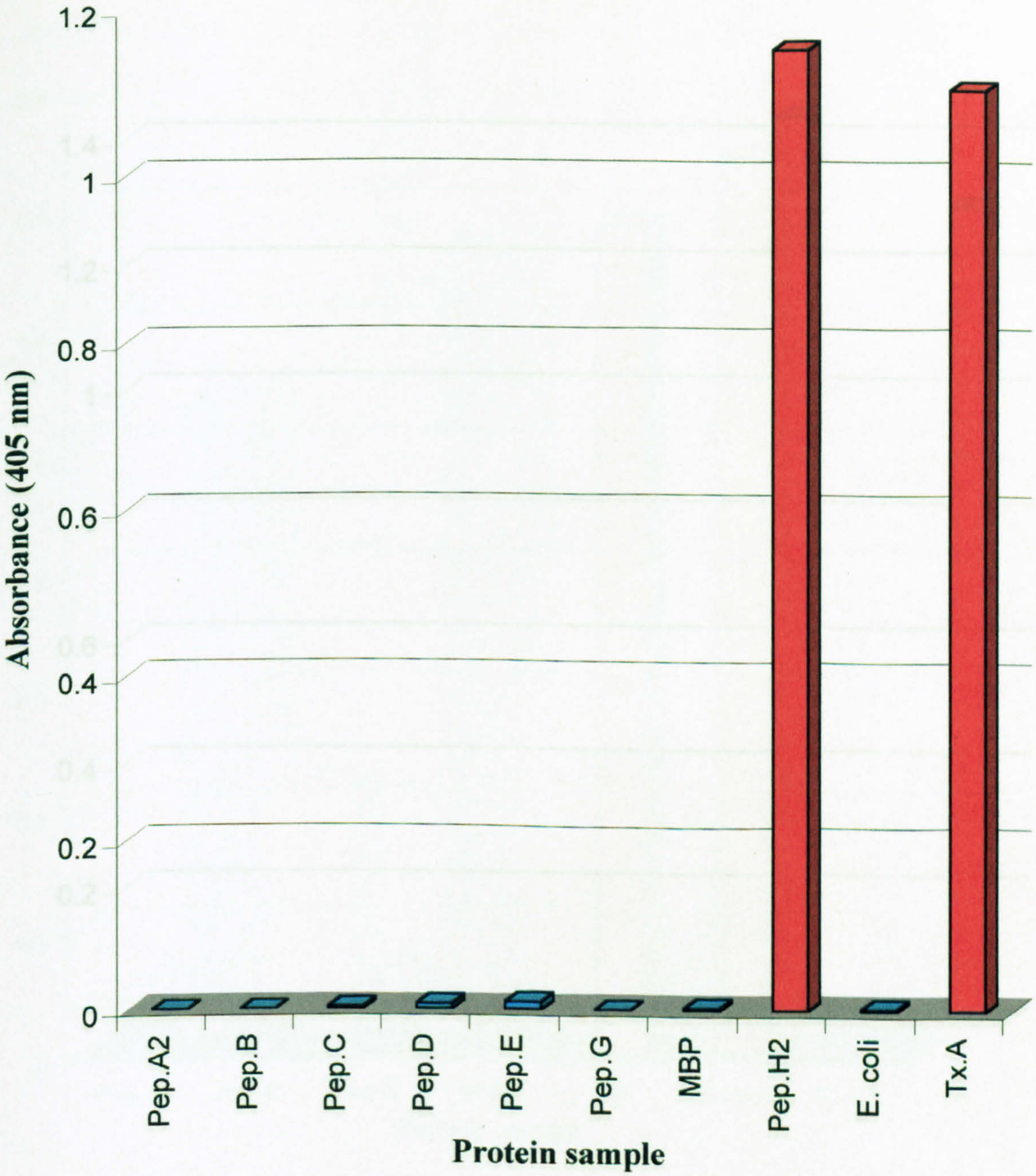


Figure 4.11
A graph to show binding of the 37B5 monoclonal antibody to the toxin A peptides to locate an active site for enterotoxicity in the toxin A molecule. Proteins shown in red bound significantly to the antibody.

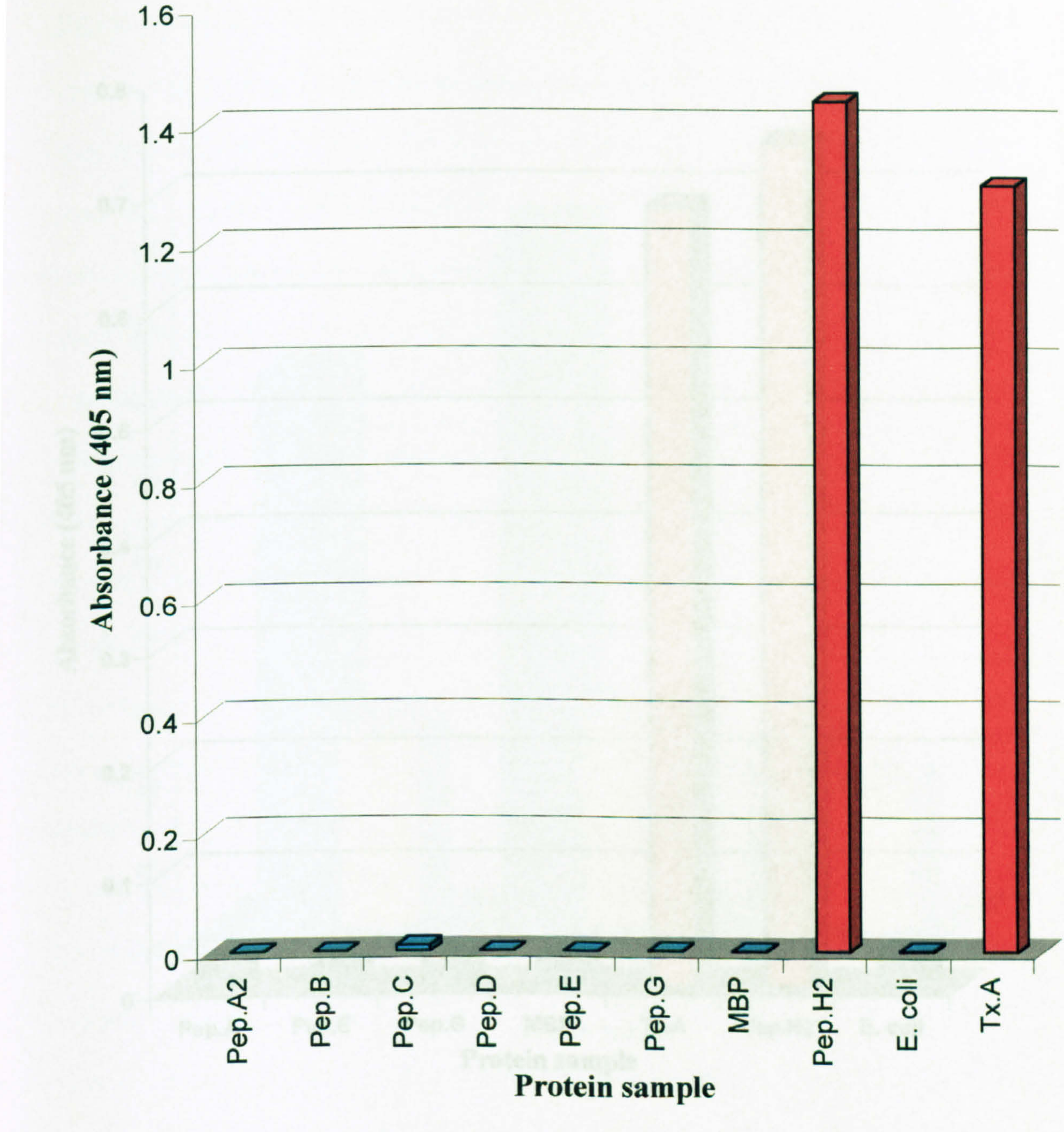


Figure 4.12
A graph to show binding of the PCG-4 monoclonal antibody to the toxin A peptides to locate a binding site for the antibody in the toxin A molecule. Proteins shown in red bound significantly to the antibody with 95% confidence limits.

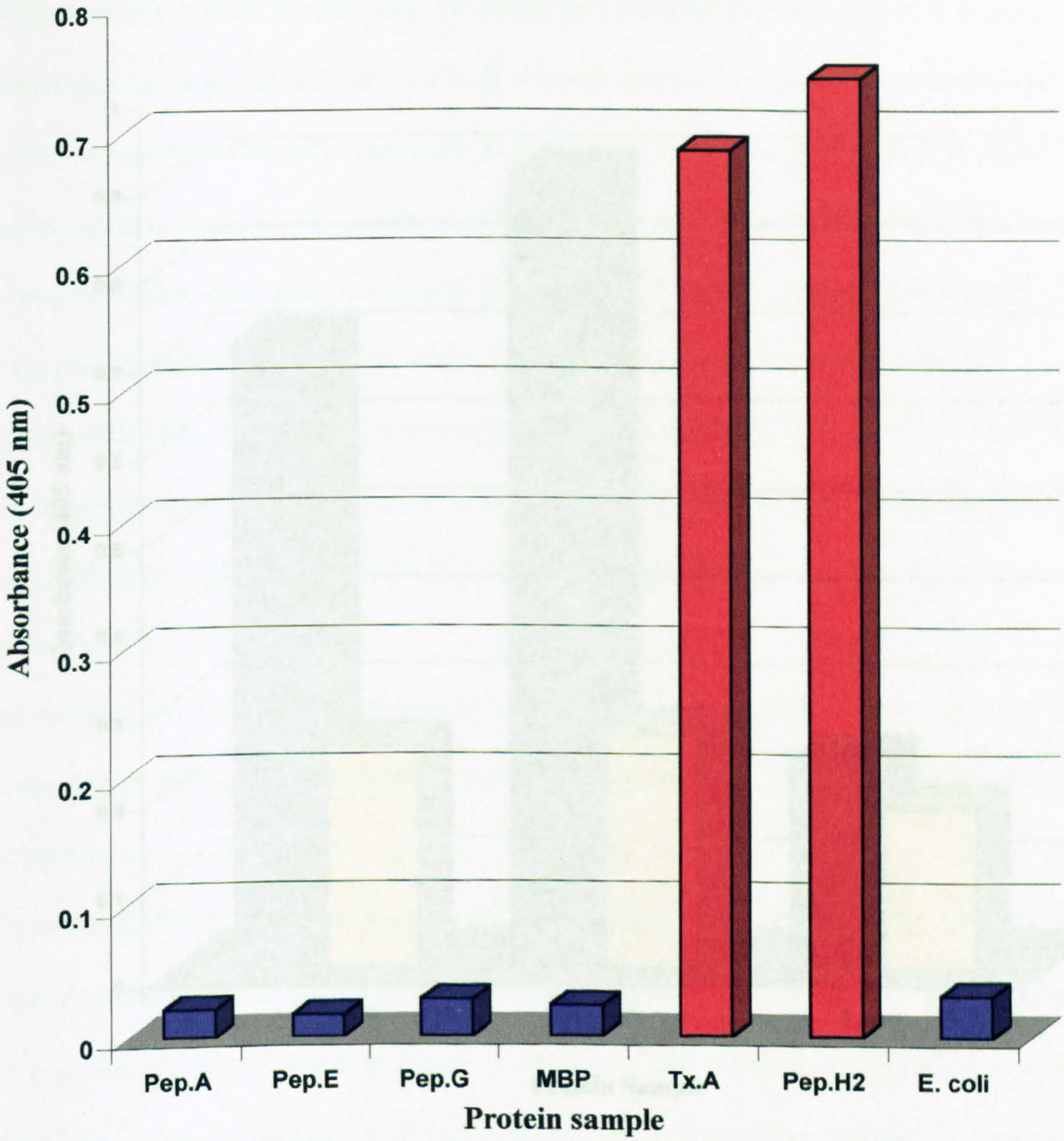


Figure 4.13
A graph to show non-specific binding of the IgG3λ murine monoclonal antibody to holotoxin A and the toxin A peptides. Protein samples shown in red bound significantly to the antibody with 90% confidence limits.

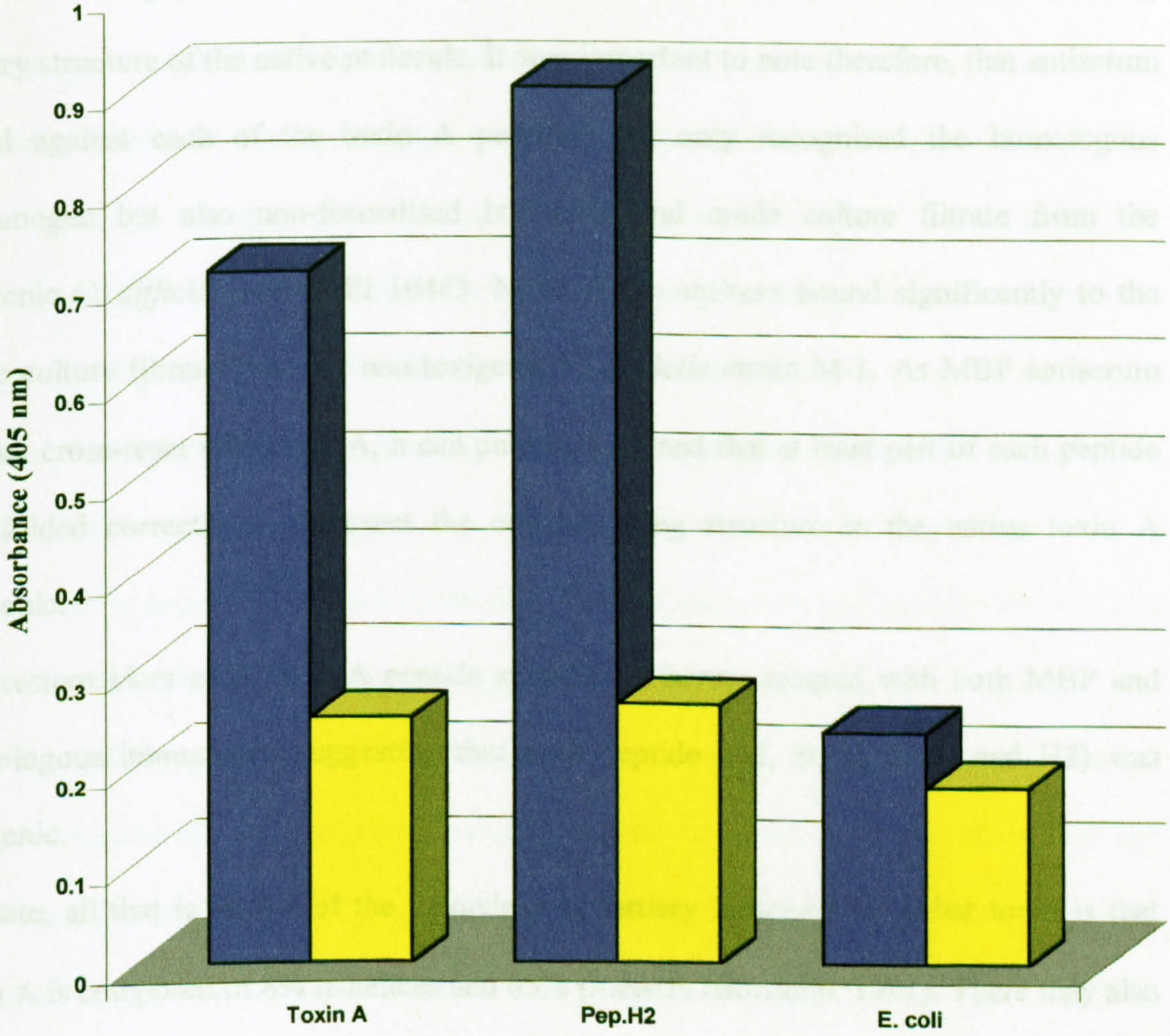


Figure 4.14

A graph to show the effect of α-galactosidase treatment on the non-specific binding of toxin A, peptide H2 and the *E. coli* negative control to the murine monoclonal antibody IgG3λ. Absorbance values shown in blue indicate non-specific binding of the antibody to the labelled peptide, whereas, those shown in yellow indicate the reduced non-specific binding to the protein due to pre-treatment of the antibody with α-galactosidase.

4.5 DISCUSSION

When a given protein is expressed as small recombinant peptides, there is always the chance that the peptides will not fold in the correct manner to represent the corresponding tertiary structure of the native molecule. It was important to note therefore, that antiserum raised against each of the toxin A peptides not only recognised the homologous immunogen but also non-formalised holotoxin and crude culture filtrate from the toxigenic *C. difficile* strain VPI 10463. None of the antisera bound significantly to the crude culture filtrate from the non-toxigenic *C. difficile* strain M-1. As MBP antiserum did not cross-react with toxin A, it can only be assumed that at least part of each peptide had folded correctly to represent the corresponding structure in the native toxin A molecule.

On western blots each toxin A peptide specific antiserum reacted with both MBP and homologous immunogen suggesting that each peptide (A2, B, D, E, G and H2) was antigenic.

To date, all that is known of the secondary or tertiary structure for either toxin is that toxin A is composed of 8% α-helices and 65% β-sheets (Borriello, 1991). There may also be a protoxin form of toxin A which is cytotoxic but which haemagglutinates poorly (Borriello *et al,* 1990) and has double the amount of α-helices (Borriello, 1991). An attempt was made to learn more about the tertiary structure of toxin A using antibody reaction profiling. The observation that toxin A antiserum recognised only recombinant peptides A2, C, D, G and H2, whereas all of the peptide antisera recognised native, non-formalised holotoxin A was initially confusing. The first assumption was that there were some antibodies in the peptide antisera that were binding non-specifically to the repeat region of holotoxin A. The results of an ELISA assay however, did not confirm this and

showed (with the exception of anti-peptide H2 antiserum) that none of the antisera bound to peptide H2 (ie repeat region of toxin A). Another consideration was that formalised holotoxin was used for the production of toxin A antiserum but non-formalised toxin A was being used for the ELISA studies. Once the ELISA assays were repeated using formalised holotoxin, antisera to peptides A2, D, G and H2 cross-reacted with the toxin, whereas antisera to peptides B and E did not. These results suggest that on an ELISA plate the structure of non-formalised toxin A alters, exposing different (previously unexposed) regions of the molecule. Formaldehyde treated toxin A however appears to be fixed in one configuration, possibly its native state. Another concern was that the formaldehyde may have altered some regions of the toxin A molecule (namely those represented by peptides B, E and F) causing a loss of antigenicity. Had time allowed this possibility could have been ruled out by formaldehyde fixing the toxin A peptides had time allowed. Cross-reactivity of a peptide antiserum with the untreated peptide but not with the formalised peptide would suggest that the formaldehyde was disrupting the structure of the peptide, causing a possible loss of antigenicity.

The antibody reaction profiles suggest that peptides B (amino acids [aa] 542-859), truncated E (aa 542-1,161) and F (aa 1,324-1,830) are unexposed in the native toxin A molecule but that peptides A2 (aa 1-536), C (aa 114-859), D (aa 869-1,330), G (aa 869-1,830) and H2 (aa 1,832-2,683) are exposed. Narrowing this down further, the exposed regions lie between aa 1-541, 1,162-1,324 and 1,832-2,683. In support of these predictions a hydropathy plot for the toxin A protein shows that the region between aa 1-541 is mainly hydrophilic. The region between aa 1,162-1,324 also shows some hydrophilic domains although it is interesting to note that the only strongly hydrophobic region in the molecule lies just upstream between aa 1,050-1,100. Finally the plot shows

that the repeat region of toxin A (aa 1,832-2,683) is the most hydrophilic part of the molecule suggesting that this portion is surface exposed (Dove *et al*, 1990). A diagrammatic representation of the predicted surface exposed regions of toxin A can be seen in appendix 8. These results provide the first indication of the tertiary structure of holotoxin A. The validity of these observations will be determined once the structure of the toxin A molecule has been deduced by X-ray crystallography.

It is interesting to note that screening of *C. difficile* genomic libraries with polyclonal toxin A antiserum only results in the isolation of clones containing the repeat region of toxin A (Price *et al*, 1987, von Eichel-Streiber *et al* 1989, Muldrow *et al* 1987, Wren *et al* 1987) although other regions of the molecule appear to be both surface exposed and antigenic. Perhaps the repeat end of toxin A is the most immunodominant part of the molecule and so the antibody response is preferentially directed against this region. This hypothesis is supported by the results seen in figure 4.2 which show that the toxin A antiserum has a high affinity for binding to peptide H2 but only binds weakly to peptides A2, D and G. Perhaps formaldehyde fixing the toxin reduced the immunodominance of the repeat region thereby allowing antibodies to be raised against other surface exposed regions. As previously mentioned, the repeat region of toxin A is extremely hydrophilic and is known to be the receptor-binding domain of the toxin (Dove *et al* 1990, Price *et al* 1987). It is also known that epitopes of the C-terminal repeat region are surface exposed due to the ability of the MAb PCG-4 to precipitate toxin A. It is likely therefore, that the repeat region is wrapped around the remainder of the native toxin A and is therefore the major part of the molecule that is exposed to the immune system. It is also possible that native toxin A dimerises in a manner that exposes predominantly the repeat structure.

When peptides A2, D and G were injected into rabbits without the immunodominant repeat region of the toxin, however, each elicited a strong antibody response.

It has recently been reported that *C. difficile* strains that produce toxin A which lacks the repeat region are not detected at all by polyclonal toxin A antiserum (Kato *et al*, 1999). Most diagnostic kits used today for the detection of toxigenic *C. difficile* strains however, make use of antiserum directed against the C-terminal repeat region of toxin A. Strains producing truncated forms of the toxin A gene that lack the C-terminal repeat region therefore maybe escaping these detection systems. It is possible that these strains are capable of causing mild disease and their detection is therefore important. It would be useful to use a polyclonal or monoclonal antiserum raised against peptide A2 to develop a more sensitive and effective diagnostic kit that would also detect these mutated but virulent *C. difficile* strains.

The 37B5 Mab, like PCG-4, recognised the C-terminal repeat end peptide H2. Although it is possible that the N-terminal peptides were not folded correctly to form an additional binding site for the 37B5 Mab, the fact that the antibody bound with such a high affinity to peptide H2 makes an additional site unlikely. These results were disappointing as it had been suggested that the 37B5 Mab was blocking an active site for enterotoxicity unrelated to toxin A binding (Kamiya *et al,* 1991). This conclusion was drawn because the 37B5 Mab, unlike PCG-4, was unable to block haemagglutination of rabbit red blood cells and therefore thought unable to block toxin A binding. Attempts to repeat the experiments of Lyerly *et al* 1986 and block haemagglutination using the PCG-4 Mab were unsuccessful in our laboratory (results not shown). Kamiya and co-workers did not use the PCG-4 Mab as a positive control in their haemagglutination neutralisation experiments with the 37B5 Mab and cannot therefore, draw the conclusion that the 37B5

antibody is not blocking enterotoxicity by blocking binding of toxin A to its target cell (Kamiya *et al* 1986). As both the PCG-4 and 37B5 Mabs bind to peptide H2, it is likely that both share the same binding site in the toxin A molecule and block enterotoxicity by blocking binding of the toxin to its target cell.

Mabs are extremely powerful tools that are frequently used to identify the functional epitopes of protein molecules. It is essential therefore, that Mabs interact specifically by a true immune reaction with their antigen. Toxin A has been shown to bind non-specifically to murine Mabs raised to antigens other than toxin A (Lyerly *et al* 1989) and this function has led to many misleading results. The ability of toxin A to non-specifically bind to antibodies, is in keeping with the ability of a number of proteins from a variety of gram-positive (Forsgren *et al*1966, Korvall 1973, Retnoningrum *et al* 1994) and gram-negative (Forsgren *et al* 1979, Labbe *et al* 1995, Mintz *et al* 1994, Yarnall *et al* 1988) microorganisms, to interact non-immunologically with immunoglobulins. Recent results have shown that unlike these other protein molecules which bind to the Fc domain of antibodies, toxin A binds to an IgG3λ murine Mab through the Fab domain (Cooke and Borriello 1998). The significance of the ability of toxin A to bind non-specifically with immunoglobulins by interaction with the Fab component is unknown.

It is well known that the repeats at the C-terminal end of toxin A are involved in carbohydrate binding (Dove *et al* 1990, Price *et al* 1987) and that they bind to the trisaccharide residue Galα1-3Galβ1-4GlcNac (Krivan *et al* 1986, Tucker *et al* 1991). The expectation was, therefore, that the repeat end peptide (H2) would be responsible for non-specifically binding to the Fab domain of murine monoclonal antibodies. The results in this section prove this to be the case and also show that binding is mediated through galactose residues on the antibody molecule. Carbohydrate is mainly localised on the

constant domain of the heavy chain of IgG Fab fragments. If it is the case, as suspected, that the C-terminal repeat region of toxin A is exposed over the surface of the molecule, there would be a maximum surface area available for toxin A to non-specifically bind to antibodies *in vivo*. At present, it is unknown whether this non-specific interaction with galactose residues on antibodies is purely coincidental or whether it is a function that has been adapted to enable toxin A to mask itself from the immune system *in vivo*. It has been suggested that this ability may promote interaction of toxin A with mucus due to the presence of secretory IgA, which contains more carbohydrate than other immunoglobulins (Cooke and Borriello 1998, Goodman 1982).

A practical outcome of these findings was the indication that Mabs raised to toxin A should be treated with α and/or β-galactosidase to confirm specific interaction. A reduction in binding to toxin A following this treatment would suggest that the Mab is binding in a non-specific manner and not by a true immune reaction. Another important consideration is that the majority of Mabs raised to toxin A that appear to bind to the C-terminal repeat region of the molecule may be doing so in a non-specific manner.

SECTION – 5

A STUDY OF THE BIOLOGICAL ACTIVITIES EXPRESSED BY THE TOXIN A PEPTIDES

SECTION – 5

A Study of the Biological Activities Expressed by the Toxin A peptides

5.4 RESULTS

5.4.1 H/A assay

5.4.2 Bovine thyroglobulin binding

5.4.3 Cytotoxicity assay

5.4.4 Rabbit ileal loop assay

5.4.5 Neutralisation of the toxin A induced CPE on Vero cells and H/A of rabbit red blood cells by polyclonal toxin A peptide antisera

5.4.6 Immunohistochemical detection of binding of peptide H2 to Vero cell receptors

5.4.7 Transmission electron microscopy (TEM) studies

5.4.7.1 Internalisation of peptide H2 into cells

5.4.7.2 Internalisation of peptide A2 into cells

5.5 DISCUSSION

Section 5

A Study of the Biological Activities Expressed by the Toxin A Peptides

5.1 SUMMARY

The eight toxin A peptides were used to localise the active sites for a range of biological activities associated with *C. difficile* toxin A. All activities related to binding of carbohydrate i.e. cold haemagglutination of rabbit erythrocytes and binding of bovine thyroglobulin were restricted solely to peptide H2 (C-terminal repeat end of toxin A). Peptide H2 also caused a cytopathic effect (CPE) on tissue culture cells which was atypical of holotoxin A. None of the other peptides had an effect. In the rabbit ileal loop assay, none of the toxin A peptides induced a visible haemorrhagic fluid secretion, whilst loops that contained either holotoxin A or cholera toxin were fully distended. Polyclonal antisera raised against both holotoxin A and peptide H2 were shown to partially inhibit the toxin A induced CPE on Vero cells and the H/A of rabbit erythrocytes. Polyclonal antisera raised against the N-terminal peptides (A2-G) however, did not protect against these toxin A induced biological activities.

Immunohistochemistry and transmission electron microscopy (TEM) studies showed that peptide H2 bound to the surface of Vero cells *in vitro* and was also internalised in membrane bound vesicles into rabbit intestinal cells in the rabbit ileal loop assay. These results suggest that the C-terminal repeat region of toxin A is solely responsible for uptake of the holotoxin into endosome-like compartments within cells. We propose that peptide H2 binds to cell surface receptors and activates intracellular transmembrane signalling pathways, which exert cellular effects (visualised as the atypical CPE) that are independent of the catalytic action of the holotoxin.

5.2 INTRODUCTION

The toxin A peptides produced in section 3 are important tools for increasing the limited knowledge on the structure-function relationships of toxin A. Various biological activities have been attributed to toxin A although the active sites responsible for many of these processes have not been identified.

Toxin A has been shown to bind with a higher affinity to hamster ileal brush border membranes at 4^0C than at 37^0C (Krivan *et al* 1986, Rolfe 1991). This cold binding has also been demonstrated with rabbit erythrocytes, bovine thyroglobulin, rabbit ileal brush borders and human intestinal epithelial cells (Krivan *et al* 1986, Pothoulakis *et al* 1991, Krivan *et al* 1987, Smith *et al*, 1997). The trisaccharide residue Galα1-3Galβ1-4GlcNac is present on rabbit erythrocytes and bovine thyroglobulin and has been revealed as the binding ligand (Krivan *et al* 1986, Krivan *et al* 1987, Clark *et al* 1987). This carbohydrate however, is not present on human cells but the findings led to the identification of the Lewis X, Y and I antigens as possible receptors for toxin A on human intestinal epithelial cells (Tucker *et al* 1991). Since this time Lewis X has been ruled out as a receptor for toxin A (Sauerborn *et al*, 1997). Also, a further study has pointed out that as the Lewis X, Y and I antigens do not possess terminal alpha-galactose units, other receptors are likely to be involved in toxin A binding to some human intestinal cells (Smith *et al*, 1997). The C-terminal region of toxin A is composed of repeating oligopeptides and it is this region of the holotoxin that has been identified as the receptor binding domain (Price *et al* 1987, Wren *et al* 1991).

Toxin A is a potent cytotoxin *in vitro* causing a characteristic rounding up of cells due to disruption of the actin cytoskeleton. As well as cytotoxic activity, toxin A also exhibits enterotoxic activity causing destruction of the colonic epithelium which results in fluid

secretion into the intestine (Lima *et al* 1988, Lyerly *et al* 1985a). The active sites responsible for these biological activities have not yet been identified, although it is believed that they reside within the N-terminal two thirds of the molecule.

The aim of this section was to determine the regions of the toxin A molecule responsible for H/A of rabbit erythrocytes, thyroglobulin binding, cytotoxicity and enterotoxicity. Peptide specific antisera would also be used in an attempt to neutralise the biological activities of toxin A. The protective potential of each peptide would therefore be determined.

5.3 MATERIALS AND METHODS

5.3.1 Haemagglutination (H/A) assay

This assay is based on a method described by Krivan *et al* (1986) with the slight modification that a 1% rabbit red blood cell (RRBC) suspension was used (Kamiya *et al*, 1988), instead of a 2.5% suspension. Where necessary the toxin A peptides were cleaved with factor Xa (see section 2.8.3). Fifty µl of each toxin A peptide (16µgml^{-1}) was diluted two-fold with Tris Buffered Saline (isotonic buffer) in the wells of a v-bottom microtitration plate (Sero-well). Holotoxin A (320µgml^{-1}) was placed in one well as a positive control. MBP and the *E. coli* protein sample were used as negative controls (see sections 3.3.7.1 and 3.3.7.5). Fifty µl of a 1% RRBC suspension was added to each well and incubated for at least 3h at 4^{0}C. H/A titres were expressed as the reciprocal of the highest dilution giving a positive H/A when viewed by eye. The experiment was repeated to compare the H/A titres of peptide H2 to holotoxin A. Peptide H2 (16µgml^{-1}) and toxin A (320µgml^{-1}) were diluted two-fold with Tris-buffered saline in the wells of a V-bottom plate. RRBCs were added to each well and the reactions were then incubated as described.

5.3.2 Thyroglobulin binding assay

The ability of each toxin A peptide to bind bovine thyroglobulin was determined using an Enzyme Linked Immunosorbent Assay (ELISA) based technique. Bovine thyroglobulin (Sigma) was dissolved in 0.05M carbonate buffer pH 9.6 to a final concentration of 10µgml^{-1}, and applied to a 96 well ELISA plate (Life Technologies Ltd., Paisley, U.K) at 4^{0}C overnight. The wells were blocked with 1% gelatin / PBS / 0.05% Tween 20 for 1h at

room temperature. Where necessary the fusion peptides were cleaved with factor Xa at 4^0C overnight (see section 2.8.3). Toxin A peptides were diluted in 0.1% gelatin / PBS / 0.05% Tween 20 to the final concentration of 10μgml^{-1}, and 50μl aliquots were applied in triplicate to the wells and incubated at 4^0C for 2h. Wells were washed with PBS / 0.05% Tween 20 at 4^0C and incubated with a 1:1,000 dilution of the relevant polyclonal peptide specific antiserum at 4^0C for 2h. Following four washes with PBS / 0.05% Tween-20, 50μl of goat anti-rabbit alkaline phosphatase conjugate (1:1000 dilution; Sigma) was applied and incubated at 4^0C for 1h. Each well was washed with PBS / 0.05% Tween 20 then sterile distilled water. 50μl of pNpp alkaline phosphatase substrate solution (Sigma) was added and absorbance values were recorded at 405nm. Controls were included to determine the background level binding of each peptide antiserum to bovine thyroglobulin. A statistical analysis was performed (see section 4.3.7).

5.3.3 Cytotoxicity Assay

This assay makes use of African Green Monkey Kidney (Vero) cells, as described by Kamiya *et al* (1988). Vero cells were grown in culture flasks in Medium 199 (Sigma, St Louis, USA) containing 5% newborn calf serum, 1% penicillin and 1% glutamine. When confluent, the cells were recovered from the flask by incubating with 3mls trypsin / EDTA solution (Sigma) for 2mins at 37^0C and were resuspended in 20mls of growth medium. Two hundred μl aliquots of cell suspension were added to each well of a 96 well sterile microtitre plate (Falcon). The cells were incubated overnight at 37^0C to reach confluency before the medium was replaced with 100μl of maintenance medium (Medium 199 containing 2% newborn calf serum, 1% penicillin and 1% glutamine). Where necessary

toxin A peptides were cleaved with Factor Xa at 4^0C overnight (see section 2.8.3). Ten-fold serial dilutions of the peptides (16μgml^{-1}) were prepared in PBS, and 100μl of each sample was added to the Vero cells in the microtitre plate. Toxin A (16μgml^{-1}) was diluted in the same way and included in the assay as a positive control. MBP (16μgml^{-1}) was included as a negative control for peptides A2-G whilst the *E. coli* protein sample (16μgml^{-1}) acted as a negative control for peptide H2. Following an incubation of 24h at 37^0C, cells were monitored for a CPE, commonly seen as cell rounding. The cytotoxicity titre (cytotoxic unit [CU]/100 μl) was expressed as the highest dilution that induced more than 50% CPE after an incubation of 24h.

For toxin A and peptide H2 the dilution giving an end point in the ten-fold dilution series was used as a starting point for a two-fold dilution series to yield a more accurate end point.

5.3.4 Enterotoxic activity of the toxin A peptides in rabbit ileal loops.

The ileal loop assay was performed at Leicester University in collaboration with Dr. Julian Ketley. Ileal loop tests were done as previously described (Evans *et al*, 1973 and Mitchell *et al*, 1986) with the following exceptions.

A method was developed for constructing closed loops in rabbit ileum. The rabbit was fasted for 24 hr and the ileum washed through with 50ml phosphate buffered saline (PBS: 4.25g/l NaCl, 2.44g/l KH_2PO_4, 8.09g/l Na_2PO_4 pH.7). From the point of ligation, 5cm ligated loops with 5cm interloop spaces were constructed and tied with cotton. Toxin A peptides were cleaved where necessary (see section 2.8.3). Five hundred μl of the toxin A peptides A2, B, C, D, E and G (72μgml^{-1}) and 0.5ml of peptide H2 (25μgml^{-1}) were

injected into different loops. The interloop spaces were injected with 0.5ml of PBSA. Toxin A (72μgml^{-1}) or cholera toxin (2μgml^{-1}) were injected and included as positive controls, whereas MBP (72μgml^{-1}) and the *E. coli* protein sample (25μgml^{-1}) were included as negative controls. The ileal loops were returned to the rabbit, which was left to recover from the anaesthetic. The loops were examined after an 18hr incubation period and the results were recorded as the volume of fluid taken from the loop / the length of the loop (V/L ; ml/cm). A small section of each loop was carefully dissected and placed in 0.5ml of glutaraldehyde for electron microscopy studies.

5.3.5 Neutralisation of the toxin A induced CPE on Vero cells by the toxin A peptide polyclonal monospecific antisera.

Using polyclonal monospecific antisera raised to toxin A and to toxin A peptides A, B, D, E, G and H2 (see section 4.3.1), an attempt was made to neutralise the toxin A induced CPE (see section 5.3.3). Vero cells were grown to confluency in 96 well tissue culture plates and the growth media replaced with 100μl of maintenance medium.

Two sets of toxin A dilutions were prepared in PBSA. In the first set toxin A was diluted to 26μgml^{-1}, 2.6μgml^{-1}, 260ngml^{-1}, 130ngml^{-1}, 65ngml^{-1}, 32.5ngml^{-1}, 16.25ngml^{-1} and 8.125ngml^{-1}. In the second set toxin A was diluted to 26μgml^{-1}, 13μgml^{-1}, 6.5μgml^{-1}, 3.25μgml^{-1}, 1.625μgml^{-1}, 812.5ngml^{-1} and 406ngml^{-1}. Each of the above mentioned polyclonal monospecific antisera and their respective pre-bleed sera were diluted 1:10 in PBSA. 100μl aliquots of each of the toxin A dilutions in set one were mixed 1:1, in separate reactions, with each of the antibody preparations. One hundred μl aliquots of each of the toxin A dilutions in set two were mixed 1:1, in separate reactions, with the

diluted preparation of either polyclonal monospecific anti-toxin A, anti-peptide H2 or their respective pre-bleed sera (the original toxin A concentrations now diluted 1:2). The tubes were incubated for 1hr at 37^0C. One hundred µl aliquots of each neutralisation reaction were applied to Vero cells in 96 well plates (the original toxin A concentrations now diluted 1:4). Each specific antiserum was limited to one column and the samples were arranged down the plate in the order of decreasing toxin A concentration.

Following an incubation of 24hr at 37^0C with 5% CO_2, the cells were observed for a CPE (commonly seen as cell rounding). The cytotoxicity titre (cytotoxic unit [CU] /100µl) was expressed as the highest dilution that induced more than 50% CPE after an incubation of 24hr.

5.3.6 Neutralisation of the toxin A induced H/A of RRBCs by polyclonal monospecific toxin A and peptide H2 antisera.

Using polyclonal monospecific antisera raised to toxin A and peptide H2 (see section 4.3.1), an attempt was made to neutralise the toxin A induced H/A of RRBCs (see section 5.3.1).

Toxin A was diluted in PBSA to the final concentrations of 65µgml^{-1}, 32.5µgml^{-1}, 16.25µgml^{-1}, 8.125µgml^{-1}, 4.0625µgml^{-1}, 2.031µgml^{-1}, 1.015µgml^{-1} and 0.505µgml^{-1}. One hundred µl aliquots of each of the toxin A dilutions were mixed 1:1, in separate reactions, with a neat preparation of either polyclonal monospecific toxin A antiserum, peptide H2 antiserum or the respective pre-bleed sera (the original toxin A concentrations now diluted 1:2). The tubes were incubated for 1hr at 37^0C. One hundred µl of each neutralisation reaction were tested for the ability to haemagglutinate RRBCs. Each specific antiserum

was limited to one column and the samples were arranged down the V-well plate in the order of decreasing toxin A concentration.

5.3.7 Detection of binding of peptide H2 to Vero cells by immunohistochemistry

5.3.7.1 Production of monolayers of Vero cells on glass coverslips.

A glass coverslip was placed in the bottom of each well of a 24 well sterile tissue culture plate (Falcon). Confluent Vero cells were recovered from a tissue culture flask by trypsinisation and resuspended in 25mls of growth medium (see section 5.3.3). One ml aliquots of the cell suspension were added to each well containing a glass coverslip. The cells were incubated at 37^0C with 5% CO_2 to reach confluency and the growth medium was then replaced with 0.3 ml of maintenance medium (see section 5.2.2.1).

5.3.7.2 Incubation of Vero cells with peptide H2

Monolayers of Vero cells were grown to confluency on glass coverslips in a 24 well tissue culture plate and the growth medium was replaced with 0.3 ml maintenance medium (see section 5.2.2.1).

Peptide H2 (0.3 ml of 18μgml^{-1} stock) was added to eight wells (A), the negative control *E. coli* protein sample (0.3 ml; directly comparable to peptide H2) was added to eight wells (B) and PBSA (0.3 ml; negative control) was added to four wells (C). The cells were incubated with the protein samples at 4^0C for 20mins. Two coverslips from each of wells A, B and C were removed and washed with 3 x 1 ml of wash buffer pH 7.5 (8mM Na_2HPO_4, 2.2mM NaH_2PO_4, 140mM NaCl). These coverslips (4^0C samples) were fixed by submerging in a glass petri-dish containing acetone for 10mins. The fixed cells were air

dried then placed in a fresh 24 well plate. The remaining cells were incubated at 37^{0}C with 5% CO_2 for 10mins. Again two coverslips were removed from A and B. These were washed, fixed and air-dried as mentioned previously (37^{0}C, 10 min samples). The remaining cells were returned to the 37^{0}C incubator. This process was repeated with two coverslips from A and B after 20mins at 37^{0}C and with two coverslips from A, B and C after 30mins at 37^{0}C. Once all of the coverslips had been placed in a fresh 24 well plate, they were covered in foil and stored at -20^{0}C ready for immunohistological examination.

5.3.7.3 Immunohistochemical detection of bound peptide H2

Immunohistochemical detection was performed as outlined by the Vectastain ® Universal *Elite* ABC Kit (Vector Laboratories) instruction manual. A brief outline is given below.

The prepared coverslips (section 5.3.7.2) were equilibrated to RT, air dried then washed with 3 x 0.5mls of wash buffer pH 7.5 (8mM Na_2PO_4, 2.2mM NaH_2PO_4, 140mM NaCl). One of each of the pairs of coverslips containing Vero cells treated with peptide H2 or the *E. coli* protein sample, for 20mins at 4^{0}C, 10mins at 37^{0}C, 20mins at 37^{0}C and 30mins at 37^{0}C were incubated with 7$\mu g ml^{-1}$ of the Mab PCG-4 (diluted in wash buffer) at 4^{0}C, overnight with shaking. One of each of the pairs of control coverslips containing untreated Vero cells incubated under identical conditions was also treated with the PCG-4 Mab as described above. The remaining coverslip from each pair was incubated with 500µl of wash buffer at 4^{0}C overnight with shaking.

All of the coverslips were washed with 5 x 1ml of wash buffer and then incubated with 0.5 ml of Biotinylated Universal Antibody (Vector Laboratories) for 30mins at RT with shaking. Any non-specific binding of the biotinylated antibody to the Vero cells or the

protein sample was evident in the second coverslip of each pair that was not incubated with primary antibody. The coverslips were washed with 5 x 1 ml of wash buffer before incubation with 0.5ml of a preformed avidin and biotinylated horse radish peroxidase macromolecular complex (Vectastain ® *Elite* ABC Reagant; Vector Laboratories) for 30mins at room temperature with shaking. Coverslips were washed with 5 x 1ml of wash buffer before incubating in diaminobenzidine tetrahydrochloride solution (DAB; Vector Laboratories) for 2mins to allow the colour to develop. A summary of the Vectastain *Elite* ABC detection system can be seen in figure 5.6.

The coverslips were rinsed in distilled water, allowed to dry then counterstained with Haemotoxylin (Nustain Inc.) for 2mins. Submerging respectively in 30%, 60% then 90% ethanol solutions for 10secs each dehydrated the cells. The cells were rehydrated by submerging in xylene solution (Nustain Inc.) for 10secs before placing on a glass slide, applying a drop of DPX mounting solution (Nustain) and overlaying with a glass coverslip.

Each sample was viewed under a bright field microscope (Orthoplan) alongside its respective negative controls at magnifications of x10, x16 and x25. Results were photographed using a Leitz-Wild MP551 camera.

5.3.8 Transmission electron microscopy (T.E.M.)

A detailed account of the methodology used in the EM studies is outlined in Powell, 1999. A brief outline is provided below.

5.3.8.1 Incubation and treatment of Vero cells with toxin A peptides for electron microscopy (E.M.) studies.

Confluent Vero (monkey kidney) cells were recovered from a tissue culture flask by trypsinisation and resuspended in 50ml growth medium (see section 5.3.3). One ml aliquots of the cell suspension were added to each well of two 24 well sterile tissue culture microtitration plates (Falcon). The cells were incubated at 37^0C with 5% CO_2 to reach confluency and the growth medium was then replaced with 0.5 ml of maintenance medium (see section 5.3.3). MBP (negative control), peptide A2, *E. coli* protein sample (negative control), peptide H2 and toxin A were each diluted to 5μgml^{-1} in maintenance medium and each sample was added in multiples of 6 x 0.5ml aliquots to the cells in the microtitration plates. A further row of six wells was incubated with 0.5ml TBS and was included as a further negative control. The microtitre plates were placed at 4^0C for 30mins. The cells in one representative well per protein sample were washed with 1ml PBSA (4.25g/l NaCl, 2.44g/l KH_2PO_4, 8.09g/l Na_2PO_4 pH.7) and then resuspended in 0.5ml 1% glutaraldehyde (fixative). The cells were gently removed from the microtitre plate, placed in 0.5ml eppendorf tubes and left to fix for 1hr at RT. These represented the 4^0C samples. The remaining cells in the plate were incubated at 37^0C with 5% CO_2 for 5mins. Again the cells in one well per protein sample were washed, transferred and fixed as above (5min samples) then the remaining cells returned to the 37^0C incubator for a further 25mins. Samples were collected, washed and fixed for each protein sample after the 25mins at 37^0C, 1hr at 37^0C, 3hrs at 37^0C and overnight at 37^0C. The appearance of the cells was monitored at each stage. After fixation the cells were gently pelleted at 6,000 xg for 2mins then resuspended in 0.5ml PBS. The cells were stored in PBSA at 4^0C until the unicryl processing procedure (see section 5.3.8.3).

5.3.8.2 Treatment of rabbit ileal tissue with toxin A peptides

Toxin A, peptide A2 and MBP (negative control) were diluted to 72μgml^{-1} in amylose buffer (20mM Tris-HCl, 200mM NaCl, 1mM EDTA). Peptide H2 and the *E. coli* protein sample (negative control) were diluted to 25μgml^{-1} in PBS. Cholera toxin was diluted to 2μgml^{-1} and was included as an additional positive control. Five hundred μl aliquots of each sample were injected into different ileal loops and incubated for an 18hr period (see section 5.3.4). The rabbit was sacrificed by injecting a lethal dose of anaesthetic drug. The loops were removed and small samples were taken and fixed in 1% glutaraldehyde overnight at 4^{0}C. The tissue samples were removed from fixative, placed in 0.5ml PBS and stored at 4^{0}C until they were processed in unicryl resin [British Biocell International, Cardiff U.K.] (see section 5.3.8.3).

5.3.8.3 Unicryl processing schedule

The cell monolayers and tissue samples prepared in sections 5.3.8.1 and 5.3.8.2 were pelleted at 6,000 xg for 2mins. They were then dehydrated by incubating in 70% ethanol for 3 x 10mins, 90% ethanol for 3 x 10mins and 100% ethanol for 3 x 10mins with intermittent centrifugation at 6,000 xg for 2mins. The dehydrated cells were infiltrated with unicryl resin (British Biocell International, Cardiff U.K.): ethanol (1:2) for 30mins, unicryl resin: ethanol (2:1) for 30mins, 100% unicryl resin for 2 x 1hr and finally with 100% resin overnight. The cells were pelleted by centrifugation at 6,000 xg for 2mins to ensure the pellets were at the bottom of the eppendorf tube. The resin was then polymerised using longwave UV (360 nm) in a UV polymerisation chamber (Agar Scientific, U.K.) for 1-2 days at 4^{0}C. Ultra-thin sections (50-100 nm) were cut using a

Reichert OMU2 ultramicrotone. Sections were collected on Formvar coated nickel grids and stored at 40°C in grid boxes prior to the post-embed immunolabelling procedure (see section 5.3.8.4).

5.3.8.4 Post-embed immunolabelling procedure

The grids containing the sections were floated face down on 30µl droplets of PBSA/1% BSA (Bovine Serum Albumin; Sigma) for 10mins. The grids were then transferred without rinsing to 30µl droplets of the relevant primary antiserum (as described below) and incubated for 1-2 hrs. Sections containing toxin A, peptide H2 or the *E. coli* protein sample were incubated with 20µgml^{-1} of the Mab PCG-4. Sections containing peptide A2 or MBP were incubated with a 1:10 dilution of peptide A2 antiserum (raised 'in house', see section 4.3.1). Sections of cells that had not been treated with any protein sample were incubated with both of the mentioned primary antisera to determine the level of non-specific binding of the antibodies to the cells. Sections were transferred to 3 x 30µl droplets of PBSA for 5mins each and were then gently jet washed in PBSA. The sections were blotted dry and then quickly transferred to 20µl droplets of gold labelled secondary antibody for 1 hr. Sections treated with the PCG-4 Mab were incubated with a 1:20 dilution of 10 or 15nm gold labelled anti-mouse conjugate (British Biocell International). Sections treated with peptide A2 primary antiserum were incubated with a 1:20 dilution of a 10nm gold-labelled protein A conjugate (British Biocell International). As further negative controls, sections were included that had been treated with each protein sample but not with the any primary antisera. These sections were subsequently incubated with

the secondary gold-labelled antibodies to determine the levels of non-specific binding of the gold conjugates to the cells or protein samples. Sections were transferred to 3 x 30µl droplets of distilled water for 2mins to remove the unbound gold conjugate. The sections were blotted dry, post stained with aqueous 4% uranyl acetate and Reynolds lead citrate (Agar Scientific U.K.) then examined at 80 KV in a Transmission Electron Microscope (JEOL JEM 100C). Sections were compared to their respective negative controls and representative areas of each section were photographed.

5.4 RESULTS

5.4.1 H/A assay

The H/A of RRBCs is dependent on the recognition of the trisaccharide residue Galα1-3Galβ1-4GlucNac. Only holotoxin A (see figure 5.1 lane 10A) and the C-terminal repeat end peptide, H2 (see figure 5.1 lane 8), acted as cold haemagglutinins for rabbit erythrocytes. The H/A titre of peptide H2 was 1:64. The N-terminal toxin A peptides, the MBP and the *E. coli* negative control sample had no effect on the RRBCs (see figure 5.1 lanes 1-7 and 9).

The assay was repeated to compare the H/A titre of peptide H2 to holotoxin A. The holotoxin ($320\mu gml^{-1}$) had an H/A titre of 1:512, whilst that of fragment H2 ($16\mu gml^{-1}$) was 1:64. This represented effects induced by $312ngml^{-1}$ and $125ngml^{-1}$ for toxin A and peptide H2 respectively.

Further evidence that peptide H2 binds to RRBCs came from the internalisation study of peptide H2 into rabbit intestinal epithelial cells (see figure 5.2). At a magnification of x 39,000 the electron micrograph shows the high affinity binding of gold labelled peptide H2 to an isolated RRBC.

5.4.2 Bovine thyroglobulin binding

Again the binding of toxin A to bovine thyroglobulin is dependant on the recognition of the trisaccharide residue Galα1-3Galβ1-4GlucNac. Apart from toxin A, only peptide H2 (C-terminal repeat region of toxin A) bound significantly to bovine thyroglobulin. In the ELISA assay, the binding of peptide H2 to thyroglobulin showed an $O.D_{405}$ of 1.723 +/- 0.023 and was significant ($p<0.001$) (see figure 5.3) which was greater than that of toxin

A which gave an $O.D_{405}$ of 0.426 +/- 0.017 with a p value of <0.0001 (see figure 5.3). None of the other toxin A peptides or negative control samples bound significantly to bovine thyroglobulin (in each case p>0.2).

5.4.3 Cytotoxicity assay

None of the peptides covering the N-terminal region of toxin A (peptides A2-G) were cytotoxic to Vero cells. In each case the cell monolayer appeared unaffected (see figure 5.4 part A). Holotoxin A (positive control) on the other hand, caused a classic CPE (see figure 5.4 part B) with a titre of $1:4x10^3$. Peptide H2, representing the C-terminal repeat region of toxin A, had an effect on the morphology of the cells (see figure 5.4 part C) which had a titre of 1:40. These findings represent an ability to induce morphological changes in cells by exposure to $2ngml^{-1}$ of toxin A and $200ngml^{-1}$ of peptide H2. The effect of peptide H2 was different to the holotoxin. The *E. coli* protein sample (negative control for peptide H2) had no effect on the Vero cell monolayer.

5.4.4 Rabbit ileal loop assay

Following incubation of the rabbit ileal loops with the toxin A peptides, each loop was monitored for the presence of an enterotoxic effect in the form of fluid accumulation. The results are shown in table 5.1. None of the toxin A peptides (A2-H2) or the negative control samples induced a visible fluid secretion into the ileal loops. The fluid accumulations recorded for the cholera toxin and *C. difficile* toxin A positive controls however, were 2.25ml/cm and 2.29ml/cm respectively.

5.4.5 Neutralisation of the toxin A induced CPE on Vero cells and H/A of RRBCs by polyclonal toxin A peptide antisera

In the cytotoxicity assay, the holotoxin A incubated with pre-bleed rabbit sera caused a classic CPE with a titre of $1:1.6x10^3$. This represented an ability to induce morphological changes in Vero cells by exposure to $4ngml^{-1}$ of toxin A.

Pre-incubation of toxin A with the different antisera raised against the N-terminal peptides (A2-G) failed to neutralise the CPE with the cytotoxicity titre remaining at $1:1.6x10^3$ in each case. A 1:10 dilution of the antiserum raised against the C-terminal repeat end peptide (H2) was successful in neutralising the toxin A induced CPE from a titre of $1:1.6x10^3$ to $1:1.6x10^1$. This represented an ability to induce morphological changes in Vero cells by exposure to $406ngml^{-1}$ of treated toxin A. A 1:10 dilution of the monospecific toxin A antiserum, however, neutralised the toxin A induced CPE causing a reduction from a titre of $1: 1.6x10^3$ to 1:4. This titre represented an end point CPE caused by $1.625\mu gml^{-1}$ of treated toxin A (results not shown).

The toxin A ($65\ \mu gml^{-1}$) treated with neat pre-immune sera gave an H/A titre of 1:16, representing an end point toxin A concentration of $2.03\mu gml^{-1}$ (see Figure 5.5, lanes 2 and 4). The toxin A treated with undiluted polyclonal monospecific toxin A antiserum was only able to haemagglutinate RRBCs when applied at a neat concentration (final concentration of toxin A of $32.5\mu gml^{-1}$) to the wells of the microtitre plate (see Figure 5.5, lane 1). When toxin A was treated with the polyclonal peptide H2 antiserum, however, the H/A titre was reduced to 1:2 representing an effect induced by exposure to $16.25\mu gml^{-1}$ of toxin A (see Figure 5.5, lane 3).

5.4.6 Immunohistochemical detection of binding of peptide H2 to Vero cell receptors

The Vectastain *Elite* ABC Kit (Vector Laboratories) was used to detect binding of toxin A peptide H2 to Vero cell surface receptors (see section 5.3.7). A diagrammatic representation of the staining procedure is shown in figure 5.6. The cell nuclei were counterstained with haemotoxylin to enable easier visualisation of the cells under the microscope. The clearest images were achieved at a magnification of x 25. A brown precipitate was evident on all of the cell monolayers treated with toxin A peptide H2 suggesting that in all conditions the peptide was binding to cell surface receptors on the Vero cells. The binding was greater following incubation at 37^0C for 30mins (see figure 5.7 B) than at 4^0C for 20mins or 37^0C for 10 or 20mins. An identical pattern was observed for cells treated with holotoxin A (results not shown).

The lack of brown precipitate on all samples incubated with the *E. coli* negative control proteins (see figure 5.7 part A) suggested that the binding seen in figure 5.7 part B was due to peptide H2 and not to a contaminating *E. coli* protein or PCG-4 binding to cell components. These results suggest, as expected, that the C-terminal repeat region of toxin A is responsible for binding to cell surface receptors.

5.4.7 Transmission electron microscopy (TEM) studies

5.4.7.1 Internalisation of peptide H2 into cells

The method outlined in section 5.3.8 was used to study early binding and internalisation of peptide H2 into Vero cells. Lowicryl resin was used in these experiments, as opposed to Spurs resin, in an attempt to retain the antigenicity of peptide H2. One of the consequences of using this resin however is that the ultrastructural preservation of the cell monolayers is often poor.

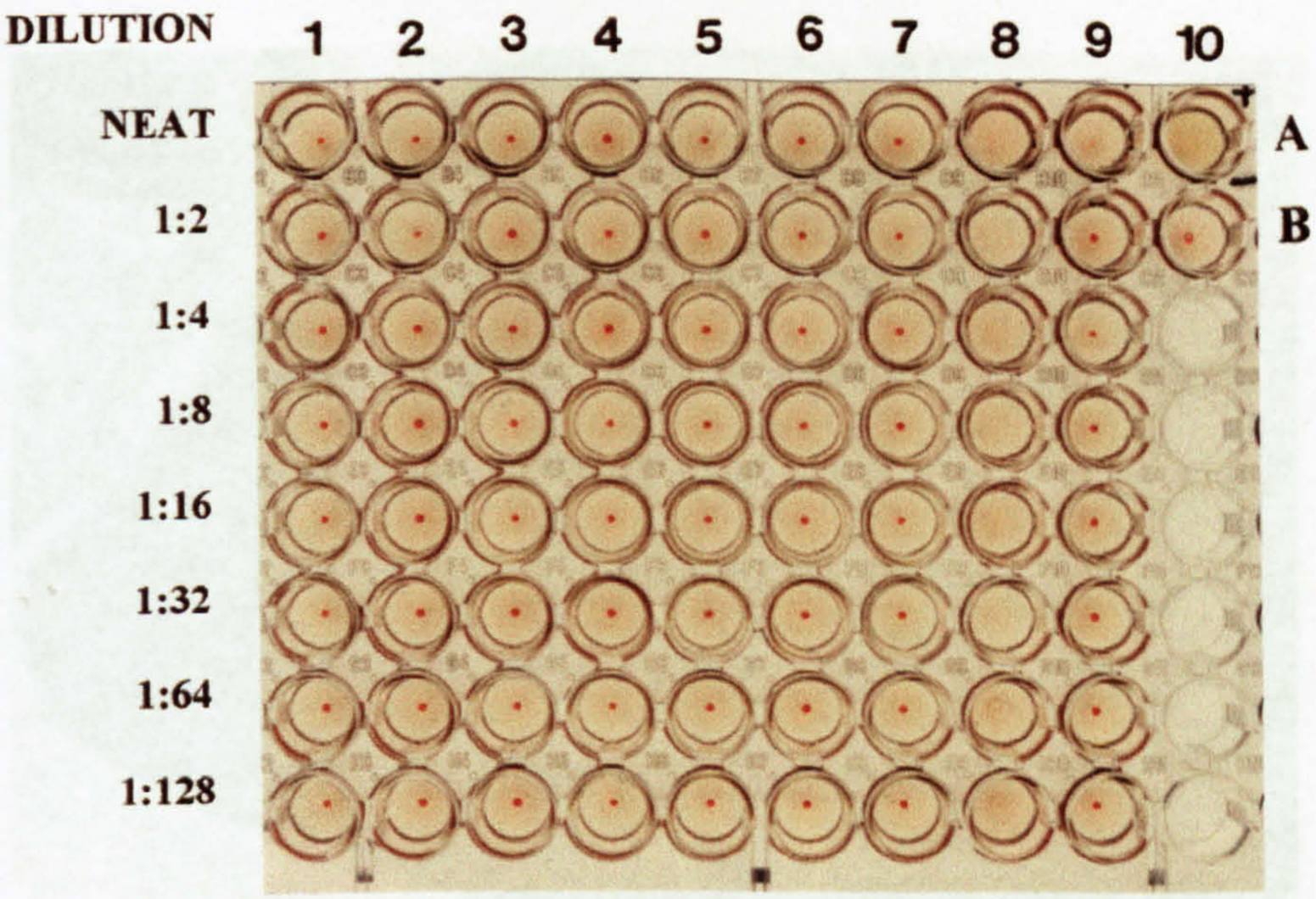
Approximately 30 x more gold particles were observed in the cell monolayers treated with peptide H2 than those treated with the *E. coli* negative control proteins (results not shown). The gold label was mainly concentrated on the cell membranes or just inside the cells and was at its greatest density in the monolayers incubated with peptide H2 for 1 hr at 37^{0}C. Unfortunately there was poor ultrastructural preservation of the tissue culture cells treated with peptide H2 and so with no time to repeat the experiment, the results were deemed inconclusive.

Peptide H2 was successfully detected however, in rabbit intestinal cells using the methods outlined in sections 5.3.8.2, 5.3.8.3 and 5.3.8.4. A large proportion of the H2 peptide was localised in membrane bound vesicles which were possibly late endosomes (see figure 5.8). The majority of the peptide was found in the late stages of internalisation (often close to the nucleus) because the rabbit ileal loop assay was incubated at 37^{0}C for 18 hrs.

There were approximately 20 x more gold labels associated with the sections treated with peptide H2 than with those treated with the *E. coli* negative control proteins. The number of gold particles associated with the negative control sections was negligible and the few particles present did not appear to be associated with membrane bound vesicles (see figure 5.9). These results suggest that the PCG-4 Mab and the gold labelled conjugate were binding specifically to peptide H2 and not non-specifically to the *E. coli* negative control proteins or the intestinal cell components. It would appear therefore, that peptide H2 can bind and be internalised into endosome-like compartments within target cells.

5.4.7.2 Internalisation of peptide A2 into cells

Attempts to localise peptide A2 in rabbit intestinal cells by TEM were inconclusive due to some non-specific binding of the polyclonal peptide A2 antiserum to the intestinal cells. The numbers of gold particles in the peptide A2 treated sections were similar to the numbers observed in the negative control sections (results not shown) suggesting that peptide A2 was not internalised efficiently into the intestinal cells.



*Neat concentration = 16μgml^{-1}

Figure 5.1

Haemagglutination of rabbit red blood cells by the cleaved toxin A peptides and their respective negative controls.

Lane 1, peptide A2; lane 2, peptide B; lane 3, peptide D; lane 4, peptide E; lane 5, peptide F; lane 6, peptide G; lane 7, MBP; lane 8, peptide H2; lane 9, *E. coli* negative control proteins; lane 10A, toxin A (positive control); lane 10B, PBSA.

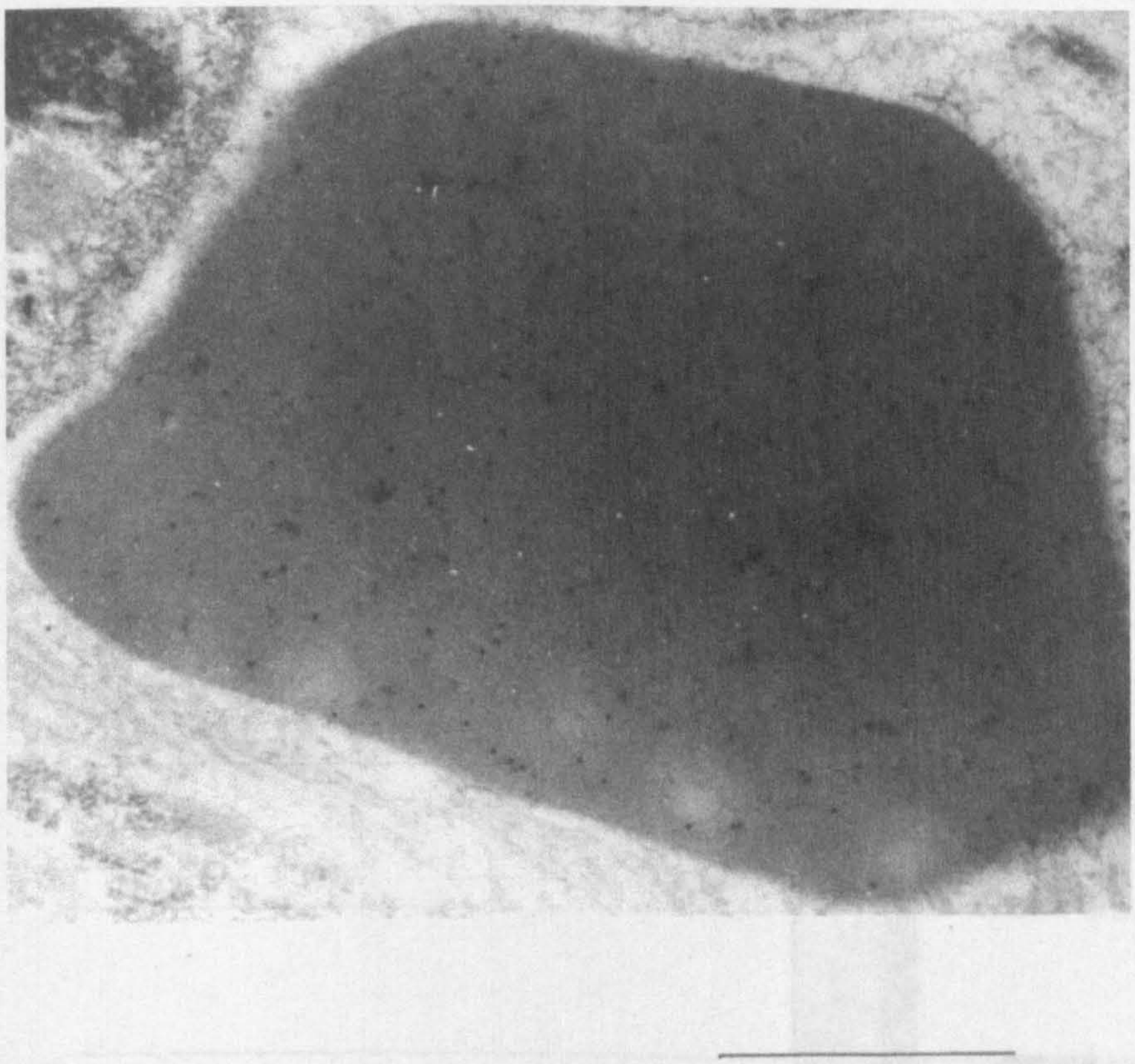


Figure 5.2

Transmission electron micrograph of a section of rabbit ileal loop tissue treated with toxin A peptide H2 showing high affinity binding of the peptide to a rabbit red blood cell (indicated by the gold particles). Taken at a magnification of x 39,000.

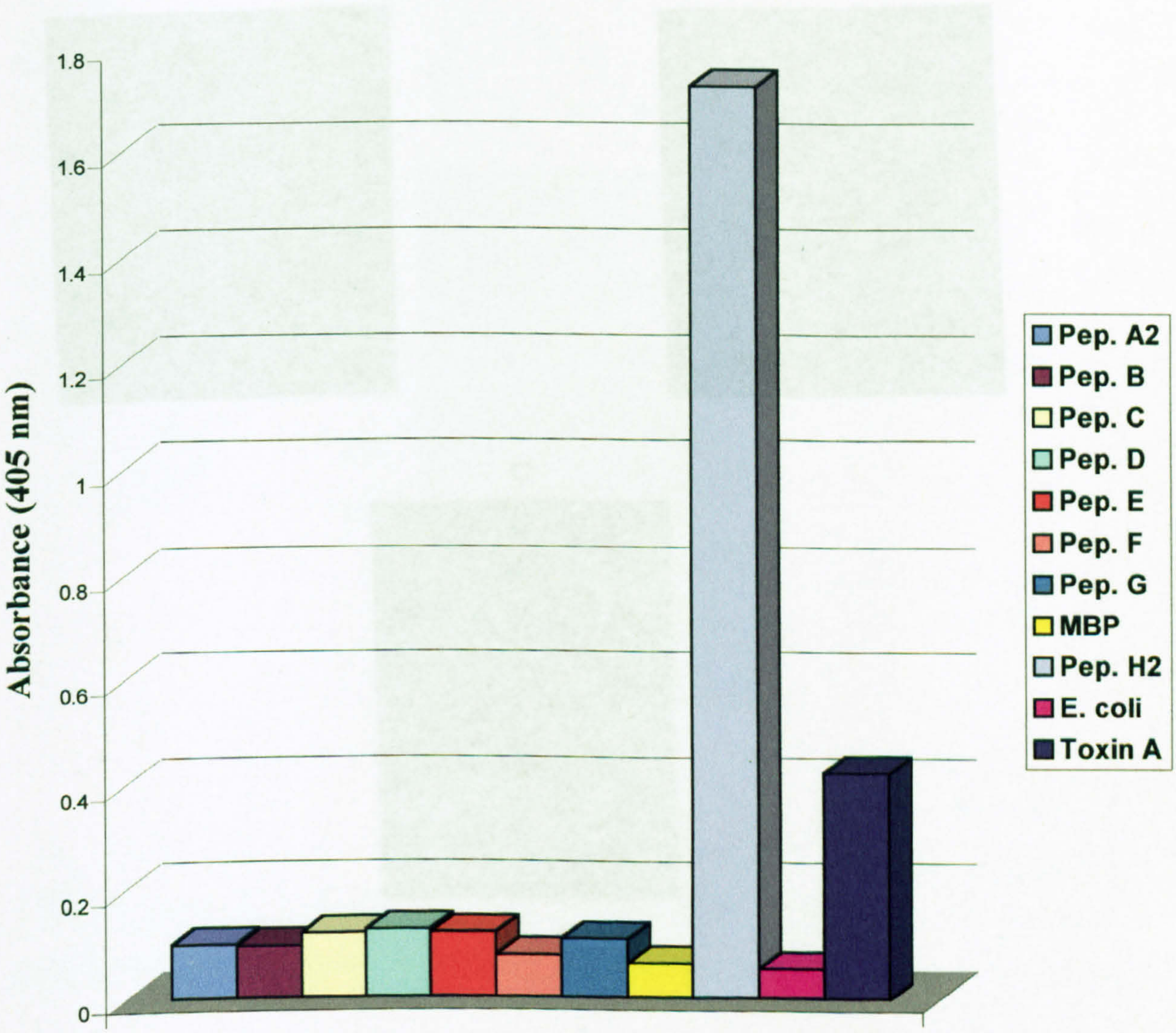


Figure 5.3
A graph to show binding of the toxin A peptides to bovine thyroglobulin in an ELISA assay. Binding was significant for holotoxin A and peptide H2 only.

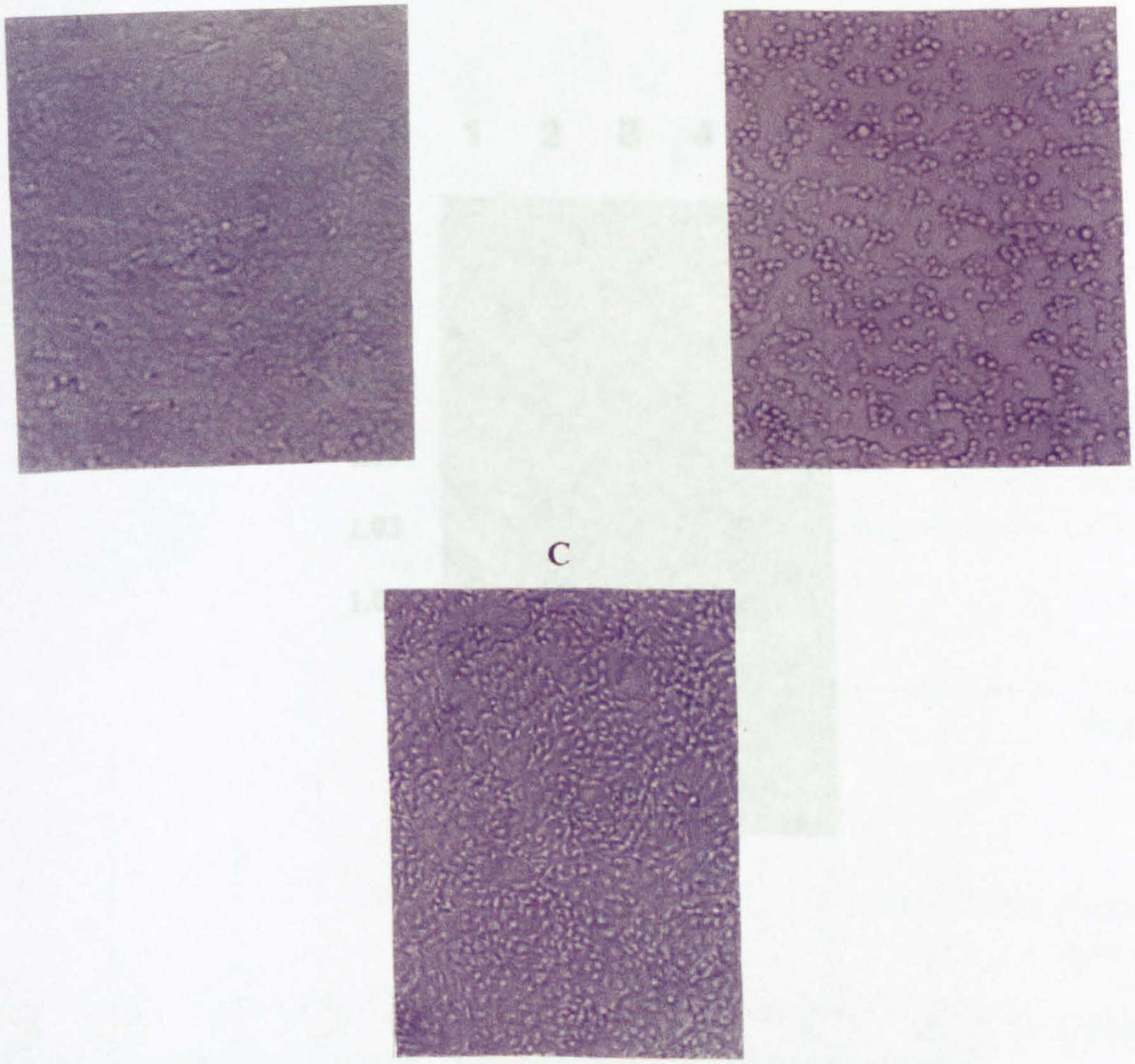


Figure 5.4

Effect of the toxin A peptides on a monolayer of vero cells after a 16 hr incubation at 37^0C with 5% CO_2. Images viewed under an inverted light microscope at a magnification of x 100. A, an unaffected monolayer; B, a toxin A treated monolayer; C, a monolayer treated with toxin A peptide H2.

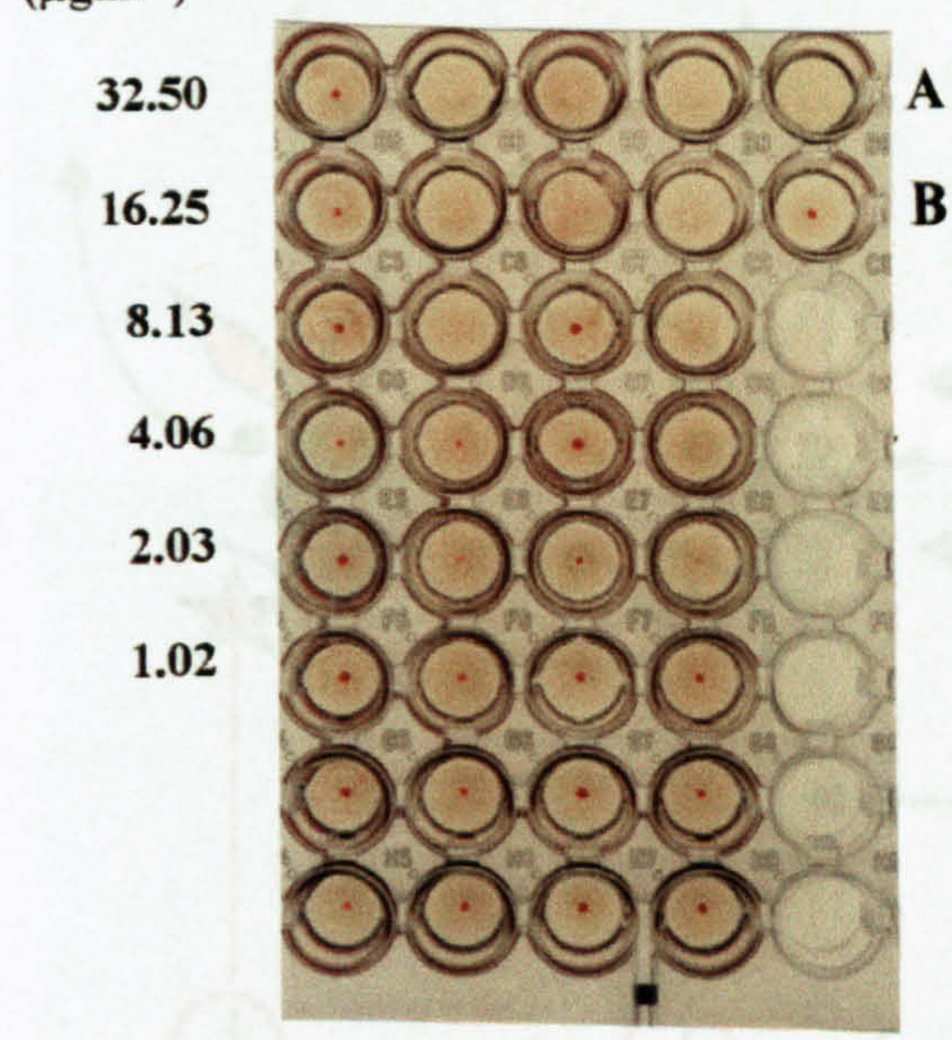


Figure 5.5

Neutralisation of the toxin A induced haemagglutination of rabbit red blood cells by pre-incubation of toxin A with polyclonal monospecific toxin A and peptide H2 antisera.

Lane 1, treated with toxin A antiserum; lane 2, treated with pre-immune serum; lane 3, treated with peptide H2 antiserum; lane 4, treated with pre-immune serum; lane 5A, toxin A (positive control); lane 5B, PBSA.

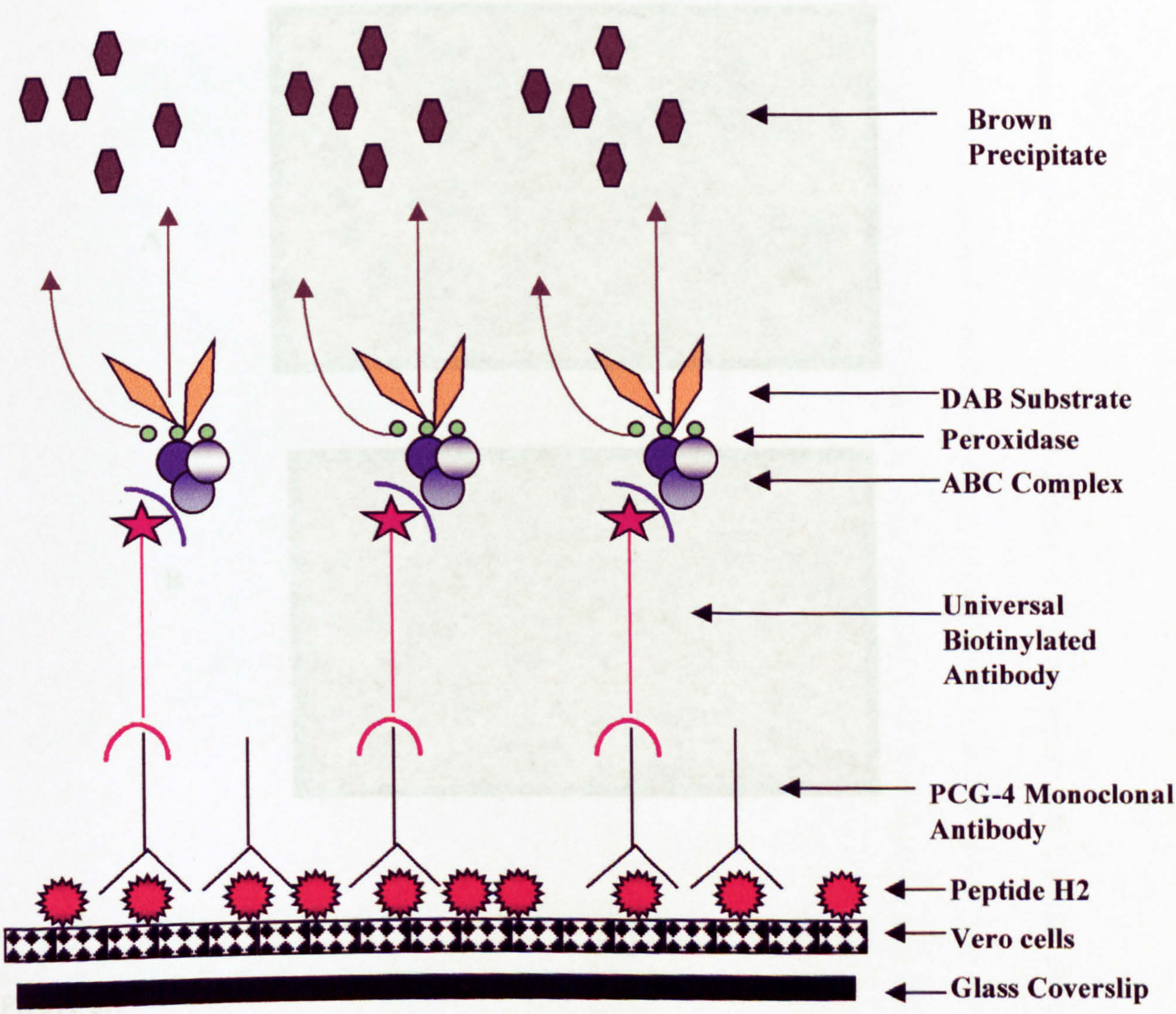
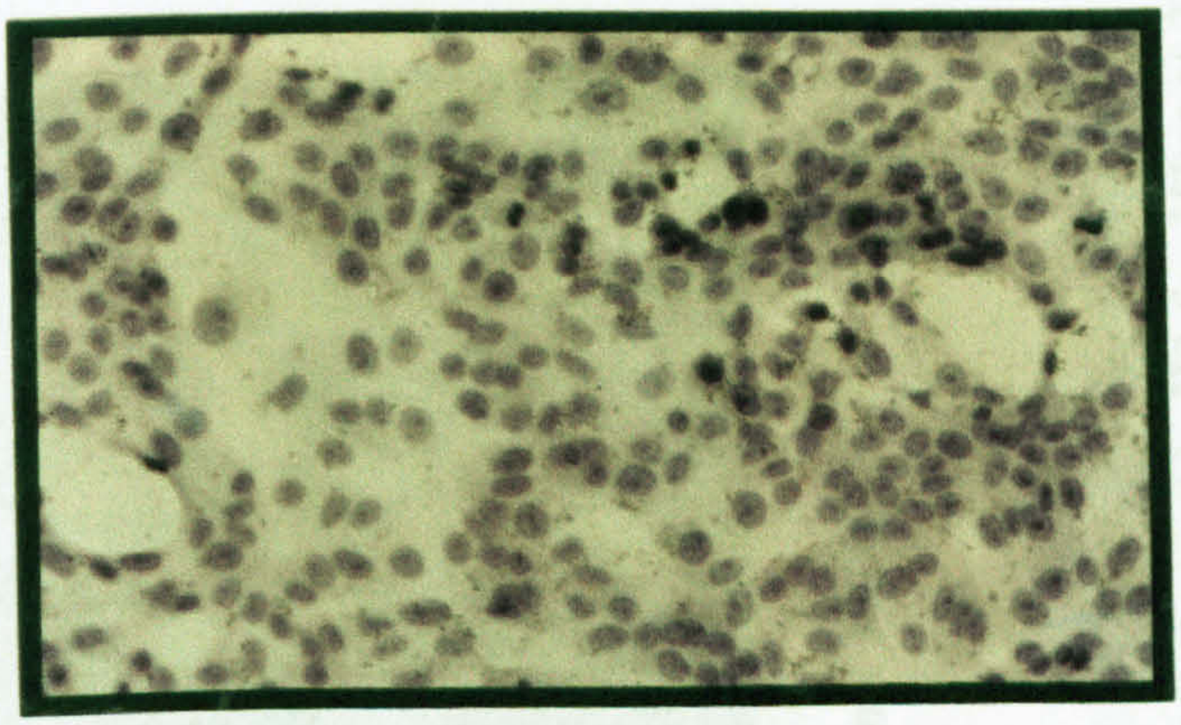


Figure 5.6

A diagrammatic representation of the Vectastain Universal Immunohistochemical staining procedure (Vector Laboratories) used for the detection of binding of peptide H2 to receptors on the surface of Vero cells. Binding of peptide H2 to Vero cells was detected primarily with the specific Mab PCG-4. Binding of the PCG-4 Mab to a biotinyated antibody followed by an avidin / biotin complex (ABC) resulted in the production of peroxidase. The DAB substrate then reacted with available peroxidase to form a brown precipitate which was visualised under a microscope.

A



B

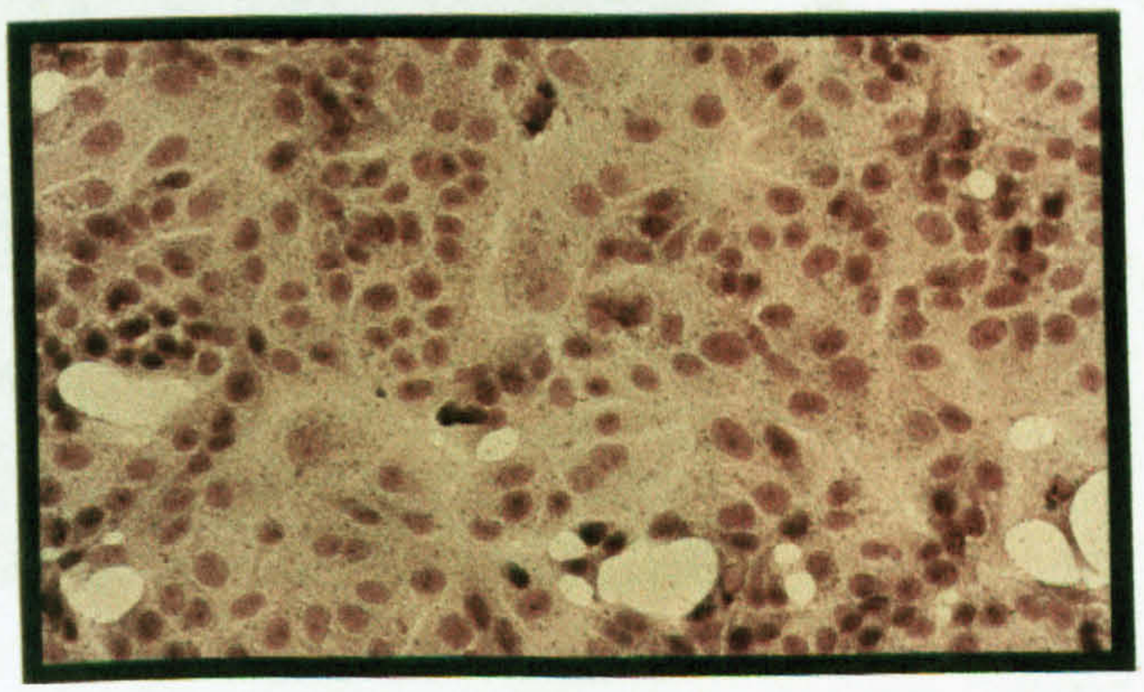
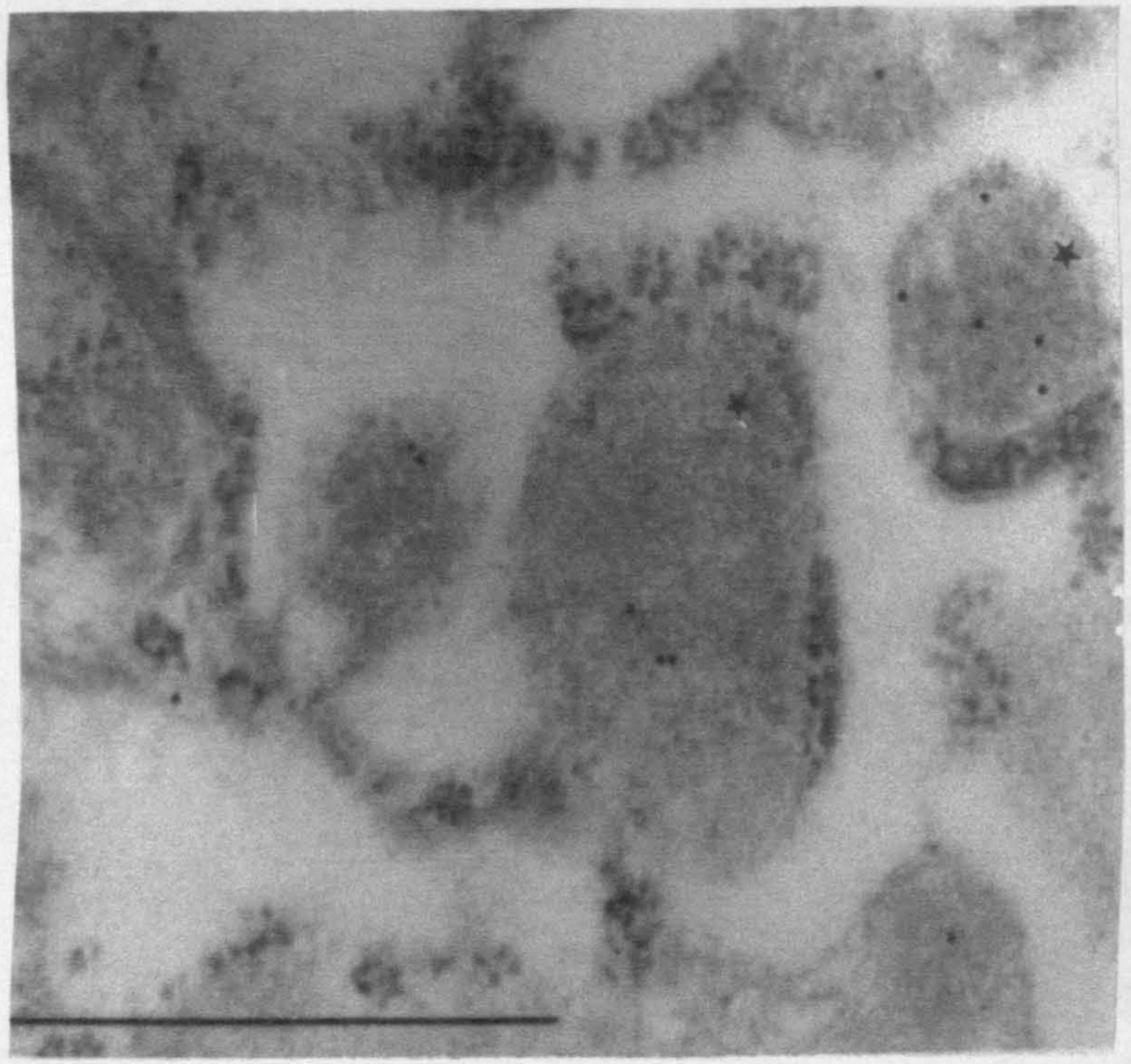


Figure 5.7

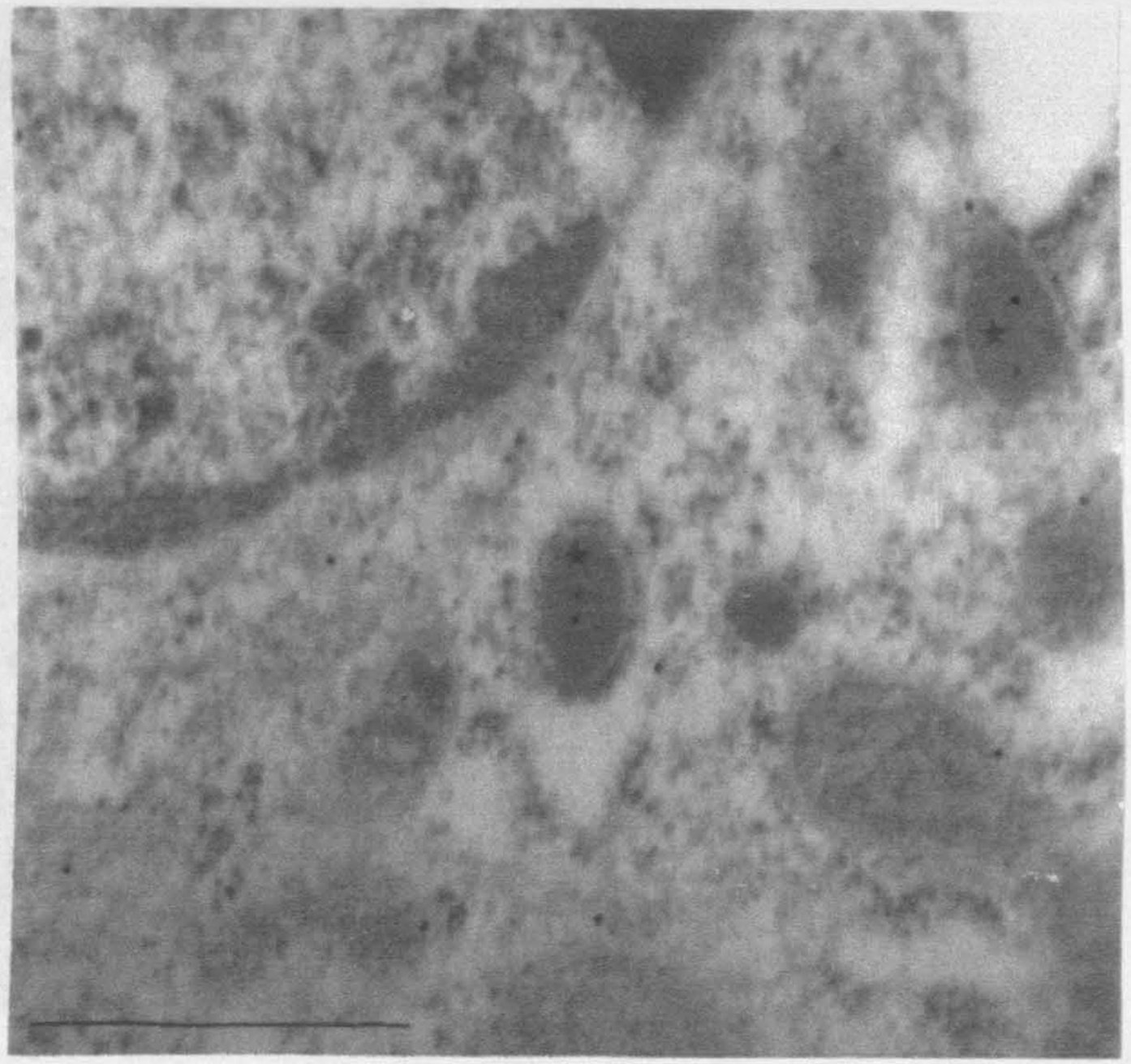
Detection of toxin A peptide H2 binding to cell surface receptors on Vero cells using immunohistochemistry. Each sample was treated with the MAb PCG-4 followed by the Vectastain ABC antibody system (Vector Laboratories). The cells were visualised under an orthoplan microscope (magnification x 25) and photographed with a Leitz-Wild MP551 camera.

A, Vero cells incubated with the *E. coli* negative control protein sample for 30 mins at 37^0C; B, Vero cells incubated with the toxin A peptide H2 protein preparation for 30 mins at 37^0C. The brown precipitate in B indicates binding of peptide H2 to Vero cells.



1μm

Magnification x 57,000

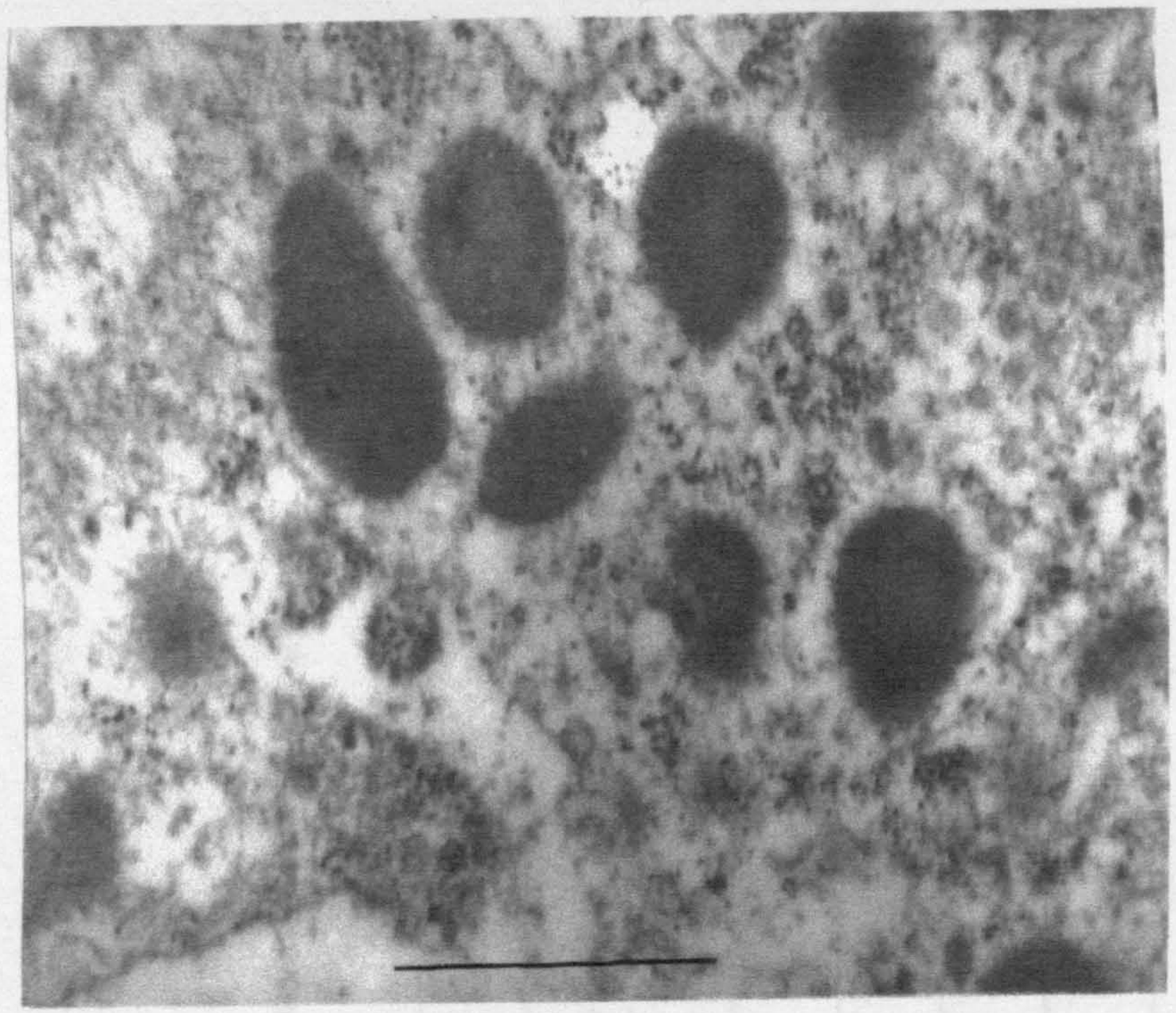


1μm

Magnification x 30,000

Figure 5.8

Transmission electron micrographs of sections of rabbit ileal loop tissue that had been incubated with toxin A peptide H2 for 18hrs at 37^0C. Peptide H2 was located using the monoclonal antibody PCG-4 followed by a gold labelled (15nm) anti-mouse antibody using a post-embed immunolabelling procedure. The peptide was found in membrane bound 'endosome-like' compartments (*) in the late stages of internalisation.



1um

Figure 5.9

Transmission electron micrographs of a section of rabbit ileal loop tissue treated with the *E. coli* negative control protein sample for 18hrs at 37^0C. The section was screened with the monoclonal antibody PCG-4 followed by a gold-labelled (15nm) anti-mouse antibody using a post-embed immunolabelling procedure. Magnification x 30,000.

SAMPLE INJECTED INTO LOOP	FLUID ACCUMULATION Vol. Fluid / Length of Loop (ml / cm)
PEPTIDE A2	NFA
PEPTIDE B	NFA
PEPTIDE C	NFA
PEPTIDE D	NFA
PEPTIDE E	NFA
PEPTIDE G	NFA
MBP (-ve cnt.)	NFA
PEPTIDE H2	NFA
E. coli (-ve cnt)	NFA
C. difficile Toxin A	18 ml / 8 cm = 2.25
CHOLERA TOXIN	16 ml / 7 cm = 2.29
PBSA	NFA

NFA – no fluid accumulation

Table 5.1

Table showing the fluid accumulation observed after each toxin A peptide was injected into a ligated rabbit ileal loop. Results are recorded as a ratio of the volume of fluid recovered from the loop divided by the length of the loop after an 18hr incubation period.

5.5 DISCUSSION

All the activities that were dependent on carbohydrate binding i.e. H/A and binding to thyroglobulin were restricted solely to peptide H2. The N-terminal peptides lacked these biological activities. These results were expected as the carboxy-terminal region of toxin A, composed of domains of oligopeptides, has been shown to be the receptor binding region (Price *et al* 1987, Wren *et al* 1991) and to specifically bind to certain tri-saccharides (Krivan *et al* 1986, Tucker and Wilkins 1991) including Galα1-3Galβ1-4NacGluc.

It was interesting to note that 2.5 times more holotoxin A than peptide H2 was required to haemagglutinate rabbit erythrocytes. This can be explained by the fact that the C-terminal repeat end of toxin A was responsible for this function and this region is approximately one third the size of the holotoxin (2.6 Kb as oppose to 8.1 Kb).

Purification of peptide H2 from inclusion bodies involved denaturing the peptide with guanidine hydrochloride followed by renaturation by gradual dialysis. A complication of this process is that some peptides will not refold properly during dialysis. It was reassuring to observe therefore, that peptide H2 was able to bind to these carbohydrates suggesting that it had retained biological activity.

None of the toxin A peptides appeared to induce a classical CPE on Vero cells or an enterotoxic effect in the rabbit ileal loops. It is possible that the peptide(s) containing the relevant active sites were not biologically active. Another, more likely explanation, is that more than one region of the toxin A molecule is responsible for the full CPE and that these regions are components of different peptides. Perhaps the N-terminal peptides, for example, could not be internalised into the Vero cells without the repeat end-binding domain.

It was interesting to observe that peptide H2 (repeat end of toxin A) caused a physical and reproducible change to Vero cells in tissue culture. The classical confluent mosaicism of the normal cell sheet was lost and the cells became 'stringy' in appearance. Peptide H2 was one hundred times less potent than toxin A at inducing morphological changes to Vero cells. This effect was dissimilar to the classical cell rounding CPE seen with toxin A and was not due to the *E. coli* negative control proteins. These results contradict the findings of Price *et al*, 1987 who concluded that a recombinant repeat end peptide had no morphological effect on CHO cells in a cytotoxicity assay.

The results obtained in this thesis do suggest that peptide H2 can have a direct effect on cells. It is possible that this binding peptide was simply clumping the cells giving them a 'stringy' appearance. Alternatively a number of experiments on cells *in vitro*, have yielded results indicating that binding of toxin A to a cell surface receptor activates intracellular transmembrane signalling pathways which may exert cellular effects independent of the catalytic action of the toxin (Pothoulakis *et al* 1988, Castagliuolo *et al* 1994). Pothoulakis *et al,* 1988 provided data suggesting that the binding of toxin A to a granulocyte membrane receptor stimulated an increase in intracellular calcium, which elicited a chemotactic response by granulocytes to toxin A. A later study used an anti-galactose antibody that binds the trisaccharide Galα1-3Galβ1-4GlcNac to mimic the effects of toxin A in rat colon. This work concluded that binding of toxin A to receptors bearing this tri-saccharide caused a series of events which resulted in fluid secretion, increased permeability and mast cell protease II release in rat colon (Pothoulakis *et al* 1996a).

If binding alone of toxin A to an intestinal cell receptor is sufficient to stimulate fluid secretion, we would have expected to observe a fluid response in the ileal loop injected

with peptide H2. The different amounts of peptide H2 and anti-galactose antibody used in these two different experiments however, could explain this discrepancy. In the rat colon experiment a 5cm loop was injected with 250μg of the anti-galactose antibody (Pothoulakis *et al* 1996a). In our experiment however, a 5cm rabbit ileal loop was injected with only 12.5μg of peptide H2 due to the poor concentration of peptide recovered by the purification procedure. It is reasonable to assume, therefore, that more peptide H2 may have resulted in fluid secretion into the ileal loop.

The demonstration that binding alone induced morphological changes in cells may partly explain why toxin A has different biological activities to toxin B although they share the same mechanism of action for induction of cytopathic effects. This observation causes concern when considering the possible use of the repeat region of toxin A as a vehicle for the delivery of oral vaccines against *C. difficile* disease.

Although other workers have demonstrated that the binding of holotoxin A to cell surface receptors activates intracellular transmembrane signalling pathways (Pothoulakis *et al* 1988, Pothoulakis *et al* 1996a) no work has been done focusing purely on binding of the C-terminal repeat region of the toxin to cells. The results in this section indicate that peptide H2 (corresponding to the repeat region of toxin A) can bind to Vero cells independently of the N-terminal portion of the holotoxin. It was interesting to observe that in the immunohistochemistry experiments the binding was greater at 37^0C than at 4^0C. These observations contradict the theory of others that have observed that holotoxin A binds to certain trisaccharides more efficiently at 4^0C than 37^0C (Krivan *et al* 1986, Tucker *et al*1991, Cooke *et al* 1998). In our experiments however, the peptide was only incubated with the cells for 20mins at 4^0C and so it is possible that the incubation time was not long enough for efficient binding.

It was unfortunate that the TEM studies on tissue culture cells for the detection of early binding and internalisation of peptide H2 were inconclusive. A high density of gold labelling was observed in the cells treated with peptide H2 but it was difficult to pinpoint label within coated pits due to poor ultrastructural preservation of the sections. The TEM studies performed on rabbit ileal tissue treated with peptide H2, however, did conclusively show that peptide H2 not only bound to cells but was also internalised into membrane bound vesicles. Some of the peptide was observed outside of vesicles, which was probably due to the way the tissue had been sectioned rather than due to the peptide escaping from endosomes. Other workers have suggested that a hydrophobic domain (amino acids 1,050-1,100), not present in peptide H2, is necessary for efficient escape of the holotoxin from endosomes (Dove *et al* 1990, von Eichel-Streiber *et al* 1995).

The low density of labelling in these peptide H2 treated sections was probably due to the small amount of peptide that was applied to the ileal loop. Only 12.5μg of peptide H2 was injected into a 5cm rabbit ileal loop, which has a vast surface area for binding and internalisation of proteins. Bearing this in mind, we were unlikely to find an area on a section showing more than 2 or 3 peptide H2 containing membrane bound vesicles. It was also possible that after incubation for 18hrs in the rabbit, a large proportion of the peptide had been degraded or had been destroyed by the immune system. Other workers that have studied internalisation of toxin A into cells by TEM have also shown a poor density of labelling, some micrographs showing only one or two gold labelled toxin molecules per endosome (Kushnaryov *et al* 1989, Sturgeon 1994, von Eichel-Streiber *et al* 1996). Although the density of labelling in the peptide H2 treated sections was low in our experiments, it was approximately 20 x greater than in the corresponding negative controls. Also in the peptide H2 treated sections, the gold label was often associated with

membrane bound vesicles suggesting uptake by a receptor mediated process, however the few gold labels found in the negative controls were not associated with vesicles suggesting non-specific binding. As a post-embed immunolabelling procedure was employed for the detection of peptide H2 there was no possibility that the gold labelled antibody seen in endosomes was taken up non-specifically by the cells. The tissue was incubated with peptide H2, fixed, sectioned and then probed with the PCG-4 and gold-labelled antibodies.

These results, taken together, suggest that the C-terminal repeat region of toxin A is independently responsible for binding and internalisation of the holotoxin into membrane bound vesicles, and also that the N-terminal region of the molecule is not necessary for these processes. This adds support to the possibility that binding alone of the holotoxin to cell surface receptors, may activate intracellular transmembrane signalling pathways which result in a change in the morphology of the target cells.

The N-terminal peptide A2, which is believed to contain the catalytic domain of the toxin (see section 6), was not internalised by the rabbit intestinal cells probably due to the fact that it was unable to associate with the cells without the receptor binding domain of the toxin molecule.

The polyclonal monospecific antisera raised against the N-terminal toxin A peptides (A2-G) failed to neutralise the holotoxin A induced CPE on Vero cells. It is possible that the catalytic domain lies inside the holotoxin A molecule and is only exposed once the toxin is in the cell cytoplasm. It is likely therefore, that microinjection of the relevant antiserum into the cell prior to internalisation of the toxin could inhibit the CPE. Polyclonal antiserum raised against peptide H2 did neutralise the toxin A induced H/A and CPE. These results compare to the data published by Wren *et al* 1991 who also found that the

antiserum raised against a small 10 amino acid repeating sequence of toxin A neutralised both H/A and the CPE. In these cases, it was assumed that the antiserum was recognising the C-terminal repeat region of the holotoxin and blocking binding of the molecule to its receptor on the erythrocyte and Vero cell.

It would appear that peptide H2 is both antigenic and immunogenic and as such, vaccination with this peptide should have a protective effect against *C. difficile* infection in an animal model. These conclusions are supported by the findings of Lyerly *et al* 1990, who found that vaccination of hamsters with a recombinant C-terminal repeat end peptide (comprising 33 of the 38 repeating units seen in holotoxin A) partially protected them against *C. difficile* disease. More recently it has also been shown that mice immunised with the C-terminal repeat region of toxin A were protected against a three-fold lethal dose of toxin A (Sauerborn *et al*, 1997).

The Mab PCG-4 has been shown to bind to the repeat region of toxin A between amino acids 2,097 through 2,141 and 2,355 through 2,398 (Frey *et al*, 1992) and blocks toxin A induced enterotoxicity but not cytotoxicity (Lyerly *et al*, 1986b). These results, together with the fact that polyclonal monospecific anti-peptide H2 neutralises cytotoxicity, suggest that different domains of the C-terminal repeat region of toxin A are responsible for binding to different cell type receptors.

SECTION – 6

BIOCHEMICAL PROPERTIES OF HOLOTOXIN A AND THE RECOMBINANT TOXIN A PEPTIDES

SECTION – 6

Biochemical Properties of Holotoxin A and the Recombinant Toxin A Peptides

6.4.2.4 Induction of expression of fragment a' from the pMal-c2 vector

6.4.2.5 Purification and cleavage of the peptide A' fusion product

6.4.3 *In vitro* glucosylation of recombinant Rho A by peptides A2 and A'

6.4.4 Binding of toxin A peptides to ATP-agarose

6.5 Discussion

Section-6

Biochemical Properties of Holotoxin A and the Recombinant Toxin A Peptides

6.1 SUMMARY

Toxin A peptides A2 (aa 1-536), B (aa 542-859), C (aa 114-859), D (aa 869-1330), E (aa 542-1161), G (aa 869-1830), H2 (aa 1832- 2683) and A' (aa 1-205) were all assayed for the ability to glucosylate recombinant RhoA (rRhoA) *in vitro*. Peptide A2 was the only peptide that possessed enzyme activity, this being approximately 45% of that observed with the holotoxin. The results obtained for peptides A', A2 and C indicate that the first 536 amino acids contain the catalytic domain for this activity, that more than the first 205aa alone are required for a peptide to be active, and that the first 113aa are essential either structurally or functionally for expression of enzymatic activity.

Only toxin A peptides B (aa 542-859) and E (aa 542-1161) were retained on ATP-agarose columns whereas peptides A2, G and H2 (covering the remainder of the molecule) were not. This indicates that the ATP binding site resides between amino acids 542-859 in the toxin A molecule.

Interestingly, the peptides that bind ATP (B and E) do not glucosylate rRhoA and the peptide that glucosylates rRhoA (A2) lacks the nucleotide-binding site. There was no evidence from these studies to suggest that a proven nucleotide-binding site was required for *in vitro* glucosylation of rRhoA.

6.2 Introduction

The cytotoxic effects of toxin A and B are characterised by the rounding up of cells which is caused by disruption of the actin cytoskeleton. Recently it has been demonstrated that both toxins A and B are enzymes that glucosylate low molecular weight GTP-binding proteins of the Rho family. Using UDP-glucose as a co-substrate, the toxins glucosylate Rho, Rac and Cdc42 thus inhibiting the biological activities of these GTPases (Just *et al*, 1994, 1995b,c). In addition, toxin A glucosylates Rap proteins (Chaves-Olarte *et al*, 1997). Rho proteins are regulators of the actin cytoskeleton, therefore glucosylation leads to destruction of the cytoskeleton and the morphological changes observed in toxin-treated cells.

Both toxins A (Lobban and Borriello, 1992) and B (Florin and Thelestam, 1984) have also been shown to bind ATP *in vitro*, and a putative nucleotide binding site has been located within the N-terminal third of toxin B based on sequence homology with an ATP binding motif (Barroso *et al*, 1994). The function of these ATP binding sites is at present unknown.

The aim of this section was to determine the regions of the toxin A molecule responsible for glucosylation of RhoA and ATP binding. The results should give some indication as to whether these two functions are connected or unrelated.

6.3 MATERIALS AND METHODS

6.3.1 Preparation of recombinant Rho A

The rRho A was provided by Dr. April Roberts and was purified at CAMR, Porton Down, Salisbury.

The *E. coli* JM101 strain containing the GST-Rho A coding sequence in the expression vector pGEX-2T was provided by Prof. Alan Hall, University College, London. Details of how the clone was produced are described by Self and Hall (1995). The culture was grown in LB broth containing 50μgml^{-1} ampicillin (2 x 1.0l) to an OD_{600} of 0.5. Expression of recombinant GST-Rho A was induced by the addition of IPTG to the final concentration of 1mM. Following induction, the culture was grown to stationary phase (A_{600} ~ 1.0), and the cells were recovered by centrifugation (5,000 x g, 10min, 4^{0}C). Following storage at -70^{0}C overnight, the cells were lysed and the GST-Rho A bound to glutathione-Sepharose (Pharmacia). Recombinant Rho A was released from the glutathione-Sepharose by treatment with thrombin (5units/1.0l culture) as described by Self and Hall (1995). Precipitation with benzamidine-agarose beads (Sigma; 10μl/1.0l) released the Rho A from the thrombin. The purified Rho A was dialysed into 50mM triethanolamine pH 7.5, 150mM KCl, 3mM $MgCl_2$ at 4^{0}C for 5hr and then stored at -70^{0}C. The protein concentration of Rho A was estimated by visually comparing the coomassie stained band intensity of the Rho A to that of a set of albumin standards on a 4-20% SDS-PAGE gel. An A_{280} value of 1.0 for Rho A was estimated to correspond to a protein concentration of 1$mgml^{-1}$.

6.3.2 *In-Vitro* glucosylation of rRho A.

This method was adapted from the glucosylation reaction described by Just *et al*, (1995a). Glucosylation reactions were carried out by myself at C.A.M.R, Porton Down, Salisbury in collaboration with Dr. April Roberts.

Briefly 33nM final concentration of toxin A and each of the toxin A peptides were assayed for glucosyltransferase activity in a 50µl reaction volume containing 50mM triethanolamine pH 7.5, 150mM KCl, 0.3mM GDP, 3mM $MgCl_2$, 10µM $ZnCl_2$, 7µM UDP- [^{14}C] glucose (Amersham; 100nCi) and 11µM (250µgml^{-1}) rRho A (see section 6.3.1).

Prior to addition to the reaction mix, the toxin A peptides were cleaved from MBP by incubation with Factor Xa as already described (see section 2.8.3). The protein concentrations of the toxin A peptides were determined using the Coomassie Plus Protein Assay Reagant Kit (Pierce) and 8% SDS-PAGE analysis (see sections 2.9.1 and 2.9.2).

The toxin A peptides were incorporated into the assay at a final concentration of 33nM based on the apparent protein concentration of the cleaved toxin A peptide without MBP. Controls were included to demonstrate that MBP and *E. coli* derived proteins could not glucosylate rRho A. Further controls were included to prove that when toxin A, toxin A peptides or Rho A were absent from the reaction mix, no glucosylation was observed.

Samples were set up in quadruplet and then incubated at 37^{0}C for 1hr. Protein samples were precipitated by incubating on ice for 3min in the presence of 0.45ml BSA (1mgml^{-1}) and 0.5ml 24% trichloroacetic acid (TCA aq; 12% final concentration). Precipitates were collected on Durapore membranes (Millipore, 25mm diameter, 0.2µm) using a vacuum filtration unit (Millipore 1225 Sampling Manifold). The filters were washed twice with 5ml 12% TCA, placed in 5ml scintillation fluid (Sigma) and soaked for 2.5hr at room

temperature. The vials were transferred to a Wallac 1410 Scintillation Counter for the detection of the Rho A-^{14}C-glucose product.

P-values were determined for each test protein by comparing the quadruplet mean cpm of the sample to the highest quadruplet mean cpm of an appropriate negative control in a two-tailed T-test. Based on the percentage of Rho A that was glucosylated, counts per minute were converted to pico moles of product (Rho A - glucose).

6.3.3 Cloning, expression and purification of the first 629bp of the toxin A gene.

6.3.3.1 PCR amplification of the first 629bp of the toxin A gene (a').

Using the method outlined in section 2.3 primers 3 and 13 (5' AGATACTAGTCGACACTATATAATA 3') (see appendix 2) were used to amplify a 629bp DNA fragment of the toxin A gene (bps 1-629; see appendix 1) from *C. difficile* strain VPI 10463 chromosomal DNA (see section 2.2.1). The thermocycling conditions were denaturation for 1 min at 97^{0}C, annealing for 1 min at 50^{0}C and extension for 1 min at 72^{0}C. The PCR product was analysed by 1.2 % agarose gel electrophoresis (see section 2.2.3).

6.3.3.2 Cloning of the 629bp PCR product (a') into the pCRII vector.

The 629bp PCR product amplified in section 6.3.3.1 was purified from an agarose gel as outlined in section 2.2.4. The purified PCR product was ligated to the pCRII T-tailing vector (see section 2.4.1) and then transformed into InvαF' competent cells (see section 2.4.2). Plasmid DNA was isolated (see section 2.2.2.1) from selected white colonies and was digested with *Bam*HI and *Sal*I (see section 2.2.6). The DNA profiles were analysed by 1% agarose gel electrophoresis (see section 2.2.3) in order to select a clone with a

629bp toxin A insert. A positive culture was mixed 1:1 with 40% glycerol and stored at –70^{0}C.

6.3.3.3 Sub-cloning of fragment a' into the pMal-c2 expression vector.

The recombinant pCRII plasmid isolated in section 6.3.3.2 and the pMal-c2 vector (New England Biolabs) were digested with *Bam*HI and *Sal*I (see section 2.2.6). Both digests were run on a 0.8% agarose gel (see section 2.2.3) and the 6.7Kb pMal-c2 and 629bp a' fragments were cut from the gel and purified (see section 2.2.4). The purified products were analysed by agarose gel electrophoresis to determine the concentration of DNA present (see section 2.2.3). The purified *Bam*HI, *Sal*I digested a' fragment was ligated to the digested pMal-c2 vector (see section 2.2.7) and the ligation was transformed into JM109 competent cells (see section 2.2.8). The transformation reaction was plated out onto LB agar plates containing 100μgml^{-1} of ampicillin (LB amp plate) and incubated overnight at 37^{0}C. Colonies were selected and sub-cultured onto a master LB amp plate and onto a LB amp plate containing 80μgml^{-1} of X-gal and 0.1mM IPTG. Again the plates were incubated overnight at 37^{0}C. Plasmid DNA was isolated from white colonies (see section 2.2.2.1), digested with *Bam*HI and *Sal*I (see section 2.2.6) and analysed on a 0.8% agarose gel (see section 2.2.3). A clone containing the 629bp a' DNA insert was incubated overnight at 37^{0}C, mixed 1:1 with 40% sterile glycerol and stored at –70^{0}C.

6.3.3.4 Induction of expression from the recombinant pMal-c2 vector.

Expression of the MBP fusion product was induced from the pMal-c2 vector containing the a' DNA insert as outlined in section 2.8.1. The presence of the toxin A peptide (A') coded for by the a' DNA fragment was determined by SDS-PAGE (see section 2.7.1) and

probing a western blot (see section 2.7.3) with a 1:10,000 dilution of anti-MBP serum (New England Biolabs). The size of the A' fusion peptide was compared to the size of the major truncated products (approx. 65 kDa) produced when toxin A peptide A2 (aa's 1-536) was expressed in *E. coli*.

6.3.3.5 Purification and cleavage of the peptide A' fusion product.

The toxin A peptide A' MBP fusion product was purified by amylose affinity chromatography as outlined in section 2.8.2. The protein in each fraction was analysed by SDS-PAGE (see section 2.7.1) and the size compared to that of the major truncated products (approx. 65 kDa) produced from the expression of peptide A2. The concentration of the fusion protein was determined as outlined in section 2.9.1. The MBP was cleaved from peptide A' by incubation with factor Xa as outlined in section 2.8.3.

6.3.4 *In vitro* glucosylation of rRho A by peptides A2 and A'.

The *in vitro* glucosylation assay outlined in section 6.3.2 was used to compare the glucosyltransferase activity of peptide A2 to any possible activity displayed by the shorter peptide A' (see section 6.3.3.5). Expression of peptide A2 in *E. coli* also results in the production of a series of truncated products the most predominant of which was approximately 65 kDa which corresponds in size and constitution to the same region of the toxin A molecule as peptide A' (see section 6.4.2.4).

Peptides A2 and A' were cleaved with factor Xa prior to addition into the glucosylation reaction (see section 2.8.3). The peptides were included in the assay at a final concentration of 33nM based on the apparent protein concentration of the full-length peptides only (i.e. truncated species and MBP not included in the concentration

estimations). As a direct comparison, an additional glucosylation assay was undertaken in which peptide A2 was incorporated at a final concentration of 33nM based on the apparent protein concentration of the peptide without MBP (i.e. truncated species included in concentration estimation).

Toxin A (33nM) and MBP (33nM) were included in the assay as positive and negative controls respectively. Other negative controls were identical to those outlined in section 6.3.2. BSA was also included in one assay in place of rRhoA to ensure that holotoxin A specifically glucosylated RhoA and not any protein. Again P-values were determined for each test protein by comparing the quadruplet mean cpm of the sample to the highest quadruplet mean cpm of an appropriate negative control in a two-tailed T-test. Based on the percentage of Rho A that had been glucosylated, counts per minute were converted to pico moles of product (Rho A - glucose).

6.3.5 Binding of toxin A peptides to ATP – agarose.

This method was adapted from that of Lobban *et al*, 1992 and Florin *et al*, 1984.

Toxin A, toxin A peptides (A2, B, E, G and H2) and MBP (1ml of a 20µg/ml solution) were each applied to one column (1cm x 2cm) of ATP-agarose (containing 1.1µmol ATP; Sigma), and to one of agarose without ATP, equilibrated with 10ml of 20mM Tris-HCl, pH 7.4. The columns were sealed and left for 30min at RT before being washed with 40ml of 20mM Tris-HCl pH 7.4. The first 6ml of wash was collected as 30 x 0.2ml fractions for dot blot analysis whilst the remaining wash was discarded. Bound protein was eluted with 1M NaCl and collected as 15 x 0.2ml fractions. 2µl of each fraction was spotted onto a nitrocellulose strip for dot blotting (see section 2.7.4). The nitrocellulose was air dried and then blocked with 1% gelatin/PBS/Tween-20 for 1hr at RT. Blots were probed with

either specific polyclonal anti-peptide serum (1:1,000 dilution), anti-toxin A serum (1:1000 dilution) (see section 4.3.1) or anti-MBP serum (1:10,000 dilution, New England Biolabs) for 2hr at RT. Blots were washed in PBS/Tween-20 and then incubated in goat anti-rabbit alkaline phosphatase conjugate (1:1,000 dilution; Sigma) for 1hr at RT. Following washing in PBS/Tween-20 and then SDW, fractions containing protein were highlighted by developing in BCIP/NBT alkaline phosphatase substrate solution (Sigma).

6.4 RESULTS

6.4.1 *In-vitro* glucosylation of rRhoA

To determine whether the PCR-generated toxin A peptides possessed glucosyltransferase activity holotoxin A and the toxin A peptides were assayed for their ability to glucosylate rRhoA in the presence of UDP-[U^{-14C}] glucose *in vitro*. Only holotoxin A and peptide A2 (aa 1-536) possessed enzyme activity giving mean counts per minute (cpm) of 66,803.0 $\pm$ 1761 ($p< 0.00001$) and 10,542 $\pm$ 627 ($p< 0.0001$) respectively, corresponding to incorporation of UDP-[U^{-14C}] glucose into RhoA (see Figure 6.1). Peptides B, C, D, E, G and H2 lacked glucosyltransferase activity and gave an insignificant mean cpm of > 208 (in each case $p> 0.1$). Other negative controls consisted of test buffer inclusive of MBP alone (85.8 $\pm$ 37.3), *E. coli* lysate alone (186.0 $\pm$ 87.9), rRhoA alone (123.1 $\pm$ 68.3) and UDP-^{14}C glucose alone (153.7 $\pm$ 78.1).

Of the 567.5 pmoles of rRhoA in the 50µl reaction volume, approximately 116.3 pmoles were converted to the RhoA-glucose product by holotoxin A as opposed to 18.16 pmoles by peptide A2. SDS-PAGE and western blot analysis demonstrated that preparations of peptide A2 contained a series of truncated species the most abundant of which were approximately 22kDa in size (65kDa including MBP) (see Figure 3.9, lanes 8 and 10). These most probably resulted from poor codon usage in *E. coli* but may also have been produced by SDS-reduction. The truncated species account for a large proportion of the total concentration of the peptide A2 preparation. At this stage it was unclear whether the enzyme activity was expressed only by full-length peptide A2 or by full-length peptide A2 plus the 22kDa truncated species.

6.4.2 Cloning, expression and purification of the first 629 bp of the toxin A gene

6.4.2.1 Introductory comment

Preparations of peptide A2 contain large amounts of truncated species of approximately 65kDa corresponding to the first 21-22kDa (629bp) of holotoxin A. Full length peptide A2 represents only about one third of the total protein concentration in the purified preparations. To ensure that the peptide responsible for glucosylation was incorporated into the assay at a final concentration of 33nM, it was important to determine whether or not the 22kDa truncated species expressed enzymatic activity.

6.4.2.2 PCR amplification of the first 629bp of the toxin A gene (a')

The primers and thermocycling conditions (see section 6.3.3.1) were successfully used to amplify a 629bp DNA product (bps 1-629 of the toxin A gene) corresponding to toxin A fragment a' (see Figure 6.2, lanes 2 and 3).

6.4.2.3 Cloning of the 629bp PCR product (a') into the pMal-c2 expression vector

The 629bp PCR product (a') was successfully cloned into the pCRII T-tailing vector (see section 6.3.3.2) (results not shown). The DNA insert was removed by digestion with *Bam*HI and *Sal*I and subsequently cloned into the pMal-c2 expression vector (see section 6.3.3.3). Seven potential transformants were screened by digestion with *Bam*HI and *Sal*I, and resolved by agarose gel electrophoresis (see Figure 6.3). Clones 1, 2, 5 and 7 all contained the 629bp toxin A fragment. The cloned DNA was sequenced (see section 3.3.4) shown to be correct and 'in frame' with the vector *malE* gene (results not shown).

6.4.2.4 Induction of expression of fragment a' from the pMal-c2 vector

Expression of the toxin A fragment a' was induced from the pMal-c2 vector (see section 6.3.3.4). Clones 1, 2, 5, and 7 all expressed a MBP-fusion product of approximately 65 kDa (peptide A') (see Figure 6.4, lanes 5 and 17, 7 and 19, 9 and 21, 11 and 23). This peptide was not produced when IPTG was omitted from the culture (see Figure 6.4, lanes 4 and 16, 6 and 18, 8 and 20, 10 and 22).

Full length peptide A' was only slightly larger in size than the 65 kDa major truncated species seen in purified preparations of peptide A2 (see Figure 6.4, lanes 12 and 24). It was decided that peptide A' was a good match for the 65kDa truncated species seen in peptide A2 preparations and was a suitable control for glucosyltransferase experiments.

6.4.2.5 Purification and cleavage of the peptide A' fusion product

The peptide A' fusion product was purified by amylose affinity chromatography (see section 6.3.3.5). The highest protein concentration for the fusion product was typically in the range of 0.8-1.0mgml^{-1}. Apart from the full-length peptide two truncated species were present in the peptide A' preparation (see Figure 6.5, lane 3). The first corresponded to the size of the MBP (see Figure 6.5, lane 5) and the second was slightly smaller than the full-length peptide. Full length peptide A' fusion product was cleaved by factor Xa to produce the 43kDa MBP and a peptide of approximately 22 kDa corresponding to the toxin A peptide (A'). A small proportion of the full-length fusion product remained uncleaved in this instance (see Figure 6.5, lane 4).

6.4.3 *In vitro* glucosylation of rRhoA by peptides A2 and A'

To determine whether the first 21kDa of holotoxin A are capable of glucosylating RhoA, the experiment was repeated (see section 6.3.2) using only peptides A2 and A' (see

section 6.3.4). Two different amounts of peptide A2 were included in the assay in separate reactions. The first reaction incorporated peptide A2 at a final concentration of 33nM dependent on the estimated concentration of the full-length peptide only in the protein mixture (≅ 65μg/ml) (A2-FL). The second reaction incorporated peptide A2 at a final concentration of 33nM dependent on the estimated concentration of both full-length peptide and truncated species in the protein mixture (≅ 195μg/ml) (A2-total).

The results (see Figure 6.6) indicated that peptide A' (aa 1-205) lacked enzyme activity giving mean cpm of 202.8 ± 76.4 ($p = 0.12$). The reaction containing holotoxin A gave mean cpm of 96,928 ± 3791 ($p < 0.0001$) corresponding to incorporation of UDP-[U^{-14C}] glucose into recombinant RhoA. Holotoxin A, however, did not glucosylate BSA proteins giving a mean cpm of 82.1 ± 43.3 ($p = 0.18$) (result not shown). In both cases, peptide A2 showed significant levels of glucosyltransferase activity. Reactions containing peptide A2-FL gave mean cpm of 24,287 ± 969 ($p < 0.0001$) whereas those containing peptide A2-total gave mean cpm of 6,486 ± 316 ($p < 0.0001$). Other negative controls consisted of test buffer inclusive of MBP alone (181 ± 114), RhoA alone (115.7 ± 28.2) and UDP-^{14}C alone (105.6 ± 95.0).

Of the 567.5 pmoles of rRhoA in the 50μl reaction volume, approximately 168.9 pmoles were converted to the RhoA-glucose product by holotoxin A as opposed to 76.1 pmoles by peptide A2-FL and 11.3 pmoles by peptide A2-total. The relevant positions of peptides A2, A' and C in the holotoxin molecule are outlined in figure 6.7.

6.4.4 Binding of toxin A peptides to ATP-agarose

The method outlined in section 6.3.5 was used to locate the ATP-binding site within the holotoxin A molecule. The positive control holotoxin A was retained on an ATP-agarose

column following stringent washing (see Figure 6.8, panel A1 row e). The negative control MBP, however, was not retained on either the ATP-agarose column or the straight agarose column (see Figure 6.8, panel A2 and B2 row e).

The toxin A peptides A2 (see Figure 6.9 panels A1 and B1 row e), G (see Figure 6.10 panels A2 and B2 row e) and H2 (see Figure 6.11 panels A and B row e) did not bind to either the ATP-agarose column or the agarose column without ATP. In each case the positive controls reacted strongly with the specified antibodies on the dot blots (panels A and B, row a on the above mentioned figures). Peptides C and A' were not tested.

The overlapping peptides B (aa 542-859) and E (aa 542-1160) were both retained on the ATP-agarose columns following stringent washing (see Figure 6.9 panel A2 row e and Figure 6.10 panels A1 row e, respectively) but were not retained on agarose columns lacking ATP (see Figure 6.9 panel B2 row e and Figure 6.10 panels B1 row e, respectively). These results suggest that the ATP-binding site is situated between amino acids 542 and 859 in the holotoxin A molecule.

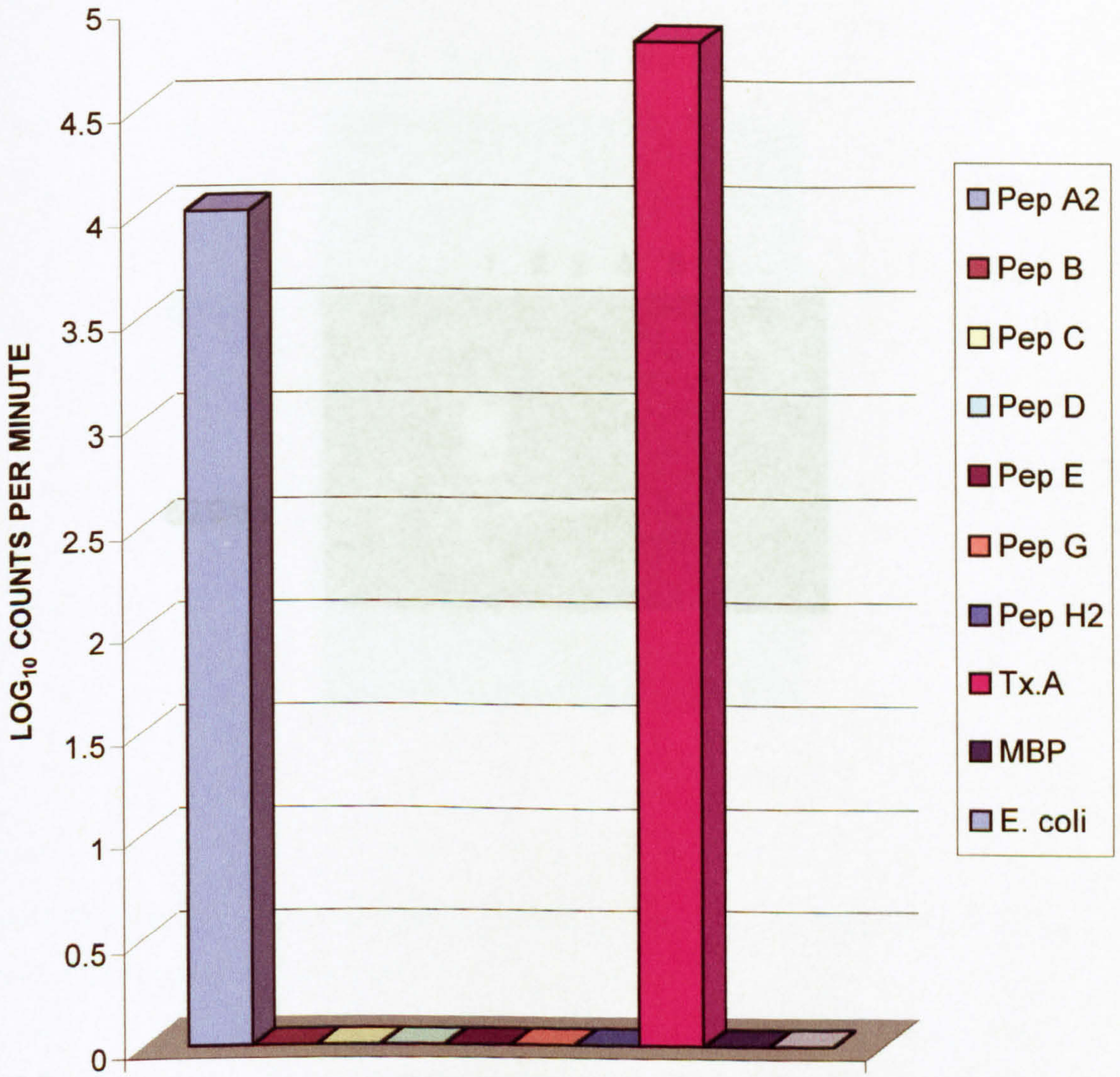


Figure 6.1
Glucosyltransferase activity of holotoxin A and the toxin A peptides. MBP was a negative control for peptides A2-G whereas *E. coli* was a negative control for peptide H2. Holotoxin A and peptide A2 significantly glucosylated rRhoA *in vitro*.

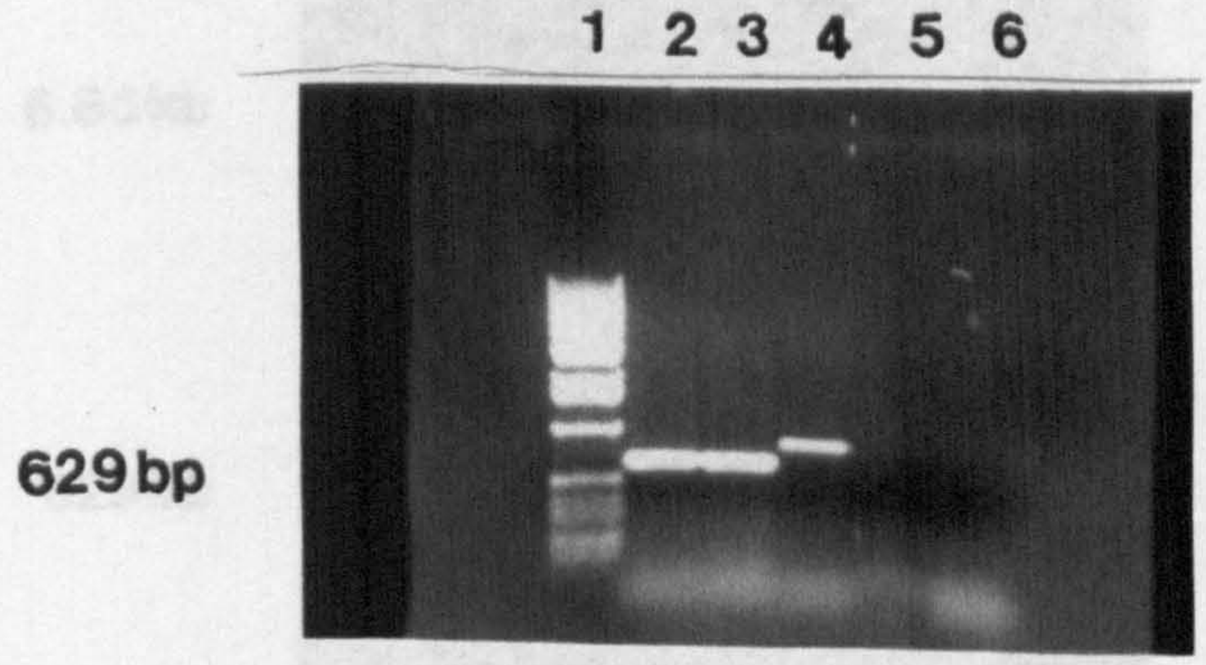


Figure 6.2

1.2% agarose gel showing the PCR amplification of toxin A fragment a' from *C. difficile* VPI10463 chromosomal DNA using primers 3 and 13.

Lane 1, 1Kb ladder; Lane 2 and 3, *C. difficile* DNA amplified with primers 3 and 13; Lane 4, *C. difficile* DNA amplified with primers 13 only; Lane 5, *C. difficile* DNA amplified with primers 3 only; Lane 6, no template.

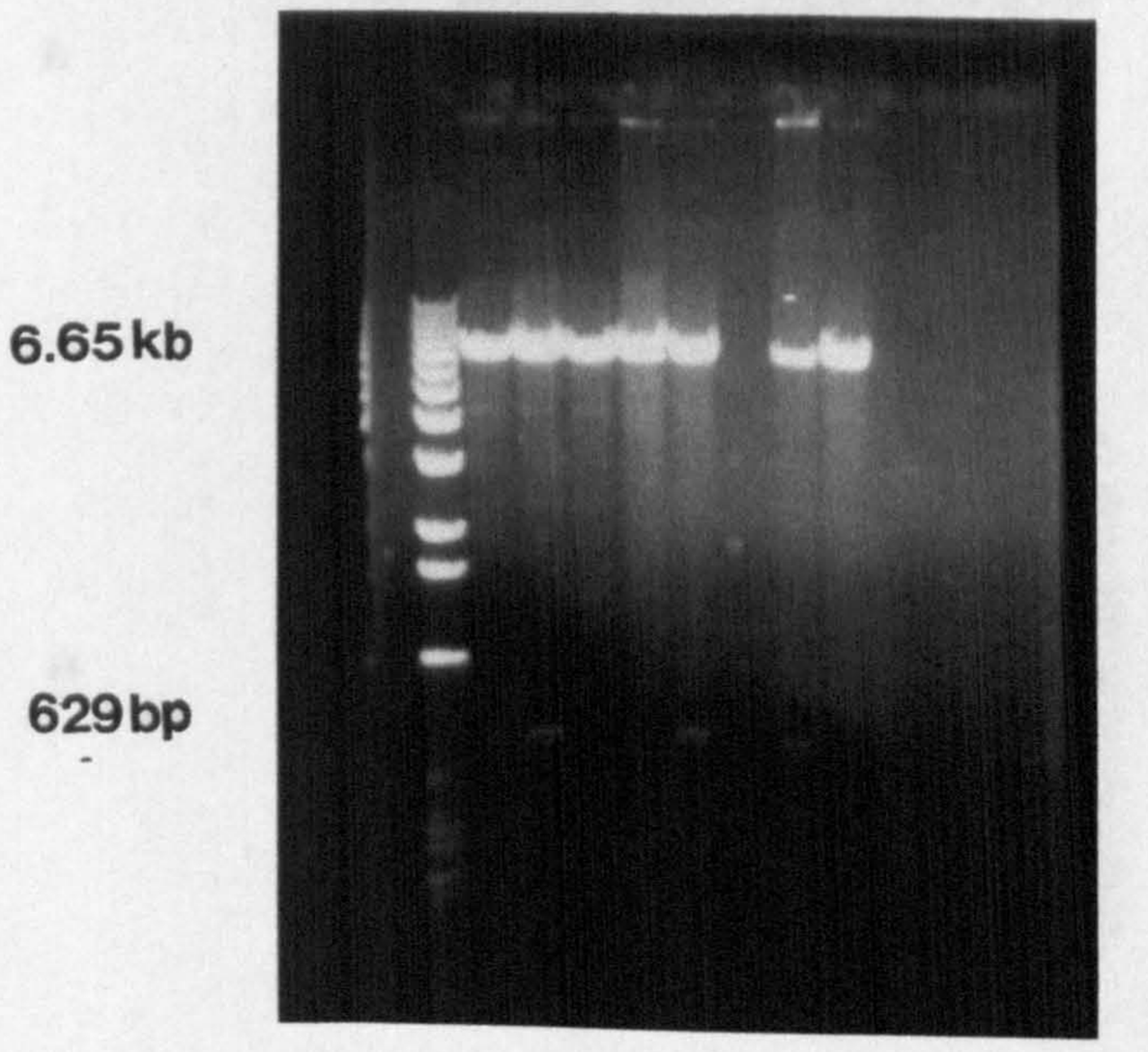


Figure 6.3

0.8% agarose gel showing the sub-cloning of toxin A fragment a' into the pMal-c2 expression vector.

Lane 1, 1Kb ladder; Lanes 2-8, potential pMal-c2 clones (clones 1-7) containing fragment a' (*Bam*HI and *Sal*I digests); Lane 9, pMal-c2 only (*Bam*HI and *Sal*I digests).

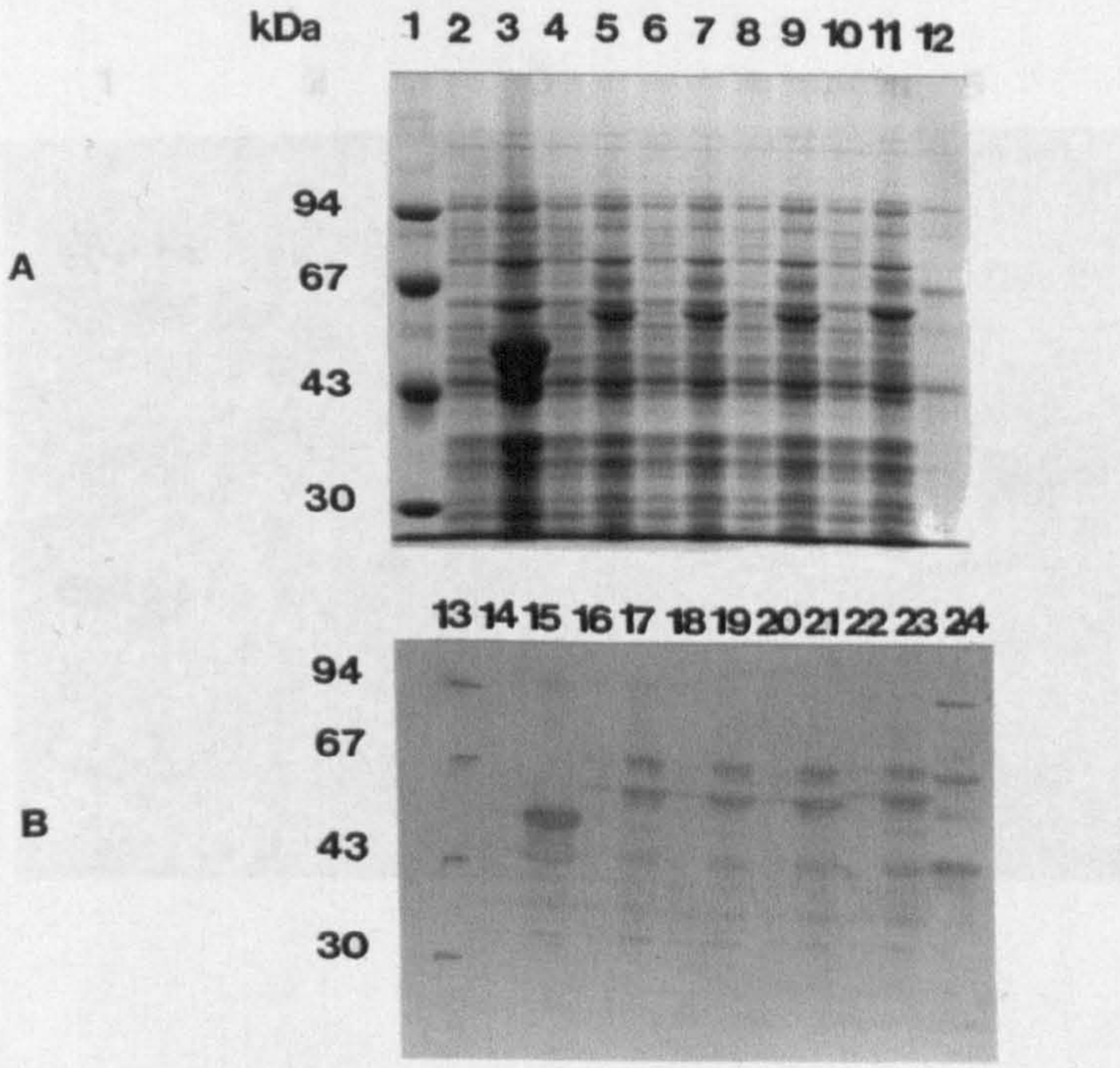


Figure 6.4

8% SDS-PAGE (A) and respective western blot (B) probed with MBP antiserum showing the induction of expression of peptide A'.

Lanes 1 and 13, LMW markers; Lanes 2 and 14, uninduced JM109 containing pMal-c2 only; Lanes 3 and 15, induced JM109 containing pMal-c2; Lanes 4 and 16, uninduced JM109 containing pMal-c2 plus fragment a' (clone 1); Lanes 5 and 17, induced JM109 containing pMal-c2 plus fragment a' (clone 1); Lanes 6 and 18, as lanes 4 and 16 (clone 2); Lanes 7 and 19, as lanes 5 and 17 (clone 2); Lanes 8 and 20, as lanes 4 and 16 (clone 5); Lanes 9 and 21, as lanes 5 and 17 (clone 5); Lanes 10 and 22, as lanes 4 and 16 (clone 7); Lanes 11 and 23, as lanes 5 and 17 (clone 7); Lanes 12 and 24, purified toxin A peptideA2

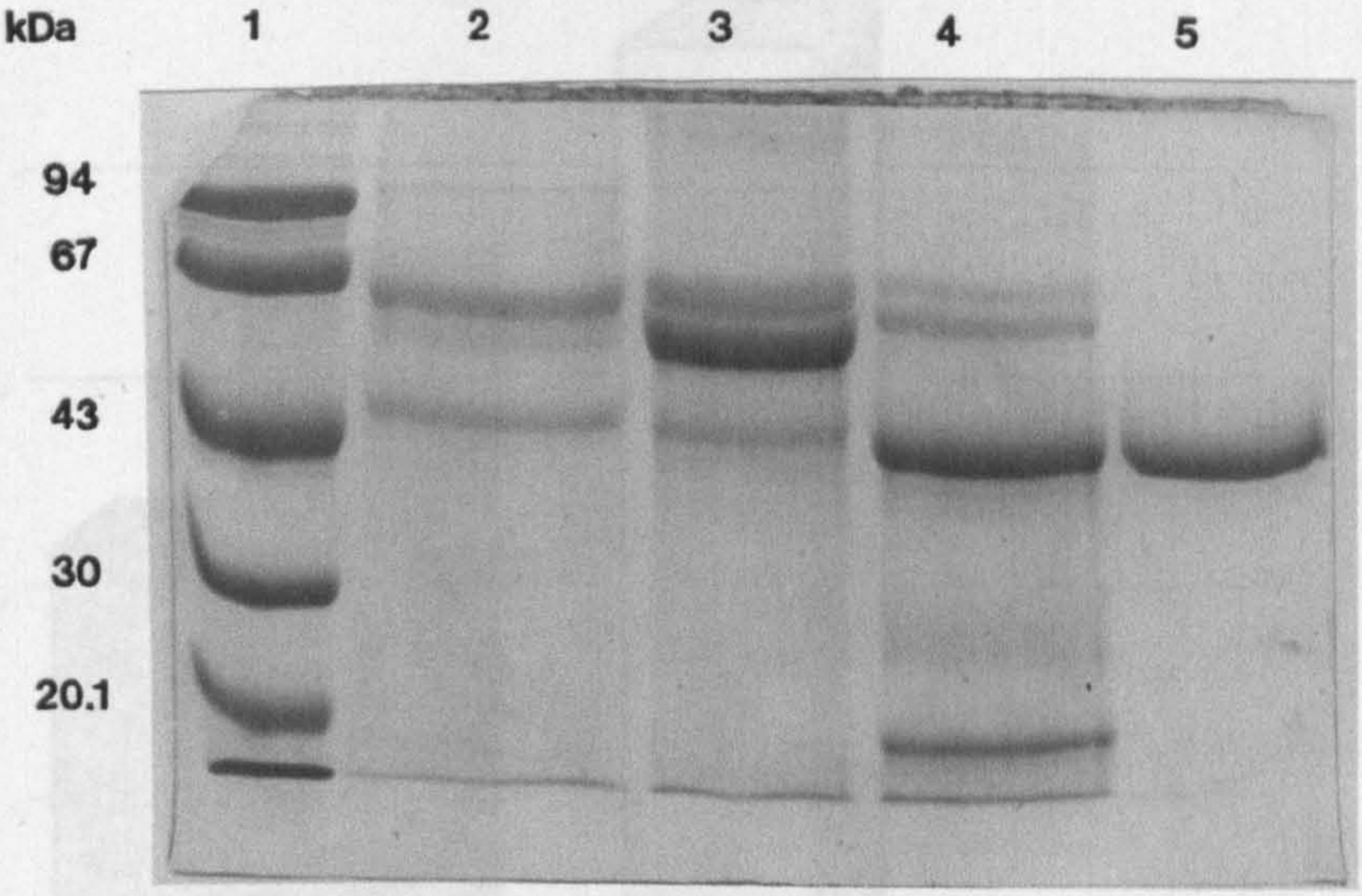


Figure 6.5

10% SDS-PAGE gel showing the amylose affinity purification and factor Xa cleavage of the peptide A' fusion product.

Lane 1, LMW markers; Lane 2, uncleaved peptide A2 fusion product; Lane 3, uncleaved peptide A' fusion product; Lane 4, factor Xa cleaved peptide A' fusion product; Lane 5, MBP.

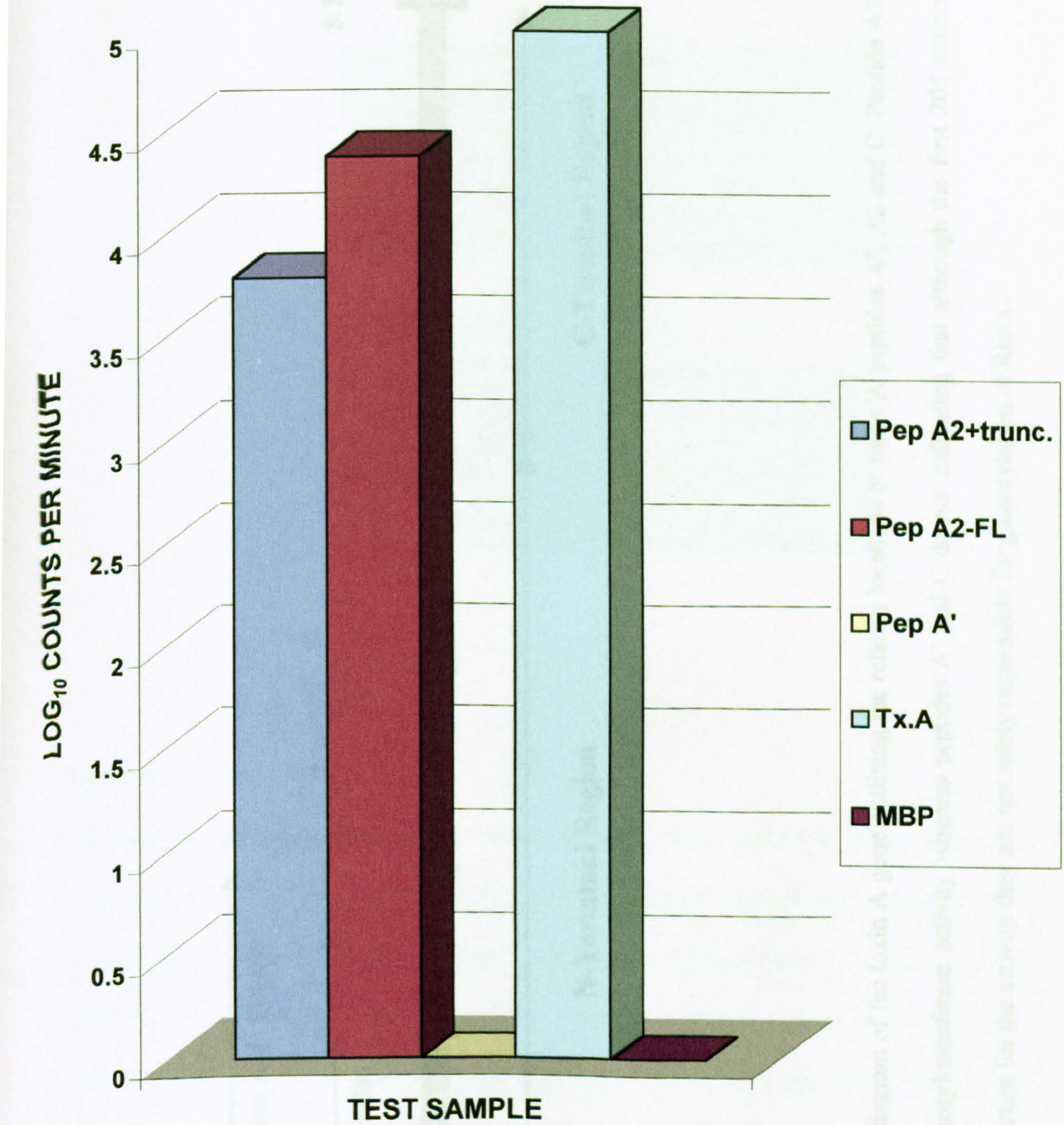


Figure 6.6

The glucosyltranserase activity displayed by preparations of peptide A2 is due to the full-length peptide and not to the 65 kDa major truncated species represented in this assay by peptide A'. The amount of peptide A2 added to the assay was calculated to take into account the concentration of full-length peptide only (Pep A2) or full-length peptide plus truncated species (Pep A2 + trunc.)

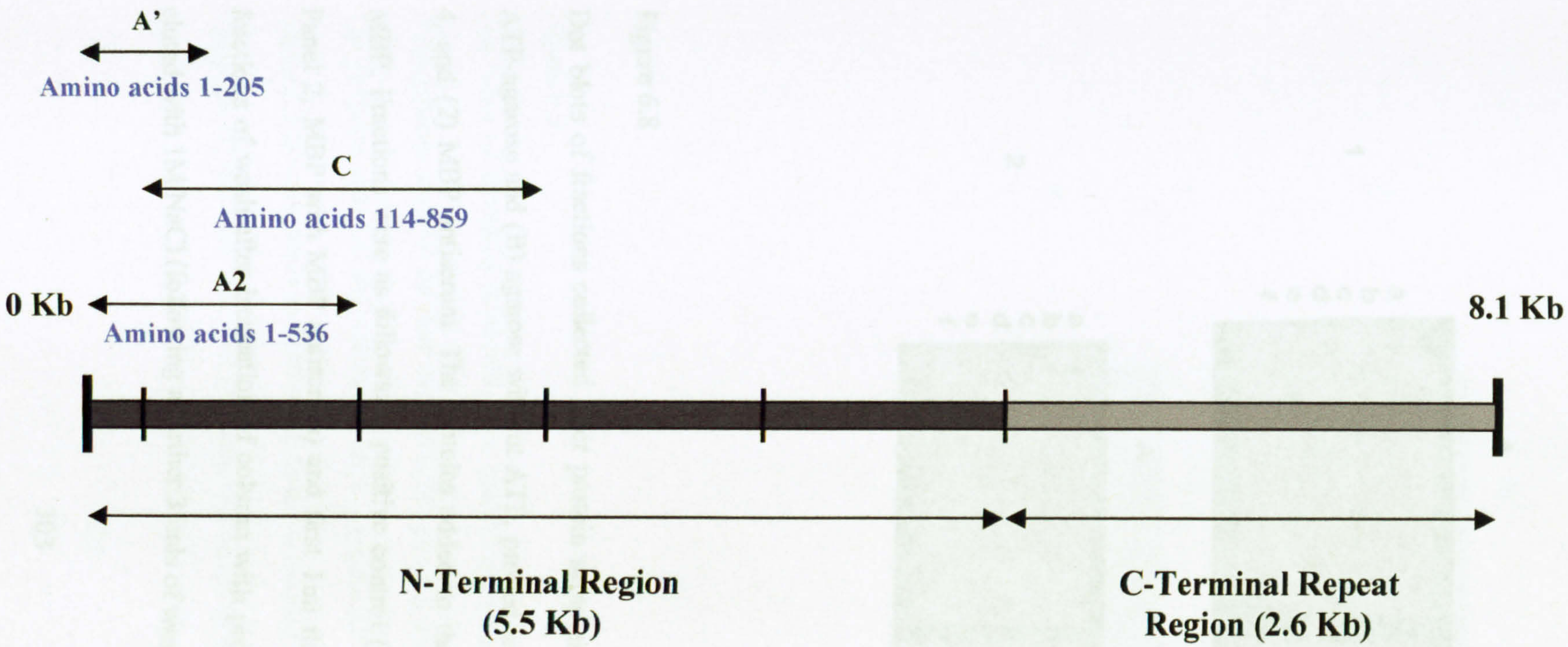


Figure 6.7

A schematic diagram of the toxin A gene outlining the relative locations of toxin A peptides A', A2 and C. Peptide A2 expresses glucosyltransferase activity whereas peptides A' and C do not indicating that although the first 205 amino acids are important for the activity they are not solely responsible for glucosylation of RhoA.

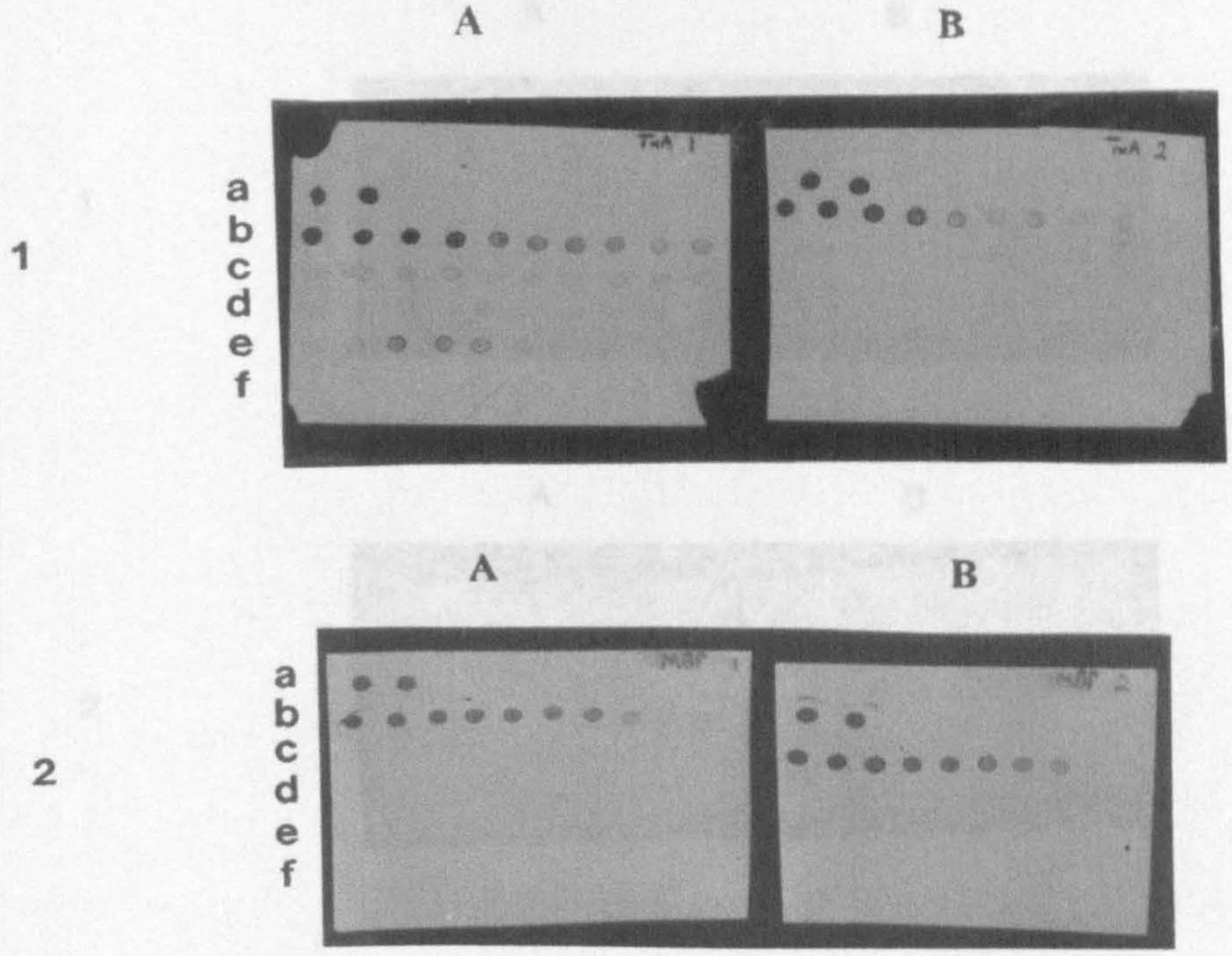


Figure 6.8

Dot blots of fractions collected after protein samples were applied to columns of (A) ATP-agarose and (B) agarose without ATP, probed with (1) monoclonal antibody PCG-4, and (2) MBP antiserum. The proteins added to the column were: 1, toxin A and 2, MBP. Fractions were as follows: a, positive control (i.e. Panel 1, toxin A with PCG-4, Panel 2, MBP with MBP antiserum) and first 1ml flow through; b,c and d, 30× 0.2ml fractions of wash after incubation of column with protein; e and f, 15× 0.2ml fractions eluted with 1M NaCl (following a further 35mls of washing).

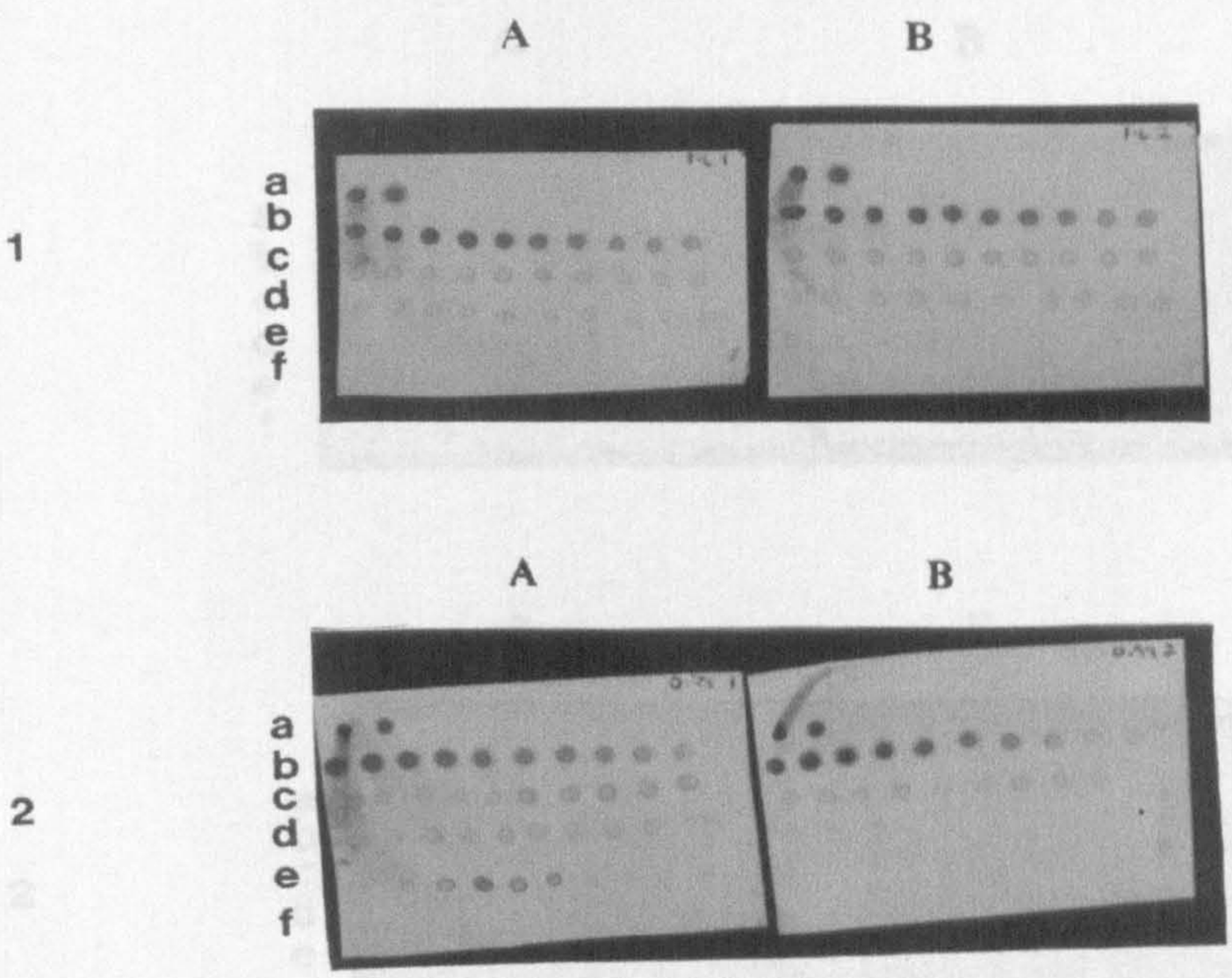


Figure 6.9

Dot blots of fractions collected after protein samples were applied to columns of (A) ATP-agarose and (B) agarose without ATP, probed with MBP antiserum.

The proteins added to the columns were 1, peptide A2 fusion product, 2, peptide B fusion product. Fractions were as follows: a, positive control (i.e. Panel 1-peptide A2, and Panel 2-peptide B) and first 1ml flow through; b-f, as b-f in figure 6.8.

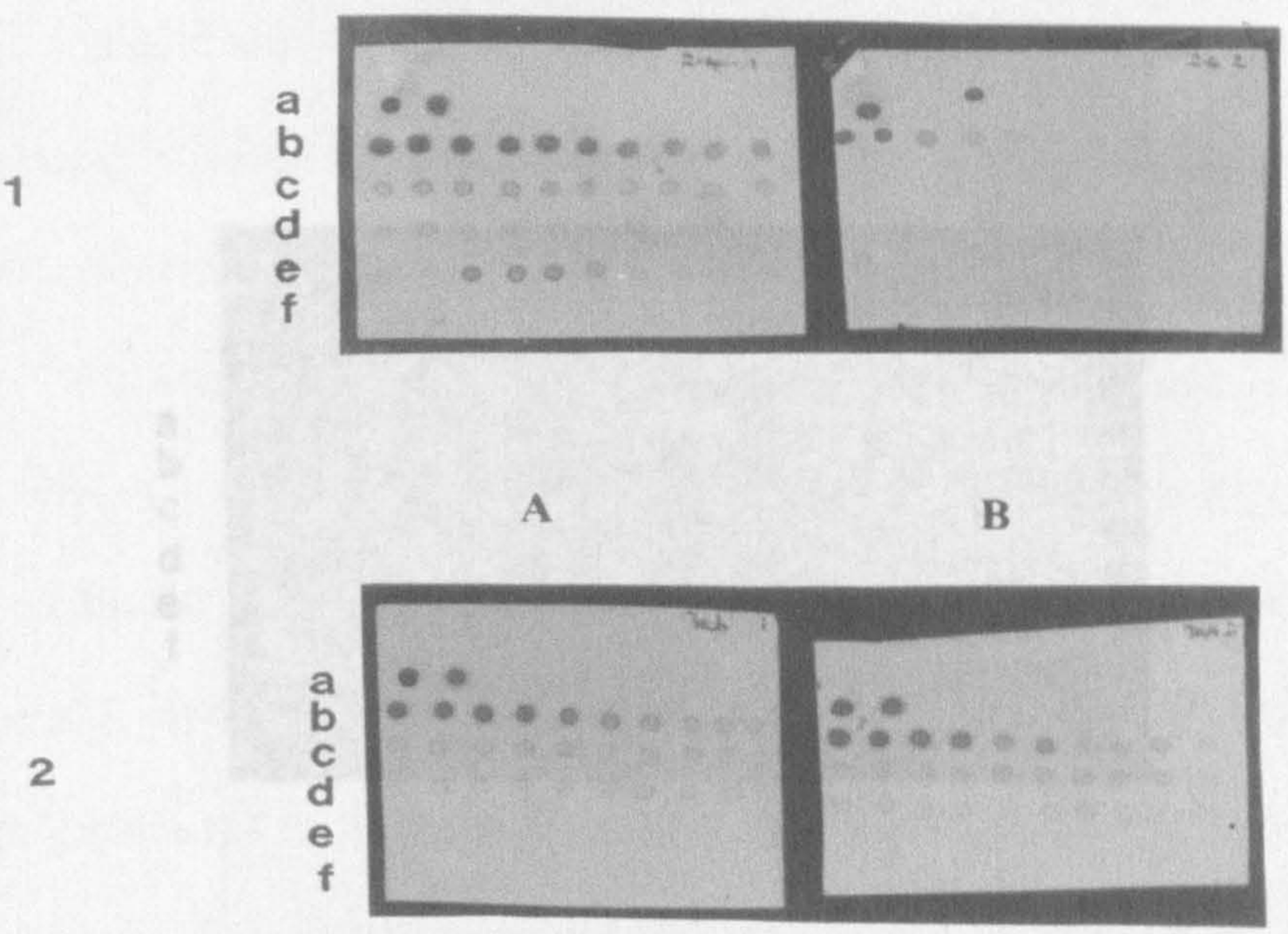


Figure 6.10

Dot blots of fractions collected after protein samples were applied to columns of (A) ATP-agarose and (B) agarose without ATP, probed with MBP antiserum.

The proteins added to the columns were 1, peptide E fusion product and 2, peptide G fusion product. Fractions were as follows: a, positive control (i.e. Panel 1-peptide E, and Panel 2-peptide G) and first 1ml flow through; b-f, as b-f in figure 6.8.

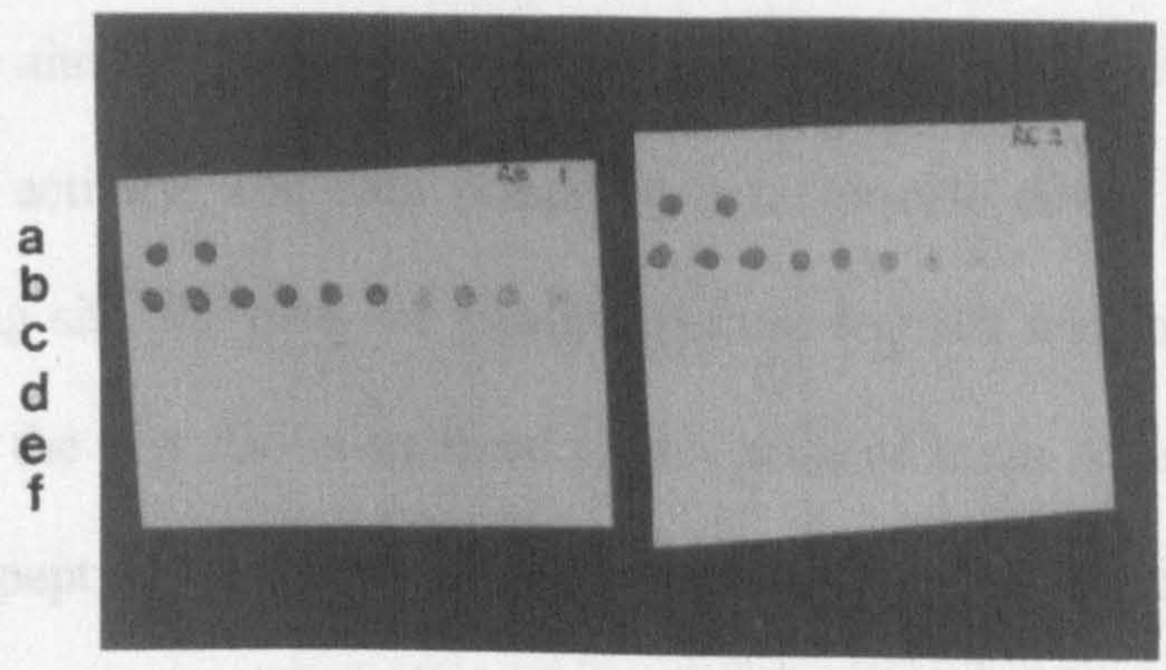


Figure 6.11

Dot blots of fractions collected after peptide H2 was applied to a column of (A) ATP-agarose and (B) agarose without ATP, probed with monoclonal antibody PCG-4.

Fractions were as follows: a, positive control (i.e. Peptide H2) and first 1ml flow through; b-f, as b-f in figure 6.8.

6.5 DISCUSSION

One of the major breakthroughs in delineating the molecular mechanism of action of *C. difficile* toxins A and B was the recent observation that they act as glucosyltransferases which monoglucosylate Rho proteins using UDP-Glucose as a co-substrate (Just *et al*, 1995b,c). One of the aims of this chapter was to locate the region of the toxin A molecule responsible for this activity. The data presented here provide direct evidence that the UDP-glucose binding site, the catalytic site for glucose transfer and the Rho interaction site all occur within the first 536 N-terminal amino acids of toxin A (peptide A2). None of the other toxin A peptides exhibited the ability to glucosylate rRhoA *in vitro*.

Analysis of the peptide A2 by SDS-PAGE showed that approximately 50% of the protein preparation consisted of a series of truncated species of about 65 kDa (inclusive of MBP) compared to the molecular mass of 99kDa (inclusive of MBP) of the full-length peptide. In order to determine whether or not this also had activity, the DNA coding for the first 205aa of the toxin A molecule was cloned and the recombinant protein product (peptide A') was examined. No activity was detected. Presuming that recombinant peptide A' was folded correctly, this result suggests that the first 205aa of the toxin A molecule alone are not sufficient for full enzymatic activity. This result also suggests that peptide A2 was more active than first anticipated as less peptide A2 was present in the initial experiments than indicated by the total purified protein measurements. Once the concentration was adjusted to incorporate only full-length peptide A2 (A2-FL) into the assay at a final concentration of 33nM, the enzyme activity expressed by peptide A2 was typically about 45% of that expressed by holotoxin A. The lower enzyme activity exhibited by peptide A2 could be explained by the fact that it was a recombinant protein expressed in a gram negative organism (i.e. not as effective as the native holotoxin). Alternatively, it was

possible that the glucosyltransferase molecule (peptide A2) had been truncated at the C-terminal end leading to a reduction in enzyme activity.

Further adding to these results, peptide C that spans the proposed catalytic domain of the toxin A molecule (aa 114-859) does not glucosylate rRhoA. Again presuming that peptide C was folded correctly, these results suggest that although amino acids 1-113 are not solely responsible for glucosylation they are essential either structurally or functionally for full enzymatic activity. Since this work was completed, other investigators have also presented data to suggest that the glucosyltransferase activity of toxins A and B resides within the N-terminus of the molecules (Hofmann *et al*, 1997, Wagenknecht-Wiesner, *et al*, 1997, Soehn *et al*, 1998, Faust *et al*, 1998).

In support of the data presented in this chapter, Hofmann and co-workers provided evidence to show that the N-terminal 546aa residues of toxin B express full glucosyltransferase activity. The similarity of these two results is not surprising considering that the homology of toxins A and B is greatest between the N-terminal two-thirds of the molecules (von Eichel-Streiber *et al*, 1992). Hofmann and co-workers also showed that a toxin B fragment encompassing the first 516aa residues was at least 1,000 fold less active than fragment 1-546 and that a fragment covering 1-468aa was completely inactive (Hofmann *et al*, 1997,). In contrast, Wagenknecht-Wiesner and co-workers have shown that a toxin B fragment covering aa 1-467 expressed extremely weak enzymatic activity (Wagenknecht-Wiesner, *et al*, 1997).

The results presented here on toxin A, together with published observations on toxin B, (Hofmann *et al*, 1997,) suggest that site directed mutagenesis of the 20aa residues (517-536) within an N-terminal peptide (aa 1-536) of toxin A or B should further delineate the catalytic domain.

In an attempt to identify the different regions of peptide A2 important for glucosylation, the peptide A2 sequence data were compared to others in a computer database (Basic Local Alignment Search Tool, BLAST search) for regions of homology. Unfortunately the programme only aligned peptide A2 with clostridial toxin sequences and did not identify any protein sequences relating to Rho glucosylation. Recent studies have shown that the amino acids 364 through 516 primarily define the substrate specificity of *C. sordellii* lethal toxin (Hofmann *et al*, 1998). In contrast to *C. difficile* toxins A and B, which exclusively modify Rho sub-family proteins, *C. sordellii* lethal toxin also glucosylates Ras sub-family proteins. The investigators showed that a chimeric fusion protein of *C. difficile* toxin B, in which amino acids 364 through 516 had been exchanged for the same region of *C. sordellii* lethal toxin also glucosylated Rho sub-family proteins (Hofmann *et al*, 1998).

A further study resulted in the identification of several amino acid residues in *C. sordellii* lethal toxin that are essential for the enzyme activity (Busch *et al*, 1998). Exchange of aspartic acid at position 286 or 288 with alanine or asparagine decreased glucosyltransferase activity by about 5,000 fold and blocked glucohydrolase activity. No enzyme activity was detected with the double mutant. Mutation of this DXD motif prevented radiolabelling by azido-UDP-glucose. Asparagine at positions 270 and 273 were also shown to be important for both glucosyltransferase and glucohydrolase activity. The investigators concluded that the DXD motif, which is highly conserved in all clostridial cytotoxins and some glucosyltransferases, is functionally essential for enzyme activity and may participate in the binding of UDP-glucose (Busch *et al*, 1998).

Results obtained in chapter 5 showed that peptide A2, although possessing glucosyltransferase activity *in vitro*, had no effect on tissue culture cells or in rabbit ileal

loops. It has been shown, however, that microinjection of the same region of toxin B into cells caused glucosylation of Rho proteins and a full CPE (Hofmann *et al*, 1997). It appears that peptide A2 is unable to affect cells due to an inability to enter the cell cytoplasm. It would be interesting, therefore, to examine the effect on cells of a chimeric protein consisting of peptide A2 fused to a protein that would deliver the peptide into cells.

Only peptides B (aa 542-859) and E (aa 542-1161) were retained on an ATP-agarose column suggesting that the ATP-binding site resides between amino acids 542 and 859 in the holotoxin molecule. These results provide the first direct identification of an ATP binding site within either the toxin A or B molecules. In these experiments the agarose was attached to the ATP via the ribose hydroxyls (Sigma cat. No. A4793). This particular attachment leaves the phosphate group of ATP particularly well exposed. Toxin A has also been shown to bind polyphosphates (Lobban and Borriello, 1992) so it is possible that the interaction between ATP agarose and peptides B and E is via a polyphosphate binding site. Based on homology with an ATP binding motif, however, a putative ATP binding site has also been identified in toxin B between amino acids 651 and 683 (Barroso *et al*, 1994). The same conserved sequence (-IGHGKDE[25×]K-) is also present in holotoxin A between amino acids 653-685. This region is covered by peptides B and E supporting the hypothesis that both peptides encode an ATP-binding site. It is also possible that the polyphosphate-binding site and the ATP binding site are coded for by the same region of the toxin A molecule. It is of interest that the peptides containing the nucleotide-binding site (B and E) were the only peptides shown to possess this ability, but these peptides lacked glucosyltransferase activity. Further, peptide A2, which possessed glucosylation activity lacked a nucleotide-binding site. There was no direct

evidence that a proven nucleotide binding site was required for *in vitro* glucosylation of rRhoA by toxin A. Microinjection of a toxin B peptide (aa 1-546) into cells resulted in glucosylation of Rho proteins and a full CPE indicating that the putative nucleotide binding site in toxin B is also unnecessary for glucosyltransferase activity (Hofmann *et al*, 1997). These observations fail to identify a role for the nucleotide binding site of toxin A and the putative one for toxin B. It is possible that the ATP binding site is important for endocytosis or translocation of toxin A into the cytoplasm. It would be interesting, therefore, to study a truncated form of toxin A, which lacked the ATP binding site but which retained its ability to bind to cells and to act as a glucosyltransferase molecule.

SECTION - 7

CYTOTOXIC ACTIVITY OF A TRUNCATED TOXIN CONSISTING OF THE GLUCOSYLTRANSFERASE AND BINDING DOMAINS OF TOXIN A

Section 7

Cytotoxic activity of a truncated toxin consisting of the glucosyltransferase and binding domains of toxin A

7.4.4 Purification of the truncated toxin A fusion peptide

7.4.5 Effect of the truncated toxin on Vero cells *in vitro*

7.4.6 H/A of RRBCs by the truncated toxin

7.5 DISCUSSION

Section 7

Cytotoxic activity of a truncated toxin consisting of the glucosyltransferase and binding domains of toxin A

7.1 Summary

Both the glucosyltransferase (bps 1 – 1,614) and receptor binding (bps 5,505 – 8,058) regions of the toxin A gene were successfully re-amplified by PCR from *C. difficile* VPI 10463 chromosomal DNA. The DNA fragment encoding the binding region (h^T) was cloned into the pCR II T-tailing vector downstream of the DNA fragment encoding the glucosyltransferase activity (a^T). The fused construct ($a^T + h^T$) was sub-cloned into the pET 30a expression vector and sequence data confirmed that the front portion of the insert (a^T) was 'in frame' with the vectors T7 promotor. IPTG induction resulted in low levels of expression of a histidine fusion protein of approximately 147 kDa consisting of the glucosyltransferase region (A^T; amino acids 1-538) fused to the binding region (H^T; amino acids 1,835-2,686) of the holotoxin molecule. The recombinant peptide, which appeared to be present in both the soluble and insoluble fractions, reacted with the PCG-4 monoclonal antibody suggesting that the C-terminal repeat region of the truncated toxin (H^T) had remained 'in frame' with peptide A^T.

Purification by histidine affinity chromatography typically yielded between 5-10 $\mu g ml^{-1}$ of soluble recombinant truncated toxin fusion protein. The truncated toxin was capable of haemagglutinating RRBCs as effectively as the holotoxin suggesting that the repeat region-binding portion (H^T) of the truncated toxin was biologically active. The truncated toxin also demonstrated classical cytotoxicity *in vitro* but was approximately 1,400 fold

less potent to Vero cells than the holotoxin. This indicates that peptide H^T (or peptide A2) is able to glucosylate cellular Rho proteins and induce a CPE suggesting that the nucleotide binding site (amino acids 651-683) is not essential for this activity. In conclusion, fusion of the glucosyltransferase and binding domains of toxin A forms an active toxin molecule. The reduced activity of the truncated toxin suggests that the central region (amino acids 539-1,834) of the holotoxin is probably involved in effective processing and / or delivery of the molecule.

7.2 Introduction

The results from section 6 indicated that peptide A2 (amino acids 1-536) contained all the structures necessary for full glucosyltransferase activity. Although this peptide was biologically active *in vitro* it was unable to induce cytotoxic effects in tissue culture cells or rabbit ileal loops, probably due to an inability to enter the cells. In order to demonstrate cytotoxic capability therefore, it would be necessary to deliver the glucosyltransferase molecule (peptide A2) into cells. As an alternative to microinjection it was decided to produce a truncated toxin consisting of peptide A2 fused to a suitable delivery peptide.

The results from section 5 indicated that peptide H2 (C-terminal repeat region of toxin A) was both able to bind to cells and to be internalised into endosome-like compartments within the cells making it a suitable delivery protein for peptide A2. The ability of peptide H2 to escape endosomes and enter the cytoplasm was unknown.

The aim of this section was to engineer a biologically active truncated toxin A ($A^T + H^T$) consisting of the glucosyltransferase molecule (amino acids 1-538) representing peptide A2, fused to the binding domain (amino acids 1,835-2,686) representing peptide H2.This

recombinant truncated toxin (see figure 7.1) would lack the central region (amino acids 539-1,834) of the holotoxin molecule. It was hoped that the truncated toxin would be used to demonstrate the cytotoxic capability of peptide A2 whilst also giving some indication as to the importance of the central region of the holotoxin molecule.

7.3 Materials and Methods

7.3.1 PCR Amplification of fragments a^T and h^T for construction of the truncated toxin construct.

The glucosyltransferase domain of toxin A (a^T) was re-amplified by PCR using the forward primer: 5' AGCCATGGCTATGTCTTTAATATCTAAAGAAG 3' (14; see appendix 2) and the reverse primer: 5' CAGAAAGAGGTCGACCAGTATAATCTCT 3' (15; see appendix 2) in a 50µl reaction volume (see section 2.3). The binding portion of toxin A (h^T) was re-amplified by PCR using the forward primer: 5' TTAGTTAAAGGTCGACTCAATATAAAT 3' (16; see appendix 2) and the reverse primer: 5' CAATCTCGAAGGATCCACCAGCTGCA 3' (17; see appendix 2) also in a 50µl reaction volume (see section 2.3). Thermocycling conditions were as follows:

FRAGMENT	DENATURE	ANNEAL	EXTEND
a^T	97^0C;1min	50^0C;1min	72^0C;2min
h^T	97^0C;1min	58^0C;1min	72^0C;3min

In both reactions there were 35 amplification cycles with the final extension time increased to 9.9min.

7.3.2 Purification of the PCR products a^T and h^T

The PCR products a^T (1.630Kb) and h^T (2.589Kb) resulting from section 7.3.1 were visualised on a 0.8% agarose gel (see section 2.2.3). The DNA fragments a^T and h^T were

excised from the gel, passed through glass beads and then Spinbind purified (see section 2.2.4).

7.3.3 Cloning of the truncated toxin construct (a^T + h^T) into the expression vector pET30a

The purified PCR products, a^T (1.630Kb) and h^T (2.598Kb), were each individually cloned into the pCRII T-tailing vector (see section 2.4). Plasmids containing the a^T DNA insert (bp's 1-1,630 of the toxin A gene; see appendix 1) were digested with *Nco*I and *Bam*HI (see section 2.2.6) and analysed on a 0.8% agarose gel (see section 2.2.3). Plasmids containing the h^T DNA insert (bps 5,485-8,074 of the toxin A gene; see appendix 1) were digested with *Sal*I and *Bam*HI (see section 2.2.6) and analysed by 0.8% agarose gel electrophoresis (see section 2.2.3). It was necessary to orientate plasmids containing fragment a^T to isolate a clone with an inverted insert. When digested with the restriction enzyme *Nco*I (see section 2.2.6), a clone with an insert in the correct orientation showed two fragments of 3.945 Kb and 1.555 Kb on a 0.8% agarose gel (see section 2.2.3) whereas a clone with an inverted insert showed two fragments of 2.345 Kb and 3.15 Kb on a 0.8% agarose gel (see section 2.2.3). These profiles were made possible by the presence of a unique *Nco*I site 1.88 kb into the pCRII vector. The recombinant plasmid with an inverted insert was linearised by digestion with the restriction enzymes *Sal*I and *Bam*HI (see section 2.2.6) to allow the h^T recombinant DNA insert (removed from the pCRII vector by digestion with the restriction enzymes *Sal*I and *Bam*HI) to be ligated (see section 2.2.7) via the *Sal*I site alongside the inverted a^T DNA fragment in the pCRII vector. The fused DNA construct was removed from the pCRII vector as a 4.172 Kb

fragment by digestion with *Nco*I and *Bam*HI (see section 2.2.6) and was analysed by 0.8% agarose gel electrophoresis (see section 2.2.3). The pET30a expression vector (Novagen Inc.) was also digested with *Nco*I and *Bam*HI then analysed by 0.8% agarose gel electrophoresis (see section 2.2.3). The fused 4.172Kb truncated toxin fragment and the 5.4 Kb-digested pET30a fragment were purified from an agarose gel (see section 2.2.4). The purified 4.172 Kb fragment was ligated with the digested pET30a vector (see section 2.2.7). Expression of constructs from the pET30a vector provides an N-terminal fusion to a histidine / S-tag (six amino acids and fifteen amino acids respectively). Recombinant plasmids were transformed (see section 2.2.9) into electrocompetent *E. coli* JM109 (Novagen Inc.) for storage at –70°C and into electrocompetent *E. coli* BL21 DE3 (Novagen Inc.) for expression. The DNA insert was sequenced from the pET30a vector using the Novagen Inc. supplied T7 promotor primer #69348-1 (TAATACGACTCACTAT) and the T7 terminator primer #69337-1 (CCGCTGAGCAATAACTA). A summary of the steps involved in cloning the truncated toxin construct can be seen in Figure 7.2

7.3.4 Expression of the truncated toxin A peptide in *E. coli* and determination of solubility

BL21 DE3 clones containing the truncated toxin A construct ($a^T + h^T$), and a pET30a plasmid alone, were grown at 37^0C overnight with shaking in LB broth containing 30μgml^{-1} kanamycin (see section 2.1.1). A 2ml aliquot of each overnight culture was inoculated into 100 ml of fresh LB broth (containing 30μgml^{-1} kanamycin) and incubated at 37^0C with shaking until the culture reached an $O.D_{600}$ of 0.6. Samples were produced

corresponding to both uninduced and induced soluble fractions (see section 2.8.4) for the truncated toxin (A^T and H^T) clone and for a pET30a clone that lacked a DNA insert.

Briefly, half of the 100ml culture was removed and pelleted at 5,000×g for 20mins (uninduced cells). Expression of the recombinant protein was induced from the T7 promotor in the remaining culture by the addition of IPTG to a final concentration of 1mM with continued incubation at 37°C for 3 hrs. These cells were pelleted at 5,000×g for 20mins (induced cells). Both the uninduced and induced cells were resuspended in 10mls PBS then sonicated in an ice-water bath for 15secs bursts at an amplitude of 10 microns until the culture was clear. The sonicates were centrifuged at 10,000×g for 20 mins at 4°C to pellet insoluble proteins. The 10ml supernatants (induced and uninduced soluble fractions) were removed and stored at 4°C until further analysis. The pellets were resuspended (by sonication) into 10mls of PBS (induced and uninduced insoluble fractions) and stored at 4°C.

Each fraction was mixed 1:1 with 2× SDS-PAGE gels (see section 2.7.1). Gels were either stained with CBB (see section 2.7.1) or transferred to nitrocellulose for western blotting (see section 2.7.3). The truncated toxin fusion peptide (A^T and H^T) was detected by incubating the blot with 3μg/ml of the PCG-4 Mab (specific for the repeat end of toxin A) followed by a 1:1,000 dilution of a goat anti-mouse IgG alkaline phosphatase conjugate (Sigma). Alternatively, the truncated toxin was detected using a 1:5,000 dilution of an anti-S-Tag antibody (Novagen Inc.) followed by a 1:1,000 dilution of a goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma).

7.3.5 Purification of the truncated toxin A fusion peptide

The truncated toxin A (fragment A^T + H^T) histidine fusion product was purified on a histidine resin affinity column using the provided buffers as outlined by manufacturers instructions (His. Bind® Buffer Kit; Novagen, Inc). A brief outline is provided below:

10mls of an overnight culture (see section 2.1.1) of BL21 DE3 cells containing the recombinant plasmid were inoculated into 1 litre of LB broth containing 30μgml^{-1} of kanamycin. The cells were incubated at 37^{0}C, with shaking (250rpm), until reaching an O.D$_{600}$ of 0.6. Expression of the truncated toxin was induced by incubating with IPTG to the final concentration of 1mM at 37^{0}C, with shaking, for a further 3 hrs. The cells were harvested at 5,000 xg for 5mins and then resuspended in 40mls of ice-cold 1x Binding buffer (Novagen Inc.). The cell sample was sonicated in an ice-water bath at amplitude of 10 microns for bursts of 15 secs until the sample was no longer viscous. Cell debris was removed from the sample by centrifugation at 39,000 xg for 20mins followed by filtration through a 0.45micron filter (Acrodisc). A 5ml sample of His. Bind Resin (Novagen Inc.) was applied to a small polypropylene column (Biorad) and allowed to pack under gravity flow. Five mls of packed resin was sufficient to purify up to 40mg of histidine fusion protein. The resin was charged and equilibrated by washing sequentially with 3 volumes of SDW, 5 volumes of 1x Charge buffer (Novagen Inc.) and 3 volumes of 1x Binding buffer (where 1 volume is equivalent to the settled bed volume). The prepared protein sample was applied to the column at a flow rate of 10 column volumes per hour. The column was washed with 10 volumes of 1x Binding buffer and 6 volumes of 1x Wash buffer (Novagen Inc.). The bound protein was eluted with 4 volumes of 1x Elute buffer (Novagen Inc.) and collected as 15 x 0.5 ml fractions. *E. coli* BL21 DE3 containing pET30a without an insert

were treated in the same way as above and the proteins also passed down a histidine column. The eluted fractions were used as *E. coli* negative controls.

The presence of protein in the fractions was determined by dot blotting (see section 2.7.4) and probing with a 1:1,000 dilution of the Mab PCG-4 followed by a 1:1,000 dilution of goat anti-mouse alkaline phosphatase conjugate (Sigma). Alternatively the dot blots were probed with a 1:5,000 dilution of an anti-S-Tag antibody (Novagen Inc.) or a 1:1,000 dilution of peptide A2 antiserum (see section 4.3.1) followed by a 1:1,000 dilution of goat anti-rabbit alkaline phosphatase conjugate (Sigma).

7.3.6 Determination of the concentration of purified truncated toxin ($A^T + H^T$)

The concentration of the purified truncated toxin peptide was determined using the Coomassie Plus Protein Assay Reagent Kit (see section 2.9.1.2) and checked using the S-Tag Rapid Assay Kit (Novagen) as outlined by the manufacturers instructions. This latter detection system is based on the interaction of the 15 amino acid S-Tag with ribonuclease S-protein which together form an enzymatically active ribonuclease. Therefore, following the addition of the purified S-protein, the assay of ribonuclease activity provides a direct measurement of the S-Tag fusion protein.

7.3.7 Effect of the truncated toxin on Vero cells *in vitro*

Vero cells were grown to confluency in 96 well sterile microtitre plates (Falcon)(see section 5.3.3). The growth medium was replaced with 50µl of maintenance medium (Medium 199 containing 2% newborn calf serum, 1% penicillin and 1% glutamine). Ten-fold serial dilutions of the truncated toxin (7.5µg/ml), holotoxin A (260µg/ml), histidine

column elution buffer (Novagen) and *E. coli* negative control (see section 7.2.5) were prepared in PBS before 50µl of each were applied to the Vero cells. Two-fold serial dilutions of truncated toxin A, *E. coli* negative control and elution buffer were also prepared in PBS and applied to the Vero cells (i.e. 50µl). The cells were monitored for a CPE, commonly seen as cell rounding after incubation at 37°C for 24 hrs. The cytotoxicity titre (Cytotoxic unit [CU]/50µl) was expressed as the highest dilution that induced more than 50% CPE after an incubation of 24 hrs.

7.3.8 Haemagglutination of RRBCs by the truncated toxin

The haemagglutination assay was performed as outlined in section 5.3.1. Briefly, 50µl of both the purified truncated toxin preparation (7.5µg/ml) and holotoxin A (100µg/ml) were diluted two-fold with TBS in the wells of a v-bottom microtitration plate (Sero-well). Holotoxin A (320µg/ml) was placed in one well as a positive control whilst the *E. coli* negative control (see section 7.2.5) was added to a second well as a negative control. Fifty µl of a 1% RRBC suspension was added to each well and incubated for at least 3 hrs at 4°C. H/A titres were expressed as the reciprocal of the highest dilution giving a positive H/A when viewed by eye.

7.4 RESULTS

7.4.1 PCR amplification of toxin A fragments a^T and h^T

It was necessary to re-design PCR primers to amplify the glucosyltransferase (a^T) and the binding region (h^T) of toxin A to enable them to be fused to eventually produce a truncated toxin which lacked the central portion of the holotoxin molecule (bp 1,630-5,485). Using the primers and thermocycling conditions outlined in section 7.3.1 both fragments were successfully amplified. Primers 14 and 15 (see appendix 2) were used to amplify the 1.630Kb product a^T (bp 1-1,630) (see figure 7.3, lanes 1 and 2) while primers 16 and 17 (see appendix 2) were used to amplify a 2.589Kb product h^T (bp 5,485-8,074) (see figure 7.3, lanes 3 and 4).

7.4.2 Cloning of the truncated toxin construct (a^T + h^T) into the pET30a expression vector

The method outlined in section 7.3.3 and represented diagrammatically in figure 7.1 was used to clone fragments a^T and h^T as a fusion into the pET30a expression vector 'in frame' with the T7 promotor. Firstly, both PCR products were cloned into the pCRII T-tailing vector. It was necessary to determine the orientation of pCRII clones containing fragment a^T. Digestion with *Nco*I allowed selection of a clone with an inverted insert. The restriction pattern required was two fragments of 1.554Kb and 4.036Kb as opposed to 3.154Kb and 2.436Kb (results not shown). Digestion of a pCRII clone containing an inverted a^T insert with *Nco*I and *Sal*I produced a 2.4Kb *Nco*I and *Sal*I vector band, a 1.554Kb *Nco*I vector band and a 1.63 Kb *Nco*I and *Sal*I band corresponding to fragment a^T (see figure 7.4, lanes 1 and 2). To visualise the cloned insert more clearly, the plasmid was first digested with *Nco*I to produce fragments of 1.554Kb and 4.036Kb. The latter

fragment was purified from an agarose gel (see section 2.2.4) then digested with *Sal*I. This resulted in two fragments, a 2.4Kb vector band and a 1.63Kb band corresponding to fragment a^T (see figure 7.4, lanes 3, 4 and 5).

Digestion of pCRII clones containing h^T with *Sal*I and *Bam*HI produced two bands of 3.9Kb (pCRII vector) and 2.589Kb (fragment h^T) (see figure 7.5, lanes 1-3).

The pCRII clone containing an inverted a^T insert was linearised by digestion with *Sal*I and *Bam*HI (see section 7.3.3). The 2.589Kb h^T fragment (released from the pCRII vector by digestion with *Sal*I and *Bam*HI) was then ligated into the opened pCRII clone containing fragment a^T. In this way fragment h^T fused to fragment a^T via a *Sal*I restriction site. Digestion of pCRII clones containing the fused truncated toxin fragment (a^T+ h^T) with *Bam*HI and *Nco*I produced a 4.2Kb band (fragment a^T+ h^T) and two vector bands of 1.5Kb and 2.4Kb (see figure 7.6, lanes 1-5).

The 4.2Kb truncated toxin fragment was sub-cloned into the pET30a expression vector via the *Bam*HI and *Nco*I restriction sites (see section 7.3.3). Digestion of pET30a clones containing the truncated toxin fragment with *Bam*HI and *Nco*I produced a 5.4Kb vector band and a 4.2Kb band corresponding to the truncated toxin fragment (see figure 7.7).

The truncated toxin DNA insert was sequenced from the pET30a vector using the primers described in section 7.3.3. The sequence data revealed that fragments a^T and h^T both corresponded to the correct regions of the toxin A molecule and that fragment a^T was in frame with the T7 promotor (results not shown).

7.4.3 Expression of the truncated toxin peptide and determination of its solubility

The method outlined in section 7.3.4 was used to isolate induced and uninduced soluble and insoluble protein fractions from *E. coli* BL21 DE3 clones containing either the

pET30a truncated toxin clone or the pET30a vector without an insert. The protein fractions were resolved by 8% SDS-PAGE then visualised by CBB staining and western blot analysis (see section 7.3.4). CBB staining did not reveal an additional recombinant protein running at approximately 147 kDa in the samples that contained the truncated toxin insert (see figure 7.8, lanes 8-14). The gel was also western blotted and probed with the PCG-4 Mab (specific for the repeat region of toxin A) and an anti-S-Tag antibody (see section 7.3.4). The PCG-4 Mab reacted with the peptide H2 positive control (see figure 7.9, lane 11) as expected. A small amount of over-spill of the positive control into lanes 9, 10 and 12 was also detected. The Mab PCG-4 did not react with the soluble or insoluble fractions from the induced BL21 DE3 cells containing the pET30a vector only (see figure 7.9, lanes 7 and 8). This further supports the theory that the weak reaction seen in lanes 9 and 10 (uninduced BL21 DE3 cells containing the pET30a vector only) was due to over-spill of lane 11 and not a specific reaction.

Prior to IPTG induction, the PCG-4 Mab reacted weakly with a protein of approximately 116 kDa in both the soluble and insoluble fractions originating from the BL21 DE3 cells which contained the truncated toxin insert (see figure 7.9, lanes 5 and 6). Following IPTG induction the PCG-4 reactive protein was increased in both the soluble and insoluble fractions although it appeared greater in the insoluble fractions (see figure 7.9, lanes 1-4). Although some truncated products were present the size of the full-length fusion protein compares well to the estimated size of 147 kDa (see figure 7.9, lanes 1-4). The fact that the PCG-4 Mab is highlighting full-length peptide by binding to the C-terminal repeat region (peptide H^T) of the construct suggests that the truncated toxin has maintained the correct reading frame throughout.

Surprisingly, the S-Tag antibody bound only to the soluble fraction taken from induced BL21 DE3 cells containing the truncated toxin insert (see figure 7.10, lane 2) and not the insoluble fraction (see figure 7.10, lane 1). When half as much of the soluble induced fraction was applied there was no reaction (see figure 7.10, lane 3). Again the highlighted protein was approximately 147 kDa as predicted. An identical western blot was also probed with polyclonal peptide A2 antiserum but the background was too high and masked the true antibody reaction (result not shown).

7.4.4 Purification of the truncated toxin A fusion peptide

The procedure outlined in section 7.3.5 was used to purify the truncated toxin histidine fusion product. The fractions eluted from the histidine column with elute buffer (Novagen) were spotted onto nitrocellulose strips for dot blotting (see section 7.3.5). Four identical blots were prepared and probed with the PCG-4 Mab, polyclonal anti-peptide A2 serum, anti-S-Tag serum or alkaline phosphatase conjugate only (secondary antibody control).

With the exception of the conjugate control, which did not react with any of the protein fractions (see figure 7.11, panel D), all of the antisera reacted with their positive controls and with the lysate before addition to the column (see figure 7.11, panels A, B and C, row a, columns 1 and 2). The positive controls were peptide H2 for the PCG-4 Mab, peptide A2 for the polyclonal A2 antiserum, and a purified S-Tag fusion product (Novagen) for the S-Tag antiserum. Only the polyclonal A2 antiserum appeared to react significantly with the 15ml of wash buffer collected prior to elution of the bound protein (see figure 7.11, panel B, row a, column 3). The results obtained with all three of the antibodies suggested that the first 1ml fraction of 12 collected contained the highest concentration of

truncated toxin histidine fusion product (see figure 7.11, panels A, B and C, rows b and c). The protein concentration of fraction one was determined as outlined in section 7.3.6 and was typically found to be in the range of 5 – 10 μgml^{-1}.

7.4.5 Effect of the truncated toxin on Vero cells

The method outlined in section 7.3.7 was used to determine the effect of the recombinant truncated toxin on Vero cells *in vitro*. Cells incubated for 24 hrs with the *E. coli* negative control sample or with the column elution buffer (see section 7.3.5) remained unaffected (see figure 7.12 panel C). Holotoxin A (260 μgml^{-1}) on the other hand caused a classic CPE (see figure 7.12 panel A) with a titre of $1:1x10^5$ which represents an ability to induce morphological changes in cells by exposure to 1.3 $ngml^{-1}$ of toxin A. The recombinant truncated toxin (7.5 μgml^{-1}) also demonstrated classical cytotoxicity (see figure 7.12 panel B) but appeared much less potent than toxin A with a titre of only 1:2 representing activity on cells by 1.858 μgml^{-1} of the protein.

7.4.6 H/A of RRBCs by the truncated toxin

Both holotoxin A and the recombinant truncated toxin haemagglutinated RRBCs. The H/A titre of holotoxin A (100 μgml^{-1}) was 1:32 whilst that of the truncated toxin (7.5 μgml^{-1}) was 1:2 (see figure 7.13 wells A1-A8 and B1-B8 respectively). This represented effects induced by 1.563 μgml^{-1} and 1.858 μgml^{-1} of holotoxin A and truncated toxin respectively. The *E. coli* negative control (see section 7.3.5) had no effect on the RRBCs.

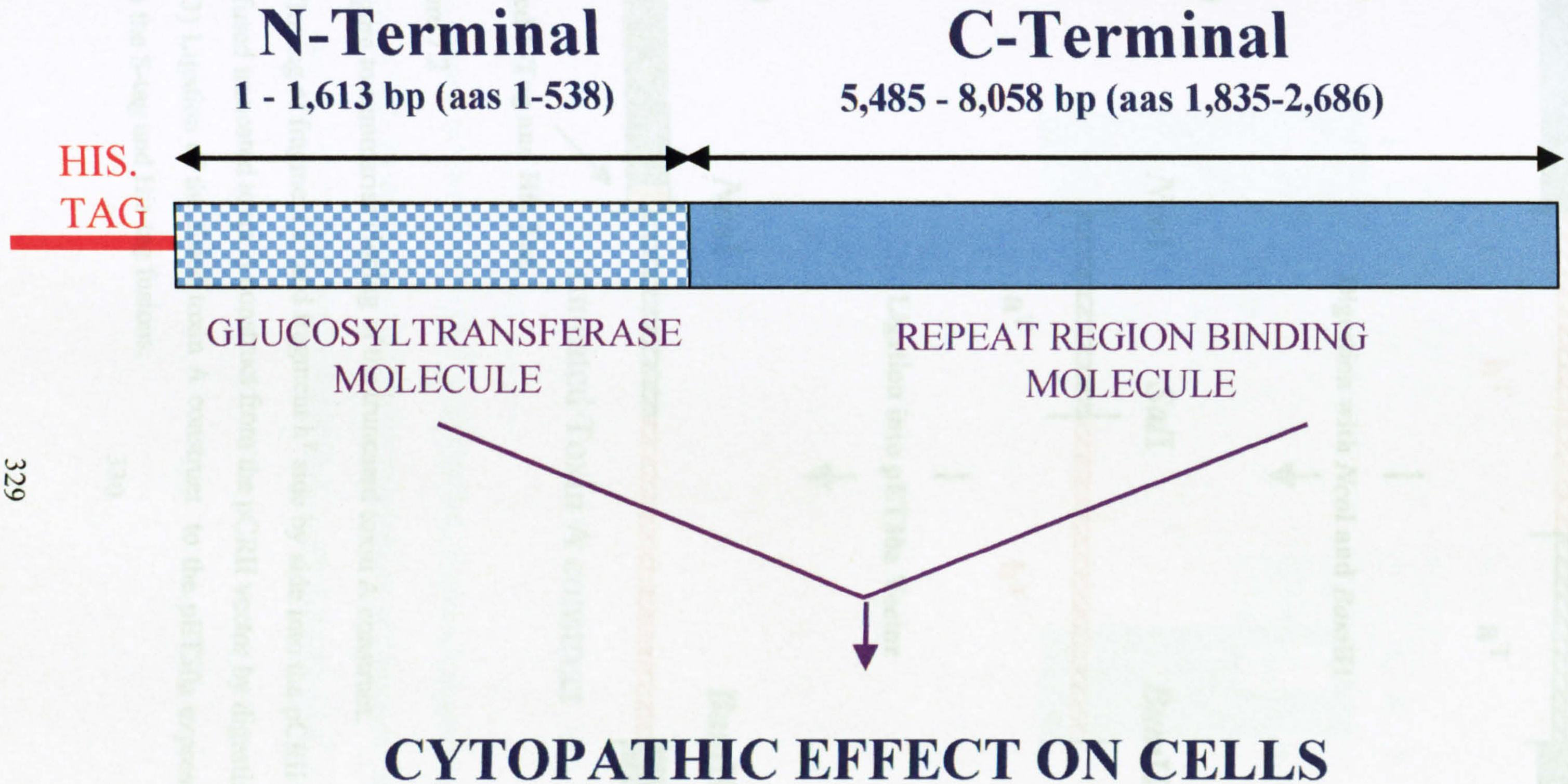


Figure 7.1

A diagrammatic representation of the truncated toxin showing fusion of the C-terminal binding component to the N-terminal toxic component of holotoxin A.

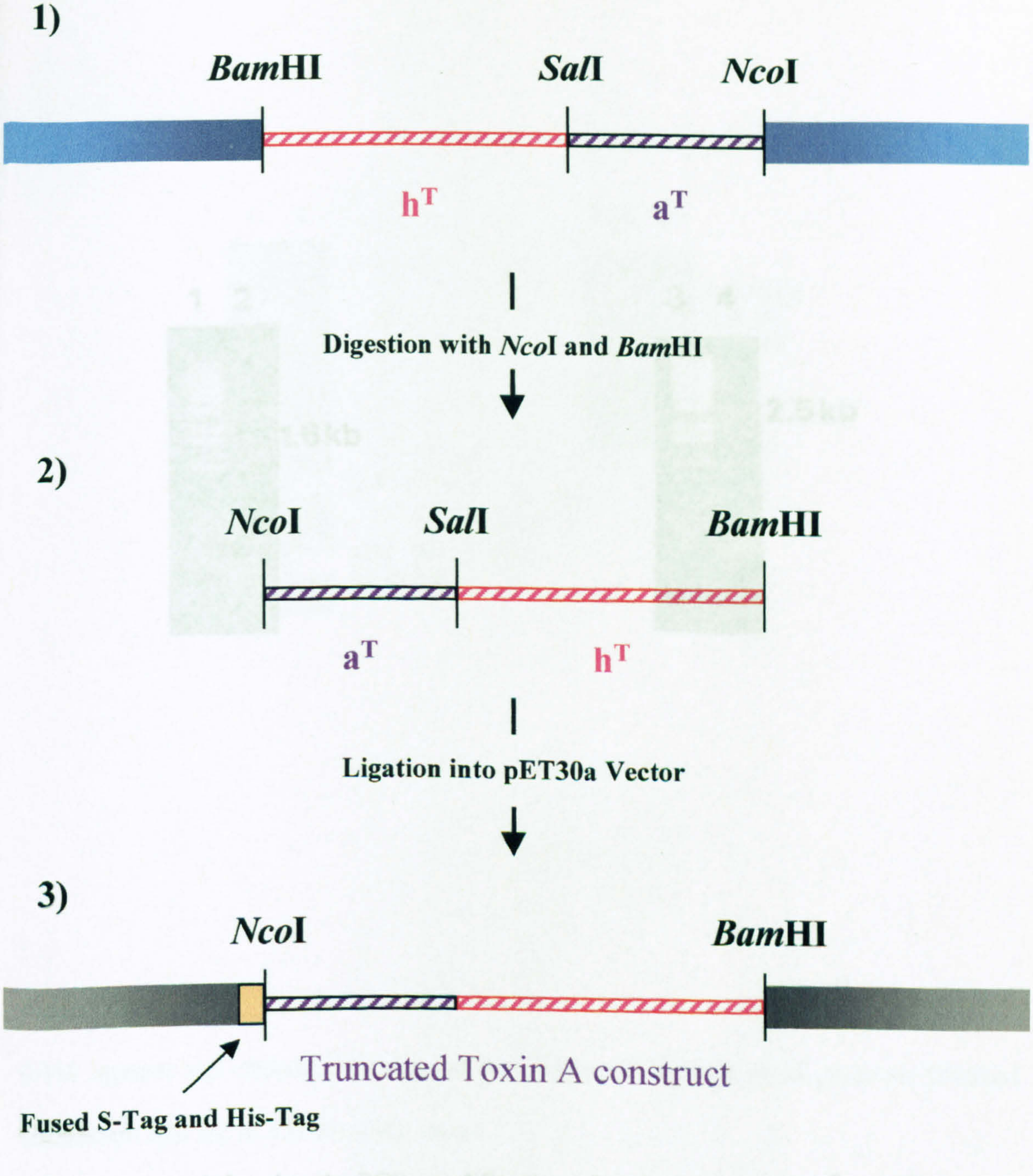


Figure 7.2

A figure to summarise cloning of the truncated toxin A construct.

1) Cloning of fragment a^T and fragment h^T side by side into the pCRII vector. 2) Removal of the fused truncated toxin A construct from the pCRII vector by digestion with *Nco*I and *Bam* HI. 3) Ligation of the fused toxin A construct to the pET30a expression vector 'in frame' with the S-tag and His-tag fusions.

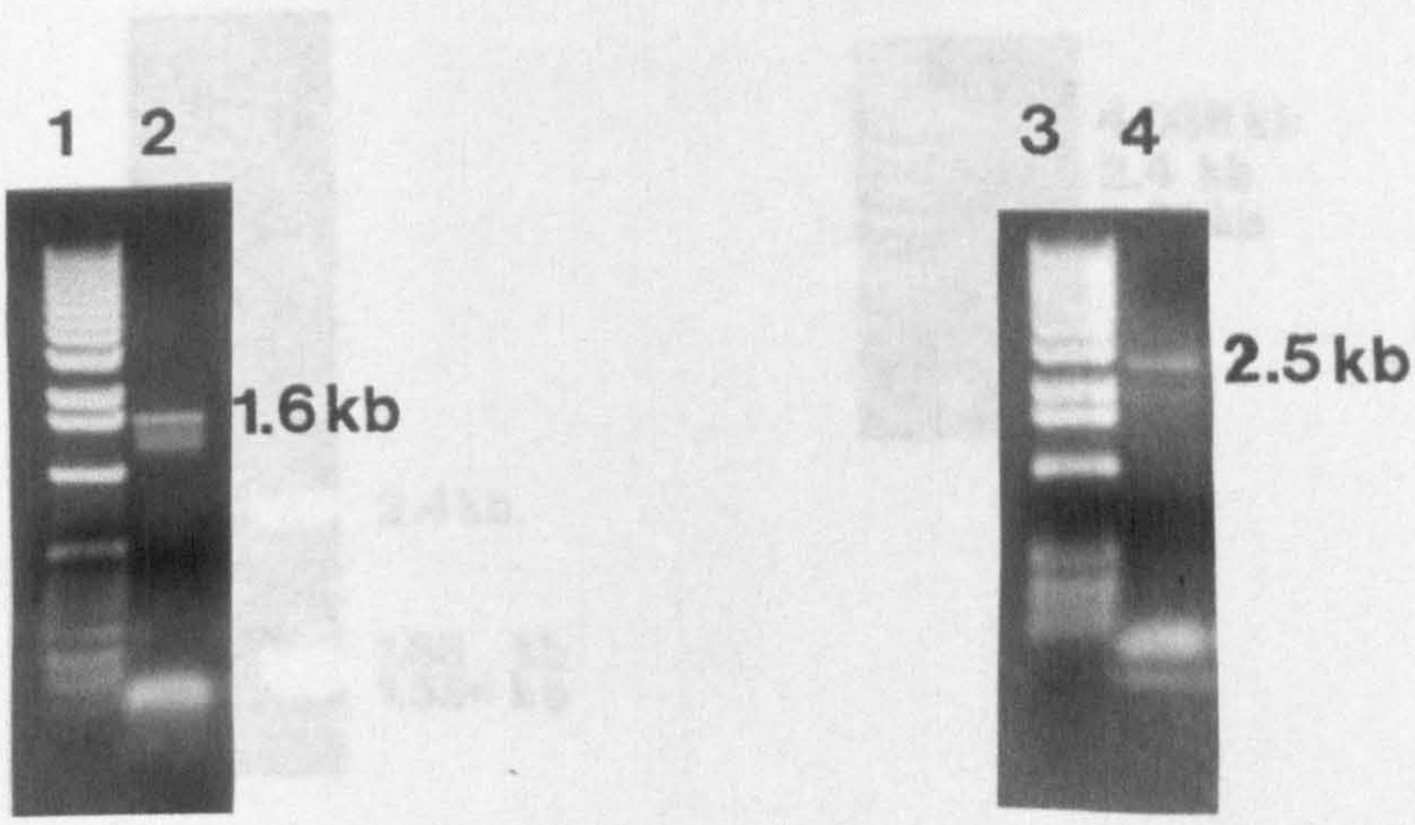


Figure 7.3

0.8% agarose gel showing the PCR amplification of toxin A fragments a^T and h^T from *C. difficile* VPI10463 chromosomal DNA for construction of truncated toxin.

Lanes 1and 3, 1 kb ladder; Lane 2, fragment a^T; Lane 4, fragment h^T.

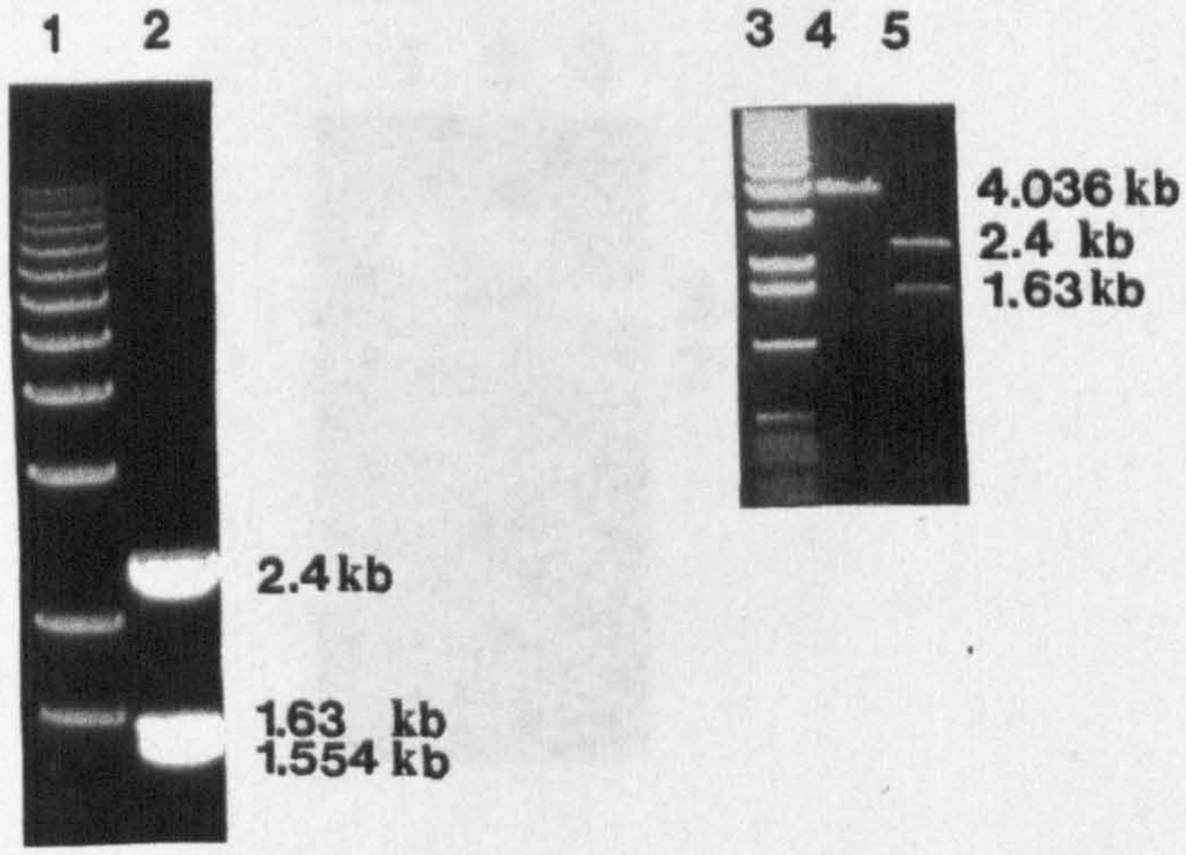


Figure 7.4

0.8% agarose gel showing cloning of the fragment a^T PCR product in an inverted orientation into the pCRII T-tailing vector.

Lanes 1 and 3, 1kb ladder, Lane 2, pCRII containing an inverted a^T insert digested with *Nco*I and *Sal*I; Lane 3, a 4.036 kb fragment produced from a pCRII plus inverted a^T insert digested with *Nco*I; Lane 5, 4.036kb *Nco*I fragment digested with *Sal*I.

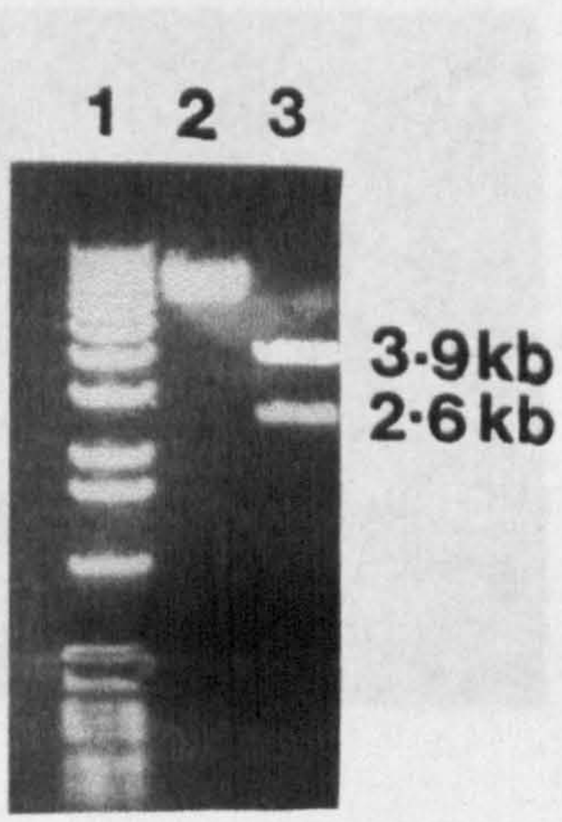


Figure 7.5

0.8% agarose gel showing cloning of the fragment h^T PCR product into the pCRII T-tailing vector.

Lane 1, 1kb ladder; Lane 2, pCRII containing fragment h^T (*Sal*I digest); Lane 3, pCRII containing fragment h^T (*Sal*I / *Bam*HI digest).

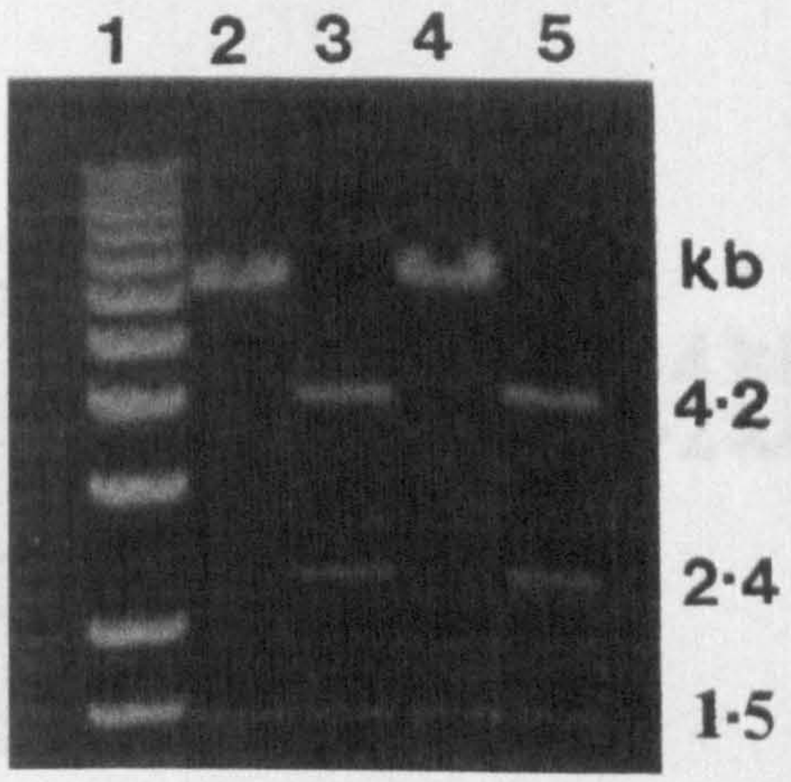


Figure 7.6

0.8% agarose gel showing fragments a^T and h^T fused together in the pCRII T-tailing vector to produce the truncated toxin fragment.

Lane 1, 1kb ladder; Lanes 2 and 4, pCRII clones containing fused truncated toxin fragment (*Nco*I digest); Lanes 3 and 5, as 2 and 4 (*Nco*I and *Bam*HI digests).

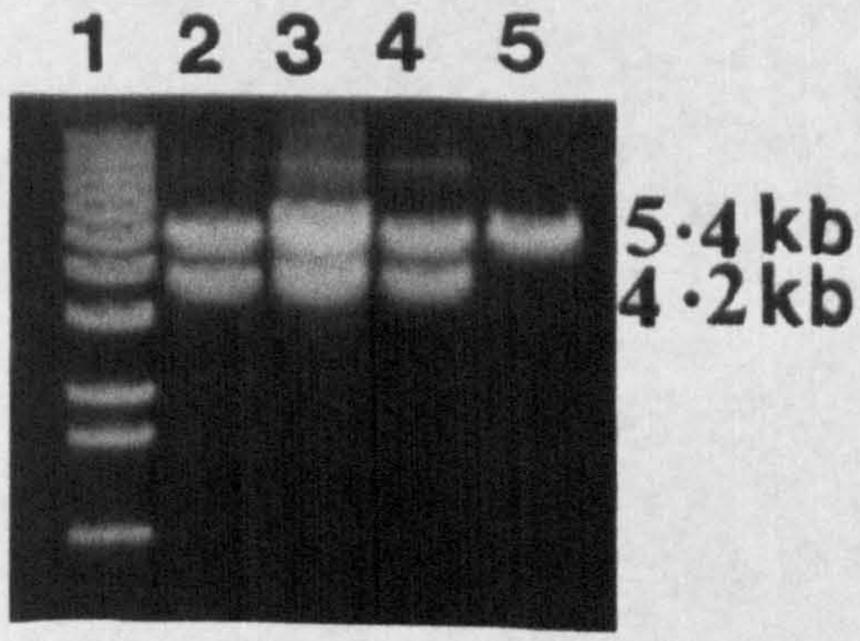


Figure 7.7

0.8% agarose gel showing sub-cloning of the truncated toxin fragment into the pET30a expression vector.

Lane 1, 1kb ladder; Lanes 2-4, pET30a containing truncated toxin A DNA insert (*Nco*I and *Bam*HI digest); Lane 5, pET30a only (*Nco*I and *Bam*HI digest).

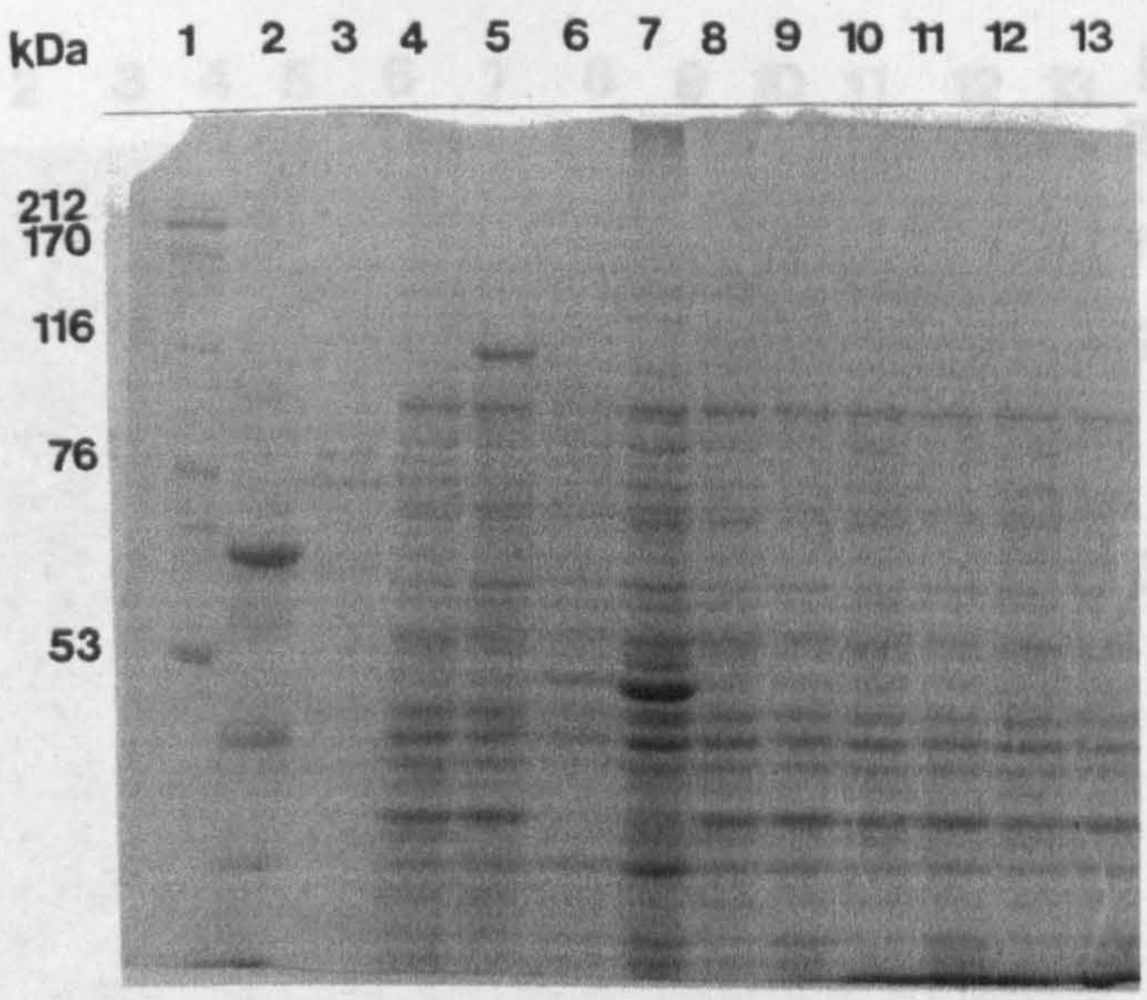


Figure 7.8

An 8% SDS-PAGE gel stained with CBB showing induction of expression of the truncated toxin peptide from the pET30a vector. Insoluble and soluble fractions are shown.

Lane 1, HMW markers, Lane 2, peptide A2; Lane 3, peptide H2; Lane 4, uninduced BL21 DE3 containing pET30a only (insoluble fraction); Lane 5, as lane 4 (soluble fraction); Lane 6, as lane 4 but induced; Lane 7, as lane 5 but induced; Lane 8, uninduced BL21 DE3 containing truncated toxin clone (insoluble fraction); Lane 9, as lane 8 (soluble fraction); Lane 10, as lane 8 but induced; Lane 11, as lane 9 but induced; Lane 12; as lane 9 (loading double); Lane 13, as lane 10 (loading double).

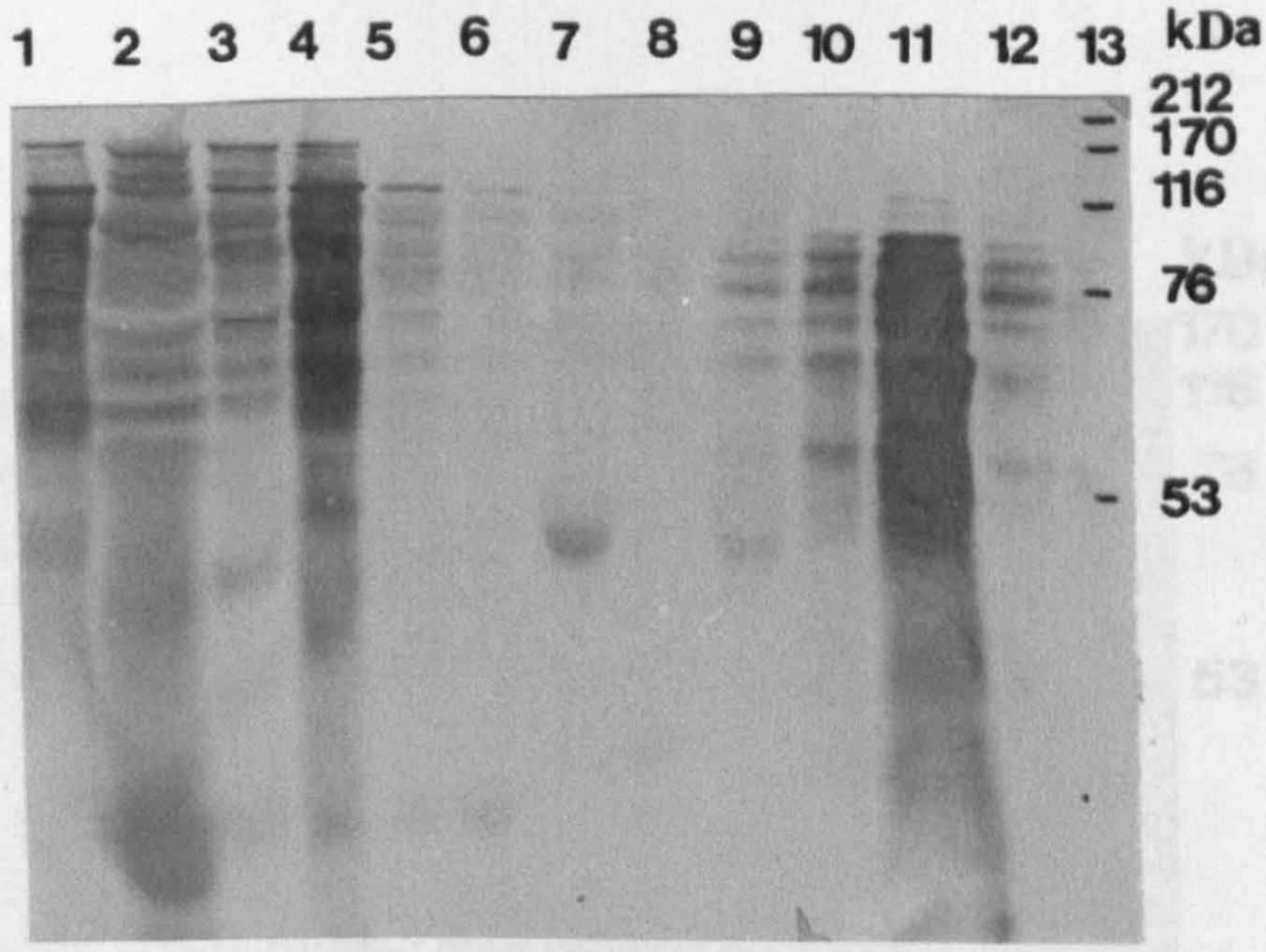


Figure 7.9

Western blot of figure 7.8 probed with the PCG-4 monoclonal antibody.

Lane 1, induced BL21 DE3 containing truncated toxin clone (insoluble fraction, 100µl); Lane 2, as lane 1 but soluble fraction (100µl); Lane 3, as lane 2 but 50µl; Lane 4, as lane 1 but 50µl; Lane 5, as lane 3 but uninduced; Lane 6, as lane 4 but uninduced; Lane 7, induced BL21 DE3 containing pET30a only (soluble fraction, 50µl); Lane 8, as lane 7 but insoluble fraction (50µl); Lane 9, as lane 7 but uninduced; Lane 10, as lane 8 but uninduced; Lane 11, peptide H2; Lane 12, peptide A2; Lane 13, HMW markers.

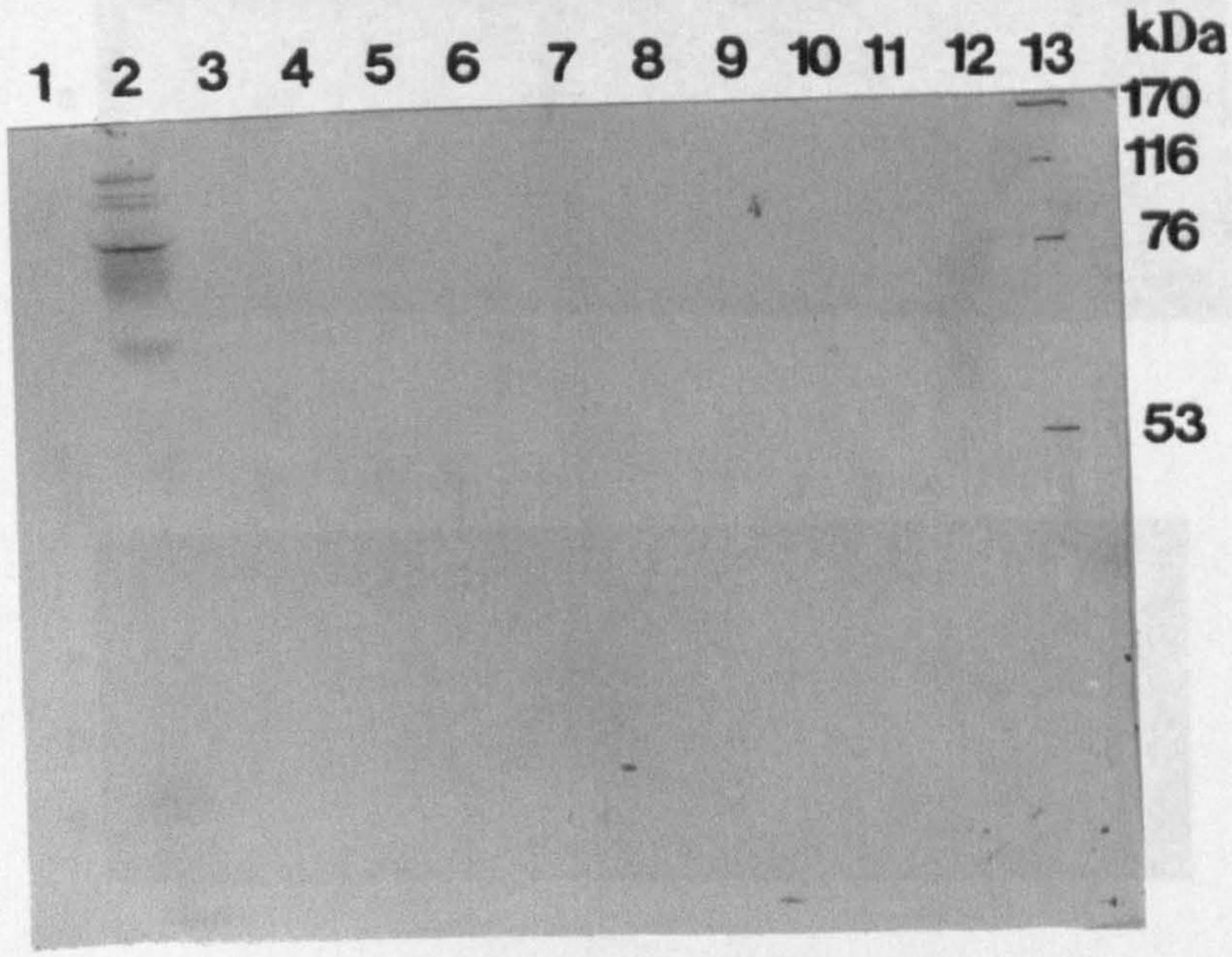


Figure 7.10

Western blot of figure 7.8 probed with the anti-S-Tag antibody (Novagen Inc.).

Lanes 1-13, identical to figure 7.9.

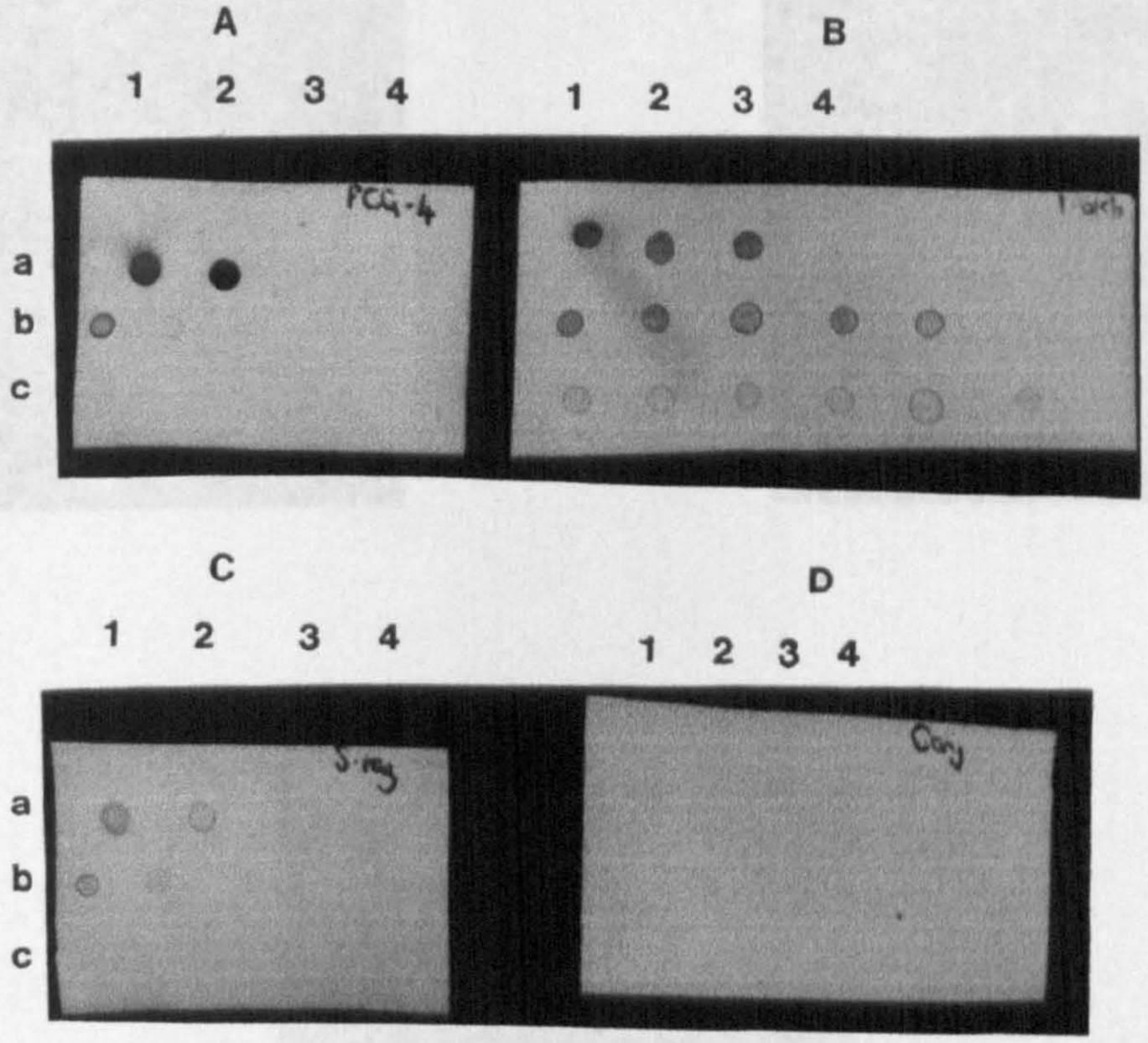


Figure 7.11

Dot blots probed with A) PCG-4 mAb, B) polyclonal anti-peptide A2 antiserum, C) anti-S.Tag antiserum and D) alkaline phosphatase conjugate only showing purification of the truncated toxin fusion product by histidine resin affinity chromatography.

Samples include a1, lysate before application to column; a2, positive control (peptide H2 for panel A, peptide A2 for panel B, S.Tag fusion product [Novagen Inc.] for panel C, all three proteins for panel D); a3, wash prior to elution; a4, elution buffer; rows b and c, 12 x 1ml fractions eluted from column following washing.

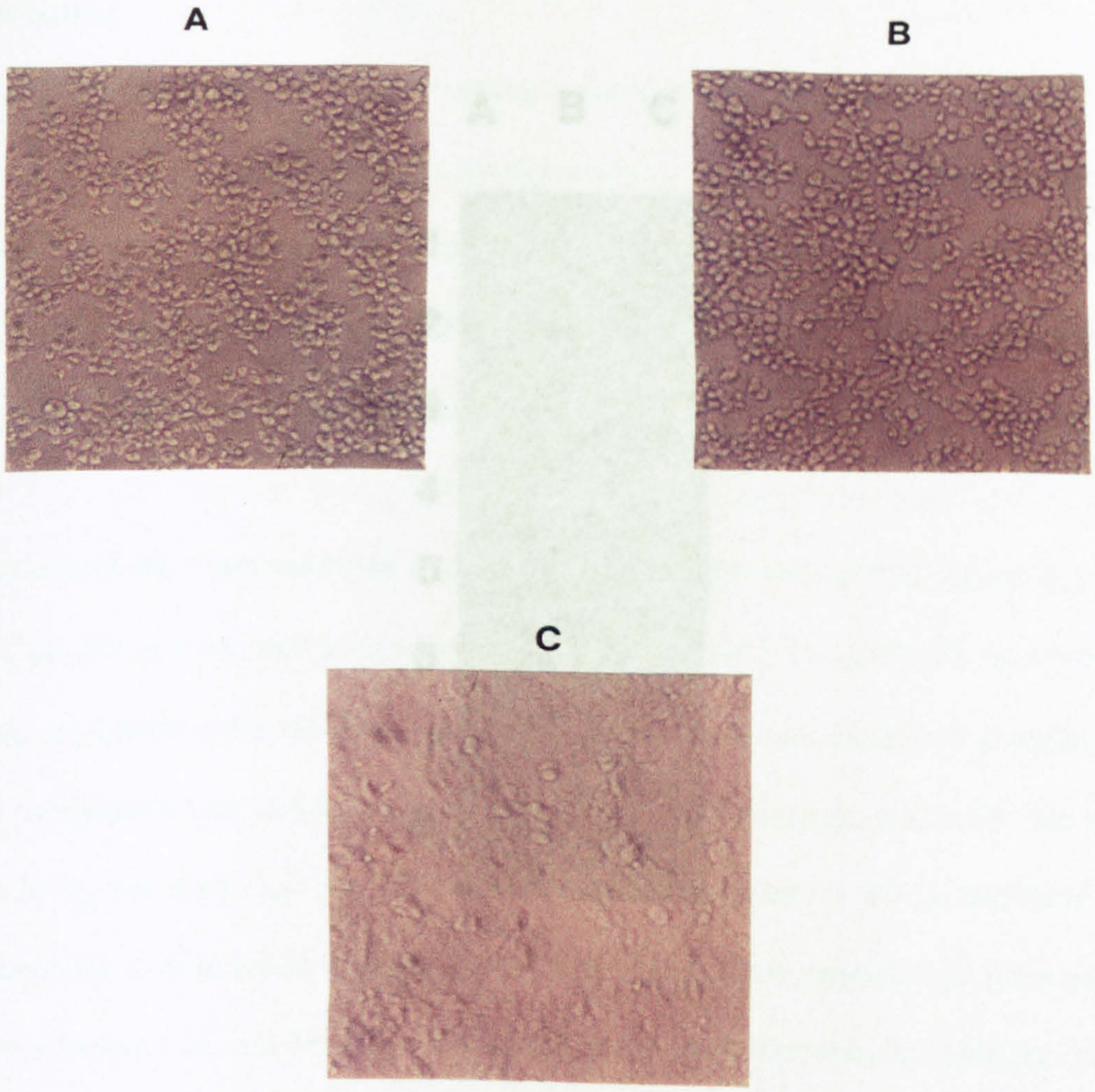


Figure 7.12

Effect of the recombinant truncated toxin on a monolayer of Vero cells after a 24 hr incubation at 37^0C with 5% CO_2. Images viewed under an inverted light microscope at a magnification of x 100.

Panel A, holotoxin A treated monolayer; panel B, recombinant truncated toxin treated monolayer; panel C, an unaffected monolayer.

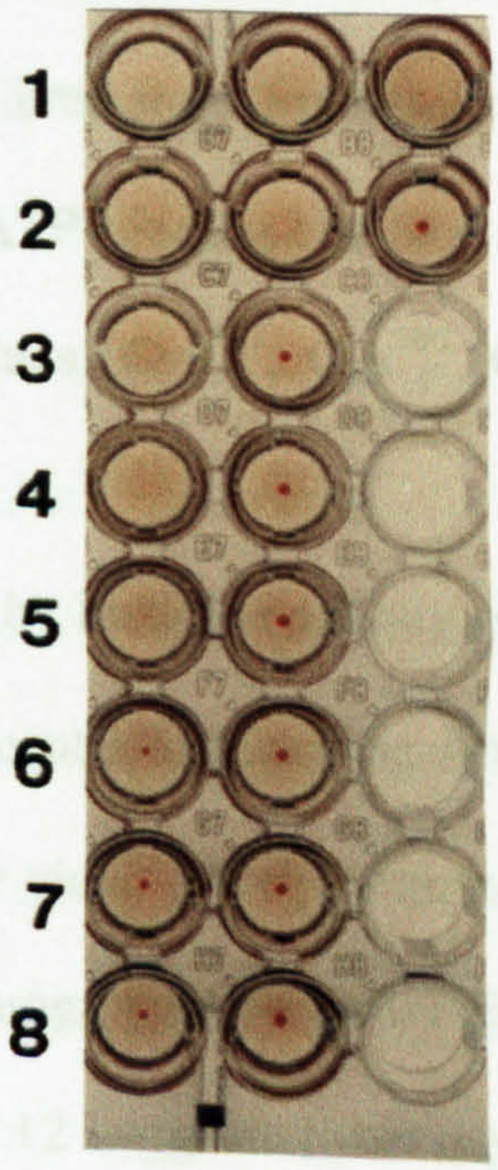


Figure 7.13

Haemagglutination of rabbit red blood cells by the recombinant truncated toxin. Two-fold serial dilutions of A, holotoxin A (100μgml^{-1}) and B, truncated toxin (7.5μgml^{-1}) can be seen going down the microtitre plate.

Wells A1-A8, 1:2 – 1:256 dilutions of holotoxin A; B1-B8, neat – 1:128 dilutions of truncated toxin; C1, holotoxin A (positive control); C2, histidine column elution buffer (negative control).

7.5 Discussion

This section was successful in constructing a biologically active truncated toxin which consisted of only the glucosyltransferase domain (A^T) fused to the binding domain (H^T) of toxin A. The truncated toxin differed from the holotoxin by lacking the central region (aa 539-1834) which contains an ATP-binding site (see section 6), a hydrophobic region and four conserved cysteine residues (Cys^{597}, Cys^{700}, Cys^{1169}, Cys^{1623}) (Barroso *et al*, 1994).

Purification of the truncated toxin by histidine affinity chromatography typically yielded 1ml of soluble protein with a concentration ranging from 5-10μg/ml. There are several possible explanations for this poor yield. Firstly, expression of clostridial proteins in *E. coli* is problematic due to codon usage (see section 3-Discussion). Secondly, the results shown in figures 7.10, 7.11 and 7.12 suggested that a proportion of the truncated toxin was insoluble. It is possible that this proportion of the fusion peptide had been put into inclusion bodies and was therefore being lost prior to purification by histidine affinity chromatography. Finally, the repeat end binding portion (H^T) of the truncated toxin may have been wrapped around the rest of the molecule (as appears to occur in the native holotoxin) masking the histidine fusion and preventing binding to the column.

The PCG-4 Mab bound to both the soluble and insoluble fractions of the truncated toxin whereas the S-Tag antibody only bound to the soluble fraction. The polyclonal peptide A2 antiserum, although appearing to bind to the truncated toxin, had a high level of background non-specific binding to other *E. coli* proteins. Both sequence data and the positive recognition of full length truncated toxin by the PCG-4 Mab indicated that the fusion protein was in the correct reading frame.

Both holotoxin A and the truncated toxin haemagglutinated RRBCs with comparable efficiency (ie. end points of 1.563 μgml^{-1} for holotoxin A and 1.858 μgml^{-1} for the truncated toxin). This suggests that the repeat end-binding portion of the truncated toxin (H^T) was biologically active and was binding to RRBCs.

The truncated toxin also induced a classic CPE on Vero cells *in vitro* but appeared approximately 1,400 fold less potent than native toxin A (ie. end points of 1.3 $ngml^{-1}$ for holotoxin A as oppose to 1.86 μgml^{-1} for truncated toxin). The truncated toxin also appeared less stable than holotoxin, with storage for more than one week at 4^0C leading to complete loss of activity (results not shown). As peptide A2 alone did not cause a CPE and peptide H2 alone did not cause a classic CPE, it is reasonable to conclude that delivery of peptide A2 into the cell resulted in the classical effect seen. These results indicate that peptide A2 contains all of the active sites necessary for cellular glucosylation and encompasses the cytotoxicity of the holotoxin. As the nucleotide binding site, the hydrophobic region and the conserved cysteine residues are all absent from the truncated toxin, it would appear that these regions are not essential for induction of cytotoxic effects. This conclusion is supported by the observations that microinjection of a corresponding peptide (amino acids 1-546) of toxin B (Hofmann *et al*, 1997), and glucosylated Rho proteins *per se* (Just *et al*, 1995c), are sufficient to cause cytotoxic effects.

The observed reduction in potency of the truncated toxin compared to the holotoxin provides important information concerning the function of the nucleotide binding site, the internal hydrophobic region and the concerned cysteine residues (see figure 1.1). Other investigators have shown that mutation of the putative nucleotide-binding site (amino acids 651-683) in toxin B caused a 99% reduction in cytotoxicity (Barroso *et al*, 1994).

Removal of the hydrophobic region and Cys^{1167} from toxin B also reduced cytotoxicity (Barroso *et al*, 1994). These results taken together with our observations on toxin A suggest that these conserved features are important for full expression of cytotoxic activity.

It would appear that together the glucosyltransferase domain (A^T) and the binding domain (H^T) of toxin A form an active toxin with a reduced potency. The results from section 5 indicate that the repeat end binding portion of toxin A (peptide H2) is able to bind to cells and be internalised into endosome-like compartments. It is reasonable to assume therefore, that this is also true of the truncated toxin. The reduction in observed cytotoxic activity is likely to be due to the truncated toxin having a reduced efficiency at escaping these compartments to enter the cytoplasm. It is also possible that the truncated toxin is folded in such a way that the repeat end-binding portion is partially masking one or all of the active sites responsible for glucosyltransferase activity. It could be argued that the CPE seen was due to uptake of some of the truncated toxin by generalised pinocytosis. If this were the only route of uptake however, it is reasonable to assume that peptide A2 alone would have been taken up in the same way enabling it also to cause a CPE. No such effect was seen with peptide A2 in Vero cells. The binding portion of toxin A therefore, appears to be aiding the cytotoxic activity of the truncated toxin, most likely by delivering the glucosyltransferase molecule into the cell. Had more time been available it would have been interesting to study the proposed internalisation of the truncated toxin into endosome-like compartments using electron microscopy studies.

The results from this section suggest that although not essential for cytotoxic activity, nucleotide binding and the internal hydrophobic region are important for toxin A stability, efficient endocytosis and / or translocation into the cytosol. The toxin B

hydrophobic region has tentatively been associated with translocation of the toxin into the cytoplasm (Barroso *et al*, 1994). Others have suggested that the putative toxin B ATP binding site may be involved with the interaction of the toxin with the cell shortly after the binding step (Florin and Thelestam, 1984).

It must be remembered that all experimentation in this section has been performed *in vitro*. The fate of the active truncated toxin *in vivo* would be of interest, and may provide evidence of an important role for the nucleotide binding sites of toxins A and B. Also, had more time been available it would have been interesting to study the effects, if any, of the truncated toxin on human intestinal cells or in the rabbit ileal loop model of the disease.

SECTION-8

GENERAL DISCUSSION

SECTION-8

GENERAL DISCUSSION

When expressing a protein as a series of overlapping recombinant peptides it is important to remember that the peptides may not fold correctly to form an active site or, in fact, an active site may be split between two peptides. As such, a given peptide may not truly resemble its homologous region in the native protein. Results from these studies therefore, must be interpreted with caution. Whereas a positive result indicates that a peptide is responsible for a given function, a negative result only implies that a particular peptide is not involved.

Having considered these points the outlined studies were successful in locating the regions of toxin A responsible for several functions. Together, these results support the three-domain model for *C. difficile* toxin A, which appears to be common to all large clostridial cytotoxins (von Eichel Streiber *et al*, 1996).

The C-terminal region of toxin A was shown to be responsible for all activity relating to carbohydrate binding, including the ability to bind non-specifically to some monoclonal antibodies. This ability probably promotes interaction of toxin A with secretory IgA in mucus or may function to mask the toxin from the immune system *in vivo*. This region of the toxin (represented by peptide H2) also demonstrated the ability to bind to cells and to be internalised, probably by receptor mediated endocytosis, into endosome-like compartments within cells. It is possible therefore, that receptor binding alone may be sufficient to stimulate endocytosis suggesting that the ATP binding site and catalytic domain of toxin A are not essential for this activity. This function makes the repeat region of toxin A an ideal delivery molecule for transporting peptides into cells. The repetitive nature of the ligand domain of toxin A allows a multipoint interaction between

the toxin and its cellular receptors, thus increasing the affinity of the toxin for the cells. Results from the antibody mapping studies suggest that native toxin A is more or less covered by its C-terminal repeat region. These results are supported by the observation that *C. difficile* strains which produce truncated toxin A which lacks the repeat region are not detected by polyclonal anti-toxin A antiserum (Kato *et al*, 1999). It is not surprising therefore, that the polyclonal antiserum raised against peptide H2 in these studies neutralised the toxin A induced CPE on Vero cells. This protection was most likely due to the antiserum blocking binding of the toxin to its target cell.

These studies were also first in locating the catalytic domain of toxin A to a 536 amino acid peptide (peptide A2) at the extreme N-terminus of the molecule. The first 113 amino acids of toxin A, although not solely responsible for glucosyltransferase activity, were shown to be essential for full catalytic activity. This region, in *C. sordellii* lethal toxin at least, does not appear to be involved in binding UDP-glucose (Busch *et al*, 1998) and does not define substrate specificity (Hofmann *et al*, 1998). It is likely therefore, that amino acids 1-113 of toxin A are essential for the correct folding of the catalytic domain and expression of its activity.

Some of these results are in keeping with those of toxin B where the enzyme activity has been located to the first 546 amino acids at the N-terminus of the molecule (Hofmann *et al*, 1997). The first 517 amino acids of toxin B had exceptionally weak enzyme activity suggesting that amino acids 517-546 are important for glucosyltransferase activity (Hofmann *et al*, 1997). The published observations on toxin B and those presented here on toxin A suggest that site directed mutagenesis of the twenty amino acids 517-536 within the N-terminal peptide A2 of toxin A would further delineate the catalytic domain. Polyclonal antibody mapping studies suggest that at least part of the fragment encoding

the catalytic domain of toxin A is exposed in the native state. The failure of peptide A2 antiserum to neutralise the toxin A induced CPE, however, suggests that the active sites responsible for glucosyltransferase activity are not fully exposed in the native state. It is likely that intracellular processing of the toxin results in a change in the tertiary structure of the molecule exposing these catalytic domains. This may explain why the induction of a CPE by microinjection and *in vitro* glucosylation requires several fold higher amounts of toxin than does intoxication by the normal route (Popoff *et al*, 1996, Mueller *et al*, 1992).

These studies also provide the first localisation of the toxin A ATP-binding site to a peptide covering amino acids 542-859 of the holotoxin. Polyclonal antibody mapping studies indicate that this region is unexposed in formalised holotoxin. The binding of toxin A to an ATP agarose column demonstrated here, and in a previous study (Lobban and Borriello, 1992) however, indicates that the ATP binding site is accessible in at least a fraction of the toxin A preparation. Therefore, there is either differential accessibility to ATP and polyclonal antibodies or unfolding of the toxin A molecule under aerobic conditions. There is some evidence for unfolding in that antibody to peptides B and E (both containing the ATP binding site) reacted with unfixed but not fixed toxin A. It is unlikely therefore that formalised toxin A would bind to ATP agarose.

The localisation of the ATP binding site in toxin A supports the identification of a putative ATP binding site in toxin B between amino acids 653-685 (Hofmann *et al*, 1997). It has been suggested that this ATP binding site may be involved in co-ordinating the UDP-glucose cofactor during modification (von Eichel-Streiber *et al*, 1996). It is of interest, therefore, that peptide A2, which possessed glucosyltransferase activity lacked the nucleotide-binding site. These observations suggest that the ATP binding site is not

involved in Rho glucosylation and fails to identify a role for the nucleotide-binding site. However, the truncated toxin A in this present study (which lacks the ATP binding site, hydrophobic region and conserved cysteine residues) was 1,000 fold less cytotoxic than the holotoxin *in vitro*. Mutation of the nucleotide-binding site in toxin B has also been shown to cause a 99% reduction in cytotoxicity (Barroso *et al*, 1994). These results suggest that the ATP binding site and the hydrophobic region are not essential for cytotoxicity but are required for stability and / or efficient translocation of the toxin into the cytosol. There are several other possible explanations that alone, or in combination, may explain the reduced activity of the truncated toxin. Firstly if the truncated toxin lacks the ability to escape endosomes, it is possible that the effect seen on Vero cells was due only to the toxin that had been internalised by pinocytosis. Another suggestion is that the glucosyltransferase domain of the recombinant truncated toxin was not as active as the corresponding region in the native holotoxin. Finally it is possible that the truncated toxin was folded in such a way that the repeat end-binding portion of the molecule was masking the catalytic domain and thus hindering glucosyltransferase activity. The effect of the truncated toxin in rabbit ileal loops, on human intestinal biopsy tissue and in the hamster model of the disease *in vivo* requires further investigation.

The major problem encountered in these studies was in producing adequate amounts of the toxin A peptides. Low levels of expression of some of the constructs in *E. coli* were a result of poor codon usage. Some regions of toxin A required amplification with two of three different sets of PCR primers before a sufficient level of expression was obtained. Although eventually successful, the main and most time consuming step of this PhD was the production of the toxin A constructs. These studies highlight the desperate need for an efficient cloning system for clostridial genes. Had time allowed, alternative-cloning

systems might have been employed for some of the more problematic regions of toxin A. Different hosts such as the gram-positive lactococcal species or *Bacillus subtilis* may be more compatible for expression of clostridial genes. Cloning systems for these organisms however are far more difficult to establish than for *E. coli*. Alternatively insect cells or yeast cells are possibilities. It should be pointed out that there is no guarantee that expression in these systems would yield better results than in *E. coli*.

The toxin A peptides provide an important tool for future research into the structure-function relationships of toxin A. Solving the 3-D structure of the holotoxin and the toxin A peptides by X-ray crystallography would clarify which regions of the molecule are exposed in the native state, and may be helpful in determining potential receptor binding sites. The data obtained should add finer detail to the preliminary tertiary structure predictions made in this thesis by antibody reaction profiling.

The involvement of the ATP binding site in toxin A induced cell damage also requires further investigation. An isogenic deletion mutant of *C. difficile* in which the ATP binding site is inactivated in the toxin A gene would be useful. Electron microscopy studies could then be used to directly compare uptake of the mutated toxin and the native holotoxin into cells. Time course studies such as these may provide data to reinforce the hypothesis that the ATP binding site is involved in efficient translocation of the toxin into the cytoplasm.

Many investigators have provided evidence to suggest that binding of toxin A to its intestinal cell receptor stimulates transmembrane signalling events which trigger fluid secretion and immune cells prior to Rho glucosylation (Pothoulakis *et al*, 1988, Castagliuolo *et al*, 1994). The observation in these studies that peptide H2 (repeat end binding portion) is not only internalised into endosome-like compartments within cells

but that it also alters the morphology of the cells tends to support this theory. Recently published data indicates that early intracellular signalling of *C. difficile* toxin A in rat ileum involves a protein tyrosine kinase-dependent pathway (Castagliuolo *et al*, 1998b). Protein tyrosine kinase activity in the ileal mucosa was increased three fold just 15 minutes after toxin A administration and significant secretory and inflammatory changes were evident after 1 hour. It would be interesting to repeat these experiments using just peptide H2 to determine whether binding alone to a cell surface receptor is sufficient to trigger these responses. In our studies no fluid accumulation was observed in rabbit ileal loops treated with peptide H2. It is possible that the concentration of peptide H2 in these experiments was too low to cause a significant fluid response. Another possibility is that binding to the cell surface receptor only stimulates a mild secretory effect because the signal is continually being removed as the toxin is down regulated into endosomes allowing the receptor to be recycled back to the membrane. It would be interesting to determine whether binding of peptide H2 to human intestinal cells also stimulates a tyrosine kinase dependent pathway. The postulated signalling capacity of toxin A probably explains the different biological activities of toxins A and B. This raises the question as to whether the toxin B repeats, in isolation, have any additional biological activity.

The apparent resistance of neonates to the damaging effects of toxin A may also be explained by intracellular signalling mechanisms. It is possible that the intestinal cell receptor for toxin A in neonates is immature, and perhaps binding to this receptor does not trigger the intracellular signalling pathways which, in adults, result in endocytosis of the toxin. Recent observations have indicated that the secretory component of secretory IgA in human milk binds to toxin A and may function as a receptor analogue, protecting

human infants from *C. difficile* associated disease (Dallas and Rolfe, 1998). Removal of the carbohydrate residues from the secretory component reduced toxin A binding. It seems likely, however, that a combination of factors are responsible for the resistance of human neonates to the damaging effects of toxin A.

There is evidence to suggest that natural immunity against toxin A does protect some humans and hamsters against *C. difficile* associated diarrhoea and PMC (Kyne *et al*, 1999, Giannasca *et al*, 1999). Antibodies to recombinant *C. difficile* toxins have also been shown to be an effective treatment for preventing relapse of *C. difficile* associated disease in hamsters (Kink and Williams, 1998). With an increasing elderly population and an expanding use of broad-spectrum antimicrobials, *C. difficile* is likely to become an increasing problem. The long-term control of this disease therefore relies on the production of an effective vaccine that protects all susceptible individuals. The vaccine potential of some of the toxin A peptides produced in this study therefore warrants investigation. Caution should be taken if considering amino acids 1-536 (peptide A2), which encompass the catalytic domain of the molecule, as a potential vaccine candidate. The C-terminal repeat-end of toxin A (peptide H2) is an obvious vaccine candidate for several reasons. Firstly, antiserum to this region protects against the toxin A induced cytopathic effect. Secondly, the repeat binding region of toxin A is known to associate with mucus and intestinal cells thus encouraging a localised immunity. Finally, this region is acid stable making it an ideal candidate for an orally delivered vaccine. This peptide is also likely to wrap around the outside of an attached peptide thus protecting it from the effects of low pH in the gut.

Recent studies have also shown that mice immunised with the repeat region of toxin A were protected against a three-fold lethal dose of toxin A (Sauerborn *et al*, 1997). The

demonstration that the repeat region of toxin A (peptide H2) alone had an effect on cells in our studies raises a note of caution over the possible use of this region as a vehicle for the formulation and delivery of oral vaccines. Recent studies, however, have been successful in using the repeat region of toxin A as a vaccine candidate against *C. difficile* associated disease (Ward *et al*, 1997, 1999). In the first of these studies the repeat region of toxin A was cloned in-frame with fragment C of tetanus toxin and was expressed in an attenuated *Salmonella typhimurium* mutant. This mutant delivered the fusion protein to the intestinal mucosa inducing an anti-toxin A serum response in mice (Ward *et al*, 1997). More recently the same fusion protein was expressed within attenuated *Salmonella typhimurium* BRD509 (*aroA aroD*). Serum from mice immunised intra-nasally or intra-gastrically with the vaccine candidate neutralised toxin A induced cytotoxicity (Ward *et al*, 1997). Intranasal administration appeared to induce higher serum and mucosal toxin A antibody responses than intragastric administration.

These studies, in mice, are extremely encouraging and indicate that the repeat region of toxin A may prove to be an ideal vaccine candidate against *C. difficile* disease. Before vaccines such as these are formulated for humans, however, it may be advisable to determine the exact intracellular signalling pathways that are stimulated by binding of the repeat region of toxin A to cell receptors.

SECTION – 9

REFERENCES

SECTION – 9

REFERENCES

Abrams, GD, Allo, M, Rifkin, GD, Fekety, R and Silva, J. (1980). Mucosal damage mediated by Clostridial toxin in clindamycin-associated colitis. Gut. 21, 493-499.

Ahlgren, T, Florin, I, Jarstrand, C, and Thelestam, M. (1983). Loss of surface fibronectin from human lung fibroblasts exposed to cytotoxin from *Clostridium difficile*. Infect Immun. 39, 1470-1472.

Aktories, K, Barman, M, Ohishi, I, Tsuyama, S, Jakobs, KH and Haberman, E. (1986). Botulinum C2 toxin ADP-ribosylates actin. Nature. 322, 390-392.

Aktories, K, Weller, V and Chatwal, GS. (1987). *Clostridium botulinum* type C produces a novel ADP-ribosyltransferase distinct from C2 toxin. FEBS Lett. 212, 109-113.

Aktories, K, and Just, I. (1995). Monoglucosylation of low-molecular mass GTP-binding Rho proteins by clostridial cytotoxins. Trends in cell biology. 5, 441-443.

Arnon, SS, Mills, DC, Day, PA, Henrickson, RV, Sullivan, NM, and Wilkins, TD. (1984). Rapid death of infant rhesus monkeys injected with *Clostridium difficile* toxins A and B: physiologic and pathologic basis. J Pediatr. 101, 34-40.

Ausubel, FM, Brent, R, Kingston, RE, Moore, DD, Seidman, JG, Smith, JA and Struhl, K (1992). Short Protocols in Molecular Biology, second edition. J. Wiley and Sons, New York.

Baldassari, L, Donelli, G, Cerquetti, M and Mastrantonio, P. (1991). Capsule-like structures in *Clostridium difficile* strains. Microbiologica. 14, 295-300.

Banno, Y, Kobayashi, T, Watanabe, K, Veno, K and Nozawa, Y. (1981). Two toxins (D-1 and D-2) of *Clostridium difficile* causing antibiotic associated colitis: purification and some characterisation. Biochem Int. 2, 629-635.

Banno, Y, Kobayashi, T, Kono, H, Watanabe, K, Veno, K and Nozawa, Y. (1984). Biochemical characterisation and biological actions of two toxins (D-1 and D-2) from *Clostridium difficile*. Revs Inf Dis. 6, 511-520.

Barclay, FE and Borriello, SP. (1982). *In vitro* inhibition of *Clostridium difficile*. European Journal of Chemotherapy and Antibiotics. 2, 155-156.

Barroso, LA, Wang, S-Z, Phelps, CJ, Johnson, JL, and Wilkins, TD. (1990). Nucleotide sequence of *Clostridium difficile* toxin B gene. Nucleic Acids Res. 18, no 13, 4004.

Barroso, LA, Moncrief, JS, Lyerly, DM and Wilkins, TD. (1994). Mutagenesis of the *Clostridium difficile* toxin B gene and effect on cytotoxic activity. Microbiol Pathogen. 16, 297-303.

Bartlett, JG, Onderdonk, AB, Cisneros, RL and Kasper, DL. (1977). Clindamycin-associated colitis due to a toxin producing species of Clostridium in hamsters. J Infect Dis. 136, 701-705.

Bartlett, JG, Chang, T-W, Gurwith, M, Gorbach, SL and Onderdonk, AB. (1978a). Antibiotic-induced enterocolitis in hamsters: studies with eleven agents and evidence to support the pathogenic role of toxin-producing clostridia. Am J Vet Res. 39, 1525-1530.

Bartlett, JG, Moon, M, Chang, T-W, Taylor, N and Onderdonk, AB. (1978b). *Clostridium difficile* in antibiotic associated pseudomembranous colitis. Gastroenterol. 75, 778-782.

Bartlett, JG, Chang, T-W, Gurwith, M, Gorbach, SL and Onderdonk, AB. (1978c). Antibiotic-associated pseudomembranous colitis due to toxin producing clostridia. N Eng J Med. 298, 531-534.

Bartlett, JG. (1984). Antibiotic-associated colitis. Dis Mon. 30, 1-54.

Bartlett, JG. (1990). *Clostridium difficile.* Clinical considerations. Rev Infect Dis. 12, 5243-5251.

Bates, RC, Buret, A, van Helden, DF, Horton, MA and Burns, GF. (1994). Apoptosis induced by inhibition of intercellular contact. J Cell Biol. 125, 403-415.

Bette, P, Mauler, F, von Eichel-Streiber, C, Popoff, MR, and Habermann, E. (1991). A comparative biochemical, pharmacological and immunological study of *Clostridium novyi* alpha toxin, *C. difficile* toxin B and *C. sordellii* lethal toxin. Toxicon. 29, 877-887.

Bisseret, F, Keith, G Rihn, B, Amiri, I, Werneberg, B, Girardot, R, Baldacini, O, Green, G, Nguyen, VK and Moteil, H. (1989). *Clostridium difficile* toxin B: characterisation and sequence of three peptides. J Chromatog. 490, 91-100.

Boquet, P and Duflot, E. (1981). Studies on the role of a nucleoside-phosphate-binding site of diphtheria toxin in the binding of toxin to vero cells or liposomes. Eur J Biochem. 121, 93-98.

Borriello, SP. (1979). *Clostridium difficile* and its toxins in the gastrointestinal tract in health and disease. Res Clin Forums. 1, 33-35.

Borriello, SP and Barclay, FE. (1984). Colonisation resistance to *Clostridium difficile* infection. In: Microecology and Therapy. 14, 75-87. Edited by Rusch, VC, Herborn-Dill, Institute for Microecology.

Borriello, SP, and Welch, AR. (1984). Detection of *Clostridium difficile* toxins. In: Antibiotic-Associated Diarrhoea and Colitis. Edited by Borriello, SP, and Martinus Nijhoff. 49-56. Boston USA.

Borriello, SP and Barclay, FE. (1985). Protection of hamsters against *Clostridium difficile* ileocaecitis by prior colonisation with non-pathogenic strains. J Med Microbiol. 19, 339-350.

Borriello, SP, Barclay, FE, Welch, AR Ketley, JM, Mitchell, TJ, Stephen, J and Griffin, GE. (1985). Host and microbial determinants of the spectrum of *Clostridium difficile* mediated gastrointestinal disorders. Microecol Ther. 15, 231-236.

Borriello, SP and Barclay, FE. (1986). An *in vitro* model of colonisation resistance to *Clostridium difficile* infection. J Med Microbiol. 21, 299-309.

Borriello, SP, Ketley, JM, Mitchell, TJ, Barclay, FE, Welch, AR, Price, AB and Stephen, J. (1987). *Clostridium difficile* – a spectrum of virulence and analysis of putative virulence determinants in the hamster model of antibiotic-associated colitis. J Med Microbiol. 24, 53-64.

Borriello, SP Barclay, FE and Welch, AR. (1988a). Evaluation of the predictive capability of an *in vitro* model of colonisation resistance to *Clostridium difficile* infection. Microb Ecol Hlth Dis. 1, 61-64.

Borriello, SP, Welch, AR, Barclay, FE and Davies, HA. (1988b). Mucosal association by *Clostridium difficile* in the hamster gastrointestinal tract. J Med Microbiol. 25, 191-196.

Borriello, SP, Davies, HA and Barclay, FE. (1988c). Detection of fimbrae amongst strains of *Clostridium difficile*. FEMS Microbiol. Lett. 49, 65-67.

Borriello, SP. (1990). Pathogenesis of *Clostridium difficile* infection of the gut. J Med Microbiol. 33, 207-215.

Borriello, SP, Davies, HA, Kamiya, S, Reed, PJ and Seddon, S. (1990). Virulence factors of *Clostridium difficile*. Revs Inf Dis. 12, suppl 2, S185-S191.

Borriello, SP. (1991). Structure and function of *Clostridium difficile* toxins. In: Molecular Pathogenesis of Gastrointestinal Infections, 161-167. Edited by Wadstrom, T, Makela, PH, Svennerholm, A-M and Wolf-Warz, H. Plenum Press, London.

Borriello, SP, Stewart, S, and Seddon, SV. (1991). Evaluation of the proposed interaction of nucleic acid with *Clostridium difficile* toxins A and B and the effects of nucleases in cytotoxicity. FEMS Microbiol Lett. 84, 51-56.

Borriello, SP, Wren, BW, Hyde, S, Seddon, SV, Sibbons, P, Krishna, MM, Tabaqchali, S, Manek, S and Price, AB. (1992). Molecular, immunological and biological characterisation of a toxin A-negative, toxin B-positive strain of *Clostridium difficile*. Infect Immun. 60, no.10, 4192-4199.

Borriello, SP. (1995). Clostridial Disease of the Gut. Clinical Infectious Diseases. 20, **suppl** 2, 5242-5250.

Branche, WC, Young, VM, Robinet, HG and Marsey, ED. (1963). Effect of colicine **production** on *Escherichia coli* in the normal human intestine. Proceedings of the Society **for** Experimental Biology and Medicine. 114, 198-201.

Braun, V, Hundsberger, T, Leukel, P, Sauerborn, M, and von Eichel-Streiber, C. (1996). **Definition** of the single integration site of the pathogenicity locus in *Clostridium difficile*. **Gene**. 181, 29-38.

Busch, C, Hofmann, F, Selzer, J, Munro, S, Jeckel, D and Aktories, K (1998). A common **motif** of eukaryotic glycosyltransferases is essential for the enzyme activity of large **clostridial** cytotoxins. J Biol Chem. 273, no.31, 19566-19572.

Calderon, GM, Torres-Lopez, J, Lin, T-J, Chavez, B, Hernandez,M, Munoz, O, Befus, **AD** and Enciso, JA (1998). Effects of toxin A from *Clostridium difficile* on mast cell **activation** and survival. Infect Immun. 66, No.6, 2755-2761.

Castagliuolo, I, LaMont, JT, Letourneau, R et al. (1994). Neuronal involvement in the **intestinal** effects of *Clostridium difficile* toxin A and *Vibrio cholerae* enterotoxin in rat **ileum**. Gastroenterol. 107, 657-665.

Castagliuolo,I, Shuck, QK, Keates, A, Kelly, CP, LaMont, JT and Pothoulakis, C. (1996). *Clostridium difficile* toxin A stimulates enterocyte and macrophage production of the **chemokine** M1P-2 in rat ileum. Gastroenterol. 110, no 4, A 878.

Castagliuolo, I, Valenick, L, Riegler, M, LaMont, JT and Pothoulakis, C (1998a). **Oligosaccharides** containing sialic acid and n-acetyl glucosamine mediate *Clostridium difficile* toxin B (TxB) binding and biological effects in human colonic mucosa. **Gastroenterol**. 114, No.4, Pt2 SS, G3889.

Castagliuolo, I, Valenick, L, Previte, GJ, Pasha, A, Wang, CC, Kelly, CP, LaMont, JT **and** Pothoulakis, C (1998b). Early intracellular signalling of *Clostridium difficile* toxin A (TxA) in rat ileum involves a protein tyrosine-kinase-dependant pathway. Gastroenterol. 114, no.4, pt.2, G3888.

Castagliuolo, I, Riegler, MF, Valenick, L, LaMont, JT and Pothoulakis, C (1999). *Saccharomyces boulardii* protease inhibits the effects of *Clostridium difficile* toxins A **and** B in human colonic mucosa. Infect Immun. 67, no.1, 302-307.

Cerquetti, M, Pantosti, A, Stefanelli, P and Mastrantonio, P. (1992). Purification and **characterisation** of an immunodominant 36kDa antigen present in the cell surface of *Clostridium difficile*. Microb Pathog. 13, 271-279.

Chang, T, Bartlett, JG, Gorbach, SL, and Onderdonk, AB. (1978). Clindamycin-induced enterocolitis in hamsters as a model of pseudomembranous colitis in patients. Infect Immun. 20, 526-529.

Chang, TW, Lauerman, M, and Bartlett, JG. (1979). Cytotoxicity assay in antibiotic-associated colitis. J Infect Dis. 140, 765-770.

Chardin, P, Boquet, P, Madaule, P, Popoff, MR, Rubin, EJ and Gill, DM. (1989). The mammalian G-protein rho C is ADP-ribosylated by *Clostridium botulinum* exoenzyme C3 and effects microfilaments in vero cells. EMBO J. 8, 1087-1092.

Chaves-Olarte, E, Florin, I, Boquet, P, Popoff, M, von Eichel-Streiber, C and Thelestam, M. (1996). UDP-Glucose deficiency in a mutant cell line protects against glucosyltransferase toxins from *Clostridium difficile* and *Clostridium sordellii*. J Biol Chem. 271, no 12, 6925-6932.

Chaves-Olarte, E, Weidmann, M, von Eichel-Streiber, C and Thelestam, M (1997). Toxins A and B from *Clostridium difficile* differ with respect to enzymatic potencies, cellular substrate specificities and surface binding to cultured cells. J. Clin. Invest. 100, 1734-1741.

Chen, GT and Inouye, M (1994). Role of AGA/AGG codons, the rarest codons in global gene expression in *Escherichia coli*. Genes Dev. 8, no.21, 2641-2652.

Clark, GF, Krivan, HC, Wilkins, TD and Smith, DF. (1987). Toxin A from *Clostridium difficile* binds to rabbit erythrocyte glycolipids with terminal Galα1-3Galβ1-4GlcNAc sequences. Arch Biochem Biophys. 257, no.1, 217-229.

Cooke, DL and Borriello, SP. (1998). Nonspecific binding of *Clostridium difficile* toxin A to murine immunoglobulins occurs via the Fab component. Infect Immun. 66, no 5, 1981-1984.

Corthier, G, Dubos, F and Raibaud, P. (1985). Modulation of cytotoxin production by *Clostridium difficile* in the intestinal tracts of gnotobiotic mice inoculated with various human intestinal bacteria. Applied and Environmental Microbiology. 49, 250-252.

Czuprynski, C, Johnson, WJ, Balish, E and Wilkins, T. (1983). Pseudomembranous colitis in *Clostridium difficile* monoassociated rats. Infect Immun. 39, 1368-1376.

Dailey, DC, Kaiser, A and Schloemer, RH. (1987). Factors influencing the phagocytosis of *Clostridium difficile* by human polymorphonuclear leukocytes. Infect Immun. 55, 1541-1546.

Dallas, SD and Rolfe, RD (1998). Binding of *Clostridium difficile* toxin A to human milk secretory component. J Med Microbiol. 47, no.10, 879-888.

Davies, HA and Borriello, SP. (1990). Detection of capsule in strains of *Clostridium difficile* of varying virulence and toxigenicity. Microb Pathogen. 9, 141-146.

Dillon, ST, Rubin, EJ, Yakubovich, M, Pothoulakis, C, LaMont, JT, Feig, LA, and **Gilbert**, RJ. (1995). Involvement of Ras-related Rho proteins in the mechanism of action **of** *Clostridium difficile* toxin A and toxin B. Infect Immun. 63, 1421-1426.

Dodson, AP and Borriello, SP. (1996). *Clostridium difficile* infection of the gut. J Clin **Pathol**. 49, 529-532.

Donta, ST and Mayers, MG. (1982). *Clostridium difficile* toxin in asymptomatic **neonates**. J Pediatr. 100, 431-434.

Donta, ST, Sullivan, N, and Wilkins, TD. (1982). Differential effects of *Clostridium difficile* toxins on tissue-cultured cells. J. Clin Microbiol. 15, 1157-1158.

Dove, CH, Wang, S-Z, Price, SB, Phelps, CJ, Lyerly, DM, Wilkins, TD, and Johnson, JL. (1990). Molecular characterisation of the *Clostridium difficile* toxin A gene. Infect **Immun**. 58, 480-488.

Duerden, BI. (1994). Virulence factors in Anaerobes. Clin Inf Dis. 18, suppl 4, S253-S259.

Eckmann, L, Jung, HC, Maly, CS, Parija, A, Wroblewska, EM and Kagnoff, MF. (1993). Differential cytokine expression by human intestinal epithelial cell lines: regulated expression of 1L-8. Gastroenterol. 105, 1689-1697.

Ehrich, M, van Tassel, RL, Libby, JM and Wilkins, TD. (1980). Production of *Clostridium difficile* antitoxin. Infect Immun. 28, 1041-1043.

Eisel, U, Jarausch, W, Goretzki, K, Henschen, A, Engels, J, Weller, U, Hudel, M, Habermann, E and Niemann, H (1986). Tetanus toxin: primary structure, expression in *Escherichia coli*, and homology with botulinum toxins. EMBO J. 5, 2495-2502.

Ellis-Pegler, RB, Crabtree, C and Lambert, HP. (1975). The faecal flora of children in the United Kingdom. J Hygiene. 75, 135-142.

Elmer, GW, Surawitz, CM, McFarland, LV and Chinn, J. (1989). An open trial of vancomycin plus *Saccharomyces boulardii* for the treatment of relapsing *Clostridium difficile* diarrhoea/colitis. In: Microecology and Therapy 19, 251. Edited by Dougherty, SH Hentges, DJ, Lyerly, DM, Rolfe, RD, Rusch, VC and Walker, RI. Herborn-Dill, Institute for Microecology.

Evans, DG, Evans, DJ and Pierce, NF (1973). Differences in the response of rabbit small intestine to heat-labile and heat stable enterotoxins of *Escherichia coli*. Infect Immun. 7, 873-880.

Eveillard, M, Fowel, V, Barc, M-C, Kerneis, S, Coconnier, M-H, Karjalainen, T, Bourlioux, P and Servin, A. (1993). Identification and characterisation of adhesive

factors of *Clostridium difficile* involved in adhesion to human colonic enterocyte-like Caco-2 and mucus-secreting HT29 cells in culture. Mol Microbiol. 7, 371-381.

Fainstein, V, Bodey, GP and Fekety, R. (1981). Relapsing pseudomembranous colitis associated with cancer chemotherapy. J Infect Dis. 143, 865.

Fairweather, NF, Lyness, VA, Pickard, DJ, Allen, G and Thomson, RO (1986). Cloning, nucleotide sequencing and expression of tetanus toxin fragment C in *Escherichia coli*. J Bacteriol. 165, 21-27.

Faust, C, Ye, B and Song, K-P (1998). The enzymatic domain of *Clostridium difficile* toxin A is located within its N-terminal region. Biochem Biophys Res. Comm. 251, 100-105.

Fekety, R Silva, J, Toshniwal, R, Allo, M, Armstrong, J, Browne, R, Ebright, J and Rifkin, G. (1979). Antibiotic-associated colitis: effects of antibiotics on *Clostridium difficile* and the disease in hamsters. Rev Infect Dis. 1, 386-397.

Finney, JM. (1893). Gastro-enterostomy for cicatrizing ulcer of the pylorus. John Hopkins, Hosp Bull. 11, 53-55.

Fiorentini, C, Malorni, W, Paradisi, S, Guiliano, M, Manstrantonio, P, and Donelli, G. (1990). Interaction of *Clostridium difficile* toxin A with cultured cells: cytoskeletal changes and nuclear polarisation. Infect Immun. 58, 2329-2336.

Fiorentini, C, and Thelestam, M. (1991). Interaction of cytopathogenic toxin from *Clostridium difficile* and it's effects on cells. Toxicon. 29, 543-567.

Fiorentini, C, Donelli, G, Nicotera, P, and Thelestam, M. (1993). *Clostridium difficile* toxin A elicits Ca^{2+}-independent cytotoxic effects in cultured normal rat intestinal crypt cells. Infect Immun. 61, 3988-3993.

Fiorentini, C, Fabbri, A, Falzano, L, Fattorossi, A, Matarrese, P, Rivabene, R and Donelli, G (1998). *Clostridium difficile* toxin B induces apoptosis in intestinal cultured cells. Infect Immun. 66, No.6, 2660-2665.

Flegel, WA, Muller, F, Daubener, W, Fischer, H-G, Haddin, V and Northoff, F. (1991). Cytokine response by human monocytes to *Clostridium difficile* toxin A and toxin B. Infect Immun. 59, 3659-3666.

Florin, I and Thelestam, M. (1983). Internalisation of *Clostridium difficile* cytotoxin into cultured human lung fibroblasts. Biochem Biophys Acta. 763, 383-392.

Florin, I and Thelestam, M. (1984). Polyphosphate-mediated protection from cellular intoxication with *Clostridium difficile* toxin B. Biochem Biophys Acta. 805, 131-136.

Florin, I and Thelestam, M. (1991). ADP-ribosylation in *Clostridium difficile* toxin-treated cells is not related to cytopathogenicity of toxin B. Biochem Biophys Acta. 1091, 51-54.

Fluit, AC, Wolfhagen, MJHM, Verdonk, GPHT, Jansze, M, Torensma, R and Verhoef, J. (1991). Non-toxigenic strains of *Clostridium difficile* lack the genes for both toxins A and B. J Clin Microbiol. 29, no 11, 2666-2667.

Forsgren, A and Sjoquest, B. (1966). 'Protein A' from *Staphylococcus aureus* 1. Pseudo-immune reaction with human γ-globulin. J Immunol. 97, 822-827.

Forsgren, A and Grubb, AO. (1979). Many bacterial species bind human IgG. J Immunol. 122, 1468-1472.

Frey, SM and Wilkins, TD. (1992). Localisation of two epitopes recognised by monoclonal antibody PCG-4 on *Clostridium difficile* toxin A. Infect Immun. 60, no 6, 2488-2492.

Furlong, J, Meighan, M, Corner, J, Murray, J and Clements, JB (1992). Methods for improved protein expression using pET vectors. Nucl Acid Res. 20, no.17, 4668.

Galili, V, Shohet, SB, Cobrin, E, Stults, CLM, and Macher, BA. (1988). Man, apes and old world monkeys differ from other mammalians in the expression of α-galactosyl epitopes on nucleated cells. J Biol Chem. 263, 17755-17762.

George, RH, Symmonds, JM, Dimmock, F, Brown, JD, Arabi, Y, Shinagawa, N, Keighley, MRB, Alexander-Williams, J and Burdon, DW. (1978). Identification of *Clostridium difficile* as a cause of pseudomembranous colitis. B Med J. 1, 695.

George, WL, Sutter, VL, Goldstein, EJC, Ludwig, SL and Finegold, SM. (1978). Aetiology of antimicrobial agent associated colitis. Lancet. 1, 802-803.

George, WL, Rolfe, RD and Finegold, SM. (1982). *Clostridium difficile* and its cytotoxin in faeces of patients with anti-microbiol agent-associated diarrhoea and miscellaneous conditions. J Clin Microbiol. 15, 1049-1053.

Giannasca, PJ, Zhang, Z-X, Lei, W-D, Boden, JA, Giel, MA, Monath, TP and Thomas, WD (1999). Serum antitoxin antibodies mediate systemic and mucosal protection from *Clostridium difficile* disease in hamsters. Infect Immun. 67, no.2, 527-538.

Goodman, JW (1982). Immunoglobulins 1: structure and function. In: Basic and Clinical Immunology, edition 4, 30-42. Edited by Stites, DP, Stobo, JD, Fudenberg, HH and Wells, JV. Lange Medical Publications, Los Altos, California.

Gorbach, SL Chang, TW and Goldin, B. (1987). Successful treatment of relapsing *Clostridium difficile* colitis with Lactobacillus GG. Lancet. 26, 1519.

Green, GA, Schue, V, and Monteil, H. (1995). Cloning and characterisation of the cytotoxin L-encoding gene of *Clostridium sordellii* : homology with *Clostridium difficile* toxin B. Gene. 161, 57-61.

Griffin, GE, McDougall, A, Ash, SA and Borriello, SP. (1984). Age related susceptibility of hamsters to clindamycin induced *Clostridium difficile* caecitis. Microecol Ther. 14, 269-270.

Guiliano, M, Piemonte, F and Manstrantonio Gianfrilli, P. (1988). Production of an enterotoxin different from toxin A by *Clostridium difficile*. FEMS Microbiol Lett. 50, 191-194.

Hafiz, S. (1974). *Clostridium difficile* and its toxins. Leeds: University of Leeds Ph.D thesis.

Hall, IC and O'Toole, E. (1935). Intestinal flora in new-born infants, with description of new pathogenic anaerobe, *Bacillus difficilis*. Am J Dis Child. 49, 390-402.

Hammarstrom, S, Perlmann, P, Gustafsson, BE and Lagercrantz, R. (1969). Autoantibodies to colon in germ free rats monocontaminated with *Clostridium difficile*. J Exp Med. 129, 747-756.

Hammond, GA, and Johnson, JL. (1995). The toxigenic element of *Clostridium difficile* strain VPI 10463. Microbiol Pathogen. 19, 203-213.

Hartwig, JH, Bokoch, GM, Carpenter, CL, Janmey, PA, Taylor, LA, Toker, A and Stossel, TP. (1995). Thrombin receptor ligation and activated Rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets. Cell. 82, 643-653.

Haslam, SC, Ketley, JM, Mitchell, TJ, Stephen, J, Burdon, DW and Candy, DCA. (1986). Growth of *Clostridium difficile* and production of toxins A and B in complex and defined media. J Med Microbiol. 21, 293-297.

Henriques, B, Florin, I and Thelestam , M. (1987). Cellular internalisation of *Clostridium difficile* toxin A. Microbiol Pathogen. 2, 455-463.

Hill, LR (1966). An index to deoxyribonucleic acid base compositions of bacterial species. J Gen Microbiol. 44, 419-437.

Hofmann, F, Hermann, A, Habermann, E and von Eichel-Streiber, C. (1995). Sequencing and analysis of the gene encoding the alpha toxin of *Clostridium novyi* proves its homology to toxins A and B of *Clostridium difficile*. Mol Gen Genet. 247, 670-679.

Hofmann, F, Busch, C, Prepens, U, Just, I and Aktories, K (1997). Localization of the glucosyltransferase activity of *Clostridium difficile* toxin B to the N-terminal part of the holotoxin. J Biol Chem. 272, no.17, 11074-11078.

Hofmann, F, Busch, C and Aktories, K (1998). Chimeric clostridial cytotoxins: identification of the N-terminal region involved in protein substrate recognition. Infect Immun. 66, no.3, 1076-1081.

Hummel, RP, Altmeier, WA and Hill, EO. (1964). Iatrogenic staphylococcal enterocolitis. Ann Surg. 160, 551.

Humphrey, CD, Condon, CW, Cantey, JR and Pitmann, FE. (1979). Partial purification of a toxin found in hamsters with antibiotic-associated colitis. Gastroenterol. 76, 478-486.

Hundsberger, T, Braun, V, Weidmann, M, Leukel, P, Sauerborn, M and von Eichel-Streiber, C. (1997). Transcription analysis of the genes *tcdA-E* of the pathogenicity locus of *Clostridium difficile*. Eur J Biochem. 224, 735-742.

Johnson, JL, Dove, CH, Price, SB, Sickles, TW, Phelps, CJ and Wilkins, TD (1988). The toxin A gene of *Clostridium difficile*. In: Anaerobes Today, chp.13, 115-123. Edited by: Hardie, JM and Borriello, SP. John Wiley and Sons Ltd.

Johnson, JL, Phelps, C, Barroso, L, Roberts, MD, Lyerly, DM, and Wilkins, TD. (1990). Cloning and expression of the toxin B gene of *Clostridium difficile*. Curr Microbiol. 20, 397-401.

Just, I, Fritz, G, Aktories, K, Giry, M, Popoff, MR, Boquet, P, Hegenbarth, S and von Eichel-Streiber, C. (1994). *Clostridium difficile* toxin B acts on the GTP-binding protein Rho. J Biol. Chem. 269, 10706-10712.

Just, I, Selzer, J, von Eichel-Streiber, C and Aktories, K. (1995a). The low molecular mass GTP-binding protein Rho is effected by toxin A from *Clostridium difficile*. J Clin Invest. 95, 1026-1031.

Just, I, Wilm, M, Selzer, J, Rex, G, von Eichel-Streiber, C, Mann, M, and Aktories, K. (1995b). The enterotoxin from *Clostridium difficile* (Tox A) monoglucosylates the Rho proteins. J Biol Chem. 270, 13932-13936.

Just, I, Selzer, J, Wilm, M, von Eichel-Streiber, C, Mann, M, and Aktories, K. (1995c). Glucosylation of Rho proteins by *Clostridium difficile* toxin B. Nature. 375, 500-503.

Just, I, Selzer, J, Hoffman, F, Green, GA and Aktories, K. (1996). Inactivation of Ras by *Clostridium sordellii* lethal toxin-catalysed glucosylation. J Biol Chem. 271, no 17, 10149-10153.

Justus, PG, Martin, JL, Goldberg, DA, Taylor, NS, Bartlett, JG, Alexander, RW and Mathias, JR. (1982). Myoelectric effects of *Clostridium difficile*: motility-altering factors distinct from its cytotoxin and enterotoxin in rabbits. Gastroenterol. 83, 836-843.

Kamiya, S, Reed, PJ, and Borriello, SP. (1988). Analysis of purity of *Clostridium difficile* toxin A derived by affinity chromatography on immobilised bovine thyroglobulin. FEMS Microbiol Lett. 56, 331-336.

Kamiya, S, Reed, PJ, and Borriello, SP. (1989). Purification and characterisation of *Clostridium difficile* toxin A by bovine thyroglobulin affinity chromatography and dissociation in denaturing conditions with and without reduction. J Med Micro. 30, 69-77.

Kamiya, S, Yamatawa, K, Meng-X, Q, Ogura, H, and Nakamura, S. (1991). Production of monoclonal antibody to *Clostridium difficile* toxin A which neutralises enterotoxicity but not haemagglutination activity. FEMS Microbiol. Lett 81, 311-316.

Kamiya, S and Borriello, SP. (1992). A non-haemagglutinating form of *Clostridium difficile* toxin A. J Med Microbiol. 36, 190-197.

Kapadia, A, Feizi, T, Jewell, D, Keening, J, and Slavin, G. (1981). Immunocytochemical studies of blood groups A, H I and i antigens in gastric mucosae of infants with normal gastric histology and of patients with gastric carcinoma and chronic peptic ulceration. J Clin Pathol. 34, 330-337.

Karjalainen, T, Barc, M-C, Collignon, A, Trolle, S, Boureau, H, Cotte-Lafitte, J and Bourlioux, P. (1994). Cloning of a genetic determinant from *Clostridium difficile* involved in adherence to tissue culture cells and mucus. Infect Immun. 62, no 10, 4347-4355.

Karjalainen, T, Poilane, I, Collignon, A, Barc, M-C, Gomez-Trevino, M, Boureau, H and Bourlioux, P. (1995). *Clostridium difficile* virulence: correlation between toxigenicity enzyme production and serotype. Microecol Ther. 25, 157-163.

Karlsson, K-A, and Larson, G. (1981). Molecular characterisation of cell surface antigens of fetal tissue. J Biol Chem. 256, 3512-3524.

Kato, H, Kato, N, Katow, S, Maegawa, T, Nakamura, S and Lyerly, DM (1999). Deletions in the repeating sequences of the toxin A gene of toxin A-negative, toxin B-positive *Clostridium difficile* strains. FEMS Microbiol Lett. 175, no.2, 197-203.

Kindon, H, Pothoulakis, C, Thim, L, Lynch-Devaney, K and Podolsky, DK. (1995). Trefoil peptide protection of intestinal epithelial barrier function: co-operative interaction with mucin glycoprotein. Gastroenterol. 109, 516-523.

Kink, JA and Williams, JA. (1998). Antibodies to recombinant *Clostridium difficile* toxins A and B are an effective treatment and prevent relapse of *C. difficile*-associated disease in a hamster model of infection. Infect Immun. 66, 2018-2025.

Koch, R. (1884). An address on cholera and it's bacillus. British Medical Journal. 30, 403-407, 453-459.

Komine, Y, Adachi, T, Inokuchi, H and Ozeki, H (1990). Genomic organisation and physical mapping of the transfer RNA gene in *Escherichia coli* K12. J Mol Biol. 212, 579-598.

Korvall, G. (1973). A surface component in group A, C and G streptococci with non-immune reactivity for IgG. J Immunol. 111, 1401-1406

Krivan, HC, Clark, GF, Smith, DF, and Wilkins, TD. (1986). Cell surface binding site for *Clostridium difficile* enterotoxin: evidence for a glycoconjugate containing the sequence Galα1-3Galβ1-4GlcNAc. Infect Immun. 53, 573-581.

Krivan, HC, and Wilkins, TD. (1987). Purification of *Clostridium difficile* toxin A by affinity chromatography on immobilised thryoglobulin. Infect Immun. 55, no.8, 1873-1877.

Kushnaryov, VM and Sedmak, JJ. (1989). Effect of *Clostridium difficile* enterotoxin A on ultrastructure of Chinese hamster ovary cells. Infect Immun. 57, No. 12, 3914-3921.

Kyne, L, Warny, M, Qamar, A, Fatimi, A, Wei, JY and LaMont, JT (1999). Natural immunity against *Clostridium difficile* toxin A protects against diarrhea and pseudomembranous colitis. Gastroenterol. 116, No.4, Pt.2, G3889.

Labbe, S and Grenier, D. (1995). Characterisation of the human immunoglobulin G Fc-binding activity of *Prevotella intermedia*. Infect Immun. 63, 2785-2789.

Laboisse, CL. (1995). Effects of *Clostridium difficile* toxin A on intestinal mucus secretion. Microbiol Ecology in Health and Disease. 8, no 4, 196-197.

Laemmli, UK. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature. 227, 680-685.

Larson, HE, Parry, JV, Price, AB, Davies, DR, Dolby, J and Tyrell, DA. (1977). Undescribed toxin in pseudomembranous colitis. Br Med J. 1, 1246-1248.

Larson, HE, Price, AB, Honour, P and Borriello, SP. (1978). *Clostridium difficile* and the aetiology of pseudomembranous colitis. Lancet. 1, 1063-1066.

Laughan, BE, Viscidi, RP, Gdovin, SL, Yolken, RH and Bartlett, JG. (1984). Enzyme immunoassays for detection of *Clostridium difficile* toxins A and B in faecal specimens. J Inf Dis. 149, 781-788.

Libby, JM and Wilkins, TD. (1982). Production of antitoxins to two toxins of *Clostridium difficile* and immunological comparison of the toxins by cross-neutralisation studies. Infect Immun. 35, no 2, 374-376.

Lima, AAM, Lyerly, DM, Wilkins, TD, Innes, DJ, and Guerrant, RL. (1988). Effects of *Clostridium difficile* toxins A and B in rabbit small and large intestine *in vivo* and on cultured cells *in vitro* . Infect Immun. 56, 582-588.

Linevsky, JK, Pothoulakis, C, Keates, S, Warny, M, Keates, AC, LaMont, JT and Kelly, CP 1997). IL-8 release and neutrophil activation by *Clostridium difficile* toxin-exposed

human monocytes. Am. J. Physiol.-Gastrointest. And Liver Physiol. 36, No.^, G1333-G1340.

Lobban, MD and Borriello, SP. (1992). Specific binding of nucleotides and NAD^+ to *Clostridium difficile* toxin A. FEBS Lett. 298, no 2,3, 185-187.

Lory, S and Collier, RJ. (1980). Diphtheria toxin: nucleotide binding and toxin heterogeneity. Proc Natl Acad Sci. 77, no 1, 267-271.

Lyerly, DM, Sullivan, NM and Wilkins, TD. (1983). ELISA for *Clostridium difficile* toxin A. J Clin Micrbiol. 17, 72-78.

Lyerly, DM, Saum, KE, MacDonald, DK, and Wilkins, TD. (1985a). Effects of *Clostridium difficile* toxins given intragastrically to animals. Infect Immun. 47, 349-352.

Lyerly, DM, Phelps, DJ, and Wilkins, TD. (1985b). Monoclonal and specific polyclonal antibodies for immunoassay of *Clostridium difficile* toxin A. J Clin Microbiol. 21, 12-14.

Lyerly, DM, Roberts, MD, Phelps, CJ and Wilkins, TD. (1986a). Purification and properties of toxins A and B of *Clostridium difficile*. FEMS Microbiol Lett. 33, 31-35.

Lyerly, DM, Phelps, CJ, Toth, J and Wilkins, TD. (1986b). Characterisation of toxins A and B of *Clostridium difficile* with monoclonal antibodies. Infect Immun. 54, no.1, 70-76.

Lyerly, DM, Krivan, HC and Wilkins, TD. (1988). *Clostridium difficile*: Its disease and its toxin. Clinical Microbiol Revs. 1, no 1, 1-18.

Lyerly, DM and Wilkins, TD (1988). Purification and properties of toxins A and B of *Clostridium difficile*. In: *C. difficile*: Its Role in Intestinal Disease, chp.9, 145-167. Edited by: Rolfe, RD and Finegold, SM. Academic Press Inc., London.

Lyerly, DM, Carrig, PE and Wilkins, TD. (1989a). Susceptibility of *Clostridium difficile* toxins A and B to trypsin and chymotrypsin. Microb Ecol Health Dis. 210, 21.

Lyerly, DM, Carrig, PE and Wilkins, TD. (1989b). Non-specific binding of mouse monoclonal antibodies to *Clostridium difficile* toxins A and B. Current Microbiol. 19, 303-306.

Lyerly, DM, Johnson, JL, Frey, SM and Wilkins, TD (1990). Vaccination against lethal *Clostridium difficile* enterocolitis with a nontoxic recombinant peptide of toxin A. Curr Microbiol. 21, 29-32.

Lyerly, DM, Barrosso, LA, Wilkins, TD, Depitre, C and Corthier, G. (1992). Characterisation of a toxin A-negative, toxin B-positive strain of *Clostridium difficile*. Infect Immun. 60, 4633-4639.

Lyerly, DM, and Wilkins, TD. (1995) *Clostridium difficile*. In: Infections of the Gastrointestinal Tract. Edited by Blaser, MJ, Smith, PD and Ravdin, JI. 867-891. Raven Press Ltd, New York.

Lyerly, DM and Allen, SD. (1997). The Clostridia, Chp. 19a, 599-623. In: Principles and Practice of Clinical Bacteriology. Edited by Emmerson, AM, Hawkey, PM and Gillespie, SH. John Wiley and Sons Ltd, England.

Machesky, LM and Hall, A. (1996). Rho: A connection between membrane receptor signalling and the cytoskeleton. Trends in Cell Biol. 6, 304-310.

Mahida, YR, Makh, S, Hyde, S, Gray, T and Borriello SP. (1996). Effect of *Clostridium difficile* toxin A on human intestinal epithelial cells: induction of interleukin-8 production and apoptosis after cell detachment. Gut. 38, 337-347.

Mahida, YR, Galvin, A, Makh, S, Hyde, S, Sanfilippo, L, Borriello, SP and Sewell, HF. (1998). Effect of *Clostridium difficile* toxin A on human colonic lamina propria cells: early loss of macrophages followed by T-cell apoptosis. Infect Immun. 66, 5462-5469.

Malamou-Ladas, H and Tabaqchali, S. (1982). Inhibition of *Clostridium difficile* by faecal streptococci. J Med Microbiol. 15, 569-574.

Maniatis, T, Fritsch, EF and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York. Cold Spring Harbor Laboratory.

Martinez, RD and Wilkins, TD. (1992). Comparison of *Clostridium sordellii* toxins HT and LT with toxins A and B of *Clostridium difficile*. J Med Microbiol. 36, 30-36.

Mastrantonio, P, Pantosti, A, Cerquetti, KM, Molinari, A, Gelosia, A and Donelli, G. (1995). *Clostridium difficile* and its surface proteins. Microecol Ther. 23, 94-101.

McFarland, LV, Elmer, GW and Stamm, WE. (1991). Correlation of immunoblot type enterotoxin production and cytotoxin production with manifestations of *Clostridium difficile* infection in a cohort of hospitalised patients. Infect Immun. 59, 2456-2462.

McFarland, L. (1995). *Clostridium difficile*–associated disease Chp. 6. In: Gastrointestinal and Hepatic Infections. Edited by Surawicz, C, and Owen, RL. WB Saunders Company, USA.

Meador, JIII and Tweten, RK. (1988). Purification and characterisation of toxin B from *Clostridium difficile*. Infect Immun. 56, 1708-1714.

Meynell, GG. (1963). Antibacterial mechanisms of the mouse gut II. The role of Eh and volatile fatty acids in the mouse gut. Brit J Exptl Pathol. 44, 209-219.

Mintz, KP and Fives-Taylor, PM (1994). Identification of an immunoglobulin Fc receptor of *Actinobacillus actinomycetemcomitans*. Infect Immun. 62, 4500-4505.

Mitchell, TJ, Ketley, JM, Haslam, SC, Stephen, S, Burdon, DW, Candy, DCA, and **Daniel**, R. (1986). The effects of toxins A and B of *Clostridium difficile* on rabbit ileum and colon. Gut. 27, 78-85.

Mitchell, TJ, Ketley, JM, Burdon, DW, Candy, DCA and Stephen, J. (1987). Biological mode of action of *Clostridium difficile* toxin A: a novel enterotoxin. J Med Microbiol. 23, 211-219.

Moncrief, JS, Barroso, LA and Wilkins, TD. (1997). Positive regulation of *Clostridium difficile* toxins. Infect Immun. 65, no 3, 1105-1108.

Mueller, H, von Eichel-Streiber, C and Habermann, E. (1992). Morphological changes of cultured endothelial cells after microinjection of toxins that act on the cytoskeleton. Infect Immun. 60, 3007-3010.

Muldrow, LL, Ibeanu, GC, Lee, NI, Bose, NK, and Johnson, J. (1987). Molecular cloning of *Clostridium difficile* toxin A gene fragment in λgt11. FEBS Lett. 213, No 2, 249-253.

Ohguchi, K, Banno, Y, Nakashima, S, Kato, M, Watanabe, K, Lyerly, DM and Nozawa, Y. (1996). Effects of *Clostridium difficile* toxin A and toxin B on phospholipase D activation in human Promyelocytic Leukaemic HL60 cells. Infect Immun. 64, no 11, 4433-4437.

Olson, MF. (1996). Guanine nucleotide exchange factors for the Rho GTPases: a role in human disease? J Mol Med. 74,563-571.

Onderdonk, AB, Cisneros, RL and Bartlett, JG. (1980). *Clostridium difficile* in gnotobiotic mice. Infect Immun. 28, 277-282.

Pantosti, A, Cerquetti, M and Mastrantonio, P. (1988). Electrophoretic characterisation of *Clostridium difficile* strains isolated from antibiotic-associated-colitis and other conditions. J Clin Microbiol. 26, 540-543.

Perelle, S, Gibert, M, Bourlioux, P, Corthier, G and Popoff, M. (1997). Production of a complete binary toxin (actin-specific ADP-ribosyltransferase) by *Clostridium difficile* CD196. Infect Immun. 65, no 4, 1402-1407.

Popoff, MR and Boquet, P. (1988). *Clostridium spiroforme* toxin is a binary toxin which ADP-ribosylates cellular actin. Biochem Biophys Res Commun. 152, 1361-1368.

Popoff, MR, Rubin, EJ, Gill, DM and Boquet, P. (1988). Actin-specific ADP-ribosyltransferase produced by a *Clostridium difficile* strain. Infect Immun. 56, 2299-2306.

Popoff, MR, Chaves-Olarte, E, Lemichez, E, von Eichel-Streiber, C, Thelestam, M, Chardin, P, Cussac, D, Anthony, B, Chavrier, P, Flatau, G, Giry, M, de Gunzberg, J and

Boquet, P (1996). Ras, Rap and Rac small GTP-binding proteins are targets for *Clostridium sordellii* lethal toxin glucosylation. J Biol Chem. 271, 10217-10224.

Pothoulakis, C, Barone, LM, Ely, R, Faris, B, Clark, ME, Franzblau, C and LaMont, JT. (1986). Purification and properties of *Clostridium difficile* cytotoxin B. J Biol Chem. 261, 1316-1321.

Pothoulakis, C, Sullivan, R, Melnick, DA, Triadfilopoulas, G, Gadenne, A-S, Meshulam, T and LaMont, T. (1988). *Clostridium difficile* toxin A stimulates intracellular calcium release and chemotactic response in human granulocytes. J Clin Invest. 81, 1741-1745.

Pothoulakis, C, LaMont, JT, Eglow, R, Gao, N, Rubins,JB, Theoharides, TC and Dickey, BF. (1991). Characterisation of rabbit ileal receptors for *Clostridium difficile* toxin A: evidence for a receptor-coupled G-protein. J Clin Invest. 88, 119-125.

Pothoulakis, C, Galili, V, Castagliuolo, I, Kelly, CP, Nikulasson, S, Dudeja, PK, Brasitus, TA, and LaMont, JT. (1996a). A human antibody binds to α-galactose receptors and mimics the effects of *Clostridium difficile* toxin A in rat colon. Gastroenterol. 110, 1704-1712.

Pothoulakis, C, Gilbert, RJ, Cladaras, C, Castagliuolo, I, Semenza, G, Hitti, Y, Montcrief, JS, Linevsky, J, Kelly CP, Nikulasson, S, Desai, HP, Wilkins, TD, and LaMont, JT. (1996b). Rabbit sucrase-isomaltase contains a functional intestinal receptor for *Clostridium difficile* toxin A. J Clin Invest. 98, No 3, 641-649.

Powell, NBL. (1999). Microscopic approaches to the study of bacterial cell associated virulence determinants. Methods in Microbiol. In press.

Price, AB and Davies, DR. (1977). Pseudomembranous colitis. J Clin Pathol. 30, 1-12.

Price, AB, Larson, HE and Crow, J. (1979). Morphology of experimental antibiotic-associated enterocolitis in the hamster: a model for human pseudomembranous colitis and antibiotic-associated diarrhoea. Gut. 20, 467-475.

Price, SB, Phelps, CJ, Wilkins, TD, and Johnson, JL. (1987). Cloning of the carbohydrate-binding portion of the toxin A gene of *Clostridium difficile*. Curr Microbiol. 16, 55-60.

Qamar, A, Warny, M, Michetti, P, Pothoulakis, C, LaMont, JT and Kelly, CP. (1999). *Sacchromyces boulardii* stimulates an intestinal IgA immune response to *Clostridium difficile* toxin A in mice. Gastroenterology. 116, No. 4, Pt. 2, G3887.

Rehg, JE. (1980). Cecal toxin (s) from guinea pigs with clindamycin-associated colitis, neutralised by *Clostridium sordellii* antitoxin. Infect Immun. 27, 387-390.

Rehg, JE and Pakes, SP. (1982). Implication of *Clostridium difficile* and *Clostridium perfringens* iota toxin in experimental lincomycin-associated colitis of rabbits. Laboratory Animal Science. 32, 253-257.

Reigler, M, Sedivy, R, Pothoulakis, C, Hamilton, G, Zacherl, J, Bischof, G, Cosentini, E, **Feil**, W, Scheissel, R, LaMont, JT, and Wenzl, E. (1995). *Clostridium difficile* toxin B is **more** potent than toxin A in damaging human colonic epithelium *in vitro*. J Clin Invest. **95**, 2004-2011.

Reiner, L, Schlesinger, MJ and Miller, GM. (1952). Pseudomembranous colitis following **aureomycin** and chloramphenicol. Arch Path. 54, 39-67.

Retnoningrum, DS and Cleary, PP (1994). M12 protein from *Streptococcus pyogenes* is a **receptor** for immunoglobulin G3 and human albumin. Infect Immun. 62, 2387-2394.

Rifkin, GD, Fekety, FR, Silva, J, (Jnr) and Sack, RB. (1977). Antibiotic-induced colitis. **Implication** of a toxin neutralised by *Clostridium sordellii* antitoxin. Lancet. 2, 1103-1106.

Rifkin, GD, Fekety, R, and Silva, J. (1978a). Neutralisation by *Clostridium sordellii* **antitoxin** of toxins implicated in clindamycin induced cecitis in the hamster. **Gastroenterology**. 75, 422-424.

Rifkin, GD, Silva, J, (Jnr) and Fekety, FR. (1978b). Gastrointestinal and systemic toxicity **of** fecal extracts from hamsters with clindamycin-induced colitis. Gastroenterol. 74, 52-57.

Rihn, B, Scheftel, JM, Girardot, R and Monteil, H. (1984). A new purification procedure **for** *Clostridium difficile* enterotoxin. Biochem Biophys Res Commun. 124, 690-695.

Rihn, B, Bisseret, F, Girardot, R, Scheftel, JM, Nguyen, VK and Monteil, H. (1988). Fast **protein** purification of *Clostridium difficile* cytotoxin. J Chromatogr. 428, 408-414.

Rocha, MFG, Maia, MET, Bezerra, LRPS, Lyerly, DM, Guerrant, RL, Ribeiro, RA and Lima, AAM. (1997). *Clostridium difficile* toxin A induces the release of neutrophil **chemotactic** factors from rat peritoneal macrophages: role of interleukin-1B, tumour **necrosis** factor alpha and leukotrienes. Infect Immun. 65, no 7, 2470-2476.

Rocha, MFG, Soares, AM, Flores, CA, Steiner, TS, Lyerly, DM, Guerrant, RL, Ribeiro, RA and Lima, AAM. (1998). Intestinal secretory factor released by macrophages stimulated with *Clostridium difficile* toxin A: role of interleukin-1B. Infect Imunn. 66, no 10, 4910-4916.

Rolfe, RD and Finegold, SM. (1979). Purification and characterisation of *Clostridium difficile* toxin. Infect Immun. 25, 191-201.

Rolfe, RD, Helebian S and Finegold, SM. (1981). Bacterial interference between *Clostridium difficile* and normal fecal flora. J Inf Dis. 143, 470-475.

Rolfe, RD and Iaconis, JP. (1983). Intestinal colonisation of infant hamsters with *Clostridium difficile*. Infect Immun. 42, 480-486.

Rolfe, RD. (1991). Binding kinetics of *Clostridium difficile* toxins A and B to intestinal brush border membranes from adult and infant hamsters. Infect Immun. 59, 1223-1230.

Rosenberg, AH, Goldman, E, Dunn, JJ, Studier, FW and Zubay,G. (1993). Effects of consecutive AGG codons on translation in *Escherichia coli* demonstrated with a versatile codon test system. J Bacteriol. 175, 716-722.

Rothman, S, Gentry, M, Brown, J, Foret, D, Stone, M and Strickler, M. (1988). Immunochemical and structural similarities in toxin A and toxin B of *Clostridium difficile* shown by binding to monoclonal antibodies. Toxicon. 26, 583-597.

Roux, E and Yersin, A. (1888). Contribution a l'etude de la dipterie. Annales de l'Institut Pasteur. 2, 629-639.

Ruoslahti, E and Reed, JC. (1994). Anchorage dependence, integrins and apoptosis. Cell. 77, 477-478.

Sauerborn, M, and von Eichel-Streiber, C. (1990). Nucleotide sequence of *Clostridium difficile* toxin A. Nucleic Acids Res. 18, 1629-1630.

Sauerborn, M, Hegenbarth, S, Laufenberg-Feldmann, R. (1994). Monoclonal antibodies discriminating between *Clostridium difficile* toxin A and B. In: Bacterial Protein Toxins. Edited by Freer, J, Aitkin, R, Alouf, JE. 510-511. Gustav, Fischer, Verlang, New York.

Sauerborn, M, Leukel, P and von Eichel-Streiber, C. (1997). The C-terminal ligand-binding domain of *Clostridium difficile* toxin A (TcdA) abrogates TcdA-specific binding to cells and prevents mouse lethality. FEMS Microbiol Lett. 155, No.1, 45-54.

Schiavo, G, Benfenati, F, Poulain, B, Rossetto,O, Polverino de Laureto, P, DasGupta, BR and Montecucco, C. (1992a). Tetanus and Botulinum-B neurotoxins block neurotransmitter release by a protoelytic cleavage of synaptobrevin. Nature. 359, 832-835.

Schiavo, G, Poulain, B, Rossetto, O, Benfenati, F, Tauc L and Montecucco, C. (1992b). Tetanus toxin is a zinc protein and its inhibition of neurotransmitter release and protease activity depend on zinc. EMBO J. 11, 3577-3583.

Schiavo, G, Rossetto, O, Santucci, A, Das Gupta, BR and Montecucco, C. (1992c). Botulinum neurotoxins are zinc proteins. J Biol Chem. 267, 23479-23483.

Schiavo, G and Montecucco, C. (1997). Clostridial Neurotoxins. Chp 14, 169-192. In: Bacterial Toxins. Edited by Aktories, K. Chapman and Hall, Weinheim.

Schmidt, M, Rumenapp, U, Bienek, C, Keller, J, von Eichel-Streiber, C and Jakobs, KH. (1996). Inhibition of receptor signalling to phospholipase D by *Clostridium difficile* toxin B : role of Rho proteins. J Biol Chem. 271, 2422-2426.

Schwan, A Sjolin, S, Trottestam, V and Aronsson, B. (1984). Relapsing *Clostridium difficile* enterocolitis cured by rectal infusion of normal faeces. Scand J Infect Dis. 16, 211-215.

Seal, D, Borriello, SP, Barclay, FE, Welch, A, Piper, M and Bonnycastle, M. (1987). Treatment of relapsing *Clostridium difficile* diarrhoea by administration of a non-toxigenic strain. Eur J Clin Microbiol. 6, 51-53.

Seddon, SV, Hemingway, I and Borriello, SP. (1990). Hydrolytic enzyme production by *Clostridium difficile* and its relationship to toxin production and virulence in the hamster model. J Med Microbiol. 31, 169-174.

Seddon, SV and Borriello, SP. (1992). Proteolytic activity of *Clostridium difficile.* J Med Microbiol. 36, 307-311.

Self, AJ and Hall, A (1995). Purification of recombinant Rho/ Rac/ G25K from *Escherichia coli.* Meth. Enzymol. 256 (B), 3-11.

Selzer, J, Hofmann, F, Rex, G, Wilm, M, Mann, M, Just, I and Aktories, K. (1996). *Clostridium novyi* α–toxin-catalysed incorporation of GlcNAc into Rho subfamily proteins. J Biol Chem. 271, no 41, 25173-25177.

Smith, H. (1995). The revival of interest in mechanisms of bacterial pathogenicity. Biol Rev. 70, 277-316.

Smith, JA, Hyde, S, Borriello, SP and Long, RJ. (1995). The effects of *Clostridium difficile* toxins A and B on the accumulation of the second messenger 1,4,5-triphosphate in membranes prepared from human duodenal biopsies. Gastroenterology. 108, no 4, A326.

Smith, JA, Cooke, DL, Hyde, S, Borriello, SP, and Long, RG. (1997). *Clostridium difficile* toxin A binding to human intestinal epithelial cells. J Med Microbiol. 46, no.11, 953-958.

Smith, LDS and King, EO. (1962). Occurrence of *Clostridium difficile* in infections of man. J Bacteriol. 84, 65-67.

Snyder, MD. (1937). Further studies on *Bacillus difficilis.* J Infect Dis. 60, 223.

Soehn, F, Wagenknecht-Wiesner, A Leukel, P, Kohl, M, Weidmann, M, von Eichel-Streiber, C and Braun, V. (1998). Genetic rearrangements in the pathogenicity locus of *Clostridium difficile* strain 8864—implications for transcription, expression and enzymatic activity of toxins A and B. Mol Gen Genet. 258, no 3, 222-232.

Sparling, PF. (1983). Bacterial Virulence and Pathogenesis: An overview. Reviews of Infectious Diseases. 5, suppl 4, S637-S645.

Steed, PM, Nagar, S and Wennogle, LP. (1996). Phospholipase D regulation by a physical interaction with the actin binding protein gelsolin. Biochem. 35, 5229-5237.

Stephen, J and Pietrowski, RA. (1986). Bacterial toxins, 2nd edition. Van Nastrand Reinhold (UK) co. Ltd.

Sugiyama, T, Mukai, M, Yamashita, R and Sunakawa, K. (1985). Experimental models of *Clostridium difficile* enterocolitis in gnotobiotic mice. 203-206. In: Germ free research: microflora control and its application to the biomedical sciences. Edited by Liss, AR. Alan R Liss, Inc, New York.

Sullivan, NM, Pellet, S, and Wilkins, TD. (1982). Purification and characterisation of toxins A and B of *Clostridium difficile* . Infect Immun. 35, 1032-1040.

Symons,M. (1996). Rho family GTPases: the cytoskeleton and beyond. TIBS. 21, 178-181.

Tabaqchali, S. (1990). Epidemiological markers of *Clostridium difficile*. Rev Inf Dis. 12, suppl 2, S192-S199.

Taylor, NS and Bartlett, JG. (1979). Partial purification and characterisation of a cytotoxin from *Clostridium difficile*. Rev Infect Dis. 1, no 2, 379-385.

Taylor, NS, Thorne, GM and Bartlett, JG. (1981). Comparison of two toxins produced by *Clostridium difficile*. Infect Immun. 34, 1036-1043.

Tedesco, FJ, Barton, FW and Alpers, DH. (1974). Clindamycin-associated colitis. Ann Intern Med. 81, 429-433.

Thall, A, Etienne-Decerf, J, Winand,R, and Galili, V. (1991). The α-galactosyl epitope on mammalian thyroid cells. Acta Endocrinol. 124, 692-699.

Thelestam, M, and Bronnegard, M. (1980). Interaction of cytopathogenic toxin from *Clostridium difficile* with cells in tissue culture. Scand J Infect Dis. 22, 16-29.

Thelestam, M, Florin, I and Chaves-Olarte, E. (1997). *Clostridium difficile* toxins. In: Bacterial Toxins, Tools in Cell Biology and Pharmacology, chp.12. Edited by: K. Aktories. Chapman and Hall, Weinheim.

Torres, JF and Lonnroth, I. (1988a). Purification and characterisation of two forms of toxin B produced by *Clostridium difficile*. FEBS Lett. 233, 417-420.

Torres, JF and Lonnroth, I. (1988b). Comparison of methods for the production and purification of toxin A from *Clostridium difficile*. FEMS Microbiol Lett. 52, 41-46.

Torres, JF and Lonnroth, I. (1989). Production, purification and characterisation of *Clostridium difficile* toxic proteins different from toxin A and toxin B. Biochim Biophys Acta. 998, 151-157.

Torres, JF. (1991). Purification and characterisation of toxin B from a strain of *Clostridium difficile* that does not produce toxin A. J Med Microbiol. 35, 40-44.

Torres, JF, Monath, TP. (1996). Antigenicity of amino acid sequences from *Clostridium difficile* Toxin B. J Med Microbiol. 44, 464-474.

Totten, MA, Gregg, JA, Fremont-Smith, P and Legg, M. (1978). Clinical and pathological spectrum of antibiotic-associated colitis. Am J Gastroenterol. 69, 311-319.

Triadafilopoulos, G, Pothoulakis, C, O'Brien, MJ and LaMont, JT. (1987). Differential effects of *Clostridium difficile* toxins A and B on rabbit ileum. Gastroenterol. 93, 273-279.

Triadafilopoulos, G, Shah, MH and Pothoulakis, C. (1991). The chemotactic response of human granulocytes to *Clostridium difficile* toxin A is age dependent. Am J Gastroenterol. 86, 1461-1465.

Trnka, YM and LaMont, JT. (1984). *Clostridium difficile* colitis. Adv Intern Med. 29, 85-107.

Tucker, KD, and Wilkins, TD. (1989). *Clostridium difficile* toxin A uses Galα1-3-Galβ1-4GlcNAc as a functional receptor. Abstracts of the Annual Meeting of the American Society for Microbiology. 63.

Tucker, KD, and Wilkins, TD. (1991). Toxin A of *Clostridium difficile* binds to the human carbohydrate antigens I, X and Y. Infect Immun. 59, no 1, 73-78.

van Ermengen. (1896) Recherches sur des cas d'accidents alimentaires. Revue Hygiene Police Sanitaire. 18, 761-769.

van Heyningen, WE. (1970). Microbial Toxins. Chp 1, 1-26. Edited by Ajl, SJ, Kadis, S and Montie, TC. Academic Press, New York and London.

von Behring, E and Kitasoto, S. (1890). Veber das Zustandekammen der diptherie-immunitat und die tetanus-immunitat bei thieren. Deutsche Medizinische Wochenschrift. 16, 1113-1114.

von Eichel-Streiber, C Harperath, V, Bosse, D and Hadding, V. (1987). Purification of two high molecular weight toxins of *Clostridium difficile* which are antigenically related. Microbiol Pathogen. 2, 307-318.

von Eichel-Streiber, C, Suckau, D, Wachter, M and Hadding, U (1989). Cloning and characterisation of overlapping DNA fragments of the toxin A gene of *Clostridium difficile*. J Gen Microbiol. 135, 55-64.

von Eichel-Streiber, C, and Sauerborn, M. (1990). *Clostridium difficile* toxin A carries a C-terminal repetitive structure homologous to the carbohydrate binding region of streptococcal glycosyltransferases. Gene. 96, 107-113.

von Eichel-Streiber, C, Laufenberg-Feldmann, R, Sartingen, S, Schulze, J, and Sauerborn, M. (1992). Comparative sequence analysis of *Clostridium difficile* toxins A and B. Mol Gen Genet. 233, 260-268.

von Eichel-Streiber, C, zu Heringdorf, DM, Habermann, E, and Sartingen, S. (1995). Closing in on the toxic domain through analysis of a variant *Clostridium difficile* cytotoxin B. Mol Microbiol. 17, no.2, 313-321.

von Eichel-Streiber, C, Boquet, P, Sauerborn, M and Thelestam, M. (1996). Large clostridial cytotoxins – a family of glycosyltransferases modifying small GTP-binding proteins. Trends in Microbiol. 4, no.10, 375-382.

Wagenknecht-Wiesner, A, Weidmann, M, Braun, V, Leukel, P, Moos, M and von Eichel-Streiber, C (1997). Delineation of the catalytic domain of *Clostridium difficile* toxin B-10463 to an enzymatically active N-terminal 467 amino acid fragment. FEMS Microbiol Lett.152, no.1, 109-116.

Ward, SJ, Douce, G, Dougan, G and Wren, BW (1997). Delivery of non-toxic fragments of *Clostridium difficile* toxin A to the mucosal immune system. Revs Med Microbiol. 8, No.S1, S34-S36.

Ward, SJ, Douce, G, Figueiredo, D, Dougan, G and Wren, BW (1999). Immunogenicity of a *Salmonella typhimurium aroA aroD* vaccine expressing a nontoxic domain of *Clostridium difficile* toxin A. Infect Immun. 67, no.5, 2145-2152.

Wedel, N, Tosselli, P, Pothoulakis, C, Faris, B, Oliver, P, Franzblau, C, and LaMont, JT. (1983). Ultrastructural effects of *Clostridium difficile* toxin B on smooth muscle cells and fibroblasts. Exp Cell Res. 148, 413-422.

Wilson, KH and Perini, F. (1988). Role of competition for nutrients in suppression of *Clostridium difficile* by the colonic microflora. Infect Immun. 56, 2610-2614.

Wren, BW, Clayton, CL, Mullany, PP, and Tabaqchali, S. (1987). Molecular cloning and expression of *Clostridium difficile* toxin A in *Escherichia coli* K12. FEBS Lett. 225, no 1,2, 82-86.

Wren, BW. (1991). A family of clostridial and streptococcal ligand binding proteins with C-terminal repeat sequences. Mol Microbiol. 5, 797-803.

Wren, BW, Russel, RRB, and Tabaqchali, S. (1991). Antigenic cross-reactivity and functional inhibition by antibodies to *Clostridium difficile* toxin A, *Streptococcus mutans* glucan binding protein, and a synthetic peptide. Infect Immun. 59, no 9, 3151-3155.

Yamagishi, T, Serikawa, T, Morita, R, Nakamura, S and Nishida, S. (1976). Persistent **high** numbers of *Clostridium perfringens* in the intestines of Japanese aged adults. **Japanese** Journal of Microbiology. 20, 397-403.

Yarnall, M, Widders, PR and Corbeil, LB. (1988). Isolation and characterisation of Fc **receptors** from *Haemophilus somnus.* Scand J Immunol. 28, 129-137.

Young, M, Minton, NP and Staudenbauer, WL (1989). Recent advances in the genetics of **the** clostridia. FEMS Microbiol Revs. 63, 301-326.

APPENDICES

APPENDIX I

The nucleotide sequence of the coding region of *Clostridium difficile* toxin A. The toxin A gene was first cloned and sequenced by Dove *et al.*, 1990.

```
ATGTCTTTAATATCTAAAGAAGAGTTAATAAAACTCGCATATAGCATTAGACCAAGAGAAAATGAGTATAAAACTATACTAACTAATTTA
                                                                                          90
 M  S  L  I  S  K  E  E  L  I  K  L  A  Y  S  I  R  P  R  E  N  E  Y  K  T  I  L  T  N  L

GACGAATATAATAAGTTAACTACAAACAATAATGAAAATAAATATTTGCAATTAAAAAAACTAAATGAATCAATTGATGTTTTTATGAAT
                                                                                         180
 D  E  Y  N  K  L  T  T  N  N  N  E  N  K  Y  L  Q  L  K  K  L  N  E  S  I  D  V  F  M  N

AAATATAAAACTTCAAGCAGAAATAGAGCACTCTCTAATCTAAAAAAAGATATATTAAAAGAAGTAATTCTTATTAAAAATTCCAATACA
                                                                                         270
 K  Y  K  T  S  S  R  N  R  A  L  S  N  L  K  K  D  I  L  K  E  V  I  L  I  K  N  S  N  T

AGCCCTGTAGAAAAAAATTTACATTTTGTATGGATAGGTGGAGAAGTCAGTGATATTGCTCTTGAATACATAAAACAATGGGCTGATATT
                                                                                         360
 S  P  V  E  K  N  L  H  F  V  W  I  G  G  E  V  S  D  I  A  L  E  Y  I  K  Q  W  A  D  I

AATGCAGAATATAATATTAAACTGTGGTATGATAGTGAAGCATTCTTAGTAAATACACTAAAAAAGGCTATAGTTGAATCTTCTACCACT
                                                                                         450
 N  A  E  Y  N  I  K  L  W  Y  D  S  E  A  F  L  V  N  T  L  K  K  A  I  V  E  S  S  T  T

GAAGCATTACAGCTACTAGAGGAAGAGATTCAAAATCCTCAATTTGATAATATGAAATTTTACAAAAAAAGGATGGAATTTATATATGAT
                                                                                         540
 E  A  L  Q  L  L  E  E  E  I  Q  N  P  Q  F  D  N  M  K  F  Y  K  K  R  M  E  F  I  Y  D

AGACAAAAAAGGTTTATAAATTATTATAAATCTCAAATCAATAAACCTACAGTACCTACAATAGATGATATTATAAAGTCTCATCTAGTA
                                                                                         630
 R  Q  K  R  F  I  N  Y  Y  K  S  Q  I  N  K  P  T  V  P  T  I  D  D  I  I  K  S  H  L  V

TCTGAATATAATAGAGATGAAACTGTATTAGAATCATATAGAACAAATTCTTTGAGAAAAATAAATAGTAATCATGGGATAGATATCAGG
                                                                                         720
 S  E  Y  N  R  D  E  T  V  L  E  S  Y  R  T  N  S  L  R  K  I  N  S  N  H  G  I  D  I  R

GCTAATAGTTTGTTTACAGAACAAGAGTTATTAAATATTTATAGTCAGGAGTTGTTAAATCGTGGAAATTTAGCTGCAGCATCTGACATA
                                                                                         810
 A  N  S  L  F  T  E  Q  E  L  L  N  I  Y  S  Q  E  L  L  N  R  G  N  L  A  A  A  S  D  I

GTAAGATTATTAGCCCTAAAAAATTTTGGCGGAGTATATTTAGATGTTGATATGCTTCCAGGTATTCACTCTGATTTATTTAAAACAATA
                                                                                         900
 V  R  L  L  A  L  K  N  F  G  G  V  Y  L  D  V  D  M  L  P  G  I  H  S  D  L  F  K  T  I

TCTAGACCTAGCTCTATTGGACTAGACCGTTGGGAAATGATAAAATTAGAGGCTATTATGAAGTATAAAAAATATATAAATAATTATACA
                                                                                         990
 S  R  P  S  S  I  G  L  D  R  W  E  M  I  K  L  E  A  I  M  K  Y  K  K  Y  I  N  N  Y  T

TCAGAAAACTTTGATAAACTTGATCAACAATTAAAAGATAATTTTAAACTCATTATAGAAAGTAAAAGTGAAAAATCTGAGATATTTTCT
                                                                                        1080
 S  E  N  F  D  K  L  D  Q  Q  L  K  D  N  F  K  L  I  I  E  S  K  S  E  K  S  E  I  F  S

AAATTAGAAAATTTAAATGTATCTGATCTTGAAATTAAAATAGCTTTCGCTTTAGGCAGTGTTATAAATCAAGCCTTGATATCAAAACAA
                                                                                        1170
 K  L  E  N  L  N  V  S  D  L  E  I  K  I  A  F  A  L  G  S  V  I  N  Q  A  L  I  S  K  Q

GGTTCATATCTTACTAACCTAGTAATAGAACAAGTAAAAAATAGATATCAATTTTTAAACCAACACCTTAACCCAGCCATAGAGTCTGAT
                                                                                        1260
 G  S  Y  L  T  N  L  V  I  E  Q  V  K  N  R  Y  Q  F  L  N  Q  H  L  N  P  A  I  E  S  D

AATAACTTCACAGATACTACTAAAATTTTTCATGATTCATTATTTAATTCAGCTACCGCAGAAAACTCTATGTTTTTAACAAAAATAGCA
                                                                                        1350
 N  N  F  T  D  T  T  K  I  F  H  D  S  L  F  N  S  A  T  A  E  N  S  M  F  L  T  K  I  A
```

CCATACTTACAAGTAGGTTTTATGCCAGAAGCTCGCTCCACAATAAGTTTAAGTGGTCCAGGAGCTTATGCGTCAGCTTACTATGATTTC 1440

P Y L Q V G F M P E A R S T I S L S G P G A Y A S A Y Y D F

ATAAATTTACAAGAAAATACTATAGAAAAAACTTTAAAAGCATCAGATTTAATAGAATTTAAATTCCCAGAAAATAATCTATCTCAATTG 1530

I N L Q E N T I E K T L K A S D L I E F K F P E N N L S Q L

ACAGAACAAGAAATAAATAGTCTATGGAGCTTTGATCAAGCAAGTGCAAAATATCAATTTGAGAAATATGTAAGAGATTATACTGGTGGA 1620

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

TCTCTTTCTGAAGACAATGGGGTAGACTTTAATAAAAATACTGCCCTCGACAAAAACTATTTATTAAATAATAAAATTCCATCAAACAAT 1710

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

GTAGAAGAAGCTGGAAGTAAAAATTATGTTCATTATATCATACAGTTACAAGGAGATGATATAAGTTATGAAGCAACATGCAATTTATTT 1800

V E E A G S K N Y V H Y I I Q L Q G D D I S Y E A T C N L F

TCTAAAAATCCTAAAAATAGTATTATTATACAACGAAATATGAATGAAAGTGCAAAAAGCTACTTTTTAAGTGATGATGGAGAATCTATT 1890

S K N P K N S I I I Q R N M N E S A K S Y F L S D D G E S I

TTAGAATTAAATAAATATAGGATACCTGAAAGATTAAAAAATAAGGAAAAAGTAAAAGTAACCTTTATTGGACATGGTAAAGATGAATTC 1980

L E L N K Y R I P E R L K N K E K V K V T F I G H G K D E F

AACACAAGCGAATTTGCTAGATTAAGTGTAGATTCACTTTCCAATGAGATAAGTTCATTTTTAGATACCATAAAATTAGATATATCACCT 2070

N T S E F A R L S V D S L S N E I S S F L D T I K L D I S P

AAAAATGTAGAAGTAAACTTACTTGGATGTAATATGTTTAGTTATGATTTTAATGTTGAAGAAACTTATCCTGGGAAGTTGCTATTAAGT 2160

K N V E V N L L G C N M F S Y D F N V E E T Y P G K L L L S

ATTATGGACAAAATTACTTCCACTTTACCTGATGTAAATAAAAATTCTATTACTATAGGAGCAAATCAATATGAAGTAAGAATTAATAGT 2250

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

GAGGGAAGAAAAGAACTTCTGGCTCACTCAGGTAAATGGATAAATAAAGAAGAAGCTATTATGAGCGATTTATCTAGTAAAGAATACATT 2340

E G R K E L L A H S G K W I N K E E A I M S D L S S K E Y I

TTTTTTGATTCTATAGATAATAAGCTAAAAGCAAAGTCCAAGAATATTCCAGGATTAGCATCAATATCAGAAGATATAAAAACATTATTA 2430

F F D S I D N K L K A K S K N I P G L A S I S E D I K T L L

CTTGATGCAAGTGTTAGTCCTGATACAAAATTTATTTTAAATAATCTTAAGCTTAATATTGAATCTTCTATTGGGGATTACATTTATTAT 2520

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

GAAAAATTAGAGCCTGTTAAAAATATAATTCACAATTCTATAGATGATTTAATAGATGAGTTCAATCTACTTGAAAATGTATCTGATGAA 2610

E K L E P V K N I I H N S I D D L I D E F N L L E N V S D E

TTATATGAATTAAAAAAATTAAATAATCTAGATGAGAAGTATTTAATATCTTTTGAAGATATCTCAAAAAATAATTCAACTTACTCTGTA 2700

L Y E L K K L N N L D E K Y L I S F E D I S K N N S T Y S V

AGATTTATTAACAAAAGTAATGGTGAGTCAGTTTATGTAGAAACAGAAAAAGAAATTTTTTTCAAAATATAGCGAACATATTACAAAAGAA 2790

R F I N K S N G E S V Y V E T E K E I F S K Y S E H I T K E

ATAAGTACTATAAAGAATAGTATAATTACAGATGTTAATGGTAATTTATTGGATAATATACAGTTAGATCATACTTCTCAAGTTAATACA 2880

I S T I K N S I I T D V N G N L L D N I Q L D H T S Q V N T

TTAAACGCAGCATTCTTTATTCAATCATTAATAGATTATAGTAGCAATAAAGATGTACTGAATGATTTAAGTACCTCAGTTAAGGTTCAA 2970

L N A A F F I Q S L I D Y S S N K D V L N D L S T S V K V Q

CTTTATGCTCAACTATTTAGTACAGGTTTAAATACTATATATGACTCTATCCAATTAGTAAATTTAATATCAAATGCAGTAAATGATACT 3060

L Y A Q L F S T G L N T I Y D S I Q L V N L I S N A V N D T

ATAAATGTACTACCTACAATAACAGAGGGGATACCTATTGTATCTACTATATTAGACGGAATAAACTTAGGTGCAGCAATTAAGGAATTA 3150

I N V L P T I T E G I P I V S T I L D G I N L G A A I K E L

CTAGACGAACATGACCCATTACTAAAAAAAGAATTAGAAGCTAAGGTGGGTGTTTTAGCAATAAATATGTCATTATCTATAGCTGCAACT 3240

L D E H D P L L K K E L E A K V G V L A I N M S L S I A A T

GTAGCTTCAATTGTTGGAATAGGTGCTGAAGTTACTATTTTCTTATTACCTATAGCTGGTATATCTGCAGGAATACCTTCATTAGTTAAT 3330

V A S I V G I G A E V T I F L L P I A G I S A G I P S L V N

AATGAATTAATATTGCATGATAAGGCAACTTCAGTGGTAAACTATTTTAATCATTTGTCTGAATCTAAAAAATATGGCCCTCTTAAAACA 3420

N E L I L H D K A T S V V N Y F N H L S E S K K Y G P L K T

GAAGATGATAAAATTTTAGTTCCTATTGATGATTTAGTAATATCAGAAATAGATTTTAATAATAATTCGATAAAACTAGGAACATGTAAT 3510

E D D K I L V P I D D L V I S E I D F N N N S I K L G T C N

ATATTAGCAATGGAGGGGGGATCAGGACACACAGTGACTGGTAATATAGATCACTTTTTCTCATCTCCATCTATAAGTTCTCATATTCCT 3600

I L A M E G G S G H T V T G N I D H F F S S P S I S S H I P

TCATTATCAATTTATTCTGCAATAGGTATAGAAACAGAAAATCTAGATTTTTCAAAAAAAATAATGATGTTACCTAATGCTCCTTCAAGA 3690

S L S I Y S A I G I E T E N L D F S K K I M M L P N A P S R

GTGTTTTGGTGGGAAACTGGAGCAGTTCCAGGTTTAAGATCATTGGAAAATGACGGAACTAGATTACTTGATTCAATAAGAGATTTATAC 3780

V F W W E T G A V P G L R S L E N D G T R L L D S I R D L Y

CCAGGTAAATTTTACTGGAGATTCTATGCTTTTTTCGATTATGCAATAACTACATTAAAACCAGTTTATGAAGACACTAATATTAAAATT 3870

P G K F Y W R F Y A F F D Y A I T T L K P V Y E D T N I K I

AAACTAGATAAAGATACTAGAAACTTCATAATGCCAACTATAACTACTAACGAAATTAGAAACAAATTATCTTATTCATTTGATGGAGCA 3960

K L D K D T R N F I M P T I T T N E I R N K L S Y S F D G A

GGAGGAACTTACTCTTTATTATTATCTTCATATCCAATATCAACGAATATAAATTTATCTAAAGATGATTTATGGATATTTAATATTGAT 4050

G G T Y S L L L S S Y P I S T N I N L S K D D L W I F N I D

```
AATGAAGTAAGAGAAATATCTATAGAAAATGGTACTATTAAAAAAGGAAAGTTAATAAAAGATGTTTTAAGTAAAATTGATATAAATAAA                4140
 N  E  V  R  E  I  S  I  E  N  G  T  I  K  K  G  K  L  I  K  D  V  L  S  K  I  D  I  N  K

AATAAACTTATTATAGGCAATCAAACAATAGATTTTTCAGGCGATATAGATAATAAAGATAGATATATATTCTTGACTTGTGAGTTAGAT                4230
 N  K  L  I  I  G  N  Q  T  I  D  F  S  G  D  I  D  N  K  D  R  Y  I  F  L  T  C  E  L  D

GATAAAATTAGTTTAATAATAGAAATAAATCTTGTTGCAAAATCTTATAGTTTGTTATTGTCTGGGGATAAAAATTATTTGATATCCAAT                4320
 D  K  I  S  L  I  I  E  I  N  L  V  A  K  S  Y  S  L  L  L  S  G  D  K  N  Y  L  I  S  N

TTATCTAATACTATTGAGAAAATCAATACTTTAGGCCTAGATAGTAAAAATATAGCGTACAATTACACTGATGAATCTAATAATAAATAT                4410
 L  S  N  T  I  E  K  I  N  T  L  G  L  D  S  K  N  I  A  Y  N  Y  T  D  E  S  N  N  K  Y

TTTGGAGCTATATCTAAAACAAGTCAAAAAAGCATAATACATTATAAAAAAGACAGTAAAAATATATTAGAATTTTATAATGACAGTACA                4500
 F  G  A  I  S  K  T  S  Q  K  S  I  I  H  Y  K  K  D  S  K  N  I  L  E  F  Y  N  D  S  T

TTAGAATTTAACAGTAAAGATTTTATTGCTGAAGATATAAATGTATTTATGAAAGATGATATTAATACTATAACAGGAAAATACTATGTT                4590
 L  E  F  N  S  K  D  F  I  A  E  D  I  N  V  F  M  K  D  D  I  N  T  I  T  G  K  Y  Y  V

GATAATAATACTGATAAAAGTATAGATTTCTCTATTTCTTTAGTTAGTAAAAATCAAGTAAAAGTAAATGGATTATATTTAAATGAATCC                4680
 D  N  N  T  D  K  S  I  D  F  S  I  S  L  V  S  K  N  Q  V  K  V  N  G  L  Y  L  N  E  S

GTATACTCATCTTACCTTGATTTTGTGAAAAATTCAGATGGACACCATAATACTTCTAATTTTATGAATTTATTTTTGGACAATATAAGT                4770
 V  Y  S  S  Y  L  D  F  V  K  N  S  D  G  H  H  N  T  S  N  F  M  N  L  F  L  D  N  I  S

TTCTGGAAATTGTTTGGGTTTGAAAATATAAATTTTGTAATCGATAAATACTTTACCCTTGTTGGTAAAACTAATCTTGGATATGTAGAA                4860
 F  W  K  L  F  G  F  E  N  I  N  F  V  I  D  K  Y  F  T  L  V  G  K  T  N  L  G  Y  V  E

TTTATTTGTGACAATAATAAAAATATAGATATATATTTTGGTGAATGGAAAACATCGTCATCTAAAAGCACTATATTTAGCGGAAATGGT                4950
 F  I  C  D  N  N  K  N  I  D  I  Y  F  G  E  W  K  T  S  S  S  K  S  T  I  F  S  G  N  G

AGAAATGTTGTAGTAGAGCCTATATATAATCCTGATACGGGTGAAGATATATCTACTTCACTAGATTTTTCCTATGAACCTCTCTATGGA                5040
 R  N  V  V  V  E  P  I  Y  N  P  D  T  G  E  D  I  S  T  S  L  D  F  S  Y  E  P  L  Y  G

ATAGATAGATATATAAATAAAGTATTGATAGCACCTGATTTATATACAAGTTTAATAAATATTAATACCAATTATTATTCAAATGAGTAC                5130
 I  D  R  Y  I  N  K  V  L  I  A  P  D  L  Y  T  S  L  I  N  I  N  T  N  Y  Y  S  N  E  Y

TACCCTGAGATTATAGTTCTTAACCCAAATACATTCCACAAAAAAGTAAATATAAATTTAGATAGTTCTTCTTTTGAGTATAAATGGTCT                5220
 Y  P  E  I  I  V  L  N  P  N  T  F  H  K  K  V  N  I  N  L  D  S  S  S  F  E  Y  K  W  S

ACAGAAGGAAGTGACTTTATTTTAGTTAGATACTTAGAAGAAAGTAATAAAAAAATATTACAAAAAATAAGAATCAAAGGTATCTTATCT                5310
 T  E  G  S  D  F  I  L  V  R  Y  L  E  E  S  N  K  K  I  L  Q  K  I  R  I  K  G  I  L  S

AATACTCAATCATTTAATAAAATGAGTATAGATTTTAAAGATATTAAAAAACTATCATTAGGATATATAATGAGTAATTTTAAATCATTT                5400
 N  T  Q  S  F  N  K  M  S  I  D  F  K  D  I  K  K  L  S  L  G  Y  I  M  S  N  F  K  S  F
```

AATTCTGAAAATGAATTAGATAGAGATCATTTAGGATTTAAAATAATAGATAATAAAACTTATTACTATGATGAAGATAGTAAATTAGTT 5490

N S E N E L D R D H L G F K I I D N K T Y Y Y D E D S K L V

AAAGGATTAATCAATATAAATAATTCATTATTCTATTTTGATCCTATAGAATTTAACTTAGTAACTGGATGGCAAACTATCAATGGTAAA 5580

K G L I N I N N S L F Y F D P I E F N L V T G W Q T I N G K

AAATATTATTTTGATATAAATACTGGAGCAGCTTTAACTAGTTATAAAATTATTAATGGTAAACACTTTTATTTTAATAATGATGGTGTG 5670

K Y Y F D I N T G A A L T S Y K I I N G K H F Y F N N D G V

ATGCAGTTGGGAGTATTTAAAGGACCTGATGGATTTGAATATTTTGCACCTGCCAATACTCAAAATAATAACATAGAAGGTCAGGCTATA 5760

M Q L G V F K G P D G F E Y F A P A N T Q N N N I E G Q A I

GTTTATCAAAGTAAATTCTTAACTTTGAATGGCAAAAAATATTATTTTGATAATAACTCAAAAGCAGTCACTGGATGGAGAATTATTAAC 5850

V Y Q S K F L T L N G K K Y Y F D N N S K A V T G W R I I N

AATGAGAAATATTACTTTAATCCTAATAATGCTATTGCTGCAGTCGGATTGCAAGTAATTGACAATAATAAGTATTATTTCAATCCTGAC 5940

N E K Y Y F N P N N A I A A V G L Q V I D N N K Y Y F N P D

ACTGCTATCATCTCAAAAGGTTGGCAGACTGTTAATGGTAGTAGATACTACTTTGATACTGATACCGCTATTGCCTTTAATGGTTATAAA 6030

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

ACTATTGATGGTAAACACTTTTATTTTGATAGTGATTGTGTAGTGAAAATAGGTGTGTTTAGTACCTCTAATGGATTTGAATATTTTGCA 6120

T I D G K H F Y F D S D C V V K I G V F S T S N G F E Y F A

CCTGCTAATACTTATAATAATAACATAGAAGGTCAGGCTATAGTTTATCAAAGTAAATTCTTAACTTTGAATGGTAAAAAATATTACTTT 6210

P A N T Y N N N I E G Q A I V Y Q S K F L T L N G K K Y Y F

GATAATAACTCAAAAGCAGTTACCGGATTGCAAACTATTGATAGTAAAAAATATTACTTTAATACTAACACTGCTGAAGCAGCTACTGGA 6300

D N N S K A V T G L Q T I D S K K Y Y F N T N T A E A A T G

TGGCAAACTATTGATGGTAAAAAATATTACTTTAATACTAACACTGCTGAAGCAGCTACTGGATGGCAAACTATTGATGGTAAAAAATAT 6390

W Q T I D G K K Y Y F N T N T A E A A T G W Q T I D G K K Y

TACTTTAATACTAACACTGCTATAGCTTCAACTGGTTATACAATTATTAATGGTAAACATTTTTATTTTAATACTGATGGTATTATGCAG 6480

Y F N T N T A I A S T G Y T I I N G K H F Y F N T D G I M Q

ATAGGAGTGTTTAAAGGACCTAATGGATTTGAATATTTTGCACCTGCTAATACGGATGCTAACAACATAGAAGGTCAAGCTATACTTTAC 6570

I G V F K G P N G F E Y F A P A N T D A N N I E G Q A I L Y

CAAAATGAATTCTTAACTTTGAATGGTAAAAAATATTACTTTGGTAGTGACTCAAAAGCAGTTACTGGATGGAGAATTATTAACAATAAG 6660

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

AAATATTACTTTAATCCTAATAATGCTATTGCTGCAATTCATCTATGCACTATAAATAATGACAAGTATTACTTTAGTTATGATGGAATT 6750

K Y Y F N P N N A I A A I H L C T I N N D K Y Y F S Y D G I

CTTCAAAATGGATATATTACTATTGAAAGAAATAATTTCTATTTTGATGCTAATAATGAATCTAAAATGGTAACAGGAGTATTTAAAGGA 6840

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

CCTAATGGATTTGAGTATTTTGCACCTGCTAATACTCACAATAATAACATAGAAGGTCAGGCTATAGTTTACCAGAACAAATTCTTAACT 6930

P N G F E Y F A P A N T H N N N I E G Q A I V Y Q N K F L T

TTGAATGGCAAAAAATATTATTTTGATAATGACTCAAAAGCAGTTACTGGATGGCAAACCATTGATGGTAAAAAATATTACTTTAATCTT 7020

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

AACACTGCTGAAGCAGCTACTGGATGGCAAACTATTGATGGTAAAAAATATTACTTTAATCTTAACACTGCTGAAGCAGCTACTGGATGG 7110

N T A E A A T G W Q T I D G K K Y Y F N L N T A E A A T G W

CAAACTATTGATGGTAAAAAATATTACTTTAATACTAACACTTTCATAGCCTCAACTGGTTATACAAGTATTAATGGTAAACATTTTTAT 7200

Q T I D G K K Y Y F N T N T F I A S T G Y T S I N G K H F Y

TTTAATACTGATGGTATTATGCAGATAGGAGTGTTTAAAGGACCTAATGGATTTGAATACTTTGCACCTGCTAATACGGATGCTAACAAC 7290

F N T D G I M Q I G V F K G P N G F E Y F A P A N T D A N N

ATAGAAGGTCAAGCTATACTTTACCAAAATAAATTCTTAACTTTGAATGGTAAAAAATATTACTTTGGTAGTGACTCAAAAGCAGTTACC 7380

I E G Q A I L Y Q N K F L T L N G K K Y Y F G S D S K A V T

GGACTGCGAACTATTGATGGTAAAAAATATTACTTTAATACTAACACTGCTGTTGCAGTTACTGGATGGCAAACTATTAATGGTAAAAAA 7470

G L R T I D G K K Y Y F N T N T A V A V T G W Q T I N G K K

TACTACTTTAATACTAACACTTCTATAGCTTCAACTGGTTATACAATTATTAGTGGTAAACATTTTTATTTTAATACTGATGGTATTATG 7560

Y Y F N T N T S I A S T G Y T I I S G K H F Y F N T D G I M

CAGATAGGAGTGTTTAAAGGACCTGATGGATTTGAATACTTTGCACCTGCTAATACAGATGCTAACAATATAGAAGGTCAAGCTATACGT 7650

Q I G V F K G P D G F E Y F A P A N T D A N N I E G Q A I R

TATCAAAATAGATTCCTATATTTACATGACAATATATATTATTTTGGTAATAATTCAAAAGCGGCTACTGGTTGGGTAACTATTGATGGT 7740

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

AATAGATATTACTTCGAGCCTAATACAGCTATGGGTGCGAATGGTTATAAAACTATTGATAATAAAAATTTTTACTTTAGAAATGGTTTA 7830

N R Y Y F E P N T A M G A N G Y K T I D N K N F Y F R N G L

CCTCAGATAGGAGTGTTTAAAGGGTCTAATGGATTTGAATACTTTGCACCTGCTAATACGGATGCTAACAATATAGAAGGTCAAGCTATA 7920

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

CGTTATCAAAATAGATTCCTACATTTACTTGGAAAAATATATTACTTTGGTAATAATTCAAAAGCAGTTACTGGATGGCAAACTATTAAT 8010

R Y Q N R F L H L L G K I Y Y F G N N S K A V T G W Q T I N

GGTAAAGTATATTACTTTATGCCTGATACTGCTATGGCTGCAGCTGGTGGACTTTTCGAGATTGATGGTGTTATATATTTCTTTGGTGTT 8100

G K V Y Y F M P D T A M A A A G G L F E I D G V I Y F F G V

GATGGAGTAAAAGCCCCTGGGATATATGGCTAAAATATATGTTTGATAAAAAATTATTCCTGTGCTACTAAGAAATTATTTTTATATAAT 8190

D G V K A P G I Y G . N I C L I K N Y S C A T K K L F L Y N

AAATATTGAG 8200

K Y .

APPENDIX 2

PCR Primers

A summary of the primers used for the PCR amplification of the toxin A fragments from *C. difficile* VPI 10463 chromosomal DNA. Engineered restriction endonuclease sites are typed in bold and stop codons are underlined.

Primer No.	Primer Sequence (5' – 3')	Location (bp's) in Toxin A Gene (see appendix 1)	Used for the Amplification of Fragments
1	TATTGCTCTT**GGATTC**ATAAAACAA	324 – 348	a1, c
2	CAGAAAGAG**GTCGAC**CAGTCTAATC	1,606 – 1,630	a1, a2
3	**GGATCC**ATGTCTTTAATATCTAAAG AAG	1 - 22	a2, a'
4	TATACTGGT**GGATCC**CTTTCTGAAG	1,609 – 1,633	b, e
5	CATTTTCAA**GTCGAC**TGAACTAATC	2,575 – 2,599	b, c
6	ACTTGAAAAT**GGATCC**GATGAATTA	2,589 – 2,613	d, g
7	TTTATATTC**GTCGAC**ATTGGCTATG	3,989 – 4,013	d, e
8	GGAGCAGGA**GGATCC**TACTCTTTAT	3,955 – 3,979	f,
9	TATATTGATT**AAGCTTTA**AACTAAT	5,484 – 5,508	f, g
10	AATTAGTTAAA**GGATCC**ATCAATAT	5,483 – 5,507	h1,
11	CTCGAAAA**GTCGAC**CAGCTTAAGCC	8,046 – 8,070	h1, h2
12	**CCATGG**GATTAATCAATATAAATAA TTCA	5,495 – 5,517	h2
13	AGATACTA**GTCGAC**ACTATATAATA	601 - 633	a'
14	AG**CCATGG**CTATGTCTTTAATATCT AAAGAA	1- 21	aT
15	CAGAAAGAG**GTCGAC**CAGTATAATC TCT	1,603 – 1,630	aT
16	TTAGTTAAAG**GTCGAC**TCAATATAA AT	5,485 – 5,511	hT
17	CAATCTCGAA**GGATCC**ACCAGCTGC A	8,049 – 8,074	hT

APPENDIX 3
Cloning and Expression Vectors

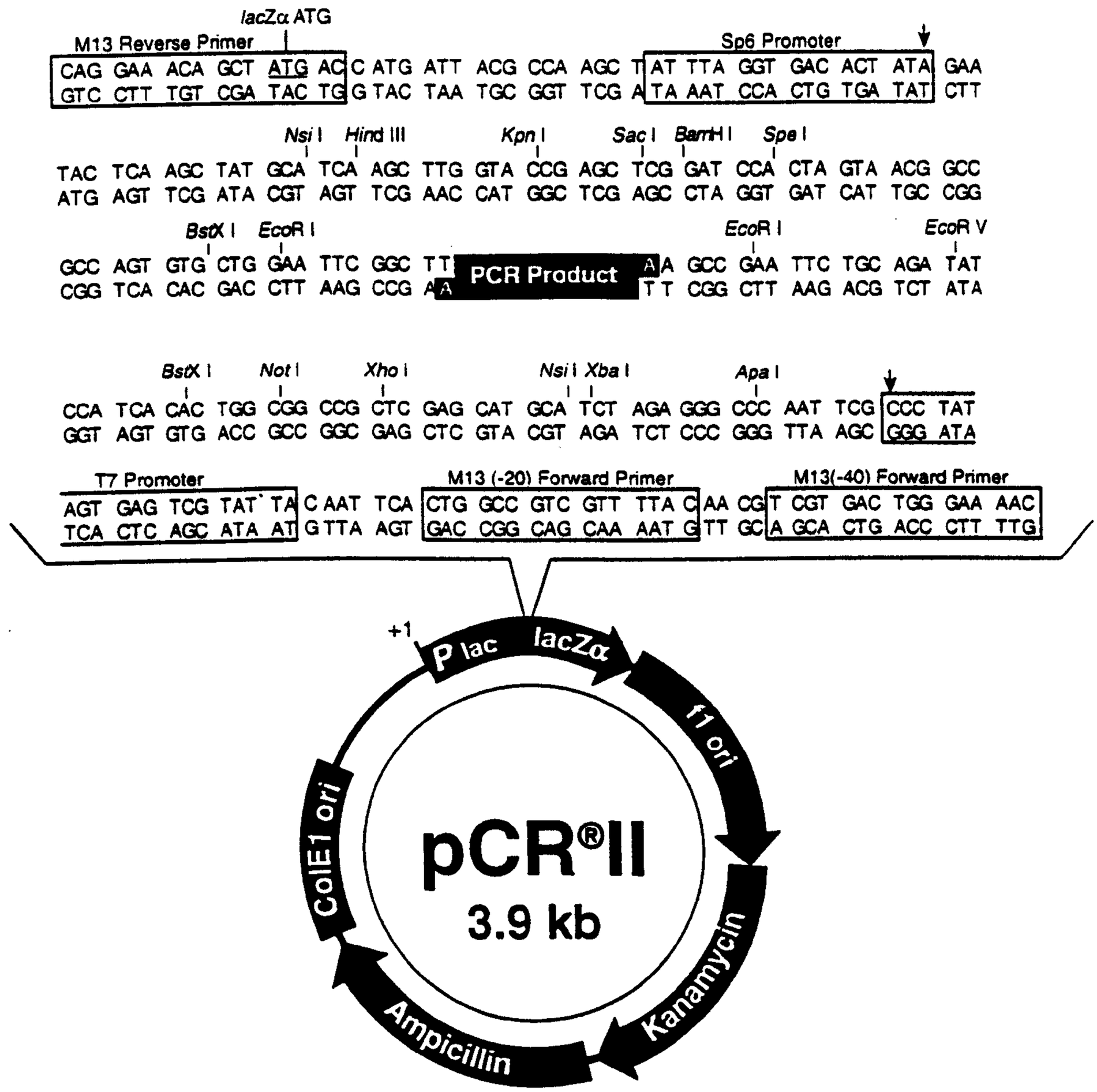
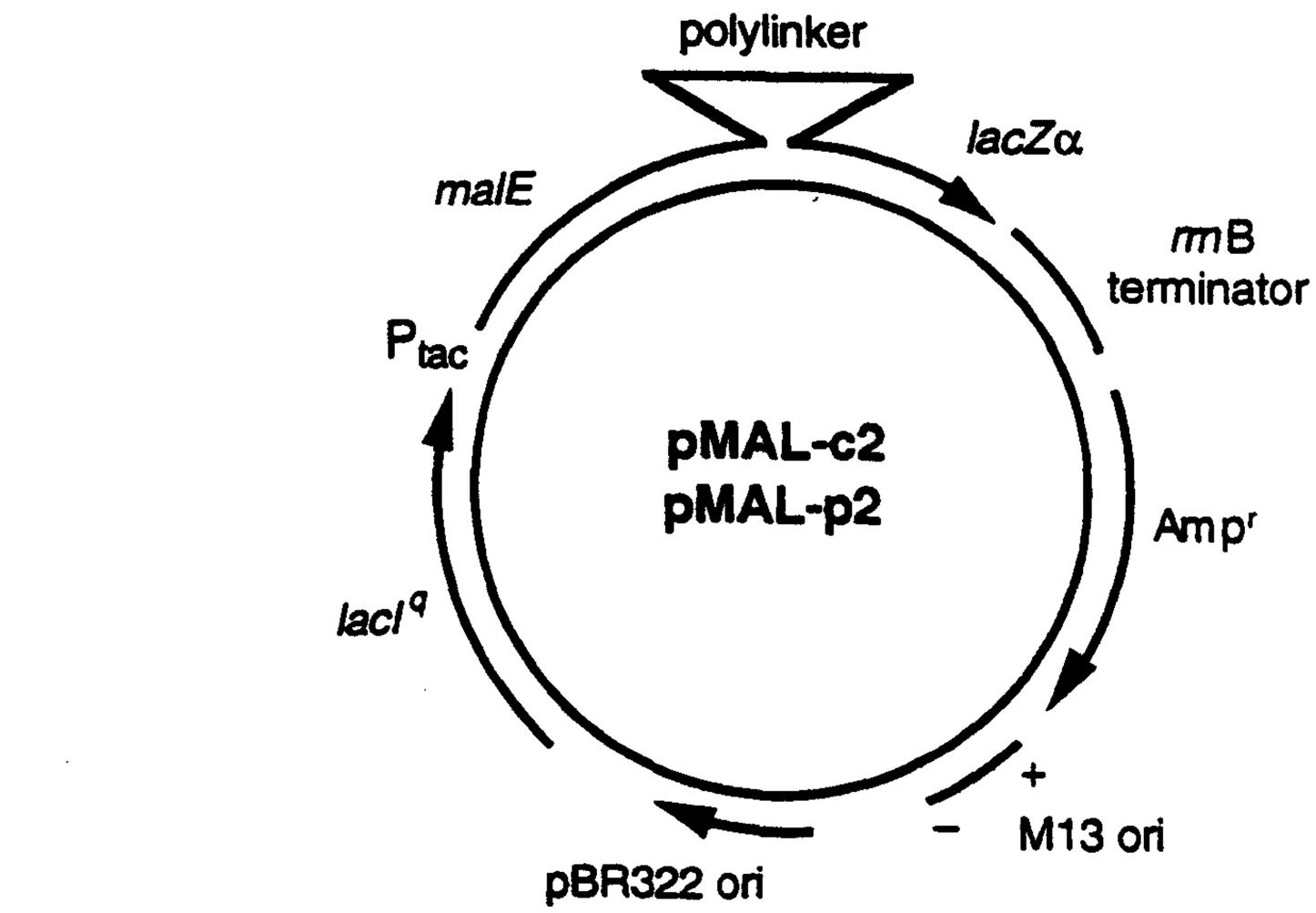


Figure showing the PCRII vector used for cloning PCR products by TA cloning. The restriction endonuclease sites surrounding the cloning region are shown along with the Sp6 and T7 primer positions. (Taken and modified from the 'TA Cloning Kit' instruction manual, Invitrogen).



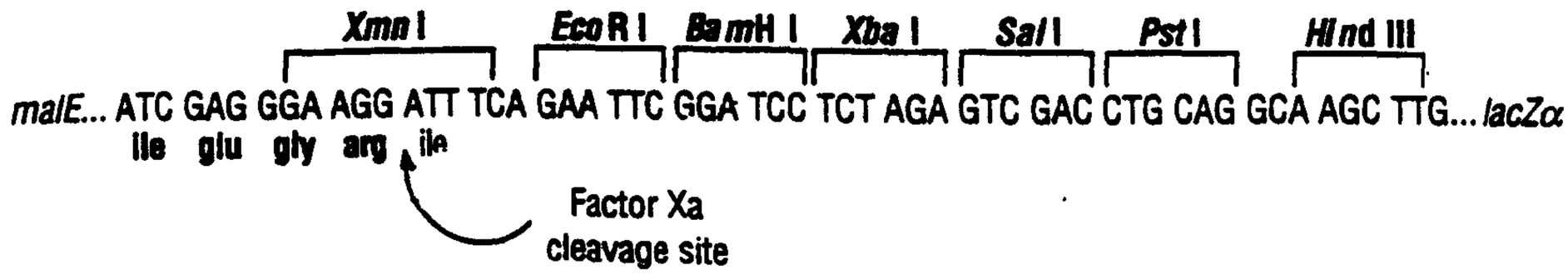


Figure showing the pMal vectors along with the polylinker sequence. PMal-c2 is 6,646 bp's and has an exact deletion of the *malE* signal sequence. PMal-p2 is 6,721 bps and has an intact *malE* signal sequence allowing periplasmic expression. The arrows indicate the direction of transcription. Expression from the pMal vectors allows fusion to a 43 kDa Maltose Binding Protein. (Taken and modified from the 'Protein Fusion and Purification System' instruction manual, New England Biolabs).

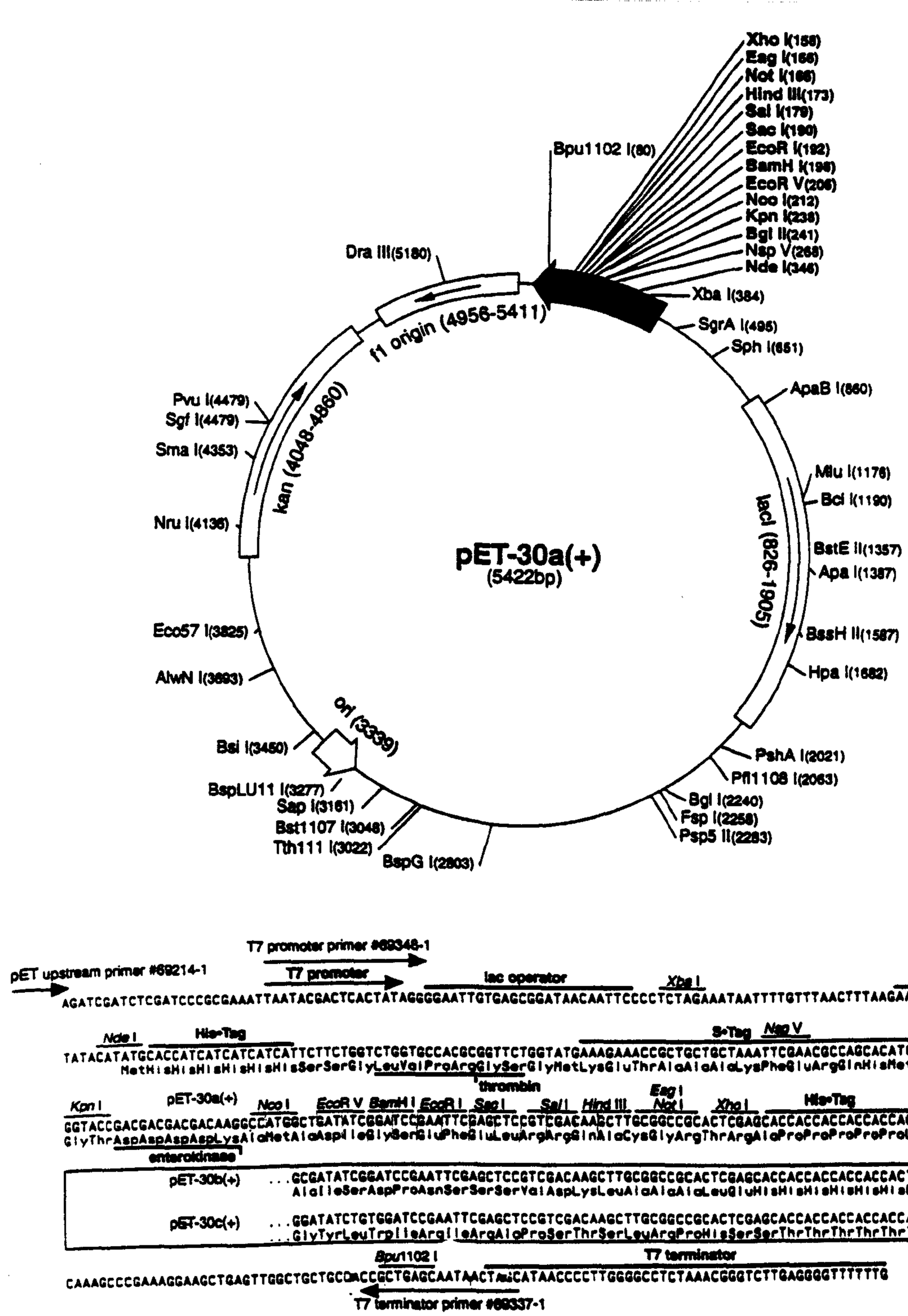
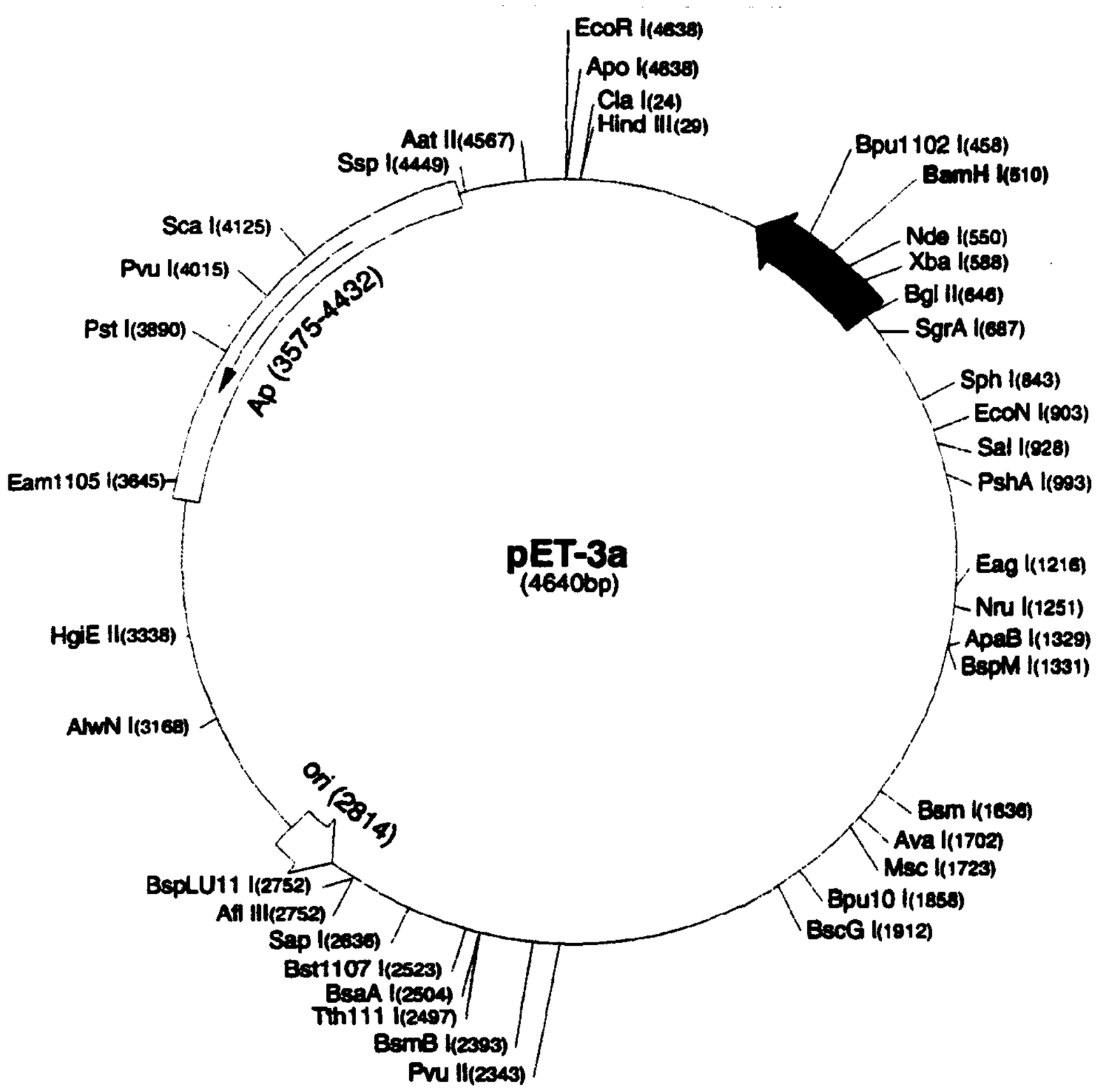
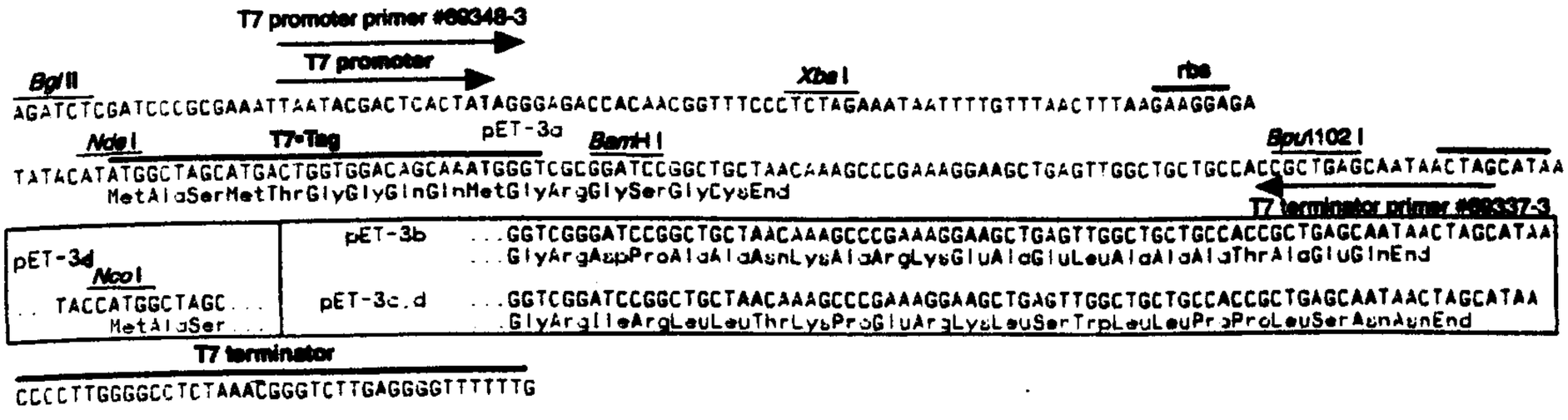


Figure showing the pET30a expression vector with its polylinker region. This vector carries an N-terminal His•Tag configuration (6 histidine residues) plus an optional C-terminal His•Tag sequence. The cloning / expression region of the coding strand is transcribed by T7 RNA polymerase. (Taken from the 'pET System Manual', Novagen Inc.).





pET-3a-d cloning/expression region

Figure showing the pET3a expression vector (Novagen Inc,). The pET3d vector is the same as pET3a with the following exceptions. It is a 4637bp plasmid and 2bp have been subtracted from each site beyond *Bam*H1 at position 510. An *Nco*1 site has also been substituted for the *Nde*1 site in pET3a. By cloning a DNA insert into the *Nco*I site it is possible to bypass the T7 tag and produce an unfused recombinant peptide.

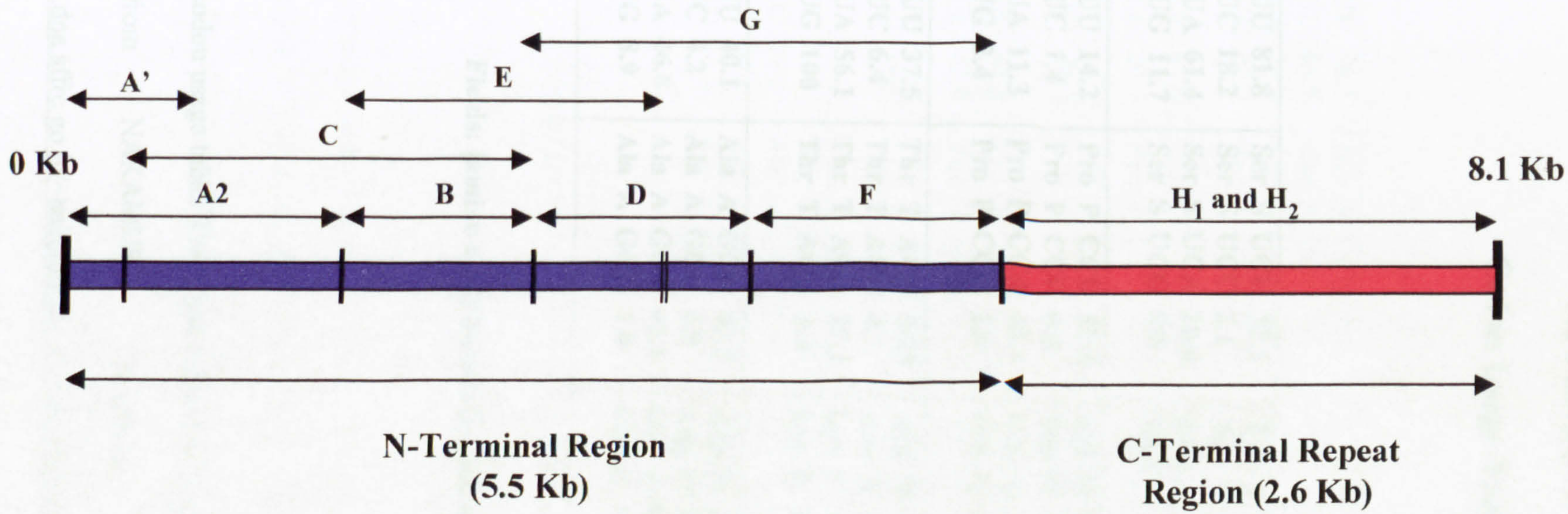
APPENDIX 4

Summary of the purified toxin A peptides

Peptide Name	Expected Peptide Size(kDa)	Actual Peptide Size(kDa)	Length of Insert DNA (Kb)	Region of the Toxin A Gene Covered (bp's)	Region of the Toxin A Protein Covered (aa's)
A2	99.28*	99.28* + 64* (truncated)	1.616	1-1,616	1-536
B	76.85*	76.85*	0.967	1,618-2,585	542-859
C	121.33*	121.33*	2.25	335-2,585	114-859
D	91.51*	91.51*	1.4	2,600-4,000	869-1,330
E	125.85*	108*	2.382	1,618-4,000	542-(~)1,161
F	96.24*	96.24*	1.528	3,965-5,493	1,324-1,830
G	144.01*	144.01*	2.893	2,600-5,493	869-1,830
H1	132.67*	132.67*	2.562	5,495-8,057	1,834-2,683
H2	89.46	89.46	2.568	5,489-8,057	1,832-2,683
A'	64.53*	64.53* + 63* truncated	0.615	1-615	1-205
A^T	56.49	56.49	1.614 Kb	1-1,614	1-538
H^T	89.46	89.46	2.556 Kb	5,505-8,058	1835-2686
Truncated Toxin:	145.95	145.95	4.17 Kb		

*** inclusive of the 43 kDa maltose binding protein.**

A table summarising the sizes of the toxin A peptides and their locations in the toxin A molecule (see appendix 1).



APPENDIX 5

A Summary of the Toxin A Peptides

A schematic diagram of the toxin A gene summarising the location of the peptides expressed from pMal and pET vectors and purified from *E. coli* cells.

APPENDIX 6

Codon Usage Tables

Phe F UUU 81.8	Ser S UCU 32.1	Tyr Y UAU 80.2	Cys C UGU 82.5
Phe F UUC 18.2	Ser S UCC 2.1	Tyr Y UAC 19.8	Cys C UGC 17.5
Leu L UUA 61.4	Ser S UCA 28.0	End - UAA 66.7	End - UGA 6.6
Leu L UUG 11.7	Ser S UCG 0.8	End - UAG 26.7	Trp W UGG 100
Leu L CUU 14.2	Pro P CCU 52.2	His H CAU 72.6	Arg R CGU 9.1
Leu L CUC 1.4	Pro P CCC 0.8	His H CAC 27.4	Arg R CGC 1.7
Leu L CUA 11.3	Pro P CCA 45.4	Gln Q CAA 83.9	Arg R CGA 5.7
Ser S CUG 2.4	Pro P CCG 1.6	Gln Q CAG 16.1	Arg R CGG 0.4
Ile I AUU 37.5	Thr T ACU 54.8	Asn N AAU 85.8	Ser S AGU 29.0
Ile I AUC 6.4	Thr T ACC 4.7	Asn N AAC 14.2	Ser S AGC 5.7
Ile I AUA 56.1	Thr T ACA 37.2	Lys K AAA 80.2	<u>Arg R AGA 75.7</u>
Met M AUG 100	Thr T ACG 3.3	Lys K AAG 19.8	<u>Arg R AGG 7.4</u>
Val V GUU 40.1	Ala A GCU 47.5	Asp D GAU 85.2	Gly G GGU 42.0
Val V GUC 4.2	Ala A GCC 4.9	Asp D GAC 14.8	Gly G GGC 6.1
Val V GUA 46.8	Ala A GCA 45.6	Glu E GAA 79.6	Gly G GGA 46.6
Val V GUG 8.9	Ala A GCG 2.0	Glu E GAG 20.4	Gly G GGG 5.3

Fields: [amino acid] [symbol] [codon] [% frequency]

C. difficile codon usage table. The rarest codons in *E.coli* gene expression are underlined.

Taken from NAKAMURA Yasukazu Official Netscape Site (http://www.dna.affrc.go.jp/~nakamura/). E-mail address: ynakamu@kazusa.or.jp

Phe F UUU 56.5	Ser S UCU 8.2	Tyr Y UAU 56.6	Cys C UGU 44.3
Phe F UUC 43.5	Ser S UCC 8.0	Tyr Y UAC 43.4	Cys C UGC 55.7
Leu L UUA 25.6	Ser S UCA 6.9	End - UAA 62.5	End - UGA 28.1
Leu L UUG 25.2	Ser S UCG 7.9	End - UAG 9.4	Trp W UGG 100
Leu L CUU 21.2	Pro P CCU 16.1	His H CAU 56.3	Arg R CGU 37.9
Leu L CUC 20.5	Pro P CCC 15.1	His H CAC 43.7	Arg R CGC 38.6
Leu L CUA 7.5	Pro P CCA 19.3	Gln Q CAA 33.6	Arg R CGA 6.5
Ser S CUG 46.9	Pro P CCG 52.4	Gln Q CAG 66.4	Arg R CGG 9.9
Ile I AUU 49.8	Thr T ACU 17.4	Asn N AAU 45.1	Ser S AGU 7.9
Ile I AUC 42.0	Thr T ACC 42.8	Asn N AAC 54.9	Ser S AGC 14.2
Ile I AUA 8.2	Thr T ACA 13.9	Lys K AAA 75.6	Arg R AGA 4.5
Met M AUG 100	Thr T ACG 25.9	Lys K AAG 24.4	Arg R AGG 2.7
Val V GUU 26.8	Ala A GCU 17.0	Asp D GAU 62.2	Gly G GGU 34.3
Val V GUC 21.0	Ala A GCC 26.7	Asp D GAC 37.8	Gly G GGC 39.6
Val V GUA 15.8	Ala A GCA 21.6	Glu E GAA 68.4	Gly G GGA 11.2
Val V GUG 36.4	Ala A GCG 34.7	Glu E GAG 31.6	Gly G GGG 14.9

Fields: [amino acid] [symbol] [codon] [% frequency]

E. coli codon usage table. The rarest codons in *E. coli* gene expression are underlined.

Taken from NAKAMURA Yasukazu Official Netscape Site

(http://www.dna.affrc.go.jp/~nakamura/). E-mail address: ynakamu@kazusa.or.jp.

APPENDIX 7

Figure showing the frequency with which the rare *E.coli* arginine codons AGA and AGG occur within toxin A fragment a2. The nucleotide sequence is shown in black, the amino acid sequence is in blue and the rare arginine codons are highlighted in red.

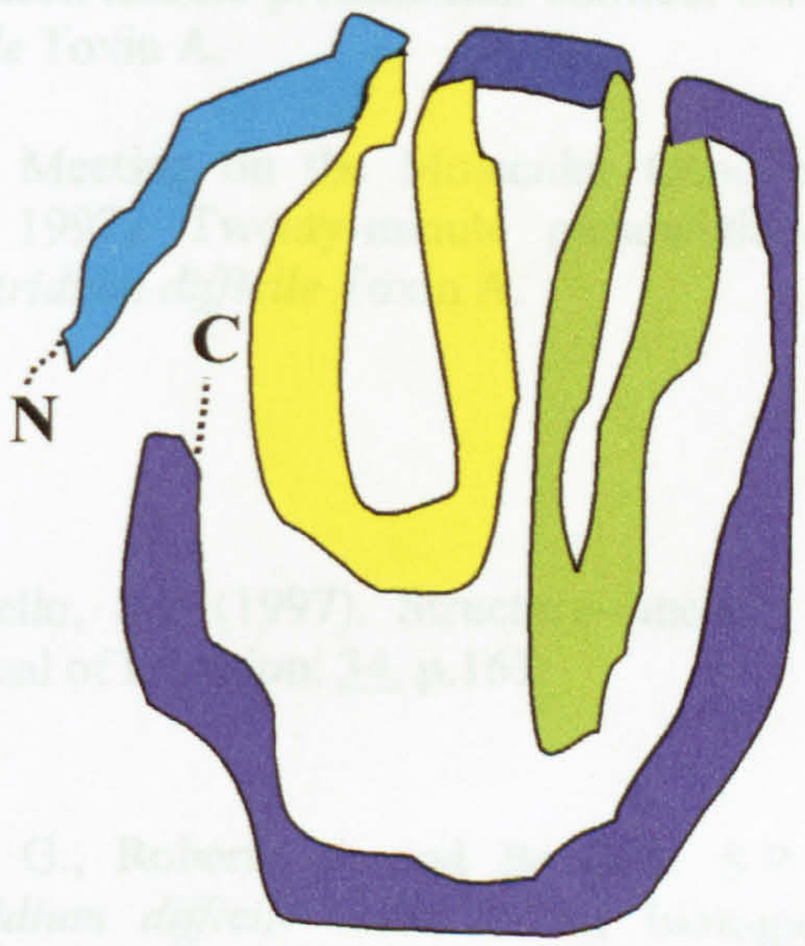
```
1    ATGTCTTTAA TATCTAAAGA AGAGTTAATA AAACTCGCAT ATAGCATTAG
       M  S  L  I   S  K  E   E  L  I   K  L  A  Y   S  I  R

51   ACCAAGAGAA AATGAGTATA AAACTATACT AACTAATTTA GACGAATATA
       P  R  E   N  E  Y  K   T  I  L   T  N  L   D  E  Y  N

101  ATAAGTTAAC TACAAACAAT AATGAAAATA AATATTTACA ATTAAAAAAA
       K  L  T   T  N  N   N  E  N  K   Y  L  Q   L  K  K

151  CTAAATGAAT CAATTGATGT TTTTATGAAT AAATATAAAA CTTCAAGCAG
       L  N  E   S  I  D  V   F  M  N   K  Y  K  T   S  S  R

201  AAATAGAGCA CTCTCTAATC TAAAAAAAGA TATATTAAAA GAAGTAATTC
       N  R  A   L  S  N  L   K  K  D   I  L  K   E  V  I  L

251  TTATTAAAAA TTCCAATACA AGCCCTGTAG AAAAAAATTT ACATTTTGTA
       I  K  N   S  N  Y   S  P  V  E   K  N  L   H  F  V

301  TGGATAGGTG GAGAAGTCAG TGATATTGCT CTTGAATACA TAAAACAATG
       W  I  G  G   E  V  S   D  I  A   L  E  Y  I   K  Q  W

351  GGCTGATATT AATGCAGAAT ATAATATTAA ACTGTGGTAT GATAGTGAAG
       A  D  I   N  A  E  Y   N  I  K   L  W  Y   D  S  E  A

401  CATTCTTAGT AAATACACTA AAAAAGGCTA TAGTTGAATC TTCTACCACT
       F  L  V   N  T  L   K  K  A  I   V  E  S   S  T  T

451  GAAGCATTAC AGCTACTAGA GGAAGAGATT CAAAATCCTC AATTTGATAA
       E  A  L  Q   L  L  E   E  E  I   Q  N  P  Q   F  D  N

501  TATGAAATTT TACAAAAAAA GGATGGAATT TATATATGAT AGACAAAAAA
       M  K  F   Y  K  K  R   M  E  F   I  Y  D   R  Q  K  R

551  GGTTTATAAA TTATTATAAA TCTCAAATCA ATAAACCTAC AGTACCTACA
       F  I  N   Y  Y  K   S  Q  I  N   K  P  T   V  P  T

601  ATAGATGATA TTATAAAGTC TCATCTAGTA TCTGAATATA ATAGAGATGA
       I  D  D  I   I  K  S   H  L  V   S  E  Y  N   R  D  E
```

```
651   AACTGTATTA GAATCATATA GAACAAATTC TTTGAGAAAA ATAAATAGTA
        T  V  L  E  S  Y  R  T  N  S  L  R  K  I  N  S  N

701   ATCATGGGAT AGATATCAGG GCTAATAGTT TGTTTACAGA ACAAGAGTTA
        H  G  I  D  I  R  A  N  S  L  F  T  E  Q  E  L

751   TTAAATATTT ATAGTCAGGA GTTGTTAAAT CGTGGAAATT TAGCTGCAGC
        L  N  I  Y  S  Q  E  L  L  N  R  G  N  L  A  A  A

801   ATCTGACATA GTAAGATTAT TAGCCCTAAA AAATTTTGGC GGAGTATATT
        S  D  I  V  R  L  L  A  L  K  N  F  G  G  V  Y  L

851   TAGATGTTGA TATGCTTCCA GGTATTCACT CTGATTTATT TAAAACAATA
        D  V  D  M  L  P  G  I  H  S  D  L  F  K  T  I

901   TCTAGACCTA GCTCTATTGG ACTAGACCGT TGGGAAATGA TAAAATTAGA
        S  R  P  S  S  I  G  L  D  R  W  E  M  I  K  L  E

951   GGCTATTATG AAGTATAAAA AATATATAAA TAATTATACA TCAGAAAACT
        A  I  M  K  Y  K  K  Y  I  N  N  Y  T  S  E  N  F

1001  TTGATAAACT TGATCAACAA TTAAAAGATA ATTTTAAACT CATTATAGAA
        D  K  L  D  Q  Q  L  K  D  N  F  K  L  I  I  E

1051  AGTAAAAGTG AAAAATCTGA GATATTTTCT AAATTAGAAA ATTTAAATGT
        S  K  S  E  K  S  E  I  F  S  K  L  E  N  L  N  V

1101  ATCTGATCTT GAAATTAAAA TAGCTTTCGC TTTAGGCAGT GTTATAAATC
        S  D  L  E  I  K  I  A  F  A  L  G  S  V  I  N  Q

1151  AAGCCTTGAT ATCAAAACAA GGTTCATATC TTACTAACCT AGTAATAGAA
        A  L  I  S  K  Q  G  S  Y  L  T  N  L  V  I  E

1201  CAAGTAAAAA ATAGATATCA ATTTTTAAAC CAACACCTTA ACCCAGCCAT
        Q  V  K  N  R  Y  Q  F  L  N  Q  H  L  N  P  A  I

1251  AGAGTCTGAT AATAACTTCA CAGATACTAC TAAAATTTTT CATGATTCAT
        E  S  D  N  N  F  T  D  T  T  K  I  F  H  D  S  L

1301  TATTTAATTC AGCTACCGCA GAAAACTCTA TGTTTTTAAC AAAAATAGCA
        F  N  S  A  T  A  E  N  S  M  F  L  T  K  I  A

1351  CCATACTTAC AAGTAGGTTT TATGCCAGAA GCTCGCTCCA CAATAAGTTT
        P  Y  L  Q  V  G  F  M  P  E  A  R  S  T  I  S  L

1401  AAGTGGTCCA GGAGCTTATG CGTCAGCTTA CTATGATTTC ATAAATTTAC
        S  G  P  G  A  Y  A  S  A  Y  Y  D  F  I  N  L  Q
```

```
1451  AAGAAAATAC TATAGAAAAA ACTTTAAAAG CATCAGATTT AATAGAATTT
        E  N  T   I  E  K   T  L  K  A   S  D  L   I  E  F

1501  AAATTCCCAG AAAATAATCT ATCTCAATTG ACAGAACAAG AAATAAATAG
      K  F  P  E   N  N  L   S  Q  L   T  E  Q  E   I  N  S

1551  TCTATGGAGC TTTGATCAAG CAAGTGCAAA ATATCAATTT GAGAAATATG
        L  W  S   F  D  Q  A   S  A  K   Y  Q  F   E  K  Y  V

1601  TAAGAGATTA TACTGGT
        R  D  Y   T  G
```

APPENDIX 8

The surface exposed and hydrophobic domains of holotoxin A



KEY:

Hydrophilic domains

- Amino acids 1-541
- Amino acids 1,162-1,324
- Amino acids 1,832-2,683

Hydrophobic domains

- Amino acids 542-1,161
- Amino acids 1,325-1,830

A diagrammatic representation of the predicted hydrophobic domains of holotoxin A as determined by antibody reaction profiles. At least part of each of the hydrophilic domains shown above is surface exposed in the native toxin A molecule having cross-reacted with polyclonal anti-formalised toxin A antiserum.

PRESENTATIONS AND PUBLICATIONS

Oral Presentations:

Third conference of the Federation of Infection Societies, Manchester, U.K (27-29 November 1996). Fifteen-minute presentation entitled: Structure-Function Relationships of *Clostridium difficile* Toxin A.

Second International Meeting on the Molecular Genetics of the Clostridia, Onzain, France (22-25 June 1997). Twenty-minute presentation entitled: Structure-Function Relationships of *Clostridium difficile* Toxin A.

Publications:

Craggs, J and Borriello, S.P (1997). Structure-function relationships of *Clostridium difficile* toxin A. Journal of Infection, 34, p.161.

Craggs, J., Choules, G., Roberts, A and Borriello S.P (1999). Analysis of cloned fragments of *Clostridium difficile* toxin A for biological activities and ability to glucosylate RhoA. Journal of Medical Microbiology: submitted for publication.