The development of live vectored vaccines targeting the alpha-toxin of *Clostridium perfringens* for the prevention of necrotic enteritis in poultry

A thesis submitted in fullfilment of the requirements for the degree of Doctor of Philosophy

By

Xenia Gatsos

BAppSc (Hons)

Biotechnology and Environmental Biology

School of Applied Science

RMIT University

Melbourne, Victoria, Australia

January 2007

Declaration

I declare that except where due acknowledgement has been made, the work is that of my own; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Xenia Gatsos

31st January 2007

Dedication

I dedicate this thesis to my loving parents, Chris and Jordana, and husband Peter, for their support, understanding and encouragement throughout the years of my study.

Acknowledgments

I would firstly like to thank my Supervisor Professor Peter Coloe, for all his support and guidance throughout my post-graduate studies. Peter, you have been an amazing mentor, and I thank you for all the advice you have passed down to me. I also thank you for your amazing ability to calm me down when my stress levels were soaring through the roof!

None of this research would have been possible without the monetary assistance from the CRC for Microtechnology, I am very grateful for their involvement.

I would like to thank Dr. Peter Smooker, my "unofficial supervisor" who took the time out to help me when I was in need. I also thank him for the ENDLESS hours he spent helping me bleed mice.

To all my friends in the Biotech lab; the constant interactions between everybody within and outside the lab ("soccer Wednesdays" pub nights, dinners and kris kringle) made my time studying within the Biotech lab a very enjoyable and memorable experience.

I would like to give special thanks to; Dr. George Moutafis, for all his worldly advice on completing a PhD, and for his help throughout my years of study; To James Holden, for his constant help throughout my animal trials; To Luke Norbury for his assistance in the countless ELISA trials; and to Grace (Kaiyan) Liu, and Karen Lodge for lending an ear when I was feeling blue.

I would like to thank my family, particularly my sisters, Helen and Petrina, for listening to my research dilemmas and supporting me in every way they could. To Mum and Dad, thank you for raising me to believe that I could do anything I set my mind to, your support and encouragement have lead me to this path today, and I am forever grateful.

Last but definitely not least, I would like to thank my husband Peter Cardassis. Thank you for your love, support and patience! With the completion of this thesis we can now begin a new chapter in our lives! You inspire and motivate me to do things I thought I could never do, and I too will always be there to support you in whatever goals you set out to achieve.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Δ	Deletion
λ	Lambda phage DNA
λ-*Pst*I	Lambda DNA digested with the restriction enzyme *Pst*I
µg	Microgram
µL	Microlitre
µm	Micrometre
aa	Amino acid
AA	Arachidonic acid
Amp	Ampicillin
Amp^R	Ampicillin resistance
ANGIS	Australian National Genomic Information Service
APC	Antigen presenting cell
bp	Base pairs
BSA	Bovine serum albumin
ºC	Degrees centigrade
C-His	Carboxy terminal hexahistidine sequence used in purification of protein via IMAC
C-terminal	Carboxy terminal domain of an amino acid sequence/protein
Ca^{2+}	Calcium ions
CE	Competitive exclusion
cfu	Colony forming units
CIP	Calf intestinal phophatase
CMI	Cell-mediated immunity
CO_2	Carbon dioxide

CTAB	Hexadecyltrimethyl ammonium bromide
CV	Column volume of resin used in chromatography
Da	Dalton
dH_2O	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DNase	Deoxyribonuclease
dNTPs	Deoxynucleoside triphosphates
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
ELIspot	Enzyme-linked immunospot assay
EtBr	Ethidium bromide
EU	The Council of European Communities (European Union)
FIA	Freund's incomplete adjuvant
g	Gram
x *g*	Gravitational acceleration
GALT	Gut-associated lymphoid tissue
GC	Guanine/cytosine content
GIT	Gastrointestinal tract
GST	Glutathione-S-transferase
h	Hour
HCl	Hydrochloric acid
H_2O	Water
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
IFN-γ	Interferon gamma
Ig	Immunoglobulin

IL	Interleukin
i.m.	Intramuscular
IMAC	Immobilised metal affinity chromatography
i.p.	Intraperitoneal
IPTG	Isopropyl-β-D-thiogalactopyranoside
i.v.	Intravenous
kbp	Kilobase pairs
kDa	One thousand Daltons
L	Litre
LA	Luria Bertani agar
LA100	Luria Bertani agar supplemented with 100 μg/mL ampicillin
LB	Luria Bertani broth
LB100	Luria Bertani broth supplemented with 100 μg/ mL ampicillin
LD_{50}	The concentration required to kill 50% of a population (50% lethal dose)
LPS	Lipopolysacharide
M	Molarity
m	Mole
MCS	Multiple cloning site
Mg^{2+}	Magnesium
mg	Milligram
mH_2O	MilliQ water
mM	Millimolar
MW	Molecular weight
N-His	Amino terminal hexahistidine sequence used in purification of protein via IMAC
N-terminal	Amino terminal domain of an amino acid sequence/protein

NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCS	New born calf serum
ng	Nanograms
NK	Natural killer cells
nM	Nanomolar
nm	Nanometer
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	PBS supplemented with 0.05% (v/v) Tween 20
PCR	Polymerase chain reaction
pH	Negative algorithm of hydrogen ion concentration
P_{htrA}	Promoter of *htr*A from *S*. Typhimurium
PNK	Polynucleotide kinase
P_{pagC}	Promoter of *pag*C from *S*. Enteritidis
P_{tac}	Hybrid promoter containing the -10 region of the *lacUV*5 promoter and the -35 region of the *trp* promoter
^{R}	Resistance to antibiotic
RBS	Ribosomal binding site
RE	Restriction enzyme
RES	Reticuloendothelial system
RNase	Ribonuclease
RT	Room temperature
s	Seconds
SBA	Sheep blood agar

SBA100	Sheep blood agar plates supplemented with 100 μg/mL ampicillin
SDS	Sodium dodecyl sulphate
SPF	Specific pathogen free
spp	Species (plural)
SRBC	Sheep red blood cells
STM1	*Salmonella* Typhimurium aroA$^-$ vaccine strain
TBS	Tris-buffered saline
TEMED	N,N,N' ,N' -tetramethyl ethylenediamine
Tet	Tetracycline
TH1	Effector T-cells involved in the initiation of cell-mediated immune responses
TH2	Effector T-cells involved in the initiation of humoral immune responses
Tm°C	Melting temperature
Tris	Tris (hydroxymethyl) amino methane
TST	Tris-saline-tween
U	Units
UV	Ultraviolet
V	Voltage
vol	Volume
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
Zn^{2+}	Zinc ions

Summary

The α-toxin of *Clostridium perfringens* is a toxin involved in numerous diseases of humans and agriculturally important animals (Songer, 1997). One of these diseases is necrotic enteritis (NE) in chickens, a sporadic enteric disease which affects chicken and other avian species world-wide (van der Sluis, 2000). This study involved the inactivation of alpha-toxin (α-toxin) for use as a potential vaccine candidate to combat NE in chickens.

The α-toxin is composed of two domains. An N-terminal domain composed of nine helices, and a C-terminal domain composed of eight anti-parallel β-sheets (Naylor *et al.*, 1998). The N-terminal domain is involved in the catalytic functions of the toxin which requires zinc for activation, and the C-terminal domain is essential for binding receptors, through the involvement of calcium ions (Naylor *et al.*, 1998; Naylor *et al.*, 1999).

During the course of this research a number of α-toxin recombinant proteins have been developed through molecular inactivation of the α-toxin gene, *plc*. Proteins plc316 and plc204 were developed by the deletion of the first three and seven α-helices of the N-terminal domain respectively. These deletions resulted in proteins which were unstable in solution and constantly aggregated into insoluble masses. They elicited lower overall antibody responses when administered to mice, which may in part have been caused by the reduction in the number of conformational epitopes present in the misfolded proteins.

A third protein, plcInv3 was developed from the deletion of part of the catalytic domain of the α-toxin. PlcInv3 was highly soluble and upon immunisation of mice elicited a significant antibody response which was also capable of protecting mice against a live challenge of *C. perfringens*.

The fourth and final protein developed was plc104. The smallest of the recombinant α-toxin proteins, it consisted entirely of the C-terminal domain of α-toxin. Its small size did not affect its ability to induce a strong antibody response when administered to mice, the antibodies of which were also protective during a challenge with *C. perfringens*.

STM1, an attenuated strain of *S.* Typhimurium was used in the development of a vectored vaccine for the expression and oral delivery of plcInv3 and plc104 within the mouse host. The use of such a vaccine is considered superior to parenteral administration of vaccine antigens targeted against enteric infections as it delivers them directly to the intestinal tract and in the case of NE, the natural site of infection (Holmgren *et al.*, 1992). Oral delivery of attenuated *S.* Typhimurium vectored vaccines induces mucosal immune responses in addition to cell-mediated and other humoral responses (Paton *et al.*, 1993; Ward *et al.*, 1999; Wang *et al.*, 1999a). Therefore this system would be beneficial for control of enteric diseases such as necrotic enteritis of chickens.

The two proteins, plcInv3 and plc104 were examined for their immune-stimulating abilities in mice when administered orally utilising the vaccine strain STM1 as a vector. The region coding for the affinity hexa-his tag of plcInv3 and plc104 was removed prior to cloning into plasmids for their expression within STM1 and hence forth the proteins were known as αInv and α104 respectively.

Proteins αInv and α104 were expressed within STM1 from expression plasmids containing the *in vivo* inducible promoters P_{htrA} and P_{pagC}. A measurable humoral immune response against α-toxin was absent following three oral vaccinations with the vectored vaccines. *In vivo* inducible plasmid expression systems allow for long-term stability of plasmids in the

absence of selective pressure (Dunstan *et al.*, 1999), although the plasmids developed in this study were fairly unstable within STM1 when examined *in vitro*, with greater than 90% plasmid loss within two days from most of the vaccines examined. This plasmid loss, which would be enhanced *in vivo* (Dunstan *et al.*, 2003; Foynes *et al.*, 2003), would have led to reduced protein expression and is probably one of the reasons why a detectable antibody response was absent.

Even though antibody responses were absent, cytokine profiling of splenocytes from vaccinated mice revealed an increase in the number of IL-4 secreting cells and the lack of IFN-γ secreting cells, indicating the stimulation of a TH2 response towards α-toxin. These responses lead to partial protection against a live *C. perfringens* challenge.

Mice immunised with toxoid (formalin inactivated α-toxin) via the intraperitoneal route elicited strong immune responses including anti-α-toxin IgG antibodies and IL-4 secretion. This vaccine was also capable of protecting mice from a live *C. perfringens* challenge.

This study demonstrated the feasibility of using STMI as a carrier for the *in vivo* expression of the *C. perfringens* α-toxin recombinant proteins αInv and α104. It is the first study to express *C. perfringens* antigens within an attenuated strain of *S.* Typhimurium, STM1. Activation of the TH2 arm of the immune system was observed, but it seems at this time, the level of activated IL-4 cells stimulated in response to vaccination with STM1 expressing the α-toxin truncated proteins αInv and α104 was not efficient enough to induce a detectable humoral response or to completely protect mice from a *C. perfringens* infection.

The partial protection of mice immunised with vaccines composed of STM1 expressing αInv and α104 indicate there is potential for this vectored vaccine system to be used in the

protection of *C. perfringens* diseases caused by the α-toxin, although the system needs to be optimised for the maximal production of a humoral immune response.

Chapter 1

Introduction

1.1 General introduction

The livestock industry is an integral part of any country's economy. The rearing of animals for human consumption is complicated and constantly fraught with problems. The outbreak and spread of disease is a re-occurring issue for farmers, particularly in areas of high density livestock.

Prevention of diseases is of utmost importance for the maintenance and production of good quality meat and can be achieved by various means. Antibiotics are one of the most popular preventative measures, but such management solutions can attract problems such as the rise of antibiotic resistant bacteria in the food chain (Molbak, 2004). There is widespread concern that once such bacteria have entered the food chain, some human diseases may stop responding to antibiotic treatments.

The advent of vaccinology has paved the way for alternative methods of prophylaxis in humans and animals alike (Walker, 1992). Vaccine research can be traced back over two centuries. In 1796 Edward Jenner injected a dose of the non-pathogenic cowpox into a child, protecting him from challenge with lethal smallpox. Although ethically times have changed, vaccine research still requires the basic knowledge of the enemy, and how it strikes. Vaccine research has advanced significantly from our traditional empirical methodology. Research is focused on creating specific products which are completely characterised despite the success of traditional vaccines over the last 100 years. With the advent of molecular and tissue

culture techniques, we are now able to create second generation vaccines which consist of defined antigens.

Not only are we constantly searching for new improved antigens, but in recent times there is an increased focus on the delivery and presentation of antigens to the correct immune cells, known as immunomodulation (Hilleman, 2000).

This introduction will focus on *Clostridium perfringens* and in particular one of the diseases it causes in poultry, necrotic enteritis. Pathogenesis of the disease will be discussed along with methods of prevention. This will lead to a discussion of vaccine research and technologies currently employed to prevent such diseases.

1.2 The Clostridia

The Genus *Clostridium* is comprised of Gram positive, spore-forming anaerobic bacilli. There are currently over 100 published species within this Genus (Quinn *et al.*, 2002). Most *Clostridium* species (spp.) ferment sugars or amino acids, but some can ferment both (Brock *et al.*, 1994). The end products of fermentation include acetone, butanol, butyric acid and other alcohols, which may give the growth medium a putrid odour (Biberstein, 1990). One method of identifying *Clostridium* spp. relies on the detection of these acids using gas-liquid chromatography (Brock *et al.*, 1994).

Clostridium spp. are widely distributed throughout the environment. They primarily inhabit the anoxic pockets of soil, in addition to sewage, freshwater and marine sediments of the world (George and Finegold, 1985; Brock *et al.*, 1994). Clostridia can also be found as commensals of the large intestine of humans and many other mammals (Allen, 1985). They have adapted to the anoxic environment of the intestine and maintain a stable balance with other organisms present, and in the majority of cases are considered harmless saprophytes

(Allen, 1985; Drasar and Roberts, 1990). *Clostridium* spp. are the only known Genus of spore-forming anaerobes associated with humans, either as non-pathogens or at infected sites (George and Finegold, 1985).

The clostridia are well known largely for their ability to produce some of the deadliest toxins known to mankind (Rood *et al.*, 1997). Botulinum and tetanus neurotoxins are two of the most potent toxins produced by clostridia followed by epsilon toxin, a product of *C. perfringens* (Payne and Oyston, 1998). Both exogenous and endogenous sources of clostridia are responsible for disease. Exogenous sources include *C. perfringens* food poisoning, gas gangrene, tetanus and botulism (Onderdonk and Allen, 1995). Endogenous (or opportunistic) infections are usually acquired through the host's own microflora. Infection is triggered through trauma, antibiotic treatment, operative procedures, immunosuppressive treatments or pre-existing conditions such as leukaemia, carcinoma, and diabetes mellitus (Allen, 1985; Onderdonk and Allen, 1995).

1.3 *Clostridium perfringens*

Bacillus welchii was first isolated by William H. Welch in 1890 from a man who had died 8 hours earlier due to a number of complications (Lucey and Hutchins, 2004). On microscopic examination, he described the bacterium as resembling anthrax bacilli, but with boxed edges, and always found it in pairs or singularly but never in chains. Its name was later changed to *Clostridium welchii* and then again to the current *Clostridium perfringens*.

C. perfringens is ubiquitous in the environment and most strains are able to grow at temperatures ranging between 20ºC and 50ºC (Hatheway, 1990). Two of its major fermentation by-products are hydrogen gas and carbon dioxide which help maintain the anaerobic environment required for its survival (Shimizu *et al.*, 2002a). Unlike most other

clostridia *C. perfringens* is amotile and one of the few aerotolerant species (Biberstein, 1990). Spores are rarely seen *in vivo* or under usual *in vitro* conditions (Krieg, 1984). Most *C. perfringens* possess a capsule that is largely composed of polysaccharides (Biberstein, 1990). *C. perfringens* has a G+C content of 28% (Shimizu *et al.*, 2002a). Most strains are sensitive to penicillins but resistant to aminoglycosides (Krieg, 1984). Resistance to tetracyclines is developing due to antibiotic use and the conjugation of tetracycline resistance plasmids to sensitive strains has been observed (Abraham and Rood, 1985).

C. perfringens is a commensal organism of the intestinal flora of humans and many animals (Tannock, 1994) and it has been isolated from virtually every animal that has ever been investigated (Krieg, 1984). Numbers tend to remain low and under control through the microfloral balance in the gastrointestinal tract. Conditions that cause instability of the gastrointestinal tract may allow for the proliferation of *C. perfringens*. This proliferation brings about the production of toxins and disease. The ability of *C. perfringens* to cause an opportunistic infection is often observed in farm-reared neonate animals who have yet to establish an intestinal microflora (Kelneric *et al.*, 1996; Songer, 1997; Bueschel *et al.*, 1998; Moxley and Duhamel, 1999; Springer and Selbitz, 1999) .

1.4 Typing of *C. perfringens* strains

Typing of *C. perfringens* is dependent upon the detection of four major toxins. These toxins are major virulence factors (Sakurai and Duncan, 1978; Tso and Siebel, 1989; Sakurai and Kobayashi, 1995; Miyamoto *et al.*, 1998). Their effects are lethal and required to cause disease. The expression of the major toxins divides strains into five toxinotypes, A to E (Meer and Songer, 1997). Their distribution amongst the type strains are listed in Table 1.1 and are represented by the Greek letters alpha (α), beta (β), epsilon (ε), and iota (ι).

Table 1.1: Classification of *C. perfringens* by toxinotype.

C. perfringens type	Toxin produced			
	Alpha (α)	Beta (β)	Epsilon (ε)	Iota (ι)
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

Based on previous tables (Rood and Cole, 1991)

Traditionally, typing of *C. perfringens* strains involved sero-neutralisation of culture filtrates *in vivo*. Mice or guinea pigs were injected with culture supernatants of *C. perfringens*, along with antitoxin, and death (mice) or dermonecrosis (guinea pigs) was assessed (Sterne and Batty, 1975). This assay was extremely time-consuming as growth of the organism was required. It was also expensive as two of the toxins, epsilon and iota, required trypsin for activation, but a third toxin, beta toxin, was inactivated by trypsin. Therefore each culture supernatant was assayed numerous times; with and without trypsin, and with and without the five different preparations of neutralising antisera (Hatheway, 1990).

Current techniques such as the polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) make the task of identification less time consuming and more economical (el Idrissi and Ward, 1992; Moller and Ahrens, 1996; Meer and Songer, 1997; Ebert *et al.*, 1999; Kadra *et al.*, 1999).

Classification by toxinotype is convenient for clinical purposes, but does not reflect the genetic relationship between strains (Tsutsui *et al.*, 1995). Genomic mapping studies have revealed that there are as many differences within a type strain as there are between type strains (Canard *et al.*, 1992). The growth characteristics of *C. perfringens* strains is also not dependant on respective type designation, but on strain-associated properties (Mollby and Holme, 1976). Even though type designation does not reflect strain properties, each type is responsible for a number of specific diseases observed in specific hosts (Section 1.6), and hence classification in this manner is satisfactory for the intended diagnostic purposes.

Some strains, predominantly type A are also capable of producing an enterotoxin responsible for gastroenteritis in humans (Narayan, 1982). This enterotoxin is a sporulation associated toxin and the only toxin not secreted from vegetative cells (Rood, 1998). It is not part of the

current classification system, due to the increased difficulties in triggering sporulation *in vitro* (Sterne and Batty, 1975), but with new diagnostic processes in place it can now be located at the molecular level via PCR (Sterne and Batty, 1975; Fach and Popoff, 1997; Meer and Songer, 1997; Kanakaraj *et al.*, 1998).

1.5 Toxins of *C. perfringens*

Toxins produced by bacteria aid in their defence and hence survival. Many bacterial species are known to produce toxins, but none as many as the species of *C. perfringens* (Hatheway, 1990). Major and minor toxins are produced, of which over 15 have been identified (Hatheway, 1990; Shimizu *et al.*, 2002a). These toxins may act in a synergistic or additive manner to produce the numerous diseases observed in its hosts, including gas gangrene and food poisoning in humans, and several enterotoxaemic diseases of animals (Awad *et al.*, 1995; Stevens and Bryant, 1997; Shimizu *et al.*, 2002a).

1.5.1 Minor toxins

Minor toxins include collagenase (κ-toxin), protease (λ-toxin), hyaluronidase (μ-toxin) and perfringolysin O (θ-toxin) (Katayama *et al.*, 1996). Many of the minor toxins alone cannot produce disease but in conjunction with the major lethal toxins can contribute to pathogenesis. In addition to their activity on the host, one of the minor toxins, λ-toxin is also able to activate the two major toxins ε- and ι-toxin (Minami *et al.*, 1997; Gibert *et al.*, 2000).

Sequencing of a type A strain of *C. perfringens* has revealed that it is capable of expressing five different μ-toxins (Shimizu *et al.*, 2002a). The sequencing also revealed a cysteine protease with sequence homology to the alpha-clostripain of *Clostridium histolyticum*

previously unknown to exist in *C. perfringens* (Shimizu *et al.*, 2002a; Shimizu *et al.*, 2002b). A detailed description of the known minor toxins produced by *C. perfringens* is listed in Table 1.2.

Minor toxins may play an essential metabolic role in the growth of *C. perfringens*. Genomic sequence analysis revealed that *C. perfringens* lacks a number of pathways required for the *de novo* biosynthesis of certain amino acids. Therefore the bacterium relies on the breakdown of extracellular products by the minor toxins to gain its nutritional requirements (Shimizu *et al.*, 2002a).

The identification of minor toxins from the culture filtrates of *C. perfringens* is made difficult by their breakdown due to the presence of other proteases (Shimizu *et al.*, 2002b). With the addition of protease inhibitors to a culture supernatant, many more proteases may be identified in the future.

1.5.2 Major toxins

As previously mentioned in Section 1.4, the four major toxins of *C. perfringens* are used in the identification of type strains. Each toxin has the ability to cause death in mice when injected in microgram quantities and each has a very different mechanism of action. Only the α-toxin gene is chromosomally located, the rest are episomal (Katayama *et al.*, 1996). The characteristics of the four major toxins are listed in Table 1.3 followed by an in depth description of each.

Table 1.2: Activities of the minor toxins of *C. perfringens*.

Minor toxin	Biological activity	Produced by
θ-toxin (theta)	Perfringolysin-O, haemolysin, cytolysin, O_2-labile	All types
κ-toxin (kappa)	Collagenase/gelatinase	All types
μ-toxin (mu)	Hyaluronidase	Types A-D
λ-toxin (lambda)	Protease	Types B, D & E
ν-toxin (nu)	DNase	All types
δ-toxin (delta)	Haemolysin	Types B & C
α-clostripain like protease	Cysteine protease	Type A (not yet tested in other types)
Neuraminidase/sialidase	N-Acetylneuraminic acid glycohydrolase	All types

Modified from Hatheway (1990) and Shimizu, *et al*., (2002b).

Table 1.3: Characteristics of the four major toxins of *C. perfringens*.

Toxin	Structural gene	Size of mature protein (kDa)	LD_{50} in mice*	Role of toxin/characterisation
α-toxin	*plc* (also known as *cpa*)	43	5 μg/kg intravenously (i.v.)	Phospholipase C enzyme, lecithinase and sphingomyelinase activity, haemolytic , cytolytic (platelets/leukocytes) dermonecrotic
β-toxin	*cpb*	34	0.3 μg/kg (i.v.) 4.5 μg/kg intraperitoneally (i.p.)	Cytolytic to human leukaemia 60 cell line, trypsin sensitive, heat labile, increases capillary permeability, increases blood pressure, decreases heart rate, neurotoxin
ε-toxin	*etx*	29-32	0.065-0.11 μg/kg (i.v)	Protoxin requiring trypsin activation, induces neurotransmitter release resulting in hippocampal damage, able to pass blood/brain barrier, cytotoxic for Madin-Darby canine kidney cell line, increases vascular permeability thus enhancing its own uptake in intestinal wall, causes swollen hyperaemic kidneys, edemic lungs, excess pericardial fluid
ι-toxin	*iab*	Ia 43-47.5 Ib 71.5-80	Ia 24 μg/kg-60 μg/kg and Ib 48 μg/kg-100 μg/kg	Binary toxin: Ia & Ib, protoxin requiring trypsin for activation, Ia; ADP-ribosylating enzyme, Ib; binding & translocation, increases vascular permeability, dermonecrotic on intradermal and lethal on i.v injection

*LD_{50} refers to the concentration of toxin required to kill 50% of mice

1.5.2.1 Alpha-toxin

Alpha-toxin is the most studied of the major toxins of *C. perfringens*, and was the first bacterial toxin established to possess enzymatic activity (MacFarlane and Knight, 1941).

It is a phospholipase C (plc) enzyme which hydrolyses the phosphate group in phosphatidylcholine, a common phospholipid of eukaryotic biological membranes to liberate free phosphorylcholine (PC) and diacylglyceride (DAG) (MacFarlane and Knight, 1941). It is also readily able to hydrolyse sphingomyelin, another common component of the eukaryotic phospholipid membrane (Saint-Joanis *et al.*, 1989). Its action is influenced by membrane factors such as cholesterol content, saturation of phospholipids, and membrane fluidity (Nagahama *et al.*, 1996) with the general rule that the lower the melting temperature of a membrane, the more sensitive it is to α-toxin attack (Nagahama *et al.*, 1998).

Alpha-toxin is a lethal toxin with an LD_{50} between 1.5-5.0 μg/kg body weight when administered intravenously to mice (Tso and Siebel, 1989). It is haemolytic, necrotic and cytolytic, lysing platelets and leukocytes, and damaging fibroblasts and muscle cell membranes (MacFarlane and Knight, 1941; McDonel, 1980; Titball *et al.*, 1993; Ninomiya *et al.*, 1994).

Characterisation of the α-toxin has previously been impeded due to degradation of the toxin in the supernatant during purification, and contamination with other extracellular proteins produced by the bacterium, notably θ-toxin, μ-toxin and κ-toxin (MacFarlane and Knight, 1941; Mollby *et al.*, 1973; Nord *et al.*, 1974; Mollby and Holme, 1976). The difficulties in its purification were overcome in 1989 when *plc* was cloned and expressed from *E. coli* (Leslie *et al.*, 1989; Titball *et al.*, 1989; Tso and Siebel, 1989).

The molecular weight (MW) of the cloned α-toxin is 43,000 dalton (Leslie *et al.*, 1989), which is synonymous to the native toxin (Fujii *et al.*, 1986). The α-toxin consists of 398 amino acids of which the first N-terminal 28 residues are cleaved upon release into the supernatant (a 3 kDa signal sequence), or in the case of *E. coli* the periplasmic space (Leslie *et al.*, 1989).

The α-toxin of *C. perfringens* is part of a family of proteins known as the zinc metalloproteases (Kurioka and Matsuda, 1976). These are a group of enzymes that require Zn^{2+} for activity. They include a number of clostridial plc's and the Gram positive *Bacillus cereus* phosphatidylcholine-preferring plc (PC-PLC) (Table 1.4). All the plc's share a higher degree of homology towards their amino (N)-terminus particularly in their zinc binding domains, which are important for catalytic function (Tso and Siebel, 1989; Tsutsui *et al.*, 1995; Karasawa *et al.*, 2003; Hauer *et al.*, 2004). This supports the hypothesis that this region is a prototype of bacterial phospholipases (Tsutsui *et al.*, 1995).

The powerful cytotoxicity observed with α-toxin results from the effects of its unique hydrophobic carboxy (C)-terminal domain, which interacts directly with the tail group of phospholipids at specific carbon atoms for enhanced hydrophobic contact (Naylor *et al.*, 1998). Many of the other plc's do not have this carboxy region (Leslie *et al.*, 1989), or if they do, their structure does not allow them the same tight affinity for the hydrophobic binding to phospholipids (Tsutsui *et al.*, 1995; Guillouard *et al.*, 1997; Naylor *et al.*, 1998). The C-terminus of α-toxin may also be involved in signalling endogenous plc activities within cells leading to disregulation of host inflammatory responses and cell lysis (Sakurai *et al.*, 1993; Sakurai *et al.*, 1994; Titball, 1997).

Table 1.4: Family of zinc metalloprotease phospholipases C

Species	Toxin	Homology with *C. perfringens* (% amino acid)	Reference
C. bifermentans	CB-PLC	51	(Tso and Siebel, 1989)
C. novyi	γ-toxin	58	(Tsutsui *et al.*, 1995)
C. sordellii	Csp	53.4	(Karasawa *et al.*, 2003)
C. haemolyticum	β-toxin	53.5	(Hauer *et al.*, 2004)
C. absonum	α-toxin	60	(Clark *et al.*, 2003)
B. cereus	phosphatidylcholine preferring plc (PC-PLC)	28	(Leslie *et al.*, 1989; Tso and Siebel, 1989)

In comparison to other bacterial plc's, over time the α-toxin has evolved in terms of both enhanced expression and activity making it one of the most toxic plc's (Tsutsui *et al.*, 1995).

Whilst the N-terminal domain is very similar when compared to a number of other bacterial plc's, the C-terminal domain is unique with no known structural similarities in prokaryotes. However, it does share a great deal of homology with the eukaryotic C2 calcium binding cell signalling proteins (Newton, 1995; Rizo and Sudhof, 1998). The α-toxin C-terminus shares 34% amino acid homology with the human arachidonate-5-lipoxygenase (HA5L) signalling protein (Titball *et al.*, 1991) and structural similarities with pancreatic lipase, and soybean lipoxygenase (Naylor *et al.*, 1998). HA5L is a member of the leukotriene synthetic pathway which is disrupted by α-toxin. The α-toxin might use its C2-like domain to bind membranes in a similar manner to this signalling protein and initiate the disregulation of host inflammatory responses as discussed below (Naylor *et al.*, 1998).

The α-toxin is not only able to damage cell membranes directly by hydrolysing phospholipids, but it is also able to trigger the disregulation of local and systemic host responses leading to tissue dysfunction, shock and death (Figure 1.3) (Bryant and Stevens, 1996; Titball, 1997). The binding of α-toxin to phospholipids may activate GTP-binding protein, which inturn activates endogenous plc's such as phosphatidylinositol plc (PI-PLC) and phospholipase D (Sakurai *et al.*, 1993; Sakurai *et al.*, 1994; Ochi *et al.*, 1996). On the other hand, product formation from direct α-toxin activity on membranes may also be able to trigger these events (Sakurai *et al.*, 1993; Sakurai *et al.*, 1994). Activation of endogenous PI-PLC by α-toxin catalyses membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol 1,4,5-triphosphate (IP_3) and diacylglyceride (DAG). In turn IP_3 induces the release of Ca^{2+} from internal reservoirs in the endoplasmic reticulum (Gustafson and Tagesson, 1989; Sakurai *et al.*, 1994).

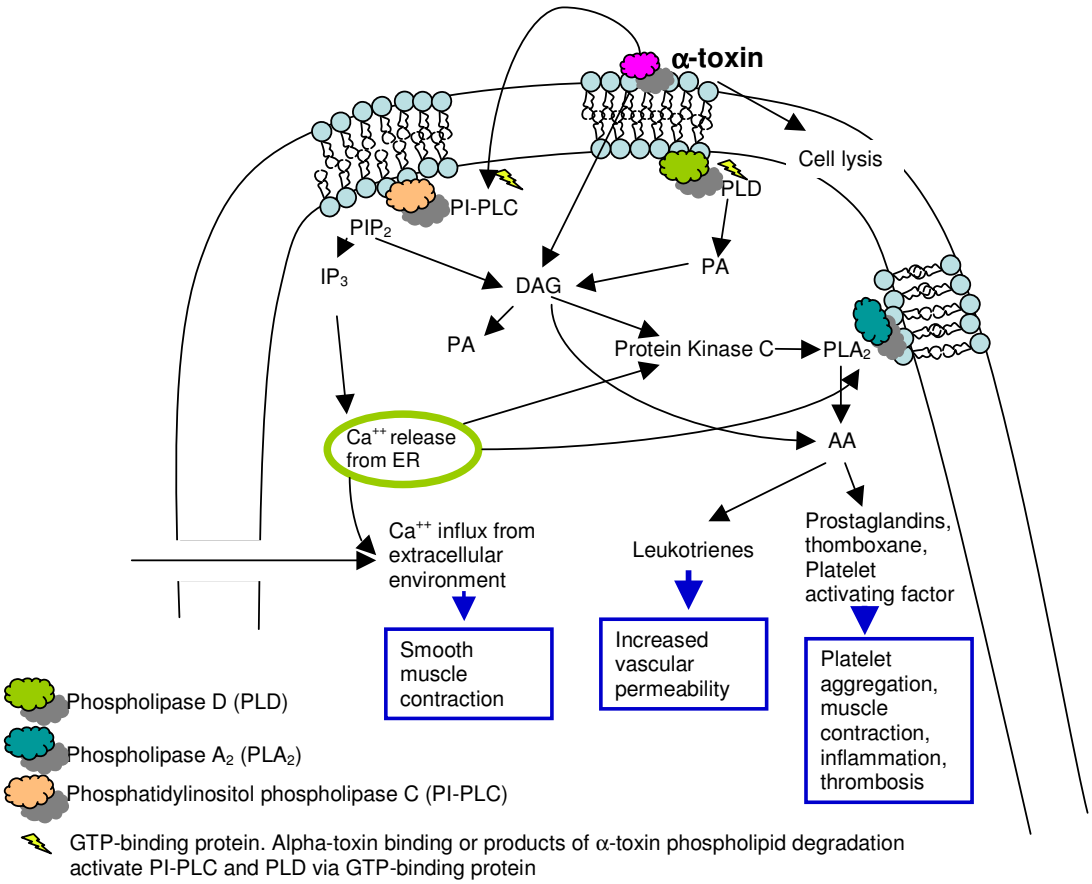


Figure 1.1: Induction of endogenous phospholipases and arachidonic acid cascade by α-toxin in a eukaryotic endothelial cell.

This burst of cytosolic Ca^{2+} mediates the influx of Ca^{2+} through membrane channels and the activation of endogenous phospholipase A_2 (PLA_2) (Gustafson and Tagesson, 1989; Bryant *et al.*, 2003). The second product of PIP_2 and phosphatidylcholine hydrolysis by PI-PLC and α-toxin respectively, DAG, is inturn directly catalysed by DAG lipase to form arachidonic acids (AA). Alternatively DAG can activate protein kinase C, which then activates PLA_2 (Gustafson and Tagesson, 1989; Sakurai *et al.*, 1994; Bunting *et al.*, 1997). The activation of PLA_2 results in the release of arachidonic acids from the membrane and the production of its metabolites which include thromboxanes, leukotrienes, and prostaglandins (Titball *et al.*, 1999), all of which play an important role in the local inflammatory response of the host.

Alpha-toxin stimulates the production of platelet activation factor and prostacyclin from AA (Bunting *et al.*, 1997), the upregulation of cell-leukocyte surface adhesion markers, and the production of interleukin-8 (Bryant and Stevens, 1996). This leads to the aggregation of platelets which remain free flowing in blood vessels, followed by the attachment of leukocytes to the platelets. These larger aggregates then bind to endothelial cells and eventually block the flow of blood (Barzaghi *et al.*, 1988; Bryant *et al.*, 2000a). Interleukin-8 may enhance leukocyte migration and enhance respiratory burst activity resulting in local vascular damage adjacent to, but not at the site of infection with *C. perfringens* (Bryant and Stevens, 1996). The products released from α-toxin induction and the AA cascade result in thrombosis, vasodilation and leukostasis, all of which restrict the blood flow in the host and provide an anoxic environment suitable for *C. perfringens* proliferation (Ninomiya *et al.*, 1994; Ellemor *et al.*, 1999). This can eventually lead to shock and death of the host (Bunting *et al.*, 1997; Stevens and Bryant, 1997).

The effects of the α-toxin in gas-gangrene are well established, however, what is not known is how big a part the α-toxin plays in other diseases of type A strains, particularly diseases of

veterinary importance (Ninomiya *et al.*, 1994; Awad *et al.*, 1995; Bryant and Stevens, 1996; Bryant *et al.*, 2000a; Bryant *et al.*, 2000b; Awad *et al.*, 2001).

1.5.2.2 Beta-toxin

The β-toxin is recognised as an important agent in necrotic enteritis of animals and humans. The β-toxin is a heat-labile, trypsin sensitive single polypeptide (Sakurai and Duncan, 1978). The structural gene *cpb* has been cloned and shown to express a polypeptide of 347 amino acids, of which the first 27 amino acids are removed upon extracellular secretion (Hunter *et al.*, 1992). The MW of the mature protein is 34,000 dalton (Sakurai and Duncan, 1978; Sakurai *et al.*, 1984; Hunter *et al.*, 1992). Beta-toxin is the second most lethal *C. perfringens* toxin following ε-toxin. Intravenous injection of β-toxin results in an LD_{50} of between 0.3 μg/kg (Sakurai and Fujii, 1987) and 1.1 μg/kg (Steinthorsdottir *et al.*, 1998).

Genetic analysis of the β-toxin reveals sequence homology with the alpha-toxin, gamma-toxin, and leukocidin of *Staphylococcus aureus* (Hunter *et al.*, 1993). These proteins are pore-forming cytolysins, and β-toxin has exhibited some cytolytic activity in a human leukaemia (HL) cell line (Nagahama *et al.*, 2003). The areas of homology suggests that β-toxin is likely to be a membrane spanning protein that oligomerises to form channels in susceptible cells (Hunter *et al.*, 1993; Steinthorsdottir *et al.*, 2000; Nagahama *et al.*, 2003; Smedley *et al.*, 2004). These channels cause a change in the membrane permeability of the cell which results in an efflux of potassium ions and an influx of calcium, sodium and chloride ions resulting in cell swelling and lysis (Steinthorsdottir *et al.*, 2000; Nagahama *et al.*, 2003). While the monomeric form of β-toxin is quite susceptible to protease and heat treatment, the multimeric form is very resistant to urea, detergent and heat treatment (Steinthorsdottir *et al.*, 2000) The process of toxin binding to receptors is irreversible, and administration of antitoxins post-injection cannot neutralise the toxin (Sakurai *et al.*, 1981).

Recent data suggests that the β-toxin may primarily act as a neurotoxin (Shatursky *et al.*, 2000), rather than a cytolysin, acting on preganglionic fibres or central nervous system components of the autonomic nervous system (Sakurai *et al.*, 1981; Shatursky *et al.*, 2000). Some neurological symptoms caused by β-toxin in agriculturally important animals includes tetany and opisthotomus (Tweten, 2001). Binding of the toxin to as yet unidentified susceptible neuronal cells causes the formation of membrane channels which allows the influx of K^{2+} and Na^{2+} ions. This may induce a rapid, irreversible depolarisation of the cell causing neuronal damage (Shatursky *et al.*, 2000).

1.5.2.3 Epsilon-toxin

Epsilon-toxin (ε-toxin) is the most potent of the *C. perfringens* toxins, and the third most potent neurotoxin from the *Clostridium* spp., following botulinum and tetanus toxins (Mantis, 2005). Intravenous delivery of 75 ng of the toxin to mice kills them within 90 minutes (Nagahama *et al.*, 1990). The rapid speed in which it is able to impart its toxicity on its host can also be attributed to the fact that it is able to penetrate the blood/brain barrier (Worthington and Mulders, 1975).

The ε-toxin is expressed as a 33 kDa protoxin (Hunter *et al.*, 1992), of which the N- and C-termini are cleaved to form the extremely potent mature ε-toxin of 29 kDa (Hunter *et al.*, 1992; Minami *et al.*, 1997). The LD_{50} in mice is between 65 and 320 ng/kg depending upon the cleavage site of the protoxin. Trypsin and chymotrypsin cleave the N-terminus after the 13^{th} amino acid, whereas λ-toxin, another product of *C. perfringens*, cleaves the protoxin after the 10^{th} amino acid (Minami *et al.*, 1997). Cleavage by the former is the more potent (Minami *et al.*, 1997).

The ε-toxin of *C. perfringens* type D causes enterotoxaemia of lambs and kids (Uzal and Kelly, 1997). The toxins' ability to increase blood pressure (Sakurai *et al.*, 1983), and vascular permeability (Worthington and Mulders, 1975) assists in its transit from the intestines to the blood system and finally to the brain, where it imparts neuronal damage (Miyamoto *et al.*, 1998; Finnie *et al.*, 1999). Neurotoxicity involves the stimulation of neurotransmitter release from the glutamatergic system. The release of glutamate from presynaptic vesicles leads to neuronal damage (Miyamoto *et al.*, 1998). It also indirectly causes contraction of muscles by activating Na^{2+} channels. Activation of these channels results in acetylcholine release from cholinergic nerve endings which in turn promotes calcium availability thereby causing contractions (Sakurai *et al.*, 1989).

The ε-toxin requires binding to specific cell receptors to impart its effects. These sialoglycoprotein receptors are thought to be present in the brain, lungs and kidney tissues, the primary targets of the toxin (Nagahama and Sakurai, 1991; Nagahama and Sakurai, 1992; Uzal and Kelly, 1997; Tamai *et al.*, 2003). The toxin oligomerises to form heptamers on the cell surface (Miyata *et al.*, 2002). These heptamers then act by forming pores in the membrane. Pore formation results in loss of transepithelial resistance and consequently an increase in ion permeability and a net influx of water creating oedema of the cell (Petit *et al.*, 1997).

1.5.2.4 Iota-toxin

The ι-toxin is a binary toxin dependent on two non-linked domains for activity (Stiles and Wilkins, 1986). The two genes coding for the binary toxin, *lap* and *Ibp*, reside on the same sequence, are expressed by the same promoter but are separated by 40 non-coding nucleotides (Perelle *et al.*, 1993; Perelle *et al.*, 1995). The sizes of the mature proteins Ia and Ib are approximately 45,000-47,500 dalton (Stiles and Wilkins, 1986; Popoff and Boquet, 1988a;

Perelle *et al.*, 1993) and 71,500-80,000 dalton (Stiles and Wilkins, 1986; Perelle *et al.*, 1993) respectively. Due to their sizes, Ia is known as the light chain, and Ib as the heavy chain. Ia functions as an ADP-ribosyltransferase, while Ib is required for cell receptor binding and translocation of component Ia across the cell membrane (Simpson *et al.*, 1987; Perelle *et al.*, 1993). Intravenous injection of Ia or Ib alone in mice and guinea pigs causes no significant changes to the animals, but simultaneous injection of the two causes death in mice and dermonecrotic lesions in guinea pigs (Stiles and Wilkins, 1986; Sakurai and Kobayashi, 1995). Lethal doses of Ia/Ib intravenously injected into mice vary between 24/48 μg/kg (Ia component) (Sirard *et al.*, 1997) and 60/100 μg/kg (Ib component) (Sakurai and Kobayashi, 1995) making it the least toxic of the major toxins of *C. perfringens*.

Both Ia and Ib proteins are secreted as protoxins which require cleavage by λ-toxin (a product of *C. perfringens*) or chymotrypsin (within the host) for activation (Gibert *et al.*, 2000). The C-terminus of activated Ib binds to sensitive cells which are most likely proteinaceous in nature (Stiles *et al.*, 2000). Once Ib is bound, docking of Ia occurs at the protease treated N-terminus of Ib (Perelle *et al.*, 1993; Perelle *et al.*, 1995). Binding of Ia to Ib triggers rapid internalisation of Ia followed by cell membrane embedment or internalisation of Ib (Stiles *et al.*, 2000). Once in the cytosol, Ia binds nicotinamide adenine dinucleotide (NAD), cleaves the ADP-ribose moiety and transfers it to arginine-177 of globular actin (G-actin) (Vandekerckhove *et al.*, 1987). The ADP ribosylation of G-actin actively interferes with actin filament polymerisation by capping of actin filament at the barbed end (fast growing end), and causes depolymerisation at the opposite end, which results in the collapse of the microfilament network (Wegner and Aktories, 1988). Rounding of cells occurs, due to the disorganisation of the actin cytoskeleton and cell death ensues (Perelle *et al.*, 1996).

The exact mechanism of Ia docking to Ib along with the specific receptors for Ib binding are still a mystery and recent X-ray crystallography of ι-toxin may shed some light on these matters (Tsuge *et al.*, 2003). Iota-toxin may become a useful biological tool for the delivery of proteins into cells. Marvaud, *et al.,* (2002) successfully delivered protein C3 of *C. botulinum* into Vero cells by using Ib for internalisation of a chimaeric C3:Ia protein (Marvaud *et al.*, 2002).

1.5.2.5 Enterotoxin

Although *C. perfringens* enterotoxin (CPE) is not classified as one of the major toxins of *C. perfringens*, it is the third most common cause of food poisoning in industrialised nations (Johnson, 1989). The cpe gene can be either plasmid-borne or found on the chromosome (Cornillot *et al.*, 1995). The chromosomally located *cpe* has been associated with food-borne gastroenteritis and the episomal *cpe* with non-food-borne gastroenteritis, such as antibiotic associated diarrhoea and sporadic diarrhoea (Collie and McClane, 1998). The plasmid-borne *cpe* may also be responsible for a number of enteric diseases observed in animals (Bueschel *et al.*, 1998).

CPE is a 35 kDa protein (McClane *et al.*, 1988) that is not secreted from the cells of growing bacteria but is released only with the sporulation of *C. perfringens* (Czeczulin *et al.*, 1996). Up to 13% of the total protein in a sporulating *C. perfringens* cell consists of enterotoxin (Czeczulin *et al.*, 1993). Even though there are no signal sequences identified at the N- or C-terminus of CPE, trypsin treatment of CPE cleaves 25 amino acids of the N-terminal sequence and creates a toxin that is 2- to 3- fold more toxic than the native CPE (Hanna *et al.*, 1992; Kokai-Kun and McClane, 1997). This enhanced activation of CPE may be physiologically important to increase the potency of CPE at its natural site of action, the small intestine of the host (Hanna *et al.*, 1992).

The mechanism of action for enterotoxin involves binding of enterotoxin to protein receptors of sensitive cells which primarily consist of enterocytes in the small intestine (Katahira *et al.*, 1997b). Two of its receptors have been identified as claudin-3 (formerly known as the human homologue of rat ventral prostate 1 protein-RVP-I) and claudin-4 (formerly known as CPE receptor) (Katahira *et al.*, 1997a; Katahira *et al.*, 1997b; Morita *et al.*, 1999). Claudins play a central role in tight junction (TJ) barrier function which controls the membrane permeability of the cell (Sonoda *et al.*, 1999).

Binding of CPE to claudin-3 and claudin-4 results in the formation of a small 90 kDa complex which destabilises the membrane barrier allowing the influx or efflux of molecules up to the size of 10 kDa (McClane et al., 1988; Wieckowski et al., 1994; Sonoda et al., 1999; Kondoh et al., 2005). Further binding of other membrane proteins to the small complex results in its membrane insertion (Wnek and McClane, 1989). This insertion results in pore formation and the onset of major membrane permeability alterations, allowing the efflux of larger molecules (Wieckowski et al., 1998). Occludin, another TJ associated protein binds to the large complex to form a 200 kDa complex which causes further structural damage to membranes (Singh *et al.*, 2000; Singh *et al.*, 2001). These changes cause rapid fluid and electrolyte loss from the gastrointestinal tract of infected individuals and animals resulting in diarrhoea (Sarker *et al.*, 1999).

1.5.2.6 Beta2-toxin

The discovery of a new cytotoxic toxin, the β_2-toxin, may affect the future of the typing of *C. perfringens* strains. This toxin was isolated and cloned by Gibert *et al.,* (1997). PCR typing studies have identified the β_2-toxin gene in many diarrhoeic piglets (Gibert *et al.*, 1997; Klaasen *et al.*, 1999; Garmory *et al.*, 2000; Waters *et al.*, 2003; Jost *et al.*, 2005) and

horses (Gibert *et al.*, 1997; Herholz *et al.*, 1999; Bacciarini *et al.*, 2003; Waters *et al.*, 2005), which may implicate this new toxin in diseases of these animals previously thought to have been caused by other toxins of *C. perfringens.* Until further studies are complete this finding is speculative, particularly for β_2-toxin involvement in horse enteritis as it is not consistently detected in isolates from diarrhoeic horses (Bacciarini *et al.*, 2003; Waters *et al.*, 2005). A more definitive role for β_2-toxin has thus far been achieved for pig enterocolitis (Bueschel *et al.*, 2003; Jost *et al.*, 2005; Waters *et al.*, 2005).

Given the name β_2-toxin due to its analogous nature with the β-toxin and its discovery from a pathogenic type C strain isolated from a piglet with lethal necrotising enterocolitis, the toxin has no significant homology with any other known proteins (Gibert *et al.*, 1997). As with most other toxins of *C. perfringens* it contains an N- terminal signal sequence for secretion to the extracellular environment (Gibert *et al.*, 1997). The size of the mature protein is approximately 28 kDa (Gibert *et al.*, 1997). The β_2-toxin is lethal to mice when 3 μg is administered intravenously, and like β-toxin is trypsin sensitive, and causes blebbing and cell death in CHO cells (Gibert *et al.*, 1997).

No further structure-function studies have been done with β_2-toxin to date but its effects mimic the effects of β-toxin suggesting that it too may be a pore-forming toxin (Smedley *et al.*, 2004).

1.6 Diseases of *C. perfringens*

C. perfringens is known widely for its ability to cause a variety of human and veterinary diseases. It is responsible for the majority of clostridial enteric diseases observed in domestic

livestock and is an important pathogen in humans (Songer, 1996). Some of these diseases are outlined below with a detailed description of necrotic enteritis, the focus of this work.

1.6.1 Human diseases

1.6.1.1 Gas-gangrene

Myonecrosis, or gas-gangrene as it is more commonly known, is an opportunistic disease caused by *C. perfringens* type A (Onderdonk and Allen, 1995; Rood, 1998). Prior to the discovery of penicillin, gas gangrene was a major cause of amputations in World War I. These days it is more common in post-operative or illegal surgeries (Allen, 1985).

The disease is relatively localised, with early symptoms including limb swelling and gross inflammation. *C. perfringens* toxin production within the host causes muscle necrosis and destruction, and haematuria, and if left untreated, the disease progresses rapidly, leading to shock and death (Awad *et al.*, 1995; Stevens and Bryant, 1997; Awad *et al.*, 2000).

The α-toxin is the major necrotising toxin responsible for the observed disease (Awad *et al.*, 1995; Awad *et al.*, 2000). It also contributes to shock by direct inhibition of myocardial contractility (Stevens and Bryant, 1997). Without the initial and continual effects of α-toxin the infection does not display signs of the fulminating disease (Awad *et al.*, 1995). Other extracellular toxins important for the spread of the disease through deeper tissues include κ-toxin, neuraminidase and θ-toxin (Shimizu *et al.*, 2002a). Gas-gangrene is controlled by the use of antibiotics, in severe cases a hyperbaric chamber is required and amputation of the affected limb(s) may be necessary.

1.6.1.2 Gastroenteritis

C. perfringens type A is responsible for a very common food-borne disease which usually appears 7-15 hours after the consumption of improperly cooled or reheated meat (Allen, 1985). The onset of the disease is associated with the sporulation of *C. perfringens* in the intestinal tract of its host (Czeczulin *et al.*, 1996). Sporulation and hence release of enterotoxin from bacterial cells triggers an accumulation of fluids and electrolytes in the intestine followed by the onset of diarrhoea. Severe abdominal cramps appear prior to diarrhoea but vomiting is absent (Narayan, 1982). Not all type A strains carry the enterotoxin gene and studies have indicated the incidence of *C. perfringens* carrying the *cpe* vary from 1.3% to 12.8% (Daube *et al.*, 1996; Meer and Songer, 1997). Due to its self-limiting nature and the voluntary reporting of the disease in most countries, *C. perfringens* gastroenteritis is an often underestimated disease worldwide (Labbe, 1991).

1.6.1.3 Enteritis necroticans

Termed Darmbrand in Germany or Pig-bel in Papua New Guinea, enteritis necroticans has been a major cause of mortality in the highland regions of Papua New Guinea and other third world countries where protein malnutrition and poor hygiene standards are common. It is caused by *C. perfringens* type C producing the β-toxin (Davis, 1984). The disease primarily occurs due to lack of the protease trypsin in the host's intestine as trypsin is an inhibitor of the β-toxin (Sakurai and Duncan, 1978). Reduction in trypsin levels result from low protein diets and diets containing high levels of trypsin inhibitors such as the sweet potato. During times of feast, ingestion of rich protein foods, particularly undercooked pork (a common food vehicle of *C. perfringens*) allows the proliferation of *C. perfringens* and the subsequent production of the β-toxin in the intestines (Davis, 1984). The term Darmbrand in German translates to “firebowels” which is an accurate description of what occurs in the intestines during infection. The bowels become inflamed and necrotic lesions develop. The incidence

of Pig-bel has decreased dramatically due to the development of a β-toxin vaccine (Lawrence *et al.*, 1990). There have been isolated incidences where enteritis necroticans has been diagnosed in the absence of β-toxin. In these cases only *C. perfringens* type A is isolated and so the term pseudo-bel has been coined (Sargeant *et al.*, 2000).

1.6.2 Animal diseases

Although *C. perfringens* primarily causes histotoxic (tissue destroying) infections in humans, most infections observed in animals are of an enteric nature, and require the production of one or more of the major toxins (Onderdonk and Allen, 1995; Songer, 1997).

A summary of the major animal diseases caused by the various type strains of *C. perfringens* is outlined in Table 1.5. For a more detailed review of clostridial enteric diseases refer to reviews by Songer (Songer, 1996; Songer, 1998). In most cases the age of the animal dictates the severity of disease. As the normal intestinal flora of a neonate is yet to be established, the colonisation and/or proliferation of enteropathogenic *C. perfringens* in the gut occurs rapidly (Timoney *et al.*, 1988).

The use of multicomponent vaccines and the prophylactic application of antitoxins prevent the spread of disease in most cases. The vaccines are targeted for use in ruminants and consist of toxoid preparations of types C and D *C. perfringens*, or formalin inactivated β-, and ε- toxins with inactivated toxins of other clostridial species (Younan *et al.*, 1995; Kelneric *et al.*, 1996). Vaccines for diseases caused by type A strains such as necrotic enteritis in poultry are yet to be developed.

Table 1.5: *C. perfringens* toxinotype and associated veterinary diseases

Toxin type	Diseases
A	Necrotic enteritis in fowl; Enterotoxaemia in cattle and lambs; Necrotizing enterocolitis in piglets; Possibly equine colitis; Canine haemorrhagic gastroenteritis
B	Dysentery in newborn lambs; Chronic enteritis in older lambs (pine); Haemorrhagic enteritis in neonatal calves and foals; Haemorrhagic enterotoxaemia in adult sheep
C	Necrotic enteritis in fowl; Haemorrhagic or necrotic enterotoxaemia in neonatal pigs, lambs, calves, goats, foals; Acute enterotoxaemia (struck) in adult sheep
D	Enterotoxaemia in lambs (pulpy kidney) and calves; Enterocolitis in neonatal and adult goats; Possibly enterotoxaemia in adult cattle
E	Enterotoxaemia likely in calves and lambs; enteritis in rabbits; Host range and disease type unclear
A-E (enterotoxin +)	Canine and porcine enteritis; Possibly bovine and equine enteritis

Reproduced from a review by Songer (1996).

1.6.3 Necrotic enteritis

Necrotic enteritis (NE) was first described by Parish in 1961 (Parish, 1961a; Parish, 1961b). It is an enterotoxaemia affecting wild and captive birds (particularly poultry and turkeys) worldwide (Gazdzinski and Julian, 1992; Stuve *et al.*, 1992; Droual *et al.*, 1994; van der Sluis, 2000). As the name suggests, NE is characterised by the destruction of villi within the small intestine accompanied by host inflammatory responses.

NE usually presents itself within 2-6 weeks of the birds' life. It is a sporadic disease (Cowen *et al.*, 1987), thought to be caused by a disruption in the chickens own microflora. The causative organism, *C. perfringens* is found in massive numbers around the site of infection, which is usually localised to the jejunum and ileum (Parish, 1961b; Long *et al.*, 1974; Al-Sheikhly and Truscott, 1977b). Both lesions and *C. perfringens* can sometimes be detected in the caeca as well (Long *et al.*, 1974; Elwinger *et al.*, 1992).

Morbidity and mortality rates of natural outbreaks range from 5-10% and 0.5-1.0% respectively (Shane *et al.*, 1985). Levels of experimentally induced NE can range from as low as 1.3-10% to as high as 5.6-37.3% (Cowen *et al.*, 1987), as the clinical disease is not very easily reproduced (Helmboldt and Bryant, 1971; Al-Sheikhly and Truscott, 1977b; Cowen *et al.*, 1987; Elwinger *et al.*, 1992; Kaldhusdal *et al.*, 1999).

Acute and chronic cases of NE have been described (Parish, 1961a). Acute NE is lethal and birds die within 3-4 days of developing the infection. Birds display a loss of appetite and usually do not eat in their last 24 hours of life. The last stages of acute NE are characterised by the lack of ability to stand, drooping of the head and wings, and closing of eyes. Death occurs within a few hours of these signs. There is no significant weight loss and internal organs are occasionally heavier than normal (Parish, 1961a). As symptoms occur within their

last few hours of life (Parish, 1961a; Helmboldt and Bryant, 1971), it is difficult to detect at any earlier stage, and birds are usually found dead on the farm. Acute NE is likely the outcome of toxins of *C. perfringens* infiltrating the internal organs and damaging these cells (Vissiennon *et al.*, 1996).

Chronic, or sub-clinical NE, as it is more commonly known, is an insidious infection often left untreated due to difficulties in its detection (Parish, 1961a). Some sub-clinical signs that may be seen during the course of the outbreak include diarrhoea, depression, ruffled feathers, and huddling (Al-Sheikhly and Truscott, 1977b; Shane *et al.*, 1985; van der Sluis, 2000).

Diagnosis is most commonly attained through on-farm necropsy (van der Sluis, 2000). Unfortunately, this retrospective diagnosis leads to high economic losses from the early stages of NE where many of the early clinical signs appear to be overlooked. This is exacerbated by the fact that most farmers (71%) do not begin treatment when any primary symptoms are observed (van der Sluis, 2000).

Economic losses are also noted throughout the course of chronic NE due to an impaired food conversion ratio observed in the birds. This causes sub-optimal performance where birds display a gradual weight loss over 1-4 weeks without a decrease in food consumption. This impacts negatively on the economy of the farm due to the increased feed expenditure (Kaldhusdal and Hofshagen, 1992; Lovland and Kaldhusdal, 1999).

C. perfringens associated hepatitis (CPH) is another sub-clinical symptom which may be associated with NE. Lovland and Kaldhusdal (1999) reported that a large number of livers with abnormalities causing carcass condemnations at a Norwegian chicken processing plant were associated with *C. perfringens* infections. Increased numbers of *C. perfringens* in the

small intestine may increase the risk of clostridia accessing the biliary ducts and possibly the portal blood stream through a damaged intestinal mucosa (Vissiennon *et al.*, 1996; Lovland and Kaldhusdal, 1999).

The cost of sub-clinical enteritis is estimated to be as high as AUD$0.10/bird and where turnover is considered (each broiler lives for about 6-7 weeks) this number turns into hundreds of thousands of dollars in revenue losses per year. These estimates are highest in Europe followed by North Africa and the Middle East (van der Sluis, 2000).

1.6.3.1 Histopathology of NE

C. perfringens primarily attaches to the apical villi of the jejunum, whereby it proliferates and produces toxins (Kaldhusdal *et al.*, 1999). These toxins, particularly the α-toxin, destroy the villous creating necrotic lesions (Al-Sheikhly and Truscott, 1977b; Shane *et al.*, 1985). The epithelial cells are sloughed off into the lumen and along with erythrocytes and lysed heterophils create a mass of fibrino-necrotic exudates also known as coagulative necrosis (Long *et al.*, 1974; Al-Sheikhly and Truscott, 1977b; Shane *et al.*, 1985). Oedema occurs in the surrounding lamina propria and this results in further detachment of the epithelial layer (Long *et al.*, 1974; Al-Sheikhly and Truscott, 1977b). In severe cases the entire mucosal surface of the duodenum and jejunum is covered by a necrotic diphtheritic membrane (a greyish thickened mucosa) and the intestines become weak and friable (Al-Sheikhly and Truscott, 1977b; Shane *et al.*, 1985). Colonisation of *C. perfringens* at necrotic regions is prominent and lesions are usually demarcated by patches of normal, healthy tissue. Sub-clinical NE is less conspicuous and on macroscopic examination the intestines appear normal, but on microscopic examination, focal lesions can be seen and are of similar character to the acute lesions (Kaldhusdal and Hofshagen, 1992).

C. perfringens is rarely found in internal organs of the infected host, but congestion of blood vessels in some internal organs is prominent, particularly the liver and spleen (Parish, 1961a; Al-Sheikhly and Truscott, 1977b; Shane *et al.*, 1985). Toxins produced by *C. perfringens*, particularly the α-toxin, may be able to enter the circulatory system through the intestinal mucosa and reach other organs, namely the liver and kidneys where it can act as an endothelio-, hepato-, and nephro- toxin (Vissiennon *et al.*, 1996) and cause other sub-clinical features such as cholangiohepatitis (Lovland and Kaldhusdal, 1999).

1.6.3.2 Host responses to NE

Host inflammatory responses observed in cases of NE include an influx of heterophils in the early stages of the infection, followed by a slight (if any) influx of monocytes (Parish, 1961a; Long *et al.*, 1974; Al-Sheikhly and Truscott, 1977b). This lack of monocyte infiltration is also observed in gas-gangrene (Ninomiya *et al.*, 1994). Leukocytes are a target of the clostridial toxin(s) and it has been shown that the α- and to a lesser extent the θ-toxin of *C. perfringens* are able to impede the migration of leukocytes to the site of infection (Awad *et al.*, 1995; Ellemor *et al.*, 1999).

Birds have been shown to "spontaneously recover" from a bout of NE and on histopathological examination regeneration of villi has been observed. The regenerated epithelial cells result in a shorter fatter villus with reduced absorptive properties (Long *et al.*, 1974; Al-Sheikhly and Truscott, 1977a; Al-Sheikhly and Truscott, 1977b).

1.6.3.3 Predisposing Factors

Most *C. perfringens* isolated from outbreaks of NE are type A strains (Das *et al.*, 1997a; Nauerby *et al.*, 2003), but type C have been known to experimentally reproduce the disease (Shane *et al.*, 1985). Type A strains are naturally present in the microflora of healthy

chickens, and since infection of chickens with *C. perfringens* alone does not consistently reproduce the disease (Cowen *et al.*, 1987) several predisposing factors are believed to contribute to the spontaneous outbreaks of necrotic enteritis.

C. perfringens is normally found in the lower, large intestines (Allen, 1985) and during necropsy high *C. perfringens* counts are seen in small intestine particularly the jejunum and ileum (Parish, 1961b; Long *et al.*, 1974; Al-Sheikhly and Truscott, 1977b). This migration suggests sufficient numbers of *C. perfringens* and minor intestinal damage to the small intestine is required for the initiation of the disease (Al-Sheikhly and Truscott, 1977a). The minor small intestinal damage provides an anaerobic environment where *C. perfringens* can multiply rapidly. This damage can be brought about by a number of factors, including diet, prior infections and inadequate husbandry practices.

1.6.3.3.1 Diet

Poor feed has been shown to damage the intestines and allow for multiplication of *C. perfringens* in the gastrointestinal tract (GIT) of birds (Stuve *et al.*, 1992). The diet of broilers is usually dictated by cost and for this reason, ingredients such as wheat and fishmeal are utilised. Unfortunately it is the inclusion of these substances in the diet that enhances the risk of NE in a flock.

A wheat based diet increases the incidence of NE, whereas the incidence of NE is decreased when a corn based diet is used (Riddell and Kong, 1992; Branton *et al.*, 1997; Craven, 2000). Barley, wheat, rye, and oats contain higher levels of indigestible non-starch polysaccharides, which are known to lead to increased digesta viscosity, decreased digesta passage rate and nutrient digestibility (Hubener *et al.*, 2002). The decrease in passage rate increases the

likelihood of anaerobe overgrowth, particularly *C. perfringens*, leading to production of toxins which can then attack the epithelial lining (Shane *et al.*, 1985).

Feeds high in protein content such as fishmeal, meat, or bonemeal also increase the incidence of NE. High levels of fish meal have been reported to predispose animals to NE or exacerbate outbreaks of NE. These high protein diets may also cause some intestinal damage which favours the subsequent establishment and multiplication of *C. perfringens* (Truscott and Al-Sheikhly, 1977).

1.6.3.3.2 Prior infections

Diet is not the only factor that damages the intestinal wall of birds leading to outbreaks of NE. Concurrent or prior infection with enteropathogenic agents, particularly coccidia (*Eimeria* spp.) can dramatically increase the risk of NE in a flock (Cowen *et al.*, 1987; Baba *et al.*, 1992; Williams *et al.*, 2003). Damage to the intestinal mucosa by subclinical infection with *Eimeria* spp. leads to retardation of intestinal motility allowing for the proliferation of *C. perfringens* and its toxins (Shane *et al.*, 1985). Control of coccidiosis is maintained through the use of coccidiostats, mainly ionophores. Unfortunately the development of drug resistance to ionophores has lead to an increased prevalence of coccidiosis and hence NE (Lister, 1996; Allen and Fetterer, 2002). Vaccines against *Eimeria* spp. are now available (Williams *et al.*, 1999) but as NE is a multifactorial disease, it can still occur in the absence of coccidiosis (Cowen *et al.*, 1987; Riddell and Kong, 1992; Branton *et al.*, 1997).

1.6.3.3.3 Other factors

NE affects both wild and captive birds, but captive birds are more likely to succumb to the disease as they are more likely to encounter stress factors which affect the balance of the GIT

flora or the immune system (Stuve *et al.*, 1992). Such stress factors include transportation, sudden environmental changes, litter conditions, high stocking density, no in-feed additives (such as antibacterials/anticoccidials) and immunocomprimising agents such as cyclophosphamide (Truscott and Al-Sheikhly, 1977; Cowen *et al.*, 1987; Stuve *et al.*, 1992; Kaldhusdal *et al.*, 1999; Lovland *et al.*, 2003).

The introduction of a new strain of *C. perfringens* through a vector such as the house-hold fly has also been shown to cause NE in chickens who consumed the flies or their deposits (Dhillon *et al.*, 2004).

1.6.3.4 Aetiology of NE

The presence of large numbers of *C. perfringens* in the small intestine and caeca during an outbreak of NE in chickens indicates the importance of this bacterium in the disease, but factors leading up to pathogenesis are still unclear. Questions that still need to be answered include:

Can all toxinotypes of *C. perfringens* cause NE?

Can all strains of the same type induce disease under the right conditions?

How large a role does α-toxin play in the pathogenesis?

1.6.3.4.1 Can all toxinotypes of *C. perfringens* cause NE?

A number of studies analysing the distribution of toxinotype amongst natural outbreaks of NE identified all *C. perfringens* isolates causing NE as toxinotype A (Das *et al.*, 1997a; Engstrom *et al.*, 2003; Nauerby *et al.*, 2003) . This may indicate that type A are the cause of NE, but this is not the case. Shane *et al.,* (1985) was able to use a type C strain of *C. perfringens* to induce NE in an experimental model in broilers. It may be plausible that any strain of *C. perfringens* producing the required amount of toxins is able to induce NE, but the fact that natural

outbreaks of NE are linked to type A strains can be explained by the bacterium's natural habitat (Allen, 1985). Type A is the only type found as a commensal in animals, and it is abundant in soil and water, therefore it has the opportunity to cause an infection when the host is stressed.

Toxin levels also differ between type strains. Type A strains generally produce more α-toxin than B-E strains (Katayama *et al.*, 1993). As α-toxin is regarded as the major virulence determinant in NE, strains producing elevated levels of this toxin would be considered more virulent than others.

1.6.3.4.2 Are all *C. perfringens* type A isolates capable of causing NE?

There is a high genetic diversity of *C. perfringens* type A between and within flocks of poultry (Nauerby *et al.*, 2003). Healthy chickens can carry numerous strains of *C. perfringens* type A at one time. A dendrogram developed from pulsed-field gel electrophoresis (PFGE) of *C. perfringens* strains isolated from healthy and diseased chickens could not separate the NE causing strains from the avirulent strains (Nauerby *et al.*, 2003). What was noted though, was the lack of strain variability in the NE affected chickens. They carried only one or two clones of *C. perfringens*, suggesting that the proliferation of these specific clones led to NE. This leads back to the importance of predisposing factors such as diet and prior infection in influencing the development of NE, particularly since experimental infection of chickens with *C. perfringens* alone does not consistently reproduce the disease (Cowen *et al.*, 1987).

Unfortunately, the study by Nauerby, *et al.,* (2003) did not detail the husbandry and dietary parameters of the chickens examined at the different farms. Therefore it cannot be known what external factors (if any) were involved in the proliferation of these particular type A

clones. Until further studies are carried out looking at the gene expression (under different conditions e.g. differential display, microarray analysis) of these different strains, it will not be known whether they all have the same potential to cause NE. No standardised methods are currently used to differentiate between normal and NE causing *C. perfringens* strains.

1.6.3.4.3 Does α-toxin alone cause NE?

There is evidence demonstrating the importance of α-toxin in the development of NE. The extracellular toxins of *C. perfringens* type A have been shown to reproduce similar histopathological effects as that obtained with a natural outbreak of NE and anti-α-toxin antibodies are able to neutralise the effects of *C. perfringens* cell-free broth cultures when administered to chickens (Al-Sheikhly and Truscott, 1977b; Fukata *et al.*, 1988). Moreover, high levels of anti-α-toxin antibodies are frequently recovered from natural outbreaks of NE implicating α-toxin in the pathology of the disease (Lovland *et al.*, 2003) or at the very least its presence as an indicator of *C. perfringens* activity and a potential indicator of NE occurrence.

The reduction of, or lack of, monocyte infiltration to the site of NE infection also suggests that α-toxin is important in the development of the disease (Parish, 1961a; Al-Sheikhly and Truscott, 1977b). Studies of α-toxin mutants in mouse gas-gangrene models indicate that α-toxin is the major toxin involved in leukostasis (Ellemor *et al.*, 1999). This impediment of monocyte migration in the mouse gas-gangrene model may also explain the lack of monocyte infiltration in NE.

Expression of α-toxin differs between strains of *C. perfringens* (Katayama *et al.*, 1993; Bullifent *et al.*, 1996) and a critical level must be exceeded in order to cause disease

(Ninomiya *et al.*, 1994). The production of α-toxin is controlled by at least two regulatory systems (Lyristis *et al.*, 1994; Ba-Thein *et al.*, 1996; Bullifent *et al.*, 1996; Matsushita *et al.*, 1996; Ohtani *et al.*, 2002a). The control of α-toxin production by these systems may be regulated by as yet unidentified external stimuli. This may also include the autoinducer 2 (AI2) like molecules, implicating quorum sensing in α-toxin production (Ohtani *et al.*, 2002b). The presence of autoinducers and their cell-cell signalling activity may co-ordinate the levels of toxins produced in certain environments or in response to increased cell density (Miller and Bassler, 2001). For example, *in vitro* polyclonal anti-α-toxin antibody co-administration with *C. perfringens* neutralised the effects of α-toxin as observed by the Nagler reaction but when the two were co-administered to mice, the antibodies were insufficient for passive protection (Traub *et al.*, 1991). One possible explanation for this observation is the enhanced expression of α-toxin in a different environmental setting (Traub *et al.*, 1991). More regulatory studies are required to better understand the regulation of the toxin genes and the environmental factors that may be involved.

C. perfringens expresses numerous other enzymes and toxins which may also play a role (albeit a minor one) in the development of NE. Das *et al.*, (1997a), demonstrated that *C. perfringens* from 12 different outbreaks of NE contained the genes for the expression of the α-toxin along with sialidase (an enzyme which causes necrotic effects on intestinal mucosa), μ-toxin (spreading factor which attacks the lamina propria) and θ-toxin (perfringolysin O, cholesterol-dependent haemolysin).

The α-toxin acts in synergy with the θ-toxin to cause gas-gangrene in mice (Awad *et al.*, 1995; Ellemor *et al.*, 1999) and with the β_2-toxin to cause bovine enterotoxaemia (Manteca *et al.*, 2002). It may also act in synergy with other toxins to cause NE in chickens.

A second generation vaccine based on recombinant toxins or toxoids will provide direct evidence regarding the exact role of the α-toxin in this disease.

1.6.4 Prevention and treatment of NE

1.6.4.1 Antibiotics

Until recently, prevention of diseases in domestic livestock relied on the heavy use of antibiotics as prophylactic agents and as growth promoters. Antibiotic growth promoters (AGP) are sub-therapeutic quantities of antibiotics administered to the animals throughout their growing season (USDA, 1999). As their name suggests, they help increase the growth of the animal by suppressing the growth of unwanted bacteria in the gut, particularly Gram positive bacteria which are known to disrupt the digestive process and slow the growth of their host (USDA, 1999; WHO, 2000). Some prophylactic, therapeutic and AGP antibiotics used to treat and prevent NE in the past have included: avoparcin (Hofshagen and Kaldhusdal, 1992; Kaldhusdal and Hofshagen, 1992), nitrovin (Wierup, 2001), virginiamycin (Wierup, 2001), bacitracin (Kondo, 1988; Watkins *et al.*, 1997), lincomycin (Watkins *et al.*, 1997), penicillin (Kondo, 1988), tetracycline (Kondo, 1988) salinomycin, tylosin, avilamycin and flavomycin (Devriese *et al.*, 1993).

Many reports indicate that overuse, or sub-therapeutic use of antibiotics is causing an increase in the number of antibiotic resistant bacteria including *C. perfringens* in the domestic livestock population (Molbak, 2004; Shea, 2004). Resistance to bacitracin, streptomycin, virginiamycin, lincomycin and the tetracyclines in *C. perfringens* and other host bacteria has been demonstrated (Kondo, 1988; Watkins *et al.*, 1997; Das *et al.*, 1997b; Williams *et al.*, 1999; Aarestrup *et al.*, 2000; Johansson *et al.*, 2004; Martel *et al.*, 2004).

In recent years the use of many AGP's and prophylactic antibiotics has ceased due to bans imposed by the regulating bodies of many countries. Antibiotics with human-use antibiotic analogues are banned from use as AGP's (Schwarz *et al.*, 2001; Aarestrup, 2002). This is to prevent the possible horizontal transfer of human-use antibiotic resistance to bacteria in humans (Linton *et al.*, 1977; Levy, 1978; Lyons *et al.*, 1980; Abraham and Rood, 1985; Simonsen *et al.*, 1998; Luh *et al.*, 2000; Jensen *et al.*, 2002), and to appease the increasing public concern on the development of antibiotic resistant bacteria (WHO, 2000).

Avoparcin is an effective AGP for the prevention of clostridial diseases in chickens (Hofshagen and Kaldhusdal, 1992; Kaldhusdal and Hofshagen, 1992). Vancomycin is an analogue of avoparcin and bacteria harbouring the gene for avoparcin resistance automatically confer resistance to vancomycin. The overuse of avoparcin has led to increased incidences of vancomycin resistant enterococci (VRE) (Aarestrup *et al.*, 2000; Wierup, 2001) and perhaps their transfer to the human population (Simonsen *et al.*, 1998; Shea, 2004). There are also indications that human isolates of VRE in Europe developed through the use of avoparcin in poultry and other food animals (Shea, 2004). Avoparcin is now banned from use as an AGP (Elwinger *et al.*, 1998; APVMA, 2001; Heuer *et al.*, 2002).

The American Association of Avian Pathologists have developed guidelines for the judicious use of antibiotics in animals, although because these are only guidelines, they are not strictly adhered to (AVMA, 2005). They also recommend the use of bacitracin, penicillin, lincomycin, and erythromycin to effectively treat clostridial infections in poultry (Watkins *et al.*, 1997) of which the last (erythromycin) is also used in human medicine (AVMA, 2005). Other countries such as Sweden, Denmark, Norway, and the European Union (EU) have gone a step further and banned all AGP use in animals and incidentally, a decrease in antibiotic

resistant bacteria has been noticed in these areas (Aarestrup, 2002; Boel and Anderson, 2002; Jensen *et al.*, 2002; Casewell *et al.*, 2003).

The Australian Pesticides and Veterinary Medicines Authority (APVMA) has recently banned the use of avoparcin for the growth promotion of poultry and severely restricted the use of virginiamycin. No other AGP's with human-use analogues are allowed in the Australian poultry industry to prevent NE. Some antibiotics that can be used to treat NE in Australia include zinc bacitracin, which is also used as an AGP, salinomycin, lincomycin and the tetracyclines (APVMA, 2006).

The lack of availability of prophylactic antibiotics has generated a number of problems in the maintenance of a healthy poultry flock. An increase in NE disease and mortality is observed (Lovland and Kaldhusdal, 1999; Lovland and Kaldhusdal, 2001; Wierup, 2001; Tornoe, 2002; Casewell *et al.*, 2003; Grave *et al.*, 2004), there are increased costs to the management of the farm as new additives may be required, a change in feed may be essential (Branton *et al.*, 1997), and a reduction in stocking densities may be unavoidable (Wierup, 2001; PoultrySite, 2006). This inevitably reduces the number and size of broilers available in the market place. The ban of prophylactic antibiotics has seen the use of therapeutic antibiotics increase markedly (Wierup, 2001; Casewell *et al.*, 2003) and unfortunately the use of therapeutic antibiotics does not prevent the reoccurrence of NE within the same flock (Lovland *et al.*, 2003).

Ionophores primarily used to treat coccidial infections in poultry have been discovered to act on Gram positive bacteria such as *C. perfringens* (Elwinger *et al.*, 1992; Elwinger *et al.*, 1998). Narasin in particular, is quite often used to prevent both coccidiosis and NE and is commonly used in the feed of poultry (Wierup, 2001; Grave *et al.*, 2004; Johansson *et al.*,

2004). Some other ionophores such as monensin, nicarbazin and salinomycin are not as effective at preventing NE (Williams *et al.*, 1999; Grave *et al.*, 2004). The development of anticoccidial vaccines (Williams *et al.*, 1999; Williams *et al.*, 2003) may see an end to the use of narasin and other ionophores which may in turn lead to an increase in NE (Tornoe, 2002). One other drawback of the use of ionophores in poultry is the possibility of resistance developing in *C. perfringens* strains as has developed in *E. faecium* (SVARM, 2002; Johansson *et al.*, 2004).

1.6.4.2 Competitive exclusion

The state of the natural intestinal flora of chickens is integral in the protection of foreign and invading pathogens. Young chickens are at higher risk of becoming overgrown or infected with opportunistic bacteria such as *C. perfringens* due to the lack of a pre-established microflora (Fukata *et al.*, 1988). Competitive exclusion (CE), first developed by Nurmi and Rantala in 1973 to control and reduce *Salmonella* colonisation in chickens, are products that contain harmless bacteria able to colonise the GIT without causing adverse effects on their host (Nurmi and Rantala, 1973). When administered to chickens, CE products help to establish an undesirable environment for pathogen colonisation or growth by a number of mechanisms such as the creation of a restrictive physiological environment by the production of volatile fatty acids, the competition for bacterial receptors, the depletion of essential substrates, the production of toxic compounds such as bacteriocins, and the stimulation of an immune response (Mead, 2000; Patterson and Burkholder, 2003; La Ragione *et al.*, 2004).

A number of CE products developed and registered for the control of *Salmonella* colonisation in chickens include Aviguard®, Broilact®, Avifree®, MSC®, and Preempt® (Schneitz, 2005). The success of these products in terms of *Salmonella* inhibition has lead to their assessment in

the prevention of *C. perfringens* colonisation and growth in chickens (Elwinger *et al.*, 1992; Hofacre *et al.*, 1998a; Hofacre *et al.*, 1998b; Craven *et al.*, 1999).

Aviguard® (Hofacre *et al.*, 1998b) and Broilact® (Elwinger *et al.*, 1992) were both able to reduce the occurrence of NE demonstrated by an increase in weight gain, a normal feed conversion ratio and a reduction in lesion scores, although complete inhibition of NE was not observed in the studies. Inconsistencies between trials was observed in the Broilact® study and these have been attributed to a number of variables including the condition of the newly hatched chicks, immune status, infection pressure and diet (Elwinger *et al.*, 1992). Another study using two undefined normal gut flora products were unable to reduce the severity of gross intestinal lesions (Hofacre *et al.*, 1998a).

Other products such as Mucosal Starter Culture (MSC®), which consists of scrapings of the caecal mucosal and intestinal tract of adult broilers (Craven *et al.*, 1999), or defined products such as *Bacillus subtilis* PY79^{hr} spores (La Ragione and Woodward, 2003) and *Lactobacillus johnsonii* FI9785 (La Ragione *et al.*, 2004) may reduce intestinal proliferation of *C. perfringens*. While CE may have a promising future in the prevention of NE, reproducible trials including protection studies using NE models are required to definitively prove its viability.

1.7 Vaccination

The development of antibiotic resistance in the developed world has rekindled an interest in vaccination as a prophylactic measure in human and agricultural communities (Walker, 1991). Prevention is better than cure and vaccination offers the advantage of life-long immunity against deadly diseases, potentially without the costs and continual administration associated with sub-therapeutic and prophylactic antibiotics (Dougan *et al.*, 1989).

There is potential for the development of a vaccine against NE. Co-administration of the supernatant of a broth culture of *C. perfringens* and anti-α-toxin antisera inhibits the characteristic NE lesions observed, providing evidence for the involvement of α-toxin in the disease (Al-Sheikhly and Truscott, 1977a; Fukata *et al.*, 1988). Levels of anti-α-toxin serum IgG are higher in broilers with a history of NE (Lovland *et al.*, 2003), suggesting chicks confronted with an increase in intestinal α-toxin levels are able to develop antibodies targeting the toxin, but not in time to prevent any observable symptoms. Investigations concerning re-infection of chickens with NE have not been reported in order to correlate the pre-existing antibodies with re-infection.

Not only may it be possible to immunise chicks, but the immunisation of laying hens may be able to protect their progeny from NE (Heier *et al.*, 2001). Naturally occurring maternal antibodies to α-toxin of *C. perfringens* have been detected using an ELISA in day old chicks (Heier *et al.*, 2001). As these antibodies were maternal, their levels decreased at the time of slaughter. Flocks with high titers of specific maternal antibodies against α-toxin had lower levels of mortality during the production period than flocks with low titers (Heier *et al.*, 2001). No NE outbreak occurred in any of the flocks in this study by Heir *et al.,* (2001), therefore the hypothesis that anti-α-toxin antibodies definitively protected chicks from developing NE could not be examined (Heier *et al.*, 2001). However, data by Lovland, *et al.,* (2004) suggested that there is a protective effect of maternal anti-α-toxin antibodies on progeny against subclinical NE and *C. perfringens* hepatitis. The short maternal IgG half-life in broilers (1-2 weeks) (Lovland *et al.*, 2003), and the lack of a maternal IgA supply (Lovland *et al.*, 2004) may not allow enough time for the progeny to remain protected from NE during their most vulnerable period (between 2-6 weeks of age). Therefore, even though vaccinating the laying hens might be a cheaper and less laborious option, it may not be plausible. Another

option is live orally delivered vaccines, which are simple to administer, and antibody responses remain life-long (Lovland *et al.*, 2003).

A great deal more is known about how the toxins of *C. perfringens* initiate gas-gangrene in humans than how they initiate NE in poultry. The α-toxin is thought to play a major role in both diseases but how it interacts in the intestines is little studied. A specific vaccine targeting the α-toxin may help to clarify the matter of whether or not α-toxin is solely responsible for the enteritis observed, and whether or not a multi-component vaccine is required.

1.7.1 Inactivated bacterial vaccines

The chemical treatment of organisms to render them immunogenic but non-viable is one of the simplest methods of producing an antigenic cocktail for use as a vaccine. Vaccines for cholera, typhoid and poliomyelitis were developed in this manner (Roit, 1994).

Inactivated clostridial vaccines such as Ultrabac® and 7/Somubac® produced by formalin preservation and adjuvanting of a collection of *Clostridium* species have been marketed world-wide for many years for use in sheep, goat and cattle (Pfizer, 2006).

Some immune responses require the activation of specific T-cells for immunity, and the administration of inactivated bacteria is ineffective at producing this specific cell-mediated immunity (Walker, 1992). Other drawbacks of using killed organisms are the potential of toxicity due to endotoxin production of Gram negative bacteria (Walker, 1992; Makela, 2000) and the development of side-effects such as pain, swelling and redness at the site of vaccination (Janeway *et al.*, 2005).

1.7.2 Subunit/toxoid vaccines

Subunit vaccines are also very effective against pathogens that rely on exotoxin production for virulence. These include the tetanus toxin of *C. tetani*, diphtheria toxin of *Corynebacterium diphtheriae,* and cholera toxin of *Vibrio cholera* (Janeway *et al.*, 2005). The immune stimulus is maximally directed to the molecule relevant for protection, and as subunit vaccines are defined in composition, the problem of unknown associated components causing adverse reactions is avoided (Makela, 2000).

Although many subunit vaccines are deemed safer than whole cell inactivated vaccines, they are not always as effective at priming the full repertoire of immune cell types required for protection against a pathogen and its toxins (Janeway *et al.*, 2005). One example is the purified polysaccharide vaccine of *Haemophilus influenzae* type B, which is unable to stimulate an immune response on its own. The discovery of linking this polysaccharide vaccine to proteins such as tetanus toxoid, diphtheria toxoid or mycobacterial heat shock protein, which elicited a T-cell response, led to the development of the highly effective conjugate vaccines (Schneerson *et al.*, 1980). This was then used in other polysaccharide vaccines such as the pneumococci and meningococci vaccines (Beuvery *et al.*, 1982; Anderson *et al.*, 1994). In other cases chemical adjuvants can be used to enhance an immune response to the subunit vaccine. Most adjuvants are composed of salts such as aluminium hydroxide, aluminium phosphate, and potassium aluminium sulphate. Mineral oils are the most potent adjuvants, however, owing to their reactivity in tissues they are unacceptable in human use and severely limited in veterinary medicine (Walker, 1992).

Many early sub-unit vaccines were comprised of purified toxins inactivated by treatment with formaldehyde and were given the name toxoid, with many still in use today (Walker, 1992). With the development of recombinant technologies a genetically defined toxoid devoid of any

toxic activities can be developed through the genetic manipulation of the recombinant protein (Makela, 2000).

1.7.3 Live attenuated organisms as vaccines and carriers of vaccines

The objective of attenuation is to provide a modified organism which mimics the natural behaviour of the original microbe without causing any significant disease (Roit, 1994). The replication of the living microbe confronts the host with a larger and more sustained dose of antigen, and in cases of intracellular pathogens, invasion of cells to mount a good cytotoxic T-cell response and memory (Roit, 1994). Another advantage of live vaccines is that the immune response largely takes place at the site of the natural infection (Roit, 1994).

Live attenuated vaccines have been shown to provide as good or better protection than their inactivated counterparts stimulating both arms of the adaptive immune system: cell-mediated and humoral (Dougan *et al.*, 1987; Maskell *et al.*, 1987; Dougan *et al.*, 1989; Brossier *et al.*, 1999; Shata *et al.*, 2000; Kochi *et al.*, 2003).

Limitations to the development of attenuated organisms include the development of attenuations that preserve the immunogenic properties of the cell, the possibility of a lethal systemic infection in the immunocompromised with the vaccine strain (de Jong *et al.*, 1998; Mastroeni and Menager, 2003; Janeway *et al.*, 2005), or the possibility of reversion to virulence (Walker, 1992). The latter may be overcome by the development of two or more unrelated attenuations in the organism (Dougan *et al.*, 1988; Makela, 2000), and some attenuated vaccines have been shown safe even in immunocompromised animals (Izhar *et al.*, 1990; Strahan *et al.*, 1992).

Toxoids can be successful at developing a good immune response in the host, but often they require large scale purification from their organism, inactivation via formaldehyde and addition of adjuvants to enhance their immunogenicity (Walker, 1992). Attenuated vaccines have the potential to "piggy-back" foreign antigens without the issues above. This has a number of advantages over administration of sub-unit or toxoid vaccines. No adjuvant is required as the host bacterium acts as a natural adjuvant and purification of high concentrations of antigen is not necessary as it is produced within the host bacterium (Brown *et al.*, 1987; Guzman *et al.*, 1991; Karem *et al.*, 1995; Matsumoto *et al.*, 1998). Delivery to the site of natural infection is possible, and parenteral administration can be avoided, as many live attenuated vaccines can be administered orally (Kochi *et al.*, 2003). This is of particular significance to the agricultural industry, where time and money can be saved by the use of an orally administered vaccine through the drinking water, feed, or as an aerosol (Coloe *et al.*, 1995).

Some live vaccines with potential as carriers of foreign antigens are listed in Table 1.6. Each live vaccine vector offers distinctive features. *Shigella flexneri* and *Listeria monocytogenes* which usually multiply in the cytosol of an infected cell, present their antigens to MHC class I molecules and hence a cytotoxic lymphocyte CD8 T cell response is primed (Goossens and Milon, 1992; Goossens *et al.*, 1995). *Salmonella*, *Mycobacterium* (BCG) and *Brucella* strains trigger a TH1 immune response as they survive or replicate within the vacuolar compartments of an infected cell and generally present themselves to MHC class II molecules (Shata *et al.*, 2000; Vemulapalli *et al.*, 2000; Gentschev *et al.*, 2001). The decision on which vector is the best one to use will be influenced by the type of immune response required, by the route of administration and the final site of expression of the heterologous antigen.

Table 1.6: Attenuated live bacterial vaccines with potential for heterologous antigen delivery

Bacterium	Attenuation(s)	Reference
Salmonella Typhimurium	Interruption of biosynthetic pathways (Δ*aro*)	(Hoiseth and Stocker, 1981)
	pur^{-} (purine mutants)	(O'Callaghan *et al.*, 1988)
S. Dublin	Δ*aro* mutants	(Smith *et al.*, 1984)
Brucella abortus	Rough strain (lack of O-chain in lipopolysaccharide)	(Vemulapalli *et al.*, 2000)
Bacillus anthracis (spores)	Sterne-Brossier strain with inactivated lethal factor, oedema factor or protective antigen	(Sirard *et al.*, 1997; Brossier *et al.*, 1999)
Salmonella Typhi (Ty21a)	galE mutant (Ty21a)	(Germanier and Fuer, 1975)
	Δ*aro* A/C	(Chatfield *et al.*, 1992b)
	Δ*aro*A/C-*htr*A	(Tacket *et al.*, 1997)
Listeria monocytogenes	*act*A mutant: lack of intra-and inter-cellular movement	(Goossens and Milon, 1992; Chakraborty *et al.*, 1994; Goossens *et al.*, 1995; Shen *et al.*, 1995)
	*plc*B mutant: prevents escape from secondary vacuoles	(Angelakopoulos *et al.*, 2002)
Mycobacterium bovis bacillus Calmette-Guerin strain (BCG)	Attenuation developed through continual passage over 10 years	(Dougan *et al.*, 1989; Matsumoto *et al.*, 1998)
Shigella flexneri	Numerous (see Phalipon and Sansonetti 1995 for review)	(Phalipon and Sansonetti, 1995; Koprowski *et al.*, 2000; Barry *et al.*, 2003; Ranallo *et al.*, 2005)
Vibrio cholerae	Partial or complete deletion of cholera toxin gene	(Butterton *et al.*, 1995; Ryan *et al.*, 1997)

1.7.4 Attenuated *Salmonella* Typhimurium as carriers of foreign antigens

Salmonella enterica serovar Typhimurium, from now on referred to as *S.* Typhimurium, is a Gram negative bacterium of the Enterobacteriaceae family. The bacterium is commonly used as a model for typhoid in mice. Its natural route of infection provides an ideal system for the delivery of heterologous antigens directly to the GIT of the target species. The bacterium is able to translocate from the intestinal lumen via the microfold cells (M-cells) of the Peyer's patches (Jones *et al.*, 1994) to the lamina propria and regional lymph nodes which then leads to colonisation of the reticuloendothelial system (RES), also known as the mononuclear phagocyte system (Dougan *et al.*, 1987; Chong *et al.*, 1996). This follows replication in the liver and spleen (Maskell *et al.*, 1987). In the mouse model, *S.* Typhimurium numbers increase and at about 7 days post-infection mice begin to die (Maskell *et al.*, 1987). *S.* Typhimurium are able to survive and replicate within eukaryotic cells including macrophages (Gahring *et al.*, 1990; Lindgren *et al.*, 1996) and have the ability to cause apoptosis in dendritic cells (Monack *et al.*, 1996). The survival of *S.* Typhimurium within macrophages assists their evasion of the immune system and perhaps their dissemination into deeper tissues (Lindgren *et al.*, 1996).

As invasion of *S.* Typhimurium through the Peyer's patches of the GIT delivers the pathogen directly to the lymphoid system of the host, development of attenuated strains of this pathogen are ideal candidates for the delivery and expression of heterologous antigens directly to the immune system.

Attenuated strains of *S.* Typhimurium effectively invade and colonise the small intestine, mesenteric lymph nodes, liver, and spleen. However, instead of replicating and causing a severe systemic infection, they grow at a much slower rate and persist at lower levels than their virulent counterparts (Dougan *et al.*, 1987; Maskell *et al.*, 1987), and are usually cleared

by the immune system within 35 days (Maskell *et al.*, 1987; Stabel *et al.*, 1990). Their survival within the host for 2-5 weeks enables the development of an immune response to the bacterium without the detrimental effects to the host (Maskell *et al.*, 1987; Stabel *et al.*, 1990; Alderton *et al.*, 1991).

Attenuated *S.* Typhimurium have the potential to be used as a combined vaccine due to their known capacity to elicit circulatory and secretory antibodies and cell-mediated immunity (Brown *et al.*, 1987; Dougan *et al.*, 1987; Guzman *et al.*, 1991; Mastroeni *et al.*, 1992; Mitov *et al.*, 1992). *Salmonella* spp., predominantly *aro*A$^-$ strains of *S.* Typhimurium, have been utilised to express and deliver a variety of foreign antigens (Appendix 1).

Some *Salmonella* attenuations include the disruption of biosynthetic pathways leading to auxotrophy (Hoiseth and Stocker, 1981; Hackett, 1990), the removal of the capacity of a strain to make or respond to cyclic AMP inhibiting growth of the strain (Hackett, 1990) and mutations leading to increased sensitivity to oxidative stress restricting their survival within macrophages (Dougan *et al.*, 1989; Hackett, 1990; Cardenas and Clements, 1992; Chatfield *et al.*, 1993).

1.7.4.1 Immune responses to *S.* Typhimurium

Live vaccination with *S.* Typhimurium attenuated strains results in both cell mediated and humoral immunity (Moser *et al.*, 1980; Brown *et al.*, 1987; Maskell *et al.*, 1987; Mitov *et al.*, 1992) whereas vaccination with heat killed strains results in a predominant humoral immune response (John *et al.*, 2002).

Interferon gamma (IFN-γ) production by natural killer cells of the innate immune system is important for the initial control of salmonellosis (Mastroeni *et al.*, 1992; Hess *et al.*, 1996;

John *et al.*, 2002; Norimatsu *et al.*, 2004). Cell mediated immune responses on the other hand are not induced by killed vaccines. The entry of live *S.* Typhimurium into antigen presenting cells (APC) such as macrophages and dendritic cells stimulates a TH1 T-cell response by inducing the expression of specific cell surface molecules and cytokines (Pashine *et al.*, 1999; Norimatsu *et al.*, 2004). Dendritic cells phagocytose and process attenuated *S*. Typhimurium molecules within their vacuolar compartments (Watts, 1997; Norimatsu *et al.*, 2004) and display the processed molecules on their cell surface via the MHC class II pathway, ultimately leading to their recognition by naïve $CD4^+$ T cells.

Along with the development of cell-mediated immunity (CMI), intragastric vaccination of attenuated *S*. Typhimurium is able to stimulate intestinal antibody production in response to a challenge (Moser *et al.*, 1980) which may assist in the prevention of initial colonisation by *S*. Typhimurium.

The predominant TH1 response leads to the clearance of *S*. Typhimurium from the host system by enhanced production and release of oxygen radicals and nitric oxide within the macrophage (Cherayil and Antos, 2001; Raupach and Kaufmann, 2001b; Janeway *et al.*, 2005), stimulation of production of new phagocytic cells and their recruitment to the site of infection (Janeway *et al.*, 2005), serum and mucosal antibody production (Moser *et al.*, 1980; Pashine *et al.*, 1999) and apoptosis of infected APC's (Monack *et al.*, 1996).

Important roles for $CD8^+$ cytotoxic lymphocytes (CTL's) have been identified in the recollection of immunity to *Salmonella* (Lo *et al.*, 1999). An infection against *S*. Typhimurium in vaccinated mice leads to the up-regulation of intracellular killing by CTL's (Mastroeni *et al.*, 1992; Lo *et al.*, 1999). B-cell responses are also important in the clearance as vaccinated mice unable to produce functional B-cells cannot survive a virulent

challenge. The role of B-cells is not confined to their ability to provide antibodies (Mastroeni *et al.*, 1993). Other B-cell processes that enhance IFN-γ and IL-12 production are required for an effective response (Mastroeni *et al.*, 2000).

1.7.4.2 Immune responses to foreign antigens delivered within *S*. Typhimurium vaccine strains

Heterologous antigen delivery and expression from attenuated *S*. Typhimurium has shown to produce both humoral and CMI responses against the antigen similar to that seen with *S*. Typhimurium alone, including high levels of secretory IgA when delivered directly to the mucosal surface (Brown *et al.*, 1987; Dougan *et al.*, 1987; Maskell *et al.*, 1987; Roberts *et al.*, 1998; Allen *et al.*, 2000; Bullifent *et al.*, 2000) (Appendix 1). In some cases protective immunity induced by *S*. Typhimurium expressing heterologous antigens in the absence of significant levels of antibodies has been noted and the protective responses observed in these studies seem to be mediated by $CD8^+$ CTL's by alternative MHC class I processing pathways (Sadoff *et al.*, 1988; Strugnell *et al.*, 1992; Pfeifer *et al.*, 1993; Turner *et al.*, 1993; Karem *et al.*, 1997; Medina *et al.*, 1999).

Many *S*. Typhimurium vaccines expressing foreign antigens induce a high level of secretory IgA when administered orally (Guzman *et al.*, 1991; Cardenas and Clements, 1992; Walker *et al.*, 1992; Ward *et al.*, 1999). Secretion of mucosal antibodies is paramount for the prevention of bacterial colonisation during a primary infection (Redman *et al.*, 1995), and attenuated *S*. Typhimurium expression of foreign antigens are capable of inducing secretory immunoglobulins even when administered at distal sites (Hopkins *et al.*, 1995; Redman *et al.*, 1995; Allen *et al.*, 2000).

1.7.4.3 Vaccination of chickens with *S*. Typhimurium carrying heterologous antigens

Immunity developed in chickens infected with *S*. Typhimurium follows a pattern similar to that observed in the mouse model, with high levels of IFN-γ production present during a primary infection (Beal *et al.*, 2004; Withanage *et al.*, 2005) and CMI responses necessary for the clearance of a secondary infection (Withanage *et al.*, 2005). *S*. Typhimurium in chickens can migrate and colonise the spleen and liver, but in most instances is primarily localised to the intestinal tissues leading to gastroenteritis, whereas in mice it causes a severe systemic infection (Henderson *et al.*, 1999; Beal *et al.*, 2004).

Chickens have been shown to respond well to vaccination with attenuated *S*. Typhimurium. The bacterium is able to colonise and invade the chicken host and develop appropriate immune mechanisms to counteract the effects of challenge with a wild type strain (Alderton *et al.*, 1991; Henderson *et al.*, 1999). These immune mechanisms include the classical production of serum IgM, followed by IgG and IgA, and secretory IgA within the mucosa of the GIT (Alderton *et al.*, 1991; Beal *et al.*, 2004; Withanage *et al.*, 2005).

Antibody responses to the vaccination of chickens with *S*. Typhimurium expressing foreign antigens has been recently demonstrated, with the significant production of IgG and IgA detected to the foreign protein, including protection against challenge (Pogonka *et al.*, 2003; Wyszynska *et al.*, 2004).

1.8 Concluding remarks

Inducing protective immunity is the goal of vaccine development and to achieve this it is necessary to induce an adaptive immune response that has both the antigen-specificity and the appropriate functional elements to combat the particular pathogen concerned. Delivery of

antigens via a live bacterial vector such as an attenuated *S.* Typhimurium, offers the advantages of delivery of antigens directly to the source of the natural infection (in this case the α-toxin of *C. perfringens* in the GIT), induction of adaptive immune responses including effector T- and B-cells, and in particular the production of secretory IgA at the mucosal sites of the GIT. The possibility exists for vaccination against two diseases such as salmonellosis and NE, within the one vaccine, offering savings on costs and time of administration within the agricultural poultry industry (Khan *et al.*, 1994; Roberts *et al.*, 1998).

Chapter 2

General materials and methods

2.1 General procedures

All chemicals used were of analytical laboratory reagent grade. All glassware, media and solutions were sterilised by autoclaving at 121^{o}C (15 lbs/in^{2}) for 20 min unless otherwise stated. All solutions were prepared with deionised water obtained from a Millipore Milli-Q® water system (mH_2O) (Liquipure, Melbourne, Australia) excluding media, which was prepared with distilled water (dH_2O).

Glassware was washed in Pyroneg detergent (Diversey-Lever, Pty. Ltd., Australia), rinsed twice in tap water, and then twice in deionised water. Glassware used for cell culture media was washed in Pyroneg detergent, rinsed twice in tap water, and twice in deionised water, and then soaked overnight in deionised water.

Solutions were dispensed using the Finnpipette® digital pipette range (Thermo Labsystems) which included a 0.5-10 µL, 5.0-40 µL, 20 µL-200 µL, 200-1000 µL, 1 mL-5 mL, 5 mL-10 mL digital pipette and a 50-300 µL multi-channel pipette.

Volumes of 1.5 mL or less were centrifuged with the Eppendorf microcentrifuge 5415C or 5415D. Greater volumes of up to 50 mL were centrifuged in the Beckman Allegra 21R centrifuge. Volumes of up to 200 mL were centrifuged in the Beckman J2-21 M/E centrifuge. All products of less than 2 g were weighed on a Sartorius analytical top-loading balance. Products of greater than 2 g were weighed on an ISSCO model 300 top loading balance.

Media containing supplements were autoclaved and cooled to 55°C before the addition of the supplement(s). All media were prepared using aseptic technique or in a lamina flow cabinet and stored at 4°C until required.

2.2 General chemicals and equipment

2.2.1 Equipment

ABI Prism BigDye Terminator Cycle	
Sequencing Ready Reaction kit:	Perkin-Elmer Corp., USA.
Anaerogen gas pack:	Oxoid, England
Balances:	
Analytical balance	Sartorius Gottingen, Germany;
Balance (0.1-500 g)	U-Lab, Australia.
BALB/c mice (female):	Animal resources centre, Canningvale, Western Australia.
Centricon ultracentrifugal devices:	Millipore, USA
Centrifuges:	
Eppendorf 5414C and 5414D bench top centrifuges	Eppendorf Geratebau, Germany
Beckman Allegra™ 21R Centrifuge	Beckaman Coulter, USA
Beckman J2-21 M/E Super Centrifuge	Beckman Coulter, USA
Centrifuge tubes:	
1.5 mL Eppendorf centrifuge tubes.	Treff AG, Switzerland.
10 mL centrifuge tubes	Sarstedt, Germany
50 mL centrifuge tubes	Greiner Labortechnik, Germany

200 mL autoclavable centrifuge bottles	Nalge Nunc International, USA
Cover slips:	Mediglass, Australia
Cryogenic tank (liquid nitrogen tank):	Cryo Biological System, USA.
Cryovials (1.8 mL):	Nalgene Company, USA.
Dry block heater:	Ratek, Australia
Electrophoresis power supply:	
PowerPac 300	Biorad Laboratories, USA.
EPS 3000xi	BioRad Laboratories, USA.
EPS600	Amersham Pharmacia LKB, Sweden.
Electrophoresis units:	
DNA:	
(a) Mini gel (GNA-100)	Pharmacia LKIB, Sweden
(b) Midi gel (wide mini-sub cell GT)	BioRad Laboratories, USA
Protein:	
(a) Mini Protean III gel system	BioRad Laboratories, USA
Electroporation cuvettes:	0.2 cm cuvettes chilled at -20°C before use, Biorad laboratories, USA
ELIspot plates:	Multiscreen™ 96-well filtration high protein binding immobilon-P membrane, Millipore, USA
Filters, 32 mm sterile	
Acrodisc 0.2 μm and 0.45μm:	Pall, USA
Gel Doc image system:	BioRad, USA
Geneclean® kit:	QBIOgene USA
Incubator for tissue culture (5% CO_2):	Forma Scientific, USA
Microplate reader (96-well) MR7000:	Dynatech (now Dynex), USA

Microscopes:

Light microscope (CH2)	Olympus Optical Co., Japan
Inverted microscope	Olympus, Japan
Microscope slides:	LOMB Scientific Co., Australia
Microtitre plate (96-well, flat bottom):	Nunc, Denmark
Needles (19, 21, and 26 gauge):	Terumo Pty, Ltd., Australia
Nitrocellulose membrane (Trans-Blot®) 0.2 μm:	BioRad, USA.
PCR machines:	
GeneAmp2400 PCR system	Perkin-Elmer, USA
PCR Express gradient thermal cycler:	Thermohybaid, UK.
Petri dishes:	BioLab, Australia.
pH meter:	Hanna, U-lab, Australia.
Platform shaker:	Ratek, Australia.
Pulse controller & Gene pulser apparatus:	BioRad, USA.
QIAprep® spin miniprep kit:	QIAGEN, Australia
Sonicator:	Branson Sonic Power Co., USA.
Syringe (1, 5, 10, 20, and 60 mL)	Terumo Pty, Ltd, Australia.
Trans-blot electrophoretic transfer cell (mini):	BioRad Laboratories, USA
Transilluminator (UV):	Novex, Australia.
Tissue culture flasks (75 cm^2, 25 cm^2):	Greiner, Germany.
Tissue culture plates (24 well, 96 well):	Greiner, Germany.
Waterbath:	Ratek, Australia.
Chromatography paper (0.18 mm thick):	Whatman, England.
Wizard® PCR prep, DNA purification kit:	Promega, USA

2.2.2 Materials

2.2.2.1 General media

Luria-Bertani broth (LB): 0.5% (w/v) Yeast extract (Oxoid, England.), 1% (w/v) tryptone (Oxoid), 0.5% (w/v) sodium chloride (BDH chemicals, UK).

Luria-Bertani agar (LA): 0.5% (w/v) Yeast extract, (Oxoid), 1% (w/v) tryptone (Oxoid), 0.5% (w/v) sodium chloride, 1.0% (w/v) bacteriological agar (Oxoid).

Brain heart infusion broth (BHIB): Brain heart infusion agar base, 3.7% (w/v) (Oxoid) dispensed in bottles to a final volume of 10 mL.

Columbia agar (CA): Columbia agar base, 3.9% (w/v) (Oxoid).

Cooked meat medium (CMM): Granules (1.0 g) of dehydrated CMM (Oxoid) were added to bottles and filled to 10 mL with distilled water.

Sheep blood agar (SBA): CA cooled to 55^{o}C and supplemented with 5% (v/v) defibrinated sheep blood (Oxoid).

Nagler plate: CA supplemented with 10% egg yolk solution (v/v). The egg yolk was first mixed with saline in a 1:1 ratio and centrifuged at 5,500 x *g* for 10 min. The supernatant was used for preparing the plates.

Thioglycollate broth: Thioglycollate (Oxoid) 2.9% (w/v) (alternative) broth was prepared and boiled to completely dissolve the medium. Ten millilitre aliquots were dispensed into 20 mL bottles.

XLD plates: 5.3% XLD (w/v) (Oxoid). The solution was sterilised by heating in a microwave set on high with frequent agitation, until it started to boil. It was then cooled to 50ºC and poured into petri dishes.

2.2.3 General solutions

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT): 5 mg/mL MTT (Sigma-Aldrich), dissolved in 10 mM Tris (pH 8.0) and stored at 4ºC away from light.

5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal): 2% (w/v) X-gal (Sigma-Aldrich) in dimethylformamide (BDH chemicals, UK).

β-mercaptoethanol: electrophoresis purity (Biorad).

λ-DNA marker: A solution of 0.1 µg/µl digested λ-DNA was prepared by incubating 20 µg λ-DNA (Promega), 18 µL Buffer H (Promega), 10U *Pst*1 (Promega) and mH_2O up to 180 µL, at 37^{o}C overnight. Twenty microlitres of 11x DNA loading buffer was then added to stop the digest. The marker was stored at -20ºC and 10 µL lots used on a DNA agarose gel.

ACK Lysing buffer: 0.15 M Ammonium chloride (NH_4Cl), 10.0 mM potassium bicarbonate ($KHCO_3$) and 0.1 mM (EDTA). The pH was adjusted to 7.2-7.4 with 1 M HCl and filter sterilised through a 0.2 µm filter prior to use.

Acrylamide/bisacrylamide solution: A 40% (w/v) prepared solution containing 38.67% (w/v) ultra pure acrylamide and 1.33% (w/v) bisacrylamide (Australia Scientific, Australia).

Agarose: 1% (w/v) DNA grade agarose (Progen Industries, Australia) in 1×TAE buffer, dissolved by heating in a microwave.

Alkaline phosphatase conjugate substrate kit: Prepared according to manufacturers instructions (Biorad).

Ammonium persulphate (APS): 10% (w/v) Ammonium persulphate (Sigma-Aldrich) stored at 4ºC.

Ampicillin: 100 mg/mL Ampicillin (CSL, Australia), filter sterilised.

Binding buffer: 25 mM Tris (Astral Scientific), pH 8.0, 0.5 M NaCl (BDH), 60 mM imidazole (Sigma-Aldrich).

Blocking solution (ELISA): Skim milk 5% (w/v) in PBST.

Bovine serum albumin fraction V (BSA): 1 mg/mL (Sigma-Aldrich), stored at -20°C.

Bradford reagent: 100 mg Coomassie Brilliant G-250 (Sigma-Aldrich) dye was dissolved in 50 mL 95% (v/v) ethanol, then mixed with 100 mL 85% (v/v) phosphoric acid, and brought up to 1 L with dH_2O. The solution was stored at 4ºC, and filtered through a 0.45 µm filter before use.

Bromophenol blue: 1% (w/v) Bromophenol blue (BDH).

Calcium chloride ($CaCl_2$): 1 M $CaCl_2$ (BDH) filter sterilised.

Cell lysis buffer I: 25 mM Tris, pH 8.0, 0.5 M NaCl, 60 mM imidazole. Lysozyme (4 mg/ml) was added to the buffer immediately prior to use.

Cell lysis buffer II: 50 mM Tris pH 8.0, 100 mM NaCl. Lysozyme (1 mg/mL) was added to the buffer immediately prior to use.

Chloroform: 100% (v/v) Chloroform (BDH).

CI: 96% (v/v) Chloroform (BDH), 4% (v/v) isoamyl alcohol (BDH).

Coating buffer: Stock A: 8.4 g of sodium hydrogen carbonate (Na_2HCO_3) (BDH) dissolved in 100 mL of dH_2O. **Stock B:** 10.6 g sodium carbonate (Na_2CO_3) (BDH) dissolved in 100 mL dH_2O. To make up the coating buffer, 45.3 mL of **A** was mixed with 18.2 mL of **B** and made up to 1 L with dH_2O. The pH of the final solutions was approximately 9.6.

Concanavalin A: A lyophilised powder (Sigma-Aldrich) reconstituted in sterile saline.

Coomassie blue destain solution: 10% (v/v) Ethanol (BDH) and 10% (v/v) glacial acetic acid (BDH).

Coomassie blue stain solution: 0.1% (w/v) Coomassie brilliant blue R-250 (Sigma-Aldrich), 50% (v/v) methanol (BDH), 10% (v/v) glacial acetic acid (BDH).

CTAB/NaCl: 4.1 g of NaCl was dissolved in 80 mL mH_2O and 10 g CTAB (hexadecyltrimethyl ammonium bromide, Sigma-Aldrich) was added and dissolved by heating at 65°C.

Dextran: 10 mg/mL stored at -20ºC (Sigma-Aldrich)

DMSO: Dimethyl sulfoxide (Sigma-Aldrich).

DNA loading buffer 11×: 10% (w/v) Ficoll (Sigma-Aldrich), 50% (v/v) glycerol (BDH), 0.5% (w/v) Orange G (Sigma-Aldrich), 1% (w/v) SDS (BDH), 10 mM EDTA (BDH), 50 mM Tris (pH 8.0).

dNTPs (deoxynucleoside triphosphates): 10 mM each of dATP, dGTP, dTTP and dCTP (Boehringer Mannheim-BM, Germany)

Dulbecco's Modified Eagle's Medium (DMEM):

100 mL 5 × DMEM (Trace, Biosciences, Castle Hill, Aust.)

10.0 mL Hepes buffer (1 M) (Trace)

13.5 mL Sodium bicarbonate (7.5%) (Trace)

4.5 mL L-Glutamine (200 mM) (Trace)

372.0 mL mH_2O.

Elution Buffer: 25 mM Tris, pH 8.0, 0.5 NaCl, 200 mM imidazole.

Ethanol: 70% (v/v), 95% (v/v), 100% (v/v) analytic ethanol (BDH).

Ethidium bromide (EtBr): A stock solution of 10 mg/mL EtBr (BM). This solution was not autoclaved.

Ethylenediaminetetra-aetate (EDTA) buffer: 0.25 M EDTA (BDH), pH 8.0.

EXTRAvidin: Purchased from Sigma and used at a 1:1000 dilution prepared in PBST.

Folin's reagent: Folin & Ciocalteaus reagent (BDH) diluted 1:1 in mH_2O, stored at 4ºC away from light.

Formaldehyde: Formaldehyde (37% w/v) was purchased from Sigma and used at a final concentration of 0.4% (w/v).

Frozen Storage Buffer (FSB) (per litre):

Manganese (II) chloride 4-hydrate ($MnCl_2.4H_2O$) (BDH)	8.91 g (45 mM)
Calcium chloride dihydrate ($CaCl_2.2H_2O$) (BDH)	1.47 g (10 mM)
Potassium chloride (KCl) (Ajax)	7.46 g (10 mM)
Hexamine cobalt chloride (Sigma-Aldrich)	0.80 g (100 mM)

Glycerol (BDH)	100 mL (10%)
Potassium acetate (KOAC) (Sigma-Aldrich)	10 mL (10 mM)

The above reagents were combined and mH_2O was added to approximately 800 mL. The pH was adjusted to 6.4 with 0.1 M HCL and mH_2O was added to a final volume of 1 L.

Glycerol: 100%, 50% (v/v) glycerol (BDH).

Haemolysin liquid assay buffer x10 (HLAB): 250 mM Tris, pH 7.4, 100 mM $CaCl_2$, 100 µM $ZnCl_2$ (BDH) 8.5% NaCl.

Hydrochloric acid: 32% (w/v) (Ajax Chemicals Ltd., Australia).

Hydrogen peroxide (H_20_2): 30% (w/v) H_2O_2 (BDH).

Imidazole: A 5 M imidazole stock solution (Sigma-Aldrich) was prepared and filtered through a 0.45 µm filter.

Isopropanol: Propan-2-ol (BDH).

Isopropyl-β-D-thiogalactopyranoside (IPTG) 1 M stock: 0.2 g/mL IPTG (Astral), filter-sterilised. One millilitre aliquots were stored at -20°C.

Lowry reagent A: 2% (w/v) Sodium carbonate (BDH), 0.4% (w/v) sodium hydroxide (BDH), 0.16% (w/v) sodium potassium tartrate (Sigma-Aldrich) and 1% (w/v) SDS.

Lowry reagent B: Copper sulphate, 4% (w/v) (Ajax, Australia)

Magnesium chloride: BDH Chemicals, UK

Methanol: 100% (v/v) (BDH).

Native PAGE running buffer (10x): 0.25 M Tris, 1.92 M glycine (Sigma-Aldrich).

Native PAGE sample buffer (5x): 312.5 mM Tris (pH 6.8), 50% (v/v) glycerol (BDH), 0.05% (w/v) bromophenol blue (Sigma-Aldrich).

Native PAGE separating gel (x%):

1.5 M Tris pH 8.8	2.5 mL
Bis/Acrylamide (40%)	x/4
H_2O	7.5-x/4 mL

Ammonium persulphate (10%)	50 µL
TEMED	5 µL

Native PAGE stacking gel (5%):

0.5 M Tris pH 6.8	1.0 mL
Bis/Acrylamide	0.67 mL
Ammonium persulphate (10%)	30 µL
TEMED	5 µL
H_2O	2.3 mL

Newborn Calf Serum (NCS): NCS (Cytosystems Pty Ltd, Australia) was dipsensed into 10 mL aliquots and heated to 65ºC for 15 min to inactive serum proteins. NCS was stored at -20ºC until required.

Nickel Sulphate (0.2 M): Nickel sulphate (2.6 g) (BDH) was dissolved in 50 mL of mH_2O.

Penicillin/Streptomycin: A stock of 5 mg/mL was purchased from Trace, and used at a final concentration of 5 µg/mL for tissue culture cell line maintenance.

Phenol (saturated): Supplied by Astral Scientific.

Phenol/chloroform-isoamyl: A ratio of 25:24:1 (BDH) of phenol, chloroform and isoamyl alcohol.

Phosphate-buffered saline (PBS): 1 tablet Dulbecco's 'A' PBS (Oxoid) in 100 mL mH_2O (sodium chloride 0.8%, potassium chloride 0.02%, disodium hydrogen phosphate 0.115%, potassium dihydrogen phosphate 0.02%).

PBST: PBS and 0.05% (v/v) Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma-Aldrich).

Plasmid isolation solution I: 50 mM Glucose (Sigma-Aldrich), 10 mM EDTA (BDH), 25 mM Tris-HCl (pH 8.0, Amresco). Lysozyme (BM) (4 mg/mL) was added immediately prior to use.

Plasmid isolation solution II: 1% (w/v) SDS (BDH), 0.2 M NaOH (BDH).

Plasmid isolation solution III: 60 mL of 5 M potassium acetate (BDH) (final concentration 3 M), 11.5 mL glacial acetic acid (BDH) (final concentration 5 M) and 28.5 mL mH_2O.

Protein lysis buffer 1: 25 mM Tris, pH 8.0, 0.5 M NaCl, 60 mM imidazole. Lysozyme (1 mg/mL) was added to the solution just before use.

Protein lysis buffer 2: 50 mM Tris, pH 8.0, 100 mM NaCl. Lysozyme (1 mg/mL) was added just before use.

RPMI/NCS: RPMI solution was purchased from Sigma. It was supplemented with 2 mM glutamine and 5% NCS prior to use.

Saline: 0.85% sodium chloride (BDH).

SDS loading buffer (5x): 60 mM Tris (pH 6.8), 25% (v/v) glycerol (BDH), 2% (w/v) SDS (BDH), 14.4 mM β-mercaptoethanol (Bio-Rad, Aust.), 0.1% (w/v) bromophenol blue (Sigma-Aldrich).

SDS-PAGE Separating gel (x%)

1.5 M Tris pH 8.8	2.5 mL
10% SDS	100 µl
Bis/Acrylamide (40%)	$x/_4$ mL
H_2O	6.5-($x/_4$) mL
Ammonium persulphate (10%)	50 µL
TEMED	10 µl

SDS-PAGE stacking gel (4%)

0.5 M Tris pH 6.8	1.0 mL
10% SDS	40 µL
Bis/Acrylamide (40%)	375 µL
H_2O	2.585 mL
Ammonium persulphate (10%)	20 µl
TEMED	5 µL

SDS running buffer stock (10x): 0.25 M Tris, 1.92 M glycine (BDH), 1% (w/v) SDS (BDH). The pH was adjusted to 8.3.

Skim milk: Bonlac Foods Limited, Australia.

Sodium acetate: 3 M (BDH), pH 4.6.

Sodium chloride: 5 M Stock solution of sodium chloride.

Sodium dodecyl sulphate (SDS): 10% (w/v) SDS (BDH).

Sodium hydroxide (NaOH): 10 M Stock solution (BDH).

Sucrose buffer I: 25% (w/v) Sucrose (BDH), 50 mM Tris (BM), pH 8.3. This solution was sterilised at 109ºC for 30 min.

Sucrose buffer II: 20% Sucrose (BDH), 0.1 mM EDTA, 30 mM Tris, pH 8.0, filter sterilised.

Sulphuric acid (H_2SO_4) (TMB stop solution): 1.0 M Sulphuric acid (BDH).

Transfer buffer: 25 mM Tris (Amresco), 192 mM glycine (BDH), 20% (v/v) methanol (BDH).

TE buffer x10: 100 mM Tris (BM), 10 mM EDTA (Amresco), pH 8.0.

TEMED (N,N,N',N'-tetramethylethylenediamine): Electrophoresis purity (Biorad).

Tetramethylbenzidine (TMB): TMB substrate reagent A (BD Biosciences, USA) and TMB substrate reagent B (BD Biosciences) were mixed in a ratio of 1:1 and used within 10 min.

Tris (hydroxymethyl) aminomethane (Tris): 2 M Stock solution (BM)

Tris-Acetate buffer (TAE) x 50: 2 M Tris, 1 M acetic acid, 0.1 M EDTA.

Tris buffered saline (TBS): 25 mM Tris, 0.18 M sodium chloride, pH 7.4.

Trypan Blue stain: Purchased from Sigma (0.4%) (w/v).

Trypsin - EDTA: Diluted by 50% in sterile PBS prior to use (Trace Biosciences, Australia.

Tryptone: Oxoid.

TST: 10 mM Tris (pH 7.4), 150 mM NaCl (BDH) and 0.05% (v/v) Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma-Aldrich).

Urea: 6 M Stock (BDH).

Wash Buffer: 25 mM Tris, pH 8.0, 0.5 M NaCl, 60 mM imidazole.

Western blot substrate solution: Chloro-1-naphthol (30 mg) (Sigma-Aldrich) was dissolved in 10 mL methanol (BDH), brought up to 50 mL with TBS, mixed with 30 µl hydrogen peroxide (H_2O_2) and used immediately for the development of Western blots probed with horseradish peroxidase (HRP) conjugates.

Whole cell lysis buffer: 0.1 M Tris, 2% (v/v) SDS and 15% (v/v) glycerol, pH 6.8.

Zinc chloride ($ZnCl_2$): 1 M Stock solution (BDH), filter sterilised.

2.2.3.1 Enzymes

All enzymes were used with their supplied buffer according to manufacturer's instructions, and were stored at -20ºC unless otherwise stated.

DNA polymerase, Expand Long Template PCR system: 5U/µL (Roche, Germany).

DNA polymerase, *Pfu*: 3U/µL (Promega, USA).

DNA polymerase, Ampli*Taq*: 5U/µL (Perkin-Elmer, USA)

Restriction enzymes: See Table 2.4

Calf intestinal phosphatase (CIP): 10U/µL (Roche, Germany), stored at 4ºC.

Polynucleotide kinase (PNK): 10U/µL (Promega, USA)

Pronase solution: 10 mg/mL (BM).

DNase I bovine pancreas: A stock of 10U/µL was prepared from a lyophilised vial of DNase (Sigma-Aldrich).

RNase solution: 10 mg/mL ribonuclease 1A bovine pancreas (Pharmacia). For DNase free RNase, the RNase solution was boiled for 30 min.

Lysozyme solution 1: 20 mg/mL lysozyme (BM) in EDTA buffer, prepared fresh.

DNA Ligase (T4): 10U/µL (Boehringer Mannheim, Germany).

2.2.3.2 Antibodies

Horse anti-α-toxin polyclonal antisera

(gas-gangrene antisera): CSL, Australia

Rabbit anti-horse IgG (H+L)-HRP: ICN, USA

Goat anti-mouse IgG-HRP: ICN, USA

Rat anti-mouse IFN-γ (capture antibody): Abcam, UK

Rat anti-mouse IL-4 monoclonal antibody: Abcam

Biotin rat anti-mouse IFN-γ: Abcam

Biotin rat anti-mouse IL-4 monoclonal antibody: Abcam

2.3 Microbiological methods

2.3.1 Bacterial strains, plasmids and recombinant proteins developed throughout the study

The description of all the bacterial strains and plasmids used throughout this study is listed in Tables 2.1. and 2.2. A description of all the recombinant proteins developed throughout the study is listed in Table 2.3.

2.3.2 Bacterial storage

E. coli and *Salmonella* strains were stored at -70^{o}C in 50% glycerol. *C. perfringens* strains were stored at -70^{o}C in a solution containing 10% skim milk, 1% tryptone, and 10 mM Tris, pH 7.5.

Table 2.1: Bacterial strains used in this study

Bacterial strain	**Genotype/Description**	**Source**
Clostridium perfringens		
61	Type A	Necrotic enteritis (NE) isolate from a chicken
60	Type A	NE isolate from a chicken
Escherichia coli	**Genotype/Description**	**Source**
TOP10F'	F´{*lac*I^{q}, Tn*10*(Tet^{R})} *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^{R}) *end*A1 *nup*G	Invitrogen
BL21	F^{-} *omp*T *hsd*S$_{B}$(r$_{B}^{-}$m$_{B}^{-}$) *gal dcm* (DE3)	Invitrogen
BL21 (DE3)pLysS	F^{-} *omp*T *hsdS*$_{B}$(r$_{B}^{-}$m$_{B}^{-}$) *gal dcm* (DE3) pLysS (Cam^{R})	Invitrogen
DH5α	*sup*E Δ*lac*U169 (ϕ80 *lac*ZΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	(Hanahan, 1983)
***Salmonella* Typhimurium**	**Genotype/Description**	**Source**
LT2-9121	*leu hsdL trpD2 rpsL120 ilv52 metE551 metA22 hsdA hsdB*	Prof. P. Reeves, Department of Microbiology, The University of Sydney
STM-1	Δ*aro*A^{-} Δ*serc*$^{-}$	RMIT University
Salmonella Typhimurium 82/6915	Wild type Inv^{+}	(Alderton *et al.*, 1991)

Table 2.2: Vectors used in this study

Plasmid	Description/Genotype	Source/Reference
pCR2.1	3.9 kb PCR cloning plasmid, Amp^R, Km^R, *lac*Z	Invitrogen
pRplc10F	Complete *plc* lacking the first 84 nucleotides from the start codon (coding for the signal sequence of the mature α-toxin) cloned into *Pst*I and *Eco*RI sites of pRSETA	Chapter 3
pCplc3	1.24 kb amplified fragment from *C. perfringens* strain 61 containing the complete *plc* with a 6xCAT (coding for 6xHis) at the 5' end, cloned into pCR2.1 using TA cloning	Chapter 3
pCplcInv3	Inverse amplified and self-ligated pCplc3 (with nucleotides 250-309 of *plc* deleted)	Chapter 3
pRSETA	2.9 kb protein expression plasmid: Amp^R, N-terminal 6xHis, T7 promoter	Invitrogen
pRplc14	Complete *plc* cloned into the *Pst*I and *Eco*RI sites of pRSETA using primers AllPstI and PlcEcoRev	Chapter 5
pRplcInv9	Inverse amplified and self-ligated pRplc14 (with nucleotides 250-309 of *plc* deleted)	Chapter 5
pRplc316	Nucleotides 246 (from ATG) to 1243 of the *plc* from *C. perfringens* strain 61 cloned into *Pst*I and *Eco*RI sites of pRSETA	Chapter 3
pRplc204	Nucleotides 582-1243 (from ATG) of the *plc* from *C. perfringens* cloned into the *Pst*I and *Eco*RI sites of pRSETA	Chapter 3
pRplc104	DNA sequence coding for the C-terminus of *plc* (nucleotides 882-1243) cloned into the *Pst*I and *Eco*RI sites of pRSETA	Chapter 3

Table 2.2 Cont.

Plasmid	Description/Genotype	Source/Reference
pBluescript SKII	2.96 kb phagemid: Amp^R, *lac*Z, ColE1 origin of replication	Stratagene, USA
pGEX4T-1	4.969 kb protein expression vector: Amp^R, $LacI^q$, N-terminal glutathione-S-transferase fusion, tac promoter (P_{tac}), pBR322 origin of replication	Amersham-Biosciences, Sweden
pBTac3	P_{tac} amplified from pGEX4T-1 inserted into pBluescript via blunt end ligation at *Eco*RV site	Chapter 5
pBPAGC21	*Pag*C promoter (P_{pagC}) from *Salmonella* Enteritidis inserted into *Eco*RV site of pBluescript via blunt ended ligation	(Moutafis, 2002)
pBTCαInv	Amplified *αInv* from pRplcInv9 cloned into the *Bgl*II and *Not*I sites of pBTac3	Chapter 5
pBTCα104	Amplified *α104* from pRplc104 cloned into the *Bgl*II and *Not*I sites of pBTac3	Chapter 5
pBPCαInv	Amplified *αInv* from pRplcInv9 and cloned into pBPAGC21 via *Bgl*II and *Not*I sites	Chapter 5
pBPCα104	Amplified *α104* from pRplc104 and cloned into pBPAGC21 via *Bgl*II and *Not*I sites	Chapter 5
pBHAαInv	Amplified *htr*A promoter (P_{htrA}) ligated to *αInv* through *Bgl*II sites and cloned into pBluescript SKII- via *Xba*I and *Not*I sites.	Chapter 5
pBHAα104	Amplified P_{htrA} ligated to *α104* through *Bgl*II sites and cloned into pBluescript via *Xba*I and *Not*I sites.	Chapter 5

Table 2.3: Description of the α-toxin recombinant proteins developed throughout this study.

Expression vector	Protein	Description	Affinity his-tag
pCplc3	plc3	Mature α-toxin (370 aa)	C-terminal
pCplcInv3	plcInv3	Plc3 with an internal deletion of amino acid (aa) residues 56-75 which encompassing two zinc binding domains essential for phospholipase C activity	C-terminal
pRplc316	plc316	The α-toxin with the deletion of the first 55 N-terminal aa residues encompassing the first three α-helical domains	N-terminal
pRplc204	plc204	Deletion of the first 166 N-terminal aa's of α-toxin encompassing 7 α-helices	N-terminal
pRplc104	plc104	Deletion of the first 266 N-terminal aa's of α-toxin encompassing the complete α-helical N-terminal domain and the first β-sheet.	N-terminal
Numerous*	αInv	Amino acid sequence identical to plcInv3 but without the affinity C-His tag	Not present
Numerous	α104	Amino acid sequence identical to plc104 but without the affinity N-His tag	Not present

*The numerous expression vectors used to express αInv and α104 were developed for use within STM1 and included plasmids pBHAαInv, pBHAα104, pBTCαInv, PBTCα104 and pBPCαInv, pBPCα104 (Table 2.2).

2.3.3 Culture conditions

All *E. coli* and *Salmonella* strains were grown on solid microbiological media under aerobic conditions at 37°C for 16 h. In instances where broth cultures were used, the strains were grown aerobically at 37°C for 16-20 h on a Ratek orbital shaker set at 120 rotations per min.

All *Clostridia* grown on solid agar were placed in airtight jars and anaerobiosis was maintained with an Anaerogen gas pack containing ascorbic acid as the active component (Oxoid). *Clostridia* were grown at 37ºC for 16-20 h.

Broths for clostridial cultivation were used immediately, once cooled from sterilisation. If not fresh, broths were boiled for ten minutes prior to *C. perfringens* inoculation to remove any dissolved oxygen.

2.4 Methods of DNA analysis

2.4.1 Chromosomal DNA extraction

Genomic DNA from *C. perfringens* was prepared by following the method described in "Current protocols in molecular biology" (Ausubel *et al.*, 1994) with a few modifications. Bacteria were grown on HBA plates overnight. Bacteria were harvested with 3 mL sucrose buffer and washed and pelleted twice in the same buffer by centrifugation at 16,000 x *g*. The pellet was resuspended in 2 mL sucrose buffer and 500 µL of lysozyme solution was added, followed by 500 µL of 10% SDS. This was incubated at 37ºC for 30 min. Thirty microlitres of RNase solution and 200 µL of pronase solution was then added, and the incubation allowed to continue for a further 45 min. The NaCl concentration of the solution was increased to 0.8 M by the addition of 640 µL of 5 M NaCl and 130 µL of Tris-EDTA buffer (TE). The

solution was thoroughly mixed and 0.1 volumes of CTAB/NaCl were added. The solution was mixed and incubated in a 65°C water bath for 10 min. An equal volume of PCI was added and the tube mixed by inversion. The phases were separated by centrifugation at 10,000 x *g* for 5 min and the upper phase was collected, mixed with an equal volume of PCI and re-spun in a centrifuge at 10,000 x *g* for 5 min. The supernatant was collected and an equal volume of isopropanol added. The solution was mixed by inversion until the DNA precipitated out of solution. The DNA was then fished out using a pasteur pipette, transferred to a 1.5 mL tube and washed in 70% ethanol. The DNA was resuspended in 100 µL TE.

Salmonella Typhimurium chromosomal DNA was extracted following the method of Ausubel, *et al.,* 1994.

2.4.2 Alkaline lysis plasmid extraction (miniprep)

Plasmids used in restriction digests were purified by using the alkaline lysis method described in "Current protocols in molecular biology" (Ausubel *et al.*, 1994).

One and a half millilitres of an overnight culture was placed in a microfuge tube and pelleted in a centrifuge at 16,000 x *g* for 2 min. The pellet was resuspended in 100 µL of plasmid isolation solution I and left at room temperature for 5 min followed by cell lysis using 200 µl of plasmid isolation solution II. The solution was placed on ice for 5 min followed by the addition of 150 µL ice cold plasmid isolation solution III to precipitate chromosomal DNA and protein. The precipitate was pelleted by centrifugation at 16,000 x *g* for 10 min at 4°C and the supernatant transferred to a new microfuge tube. Fifty microlitres of PCI was added to the tube, mixed by vortex and centrifuged for 2 min at 16,000 x *g*. The upper aqueous phase was collected and mixed with 50 µL of CI. The solution was mixed by vortex and

pelleted by centrifugation as above. The aqueous phase was collected and 2 volumes of 96% ethanol added to precipitate the plasmid DNA. Plasmid DNA was pelleted by centrifugation at 16,000 x *g* for 5 min, the supernatant decanted and the pellet air-dried before the addition of 40 µL 10 mM Tris, pH 8.0 and stored at -20ºC.

2.4.3 Plasmid extraction for DNA sequencing

Plasmid DNA required for sequencing was purified using the QIAprep® spin miniprep kit as per the manufacturer's instructions.

2.4.4 Extraction of DNA from agarose

DNA was excised from the agarose gel and extracted using the GeneClean® kit according to the manufacturer's instructions.

2.4.5 Purification of DNA from PCR

Amplified DNA fragments were desalted by use of the Wizard® PCR prep DNA purification kit according to the manufacturer's instructions.

2.5 Agarose gel electrophoresis

Ten microlitres of PCR or extracted DNA product and 2.0 µL of 11x DNA loading buffer were combined and separated on a 1-2% DNA grade agarose gel in a minigel or midigel unit filled with 1xTAE buffer. A current of 100 V was applied to the gel, and the DNA fragments separated for 1 h. The gel was then stained in a 3 µL/mg EtBr bath for 5 min, followed by a 20 min destaining period in running tap water.

The DNA products were visualized with an UV illuminator and photographed using the Geldoc imaging system (Biorad). Sizes of DNA fragments were estimated by the addition of lambda (λ) DNA previously digested with *Pst*I (λ-*Pst*I) to the gel prior to electrophoresis. A diagram of the λ-DNA sizes is located in Appendix 2.

2.6 Quantitation of DNA

A number of methods were used in the quantitation of DNA. For the exact quantitation of well-purified DNA, a spectrophotometer was used, and for approximate quantitation of DNA, an ethidium bromide plate was used or DNA was separated on an agarose gel and compared along known standards of λ-*Pst*I.

2.6.1 Spectrophotometric quantitation of DNA

The Shimadzu UV-160 Spectrophotometer was used to determine the optical density (OD) of a solution of DNA at a wavelength of 260 nm, which is the optimal wavelength for nucleic acids. Protein contamination was detected by an OD reading at 280 nm. An OD ratio of DNA/protein was then used to measure the quality of the DNA. Ratios of 1.8-2.0 indicated good quality DNA (Sambrook and Russell, 2001). An OD of 1 corresponded to 50 μg/mL double stranded DNA and the amount of DNA present was determined using this standard.

2.6.2 Ethidium bromide plates for the quantitation of DNA

Ethidium bromide (EtBr) plates were used for the quantitation of samples containing between 1-250 ng of DNA. One percent DNA grade agarose (Progen) in TAE buffer was dissolved in a microwave. After cooling slightly, the agarose was poured into a petri dish and EtBr to the

final concentration of 0.5 μg/mL was added. The plates were swirled to evenly distribute the EtBr and covered with aluminium foil to cool. The plates were stored at 4°C away from light until required. Plates were dried at 37ºC prior to use. One microliltres quantities of varying λ DNA concentrations were placed on the EtBr plates, 1.0 μL of sample DNA was added and quantified by comparing with the λ standards under a UV illuminator.

2.6.3 DNA gel electrophoresis quantitation of DNA

Lambda-DNA previously digested with *Pst*I was separated on an agarose gel along with sample DNA fragments. The quantity of DNA within each digested fragment of λ-DNA is listed in Appendix 2 and was compared with intensities of unknown DNA samples.

2.7 DNA manipulation

2.7.1 Restriction enzymes

The restriction enzymes used throughout the study are listed in Table 2.4.

Approximately 1 μg of plasmid DNA was digested with 1U of enzyme containing 1x buffer in a total volume of 10-20 μL at 37°C for 2 h. Enzymes were heat inactivated at 65ºC for 15 min when DNA was to be used in cloning experiments. All restriction enzymes were purchased from Promega, USA.

Table 2.4: List of restriction enzymes (RE) used in this study

Restriction enzyme	Recognition sequence
*Bam*HI	G⇓GATCC
*Bgl*II	A⇓GATCT
*Dpn*I	GA⇓TC
*Eco*RI	G⇓AATTC
*Eco*RV	GAT⇓ATC
*Not*I	GC⇓GGCCGC
*Pst*I	CTGCA⇓G
*Sac*I	GAGCT⇓C
*Xba*I	T⇓CTAGA
*Xho*I	C⇓TCGAG

2.7.2 Amplification of DNA

2.7.2.1 Primers

Primers were designed with the aid of the software program Sci Ed Central for Windows 95. Primers were designed to have a guanine/cytosine (GC) content between 40-60% with a melting temperature (TmºC) in the range of 50-80ºC where possible. Primers were obtained as lyophilised samples from a number of companies (Table 2.5).

2.7.2.2 Polymerase chain reaction (PCR)

General PCR was performed according to the polymerase manufacturer's instructions. The general reaction mix and programs used are listed in Table 2.6, 2.7 and 2.8. The annealing temperature is specified in the relevant chapters. PCR products for cloning purposes were amplified using *Pfu* polymerase (Promega, USA) or Expand Long template PCR system (Roche, Germany) to reduce errors. PCR for general detection of fragments was prepared with Ampli*Taq* polymerase.

The reagents were prepared in the sterility of a Biological Safety Cabinet, Class II. A Perkin-Elmer DNA Thermal Cycler or a ThermoHybaid gradient cycler was used to amplify the DNA. Approximately 10 and 100 ng of template DNA was used from plasmid and chromosomal DNA respectively. Reactions were prepared in a final volume of 25 µL, when a high amount of amplified DNA was required the reaction was prepared in a final volume of 50 µL.

Table 2.5: List of primers used throughout this study

Primer name	Sequence	Description	TmºC*
ALLPstI	TAACGCTGCAGATAAAAAATGAAAAGAAAGA	Forward primer for *plc*	55
253PLCf	GGTTCCTGCAGTCCAGATTATGATAAGAACG	Starts at base pair (bp) 253 of *plc*	62
590PLCf	AATCTGCAGAGCAGGTTGCAAAACTAATGAGGA	Starts at bp 590 of *plc*	62
Pst891F	CAACCTGCAGTGAAAAAGATGCTGGAAC	Starts at bp 891 of *plc*	60
PlcEcoREV	GCTTTGAATTCGTAAATACCACCAAAACC	Reverse primer for complete *plc*	57
plcHisEcoREV	CGCGAATTCTTAATGATGATGATGATGATGTTTT ATATTATAAGTTGA	Reverse primer for the introduction of a 6xCAT at the 3' of *plc*.	
Inv1	AATAATTTCTCAAAGGATAATAGTTGG	Forward primer for inverse PCR of *plc* starting at bp 310 and ending at bp 336	51
Inv2	TGGATAAGTAGAACCTAATTGAAGCTC	Reverse primer for inverse PCR of *plc* starting at bp 249 and ending at bp 223	55
Fpst2	AGTCTGCAGTTGGGATGGAAAGATTGATGGA	Amplification of *plc* without signal sequence	
pRSETNotI	CTTCGCGGCCGCCTTTGTTAGCAGCCGGATCAA	Reverse primer for the amplification of *plc* truncates from vector pRSETA for cloning into pBluescript	86.7
890BglII	TCAAGATCTGGTGAAAAAGATGCTGGAACAGAT	Forward primer for the amplification of *plc104* from pRplc104 for cloning into pBluescript	74.6

Primer name	Sequence	Description	TmºC*
plcBglII	TAA**AGATCT**AAAAGAAAGATTTGTA	Forward primer for the amplification of *plcInv9* from pRplcInv9 for cloning into pBluescript	65.0
TacR	GTAT**AGATCT**CATGAATACTGTTTCCTGTGT	Reverse primer for tac promoter	64.2
TacF	GTT**TCTAGA**TGCACGGTGCACCAATGCTT	Forward primer for tac promoter	75.5
HtrAF	AAG**TCTAGA**GCTTAACGACTTTCGCGA	Forward primer for *htr*A promoter	68.6
HtrAR	TGGT**AGATCT**CATGTGTTTCAATCTCGATTA	Reverse primer for *htr*A promoter	69.3
pRSET Rev	TAGTTATTGCTCAGCGGTGG	Sequencing frompRSETA	52
T7 Promoter	TAATACGACTCACTATAGGG	Sequencing from for pRSETA	48
M13 -20	GTAAAACGACGGCCAG	Sequencing from pBluescript, pCR2.1	45.8
M13 Reverse	CAGGAAACAGCTATGAC	Sequencing from pBluescript, pCR2.1	44.5

*Calculated by company of synthesis

Bases highlighted are enzyme recognition sites with:

Pink sequence indicating a *Pst*I

Green sequence indicating an *Eco*RI

Blue sequence indicating a *Bam*HI

Red sequence indicating a *Not*I

Orange sequence indicating a *Bgl*II

Purple sequence indicating an *Xba*I

Table 2.6: Standard reaction conditions for amplification of DNA by PCR

PCR reagent	Final concentration
DNA template	10-100 ng
dNTP	0.2 mM
Primer A	0.2 µM
Primer B	0.2 µM
10 x PCR buffer (supplied with polymerase)	1 x
$MgCl_2$ (Ampli*Taq* only)	2.5 mM
Polymerase:	
pfu	1.5U/50 µL
Ampli*Taq*	1.0U/50 µL
Expand Long range	2.5U/50 µL

Table 2.7: Standard amplification conditions of PCR with *Taq* and Expand Long range polymerase.

Temperature (^oC)	Time (s)	Cycles
94	60	1
94	10	
Specific Annealing ºC	30	
72	*Taq* 60/kbp	35
68	Expand 60/kbp	
72	10 min	1

Table 2.8: Standard amplification conditions with *pfu* polymerase.

Temperature (°C)	Time (s)	Cycles
94	60	1
94	45	
Specific Annealing ºC	45	35
72	120/kbp	
72	10 min	1

2.7.3 Phosphatase treatment of vector DNA

Plasmids were treated with alkaline phosphatase by incubating 4-6 μg of restriction-digested plasmid DNA with 0.06U of calf-intestinal alkaline phosphatase in the supplied buffer for 1-2 h in a total volume of 30 μL. The hydroxylated plasmid DNA was then purified using the Geneclean® kit according to the manufacturer's instructions.

2.7.4 Kinase treatment of PCR products

Prior to self-ligation, 150-250 ng of PCR amplified DNA was incubated with 10U of T4 polynucleotide kinase (PNK), 10 μM ATP, 1x PNK buffer in a total volume of 50 μL. The reaction was incubated at 37°C for 2 h and then heat inactivated at 70°C for 10 min.

2.7.5 Ligation

Ligations were performed with purified digested DNA at a ratio of 1 vector molecule to 3 molecules of insert with the addition of 2U T4 ligase and buffer in a total volume of 10-20 μL. The reaction was incubated at 14-16°C overnight. In some instances digested DNA was ligated without further purification. In these cases the volume of digested DNA never exceeded 1/3 the total reaction volume. The ligated DNA was then purified prior to transformation via electroporation, or used directly for transformation with chemically competent cells.

2.7.5.1 Purification of DNA from ligation for electroporation

DNA ligations were precipitated from their buffers in order to prevent arcing during electrotransformation. This was done by the addition of 0.1 vol of 3 M sodium acetate, 2.5 vol of 100% ethanol, and 1 μL dextran (of a 10 μg/μL stock) directly into the ligation. The

reaction was mixed by vortex and left on ice for 10 min. The DNA was then precipitated by centrifugation at 16,000 x *g* for 5 min. The sample was washed in 70% ethanol and the centrifugation process repeated. The DNA was resuspended in 10 µL water and transformed via electro-transformation as described below.

2.7.6 Electro-competent cell preparation

Bacterial cells were prepared for electro-transformation by the method supplied with the Gene Pulser apparatus user's manual (BioRad). Briefly, 200 mL of Luria-Bertani broth (LB) was inoculated with 1/100 volume of an overnight bacterial culture. The cells were grown with vigorous shaking at 37ºC to an OD_{600} of 0.5-0.7 (early to mid-log phase). The culture was chilled on ice for 15 to 30 min and the culture pelleted by centrifugation in 50 mL tubes at 5,445 x *g* for 15 min at 4ºC. The supernatant was drained and the pellet resuspended in an equal volume of ice-cold mH_2O. The centrifuge process was repeated and the cells resuspended in a total volume of 100 mL of ice-cold mH_2O. The cells were pelleted as above and resuspended in 4 mL ice-cold 10% glycerol. The cells were pelleted once again and resuspended with 10% glycerol in a final volume of 400 µL. Aliquots of 40 µL were distributed to 1.5 mL tubes and stored at -70ºC until required.

2.7.7 Electro-transformation

The method supplied with the Gene Pulser apparatus user's manual (BioRad) was employed for the high efficiency electro-transformation of *E. coli* and *S.* Typhimurium. Frozen electro-competent cells were thawed on ice for 15 min and mixed with up to 2 µL of unpurified DNA, or 10 µL of purified DNA and transferred to an ice-cold electrocuvette with a 0.2 cm gap. The pulse settings used to deliver DNA into the cells were 2.5 kV, 25 µF and 200 Ω. After the pulse, LB was immediately added to the cells to a total volume of 1 mL and were

incubated at 37°C for 1 h. One hundred microlitres of transformed culture was plated out onto Luria-Bertani agar (LA) containing the appropriate selective agents. In some instances the remaining contents of the transformation mixture were concentrated by centrifuging at 5,455 x *g* for 5 min and also plated onto LA containing the appropriate selective agents.

2.7.8 Preparation of chemically competent cells

Bacteria were grown overnight on LA plates and 4-5 isolated colonies were selected and used to inoculate a 100 mL flask of LB. The cells were incubated at 37ºC with vigorous shaking and when mid-log growth reached were placed in 50 mL tubes and allowed to cool on ice for 10 min. The cells were pelleted by centrifugation at 5,445 x *g* for 10 min at 4ºC. The supernatant was drained and the cells resuspended in 20 mL of frozen storage buffer (FSB). The cells were pelleted once again and resuspended in 1 mL of FSB. One to two hundred microlitre aliquots were prepared in 1.5 mL tubes and stored at -70ºC until required for transformation.

2.7.9 Chemical transformation

Up to 10 µL of a ligation (5% of the total competent cell volume) was added to the chemically competent cells. This was incubated on ice for 30 min. The cells were then heat shocked in a 42ºC water bath for exactly 90 s, followed by incubation on ice for 1-2 min. LB broth was added to a final volume of 1 mL and the cells incubated at 37ºC for 1 h. Ten to 100 µL of the transformed cells were plated onto LA plates containing appropriate selective agents.

2.7.10 Sequencing

DNA was amplified using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer). DNA from pRSETA was amplified using primers pRSETRev or T7promoter, and DNA from plasmids pBluescript and pCR2.1 was amplified using primers M13-20 or M13reverse. The amplified DNA was then precipitated with 2.5 vol of absolute ethanol and 0.1 vol of 3 M sodium acetate as described in the manufacturer's protocol and was sequenced using the ABI Prism 377 DNA Sequencer with XL Upgrade (Perkin-Elmer) at Monash University (Clayton Campus), Victoria, Australia.

2.8 Protein methods

2.8.1 Preparation of whole cell lysates

Samples of *E. coli* and *Salmonella* from broth cultures were pelleted by centrifugation at 16,000 x *g* for 2 min. The pellet was resuspended in 0.1 vol of whole cell lysis buffer and the sample boiled for 5 min. Debris was pelleted by centrifugation and the protein content of the supernatant was determined using the Lowry method.

2.8.2 Protein determination

2.8.2.1 Bradford assay

Protein content determination in samples containing no traces of detergent was performed using the Bradford method (Bradford, 1976; Bollag and Edelstein, 1991).

One millilitre of Bradford reagent was added to 100 µL of sample, or standard, mixed by inversion and left to stand for 2 min. Where required samples and standards were diluted in 0.15 M NaCl. Bovine serum albumin fraction V (BSA) was used as the standard protein

solution. Two hundred microlitres of processed sample was added to the wells of a 96-well microtitre plate. The OD of samples was determined with a Dynatech ELISA plate reader at 600 nm (OD_{600}), and a standard curve constructed by plotting the concentrations of the bovine serum albumin standards (μg) versus absorbance was used to determine the protein content of unknowns.

2.8.2.2 Lowry assay

The Lowry assay was performed on samples containing the detergent SDS. Lowry reagent A and Lowry reagent B were mixed in a ratio of 100:1 to produce Lowry reagent C. Six hundred microlitres of reagent C was mixed with 200 μL of bacterial cell lysate (or BSA protein standards) then incubated at room temperature for 20 min. Sixty microlitres of Folin's reagent (Folin-Ciocalteau reagent diluted 1:1 in water) was added then mixed rapidly and left to stand for 30 min. Two hundred microlitres of the mixture was dispensed into wells of a 96-well microtitre tray and the OD_{600} was determined. A standard curve was constructed by plotting the concentrations of the bovine serum albumin standards (μg) versus absorbance, and the concentration of unknown samples determined.

2.8.3 SDS-PAGE

SDS-PAGE was performed using a discontinuous buffer system (Bollag and Edelstein, 1991). The gels were prepared using SDS stacking gel buffer and SDS separating gel buffer. Proteins were mixed with SDS loading buffer and heated to 100ºC in a dry heating block for 5 min. The proteins were separated by electrophoresis at 80 V for 30 min and then at 200 V for 35 min in an electrophoresis unit (Biorad) containing SDS running buffer. Two gels were run at the same time in this system. Gels that were not transferred onto nitrocellulose membranes for immunoblotting were stained with Coomassie blue. The SeeBlue protein marker was

separated along side protein samples and used for the determination of protein size (Appendix 3).

2.8.4 Native PAGE

Native PAGE was prepared according to the methods in Bollag and Edelstein (1991). Native PAGE gel stacking buffer and native PAGE gel separating buffer was used to prepare the polyacrylamide gel. Proteins were mixed with native PAGE sample buffer, loaded onto the gel and separated by electrophoresis for up to 3 h at 120 V at 4ºC in native PAGE running buffer. Gels were then stained by Coomassie blue.

2.8.5 Coomassie staining

SDS PAGE gels were stained in Coomassie blue staining solution for 30 min. Gels were rinsed under dH_2O and destained in the Coomassie destain solution for up to 2 h. A tissue was folded and placed into the destaining solution to hasten the destaining of the gels.

2.8.6 Immunoblotting

2.8.6.1 Adsorption of antisera to *E. coli*.

Four millilitres of TE buffer were used to flood an agar plate of *E. coli* grown for 18 h at 37ºC. The cells were harvested and pelleted by centrifugation for 10 min at 5,445 x *g*. The cells were washed in TE buffer and sonicated using 6 x 20 s bursts with a 30 s cooling off period between each sonication. Insoluble matter was pelleted by centrifugation at 14,000 x *g* for 5 min and the supernatant filtered through a 0.45 µm filter. Two millilitres of the supernatant was used to adsorb 15 µL of anti-α-toxin antisera (anti-gas-gangrene) (CSL, Australia) previously diluted in 1 mL TST. This was left shaking at room temperature for 4 h

or at 4°C for 24 h. The adsorbed antisera was diluted to 25 mL in Tris buffered saline (TBS) and 1% skim milk and stored at -20°C until required.

2.8.6.2 Protein electrophoretic transfer and immunoblotting

Proteins resolved by SDS-PAGE gels were transferred by electrophoresis onto a nitrocellulose membrane padded with 4 sheets of Whatman chromatography paper and a scotch-brite pad on both sides of the cassette. Transfer buffer was added to the tank and the electrophoretic transfer was carried out at 70 V for 1.5 h. A container of ice was placed in the buffer to maintain a cool temperature to prevent overheating of sample transfer.

After electrophoretic transfer, the nitrocellulose membrane was blocked by incubation with 5% (w/v) skim milk in TBS for 1 h on a rotary shaker. The skim milk solution was removed and the membrane washed twice by gentle agitation in TBS for 2 min. The membrane was then incubated overnight at 4°C on a rotary shaker with 25 mL of TST containing adsorbed antiserum. The membrane was then washed 2 times in TBS buffer for 5 min. Horseradish peroxidase-conjugated (HRP) antibodies diluted 1:5000 in TBS containing 1% skim milk were used as the secondary antibodies and were incubated with the membrane for 1 h shaking at 22ºC. The membranes were washed 3 times in TBS for a total of 5 min and the bound peroxidase was visualised by incubating in the Western blot substrate solution for about 5–15 min. The reaction was stopped by washing the blot in dH_2O.

2.8.7 Enzyme-Linked Immunosorbent Assay

Ninety-six well flat bottomed sterile plates (Greiner, Germany) were coated with 100 µL of 3 µg/mL purified protein or 10 µg/mL of a mixed protein sample diluted in PBS and incubated for 1 h at 37ºC or 4ºC overnight. Unbound protein was removed with 3 washes in

PBS/Tween20 and the plate coated with 200 µL of blocking buffer. After a further hour incubation at 37ºC, plates were washed as above, patted dry on absorbent paper towelling, and 100 µL of primary antibody two-fold serially diluted in diluent (1% skim milk, PBST) was added. Plates were incubated at 37ºC with vigorous shaking on an ELISA shaker platform for 2 h. The solution was discarded, wells washed 4 times in PBST and patted dry. Secondary antibody conjugated to HRP was diluted 1:5000 in PBST (1% skim milk) and 100 µL added to the wells of the ELISA plate. Following a 1 h incubation at 37ºC on a shaking platform, the plates were washed 4 times in PBST followed by a wash in dH_2O and then patted dry on absorbent paper. Tetramethylbenzidine (TMB) substrate was prepared and 100 µL added to all wells. The development of the reaction was allowed to proceed for up 30 min and the reaction was stopped with 50 µL of 1 M sulphuric acid. The OD_{450} of the wells was determined in a Dynatech ELISA microplate reader. The endpoint was determined as the dilution at which the OD_{450} was 3 times the background level ($OD \geq 0.2$). All samples were tested in duplicate.

2.9 Tissue culture techniques

The Chinese hamster ovary (CHO) cell line was purchased from CSL, Australia.

2.9.1 Maintenance and storage of cell lines

Cell lines were maintained in Dulbecco's Modified Eagles Medium (DMEM) with 10% (v/v) newborn calf serum (NCS) and 5 µg/mL penicillin and streptomycin (P/S) (Trace) in a tissue culture incubator containing 5% CO_2, 95% air at 37ºC. Upon reaching cell confluence, the tissue culture medium was discarded and the cells washed twice with sterile PBS. Cells were then treated with 50% trypsin/EDTA (Trace Biosciences, Aust.) for 5 min. Cells were

harvested from flasks by vigorous pipetting and transferred to 10 mL tubes. Cells were pelleted by centrifugation at 1,800 x *g* for 5 min and resuspended in 1 mL DMEM with 10% (v/v) NCS. The cell suspensions were used for subculture, or long term storage (with 10% (v/v) methyl sulphoxide) in liquid nitrogen.

2.10 Animal studies

Prior to the commencement of any animal studies, animal ethics approval was gained via the RMIT Animal Ethics Committee under apporval numbers AEC 0243 and AEC 0404.

Specific pathogen free (SPF) six to eight week old female BALB/c mice were purchased from the Animal Resource Centre, Canningvale, Western Austalia. Mice were acclimatised for a minimum of 1 week prior to the start of any experimentation.

Mice were bled from the retro-orbital vein using a capillary tube coated with heparin. Blood samples were pelleted by centrifugation at 6,000 x *g* for10 min and the sera was collected and stored at -20ºC until required for assays.

2.11 Bioinformatics

All the Bioinformatics programs used in this investigation were located at the Australian National Genomic Information Service (ANGIS) within WebANGIS and BioManager (www.angis.org.au). The program Sci Ed Central was used for the design of primers and vectors.

Chapter 3

Cloning, mutation and characterisation of the alpha-toxin

3.1 Introduction

The *C. perfringens* α-toxin is a co-catalytic zinc binding enzyme present in all *C. perfringens* type-strains (Kurioka and Matsuda, 1976; Rood and Cole, 1991; Vallee and Auld, 1993b). It is the major toxin responsible for the production of gas-gangrene in humans and is intimately involved in the production of a number of veterinary diseases (Awad *et al.*, 1995; Songer, 1996; Songer, 1998; Awad *et al.*, 2001).

The α-toxin is composed of 398 amino acids, of which the first 28 N-terminal residues are cleaved upon translocation and release of the toxin into the extracellular environment (Leslie *et al.*, 1989; Saint-Joanis *et al.*, 1989; Tso and Siebel, 1989). The mature 43 kDa α-toxin is highly toxic, and the administration of as little as 100 ng intravenously and 1 μg intraperitoneally is lethal to mice (Fujii *et al.*, 1986; Tso and Siebel, 1989; Ginter *et al.*, 1996).

The α-toxin is a phospholipase C (plc) enzyme, but unlike many other bacterial plc's it is highly toxic (MacFarlane and Knight, 1941; Leslie *et al.*, 1989; Tso and Siebel, 1989; Tsutsui *et al.*, 1995). Its toxicity is attributed to its ability to attack biological membranes from cells such as erythrocytes, platelets, leukocytes and sphingomyelin rich membranes (MacFarlane and Knight, 1941; McDonel, 1980; Krug and Kent, 1984; Saint-Joanis *et al.*, 1989). Its indirect effects include the activation of the arachidonic acid cascade leading to unmitigated

host inflammatory responses (Gustafson and Tagesson, 1989; Ninomiya *et al.*, 1994; Bunting *et al.*, 1997; Ellemor *et al.*, 1999; Titball *et al.*, 1999).

The precise nature of the receptor targeted by α-toxin remains unknown, although the nature of the phospholipid moiety is integral to recognition by α-toxin and is influenced by cholesterol content, saturation of phospholipids and membrane fluidity (Nagahama *et al.*, 1996; Naylor *et al.*, 1998).

X-ray crystallography of the *C. perfringens* α-toxin has revealed that it is composed of two domains, an α-helical N-terminal domain (residues 1-249) containing nine helices, and an eight-stranded β-sandwich C-terminal domain (residues 256-370), linked together by a number of highly mobile residues (247-255) (Figure 3.1) (Naylor *et al.*, 1998). The N-terminal domain contains the catalytic site whilst the C-terminal consists of the phospholipid membrane binding domain (Titball *et al.*, 1991; Titball *et al.*, 1993; Nagahama *et al.*, 1998). The catalytic site is maintained by the presence of two zinc molecules. A third exchangeable ion which can accommodate zinc or another divalent cation such as cobalt is liganded to a third site within the active site cleft (Krug and Kent, 1984; Nagahama *et al.*, 1995). The membrane binding domain of the C-terminal region requires Ca^{2+} for activation and binding to phospholipids (Fujii *et al.*, 1986; Guillouard *et al.*, 1997). The N-terminal domain retains phospholipase activity when separated from its C-terminal domain, but is devoid of its sphingomyelinase, haemolytic, cytotoxic, myotoxic and lethal properties (Titball *et al.*, 1991; Titball *et al.*, 1993; Nagahama *et al.*, 1998; Jepson *et al.*, 1999).

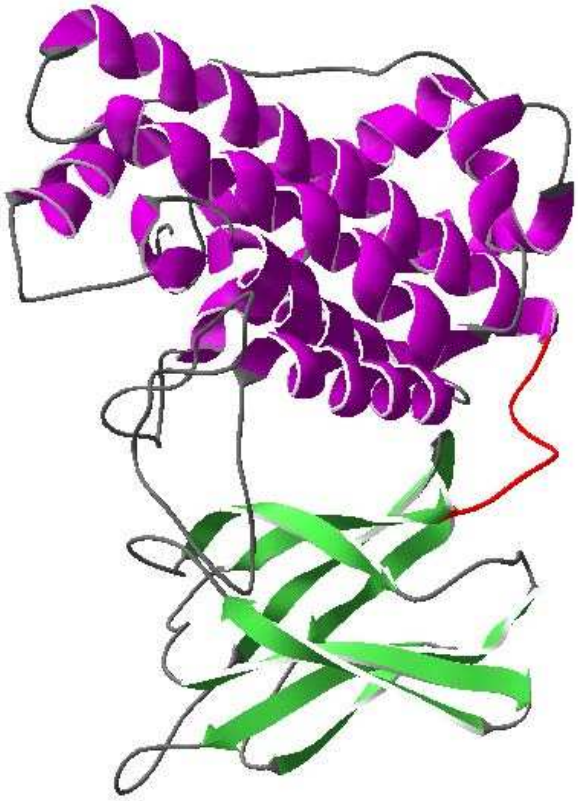


Figure 3.1: Cartoon representation of the α-toxin. The N-terminal α-helical domain is shaded purple and the C-terminal β-sandwich is shaded green. The flexible linker joining the two regions is shaded red (Naylor *et al.*, 1998).

The α-toxin is found in two conformational states, a closed inactive state, and an open active state. Activation of the α-toxin relies on communication between the N- and C-terminal domains (Eaton *et al.*, 2002). Calcium mediated binding of the C-terminal domain to phospholipids may trigger the transition of α-toxin from the closed inactive state to the open active state by the movement of two loops encompassing amino acid (aa) residues 50-90 and 135-150. In the closed form of α-toxin, loop 50-90 is thought to keep the active site away from the membrane binding plane, while loop 135-150 prevents residues H-148 and His-136 from forming the third zinc binding domain (Naylor *et al.*, 1998; Naylor *et al.*, 1999; Eaton *et al.*, 2002).

Inactivation of the α-toxin has been achieved via site-directed mutagenesis of aa residues essential to membrane binding, and catalytic activity of the toxin. Five of the nine histidines within the toxin are required for zinc binding (His-11, His-68, His-126, His-136, His-148) (Nagahama *et al.*, 1995; Guillouard *et al.*, 1996) whilst a number of residues located on protruding loops between β-sheets are required for calcium binding and membrane recognition (Naylor *et al.*, 1998; Naylor *et al.*, 1999; Walker *et al.*, 2000; Jepson *et al.*, 2001).

Separation of the two domains reveals that the C-terminal domain, although non-toxic is able to protect against the lethal effects of the α-toxin (Titball *et al.*, 1991; Titball *et al.*, 1993; Williamson and Titball, 1993; Stevens *et al.*, 2004). Therefore the presence of the C-terminal domain is essential for the development of protection against the effects of α-toxin.

This study aims to develop a number of truncated α-toxin structures, via graduated deletion from the catalytic N-terminal domain, or via a deletion of a region required for substrate binding in order to remove cytotoxic properties and evaluate the truncates as potential vaccine candidates. A protein based on $cpa_{247-370}$ previously developed by Titball *et al.*, (1993) will

also be created although this protein will have an additional deletion at the C-terminal domain in order to determine what, if any, changes in immunological and structural properties occur.

3.2 Materials and methods

3.2.1 Analysis of *plc* sequences

Primers for the amplification of the full *plc* were designed from *plc* sequences published in the ANGIS database and are listed in Table 2.5.

Multiple *plc* sequences were aligned and analysed using the program ClustalW (Thompson *et al.*, 1994)(obtained within BioManager by ANGIS) and areas of homology were chosen for primer design. Primers developed for the truncation of the *plc* were designed from the cloned and sequenced *plc* gene in this study.

3.2.2 Cloning of *plc*

The plasmids developed for the cloning and truncation of the α-toxin gene are outlined in Table 3.1.

3.2.2.1 PCR of the *plc* gene (pRSETA)

The forward primers Fpst2, 253PLCf, Pst891F and 590PLCf were used in conjunction with the reverse primer PLCecoREV to create nested deletions from the 5' terminus of *plc*. The plc gene from the chromosomal DNA of *C. perfringens* 61 (chromosomal DNA extraction method in Chapter 2.4.1 was amplified using *pfu* polymerase (Promega, USA) according to the methods described in Chapter 2.7.2.2. The annealing and elongation conditions for each amplification were as follows:

Fpst2, annealing at 52ºC, elongation of 2 min;

253PLCf, annealing at 62ºC, elongation of 2 min;

590PLCf, annealing at 55ºC, elongation of 1.5 min; and

891PLCf, annealing at 60ºC, elongation of 1 min.

Table 3.1: Description of vectors used to clone and mutate the α-toxin gene of *C. perfringens.*

Vector	Description	Source/Reference
pCR2.1	Amp^R, Kan^R, *lac*Z	Invitrogen
pCplc3	1.24 kb amplified fragment from *C. perfringens* strain 61 containing the complete *plc* with a 6xCAT (coding for 6xHis) at the 5' end, cloned into pCR2.1 using TA cloning	This study
pCplcInv3	Inverse amplified self-ligated pCplc3 (with nucleotides 250-309 of *plc* deleted)	This study
pRSETA	2.9 kbp protein expression plasmid: Amp^R, N-terminal 6xHis, P_{T7}	Invitrogen
pRplc316	Nucleotides 246 (from ATG) to 1243 of the *plc* from *C. perfringens* strain 61 cloned into *Pst*I and *Eco*RI sites of pRSETA	This study
pRplc204	Nucleotides 582-1243 (from ATG) of the *plc* from *C. perfringens* cloned into the *Pst*I and *Eco*RI sites of pRSETA	This study
pRplc104	DNA sequence coding for the C-terminus of *plc* (nucleotides 882-1243) cloned into the *Pst*I and *Eco*RI sites of pRSETA	This study
pR10plc2F	Complete *plc* lacking the first 84 nucleotides from the start codon (coding for the signal sequence of the mature α-toxin) cloned into *Pst*I and *Eco*RI sites of pRSETA	This study

Ten microlitres of each amplified product was analysed on a 1% agarose gel, and the remaining PCR product desalted using the Wizard PCR prep DNA purification kit (Promega, USA). The purified DNA was digested overnight with *Eco*RI and *Pst*I. pRSETA previously isolated from *E. coli* BL21, was also digested with the aforementioned enzymes. The two were heat inactivated and then ligated overnight without further purification. Following the purification of the ligation, 10 µl was transformed via electroporation into electrocompetent *E. coli* BL21 pLysS. When electro-cuvettes were unavailable, the chemical transformation of *E. coli* followed the ligation (Chapter 2.7.9). One millilitre of LB was added and the transformation further incubated at 37ºC for 1 h. One hundred microliters were plated out onto LA plates and SBA plates supplemented with 100 µg/mL ampicillin (from now on referred to as LA100 and SBA100 respectively). Colonies surrounded by a distinct zone of haemolysis (presence and expression of complete *plc*) or 10-20 randomly selected colonies (truncated *plc*) were selected and screened for the presence of *plc* using the alkaline lysis miniprep method as outlined in Chapter 2.4.2. Plasmid inserts were screened with a number of enzymes including *Sac*I, *Bam*HI or double digested with *Xho*I and *Eco*RV and the presence of *plc* and its truncates was confirmed by DNA sequence characterisation.

3.2.2.2 TA cloning of *plc*

Primers ALLPstI and PlcHisecoREV (Table 2.5) were used to amplify *plc* from *C. perfringens* strain #61. The Expand long range polymerase (Roche, Germany) was used and the conditions used to amplify the sequence were as described in Chapter 2.7.2.2.with an annealing temperature of 48ºC for 30 s and an elongation time of 2 min. The concentration of primer PlcHisecoRev was increased to 0.6 µM.

The PCR product was analysed on a 1% agarose gel, and the PCR product was cloned directly into the TA cloning vector pCR2.1 according to the manufacturer's instructions (Invitrogen).

Briefly, 30 ng of PCR product was mixed with 50 ng of pCR2.1 vector along with 4U of T4 DNA ligase and ligase buffer. This was incubated overnight in a 14ºC waterbath. Two microlitres of the ligation product was then transformed into chemically competent *E. coli* TOP10F' cells, supplied with the TA cloning kit. One hundred microlitres of the transformed cells were plated onto SBA100 and LA100 previously spread with 40 µL of 40 mg/mL isopropyl-β-D-thiogalactopyranoside (IPTG) and 40 µL of 40 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Clones with a distinct zone of haemolysis were selected for further analysis.

3.2.2.3 Inverse PCR of *plc*:

Primers Inv1 and Inv2 were used for inverse PCR of *plc*. The vector pCR2.1 carrying *plc* was used as a template for the inverse PCR. *Pfu* polymerase was used in the PCR to allow for the direct blunt-ended self-ligation of the vector. The pCR2.1 vector carrying the *plc* insert was amplified with an annealing temperature of 50ºC for 30 s followed by an elongation of 11 min.

Amplification of the vector was confirmed by gel electrophoresis and the remaining PCR product was digested with *Dpn*I to degrade any template DNA present. Forty microlitres of PCR product was digested with 20U of *Dpn*I with its corresponding buffer in a total volume of 100 µL. The digestion was stopped after a 2 h incubation at 37°C by heat inactivation of *Dpn*I at 65ºC for 15 min. The PCR product was purified using the Wizard® PCR prep DNA purification kit and resuspended in a final volume of 25 µL of mH_2O and the DNA concentration determined with a spectrophotometer.

Prior to self-ligation, phosphate groups were added to the 5' end of the PCR product by incubation with T4 polynucleotide kinase as described in Chapter 2.7.4. The ligation was performed directly on the kinase treated PCR product without further purification. To determine the optimum intramolecular ligating conditions, two concentrations of DNA were used in the self-ligation. Fifty and 100 ng of DNA were used along with 2U of T4 ligase and buffer in a total volume of 20 μL. The ligation was incubated at room temperature (about 22ºC) overnight. The self-ligated vector was transformed into TOP10F' cells as described in the TA cloning Kit manual. Transformants were grown on SBA100 and colonies lacking a zone of haemolysis were selected for plasmid analysis. Clones were screened using the alkaline lysis miniprep method followed by digestion with the restriction enzyme *Bam*HI.

3.2.3 Characterisation of *plc* clones

3.2.3.1 Expression of α-toxin and α-toxin truncates

One percent (v/v) of an overnight culture of *E. coli* harbouring *plc* or its truncates was used to inoculate 10 mL LB100. The broth was incubated at 37ºC with vigorous shaking until mid-log phase of growth was reached (an OD_{600} of between 0.4-0.6). IPTG to a final concentration of 1 mM was added to the culture and the culture incubated for a further 16 h. One millilitre hourly samples of culture were taken pre and post induction, pelleted by centrifugation and lysed using the whole cell lysis method (Chapter 2.7.1). Fifteen to thirty micrograms of lysed cell supernatant was analysed using SDS-PAGE and immunoblotting (Chapter 2.7.3)

3.2.3.2 Isolation and localisation of foreign protein

Periplasmic fractions were isolated by osmotic shock. Pelleted bacteria were resuspended in 1/20 the original volume of sucrose buffer II. This suspension was left on ice for 10 min and the cells resuspended every few minutes by flicking the tube. The cells were then pelleted by

centrifugation at 17,200 x *g* for 10 min at 4ºC. The supernatant was discarded and the pellet resuspended in 1/10 the original culture volume of ice cold 0.1 mM $MgCl_2$. The suspension was continually resuspended by flicking the tube every few minutes while keeping it at 4ºC for 10 minutes. The cells were pelleted by centrifugation as before and the supernatant (containing the periplasmic fractions) was collected.

The remaining spheroplasts were resuspended in 1/10 the original culture volume of 0.1 M Tris, pH 8.0 containing 1 mg/mL lysozyme. The spheroplasts were lysed by up to four freeze/thaw cycles. This consisted of freezing the sample in a liquid nitrogen bath, followed by thawing the sample at 42ºC. In the final thaw cycle 5U of DNase was added. The cytoplasmic fractions were collected following centrifugation, and the insoluble matter resuspended in the same volume of 1x SDS PAGE sample buffer. Equal volumes of each fraction were loaded onto an SDS-PAGE and separated by electrophoresis. Separated protein samples were stained using Coomassie blue reagent or transferred onto a nitrocellulose membrane and detected via an immunoblot (Chapter 2.7.3).

3.2.3.3 Isolation of inclusion bodies

A bacterial culture grown and induced as described in Chapter 3.2.3.1 was used as the starting point for inclusion body isolation. The bacteria were pelleted by centrifugation at 14,000 x *g* for 5 min. The pellet was resuspended in 3 mL of protein lysis buffer II per gram of *E. coli* and left shaking on a rotating platform for 20 min. Ten millimolar benzamidine and 10 µg/mL leupeptin were added followed by 4 mg of deoxycholic acid per gram of *E. coli* and the suspension incubated at 37ºC for a further 20 min on a rotating platform. The solution was frozen in liquid nitrogen and thawed at 42ºC. Upon thawing DNase was added to the suspension to a final concentration of 5 µg/mL and incubated at 37ºC until the solution was no longer viscous. The freeze/thaw process was repeated once again. The sample was

pelleted by centrifugation at 16,000 x *g* for 15 minutes at 4ºC. The supernatant was collected for further analysis on an SDS-PAGE and the pellet resuspended in 1 mL mH_2O/gram *E. coli*. One hundred microlitre aliquots of the solution were dispensed into 1 mL tubes and pelleted by centrifugation as above. The supernatant was discarded and the pellets resuspended in 0.1 M Tris, pH 8.5 containing different concentrations of urea ranging from 0.5 to 5 M. Insoluble debris was once again pelleted as above and the supernatant of each different concentration of urea analysed by SDS-PAGE and immunoblots.

3.2.4 Protein purification

3.2.4.1 Isolation of soluble proteins

One percent of an overnight culture (v/v) was used to inoculate 100-500 mL fresh LB. The culture was grown until an OD_{600} of 0.4-0.6 was reached and protein expression induced with 1 mM IPTG. The culture was incubated for a further 3 h and then pelleted by centrifugation. The bacteria were resuspended in 1/25 the volume of protein lysis buffer I and incubated at 4ºC for 30 min on a rocking platform. The bacteria were then treated with a repeated freeze/thaw cycle up to 4 times, with the addition of 5U of DNase following the first thaw cycle. Debris was pelleted by centrifugation at 5,445 x *g* for 40 min at 4ºC. The supernatant was filter sterilised through a 0.2 µm membrane and loaded onto an immobilised metal affinity chromatography (IMAC) gravity flow column.

3.2.4.2 Immobilised Metal Affinity Chromatography (IMAC)

Chelating sepharose fast flow was prepared according to the manufacturer's instructions (Amersham Biosciences) using 1 and 5 mL gravity flow columns (QIAGEN). Once packed and washed with H_2O, the column was charged using half a column volume (CV) of a 0.2 M solution of the transitional metal nickel sulphate ($NiSO_4$) (BDH). The column was washed with at least 5 CV of 0.45 µm filtered mH_2O and equilibrated with 5 CV of binding buffer.

Up to 60 mL of supernatant containing his-tagged protein was applied to the column. The column was washed with 10 CV of wash buffer containing 80 mM imidazole, or 60 mM imidazole for plcInv3, following protein binding and eluted with 10 CV of elution buffer. One millilitre fractions were collected during the elution step, and analysed using SDS-PAGE. The flow through from the column was then placed into a second 1 mL IMAC column and the process repeated.

3.2.4.3 On-column refolding of urea-solubilised inclusion bodies

Proteins resolubilised with urea were filtered through a 0.2 μm filter and loaded onto an IMAC column previously charged with $NiSO_4$ and washed with 5 M urea in refolding buffer (25 mM Tris, pH 8.0, 25% glycerol, 0.5 M NaCl). Once bound, his-tagged proteins were subjected to a reducing gradient of urea concentrations in refolding buffer from 5 M to 0 M in 1 M decrements to aid in refolding of the protein. The column was washed in refolding buffer containing 60 mM imidazole and his-tagged protein eluted with refolding buffer containing 200 mM imidazole.

3.2.4.4 Cleaning in place and regeneration of column

Gravity flow columns were re-used up to 5 times and cleaned between each use to remove hydrophobic and ionic bound contaminants. Cleaning in place involved the application of 10 CV of 2 M NaCl, followed by 10 CV of 1 M NaOH, and finished with 10 CV of 70% ethanol (v/v). Columns were washed with H_2O between each solution and Ni^{2+} was stripped with 2 CV 0.05 M EDTA, 0.5 M NaCl, pH 7.0. A 20% ethanol solution was added to the sepharose when stored for later use.

3.2.4.5 Buffer exchange of proteins

Eluted proteins were concentrated using Centricon centrifugal devices (Millipore) of cut-off pore size 3 kDa and 10 kDa according to the manufacturer's instructions. Buffer exchange of proteins was also done in the Centricon devices. Salts and metals were removed by continual concentration and dilution of the protein sample in the new buffer.

3.2.5 Alpha-toxin assays

3.2.5.1 EYA reaction

Wells were bored into Nagler plates using a 5 mm metal borer. Plates were warmed and dried at 37ºC and 20 µL volumes of protein were added to the wells. Twenty-five millimolar Tris, pH 7.4 was used as a control. The plates were incubated at 37ºC and the diameter of the zone of turbidity developed was measured every half hour (in mm). Samples with no observable signs of hydrolysis were left at 37ºC for up to 24 hours.

3.2.5.2 Haemolysin assay

SBA was prepared as described in Chapter 2.2.2.1 with the addition of 100 µg/mL of ampicillin to the molten agar. Fifty microlitres from a stock solution of 100 mM IPTG was spread onto the SBA plates and *E. coli* colonies transformed with vector containing *plc* or truncates were streaked onto the agar and incubated for 18 h at 37ºC. Plates were then incubated at 4ºC for 2 h prior to determining the presence of incomplete haemolysis surrounding the colonies.

3.2.5.3 Haemolysin liquid assay (HLA)

Five millilitres of a sheep red blood cell (SRBC) suspension was pelleted by centrifugation at 3,000 x *g* for 10 min. The SRBC were washed and pelleted repeatedly in HLA buffer until

the supernatant was clear of lysed blood cells. A 1% solution (v/v) of SRBC was prepared in HLA buffer.

Alpha toxin (100 µL) was diluted two-fold in HLA buffer along the wells of a 96-well microtitre plate. One hundred microlitres of H_2O or HLA buffer were used as the positive and negative controls respectively. One hundred microlitres of the 1% SRBC suspension was added to each well and the plate incubated at 37ºC on a shaking platform for 2 h. Following a further 1 h incubation at 4°C the plate was spun in a centrifuge at 3,000 x *g* for 15 min at 4ºC and 100 µL of the supernatant dispensed into a new 96-well plate. The optical density was determined at a wavelength of 550 nm in a Dynatech ELISA plate reader. The percent of cell lysis was determined using the following equation:

(OD test well /mean OD positive well) X 100

The number of haemolytic units (HU_{50}) was expressed as the reciprocal of the dilution of toxin required to cause 50% haemolysis. The specific haemolytic activity of the protein was defined as the amount of protein in nanograms required to cause 50% haemolysis.

3.2.5.4 MTT cytotoxicity assay

The CHO cell line was cultured in 25cm^2 tissue culture flasks in DMEM supplemented with 10% (v/v) NCS and 5 µg/mL penicillin/streptomycin at 37^{o}C in air with 5% CO_2 until confluent. Cells were harvested by trypsin-EDTA treatment and counted using a haemocytometer cell counting chamber (Chapter 2.9.1). Trypan blue was added to the cells to distinguish viable from dead cells.

Ninety-six well tissue culture plates were seeded with 2 x 10^4 CHO cells in 100 µL DMEM/10% NCS and incubated overnight to form a monolayer. One hundred microlitres of protein in two-

fold serial dilutions (made with 25 mM Tris, pH 7.4) was added to the wells. One hundred microliltres of 25 mM Tris pH 7.4 was added to control wells. Test fractions were performed in duplicate. After 18 h incubation, the percentage of cell death was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).

Twenty microlitres of 5 mg/mL MTT in 10 mM Tris-Cl, pH 8.0, was added to each well and incubated at 37°C for 4 h. The overlaying medium was carefully removed using a multichannel pipette, and 100 µL of 0.04 M HCl in dimethylsulphoxide (DMSO) was added to dissolve the formazan product. The plates were placed on a shaking platform for 5-15 min (until all products were solubilised) and then placed in a Dynatech ELISA plate reader to determine the absorbance at a wavelength of 600 nm. One hundred microlitres of 0.04 M HCl in DMSO was used as a blank. The percentage of cell death was calculated using the following equation.

$$\% \text{ cell death} = \left(1 - \frac{OD_{600} \text{ of test well}}{OD_{600} \text{ of negative control well}}\right) \times 100$$

The cytotoxicity was expressed as the tissue culture dose 50 (TCD_{50}). A TCD_{50} was defined as the dilution of toxin that caused death in 50% of the cells at the chosen time point. The inverted value of this dilution gave the titre - the number of TCD_{50} units in the stock solution of the toxin, or as specific toxicity, which was defined as the amount of the toxic fraction, in nanograms of protein, required to cause 50% cell death.

In order to determine the cytotoxic effects of the truncated α-toxin proteins on CHO cells, 100 µg/mL of each protein was assayed in the MTT assay, along with 50 µg/mL of α-toxin, a level which results in approximately 80% cell death.

3.2.5.5 *p*-NPPC assay

The activity of α-toxin on the substrate para-nitrophenylphosphorylcholine (*p*-NPPC) was determined using the method of Kurioka and Matsuda (1976) with a few modifications. Briefly, a reaction mix (200 µL) consisting of 10 mM *p*-NPPC substrate (Sigma), 10 µM $ZnCl_2$, 60% glycerol, 50 mM Tris, pH 7.2 and toxin was dispensed in a 96-well plate and incubated in a fluoSTAR OPTIMA plate reader (BMG Labtechnologies) at 37°C. The plate was shaken for 1 min prior to each reading. The absorbance was determined every 5 min and the concentration of *p*-nitrophenol liberated determined using a standard curve of various *p*-nitrophenol concentrations in the same reaction buffer. One Unit was defined as the hydrolysis of 1 nanomole of *p*-nitrophenol per min under the specified conditions.

3.2.5.6 Gel mobility assay

Tris buffered saline (20µL) containing 0.15 µg/µL of α-toxin or truncated α-toxin proteins was incubated with 20 mM $CaCl_2$ and 10 mM EDTA, or EDTA alone at 22ºC for 5 min. Native PAGE sample buffer was added and the samples loaded onto an 8.5% (α-toxin and plcInv3) or 12.5% (plc104) native-PAGE gel and separated by electrophoresis at 120 V for 3 h at 4ºC. The gels were stained in Coomassie blue dye followed by destaining solution.

3.2.5.7 Erythrocyte binding assays:

Qualitative assay:

This assay was adapted from Nagahama *et al*., (1995). Membranes were prepared by pelleting 2 mL of sheep red blood cells in a centrifuge for 5 min at 5,445 x *g* and resuspending them in 10 mL of 5 mM Tris, pH 8.0. The membranes were pelleted at 14,000 x *g* and washed twice more with a final resuspension in 5 mL of TBS. The protein content of

the membranes was determined using the Bradford assay, and the suspension was adjusted to 100 μg/mL with TBS.

One hundred microlitres of the 100 μg/mL erythrocytic membrane suspension was mixed with 1 μg of protein. Each protein sample was assayed in the presence and absence of 5 mM $CaCl_2$. Following a 30 min incubation at 37°C on a orbital shaker, the membranes were pelleted by centrifugation at 14,000 x *g* for 20 minutes at 4°C. The supernatants were collected for analysis and the membranes washed in TBS and pelleted as above. This wash was repeated three times and the membranes were finally resuspended in 100 μL SDS loading buffer. Thirty microlitres of supernatant and membrane suspension was analysed via SDS-PAGE followed by an immunoblot using horse polyclonal anti-alpha toxin antisera.

Quantitative assay:

Membranes were prepared as above and 100 μL of the 100 μg/mL membrane suspension was used to coat a 96-well microtitre plate. All following incubations were completed at 37ºC for 1 h and microtitre plates were washed three times in TBS between each step. After a 1 h incubation, the wells of the plate were washed and unbound sites blocked with 200 μL of TBS containing 5% skim milk (v/v). Following incubation and washing, 100 μL of a 10 μg/mL protein sample (α-toxin or truncated α-toxin protein) diluted in TBS and 1% skim milk was added to the wells of the plate in the presence or absence of 10 mM $CaCl_2$. The plate was incubated at 37ºC on a rotating platform. Following washing, mouse anti-α-toxin sera diluted 1:2,000 in TBS(1% skim milk) was prepared and 100 μL added to the wells. After incubation and washing, wells were incubated with 100 μL of a 1:5000 dilution of goat anti-mouse IgG-HRP. Plates were washed four times in TBS and 100 μL of TMB substrate added to each well. Following a 15 min incubation at room temperature 50 μL of 1 M sulphuric acid was added and the absorbance of the wells at an OD_{450} was determined using a Dynatek plate

reader. Three wells were treated the same as the test wells except that no α-toxin was added, and these were used as the background reading for the assay and were automatically subtracted by the Dynatek plate reader. All test samples and controls were performed in triplicate.

3.3 Results

3.3.1 Cloning of the *plc* gene

A number of *plc* sequences have been published in the Genbank database and were obtained through the ANGIS website. These sequences were used to design primers for the amplification of *plc*. Two strategies were developed for the cloning and expression of *plc*. One primer, Fpst2, was designed to bind 84 base pairs downstream of the *plc* start codon in order to avoid amplifying the signal sequence of *plc* usually required for the secretion of the α-toxin into the extracellular environment of *C. perfringens*. The amplified *plc* was directionally cloned into pRSETA and transformed into *E. coli* BL21pLysS to create clone BL21pLysS(pR10plc2f) (Figure 3.2 B,C). Sequencing of the clone confirmed that *plc* was in frame with the vector start codon and produced a product of 412 aa's which equated to a theoretical size of approximately 47.5 kDa. Its size was estimated at 57 kDa when calculated from its migration on a SDS-PAGE, approximately 10 kDa higher than the theoretical weight. The expressed α-toxin was designated 10plc2f and contained a 5 kDa region upstream of its N-terminus which included the His x 6 sequence required for downstream processing and was recognised by horse polyclonal anti-α-toxin antibodies (Figure 3.3).

The second strategy of cloning and expressing the complete *plc* gene involved engineering a 6 x CAT sequence (to code for the protein His x 6) to the reverse *plc* primer (primer PlcHisEcoRev) in order to purify the α-toxin using IMAC from a C-terminal His-tag (C-His tag) as opposed to the N-terminal His-tag (N-His tag) as for 10plc2f. The *plc* was amplified and cloned into pCR2.1 using the TA cloning strategy and transformed into *E. coli* TOP10F' cells (Figure 3.4).

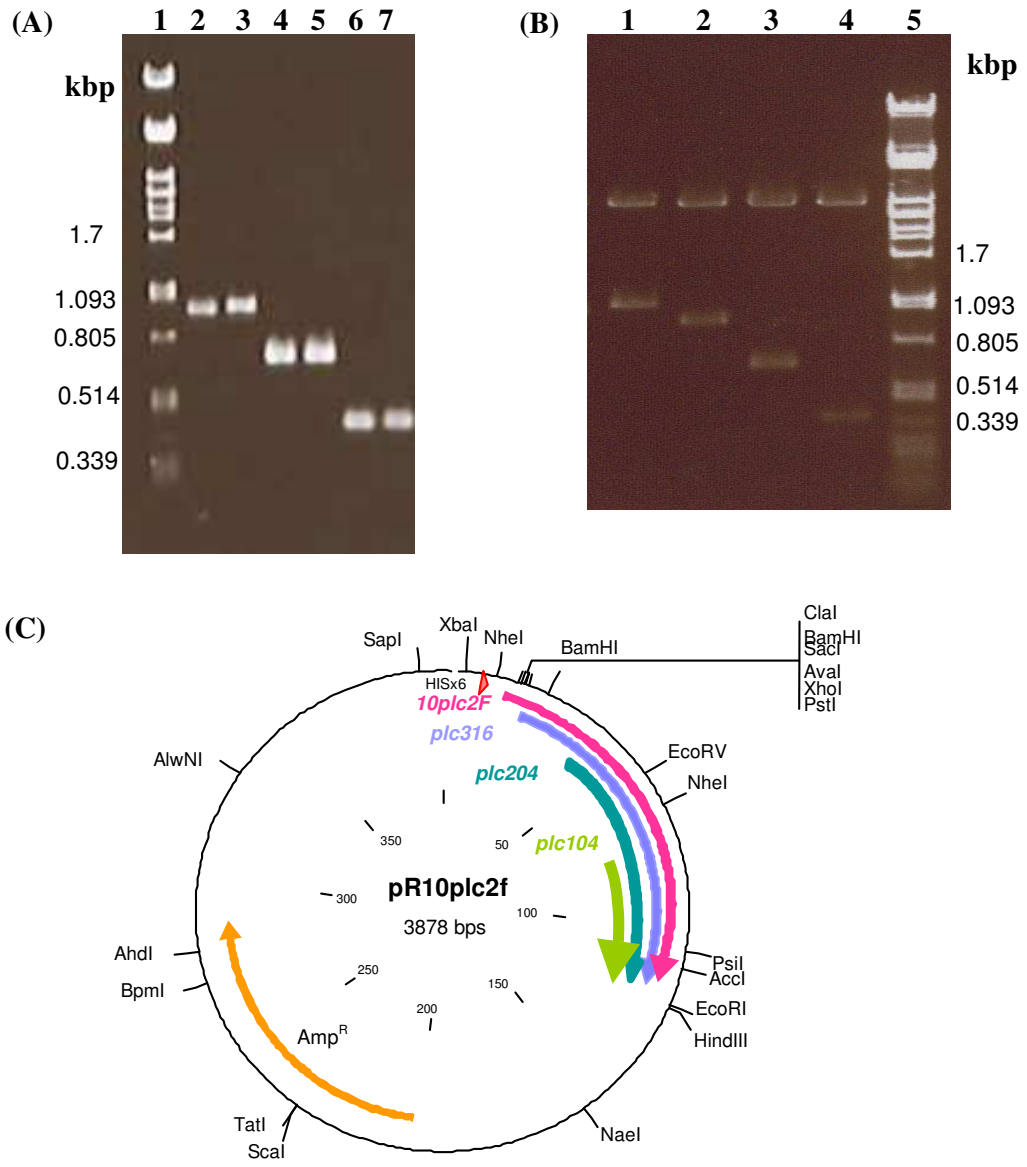


Figure 3.2: The development of α-toxin expression systems using the commercial vector pRSETA (Invitrogen). (A) PCR of truncated *plc*. Lane 1, λ-*Pst*I; Lane 2+3, PCR of *plc316*; Lane 4+5, PCR of *plc204*; Lane 6+7, PCR of *plc104*. **(B)** Cloning of *plc* and truncated *plc* genes into pRSETA expression vector. Lane 1, pR10plc2f; Lane 2, pRplc316; Lane 3, pRplc204; Lane 4, pRplc104; Lane 5, λ-*Pst*I. Lanes 1-4 digested with *Pst*I and *Eco*RI, Lane 5 digested with *Pst*I. **(C)** Restriction map of pR10plc2F. The *10plc2F* gene was cloned into the *Pst*I and *Eco*RI sites of pRSETA to create pR10plc2F. The same methodology was used for the cloning of the other three truncated *plc* genes into pRSETA.

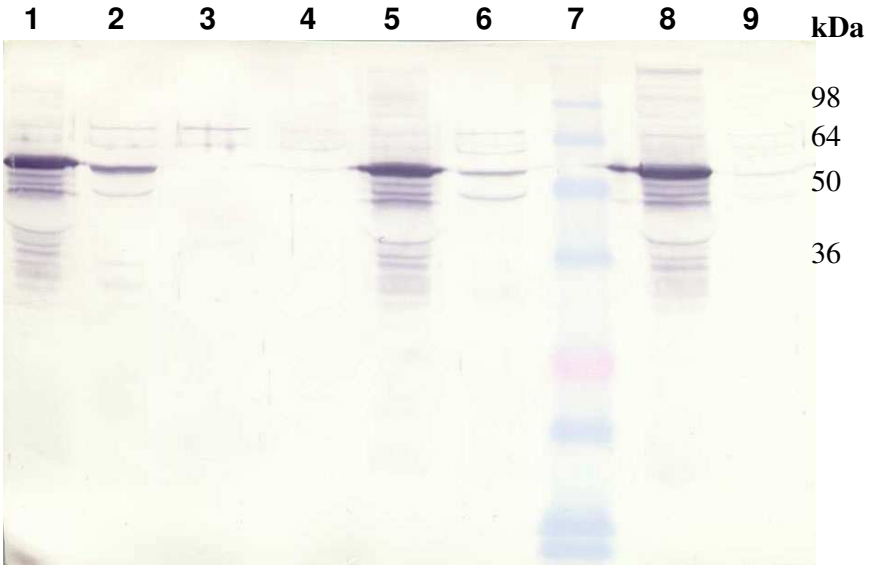


Figure 3.3: Expression of α-toxin from BL21pLyS(pR10plc2f) as detected by an immunoblot using horse anti-α-toxin antibodies. Lane 1, 3 h insoluble pellet of BL21pLysS(pR10plc2f); Lane 2, 3 h cytoplasmic fraction of BL21pLysS(pR10plc2f); Lane 3, 6 h insoluble pellet of BL21pLysS containing empty vector; Lane 4, 6 h cytoplasmic fraction of BL21pLysS containing empty vector; Lane 5, 4 h insoluble pellet of BL21pLysS(pR10plc2f); Lane 6, 4 h cytoplasmic fraction of BL21pLysS(pR10plc2f); Lane 7, Seeblue protein marker; Lane 8, 6 h insoluble pellet of BL21pLysS(pR10plc2f); Lane 9, 6 h cytoplasmic fraction of BL21pLysS(pR10plc2f).

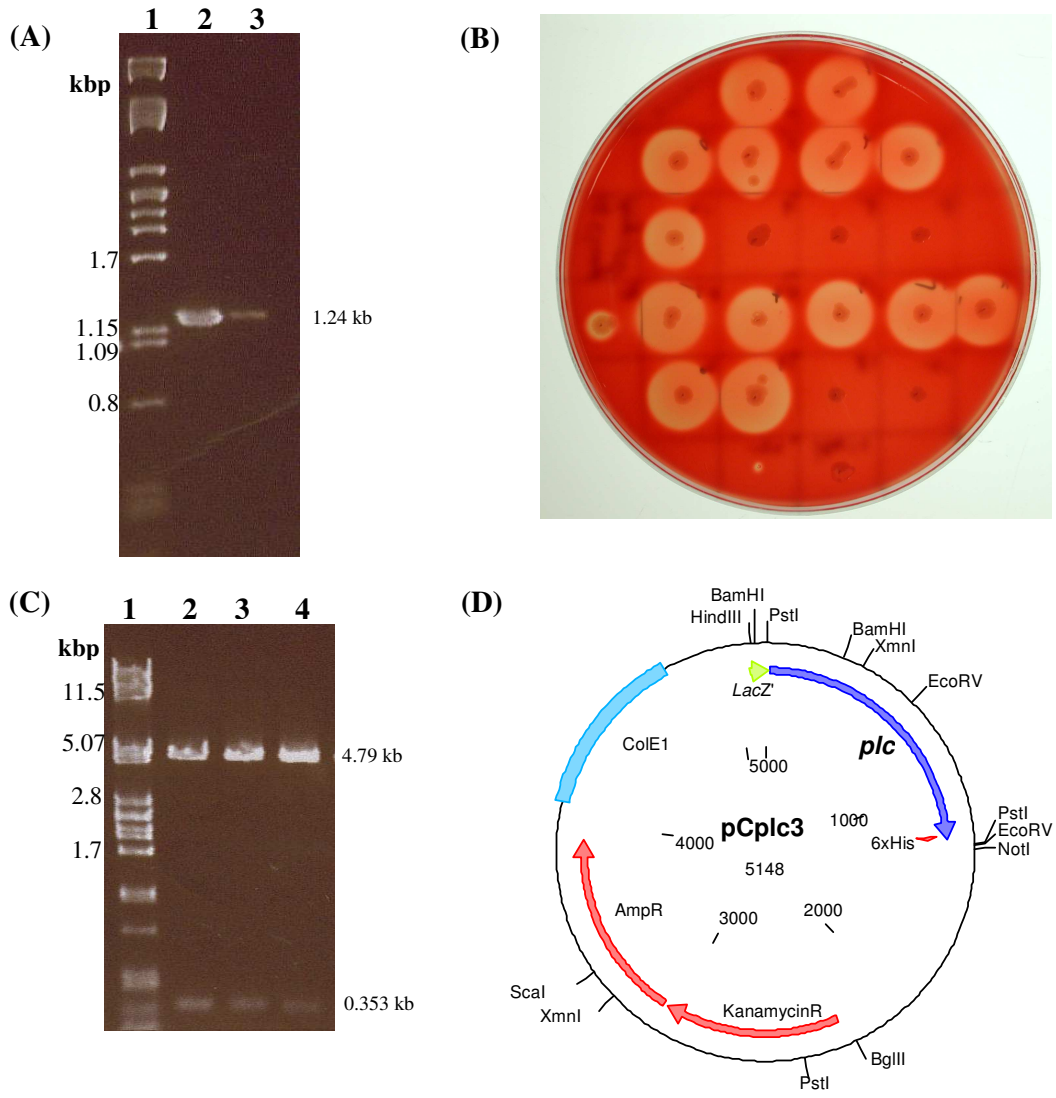


Figure 3.4: The development of the *plc* expression vector pCplc3. (A) PCR of *plc* using reverse primer containing internal CAT x 6 sequence. Lane 1, λ-*Pst*I; Lane 2+3, PCR of *plc* at a 45°C and 50°C annealing temperature respectively. **(B)** Haemolytic transformants following ligation of pCR2.1 and *plc*. Distinct zone of incomplete haemolysis observed around colonies which have acquired the complete *plc* sequence. **(C)** Cloning of *plc* into pCR2.1 vector. Lane 1, λ-*Pst*I; Lane 2-4, clone 1 (pCplc1), clone 2 (pCplc2) and clone 3 (pCplc3) digested with *Bam*HI. **(D)** Restriction map of vector pCplc3 with the *plc* insert. The *plc* was cloned directly into the vector by the use of the TA cloning strategy.

Transformants were grown overnight on HBA100 and IPTG, and haemolytic colonies (Figure 3.4) were isolated for further analysis by restriction enzyme digests (Figure 3.4). Clone TOP10F'(pCplc3) was sequenced (Appendix 3) and used for the expression and purification of the α-toxin, named plc3. Sequencing results showed that *plc* cloned into the pCR2.1 vector was not in frame with the upstream *lac*Z gene and hence its expression was void of any β-galactosidase amino acids. The initiation codon of *plc* was 95 bp downstream of the *lac*Z initiation codon, and there was no identifiable consensus Shine-Dalgarno (S-D) 8-13 bp upstream of the *plc* initiation site. The theoretical molecular weight (MW) of the expressed mature plc3 was calculated as 43.4 kDa, and after its migration on an SDS-PAGE was calculated as 47 kDa.

Expression of α-toxin from BL21pLysS(pR10plc2f) and TOP10F'(pCplc3) clones revealed that 10plc2f was predominantly expressed and localised to the cytoplasm in the form of inclusion bodies (Figure 3.3) rather than soluble cytoplasmic protein, whereas plc3 was expressed and secreted into the periplasmic space of *E. coli* (Figure 3.5). Furthermore attempts to purify the soluble component of 10plc2f were unsuccessful. The protein did not bind very well to Ni^{2+} charged IMAC and was constantly eluted in the wash buffer in the presence of low levels (10 mM) of imidazole. Clone TOP10F'(pCplc3) expressed α-toxin in a soluble form. The C-His tag of plc3 was used to purify the protein from the cell lysate via IMAC (Figure 3.6). Concentration of up to 80 mM imidazole did not affect the affinity of plc3 to the Ni^{2+} column, and the protein was eluted with 200 mM imidazole. Approximately 3.5 mg of plc3 was obtained per litre of *E. coli* following IMAC purification.

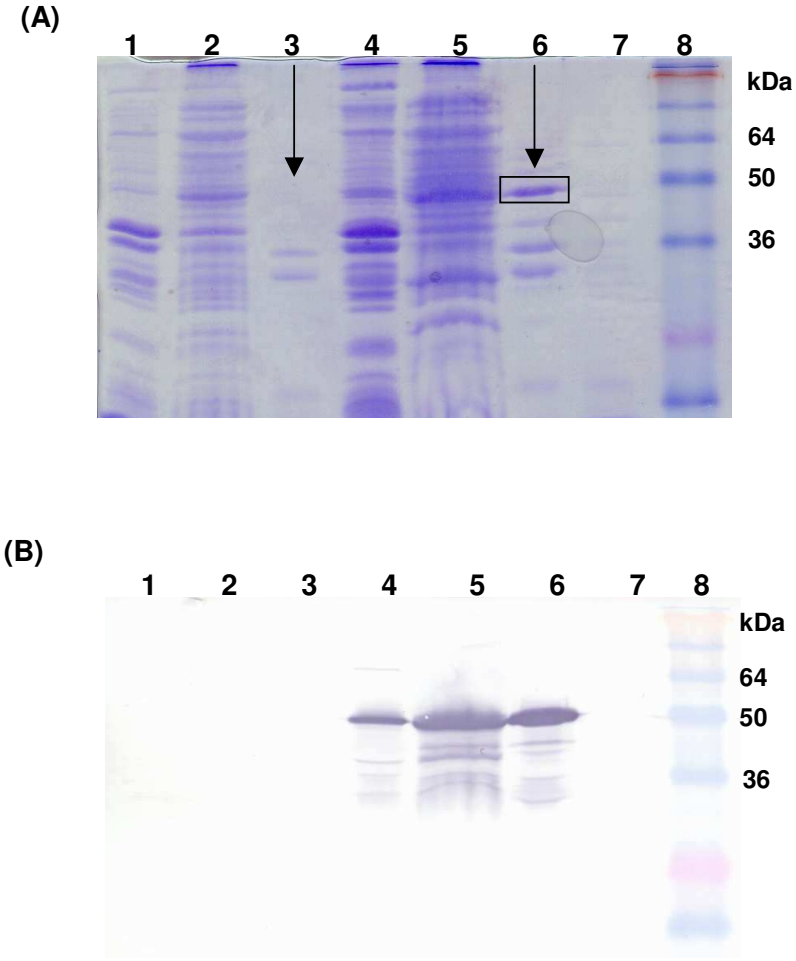


Figure 3.5: Location of plc3 3 h post-induction as analysed on an SDS-PAGE. (A) Coomassie blue stained gel **(B)** Following an immunoblot using anti-α-toxin horse polyclonal antisera. Lane 1, resolubilised pellet of TOP10F' cells (negative control); Lane 2, cytoplasmic fraction of TOP10F'cells; Lane 3, periplasmic fraction of TOP10F' cells; Lane 4, resolubilised pellet of TOP10F'(pCplc3); Lane 5, cytoplasmic fraction of TOP10F'(pCplc3); Lane 6, periplasmic fraction of TOP10F'(pCplc3); Lane 7, empty well; Lane 8, SeeBlue protein marker.

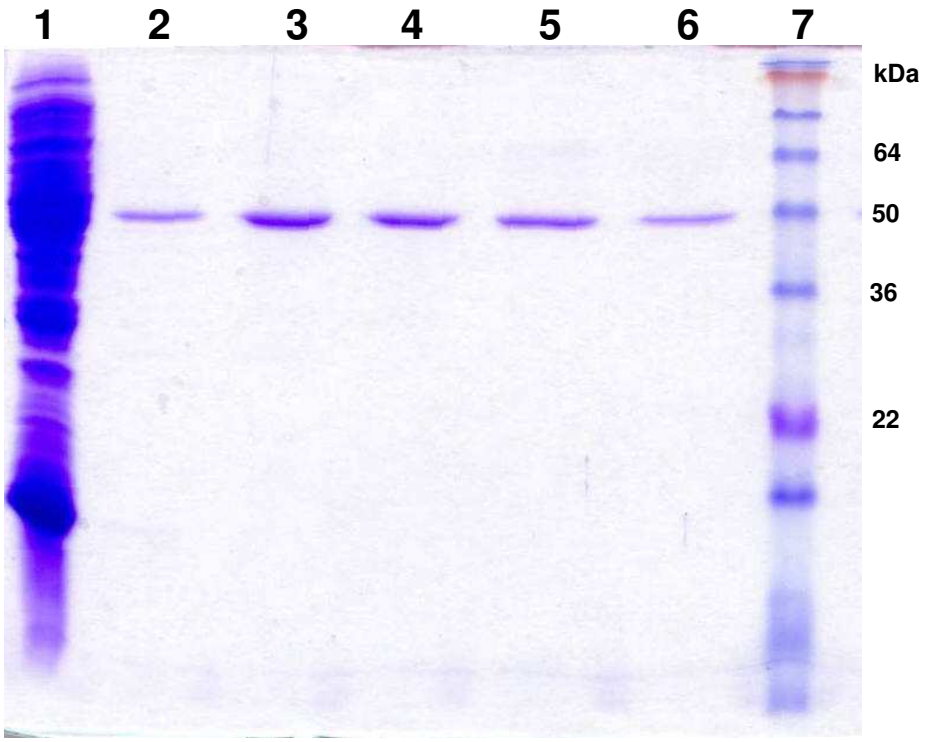


Figure 3.6: IMAC purification of alpha-toxin (plc3) eluted with 200 mM imidazole. Lane 1, pre-column whole cell lysate; Lane 2-6, elution 1-5 respectively; Lane 7, SeeBlue protein standard.

3.3.2 Cloning of *plc* truncated genes

The truncation of *plc* was based on two strategies; nested deletions occurring at the 5' end of the *plc*, or as an internal deletion of *plc*. Three products were created by the amplification of nested deletions, a 1.01 kb, 0.67 kb and 0.37 kb product (Figure 3.2 A) which were all digested with *Pst*I and *Eco*RI and directionally cloned into expression vector pRSETA, and following transformation into BL21pLysS produced clones BL21pLysS(pRplc316), BL21pLysS(pRplc204) and BL21pLysS(pRplc104) (Figure 3.2 B, C) respectively. The number in each vector name is representative of the number of α-toxin amino acids present in the expressed sequence.

Deletion of an internal fragment of *plc* was achieved via inverse PCR of plasmid pCplc3. Primers Inv1 and Inv2 bound within the *plc* region 60 base pairs apart amplifying a fragment of 5.088 kb in size. This fragment was then self-ligated, transformed into TOP10F' cells and grown on SBA100. Non-haemolytic colonies were selected, and their plasmids screened via RE digestion for the absence of a second *Bam*HI site deleted during inverse PCR (Figure 3.7). One such clone, TOP10F'(pCplcInv3) was selected for protein expression of the truncated α-toxin, plcInv3.

The amino acid alignment in Figure 3.8 illustrates the amino acids deleted within the α-toxin from each construct with reference to the complete mature α-toxin. The properties of each construct are described in Table 3.2. The 3-dimensional structure of the α-toxin has been solved by Naylor *et al*., (1998) and its structure is presented in Figure 3.1 with the N-terminal and C-terminal domains outlined. Proteins plc316, plc204 and plc104 were developed with deletions from the N-terminal domain of the α-toxin, with plc104 composed only of the C-terminal β-sheets.

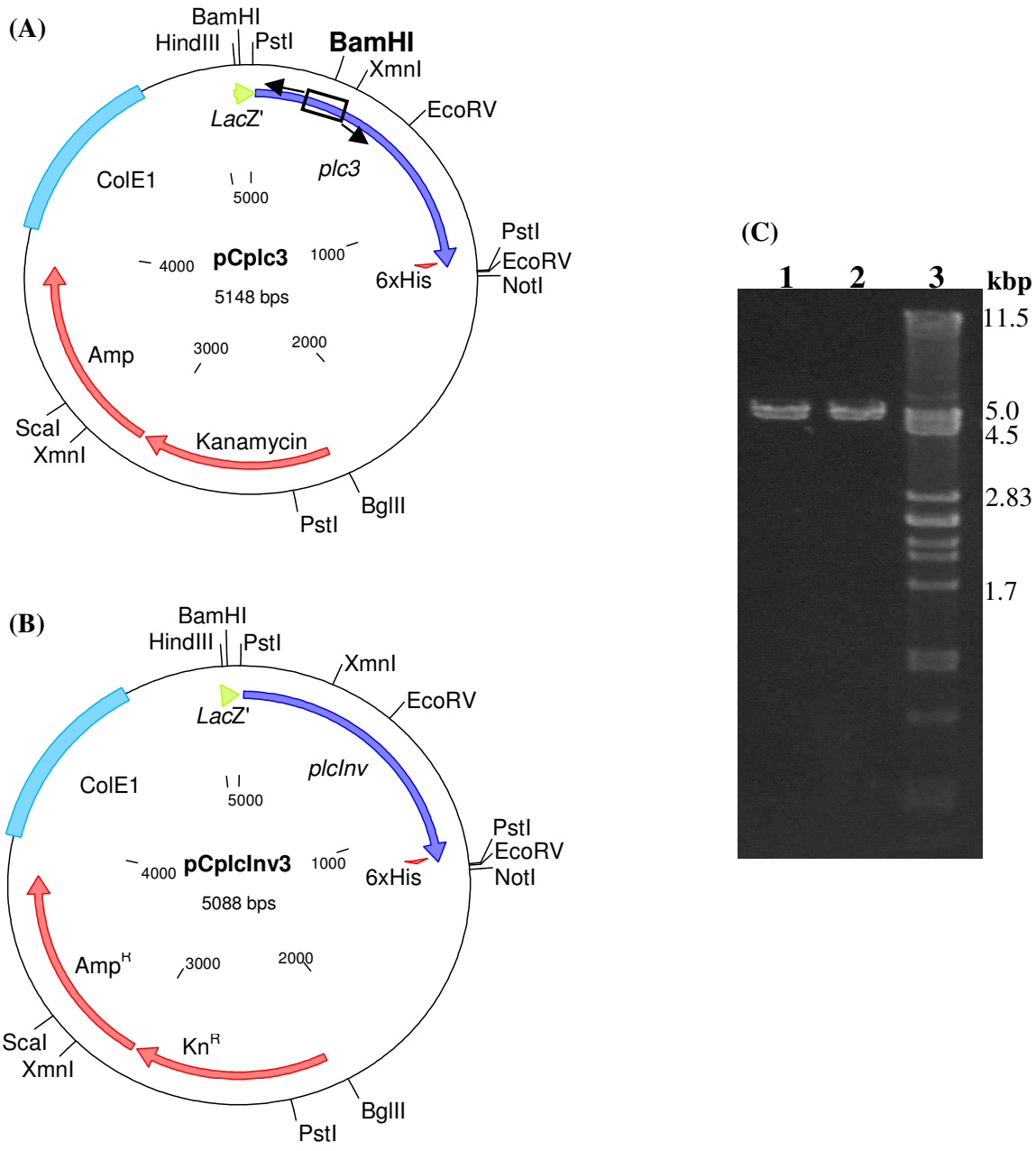


Figure 3.7: The development of vector pCplcInv3. **(A)** Restriction map of pCplc3. Outward facing black arrows indicate point of primer binding for inverse PCR. Open box indicates region deleted via PCR which includes a *Bam*HI site. **(B)** Restriction map of pCplcInv3. The *Bam*HI site is no longer present. **(C)** Clones of transformants carrying the pCplcInv3 self-ligated vector. Lane 1, pCplcInv2; Lane 2, pCplcInv3; Lane 3, λ -*Pst*I. All clones were digested with *Bam*HI.

```
1   WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL
1   WDGKIDGTGT HAMIVTQGVS ILENDLSKNE PESVRKNLEI LKENMHELQL

51  GSTYPDYDKN AYDLYQDHFW DPDTDNNFSK DNSWYLAYSI PDTGESQIRK
51  GSTYP                      NNFSK DNSWYLAYSI PDTGESQIRK
51      PDYDKN AYDLYQDHFW DPDTDNNFSK DNSWYLAYSI PDTGESQIRK

101 FSALARYEWQ RGNYKQATFY LGEAMHYFGD IDTPYHPANV TAVDSAGHVK
101 FSALARYEWQ RGNYKQATFY LGEAMHYFGD IDTPYHPANV TAVDSAGHVK
101 FSALARYEWQ RGNYKQATFY LGEAMHYFGD IDTPYHPANV TAVDSAGHVK

151 FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG
151 FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG
151 FETFAEERKE QYKINTAGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG
151                  AGCK TNEDFYADIL KNKDFNAWSK EYARGFAKTG

201 KSIYYSHASM SHSWDDWDYA AKVTLANSQK GTAGYIYRFL HDVSEGNDPS
201 KSIYYSHASM SHSWDDWDYA AKVTLANSQK GTAGYIYRFL HDVSEGNDPS
201 KSIYYSHASM SHSWDDWDYA AKVTLANSQK GTAGYIYRFL HDVSEGNDPS
201 KSIYYSHASM SHSWDDWDYA AKVTLANSQK GTAGYIYRFL HDVSEGNDPS

251 VGKNVKELVA YISTSGEKDA GTDDYMYFGI KTKDGKTQEW EMDNPGNDFM
251 VGKNVKELVA YISTSGEKDA GTDDYMYFGI KTKDGKTQEW EMDNPGNDFM
251 VGKNVKELVA YISTSGEKDA GTDDYMYFGI KTKDGKTQEW EMDNPGNDFM
251 VGKNVKELVA YISTSGEKDA GTDDYMYFGI KTKDGKTQEW EMDNPGNDFM
251                  EKDA GTDDYMYFGI KTKDGKTQEW EMDNPGNDFM

301 TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
301 TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
301 TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
301 TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK
301 TGSKDTYTFK LKDENLKIDD IQNMWIRKRK YTAFPDAYKP ENIKIIANGK

351 VVVDKDINEW ISGNSTYNIK
351 VVVDKDINEW ISGNSTYNIK
351 VVVDKDINEW ISGNSTYNIK
351 VVVDKDINEW ISGNSTYNIK
351 VVVDKDINEW ISGNSTYNIK
```

Figure 3.8: Amino acid sequence of mature α-toxin. Region shaded grey represents N-terminal α-helical domain and open boxed region represents C-terminal β-sheet domain. Each truncated α-toxin created in this study is represented by a different colour: **Black; plc3, plum; plcInv3, blue; plc316, orange; plc204, green; plc104.**

Amino acids highlighted pink are important for zinc binding at active site.

Amino acids highlighted teal are important for calcium binding.

Amino acids highlighted green are important for binding of active site to membranes.

Table 3.2: Description of α-toxin gene products

Expression vector	Protein	No. of aa in protein[a]	Deletion(Δ)[b]	Purification (mg/L of culture)	Calculated MW (kDa)
pR10plc2f	10plc2f	370	Signal sequence	-[c]	57.9+/-0.602
pCplc3	plc3	370	-	3.5	46.9+/-0.478
pCplcInv3	plcInv3	350	56-75 (Δ of 2 zinc binding domains)	1.5	44.0+/-1.08
pRplc316	plc316	316	1-55 (Δ of the first 3 α-helices from N-terminus)	1.4	47.3+/-0.434
pRplc204	plc204	204	1-166 (Δ of the first 7 α-helices from N-terminus)	2.0	30.6+/-0.331
pRplc104	plc104	104	1-266 (Δ of complete α-helical N-domain and the first β-sheet)	3.0	18.3+/-1.01

[a] His tag region not included

[b] Amino acid (aa) position number of mature α-toxin

[c] Not determined

PlcInv3 has an internal deletion at the N-terminal domain within the catalytic cleft of the α-toxin.

All four truncated α-toxins were successfully expressed from *E. coli* and recognised by anti-α-toxin horse polyclonal antisera (Figure 3.9). Protein plc316 was expressed at a high level but the majority of the protein was isolated as inclusion bodies (Figure 3.10). Protein plc204 was slightly more soluble, but again the majority of it was isolated as inclusion bodies (Figure 3.11). Protein plc104 was highly soluble and isolated in both the cytoplasm and as inclusion bodies (Figure 3.12). Protein plcInv3 was not expressed as inclusion bodies to a high degree and like its parent protein plc3, was translocated to the periplasmic space of *E. coli* (Figure 3.13). Protein plcInv3 IMAC purification required a milder wash of 60 mM imidazole as 80 mM imidazole eluted the protein from the column. TOP10F' cells harbouring pCplcInv3 also grew much slower than cells harbouring pCplc3 even prior to induction. It took approximately 3 hours for TOP10F'(pCplc3), and 5 hours for TOP10F'(pCplcInv3) cells to reach the mid-log phase of growth (as determined by an OD_{600} of 0.5)

As plc316 and plc204 were isolated in an insoluble form, 5 M urea was required to solubilise them. The proteins were purified via their N-His tag using IMAC. Removal of urea and refolding of plc316 and plc204 was achieved during IMAC via on-column refolding using a gradient reduction of urea, and then finally eluted with 200 mM imidazole (Figure 3.14). Protein plc104 required no such treatment and was much simpler and quicker to purify. In general, about 1.4, 1.5, 2, and 3 mg of protein per litre of *E. coli* was purified from BL21pLysS(pRplc316), TOP10F'(pCplcInv3), BL21pLysS(pRplc204) and BL21pLysS(pRplc104) respectively (Table 3.2).

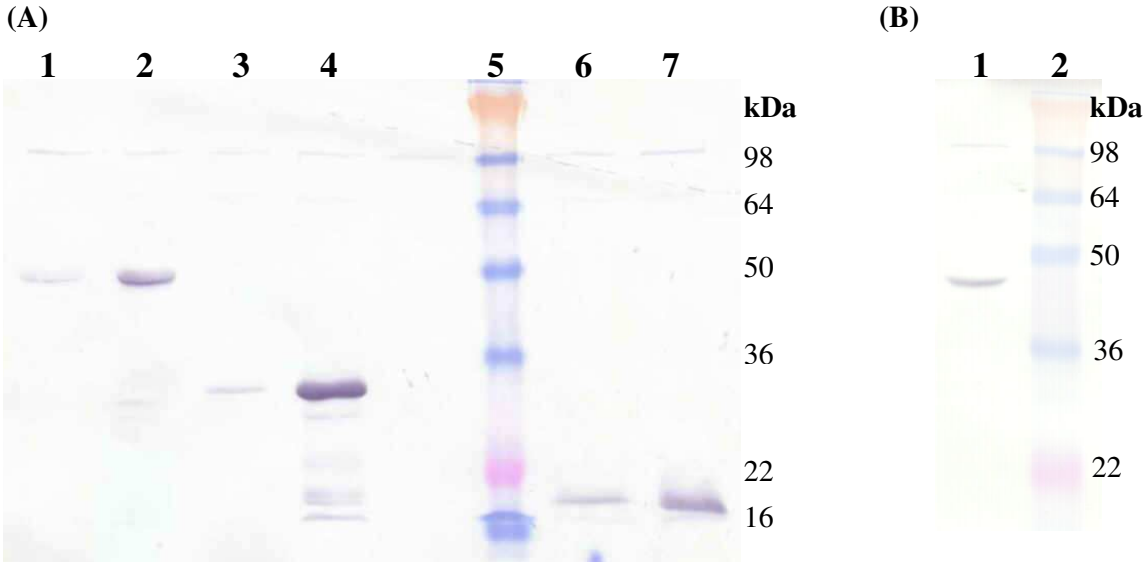


Figure 3.9: Expression of α-toxin truncates plc316, plc204, plc104 and plcInv3 as assessed by SDS-PAGE and immunoblotting using *E. coli* adsorbed horse anti-α-toxin antisera. (A) Lane 1, uninduced cell lysate of BL21pLysS(pRplc316); Lane 2, 3 h post-induced lysate of BL21pLysS(pRplc316); Lane 3, uninduced cell lysate of BL21pLysS(pRplc204); Lane 4, 3 h post-induced lysate of BL21pLysS(pRplc204); Lane 5, Seeblue protein marker; Lane 6, uninduced cell lysate of BL21pLysS(pRplc104); Lane 7, 3 h post-induced lysate of BL21pLysS(pRplc104). **(B)** Whole cell lysate of TOP10F'(pCplcInv3) 3 h post-induction with IPTG. Lane 1, TOP10F'(pCplcInv3); Lane 2, SeeBlue protein marker.

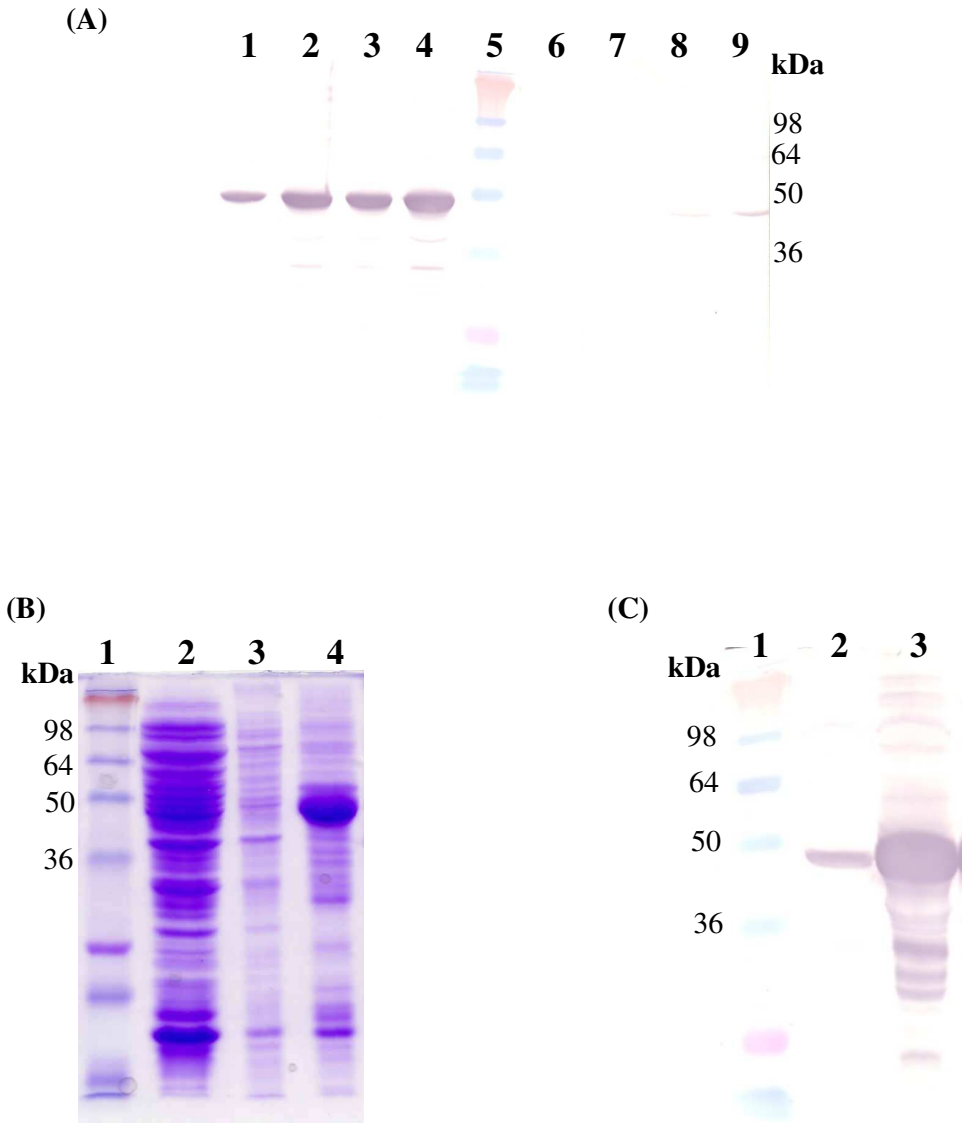


Figure 3.10: The localisation of plc316 expressed from BL21pLysS(pRplc316). (A) Immunoblot using anti-α-toxin horse polyclonal antisera to detect the expression of plc316 at 0, 1, 2 and 3 h post-induction in the insoluble matter (Lanes 1-4) or in the cytoplasm of the cell lysate (lanes 6-9). **(B)** Solubilisation of BL21pLysS(plc316) with; standard protein lysis buffer (Lane 2); deoxycholate (Lane 3); 5 M urea (Lane 4). **(C)** Immunoblot of BL21pLysS(plc316) with anti-α-toxin polyclonal antisera after solubilisation in 5 M urea. Lane 1, SeeBlue protein marker; Lane 2, deoxycholate in protein lysis buffer; Lane 3, 5 M urea.

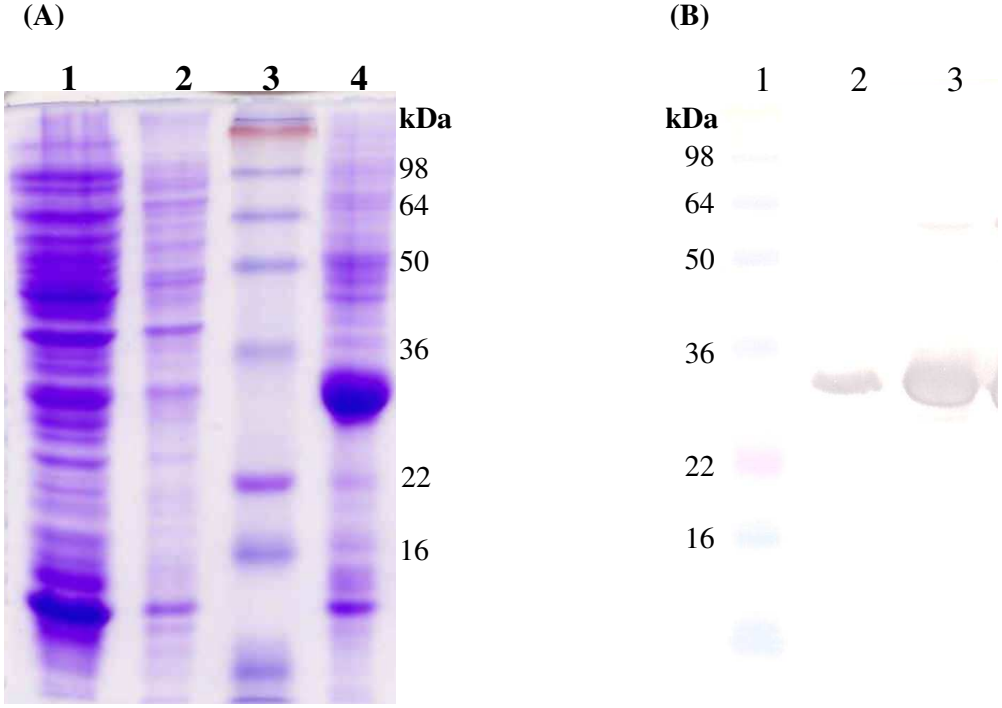


Figure 3.11: The localisation of plc204 expressed from BL21pLysS(pRplc204). (A) Solubilisation of BL21pLysS(plc204). Lane 1, standard protein lysis buffer; Lane 2, deoxycholate in protein lysis buffer; Lane 3, SeeBlue protein marker; Lane 4, 5 M urea. **(B)** Immunoblot probed with anti-α-toxin polyclonal antisera after solubilisation in 5 M urea. Lane 1, SeeBlue protein marker; Lane 2, deoxycholate in protein lysis buffer; Lane 3, 5 M urea.

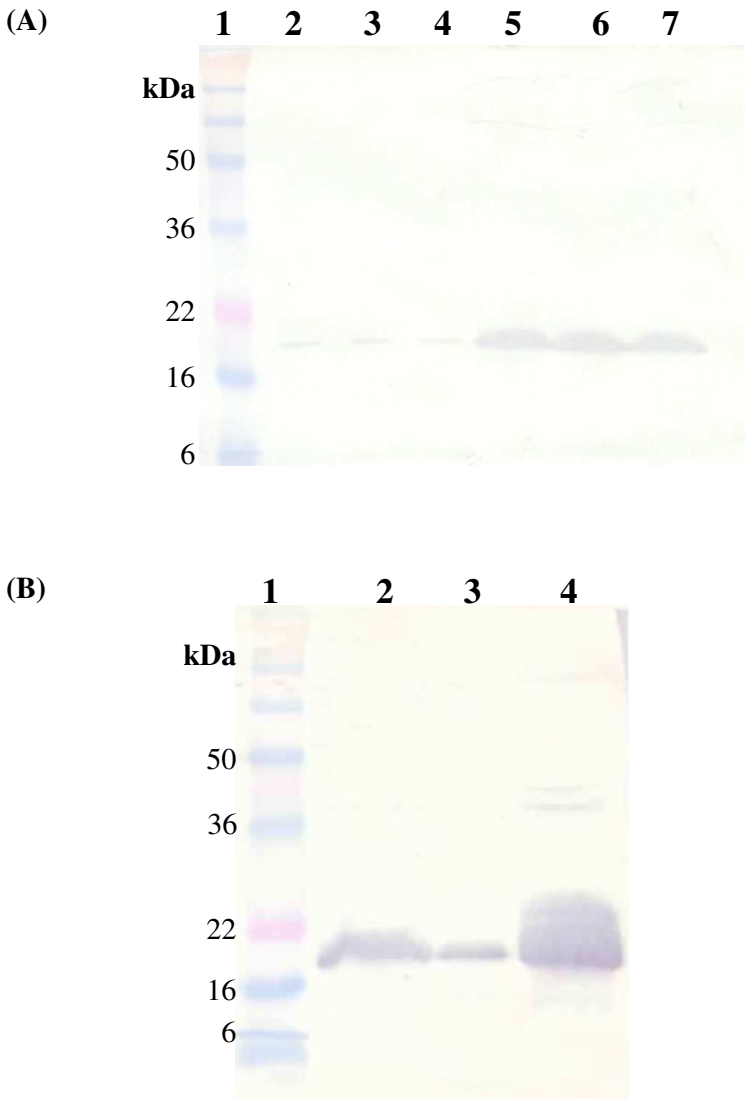


Figure 3.12: The localisation of plc104 expressed from BL21pLysS(pRplc104). The detection of plc104 using horse anti-α-toxin polyclonal antisera as the primary antibody in the **(A)** periplasm (Lanes 2-4) or cytoplasm (Lanes 5-7) and **(B)** cytoplasm (Lanes 2 & 3) or inclusion bodies solubilised in 5 M urea (Lane 4).

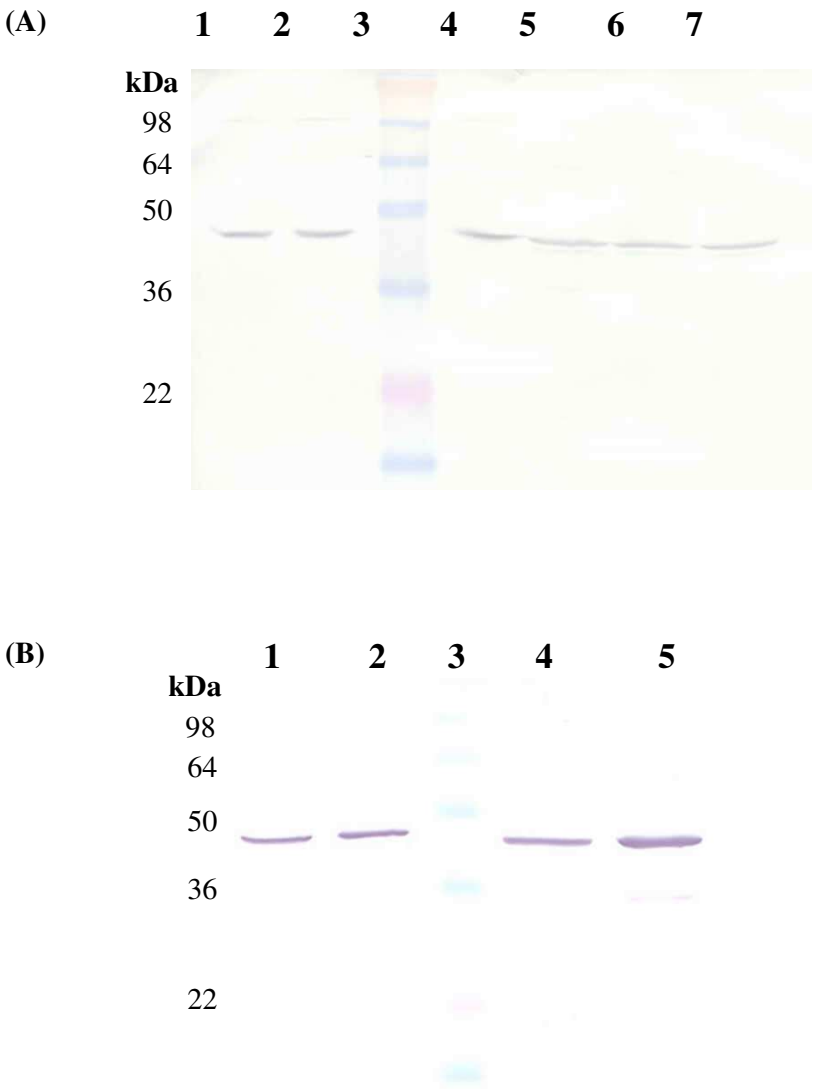


Figure 3.13: The localisation of plcInv3 from TOP10F'(pCplcInv3). **(A)** Immunoblot of periplasmic fractions (Lanes 1, 2 & 4), or cytoplasmic fractions (Lanes 5-7); Lane 3, SeeBlue protein marker; **(B)** Immunoblot of cytoplasmic and solubilised inclusion bodies of TOP10F'(pCplcInv3). Lane 1, cytoplasmic fraction pre-induction; Lane 2, insoluble fraction preinduction; Lane 3, SeeBlue protein marker; Lane 4, cytoplasmic fraction 1 h post-induction; Lane 5, insoluble fraction 1 h post-induction.

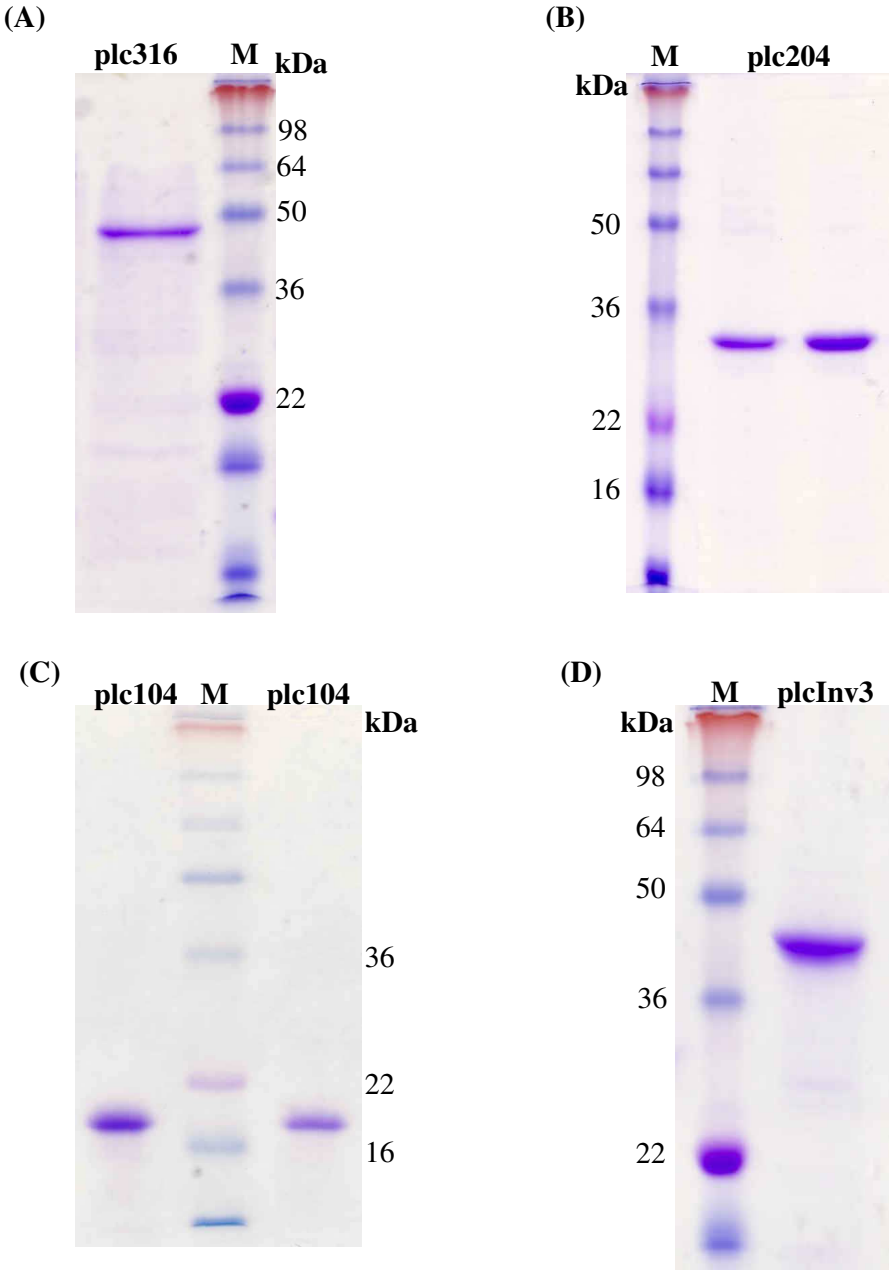


Figure 3.14: IMAC purification of α-toxin truncates. Cell lysates were loaded onto a nickel-charged 1 mL sepharose column, washed with 80 mM imidazole (60 mM when using TOP10F'(pCplcInv3) cell lysate) and eluted with 200 mM imidazole. About 1-2 µg were loaded onto a SDS-PAGE, separated and stained with Coomassie blue. **(A)** Protein plc316 **(B)** Protein plc204 **(C)** Protein plc104 **(D)** Protein plcInv3. SeeBlue protein marker (Labelled M) loaded with samples.

Once purified from the cell lysates, imidazole was removed from the proteins by buffer exchange with 25 mM Tris using Centricon centrifugal filter units. The proteins were concentrated using the same devices. Concentration of refolded plc316 and plc204 in this manner resulted in the formation of protein aggregates. This lead to a decrease in protein solubility by 12 and 7 fold in the case of plc316 and plc204 respectively. Protein plc104 was concentrated to a higher degree without protein aggregation, but upon storage in 30% glycerol, 25 mM Tris at -20ºC, the protein began to aggregate over time. Samples of plc104 prepared in SDS loading buffer and stored at -20ºC for a couple of weeks were found to form aggregates of varying sizes on subsequent SDS-PAGE separation (Figure 3.15). Three distinct bands were observed of approximately 56.6, 39.3 and 18.5 kDa in size. Protein plc3 and plcInv3 were soluble during concentration and buffer exchange and were stored at concentrations of up to 4 mg/mL.

3.3.3 Properties and activities of α-toxin (plc3) and truncated proteins

The activities of the cloned α-toxin were assessed with a number of assays. Haemolytic, cytotoxic, and phospholipase activities were assessed using the HLA, MTT and p-NPPC/EYA assays respectively. The assays were optimised to maximise the α-toxin activity and also used to assess the activities of the truncated α-toxin proteins.

The rate of activity of plc3 on the hydrolysis of egg yolk was determined to be 1.07 mm/h when a 100 μg/mL solution of plc3 was dispensed into wells of an EYA plate. When phospholipase activity of the truncated α-toxins on EYA was assessed, no activity was observed at concentrations up to 100 μg/mL of protein, or when clones carrying the vectors were streaked onto EYA plates and incubated overnight in the presence of IPTG.

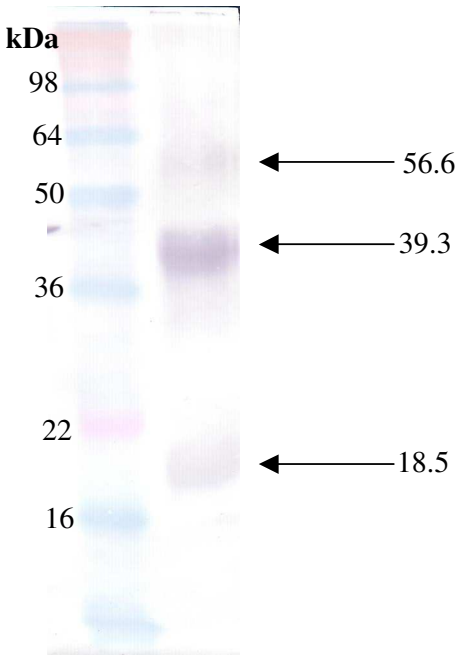


Figure 3.15: Oligomerisation of plc104 following heating (5 min, 100ºC) and cooling in SDS sample buffer.

The haemolytic activity of the α-toxin and truncated proteins was assessed with the haemolysin liquid assay (HLA). On optimisation of the assay using plc3, $ZnCl_2$ alone between 1 μM and 1 mM had no effect on haemolysis of sheep erythrocytes and 10 mM $CaCl_2$ alone resulted in minimal activity (Figure 3.16), whereas the addition of 10 μM $ZnCl_2$ to the reaction containing 10 mM $CaCl_2$ resulted in a 12-fold increase in activity. The combination of 10 μM $ZnCl_2$ and 10 mM $CaCl_2$ was taken as 100% activity and all other determinations were made from a comparison to this reference. An increase to 100 μM $ZnCl_2$ in the presence of 10 mM $CaCl_2$ again enhanced α-toxin activity by 2.6 times the reference activity. Zinc chloride at levels of 1 mM were found to be inhibitory to the assay, and when a control sample of erythrocytes prepared in TBS was lysed in water in the presence of 1 mM $ZnCl_2$ haemolysis was not observed.

All α-toxin truncates were unable to lyse erythrocytes when concentrations of up to 100 μg/mL were tested. Whereas as little as 0.6 μg/mL of α-toxin was able to cause the lysis of 50% (HU_{50}) of the erythrocytes in the assay.

Another assay used to determine the effects of calcium and zinc on α-toxin activity was the *p*-NPPC assay first developed by Kurioka and Matsuda (1976). In this assay, α-toxin in the presence of calcium was not effective at causing any *p*-NPPC hydrolysis at any concentration tested (between 0.1-10 mM) but zinc was most effective between 1 and 10 μM with α-toxin activity decreasing significantly ($p<0.05$) in the presence of 1 mM $ZnCl_2$ (Figure 3.17).

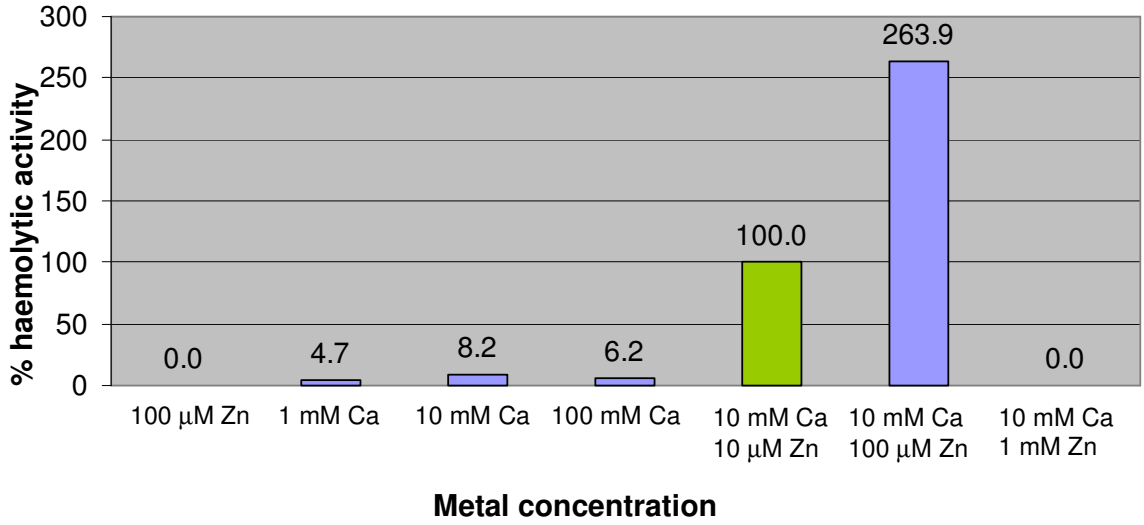


Figure 3.16: The effects of zinc and calcium ions on the haemolysin activity of the alpha-toxin (plc3). The percent haemolytic activity was defined as a percentage of the ratio of the test HU_{50} over HU_{50} of plc3 in the presence of 10 mM $CaCl_2$ and 10 μM $ZnCl_2$ (shaded green). HU_{50} was defined as the reciprocal of the toxin dilution required to lyse 50% of the sheep red blood cells under the experimental conditions. The starting dilution for all reactions was 50 μg/mL.

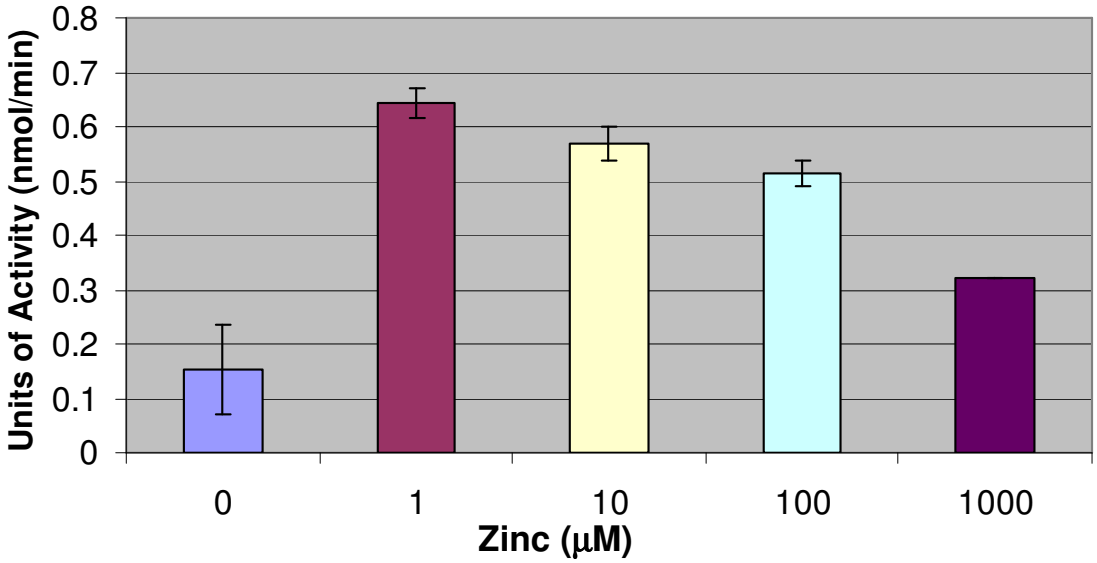


Figure 3.17: The effect of zinc on the rate of *p*-NPPC hydrolysis by α-toxin (plc3). A reaction mix of 200 μL was prepared containing 25 mM Tris, 0.15 M NaCl, 60% glycerol, 20 mM *p*-NPPC, and 50 μg/mL plc3. Samples were incubated for an hour at 37ºC and the units of activity were calculated as the number of nmol of nitrophenol liberated per minute under the experimental conditions.

The cytotoxic effects of the α-toxin and its mutants were assessed via the MTT tissue culture assay. The TCD_{50} for α-toxin was found to be 48.2 ng (or 0.24 μg/mL), whereas all α-toxin truncated proteins (plcInv3, plc316, plc204 and plc104) were unable to cause any cytotoxic effects in the CHO cell line when tested at concentrations up to 30 μg/mL.

3.3.4 Further studies of plc104 and plcInv3

The two soluble proteins, plcInv3 and plc104, were further characterised. Their calcium and membrane binding abilities were examined in a number of assays. Gel mobility assays of the α-toxin in the presence of calcium showed that both plc3 and plcInv3 bound calcium as observed by their reduced mobility through an 8.5% native PAGE gel (Figure 3.18). Mobility of plc104 was harder to examine due to its small size and on a 12.5% native PAGE gel, no differential migration was observed.

The binding of the toxins to erythrocytic membranes was analysed by two methods, a qualitative method involving α-toxin binding to a suspension of erythrocytic membranes in the presence or absence of Ca^{2+}, followed by thorough washing and SDS-PAGE separation of the membrane suspension, and a quantitative method whereby the erythrocytic membranes were fixed onto a plastic support, followed by the addition of α-toxin, plcInv3 or plc104, in the presence or absence of calcium ions and the quantitation of binding assessed using an ELISA.

The results of the qualitative calcium binding assay revealed that binding of plc3 and plcInv3 was enhanced in the presence of Ca^{2+}, whereas plc104 was isolated in the wash solution (Figure 3.19). Its presence associated with erythrocytic membranes could not be seen in the presence or absence of Ca^{2+}.

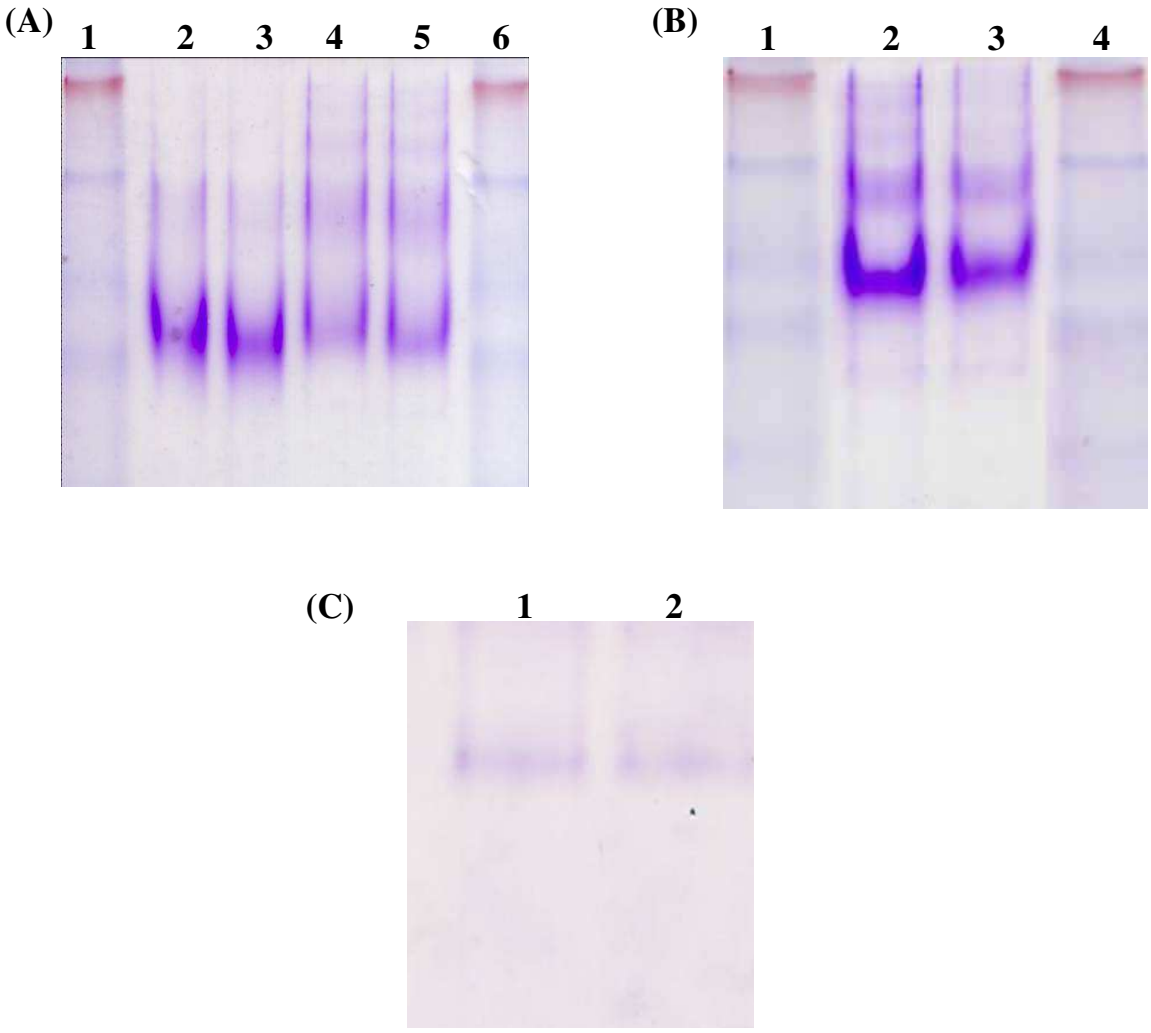


Figure 3.18: Gel mobility of α-toxin (plc3), plcInv3 and plc104 in the presence or absence of Ca^{2+}. **(A)** Native PAGE of α-toxin. Lane 1 & 6, SeeBlue protein marker; Lane 2 & 4, α-toxin + 20 mM $CaCl_2$ + 10 mM EDTA; Lane 3 & 5, α-toxin + 10 mM EDTA. **(B)** Native PAGE of plcInv3. Lane 1 & 4, SeeBlue protein marker; Lane 2, PlcInv3+ 10 mM EDTA; Lane 3, PlcInv3 + 20 mM $CaCl_2$ + 10 mM EDTA **(C)** Native PAGE of plc104. Lane 1, Plc104 + 10 mM EDTA; Lane 2, Plc104 + 20 mM $CaCl_2$ + 10 mM EDTA.

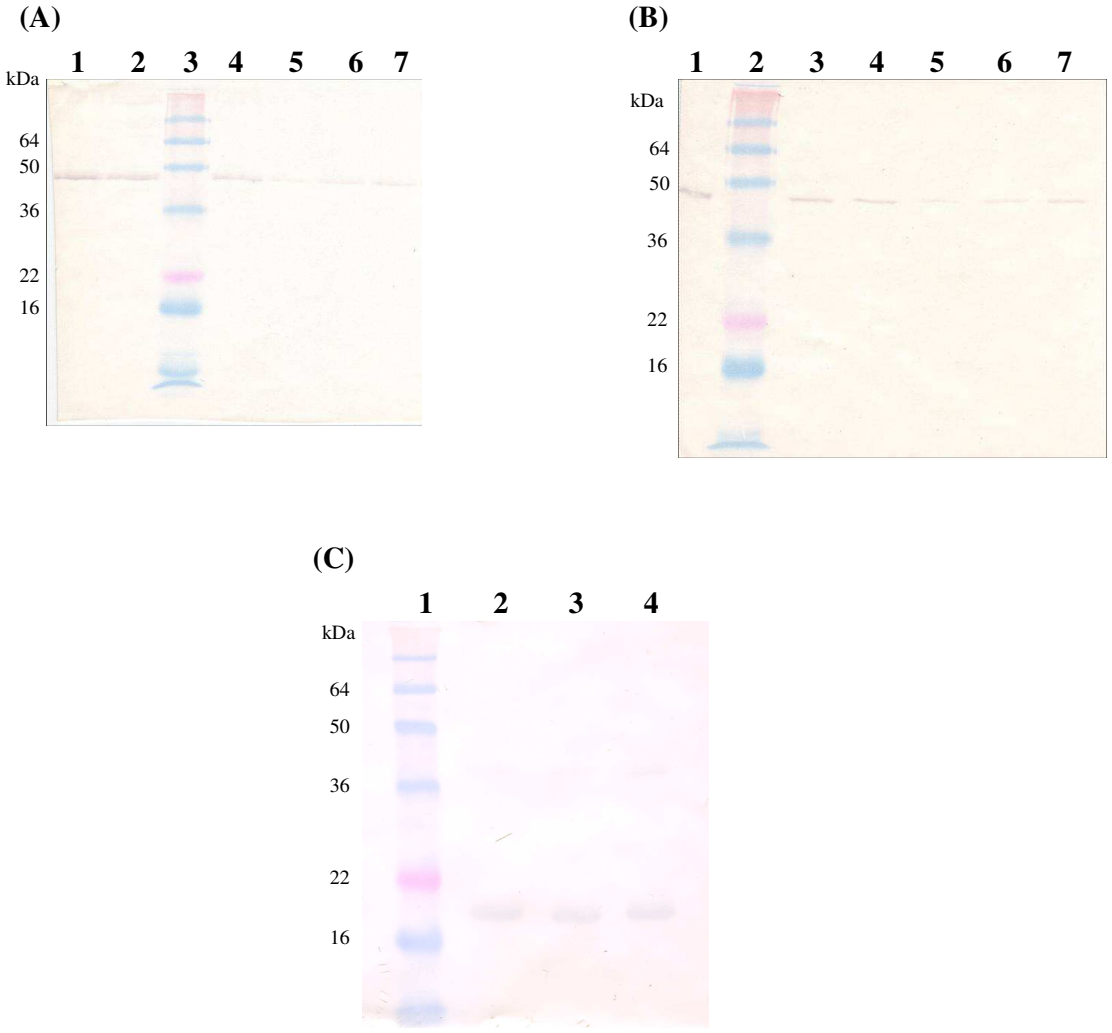


Figure 3.19: Erythrocyte membrane qualitative binding assay of α-toxin, plcInv3 and plc104. Proteins were incubated in an erythrocytic membrane suspension in the presence or absence of Ca^{2+} followed by thorough washing of the suspension and analysis by SDS-PAGE. Following transfer to a nitrocellulose membrane bound proteins were detected using horse anti-α-toxin polyclonal antisera. **(A)** Plc3 in the pelleted membranes. Lanes 1, 2, and 4, erythrocytic membranes with plc3 and Ca^{2+}; Lanes 5, 6, and 7, membranes and plc3 without Ca^{2+}; Lane 3, SeeBlue protein marker. **(B)** PlcInv3 in the pelleted membranes. Lanes 1, 3, and 4, membranes and plcInv3 with Ca^{2+}; Lanes 5, 6, and 7, membranes and plcInv3 without Ca^{2+}; Lane 2, SeeBlue protein marker. **(C)** Plc104 in the supernatant of pelleted membranes. Lanes 2, 3 and 4, supernatant of membranes with Ca^{2+}; Lane 1, SeeBlue protein marker.

To confirm the binding of plcInv3 to membranes and the absence of binding of plc104, the quantitative ELISA was done. The results of the assay are displayed in Figure 3.20. Binding of plc3 and plcInv3 to membranes was significantly enhanced ($p<0.05$) in the presence of Ca^{2+}, whereas binding of plc104 to membranes was not enhanced in the presence of Ca^{2+}.

The results of membrane binding between protein samples could not be directly compared as polyclonal, and not monoclonal antibodies were used in the ELISA, which would result in misconstrued data for protein plc104 as this protein likely lacks a number of epitopes recognised by the horse polyclonal anti-α-toxin antisera. Therefore the difference in binding within a protein sample in the presence and absence of Ca^{2+} was determined, and the difference obtained compared to the other protein samples. Binding properties of plc3 and plcInv3 did not differ significantly, but the binding of plc104 was significantly less ($p<0.05$) compared to both plc3 and plcInv3 suggesting that this protein has lost its ability to bind to erythrocytic membranes.

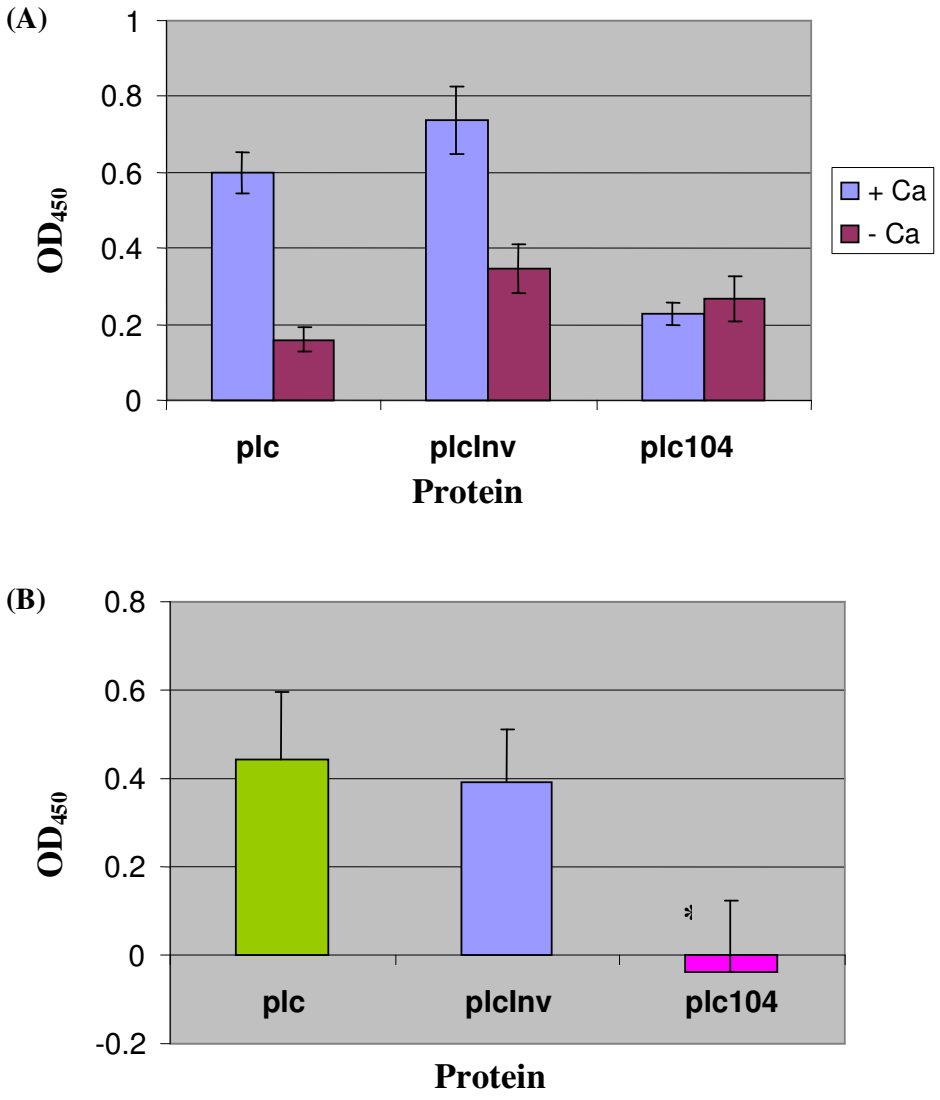


Figure 3.20: Quantitative erythrocyte-membrane binding assay. Binding of proteins to membranes was assessed in the presence and absence of Ca^{2+} and detected using an ELISA assay with mouse polyclonal anti-α-toxin antisera. **(A)** Binding of proteins to membranes with and without Ca^{2+}. **(B)** Calculated binding differences within each sample for direct comparison between proteins. The OD_{450} result from within a sample containing Ca^{2+} was subtracted from the OD_{450} reading of the same sample without Ca^{2}. Plc3 and plcInv3 were not significantly different in their binding capacities. *Binding to erythrocytic membranes by plc104 was significantly lower than both plc3 and plcInv3 ($p<0.05$).

3.4 Discussion

Cloning of *plc* has previously been achieved by a number of researchers (Leslie *et al.*, 1989; Saint-Joanis *et al.*, 1989; Titball *et al.*, 1989; Tso and Siebel, 1989). In this study, the *plc* of *C. perfringens* was cloned and expressed in *E. coli*, as a first step in creating non-toxic variants, for potential use as vaccines. Expression and purification of the complete *plc* was aided by the presence of a 6xHis-tag located either at the N- (10plc2f) or C- (plc3) terminus of the α-toxin.

The first of these proteins, 10plc2f, with the N-His tag, was isolated in inclusion bodies within the cytoplasm of the host. This could suggest that the over-expression of α-toxin led to misfolding of the protein, but based on the literature of the structure of α-toxin, it is more likely that the integrity of the amino-terminus was compromised by the addition of the amino-terminus His-tag (including the 36 vector coded aa's) that replaced the α-toxin signal sequence.

The binding of Zn^{2+} to the active site of α-toxin involves a number of residues scattered throughout different regions of the toxin (Nagahama *et al.*, 1995; Guillouard *et al.*, 1996; Naylor *et al.*, 1998). One of these residues, tryptophan 1 (Trp-1), is responsible for liganding one zinc molecule in the active site (Naylor *et al.*, 1998) and in the mature toxin is buried within the molecule protected from attack by aminopeptidases (Nagahama *et al.*, 1994). The spatial position and not the nature of the side chain of Trp-1 is crucial for binding of the toxin to the zinc ion (Nagahama *et al.*, 2000) and this spatial arrangement could have been disrupted by the addition of 36 vector encoded amino acids (which also included the 6xHis) to the N-terminus of Trp-1. Trp-1 is localised to a hydrophobic pocket within the active site (Guillouard *et al.*, 1996), and hindrance of Trp-1 to this position may have exposed the

hydrophobic active site to solvent leading to the formation of insoluble aggregates. Nagahama *et al*. (2000) examined the effects of the addition of two amino acid residues preceding Trp-1 (glycine and isoleucine) and although the protein remained soluble, a marked reduction in phospholipase C activities and the abolishment of haemolytic activities was observed. In the current study, the addition of 36 instead of 2 aa's may have lead to a more pronounced effect on toxin structure and stability.

As can occur during high levels of expression of heterologous proteins in *E. coli*, a small proportion of the toxin remained soluble during expression (Fahnert *et al.*, 2004) and was isolated in the cytoplasm. This fraction was no longer able to bind to the IMAC column even at relatively low imidazole concentrations. Lack of binding to the column may have been due to cleavage of the N-His tag by proteases. As soluble protein expression from vector pR10plc2f was very low, further characterisation of α-toxin was obtained from protein plc3.

Expression of plc3 was successfully achieved from *E. coli* TOP10F'cells harbouring pCplc3, a plasmid based on the TA cloning vector. This vector is not an ideal vector for protein expression, as its *lac*Z promoter is not as efficient as other systems at over-expressing recombinant proteins (Amann *et al.*, 1983; de Boer *et al.*, 1983). On analysis of the cloned *plc* sequence, a consensus Shine-Dalgarno sequence (S-D) such as GGAGG, GGAG, GAGG, AGGA 8-12 bp upstream of the initiation of translation codon was not present (Appendix 4). The lack of an S-D suggests that ribosomal RNA (rRNA) was able to bind directly to the *plc* initiation codon and begin translation. This has been noted in a number of research articles, and what is becoming clear is that the S-D sequence is not obligatory for the successful expression of genes (Nakamoto, 2006), rather, accessibility of rRNA to the initiation codon is more important than the presence of a S-D sequence (Van Etten and Janssen, 1998). The low level of purification (3 mg/L) of plc3 achieved in this study may have resulted from the low

level of translation achieved, on the other hand, the low level of translation may have prevented the formation of inclusion bodies as is so commonly seen in high-level expression systems where the dynamics of protein folding cannot keep up with the transcription/translation efficiency of the cell (Ellis, 1997). The incorporation of a S-D sequence 8-12 bp upstream of the *plc* initiation codon could be used to determine if its presence alters expression levels.

3.4.1 Alpha-toxin activities

The haemolytic activity of α-toxin provides a strong indication of its toxicity in living organisms (Jepson *et al.*, 1999) therefore a haemolytic assay was established to determine the presence of residual toxicity of the truncated α-toxins. On optimisation of this assay using plc3, Ca^{2+} in the form of $CaCl_2$ at a level of 10 mM was required for efficient lysis of sheep red blood cells in the presence of 100 μM Zn^{2+}. Calcium is required at much higher levels than Zn^{2+} as it binds to α-toxin with a very low affinity (Guillouard *et al.*, 1997; Naylor *et al.*, 1999). On the other hand excess Zn^{2+} at levels of 1 mM was inhibitory to the assay. On closer examination of this inhibitory effect, it was found that 1 mM Zn^{2+} inhibited the osmotic lysis of a 1% suspension of sheep red blood cells prepared in TBS, therefore this inhibition was unrelated to the α-toxin activity and was a consequence of reversible binding of zinc to the erythrocyte cells (Avigad and Bernheimer, 1976). Osmotic lysis of erythrocytes is not suppressed by high levels of Zn^{2+} when only in the presence of water (Avigad and Bernheimer, 1976) but the presence of Tris (>10 mM) precipitates released hemoglobin (Takeda *et al.*, 1977) projecting the illusion of inhibited lysis.

As inhibition of haemolysis by α-toxin in the presence of Zn^{2+} was found to be unrelated to the inhibition of α-toxin activity, hydrolysis of *p*-NPPC was used to assess the effects of Zn^{2+}

and Ca^{2+}on the α-toxin (Kurioka and Matsuda, 1976). Calcium ions had no effect on the hydrolysis of *p*-NPPC as would be expected as Ca^{2+} is only necessary for the binding of α-toxin to membrane packed and not mono-dispersed phospholipids (Titball *et al.*, 1991). Zinc was very effective at increasing the rate of *p*-NPPC hydrolysis between 1 and 10 μM, and there was a significant decrease in the rate of *p*-NPPC hydrolysis when 1 mM Zn^{2+} was added to the assay (as $ZnCl_2$).

It appears from the results that Zn^{2+} are stimulatory at low concentrations but inhibitory at high concentrations in the *p*-NPPC assay. A possible explanation for this involves the activation of the active site. Two molecules of zinc are tightly bound to the active site of α-toxin (Krug and Kent, 1984; Nagahama *et al.*, 1995; Nagahama and Sakurai, 1996). A third molecule of zinc is also regularly found liganded to the toxin, but this site is a loosely bound interchangeable region that can accommodate the binding of other divalent cations such as cobalt or manganese (Nagahama *et al.*, 1995; Nagahama and Sakurai, 1996). This third loosely bound cation is necessary for activity of the toxin (Nagahama *et al.*, 1995; Nagahama *et al.*, 1997). Closed and open forms of α-toxin have been observed using crystallography and each contains two and three atoms of zinc respectively (Naylor *et al.*, 1998; Titball *et al.*, 2000; Eaton *et al.*, 2002). Catalysis of substrate may require continual exchange between the two forms for efficient hydrolysis, and the presence of excess zinc may hinder the efficient catalysis of *p*-NPPC (a mode of direct inhibition) by saturating the cocatalytic sites.

Similar findings of zinc inhibition at high Zn^{2+} concentrations have been reported for some mono-zinc metalloenzymes such as thermolysin, carboxypeptidase and endopeptidase (Holmquist and Vallee, 1974; Kerr and Kenny, 1974; Mallya and Van Wart, 1989; Gomez-Ortiz *et al.*, 1997).

Further enzyme studies are required to determine the kinetics of the inhibition observed in the *p*-NPPC assay.

3.4.2 Characterising deletions

Truncation of the *plc* was based on two strategies. Deletion of bases from the 5' end (N-terminal end of translated protein) and the truncation of areas within the *plc* known to be important for the catalytic activity of the expressed mature enzyme.

Deletion of the first 54 or 166 amino acids of α-toxin resulted in the production of proteins plc316 and plc204 respectively. Expression of both these proteins led to inclusion body formation within the cytoplasm of BL21pLysS cells. pRSETA is a strong expression vector and it is likely that inclusion bodies formed due to the high expression levels of the vector. Inclusion body formation of recombinant proteins from high expression vector systems is very common and occurs due to a highly unfavourable protein-folding environment caused by the high macromolecular crowding of recombinant protein (Ellis, 1997; Sorensen and Mortensen, 2005). The level of translation far exceeds the process of protein folding leading to aggregation of intermediates with surface exposed hydrophobic patches normally buried within the interior of the protein molecule (Ellis, 1997; Fahnert *et al.*, 2004). Methods to decrease inclusion body formation include reducing the growth temperature of the recombinant bacterium and reducing the induction process, both of which were attempted in this study but to no avail. The co-expression of chaperones has had some success in increasing the rate of soluble protein present in a cell, and this could be further examined in the future (Ellis, 1997; Sorensen and Mortensen, 2005).

The formation of inclusion bodies does have its benefits, such as ease of purification, high level of production, and resistance to protease cleavage (Fahnert *et al.*, 2004) and both

proteins were isolated from inclusion bodies and solubilised in urea to a relatively high level. The disadvantage of isolating and purifying proteins from inclusion bodies is that a relatively low yield is obtained following refolding of protein in non-denaturing solutions. Refolding of urea-solubilised plc316 and plc204 was attempted whilst bound to a Ni^{2+} charged resin column. Refolding of the proteins by this manner was successful but over time both proteins began to aggregate whilst in storage and during buffer exchange with the Centricon centrifugal devices. The cause for the re-aggregation of both proteins is uncertain, but it may be that the deletions of α-helices from the N-terminus of α-toxin which are also involved in the stability of the catalytic domain of the toxin led to the destabilisation of the toxin moiety, with the exposure of the hydrophobic pocket, eventually leading to aggregation of monomers with exposed hydrophobic residues (Nagahama *et al.*, 1995; Guillouard *et al.*, 1996; Nagahama *et al.*, 1997; Naylor *et al.*, 1998).

The third truncate, plc104 is composed of the C-terminal β-barrel domain. Truncates composed entirely of the C-terminal domain ($cpa_{247-370}$) have previously been shown to retain their ability to bind membranes, but lack any detectable enzymic activities (Titball *et al.*, 1993; Nagahama *et al.*, 1998; Naylor *et al.*, 1998). Truncate plc104 is smaller than these previously expressed truncates in that it is only composed of 7 of the 8 β-sheets, as the first β-sheet comprising aa's 256-265 has been deleted (Titball *et al.*, 1993).

One major difference between plc104 and the previously cloned and characterised C-terminal domain is the inability of plc104 to bind calcium. Gel mobility assays of plc104 exhibited no observable differential migration in the presence and absence of Ca^{2+}. Calcium ions bind to α-toxin with a low affinity, but in association with phospholipids, this affinity can be enhanced 1000-fold (Rizo and Sudhof, 1998; Naylor *et al.*, 1999), therefore a number of membrane binding assays were attempted.

Membrane binding of α-toxin can only occur in the presence of Ca^{2+} , and the presence or absence of Ca^{2+} in membrane binding assays with plc104 did not lead to enhanced membrane binding abilities suggesting that plc104 has lost its ability to bind Ca^{2+} and phospholipid membranes. Lack of membrane binding can be attributed to the deletion of the first β-sheet encountered in the C-terminal domain, β1. The β-sandwich sheets of the C-domain are structured to resemble a scaffold which allows the emergence of variable loops at the top and bottom of the domain (Rizo and Sudhof, 1998). These loops are involved in calcium binding and determine the functional specificity of the domain (Naylor *et al.*, 1998; Rizo and Sudhof, 1998; Naylor *et al.*, 1999). Residues required for calcium binding are located on loop-β1/β2 (located between sheets β1 and β2), loop-β3/β4 (between sheets β3 and β4) and loop-β5/β6 (between sheets β5 and β6) (Guillouard *et al.*, 1997; Naylor *et al.*, 1998; Naylor *et al.*, 1999; Walker *et al.*, 2000; Jepson *et al.*, 2001) and all loops are found in a cluster at one end of the structure, held together by the β-sheets. Loop-β1/β2 is involved in the liganding of two Ca^{2+}, the highest affinity calcium binding site Ca1, and the lower affinity Ca3 site (Guillouard *et al.*, 1997; Naylor *et al.*, 1998; Naylor *et al.*, 1999; Jepson *et al.*, 2001). The deletion of β1 in the development of plc104 may have disorganised the structure of this loop, creating a linear rather than a loop topology, leading to the disruption of calcium liganding at the two sites, possibly three if the tertiary structure of truncate has been compromised. Probe studies with monoclonal antibodies could be used to determine the degree of structural change in plc104.

One other consequence of deleting β1 may have been the destabilisation of the protein. Polymers of plc104 were observed after native and SDS-PAGE analysis of the sample (Figure 3.15). The three bands observed on the gel equate to dimers and trimers of the plc104. Deletion of β1 may expose non-polar residues of β2 which include methionine, phenylalanine

and isoleucine to solvent (Naylor *et al.*, 1998). Over time, clustering of plc104 molecules may occur in these regions, leading to the oligomer formation observed after gel analysis.

Despite the lack of membrane binding and slight insolubility developed over time, plc104 retained epitopes recognised by polyclonal antisera against the α-toxin, whilst remaining non-haemolytic and non-cytotoxic.

The final truncate, plcInv3 was created from an internal deletion of *plc* previously cloned into vector pCR2.1. Unlike plc316 and plc204, where the deletion of the N-terminus resulted in misfolding of the toxin, a deletion of an internal region of the N-terminus important in the functioning of the activity of the α-toxin did not result in an insoluble mass, and retained its structural integrity. Evidence to support this includes the recognition of plcInv3 by polyclonal antisera against the α-toxin, the ability to bind calcium and migrate differentially on a native gel and the ability to bind erythrocytes in the presence of excess Ca^{2+}. All these features were present in the absence of enzymic activity and hence toxicity. The use of monoclonal antibodies to confirm the integrity of epitopes could also be done to further assess the conformational differences between plc3 and plcInv3.

PlcInv3 lacks aa residues 56-75 of the mature α-toxin. This region forms part of a mobile loop encompassing aa residues 55-90 and is important for communicating between the N- and C-terminal domains (Eaton *et al.*, 2002). In the closed form of the α-toxin this loop region folds into an α-helix not present in the open form. Aspartate (Asp)-71 is hydrogen bonded to lysine-201, and a salt bridge is formed between the side chains of aspartate-75 and arginie-99. These interactions hold the loop in place away from the membrane binding plane and stabilise the newly created α-helix composed of aa's 73-80 (Eaton *et al.*, 2002) in order to obstruct the active site. On binding of the C-terminal domain to membranes a conformational shift

triggers the collapse of the α-helix, the reformation of the complete loop, which then exposes the active site to the membrane. The alignment and insertion of the active site cleft into the phospholipid membrane occurs via polar residues followed by the cleavage of the phospholipid tail (Nagahama and Sakurai, 1996; Eaton *et al.*, 2002; Nagahama *et al.*, 2006). Upon cleavage, α-toxin is released, and returns to its closed conformation (Eaton *et al.*, 2002).

Protein plcInv3 lacks part of this flexible loop, and the shortened loop present may be unable to alternate between the open and closed forms of the toxin rendering the toxin inactive. The absence of the essential residues, tyrosine-57, tyrosine-65 , phenylalanine-69, threonine-74 (highlighted in Figure 3.8) would prevent binding and insertion of α-toxin into biological membranes (Guillouard *et al.*, 1996; Nagahama and Sakurai, 1996; Nagahama *et al.*, 1997; Nagahama *et al.*, 2006). The deletion of histidine-68 and aspartate-56 would also alter the binding efficiency of at least 2 zinc molecules (Nagahama *et al.*, 1995; Nagahama *et al.*, 1997; Eaton *et al.*, 2002; Nagahama *et al.*, 2006).

Indirect evidence that plcInv3 binds less (if any at all) Zn^{2+} is seen during the washing steps of IMAC. The reduced ability of plcInv3 to bind Ni^{2+} on the IMAC reflects the reduction in the number of zinc binding domains present. The exact number of Zn^{2+} ions able to bind plcInv3 was not calculated, and could be determined in the future with the use of atomic absorption spectrophotometry.

As plcInv3 mimics the binding properties of α-toxin, it may have potential use as a probe to further determine the nature of the α-toxin receptor on biological membranes, without the lytic and toxic side-effects.

One interesting feature of TOP10F' cells harbouring vector pCplcInv3 was the difference in growth rate observed when compared to pCplc3, its parent vector. Apart from a 60 bp deletion within *plc*, cells harbouring pCplc3 and pCplcInv3 were identical, yet the latter transformant constantly took almost twice as long to grow to mid-log phase, and produced much less protein than pCplc3 harbouring cells (1.5 mg/mL vs. 3.5 mg/mL).

Usually an impaired growth rate is observed in response to high levels of heterologous protein expression within *E. coli* (Hoffmann and Rinas, 2004). This is a direct response to the high-energy requirements induced by recombinant protein synthesis, leading to the synthesis of stress proteins and elevated respiration rates (Hoffmann and Rinas, 2004). High levels of plcInv3 expression were not observed following induction, and strict control of expression was not maintained due to the expression system used, therefore the reduced growth rate observed with cells harbouring plcInv3 can not be attributed to high levels of protein expression, but is more likely due to the characteristic properties of protein plcInv3 taking effect during leaky expression.

PlcInv3 may be much more unstable than plc3, leading to increased protease activity by the *E. coli* host, in effect leading to enhanced respiration and the observed decrease in growth rate (Schmidt *et al.*, 1999; Rozkov and Enfors, 2004). The complete degradation of plcInv3 did not occur as the protein was evident during induction and growth phases of bacteria, unlike the protein degradation observed by Schmidt *et al*., (1999) where protein was not evident in an immunoblot after an hour induction with IPTG. Future studies such as pulse labelling experiments could be used to show whether the majority of plcInv3 is degraded upon its expression (Viaplana *et al.*, 1997). Another cause for the up-regulation of stress-responses leading to increased respiration could be due to the interaction of plcInv3 with the host cell membranes. During its translocation into the periplasm, plcInv3 may interact with the

membranes in a way that more energy is required for maintenance of the membrane potential of the host, leading to increased protease activity and degradation of the protein (Viaplana *et al.*, 1997).

Reduced plcInv3 expression levels could also be caused at the translational level by the formation of secondary mRNA structures that obstruct rRNA binding to the initiation codon of plcInv3. This would reduce translation efficiency resulting in lowered plcInv3 expression levels (Wikstrom *et al.*, 1992).

Analysis of the host gene expression pattern via its transcriptome or proteome could be used to identify changes in synthesis of host cell proteins during the recombinant protein production phase (Hoffmann and Rinas, 2004; Schweder and Hecker, 2004). This knowledge could then lead to determining what and where this recombinant expression-specific host stress response is occurring.

In conclusion, an active form of α-toxin engineered with a C-His tag was created and successfully purified from *E. coli*. The α-toxin, named plc3 retained all of its enzymic properties and was recognised by polyclonal antisera against the α-toxin. A number of α-toxin mutants were also successfully cloned and purified from *E. coli*. The level of solubility of the concentrated proteins varied somewhat, but none of them retained any haemolytic, cytotoxic or phospholipase C activities whilst still being recognised by polyclonal antisera. The immunogenicity of these proteins will be further assessed in animal studies as a first step in determining their potential as vaccine candidates.

Chapter 4

Immunogenicity of α-toxin mutants and their potential as protective vaccines

4.1 Introduction

The α-toxin of *C. perfringens* is a major contributing factor in the development of necrotic enteritis (NE) in avian species (Al-Sheikhly and Truscott, 1977b; Al-Sheikhly and Truscott, 1977c; Fukata *et al.*, 1988; Lovland *et al.*, 2003; McCourt *et al.*, 2005). It acts by arresting the migration of neutrophils and platelets to the site of infection by promoting their aggregation adjacent to but not at the infectious site (Bunting *et al.*, 1997; Bryant *et al.*, 2000a; Bryant *et al.*, 2000b), and reduces sodium dependant glucose absorption from the gut contributing to the diarrhoea observed in cases of NE (Rehman *et al.*, 2006). Antibodies developed against the cells of *C. perfringens* alone do not afford protection against a *C. perfringens* infection (Traub *et al.*, 1991) and antibodies directed towards the toxins, in particular α-toxin, are necessary (Traub *et al.*, 1991). Therefore prevention of NE and other diseases of *C. perfringens* would require vaccination against the exoenzymes it produces.

Retrospective immunological analysis of commercial poultry flocks reveals a significant association between the occurrence of *C. perfringens* associated hepatitis (CPH) or sub-clinical NE at slaughter and the increased presence of a specific antibody response to α-toxin (Lovland *et al.*, 2003). The presence of higher anti-α-toxin antibodies also results in less mortality (Heier *et al.*, 2001). Protection from the lethal effects of the α-toxin have also been demonstrated in birds administered anti-α-toxin antibodies prior to challenge with *C. perfringens* (Fukata *et al.*, 1988). These studies demonstrate the ability of birds to develop

an antitoxin response against the α-toxin and the presence of these anti-α-toxin antibodies results in a decreased mortality rate. The development of vaccines targeting the α-toxin could reduce the risks of NE in chickens, and save thousands of dollars in revenue lost from this disease (van der Sluis, 2000).

Experimental reproduction of NE in chickens is quite complex and involves a number of predisposing factors, which even when present cannot guarantee the reproduction of the disease (explored in Chapter 1.6.3.3) (Cowen *et al.*, 1987; Elwinger *et al.*, 1992; Kaldhusdal *et al.*, 1999), therefore, another model to determine the effectiveness of an α-toxin vaccine is necessary. One such model is the gas-gangrene model in mice. Studies have shown that α-toxin is the major contributing factor in the disease, and therefore this model could be used to examine the efficacy of recombinant α-toxin proteins as potential vaccine candidates (Ninomiya *et al.*, 1994; Awad *et al.*, 1995; Awad *et al.*, 2000) .

Gas-gangrene is characterised by the production of gas within the tissues as a result of *C. perfringens* metabolism, accompanied by the production of numerous toxins (Shimizu *et al.*, 2002a). The toxins act by reducing the oxygen tension in the infected area, allowing the proliferation of *C. perfringens*, and also as a spreading factor, degrading tissues and allowing for the migration of the bacterium throughout the body (Shimizu *et al.*, 2002a). The α-toxin causes intravascular coagulation (Barzaghi *et al.*, 1988; Ninomiya *et al.*, 1994; Bunting *et al.*, 1997; Ellemor *et al.*, 1999), reduced perfusion and the development of thrombosis, all of which lead to a reduced oxygen flow to the infected area (Bryant *et al.*, 2000a; Bryant *et al.*, 2000b). Cyanosis ensues followed by the necrosis of deoxygenated tissues, leaving the area swollen, necrotic and black. The synergistic activity of the toxins decreases the viscosity of ground substances in connective tissue and allows the migration of *C. perfringens* into deeper

tissues, where the continuous production of α-toxin eventually leads to shock and death (Stevens and Bryant, 1997; Shimizu *et al.*, 2002a).

A number of truncated α-toxin proteins have been developed (Chapter 3) and their residual toxin activity determined to be non-existent making them ideal candidates for immunisation and challenge trials in mice. This chapter explores their potential for use as vaccines targeted against the α-toxin of *C. perfringens* using a Murine gas-gangrene model.

4.2 Materials and methods

4.2.1 Protein preparation

Proteins plc3 (α-toxin), plcInv3, plc316, plc204 and plc104 developed in Chapter 3 (Table 4.1) were purified to homogeneity as described in Chapter 3.2.4. Tris was removed from protein samples by buffer exchange with PBS using Centricon centrifugal devices and the proteins were concentrated using the same devices.

4.2.2 Toxoid preparation

Formaldehyde (Sigma-Aldrich) was added directly to a solution of plc3 (prepared in PBS at a concentration of 1 mg/mL) to give a final concentration of 0.4% (v/v) formaldehyde. The mixture was incubated at 37ºC overnight on a rotating platform. Toxin inactivation was confirmed by the addition of 40 µL of formaldehyde treated sample to wells bored into a Nagler and SBA plate in the presence of 10 mM $CaCl_2$. Residual formaldehyde was removed by buffer exchange with PBS in Centricon centrifugal filters and the protein content of the solution was reconfirmed by the Bradford method, as protein losses during buffer exchange could be quite high.

4.2.3 Adjuvant preparation

Prior to vaccinations, inactivated plc3 (toxoid) and truncated α-toxin proteins plcInv3, plc316, plc204 and plc104 were prepared in an equal volume of Freund's incomplete adjuvant (FIA) (Sigma) following the protocol outlined in Ausubel, (1994).

Table 4.1: Description of the α-toxin truncated proteins used in this study as potential vaccine antigens for the development of immune responses targeted against the α-toxin.

Protein	Description	Location of His tag
plc3	The complete α-toxin of *C. perfringens*.	C-terminus
plcInv3	Plc3 with an internal deletion of aa residues 56-75 which encompass two zinc binding residues essential for phospholipase C activity.	C-terminus
plc316	The α-toxin with the deletion of the first 55 aa's of the mature toxin (deletion of the first three α-helices of the N-terminal domain).	N-terminus
plc204	The α-toxin with a deletion of the first 166 aa's of the mature toxin (deletion of the first 7 α-helices of the N-terminal domain).	N-terminus
plc104	The α-toxin with the deletion of the first 266 aa's of the mature toxin (deletion of the complete N-terminal α-helical domain and the first β-sheet of the C-terminal domain)	N-terminus

In brief, an emulsion was prepared by drawing up and expelling equivalent volumes of protein and FIA through a 21 gauge needle in a 3 mL glass syringe until the fluidic suspension increased markedly in viscosity and was no longer able to disperse when a drop was placed in a glass beaker of cold water. Vaccines prepared in adjuvant were used within 6 h of preparation.

4.2.4 Mouse immunogenicity trial

Six groups, each containing seven female BALB/c mice (6-8 week old) were vaccinated via the intraperitoneal route using a 1 mL syringe and 21 gauge needle with 50 μL of protein:adjuvant emulsion. The groups consisted of a PBS:FIA control, toxoid(plc3):FIA, plcInv3:FIA, plc316:FIA, plc204:FIA and plc104:FIA. Mice were vaccinated on days 1, 15, and 37 with 5, 25, and 50 μg of protein respectively. Mice were bled prior to each vaccination and thrice more following the final vaccination at intervals between 14-20 days.

4.2.4.1 ELISA

Antibody responses of vaccinated mice were measured using the ELISA as outlined in Chapter 2.8.8 with a few modifications. Wells were coated with 3 μg/mL of purified α-toxin (plc3). Doubling dilutions of sera obtained from vaccinated mice were used as the primary antibody, and goat anti mouse IgG-HRP (diluted 1:5000 in PBST/1% skim milk) was used as the secondary conjugated antibody. Following the addition of the substrate TMB, plates were incubated for 15 min before the addition of 50 μL of a 1 M solution of sulphuric acid. The endpoint was determined as the dilution at which the optical density (OD) at a wavelength of 450 (OD_{450}), was 3 times the background level (determined as an approximate OD of 0.2).

4.2.5 Antibody purification

One millilitre gravity flow columns were packed with a combination of 250 µL of Protein G Sepharose 4 Fast Flow (Amersham Biosciences) and 250 µL of Protein A Sepharose 4 Fast Flow (Amersham Biosciences). The columns were washed with PBS prior to loading of serum samples. Sera obtained from mice within the same group were pooled and diluted by half with 2 x PBS. Diluted sera were loaded on the column, unbound protein was washed out with 10 column volumes (CV) of PBS and antibodies were eluted with 0.1 M glycine, pH 3.0. Eluted antibody was collected in 500 µL lots into 1 mL tubes containing 50 µL 1 M Tris, pH 9.0 to neutralise the effects of the acidic elution buffer. The sepharose was regenerated by washing with an additional 3-4 CV of elution buffer followed by re-equilibration with 3-4 CV of PBS. Strongly bound hydrophobic proteins, lipoproteins and lipid were removed by cleaning in place procedures which included washing the sepharose column with 2 CV of 50 mM NaOH in 1.0 M NaCl and 2 CV of 70 % ethanol. Five CV of PBS was added between the two cleaning steps.

Removal of glycine from immunoglobulins isolated by this procedure was achieved by buffer exchange with 25 mM Tris, pH 7.4, in VivaSpin 0.5 mL 10 kDa cut-off concentrators (Satorius group) according to the manufacturer's instructions. Protein content of the immunoglobulins was determined using the Bradford assay (Chapter 2.8.2.1)

4.2.6 Toxin neutralisation assays

The MTT cytotoxicity assay (Chapter 3.2.5.4) was used to test the α-toxin neutralising abilities of the antisera obtained from vaccinated mice. Antiserum (50 µL lots containing 0.15 mg/mL to 20 mg/mL protein) was heat inactivated at 56ºC for 30 min prior to use and then mixed with 50 µL of α-toxin (20 µg/mL). After 30 min at 37ºC the 100 µL samples

were dispensed into wells of a 96-well plate previously seeded with 2 x 10^4 CHO cells. The MTT assay was performed as described in Chapter 3.2.5.4.

The assay was unsuccessful (see Section 4.3.2) and therefore the neutralising effects of the antiserum was assessed using the *p*-NPPC assay described in Chapter 3.2.5.5. Serum (40-160 μg) inactivated at 56ºC for 30 min or purified polyclonal antibodies (49-114 ng) were mixed with 2 μg (for serum samples) or 4 μg (for purified immunoglobulins) of α-toxin (plc3), in a final volume of 20 μL, for 30 min at 37ºC. Following the incubation, the toxin activity was assessed with the *p*-NPPC assay in a fluoSTAR OPTIMA plate reader set to 37ºC with a shaking interval of 1 minute prior to each 5 min reading. Samples were repeated in triplicate for the unpurified sera and quadruplicate for the purified immunoglobulins. One unit was equivalent to the hydrolysis of 1 nmol of *p*-NPPC in 1 min under the specified conditions.

4.2.7 Mouse challenge trials

4.2.7.1 Vaccination regime

Groups of seven BALB/c female mice (6-8 week old) were vaccinated on days 1, 14 and 28 with 14, 25, and 50 μg of a protein:FIA emulsion in a total volume of 100 μL via the intraperitoneal route. Proteins plcInv3 and plc104 (developed in Chapter 3) were used as the test vaccines, and plc3 toxoid was used as a positive control. A fourth group consisting of vaccination with an unrelated bacterium (*S.* Typhimurium *aro*A$^-$) was used as the negative control throughout this study. The control group was previously vaccinated with 10^9 attenuated *S.* Typhimurium (STMI) cells via the oral route using a gavage needle (Alderton *et al.*, 1991). Mice were challenged with either α-toxin or *C. perfringens* two weeks following the final vaccination as described below.

4.2.7.2 Toxin challenge

Mice vaccinated as in Section 4.2.7.1 were administered five times the lethal dose (5 μg) of purified α-toxin (plc3) via the intraperitoneal route in a total volume of 100 μL (Williamson and Titball, 1993; Schoepe *et al.*, 1997; Jepson *et al.*, 1999). Mice were observed continuously for the first 4 h, hourly for the next 6, and twice daily thereafter. When three or more major signs of toxicity were observed (Table 4.2) mice were sacrificed by cervical dislocation. In most circumstances many of these clinical signs were not observed, therefore when mice reached recumbency they were sacrificed immediately and time of death was recorded for a comparison to other groups.

4.2.7.3 *C. perfringens* challenge

C. perfringens strain 60, a field isolate obtained from a chicken with clinical signs of necrotic enteritis was used as the challenge organism (Table 2.1). One percent (v/v) of an overnight culture of *C. perfringens* 60 grown in thioglycollate broth was used to inoculate bottles containing 500 mL of freshly prepared and autoclaved thioglycollate broth. To avoid oxygen exposure to the bacterium, a lid with a rubber septum was used to seal the bottle, and the 1% inoculum was injected into the bottles with a syringe. The rubber septum was first sterilised with 80% (v/v) ethanol. The culture was grown for 4 h at which time approximately 5 x 10^8 cfu/mL was reached.

Table 4.2: Signs of toxicity observed in mice following intraperitoneal injection of 5 μg of α-toxin (plc3).

	Acute clinical signs
1	Increased respiration (panting)
2	Impaired eye and pedal reflexes
3	Pallid mucous membranes or ears (paleness)
4	Recumbency (animals lay limp)

The culture was pelleted by centrifugation in sterilised 250 mL autoclave bottles at 10,000 x *g* for 10 min, the supernatant discarded and the pellet washed in sterile saline, repelleted as above and resuspended in a final volume of 3 mL saline (including bacterial volume). Mice received 50 µL containing approximately 4 x 10^9 cells by an intramuscular injection to the left thigh. The right thigh was left unchallenged as an internal control. Mice were monitored closely for the first 4 h, and every 6-8 h for the following 72 h. Severity of gangrene in the mice was determined using an index calculating the severity of infection of challenged mice (Table 4.3). Mice were examined and scored according to Table 4.3 every 8 h for the first 48 h post-challenge and the examination of mice was completed by an individual unaware of the vaccination or challenge status of the mice.

4.2.8 Statistical analysis

All statistical analysis was calculated using the program SPSS v13.0. The results of all ELISA assays for the detection of anti-α-toxin antibodies were transformed by log2 in order to produce a data set with an equal variance (Dytham, 2003). The transformed data of the immunogenicity trial (Section 4.2.4) were analysed by an ANOVA and included the Tukey's HSD (Tukey, 1953) post-hoc analysis to determine differences between groups of vaccines.

Independant Student's *t*-tests were used to analyse the results of the *p*-NPPC assays.

Log2 transformed titres obtained from challenge trials (Section 4.2.7) were analysed using the independent Student's *t*-test ("STUDENT", 1908) and data obtained from mice challenged with α-toxin were analysed using the nonparametric Mann-Whitney U rank test (Mann and Whitney, 1947) as data could not be transformed to obtain a homogenous variance (Dytham, 2003).

Table 4.3: The index used to score mice infected with *C. perfringens*. The reduced perfusion of vessels and migration of *C. perfringens* is inadvertently observed via the swelling (oedema of infected thigh and gas production by *C. perfringens*) and blackening (cyanosis) of the injection site, and eventual gas-gangrene development towards the foot pad.

Score	Clinical signs
0	Swelling/limping of infected thigh
1	Cyanosis (blackening) at the injection site
2	Spread of cyanosis to the thigh surrounding injection site
3	Spreading of cyanosis to ankle/Swelling of foot pad
4	Blackening of complete foot pad (sacrifice)/mouse found dead

4.3 Results

4.3.1 Immunogenicity of vaccines

The mouse immunogenicity trials were undertaken to determine the ability of each of the α-toxin truncates to produce antisera directed towards the α-toxin. A control vaccination of PBS alone was included to confirm the antibody response developed was specific to the vaccine in question. The antibody responses of mice to the different proteins over an 11 week period are presented in Figure 4.1. Levels of IgG were not seen prior to the second vaccination with any of the vaccines, including the plc3 toxoid. Overall, the antibody responses to the vaccines peaked between days 46 and 60, with a decline thereafter. Antibody responses at day 60 are shown in Figure 4.2. The immunogenicity of the different vaccines can be separated into two groups, those that stimulate a high antibody production and low antibody production. Proteins plc316 and plc204, showed the lowest production of anti-α-toxin IgG at a significantly reduced level ($p<0.01$) when compared to all other vaccines in an ANOVA (with Tukey's HSD post-hoc analysis). This reduced antibody detection was observed at all other time-points as well.

4.3.2 Neutralising activities of antibodies

Complement inactivated mouse serum from mice immunised with the various α-toxin truncated proteins (Table 4.1) was assessed for its α-toxin neutralising effects on the CHO cell line using the MTT cytotoxicity assay. Neutralisation of α-toxin activity was unsuccessful when unpurified sera was used in this assay.

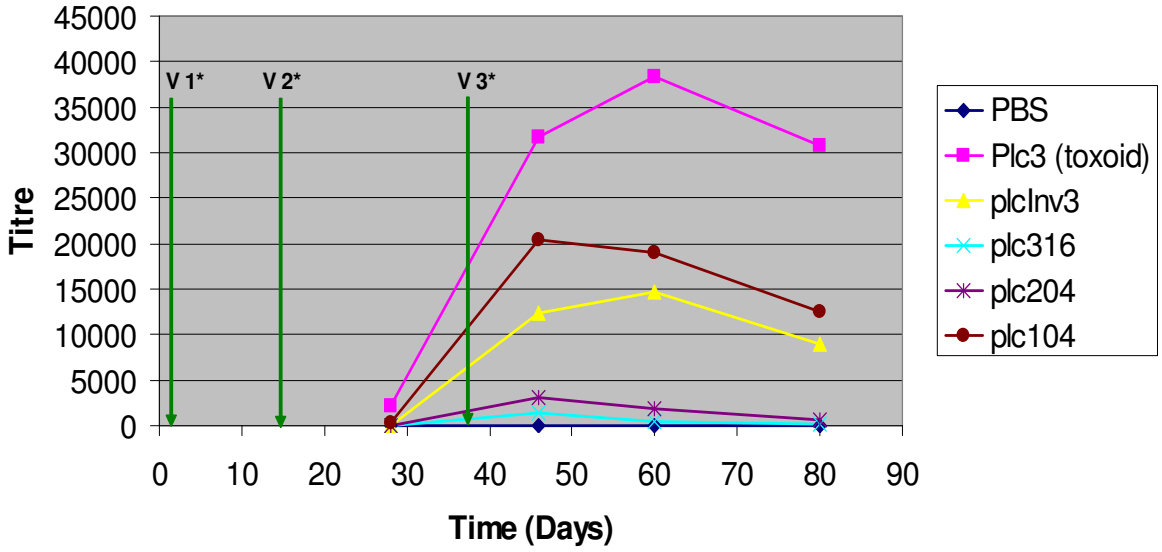


Figure 4.1: IgG responses of BALB/c mice vaccinated with a number of different alpha-toxin truncated vaccines. Sera obtained from mice were assessed with an ELISA for the production of anti-α-toxin specific IgG. The titres are representative of the reciprocal dilution of the end-point (given as an OD_{450} of 0.2). Green arrows indicate days of vaccination (Day 1, Day 15, and Day 37). Antibodies were not measurable before day 30.

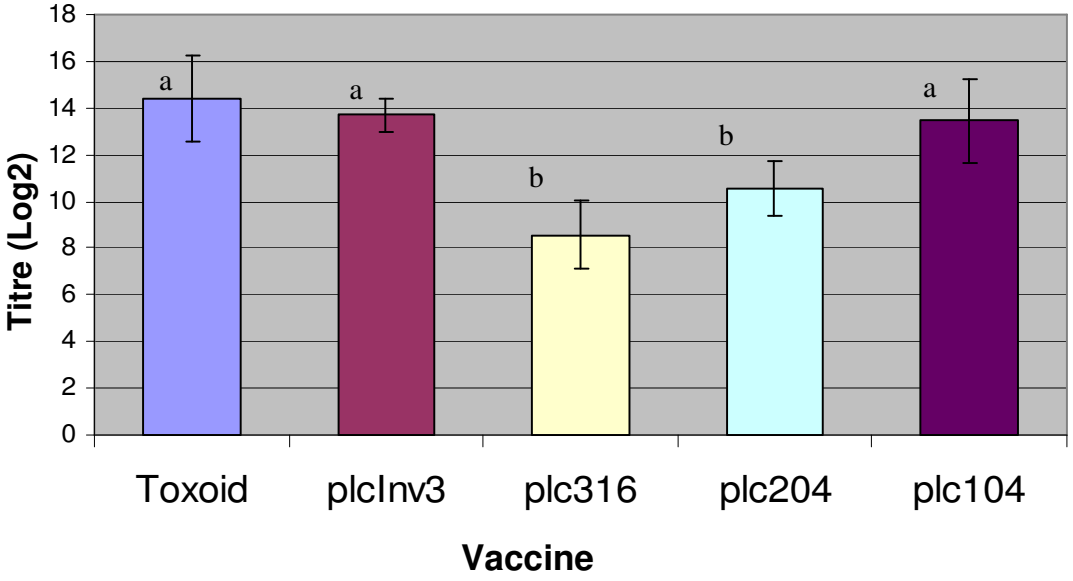


Figure 4.2: Day 60 IgG ELISA results of immunogenicity trial. The titre was calculated as the reciprocal (log2) dilution of the end-point (given as an OD_{450} of 0.2). Bars with different letters are significantly different from each other ($p<0.05$) when analysed using an ANOVA with Tukey's HSD post-hoc analysis. Each histogram is the average result and range of titres from 6 mice in a group. The control (PBS vaccinated mice) as per Figure 4.1 showed no measurable response and is omitted from the graph.

The presence of serum components (such as hormones, proteins etc.) enhanced the growth of the cell-line making it impossible to detect any neutralising affects of antiserum from vaccinated mice, therefore, the *p*-NPPC assay was trialled as this assay is specifically designed to measure the phospholipase C activity of the α-toxin (Kurioka and Matsuda, 1976).

When heat inactivated serum from immunised mice was used to neutralise α-toxin in the *p*-NPPC assay, a change in the rate of hydrolysis was observed, but in the opposite direction from that expected. The presence of serum components in the *p*-NPPC assay significantly enhanced ($p<0.05$) the rate of hydrolysis of *p*-NPPC by α-toxin whether serum from vaccinated mice or control serum was used (Figure 4.3). This effect was seen irrespective of whether a smaller (40 μg) or larger (160 μg) serum sample was tested (Figure 4.4).

As serum components were influencing the assay, serum from mice in the same groups were pooled, and the immunoglobulins (Ig's) isolated using Protein A/G columns. Low levels of Ig's were obtained and assuming the Ig's were mostly composed of IgG of MW 146,000 Da, the total amount of Ig's obtained was between 150 and 342 ng. The molar ratio of purified Ig to alpha-toxin (plc3) used in the *p*-NPPC assay was was; 1:109 for plcInv vaccinated mice; 1:254 for plc104 vaccinated mice; and 1:152 from toxoid vaccinated mice. Even though there were not enough immunoglobulins to saturate and completely neutralise the α-toxin, each sample significantly reduced ($p<0.001$) the rate of *p*-NPPC hydrolysis over the period tested (Figure 4.5) indicating the presence of neutralising immunoglobulins.

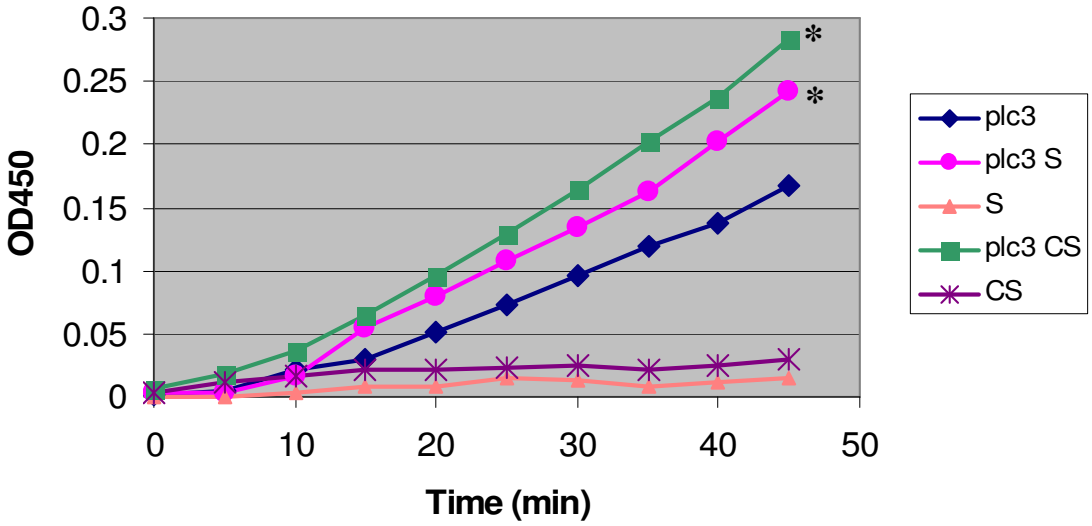


Figure 4.3: The influence of heat inactivated antisera on α-toxin (plc3) in the hydrolysis of *p*-NPPC. Release of the chromogenic product nitrophenol was detected by an increase in absorbance at OD_{450}. Plc3 (2 μg) was incubated alone (plc3), in the presence of serum obtained from mice previously immunised with toxoid (plc3 S), or incubated with serum from mice immunised with PBS (plc3 CS). The symbols 'S' and 'CS' are serum from plc3 and PBS immunised mice respectively in the absence of plc3 (control samples). *A significant enhancement of the hydrolysis of *p*-NPPC in the presence of serum was observed ($p<0.05$) when compared to plc3 alone.

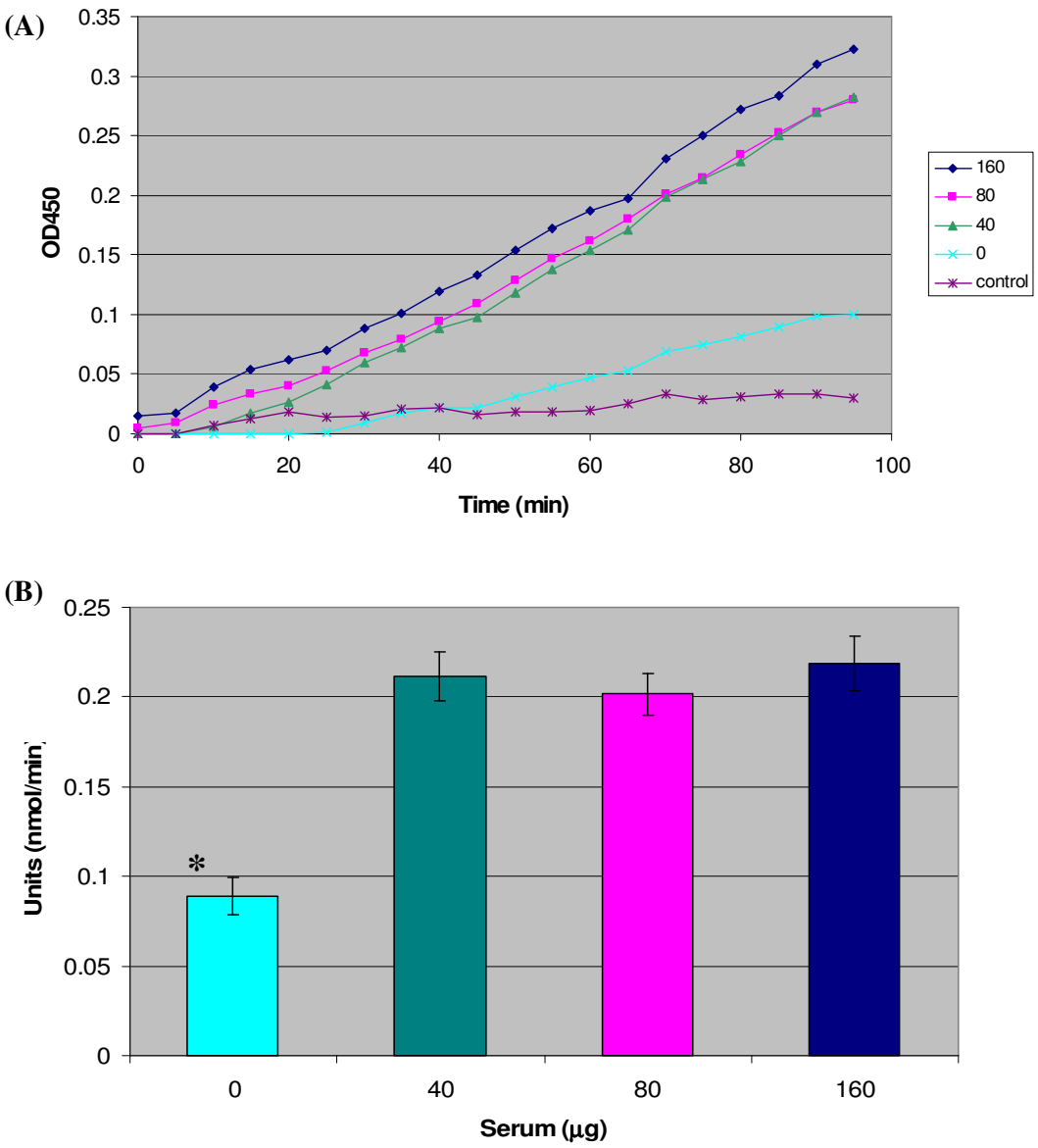


Figure 4.4: The influence of various concentrations of heat inactivated antisera on the activity of α-toxin (plc3) in the *p*-NPPC assay. (A) The rate of *p*-nitrophenol liberation by plc3 as determined by an increase in the absorbance at OD_{450}. Plc3 (2 µg) was incubated alone (0), or in the presence of 40, 80 and 160 µg of serum obtained from mice immunised with toxoid. The "control" consisted of 160 µg of serum from plc3 immunised mice, in the absence of plc3. **(B)** A bar graph representation of the calculated rate of *p*-NPPC hydrolysis in nmol/min of each sample in (A). Results display means and standard errors of triplicate data. *A significant reduction in the rate of *p*-NPPC hydrolysis was observed in the absence of sera ($p<0.05$)

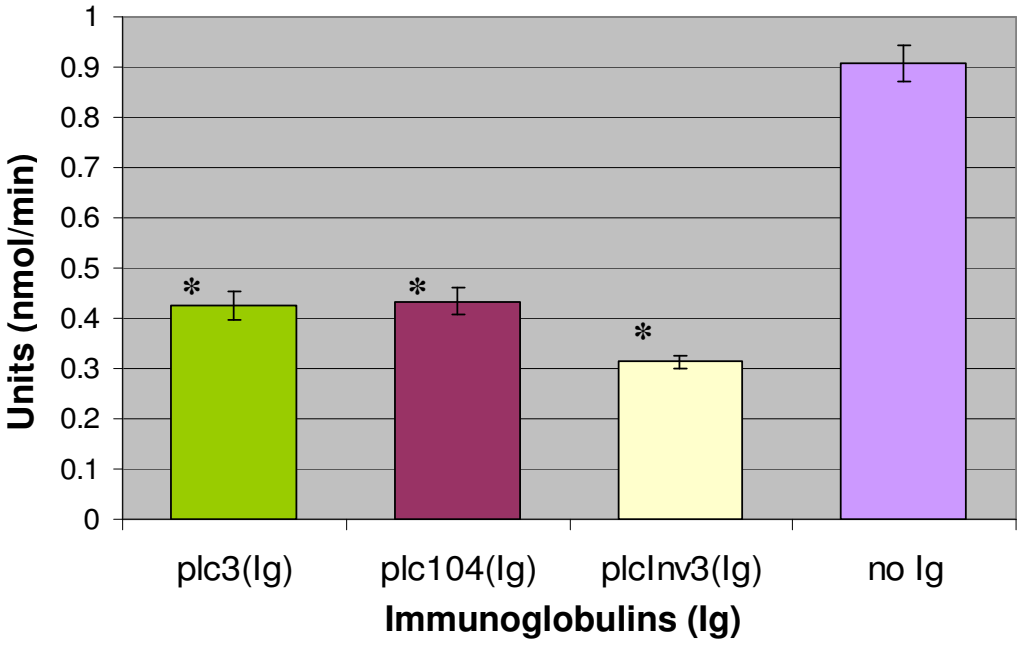


Figure 4.5: The neutralisation of the phospholipase C activity of α-toxin by purified immunoglobulins (Ig). Alpha-toxin (plc3) (4 μg) was incubated with Ig isolated from mice immunised with: plc3 toxoid (82 ng), plc104 (49 ng), and plcInv3 (114 ng) in a final volume of 20 μL for 30 min at 37ºC, followed by the *p*-NPPC reaction. Rates of *p*-NPPC hydrolysis was calculated (nmol/min) from quadruplicate samples and are displayed as means and standard errors. Molar ratio of Ig to plc3 in all samples tested was very low^ (1:270 for plc104 Ig) therefore complete inhibition of plc3 activity was not achieved. Although there was a highly significant reduction * ($p<0.001$) in *p*-NPPC hydrolysis when all Ig isolated from plc3 toxoid, plcInv3 and plc104 vaccinated mice incubated with plc3 was compared to plc3 activity in the absence of Ig (labelled "no Ig").

^ With the assumption that the majority of the Ig's present are IgG (estimated size of 146 kDa).

4.3.3 Challenge of mice

The results of the immunogenicity trials outlined in Section 4.2.4 indicated that optimum IgG responses were observed 1-3 weeks after final vaccinations (Fig 4.1) and antibody levels started to stabilise or decline thereafter, therefore mice were challenged with α-toxin or *C. perfringens* 2-weeks following the final vaccination (Day 43).

4.3.3.1 Toxin challenge

Prior to challenge, serum was collected from vaccinated mice and was screened to confirm the presence of anti-α-toxin antibodies in an ELISA. All mice vaccinated with plcInv3, plc104 and toxoid developed significant antibody responses against the α-toxin, whereas anti-α-toxin antibodies from sham-immunised mice were undetectable in the ELISA. Mice vaccinated with plcInv3 and plc104 had significantly lower antibody responses when compared to the plc3 toxoid vaccinated group ($p<0.01$) (Figure 4.6).

Signs of toxicity in control mice injected with 5 mouse lethal doses (MLD) (5 μg) of α-toxin (plc3) were observed within 2 h of the injection with a mean time to death of 2.1 h (Table 4.4). All plc3 toxoid vaccinated mice survived the α-toxin challenge without any observable signs of toxicity. Plc104 and plcInv3 vaccinated mice developed signs of acute toxicity and died within 3-4 h of challenge. One mouse in each group survived the challenge without any observable signs of acute toxicity (Table 4.4).

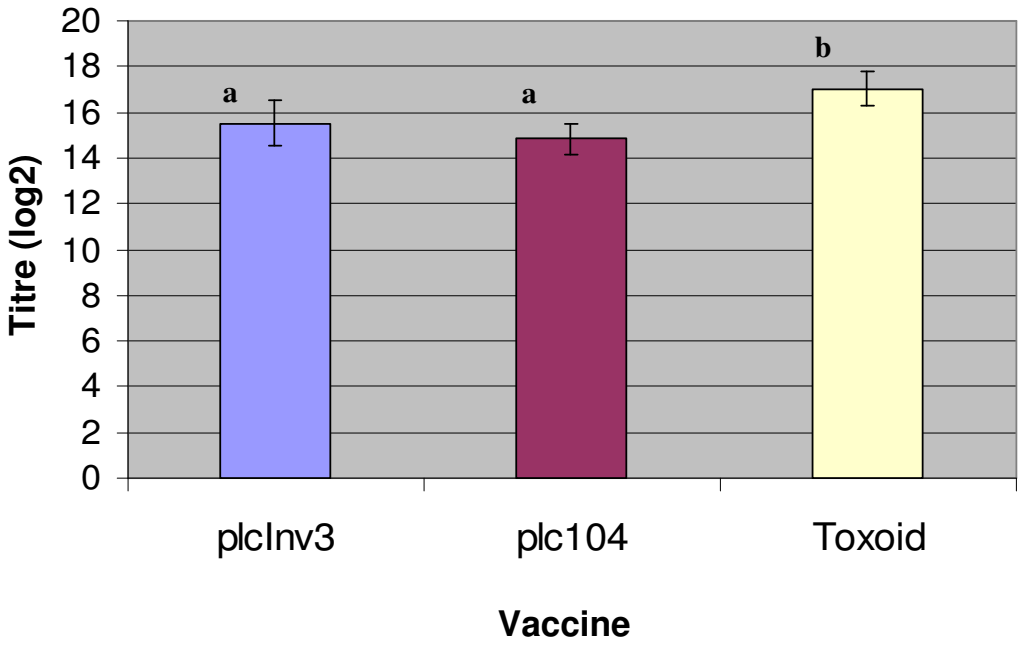


Figure 4.6: Anti-α-toxin IgG responses of mice following vaccination with plcInv3, plc104 or plc3 toxoid prior to α-toxin challenge. Mice were vaccinated 3 times over a 6 week period. Each histogram is the average result and range of titres from 7 mice in a group. The titre was calculated as the reciprocal (log2) dilution of the end-point (determined as an OD_{450} of 0.2). Bars with different letters were significantly different ($p<0.01$).

Table 4.4: Survival of vaccinated mice following a lethal i.p injection of 5 μg of α-toxin (plc3). Values with different superscript were significantly different from each other ($p<0.05$).

Vaccine	No. mice survived/total	Mean time to death (h +/- SD)
plcInv3	1/7	3.083 +/- 0.576[a]
plc104	1/7	3.747 +/- 0.295[a]
toxoid	7/7	-
control	0/7	2.108 +/- 0.36[b]

The Levene's test for homogeneity of variance showed that the variance in time to death of groups of mice was not homogenous, and one group, the plcInv3 vaccinated group was not normally distributed, therefore the non-parametric Mann-Whitney test was used to determine the differences in time to death of vaccinated mice compared to the control group (mice vaccinated with PBS in FIA). Plc104 and plcInv3 vaccinated mice survived for a significantly longer time when compared to control mice ($p<0.05$). Differences in time of death between plcInv3 and plc104 did not differ significantly ($p>0.05$).

4.3.3.2 *C. perfringens* challenge

Prior to challenge serum collected from vaccinated mice was screened to confirm the presence of anti-α-toxin antibodies in an ELISA. All mice vaccinated with plcInv3, plc104 and toxoid developed significant antibody responses against the α-toxin, without any residual signs of toxicity, whereas anti-α-toxin antibodies from sham immunised mice were undetectable in the ELISA. Mice vaccinated with plc104 produced an anti-α-toxin response significantly lower than plcInv3 and plc3 toxoid immunised mice ($p<0.01$) (Figure 4.7).

The index of gross pathology of *C. perfringens* infected mice used in this trial was based on previous challenge trials (Awad *et al.*, 2000; Awad *et al.*, 2001) and is outlined in Table 4.3. All mice including plc3 toxoid vaccinated mice developed localised swelling and limping in the injected thigh muscle, therefore these clinical signs were not included in the scoring system.

Retrospective plate counting of the challenge inoculum on SBA revealed that mice were only given 4.05×10^6 *C. perfringens* cells as opposed to the calculated 5×10^8 cells.

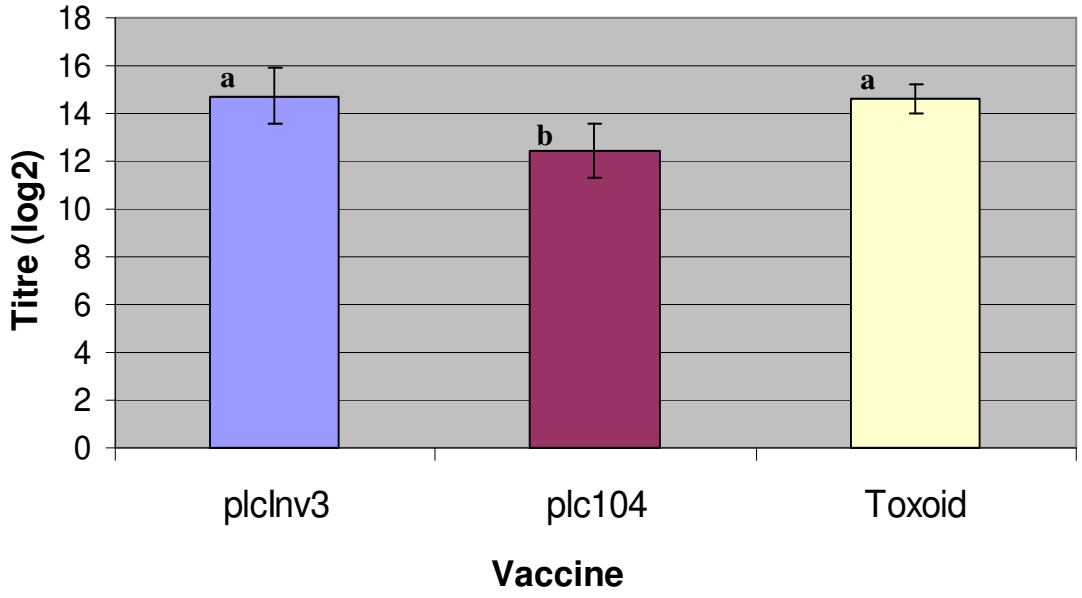


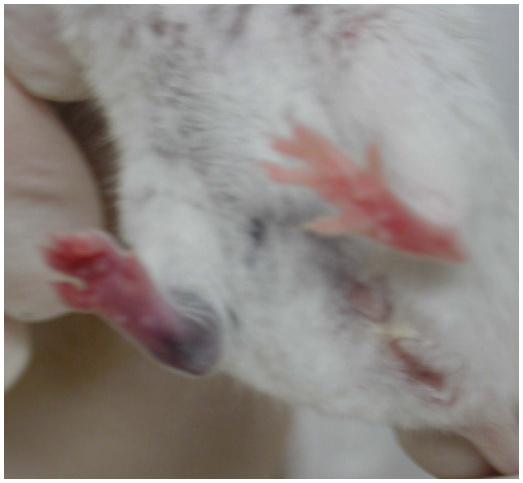
Figure 4.7: The development of anti-α-toxin IgG antibodies following vaccination of mice with plcInv3, plc104 or plc3 toxoid prior to *C. perfringens* challenge. Mice were vaccinated 3 times over a 6 week period. IgG responses were assessed using an ELISA. Each histogram is the average result and range, of titres from 7 mice in a group. The titre was calculated as the reciprocal (log2) dilution of the end-point (given as an OD_{450} of 0.2). Bars with different letters were significantly different ($p<0.01$).

Even with the reduced inoculum, all sham-immunised control mice developed severe signs of gas-gangrene as described by the development of cyanosis from the site of infection towards the footpad, with the death of one mouse between 24 and 32 h after challenge. When gangrene encompassed the footpad mice were sacrificed due to animal welfare considerations (Figure 4.8 B).

Toxoid vaccinated mice did not develop any further clinical signs of disease other than localised swelling and limping. All seven plcInv3 vaccinated mice survived equally as well as plc3 toxoid vaccinated mice, and other than swelling and limping, the presence or progression of gas-gangrene was absent (Figure 4.9). Three of six mice immunised with plc104 succumbed to the clostridial challenge, with gas-gangrene developing around the site of injection in two mice, and in a third case progressed towards the ankle. The 7th mouse died prior to the challenge commencing from unrelated causes.

The clinical signs of gas gangrene in both the negative control group and the plc104 immunised mice became obvious at about 16 h following the *C. perfringens* challenge and the clinical sign became more severe as time progressed (Figure 4.10).

(A)



(B)

(C)

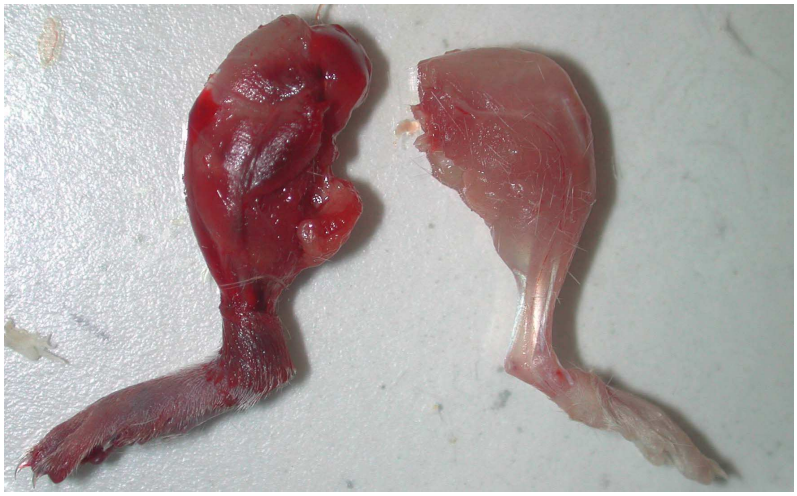


Figure 4.8: The clinical signs of gas-gangrene development in mice infected with *C. perfringens.* **(A)** The spread of gangrene at the ankle and swelling of the foot pad of a mouse indicative of a score of 3 according to the index of gross pathology outlined in Table 4.3. **(B)** The spread of gas-gangrene to the foot-pad indicative of a score of 4 on the index of gross pathology outlined in Table 4.3. **(C)** Comparisons of left and right thighs of a mouse infected with *C. perfringens* 68 h earlier. Inflammation and swelling is prominent in the infected thigh (left).

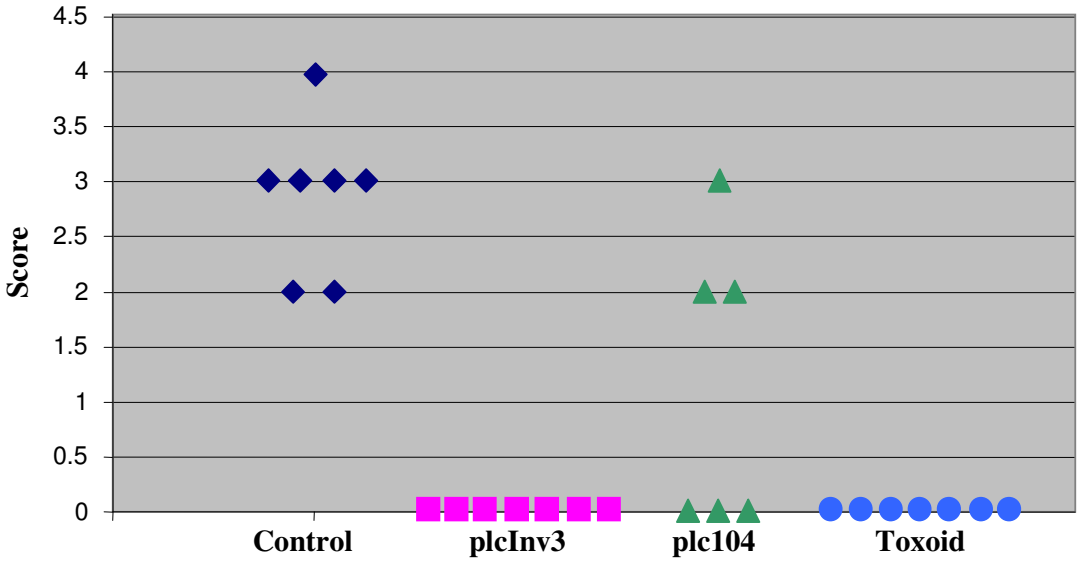


Figure 4.9: Distribution of infection within immunised groups of mice at 48 hours post *C. perfringens* challenge. Individual mice were scored according to the severity of the clinical signs of gas gangrene; 0 = swelling/limping; 1 = cyanosis at the site of injection; 2 = spread of cyanosis to the thigh surrounding injection site; 3 = cyanosis spread to the ankle/swelling of the foot pad; 4 = blackening of complete foot pad/mouse found dead.

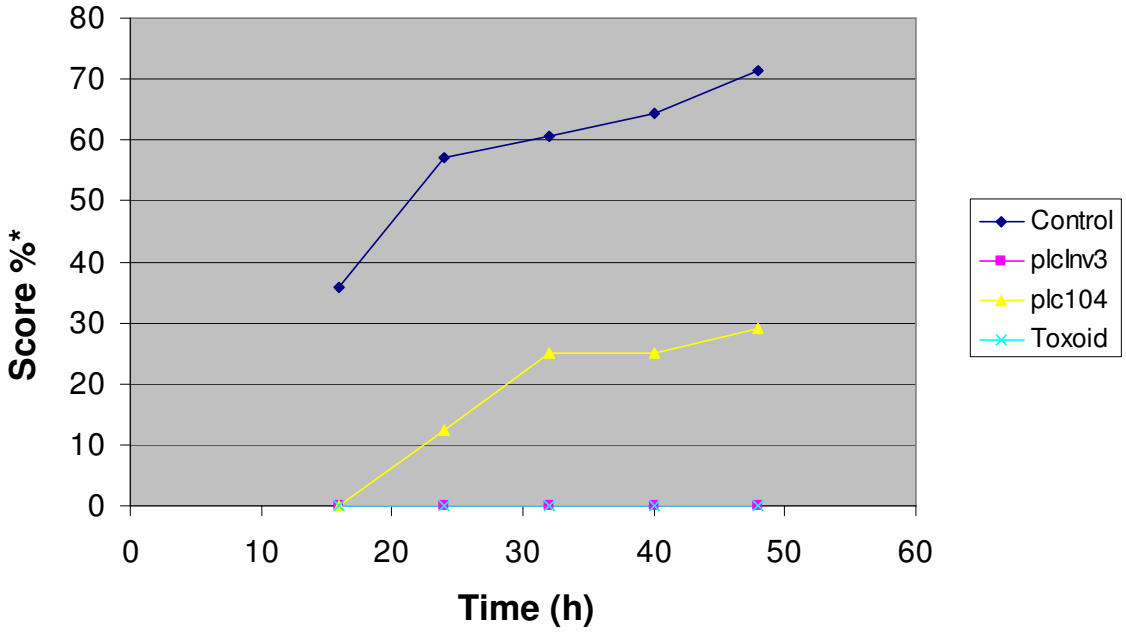


Figure 4.10: Gross pathology of mice infected with 4.05 x 10^6 *C. perfringens* cells following vaccination with plcInv3, plc104, plc3 toxoid or an unrelated bacterium (control). A 50 µL dose of *C. perfringens* cells was administered to the left thigh of mice which were then closely observed over a 72 h period for overt signs of disease which included swelling and cyanosis of the thigh and footpad. The data reflect the percentage of; the sum of scores per group divided by the total possible score.

*See Table 4.3 for description of scoring system.

4.4 Discussion

Four truncated α-toxin proteins developed, expressed and purified as described in Chapter 3, were examined for their potential as vaccines targeting the α-toxin of *C. perfringens*. Their immunogenicity was determined in BALB/c mice when administered i.p with FIA. All four vaccines plcInv3, plc316, plc204, plc104 and the positive control plc3 toxoid produced a significant titre of anti-α-toxin IgG. Mice vaccinated with the proteins plcInv3 and plc104 responded with the production of a much higher IgG titre than did mice vaccinated with plc204 and plc316. The high level of antibody titre produced against vaccination with plc104 and plcInv3 as assessed in an ELISA coated with native plc3 indicated that the vaccines were strong immunogens that were capable of inducing antibodies that reacted with the holotoxin. The reduced antibody responses of plc204 and plc316 vaccinated groups, could be attributed to their altered physical states. Purification and preparation of both proteins resulted in decreased solubility (Chapter 3). The misfolding of the two proteins may have affected epitope presentation during vaccination of mice, resulting in reduced production of antibodies essential for specific recognition of the α-toxin. These two vaccine prototypes were omitted from further examination, and additional work focussed on the truncated proteins plcInv3 and plc104 which were both soluble and highly immunogenic.

The phospholipase C (plc) neutralising abilities of antisera from mice vaccinated with plcInv3, plc104 and plc3 toxoid were investigated using the *p*-NPPC assay. When antisera from PBS vaccinated mice (negative control group) or plc3 toxoid vaccinated mice was tested, an enhancement of α-toxin plc activity was observed. The antiserum was heat treated for 30 min at 56ºC to inactivate complement and other heat labile enzymes and was tested in the absence of α-toxin to eliminate the possibilities that a component of the antiserum was itself hydrolysing the substrate. When this was done, only a baseline response was observed.

The results suggest one of two possibilities, that component(s) of the sera could be enhancing the plc activities of the α-toxin or, the effects of the sera could be acting directly on the substrate itself by improving the characteristics of the reaction media (Kurioka and Matsuda, 1976).

Enhanced plc activities of α-toxin have been demonstrated where site-directed mutations or the deletion of the complete C-terminal domain have lead to the increased accessibility of the active site of α-toxin to *p*-NPPC (Titball *et al.*, 1991; Walker *et al.*, 2000). Some proteins in eukaryotic cells require activation via regulatory proteins such as calmodulin (Alberts *et al.*, 2002). The proteins in the serum could be regulating the α-toxin plc activity in a similar regulatory manner, by binding to regions of the α-toxin and enhancing the accessibility of substrate to the active site. If this is found to be true, it has interesting consequences on the fate of α-toxin once inside the host, as the hosts own vascular system could be inadvertently up-regulating the activity of the α-toxin. If the latter is found to be true, that serum helps to improve the characteristics of the reaction media, future use of the *p*-NPPC assay could be improved by the addition of a small amount of sera to enhance plc activities when only small a concentration of α-toxin is available. Rate limiting effects were not seen at the serum concentrations used and further enzyme kinetic studies would be required to further assess the behaviour of the mouse serum on α-toxin in the *p*-NPPC assay. Studies should also include other substrates such as egg yolk and erythrocytes.

Since the antiserum was causing an increase in plc activities, it was necessary to purify the immunoglobulins from the antisera. The neutralising ability of Ig isolated from mice vaccinated with plcInv3, plc104 and plc3 toxoid was examined. All three Ig samples were able to reduce the activity of α-toxin in the *p*-NPPC assay indicating the presence of plc neutralising antibodies (Sato *et al.*, 1989; Titball *et al.*, 1991).

Protein plc104 does not contain any N-terminal α-helical regions and antibodies produced in mice immunised with plc104 would also be devoid of antibodies targeted towards this catalytic region, yet these antibodies were able to neutralise the *p*-NPPC hydrolysing activities of the α-toxin. A mutation in the C-terminal region of α-toxin is most likely to affect the binding abilities of the α-toxin and hence haemolytic and cytotoxic, but not phospholipase C activities (Titball *et al.*, 1991; Guillouard *et al.*, 1997). Contrary to this, evidence exists that mutations in the C-terminal domain affect the phospholipase C activity of the α-toxin on substrates such as *p*-NPPC which contain no membrane binding surfaces onto which the C-terminal domain of the α-toxin can anchor (Guillouard *et al.*, 1996; Walker *et al.*, 2000; Jepson *et al.*, 2001).

Mutations of residues in the C-terminus alters the active site of the toxin by reducing the access of substrate to the active site, reducing binding of the toxin to the phospholipid head group of the substrate, altering the structural integrity of the toxin and by interrupting the communication between the N-and C-termini which is important for correct catalytic activity (Sato *et al.*, 1989; Guillouard *et al.*, 1997; Naylor *et al.*, 1999; Walker *et al.*, 2000; Jepson *et al.*, 2001). Therefore antibodies directed to epitopes in this region would also reduce or abolish catalytic activity of the α-toxin via steric hindrance or direct blockage of the active site, as was observed in the *p*-NPPC neutralisation assay.

The positive results of the neutralisation assays suggest that plc activity can be inhibited from antibodies produced by vaccination with plc104 and plcInv3, but this does not necessarily assume that these antibodies will be protective *in vivo* as antibodies preventing the binding of α-toxin to biological membranes are also necessary for complete protection against the toxin

(Sato *et al.*, 1989; Williamson and Titball, 1993). Therefore the vaccines abilities to protect mice against a challenge with α-toxin or *C. perfringens* were assessed.

Mice were vaccinated and challenged with a 5 x mouse lethal dose (MLD) of α-toxin (5μg) (Williamson and Titball, 1993; Schoepe *et al.*, 1997; Jepson *et al.*, 1999) or 4 x 10^6 cfu/mL of *C. perfringens*, a dose large enough to cause gas-gangrene in this study.

Results of the toxin challenge suggest antibodies were produced (Figure 4.6) and did protect mice for a longer time than control groups (Table 4.4), although mice did not survive, unlike the plc3 toxoid vaccinated group where all mice survived the α-toxin challenge with no observable signs of acute toxicity. However, one mouse from groups plcInv3 and plc104, did survive the challenge. The mice used in the study were inbred Balb/c mice therefore no genetic differences would be present amongst the mice. Although animal to animal variation does occur (Moutafis, 2002). The pre-challenge antibody responses as measured in the ELISA showed no reason why these two mice may have survived, in that the antibody responses in these mice prior to challenge were not significantly correlated to protection against α-toxin challenge. These two mice may have developed an enhanced immune response upon challenge or have been given a slightly smaller dose of toxin which they were able to neutralise. Unfortunately serum from these two mice was not taken post-challenge to confirm a boosted immune response occurring.

A publication by Williamson and Titball (1993), suggests that vaccination with the C-terminal domain protects mice against α-toxin challenge, whilst the data obtained from the vaccination with plcInv3 which contains the complete C-terminus suggests otherwise. It is difficult to compare the survival studies with other authors as in most publications, the quantity of protein rather than the unit of activity of α-toxin is mentioned in a challenge trial, and the assays used

for unit determination are not standardised. Therefore even though the protein content is similar, the rate of activity may not be. One other difference between their study and this current one is that Williamson and Titball (1993), immunised mice as required until the specific antibody titre to α-toxin plateaued or for a maximum of 6 times, therefore the antibody levels in their mice may have been much higher during challenge with α-toxin which lead to protection against the acute effects of a highly lethal dose of α-toxin, whereas this trial was limited to 3 vaccinations. In the field it would not be realistic to continually boost animals as often as required which is why the immunisation regime was kept to a 3 dose maximum. Freund's complete adjuvant was also omitted because this is not an adjuvant that would be used in the veterinary field or in humans. Indeed it is possible that antibodies directed against specifc domains may have been lower in the truncated vaccines.

Even though plcInv3 and plc104 vaccinated mice did not survive the α-toxin challenge, the dose administered was much higher than would occur naturally where small pulsed doses would be released from actively growing *C. perfringens* cells. Therefore a live bacterial challenge was administered to determine the overall performance of the proteins as vaccines when encountering *C. perfringens*.

All mice including toxoid vaccinated mice developed swelling and limping. A high dose of bacterial cells, whether dead or alive, particularly into the thigh muscle would induce an acute inflammatory response which would include limping and swelling (Awad *et al.*, 2000), therefore these clinical signs of disease were omitted during the scoring of challenged mice.

The performance of all three vaccines, plcInv3, plc104 and plc3 toxoid was much better than the sham-immunised control group.

Some breakthrough in protection was observed in mice vaccinated with plc104. Antibodies against the C-terminal domain are essential for the complete neutralisation of the α-toxin, probably by preventing the docking of α-toxin to receptors on phospholipids (Williamson and Titball, 1993). Vaccine plc104 may have a few epitopes missing which are necessary for the prevention of anchoring of α-toxin to the membrane surfaces. The protein itself is no longer able to bind erythrocytes (Chapter 3), and the absence of the 1st β-sheet and hence the disruption of the 1st loop contains an epitope recognised by monoclonal antibody 9F3A6 which has some lethal neutralising abilities (Sato *et al.*, 1989; Guillouard *et al.*, 1997). Its absence may have contributed to the breakthrough in protection of *C. perfringens* infected mice. Protein plc104 is also missing the linear epitope region encompassing residues 192-199 (ARGFAK). This linear epitope has shown to be partially protective in α-toxin challenges of mice (Logan *et al.*, 1991). The future production of more plc104 antibodies could be used to assess the antisera in other assays such as the haemolysin liquid assay to determine if the antibodies present are able to bind to the C-terminal domain of α-toxin, and hence prevent cell lysis, which would be indicative of lethality in mice (Schoepe *et al.*, 1997). From the study thus far, it seems that the missing β-sheet is important for the complete protection of mice against gas-gangrene.

However even with the deletion of the 1st β-sheet of the C-terminus from α-toxin, mice were protected from challenge against live *C. perfringens*, suggesting other epitopes located in the remaining loops are important for protection against infection.

The protection afforded by plcInv3 vaccination was as good as that by plc3 toxoid, which has previously been shown to be highly immunogenic and protective in guinea pig trials (Ito, 1970). The truncate $cpa_{247\text{-}370}$ developed by Williamson and Titball (1993) also shows promising results as a vaccine with only a few mice developing clinical signs of infection

when challenged with 3 x 10^7 to 3 x 10^9 cfu of *C. perfringens* (Stevens *et al.*, 2004). It would be interesting to see if both plcInv3 from this study and $cpa_{247-370}$ from the study by Williamson and Titball (1993) would fare just as well as each other in a challenge trial. PlcInv3 contains more epitopes targeted against the plc activities of the α-toxin and may therefore provide better protection than $cpa_{247-370}$, as the presence of the non-toxic C-terminal region alone, which is responsible for binding receptors, may not protect against an overwhelming challenge in the absence of plc neutralising antibodies (Walker, 1992).

The strain of *C. perfringens* used in this study was a field isolate from a case of NE in a chicken. The strain was able to cause severe gas-gangrene and the death of one mouse when administered at doses as low as 4 x 10^6 cfu. Other authors (Stevens *et al.*, 1987; O'Brien and Melville, 2004) suggest that the inoculum required to cause an observable clinical sign of gas gangrene in the Murine model is between 10^8 and 10^9 in order to provide enough tissue damage and localised blood clotting to bring about ischemia at the site of infection and the subsequent development of gangrene. They suggest that mice are more resistant to the development of ischemia than are humans. It may be that the strain used in this study is more pathogenic producing higher levels of α-toxin and other associated toxins.

The overall results of this study describe a new α-toxin vaccine protein, plcInv3, capable of protecting mice against the observable clinical signs of gas-gangrene. Whilst plcInv3 has the aa's 56-75 deleted, the antibodies developed in immunised mice were effective in neutralising the plc activity *in vitro* and protecting mice against a live *C. perfringens* challenge. Clearly whilst the deleted region has been identified as the active site for plc activity, it is not the only region on the α-toxin that can lead to neutralisation of the plc activity (Figure 4.5). Perhaps the binding of antibodies to other epitopes induces a change in folding of α-toxin which leads to its neutralisation. Further protection is provided by the presence of the complete C-

terminus which leads to the production of antibodies able to prevent the initial binding of the α-toxin to eukaryotic membranes.

Vaccine plc104 was also able to protect mice against the major signs of gas-gangrene although some breakthrough in protection was observed which may be attributed to the lower immune responses observed in these mice or the reduced epitopes present in the vaccine which were not enough to provide complete protection. The neutralisation of plc activity that was evident when using immunoglobulins from plc104 immunised mice suggests, as with plcInv3, that binding of these immunoglobulins alters the conformation of the α-toxin so that it neutralises its plc activity and binding ability and hence leads to reduced pathogenicity with a live *C. perfringens* challenge.

Chapter 5

Vaccination against the α-toxin of *C. perfringens* by use of the live orally delivered vaccine vector STM1

5.1 Introduction

A clostridial vaccine to aid in the prevention of necrotic enteritis (NE) in chickens has yet to be developed. Clostridial vaccines are currently only available for ruminants and these consist of toxoid preparations of types C and D *C. perfringens* bacteria, or a cocktail of formalin inactivated β- and ε-toxins (Younan *et al.*, 1995; Kelneric *et al.*, 1996). NE is a multifactorial disease, but the α-toxin of *C. perfringens* is thought to be a major contributing factor to the development of the disease (Chapter 1.6.3). Vaccination of mice with proteins plcInv3 and plc104 (Chapter 3) has shown to protect against the lethal effects of α-toxin produced by *C. perfringens* in a live clostridial challenge (Chapter 4). These proteins are currently administered parenterally in an emulsion of FIA. The development of these proteins into effective veterinary vaccines for chickens would require a system whereby they are easy to produce, purify and deliver to the host. The development of live attenuated bacterial vectors that can survive the harsh intragastric environments of the host for use as delivery vehicles of foreign antigens meets these criteria. They are relatively easy to produce, there is no need to purify end products, and delivery to chickens can be achieved with mass vaccination via the drinking water, feed, or as an aerosol (Coloe *et al.*, 1995). They also have the ability to induce mucosal immune responses (Brown *et al.*, 1987; Maskell *et al.*, 1987; Walker *et al.*, 1992; Bullifent *et al.*, 2000), an added benefit in the prevention of diseases of the gastrointestinal tract (GIT) such as NE of chickens.

One such bacterial vector is the attenuated *S*. Typhimurium. It has the potential to be used as a combined vaccine due to its known capacity to elicit circulatory and secretory antibodies as well as cell mediated immunity (Brown *et al.*, 1987; Dougan *et al.*, 1987; Guzman *et al.*, 1991; Mastroeni *et al.*, 1992).

Many investigations have often used plasmid-encoded heterologous genes for expression within attenuated *S*. Typhimurium (Poirier *et al.*, 1988; Sadoff *et al.*, 1988; Coulson *et al.*, 1994a; Londono *et al.*, 1996; Dalla Pozza *et al.*, 1998; Pacheco *et al.*, 2005). A disadvantage of this is the ability of *S*. Typhimurium to retain the plasmid in the absence of selective agents such as antibiotics, as would be found *in vivo* (Maskell *et al.*, 1987; Dougan *et al.*, 1989; Molina and Parker, 1990; Strugnell *et al.*, 1990; Coulson and Titball, 1993). This plasmid instability can lead to the lack of immune responses within the host if the plasmid is removed from the system before enough recombinant protein can be accumulated *in vivo* (Fairweather *et al.*, 1990; Dalla Pozza *et al.*, 1998). One system to overcome plasmid instability is the chromosomal integration of the heterologous antigen gene, although the reduced copy number of the gene present could also result in a reduced immune response (Strugnell *et al.*, 1992; Cardenas and Clements, 1993).

Another system of controlling plasmid stability, while maintaining a relatively high-copy number is the use of *in vivo* inducible promoters. Such promoters would only induce the expression of the recombinant gene under specific environmental conditions (Dunstan *et al.*, 1999). This system also alleviates the need for specialised growth conditions and supplements in order to induce expression of a foreign antigen (Maskell *et al.*, 1987). Two of the most characterised *in vivo* inducible promoter systems include the *pag*C and *nir*B promoters (Chatfield *et al.*, 1992a; Karem *et al.*, 1995; Dunstan *et al.*, 1999; Chen and Schifferli, 2000; Chen and Schifferli, 2001; Ruiz-Perez *et al.*, 2002). Recently the *htr*A

promoter was compared to the *nir*B and *pag*C promoters and found to induce a higher immune response in addition to protection against challenge (Roberts *et al.*, 1998; Foynes *et al.*, 2003).

In this study the *htr*A promoter (P_{htrA}), along with the *pag*C (P_{pagC}) and the constitutively expressed tac promoter (P_{tac}) were examined for their ability to express the *plcInv3* and *plc104* from within STM1. Immune responses were documented and the protective capacities of the vaccine were examined following a *C. perfringens* challenge of vaccinated mice.

5.2 Materials and methods

The organisms and vectors used and created in this study is listed in Table 5.1 and 5.2 with a detailed description of their construction followed below.

5.2.1 Construction of the vector pBTac3

The tac promoter (P_{tac}) was amplified from vector pGEX-4T-1 (Amersham Biosciences, Sweden) using primers TacF (forward primer) and TacR (reverse primer) (Table 2.5). An *Xba*I site was added to primer TacF for ease of determining the orientation of the insert, and a *Bgl*II site was added to primer TacR for downstream cloning of the α-toxin gene truncates.

P_{tac} was amplified using *pfu* polymerase (Table 2.6 and 2.8) with an annealing temperature of 58ºC and an extension time of 2 min. The amplification of P_{tac} was confirmed by gel electrophoresis (Chapter 2.5) using a 1.5% agarose gel.

The amplified product was treated with polynucleotide kinase (PNK) (Chapter 2.7.4), in a total volume of 30 µL. The reaction was incubated at 37ºC for 2 h and heat inactivated at 70ºC for 10 min.

Vector pBluescript (8-10 µg) was digested with *Eco*RV in a total volume of 10 µL, for 4 h. Following heat inactivation, 8 µL of the restriction digested vector was treated with calf intestinal phosphatase (CIP) to prevent its self-ligation (Chapter 2.7.3). The hydroxylated vector was then purified using the GeneClean kit (QBIOgene, USA) and resuspended in a final volume of 20 µL.

Table 5.1: Organisms used in the development of orally delivered vaccines.

Organism	Genotype	Source
E. coli DH5α	*supE ΔlacU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	(Hanahan, 1983)
Salmonella Typhimurium 82/6915	Wild type Inv^+	(Alderton *et al.*, 1991)
Salmonella Typhimurium 9121 (LT2)	*leu hsdL trpD2 rpsL120 ilv452 metE551 metA22 hsdA hsdB*	Prof. P. Reeves, Department of Microbiology, The University of Sydney
STMI	$\Delta aroA^-$ $\Delta serC^-$	(Alderton *et al.*, 1991; Coloe *et al.*, 1995)

Table 5.2: Plasmids used in the development of STMI orally delivered vaccines.

Plasmid	Description/genotype	Source
pGEX4T-1	4.969 kb protein expression vector: Amp^R, *lac*I^q, N-terminal glutathione-S-transferase fusion, tac promoter (P_{tac}), pBR322 origin of replication	Amersham Biosciences, Sweden
pBluescript SKII-	2.96 kb phagemid: Amp^R, *lac*Z, ColE1 origin of replication	Stratagene, USA genbank #X52330
pBTac3	P_{tac} amplified from pGEX4T-1 and inserted into pBluescript via blunt end ligation at *Eco*RV site	This study
pBPAGC21	*Pag*C promoter (P_{pagC}) from *Salmonella* Enteritidis inserted into *Eco*RV site of pBluescript via blunt ended ligation	(Moutafis, 2002)
pRSETA	2.9 kb protein expression plasmid: Amp^R, N-terminal 6 x His, T7 promoter	Invitrogen
pRplc14	Complete *plc* cloned into the *Pst*I and *Eco*RI sites of pRSETA using primers AllPstI and PlcEcoRev (Table 2.5)	This study
pRplcInv9	Inverse amplified pRplc14 (using primers Inv1 and Inv2) self-ligated (nucleotides 250-309 of *plc* deleted)	This study
pRplc104	DNA sequence coding for the C-terminus of *plc* (nucleotides 882-1243) cloned into the *Pst*I and *Eco*RI sites of pRSETA	Chapter 3 (3.2.2.1)
pBTCαInv	Amplified *αInv* from pRplcInv9 cloned into the *Bgl*II and *Not*I sites of pBTac3	This study
pBTCα104	Amplified *α104* from pRplc104 cloned into the *Bgl*II and *Not*I sites of pBTac3	This study

Table 5.2 Continued.

Plasmid	Description/genotype	source
pBPCαInv	Amplified *αInv* from pRplcInv9 and cloned into pBPAGC21 via *Bgl*II and *Not*I sites	This study
pBPCα104	Amplified *α104* amplified from pRplc104 and cloned into pBPAGC21 via *Bgl*II and *Not*I sites	This study
pBHAαInv	Amplified *htr*A promoter (P_{htrA}) ligated to *αInv* through *Bgl*II sites and cloned into pBluescript SKII- via *Xba*I and *Not*I sites.	This study
pBHAα104	Amplified P_{htrA} ligated to *α104* through *Bgl*II sites and cloned into pBluescript via *Xba*I and *Not*I sites.	This study

The PNK treated amplified P_{tac} and CIP treated pBluescript were combined in a 3:1 molar ratio (as determined by quantisation on an agarose gel) and ligated overnight (Chapter 2.7.5) at 22ºC (RT) in a final volume of 20 µL.

The ligated product was precipitated and purified with ethanol and sodium acetate (Chapter 2.7.5.1) and resuspended in 10 µL of milliQ water followed by electro-transformation into DH5α electro-competent cells (Chapter 2.7.6 and 2.7.7). Following a 1 h incubation at 37ºC, 10, 50 and 100 µL of the transformation was spread onto LB agar supplemented with 100 µg/mL ampicillin (LA100), and previously spread with 40 µL of 40 mg/mL IPTG and 40 µL of 40 mg/mL X-gal.

Non β-galactosidase producing colonies (white) were selected for further analysis by RE digestion following plasmid miniprep extractions (Chapter 2.4.2). Isolated plasmids were digested with *Xba*I for the confirmation of the P_{tac} insert and determination of orientation.

5.2.1.1 Expression plasmid pBPAGC21

Vector pBPAGC21 was kindly provided by G. Moutafis, and contained the *pag*C promoter (P_{pagC}) amplified from *S.* Enteritidis cloned into the *Eco*RV site of the pBluescript vector by the same procedure as described for the creation of vector pBTac3, except that a *Bam*HI was site was incorporated into its 5' terminus during PCR instead of a *Xba*I site.

5.2.1.2 Construction of plasmid pRplcInv9

Construction of the pRplcInv9 vector began with the amplification of the complete *plc* from *C. perfringens* 61 using primers AllPstI and PlcEcoREV (Table 2.5) and *pfu* polymerase with an annealing temperature of 55ºC and an elongation time of 2 min, followed by *Pst*I and

*Eco*RI digestion and cloning into the *Pst*I and *Eco*RI sites of pRSETA, creating vector pRplc14 (procedures for cloning and transformation essentially as outlined in Chapter 3.2.2.1). The pRplcInv9 vector was then constructed using the same methodology as that outlined for the construction of pCplcInv3 (Chapter 3.2.2.3), via inverse PCR of pRplc14 using primers Inv1 and Inv2 (Table 2.5).

5.2.1.3 Amplification of the *αInv* gene

Vector pRplcInv9 was used as the template for amplification of *αInv*, identical to the plcInv3 (Chapter 3) gene except that the CAT x 6 sequence coding for the His-tag was removed. Primers plcBglII and pRSETANotI were used in the PCR. Expand polymerase (Table 2.6 and 2.7) was used to amplify the αInv gene with an annealing temperature of 58ºC and an extension of 2 min. Amplification of *αInv* was confirmed by agarose gel electrophoresis.

5.2.1.4 Amplification of the *α104* gene

Vector pRplc104 (constructed in Chapter 3) was used as the template DNA for amplification of the α104 gene. The forward primer 890BglII and reverse primer pRSETANotI were used to amplify *α104* using *pfu* polymerase with an annealing temperature of 58ºC and an extension of 2 min. The α104 gene was identical to the plc104 gene (Chapter 3) except that the CAT x 6 sequence coding for the His-tag was removed. Amplification of *α104* was confirmed by agarose gel electrophoresis.

5.2.1.5 Cloning of *αInv* and *α104* into pBTac3 and pBPAGC21

The amplified products, *α104* and *αInv* were purified using the GeneClean kit and along with the vectors pBTac3 and pBPagC21 were digested with *Not*I and *Bgl*II for 3-4 h. After heat

inactivation of the digestions, *α104* was ligated with pBTac3, in a 3 to 1 molar ratio (Chapter 2.7.5). In a separate reaction *α104* was ligated to pBPagC21. The αInv gene was also ligated to pBTac3 and pBPAGC21 using the same methodology.

After allowing the fragments to ligate for 18 h at 14-16ºC the products were purified with sodium acetate and ethanol (Chapter 2.7.5.1), resuspended in 10 µL of milliQ water, and added to electro-competent DH5α cells for electro-transformation (Chapter 2.7.6 and 2.7.7). Transformants were grown on LA100, and following 18 h of growth at 37ºC, 10-20 colonies were isolated and incubated in 2 mL of LB broth supplemented with Amp100 (LB100) for 18 h. Following the incubation, plasmids were isolated (Chapter 2.4.2) and digested with *Xba*I and *Not*I (pBTac3:*α104* inserts) or *Hind*III (pBPAGC21:*α104*, pBPAGC21:*αInv*, and pBTac3:*αInv* inserts) and analysed on an 1.4% agarose gel.

5.2.2 Construction of expression plasmids pBHAαInv and pBHAα104

Cloning of the αInv and α104 genes into the *htr*A promoter (P_{htrA}) was completed prior to the cloning of P_{htrA} into pBluescript.

P_{htrA} was amplified with primers HtrAF and HtrAR (Table 2.5) using *pfu* polymerase (Table 2.6 and 2.8). A 58ºC annealing temperature was used and the elongation time was kept to 2 min 50 sec. Amplification of the αInv and α104 genes was as described in the sections above (5.2.1.1 and 5.2.1.2).

The αInv, α104 and P_{htrA} PCR products were purified using the Wizard PCR Prep DNA purification Kit (Promega) and digested with the restriction enzyme *Bgl*II. A sample of each digestion was loaded onto an agarose gel to calculate the concentration of each of the

fragments prior to ligation. Equal concentrations of P_{htrA} and *αInv* or P_{htrA} and *α104* were added to a reaction mix containing T4 ligase and buffer and incubated for 18 h at 14-16ºC (Chapter 2.7.5). One microliter of each ligation was used in a PCR with primers HtrAF and pRSETNotI and *pfu* polymerase with an annealing temperature of 58ºC and an elongation time of 3 min (Table 2.6 and 2.8).

Specific amplification of the ligated fragments P_{htrA}:*αInv* or P_{htrA}:*α104* was confirmed by separating the samples by electrophoresis on a 1.2% agarose gel. The PCR products were purified from the PCR buffers using the Wizard PCR prep DNA purification Kit and digested with the enzymes *Not*I and *Xba*I. Vector pBluescript was also digested with the aforementioned enzymes. The reactions were stopped by heat inactivation of the enzymes which was then followed by the ligation of P_{htrA}:*αInv* to pBluescript, and P_{htrA}:*α104* to pBluescript in a 3 to 1 molar ratio. Each ligation was purified using sodium acetate and ethanol (Chapter 2.7.5) and used in the electro-transformation of DH5α *E. coli* electro-competent cells.

Fifty and 100 µL of the transformations was plated onto LA100 and following overnight incubation, 10-20 colonies were isolated for plasmid analysis using the restriction enzyme *Hind*III.

5.2.3 Plasmid transformation into vaccine strain STMI

The vectors pBPAGC21, pBPCαInv, pBPCα104, pBTCαInv, pBTCα104, pBHAαInv and pBHAα104 were isolated from their DH5α hosts using the Wizard Miniprep Kit (Promega) and electro-transformed into the electro-competent restriction negative modification positive *S.* Typhimurium strain 9121 (LT2) as outlined in Chapter 2.7.6 and 2.7.7.

Colonies of *S.* Typhimurium LT2 harbouring plasmids were isolated, inoculated into LB100 and following 18 h incubation on an orbital shaker at 37ºC plasmids isolated using the Wizard Miniprep Kit. Plasmids were transformed into electro-competent STMI cells via electroporation as above and isolated colonies screened via restriction enzyme digestions for the presence of the correct plasmids.

5.2.4 Expression of proteins

STMI(pBTCαInv) and STMI(pBTCα104) were grown in LB100 at 37ºC on an orbital shaker till an OD_{600} of 0.5 was reached, at which time 1 mL samples were taken, pelleted by centrifugation and resuspended in 100 µL of lysis buffer or 1 X SDS loading buffer.

STMI(pBPCαInv) and STMI(pBPCα104) were grown in LB100 in the presence of 100 mM $MgCl_2$. When the cells reached mid-log phase of growth (an approximate OD_{600} of 0.5) 1 mL aliquots were taken, pelleted at 14,000 x *g* for 5 min and resuspended in fresh LB100 (1 mL volumes) containing either 0, 1, 10 or 100 mM $MgCl_2$, and grown for a further 4 h. The 1 mL samples were pelleted and resuspended in 100 µL of whole cell lysis buffer. Protein content was determined using the Lowry protein assay (Chapter 2.8.2.2).

STMI(pBHAαInv) and STMI(pBHAα104) were grown in LB100 at 37ºC on an orbital shaker till an OD_{600} of 0.5 was reached, at which time 1 mL samples were taken and dispensed into 1.5 mL tubes with the addition of 0, 1 or 10 mM H_2O_2, or the samples were placed in a 42ºC waterbath for a further 2 h. Following the incubation, the 1 mL samples were pelleted by centrifugation and resuspended in 100 µL of 1 X SDS loading buffer.

Protein samples were loaded onto an SDS-PAGE and separated by electrophoresis (Chapter 2.8.3). Following separation, proteins were transferred onto a nitrocellulose membrane (Chapter 2.8.7.2), probed with horse anti-α-toxin polyclonal antisera (gas-gangrene antisera) (CSL), and then probed with the secondary conjugated rabbit anti-horse IgG-HRP.

5.2.5 Plasmid stability assay

Stocks of STM1 harbouring the vectors were streak plated onto LA100 to obtain single colonies. Three to four well isolated colonies were selected for plasmid stability assays and incubated in 10 mL of LB100 at 37ºC on an orbital shaker for 18 h. The culture was 10-fold diluted from 10^{-1} to 10^{-6} in LB and 100 µL of each dilution spread plate onto LA100, in duplicate, to determine the initial plate count. The 10^{-6} dilution (10 mL) was incubated for 24 h at 37ºC in the absence of ampicillin. This culture was again serially 10-fold diluted to 10^{-6} and 100 µL of each dilution spread plate onto LA100 and LA, in duplicate. The 10^{-6} dilution (10 mL) was incubated at 37ºC for 24 h and the process of dilution and spread plating repeated. The whole process was repeated for up to 5 days (approximately 100 generations) or until less than 0.05-0.002% of the culture retained the plasmid.

5.2.6 Vaccine trials

5.2.6.1 Preparation of test vaccines

One percent (v/v) of an overnight culture of STMI harbouring the different plasmid constructs was used to inoculate 10-50 mL of freshly prepared LB100. The culture was incubated in an orbital shaker at 37ºC until an OD_{600} of 0.15-0.2 (as determined by adding 200 µL of culture to a 96-well plate and measuring the absorbance in a Dynatek plate reader set to a wavelength of 600 nm) was

reached. Cells were concentrated by centrifugation, and using the formula below, resuspended in sterile PBS to a final cell concentration of 1.0 x 10^{10} cfu/mL.

$(OD_{600} \times 7.65) - 0.3 = \text{result} \times 10^9$ cfu/mL

1.0 x 10^9 cfu STM1 were used for oral vaccinations of mice. This has previously been shown to be a safe and effective level with STM1 (Alderton *et al.*, 1991).

5.2.6.2 Delivery of vaccines

STMI vaccines prepared as above (Section 5.2.6.1) were administered orally to mice using an autoclaved gavage needle and a 1 mL syringe. The vaccine was drawn through the gavage needle, which was then dipped in glycerol for a smoother passageway down the mouse esophageus. The needle was rinsed in 80% ethanol (v/v) between the vaccinations of different groups of mice. A dose of 100 μL containing approximately 1 x 10^9 cells was administered orally to each mouse. The vaccine dose was diluted up to 10^{-7} in PBS and spread plate onto LA100 for retrospective counting of the exact vaccine doses administered.

5.2.7 Immunogenicity assays

Balb/c mice (5/group) were vaccinated with STM1 harbouring the plasmid construct vaccines, or with α-toxin (plc3) toxoid on days 1, 15, and 29. Cells (between 10^7-10^9) were administered to all mice, with the exact dose determined following retrospective spread plate counting of 10^{-5}, 10^{-6} and 10^{-7} diluted cultures on LA100 plates (Table 5.3). Twenty days following final vaccination, mice were sacrificed by cervical dislocation and their spleens removed and placed in RPMI/NCS, supplemented with penicillin and streptomycin (5 μg/mL).

Table 5.3: Vaccination of mice via the delivery vector STM1 harbouring plasmids containing the various α-toxin truncated genes.

Vaccine	Dose administered (cfu)		
	Day 1	Day 15	Day 29
pBPAGC21	2.3×10^8	4.2×10^8	1.5×10^8
pBPCαInv	2.6×10^8	5.6×10^8	3.3×10^8
pBPCα104	3.2×10^8	4.5×10^8	2.61×10^8
pBHAαInv	2.8×10^8	4.4×10^8	2.78×10^8
pBHAα104	9.24×10^7	8.0×10^8	1.3×10^7
pTCαInv	7.52×10^7	3.5×10^8	4.1×10^7
pBTCα104	8.4×10^7	6.0×10^6	1.52×10^8
Toxoid *	10 μg	25 μg	50 μg

*Toxoid was administered via the i.p route using a 25 gauge needle and 1 mL syringe. The amount stated was administered in a total volume of 100 μL in a 1:1 ratio with FIA (see Chapter 4.2.2 for detailed description of toxoid vaccine preparation).

5.2.7.1 Splenocyte preparation

The splenocytes were homogenised by mashing the spleen using the back of a 3 mL syringe. The homogenised cells were resuspended in 5 mL of RPMI/NCS and filtered through a cell strainer (BD Falcon). The splenocytes were pelleted by centrifugation at 1,600 x *g* for 5 min at 4ºC, the RPMI/NCS solution decanted and cells resuspended in 5 mL of ACK lysing buffer to lyse any erythrocytes present. Following a 5 min incubation on ice, 5 mL of RPMI/NCS was added to the lysing solution and the cells pelleted by centrifugation (1600 x *g,* 5 min, 4ºC). The cells were washed 3 times in RPMI/NCS and resuspended in 1 mL of RPMI/NCS. Cell viability was calculated using Trypan Blue exclusion. Ten microliters of cell suspension, 10 µL of Trypan blue (Sigma) and 80 µL of PBS were mixed and viable cells counted in a haemocytometer chamber using an inverted microscope. Cell concentration was adjusted to 10^6 cells/90µL in RPMI/NCS. Ninety microliters was dispensed into nitrocellulose backed 96-well plates previously incubated with anti-cytokine specific antibodies and blocked with 5%NCS (see Section 5.2.7.2).

5.2.7.2 ELIspot assay

Preparation of this assay required a sterile work environment. Multiscreen™-IP Filter 96-well plates (Millipore) were soaked with 100 µL methanol for 5 min prior to use. Plates were rinsed 3 times with about 200 µL sterile PBS and patted dry with autoclaved paper towelling. Wells were then coated with 100 µL of anti-cytokine specific capture antibody diluted in coating buffer. IFN-γ capture antibody was used at a concentration of 1.0 µg/mL and IL-4 at 0.5 µg/mL. Plates were incubated overnight at 4ºC. Unbound capture antibody was discarded and the plates rinsed 3 times in filter sterilised PBST. Excess liquid was removed from the plate by patting the bottom of the plate onto sterilised dry absorbent paper, and wells were blocked with 200 µL of 5% NCS (prepared in PBS) for 2 h at room temperature (RT). Wells

were rinsed in PBST (x3) following the blocking step, patted dry and 100 µL of RPMI/NCS solution added to each well and incubated in a humidifier for 10 min. The RPMI/NCS solution was discarded, plates were patted dry and 90 µL of splenocytes added to each well (containing 10^6 cells/90 µL). Wells were seeded in triplicate and a fourth well (for each sample tested) served as an internal control (no stimulatory mitogen was added). An extra 8 wells were inoculated with spleen cells for use as positive and negative controls. Four of the wells remained unstimulated (negative control) and four wells were stimulated with the addition of 10 µL of a stock (10 µg/mL) of concanavalin A (Sigma-Aldrich), a mitogen (positive control). Alpha-toxin (plc3) was prepared in RPMI/NCS at a concentration of 250 µg/mL and 10 µL added to each well (except positive and negative control wells). Plates were incubated in a humidifier (37ºC, 5%CO_2) for 21 h wrapped in aluminium foil. Following this incubation, plates no longer required to be kept in a sterile environment. The solution in the wells was decanted and wells washed 8 times with PBST and twice in milliQ water. Biotin rat anti-mouse IFN-γ or IL-4 (Abcam) were prepared at a concentration of 0.5 µg/mL in PBS containing 1% NCS and 100 µL added to all wells. The plates were incubated at RT for 2 h. Plates were washed with PBST (4x) and PBS (x2), patted dry and 100 µL of EXTRAvidin® (Sigma) diluted 1:1000 in PBS/1% NCS added to each well. The plates were again incubated at RT for 2 h. The plates were rinsed in PBST (x4) and PBS (x2), patted dry, and then 50 µL of alkaline phosphatase conjugate substrate (Biorad) added. The reaction was allowed to proceed until spots began to develop (usually about 10 min) and the reaction stopped by rinsing the wells with milliQ water (3x). The plates were dried and stored in the dark (wrapped in aluminium foil) until counting of spots was done. Spots were enumerated using a dissecting microscope.

The independent Student's *t*-test was used to determine any statistical differences between vaccine groups ("STUDENT", 1908).

5.2.7.3 Detection of antibody responses

5.2.7.3.1 Detection of anti-α-toxin IgG antibodies

Wells were coated with 3 μg/mL of α-toxin (plc3) antigen. The ELISA assay was as described in Chapter 2.8.8 but following the serial two-fold diluting of sera in the wells, plates were incubated at 4ºC for 24 h.

Detection of anti-α-toxin antibodies was also examined in immunoblots. Two micrograms of α-toxin was loaded onto a polyacrylamide gel and the protein separated by electrophoresis (Chapter 2.8.3). The α-toxin was then transferred onto a nitrocellulose membrane (Chapter 2.8.7.2), blocked with 5% skim milk (in TBS), and probed with pooled mouse sera (overnight, 4ºC). The presence of bound anti-α-toxin antibodies was detected by probing with a goat anti-mouse IgG-HRP conjugate diluted 1 in 5000 in TBS (1% skim milk). Bound anti-mouse IgG-HRP was visualised with incubation in Western blot substrate solution (Chapter 5.2.7.3.2).

5.2.7.3.2 Detection of anti-*Salmonella* IgG antibodies

S. Typhimurium (82/6915) whole cell antigen was prepared by pelleting a 10 mL overnight culture of *S*. Typhimurium (14,000 x *g* 5 min) and resuspending it in 5 mL TE buffer. The cells were washed twice in TE buffer and sonicated using 6 x 20 s bursts with a 30 s cooling off period between each sonication. Insoluble matter was pelleted by centrifugation at 14,000 x *g* for 5 min and the supernatant filtered through a 0.45 μm filter. Protein content was determined using the Lowry assay (Chapter 2.8.2.2).

The wells of 96-well plates were coated with 10 μg/mL of *S.* Typhimurium whole cell antigen and the ELISA protocol for the detection of anti-*S.* Typhimurium antibodies was as described in Chapter 2.8.8. The primary antibody consisted of pooled sera from each vaccine group.

5.2.8 Mouse challenge trial

Mice (6/group) were orally vaccinated with 100 μL of STM1 harbouring plasmid constructs in PBS, on days 1, 15, and 25 by gavage needle and a 1mL syringe. Cells (between 10^7-10^9) were administered to all mice, with the exact dose determined following retrospective spread plate counting of 10^{-5}, 10^{-6} and 10^{-7} diluted cultures on LA100 plates (Table 5.4). Toxoid plc3 was used as a positive control throughout the trial, and this was prepared with FIA in a 1:1 emulsion and 100 μL administered i.p to each mouse (preparation of toxoid described in detail in Chapter 4.2.2).

Mice were bled by retro-orbital puncture prior to challenge (Day 36) and challenged with 5 x 10^6 *C. perfringens* cells in 50 μL saline in the left thigh on day 40 as outlined in Chapter 4.2.7.3. Monitoring and scoring of infected mice was as described in Chapter 4.2.7.3.

Table 5.4: The vaccination regime for the *C. perfringens* challenge trial. Mice were vaccinated with the delivery vector STMI harbouring plasmids expressing the various α-toxin gene truncates.

	Dose administered (cfu/100 μL) on day*:		
Vaccine	Day 1	Day 15	Day 25
pBPAGC21	4.4×10^8	2.2×10^8	2.36×10^8
pBPCαInv	4.4×10^8	2.52×10^8	3.0×10^8
pBPCα104	3.14×10^8	2.94×10^8	2.32×10^8
pBHAαInv	3.5×10^8	2.16×10^8	2.48×10^8
pBHAα104	4.6×10^8	8.4×10^7	2.3×10^8
pBTCαInv	2.76×10^8	1.04×10^8	1.32×10^8
pBTCα104	8.0×10^7	2.9×10^8	1.64×10^8
Toxoid*	20 μg (i.p)	40 μg	50 μg

*Toxoid was administered via the i.p route using a 25 gauge needle and 1 mL syringe. The amount stated was administered in a total volume of 100 μL in a 1:1 ratio with FIA.

5.3 Results

5.3.1 Construction of vectors for the inducible expression of the α-toxin truncated genes *α1nv* and *α104*

Three different promoter systems were developed for the differential expression of α-toxin truncated genes using the commercial vector pBluescript SKII as the template. Vector pBPAGC21 was previously created by Moutafis (2002) and contains the *pag*C promoter (P_{pagC}) of *S.* Enteritidis blunt-end ligated into the *Eco*RV site of pBluescript SKII in the opposite orientation to the *lac*Z reading frame. The cloned P_{pagC} region contained the -10 and -35 regions, the upstream regulatory binding sites, the Shine-Dalgarno ribosome biding site (RBS), and the ATG start signal of *pag*C.

Two other plasmids with different promoters were also constructed to examine and compare their ability to express the α-toxin truncated genes from STM1.

The tac promoter (P_{tac}) was amplified from pGEX4T-1 and included all the necessary components to initiate transcription, the -10 and -35 regions, the RBS site and the initial ATG start site followed by a *Bgl*II site for directional cloning of the α-toxin truncated genes. The amplified P_{tac} was cloned into pBluescript SKII via the *Eco*RV site. Restriction enzyme analysis of plasmids with *Xba*I was used to identify inserts of P_{tac} ligated in the opposite direction of *lac*Z. Such a plasmid, pBTac3 was isolated, sequenced and used in the cloning of the α-toxin gene truncates (Figure 5.1).

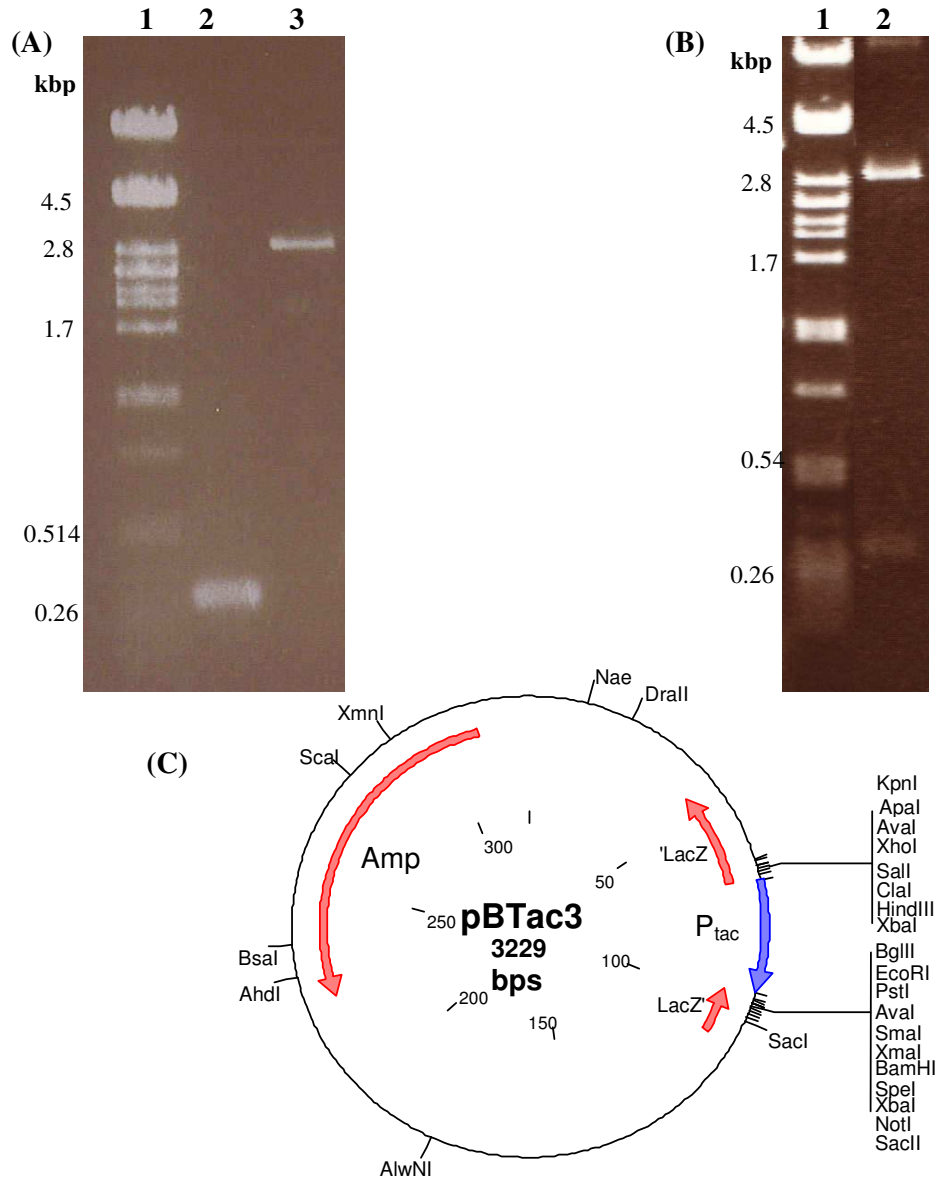


Figure 5.1: The development of the pBTac3 expression vector. (A) Amplification of P_{tac} from pGEX4T-1. Lane 1, λ-*Pst*I, Lane 2, PCR of P_{tac}; Lane 3, purification of pBluescript from DH5α. **(B)** Cloning of P_{tac} into pBluescript. Lane 1, λ-*Pst*I; Lane 2, pBTac3 digested with *Xba*I. **(C)** Restriction map of pBTac3. P_{tac} was cloned into the *Eco*RV site of pBluescript.

The α-toxin truncated genes *plcInv3* and *plc104* (developed in Chapter 3), of which their gene products have previously shown to protect mice from a challenge against *C. perfringens* (Chapter 4) were amplified without their CAT x 6 sequences (coding for affinity His-tags) with primers containing *Bgl*II and *Not*I sites at their 5' and 3' termini respectively. As the amplified products lacked a CAT x 6, their names were changed to *αInv* (for *plcInv3*) and *α104* (for *plc104*). The two products were cloned downstream of the two expression vectors pBPAGC21 and pBTac3 via the same RE sites of the vectors (Figures 5.2 to 5.5) creating 4 new expression vectors, pBTCαInv (Figure 5.2) and pBTCα104 (Figure 5.3) from the original pBTac3 vector and pBPCαInv (Figure 5.4) and pBPCα104 (Figure 5.5) from the original pBPAGC21.

The third promoter, the *htr*A promoter (P_{htrA}), was amplified from the genome of *S.* Typhimurium. Primers for its amplification were designed from the sequenced *S.* Typhimurium *htr*A deposited in GenBank (accession number 18802). The amplified P_{htrA} was first ligated to either the amplified *αInv* or *α104* via the common *Bgl*II sites present on the primers. Following confirmation of ligation via PCR using the forward P_{HtrA} primer (HtrAF), and the reverse α-toxin gene truncate primer (pRSETNotI), the newly created gene products were digested with *Xba*I and *Not*I, and directionally cloned into pBluescript to create expression vectors pBHAαInv and pBHAα104 (Figures 5.6 and 5.7).

5.3.2 Expression of α-toxin truncates in STMI

All recombinant plasmids were transformed into STM1 via the restriction minus, modification positive host *S.* Typhimurium LT2. Plasmids were purified using the Wizard miniprep kits (Figure 5.8) as the standard alkaline lysis miniprep procedure constantly resulted in poorly purified plasmid extracts, due to the high protein content of the bacterium.

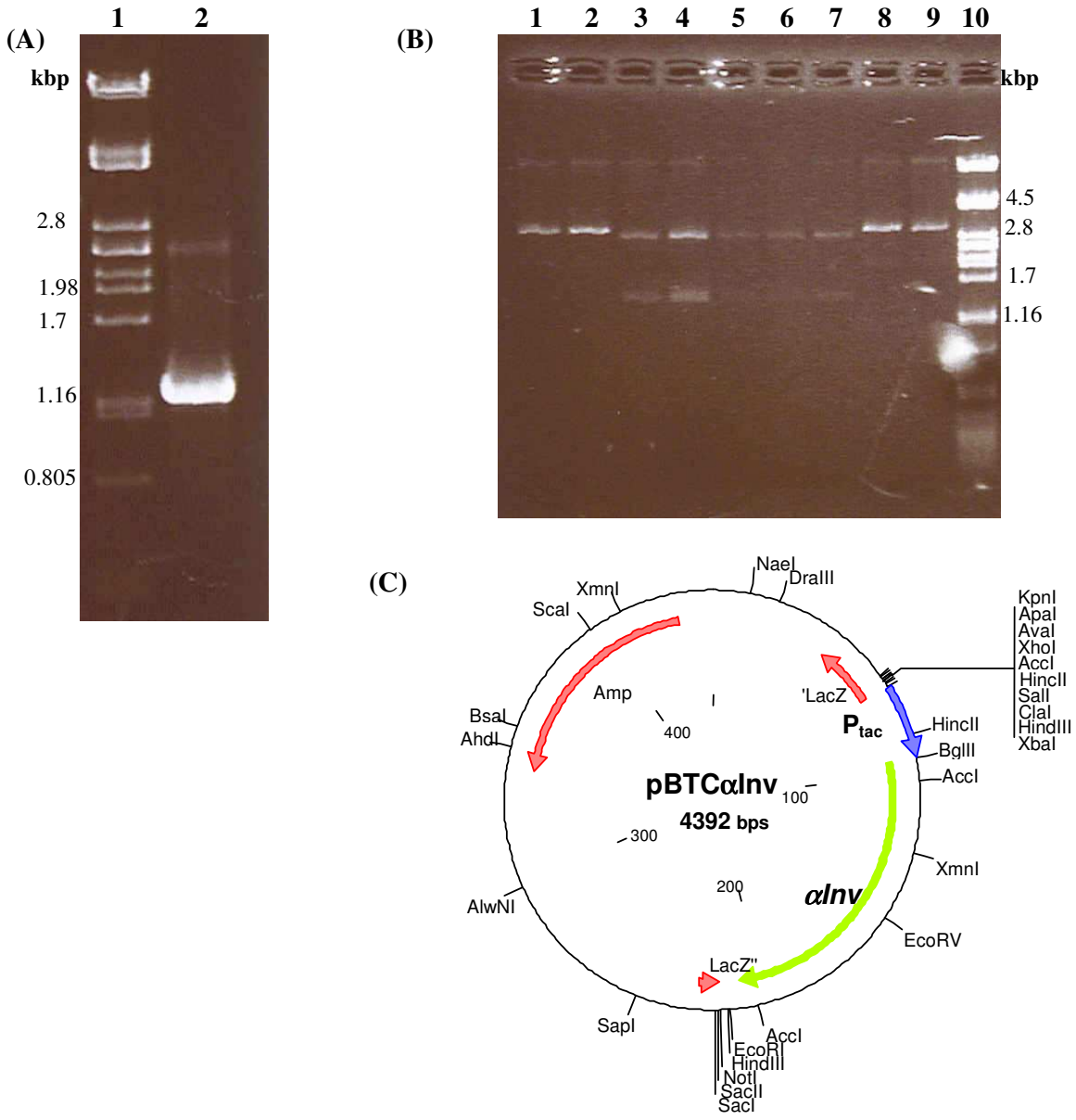


Figure 5.2: Construction of plasmid pBTCαInv. (A) The αInv gene fragment was amplified from plasmid pRplcInv9. Lane 1, λ-*Pst*I; Lane 2, amplification of αInv gene. **(B)** Cloning of *αInv* into pBTac3. Lanes 1-2 and 8-9, empty pBTac3 digested with *Hind*III; Lanes 3-7, pBTCαInv digested with *Hind*III; Lane 10, λ-*Pst*I. **(C)** Restriction map of pBTCαInv.

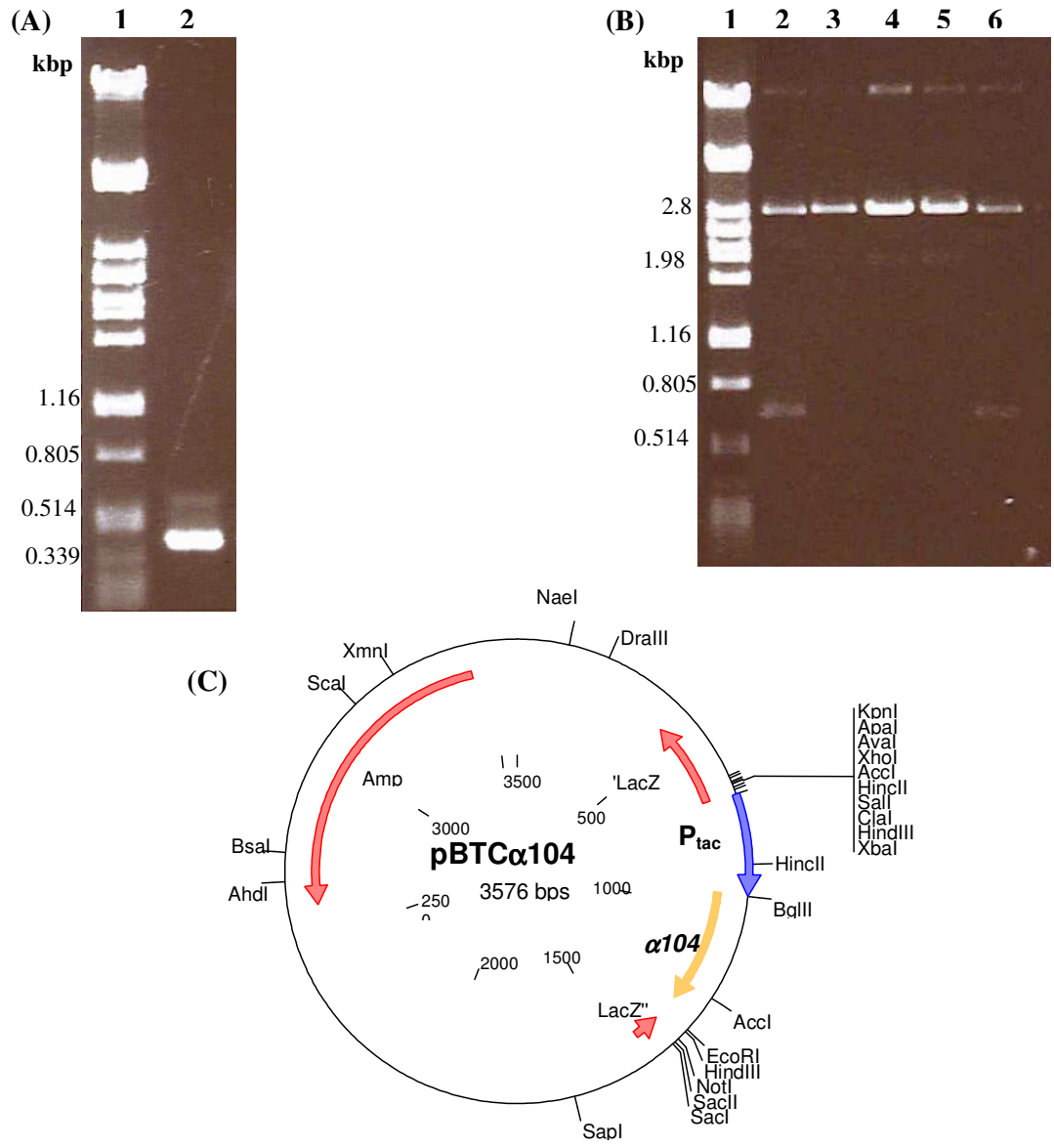


Figure 5.3: Construction of plasmid pBTCα104. **(A)** The *α104* was amplified from plasmid pRplc104. Lane 1, λ-*Pst*I; Lane 2, amplification of *α104*. **(B)** Cloning of *α104* into pBTac3. Lane 1, λ-*Pst*I; Lanes 2 and 6, pBTCα104 digested with *Xba*I and *Not*I, Lanes 3-5, empty pBTac3 digested with *Xba*I and *Not*I. **(C)** Restriction map of pBTCα104.

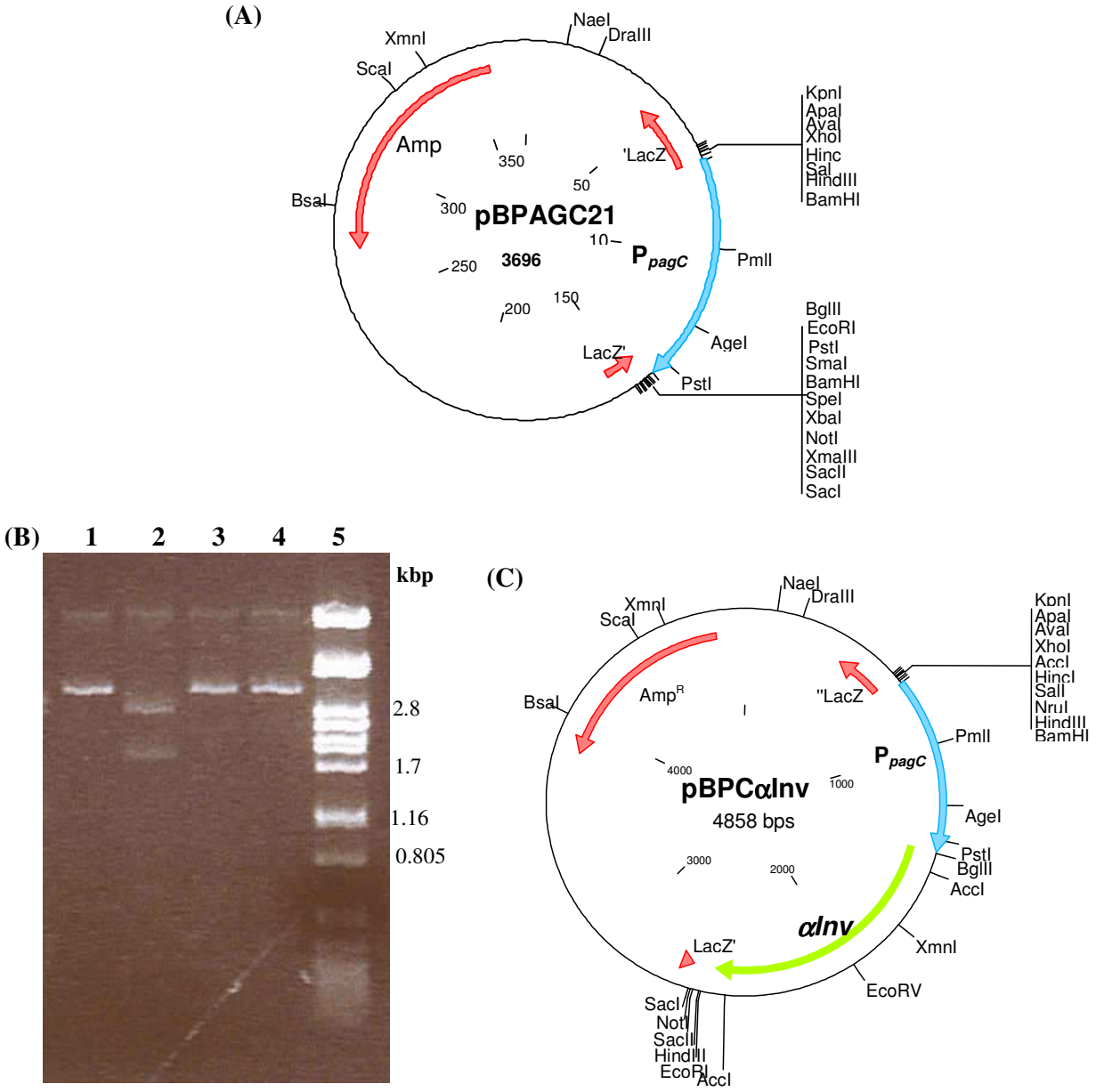
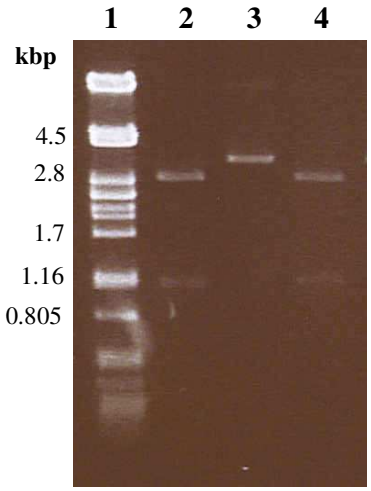


Figure 5.4: Construction of plasmid pBPCαInv. (A) Restriction map of pBPAGC21. **(B)** Cloning of *αInv* into pBPAGC21. Lanes 1, 3 and 4, empty pBPAGC21 digested with *Hind*III; Lane 2, pBPCαInv digested with *Hind*III. **(C)** Restriction map of pBPCαInv.

(A)



(B)

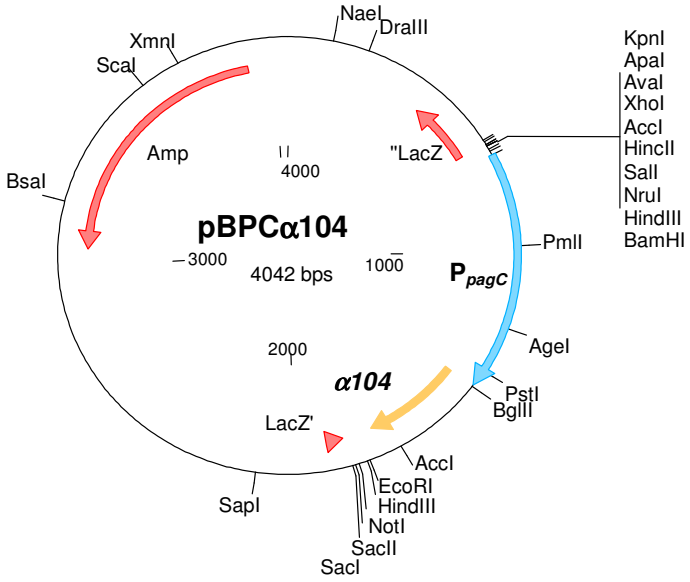


Figure 5.5: Construction of plasmid pBPCα104. **(A)** Cloning of *α104* into pBPAGC21. Lane 1, λ-*Pst*I; Lanes 2 and 4, pBPCα104 digested with *Hind*III; Lane 3, empty pBPAGC21 digested with *Hind*III. **(B)** Restriction map of pBPCα104.

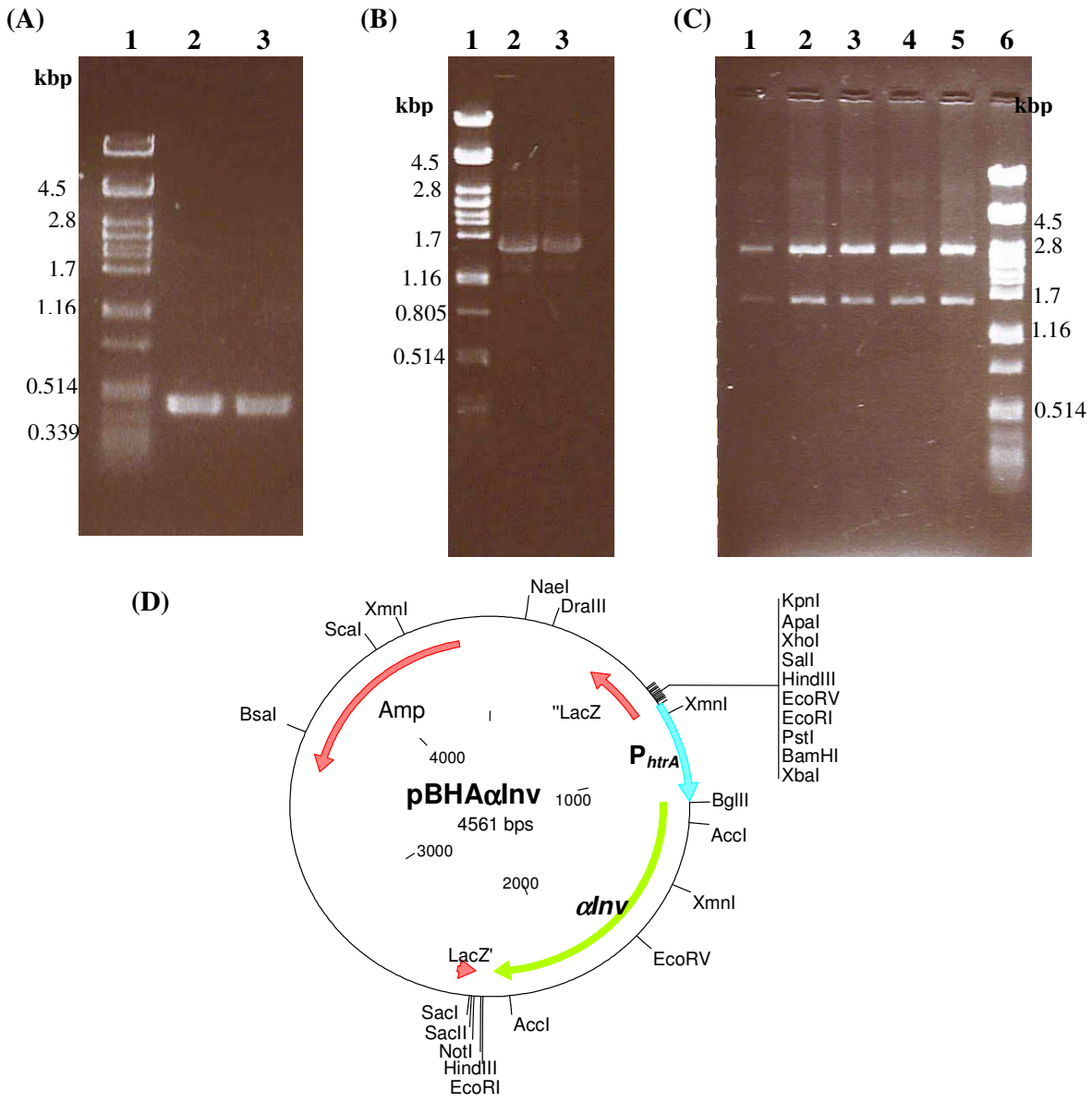


Figure 5.6: Construction of plasmid pBHAαInv. (A) PCR of P_{htrA} from *S.* Typhimurium. Lane 1, λ-*Pst*I; Lane 2-3, PCR of P_{htrA}. **(B)** PCR of the P_{htrA}:*αInv* ligation. Lane 1, λ-*Pst*I; Lanes 2-3, PCR of P_{htrA}:*αInv* ligation. **(C)** Directional cloning of the P_{htrA}:*αInv* fragment into pBluescript. Lanes 1-5, pBHAαInv digested with *Hind*III; Lane 6, λ-*Pst*I. **(D)** Restriction map of the plasmid pBHAαInv.

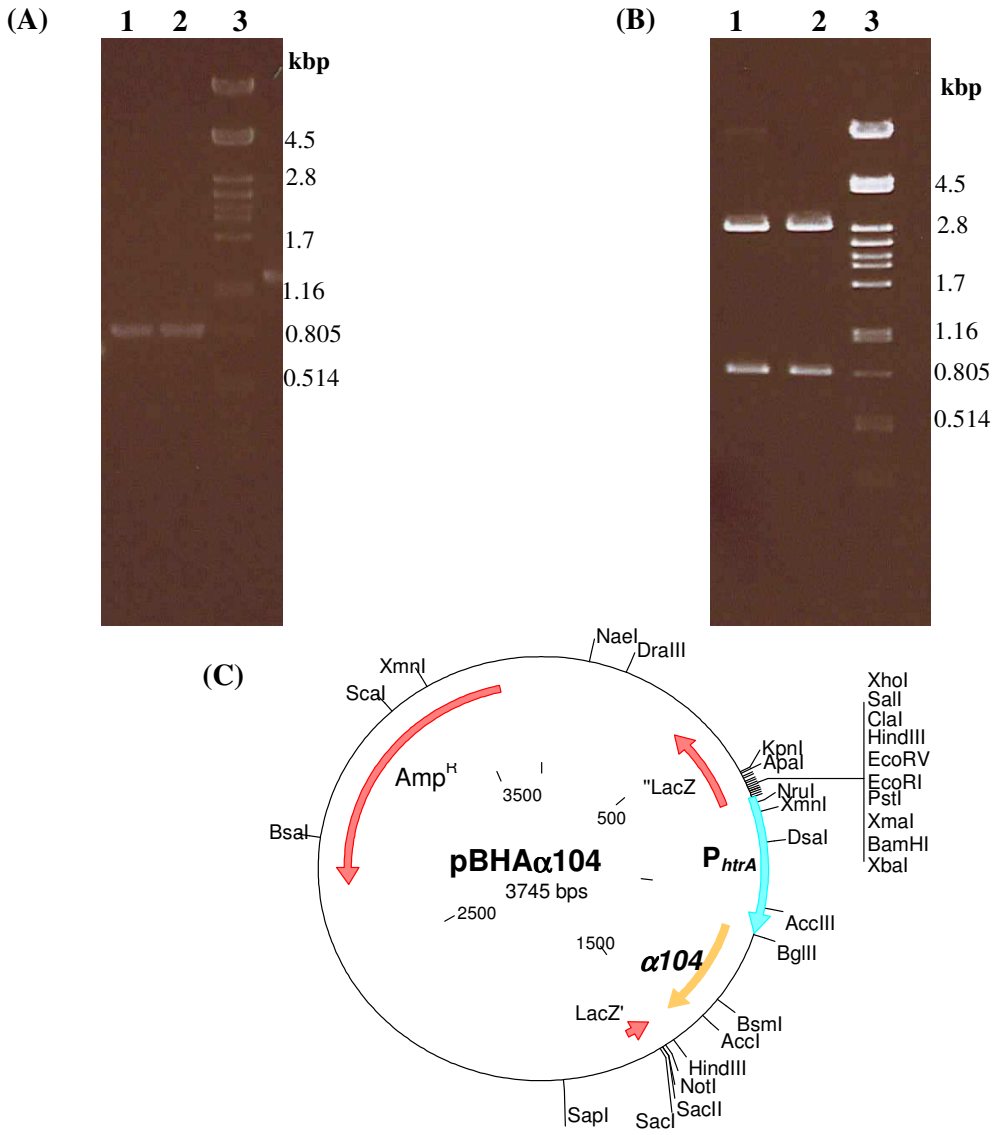


Figure 5.7: Construction of plasmid pBHAα104. **(A)** PCR of P_{htrA}:*α104* ligation. Lanes 1-2, PCR of P_{htrA}:*α104* ligation; Lane 3, λ-*Pst*I marker. **(B)** Directional cloning of P_{htrA}:*α104* fragment into pBluescript. Lanes 1-2, pBHAα104 digested with *Hind*III; Lane 3, λ-*Pst*I. **(C)** Restriction map of the plasmid pBHAα104.

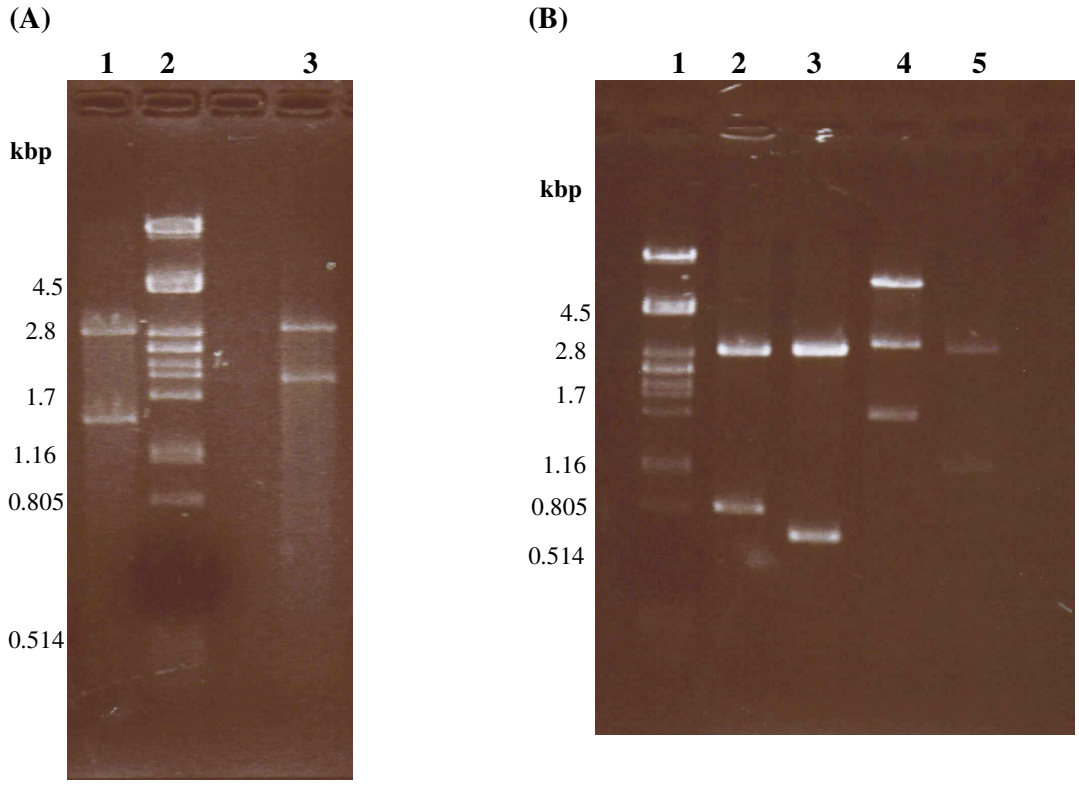


Figure 5.8: Isolation of expression plasmids containing α-toxin truncated gene inserts from vaccine strain STM1. (A) Plasmids pBTCαInv (Lane 1) and pBPCαInv (Lane 3) digested with *Hind*III; Lane 2, λ-*Pst*I. **(B)** Plasmids pBHAα104 (Lane 2), pBTCα104 (lane 3), pBHAαInv (Lane 4) and pBPCα104 (Lane 5) digested with *Hind*III; Lane 1, λ-*Pst*I.

Transformation efficiency of pBHAαInv into STM1 was very low (4-6 colonies) and growth of the resultant transformant STM(pBHAαInv) was much poorer than the other vaccines developed. Mid-log phase was constantly reached at about 6 h of growth as opposed to 3-4 h for the other STM1 vaccines.

5.3.2.1 Expression of α-toxin truncated proteins from pBPCαInv and pBPCα104

Expression of αInv and α104 from the P_{pagC} was controlled by the presence or absence of $MgCl_2$. Expression of αInv from STM1(pBPCαInv) was induced in the absence of $MgCl_2$, and in the presence of low levels of $MgCl_2$ (1 and 10 mM) whereas high levels (100 mM) of $MgCl_2$ repressed the expression of protein αInv (Figure 5.9A).

Expression of α104 from STM1(pBPCα104) could not be detected in the immunoblot in the absence or presence of $MgCl_2$.

5.3.2.2 Expression of α-toxin truncated proteins from pBTCαInv and pBTCα104

Expression of αInv from STM1(pBTAαInv) was constitutive from P_{tac} (Figure 5.9C) and did not require specific induction of any kind. As with STMI(pBPCα104), expression of α104 from STM1(pBTCα104) was not detected in the immunoblots probed with horse anti-α-toxin polyclonal antisera.

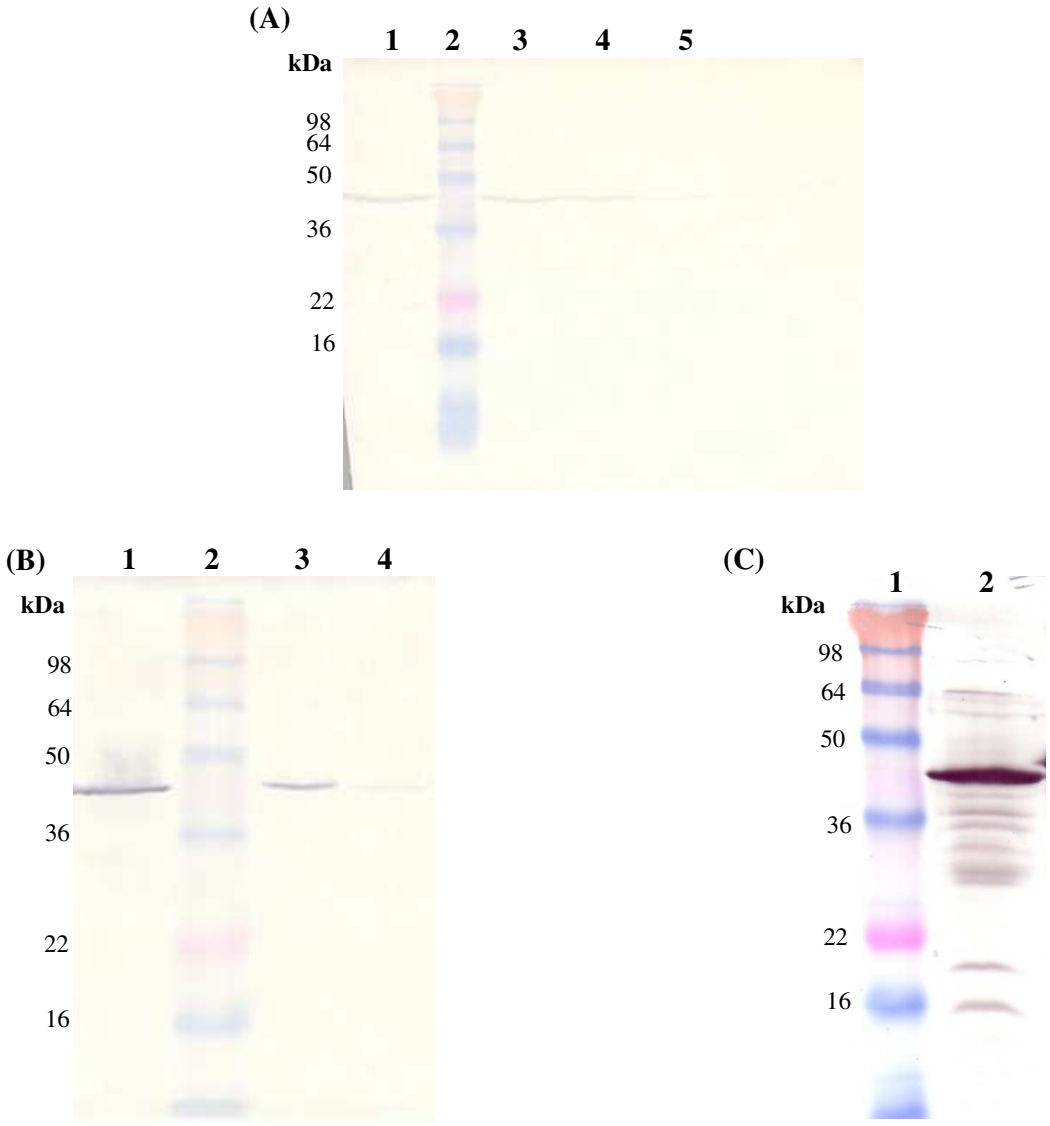


Figure 5.9: The detection of αInv expression from STM1 harbouring expression plasmids via immunoblots probed with horse anti-α-toxin polyclonal antibodies. (A) STM1(pBPCαInv) induced with: 0 mM $MgCl_2$ (Lane 1), 1 mM $MgCl_2$ (Lane 3), 10 mM $MgCl_2$ (Lane 4), 100 mM $MgCl_2$ (Lane 5); Lane 2, SeeBlue protein marker **(B)** STM1(pBHAαInv) induced with: 1 mM H_2O_2 (Lane 1), 10 mM H_2O_2 (Lane 3), or 0 mM H_2O_2 (Lane 4); Lane 2, SeeBlue protein marker. **(C)** STM1(pBTCαInv): Lane 1, SeeBlue protein marker; Lane 2, STM1(pBTCαInv).

5.3.2.3 Expression of α-toxin truncated proteins from pBHAαInv and pBHAα104

Expression of αInv from P_{htrA} was controlled by the addition of H_2O_2. In the absence of H_2O_2, a very low level of αInv expression could be detected from STM1(pBHAαInv) in immunoblots probed with polyclonal anti-α-toxin antisera, but low levels (1 mM) of H_2O_2 enhanced the expression and detection of αInv from the same strain (Figure 5.9B). The addition of 10 mM H_2O_2 dramatically reduced the level of expression of αInv when compared to the addition of 1 mM H_2O_2 but expression was still higher than in the absence of H_2O_2.

As with expression from plasmids pBPCα104 and pBTCα104, expression of α104 from STM1(pBHAα104) could not be detected in the immunoblots.

5.3.3 Stability assays

The stability of the recombinant plasmids within STM1 in the absence of the selective antibiotic ampicillin was determined *in vitro*. STM1 harbouring recombinant plasmids were incubated in LB in the absence of ampicillin, and then spread plate onto HA with or without ampicillin.

The percentage of STM1 cells retaining the different recombinant plasmids was determined over a 5 day period (Figure 5.10).

STM1(pBPAGC21) was used as a reference between each stability assay as this plasmid contains no gene downstream of P_{pagC}, and therefore represents an expressionless plasmid. Although, pBPAGC21 is lost over time, it is a gradual loss, with some colonies tested retaining the plasmid longer than others (as depicted by the standard deviation bars).

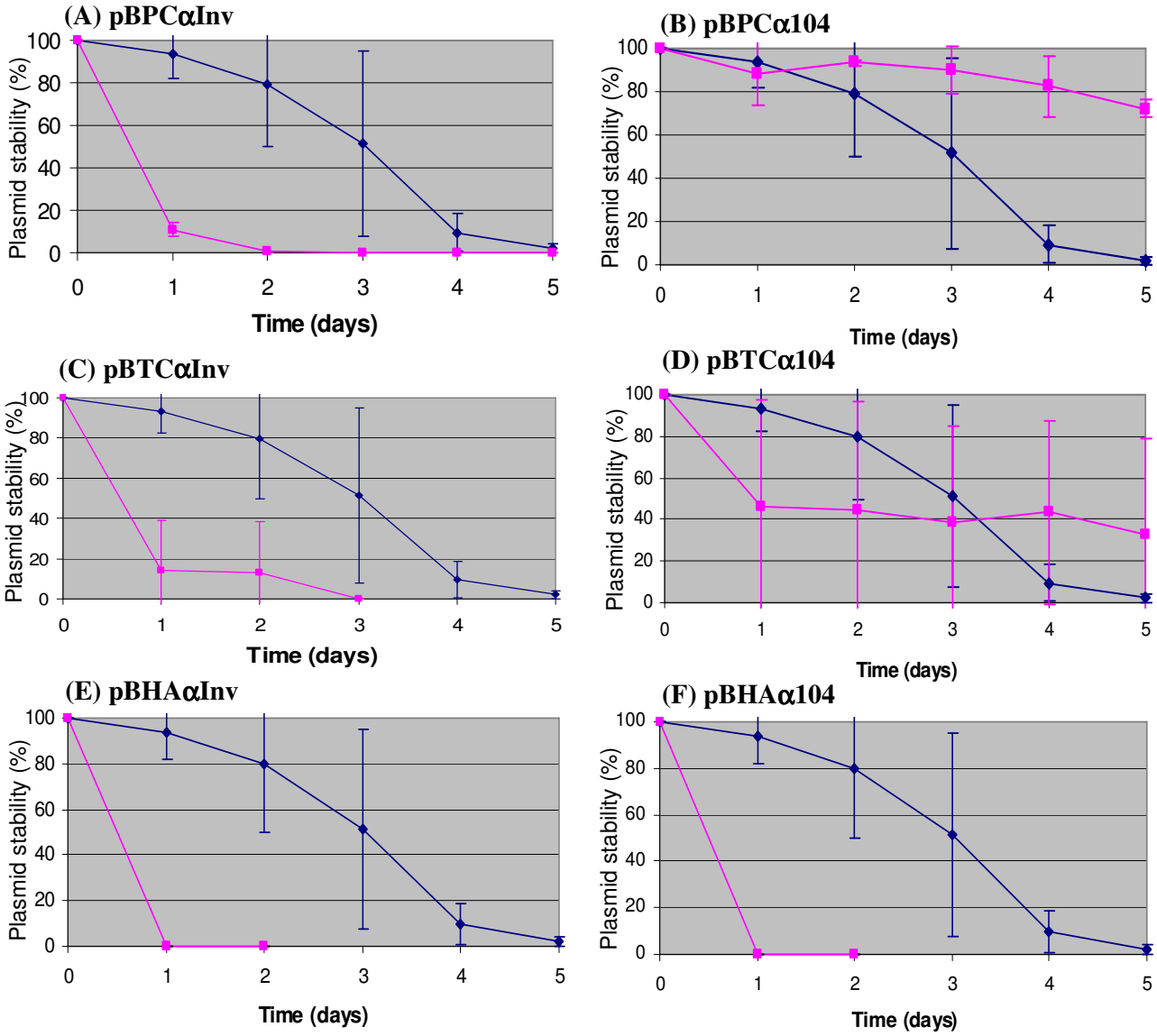


Figure 5.10: Stability of expression plasmids within STM1 in the absence of the selective antibiotic ampicillin. The control plasmid pBPAGC21 (no α-toxin gene insert) was included with each graph (dark blue) as a reference for comparison between plasmids. The pink line within the graphs represents the mean results of triplicate plasmid stability assays of: (A) pBPCαInv (B) pBPCα104 (C) pBTCαInv, (D) pBTCα104, (E) pBHAαInv or (F) pBHAα104. Standard deviations are shown as positive and negative bars.

By the end of day one most of the STM1 colonies retained the plasmid, and by day two there was a slight drop with approximately 80% of the colonies retaining the plasmid.

Both recombinant plasmids pBHAαInv and pBHAα104 carrying P_{htrA} were lost within 1 day (approximately 20 generations) in all 3 colonies selected (Figure 5.10E & F).

STM1 strains carrying recombinant plasmids controlled by P_{tac} were also not very stable. A large deviation can be seen in the pBTCα104 graph (Figure 5.10D). This deviation is due to the fact that 2/4 colonies examined were more stable over a longer period of time than the other 1 examined. This prompted the examination of a fourth colony, which also resulted in the rapid loss of the plasmid.

Plasmid pBTCαInv was also unstable (Figure 5.10C). Two of three colonies examined lost their plasmid within 1 day, whereas 6% of cells from the third colony examined retained the plasmid. A fourth colony was examined and found to retain the plasmid for up to 2 days (50%) which was then was completely lost by the following day.

Even though plasmid pBPCαInv was being expelled from STM1 as early as 1 day (20 generations), this plasmid was still present up until day 3 (0.05%). Each colony examined gave a similar result (small deviations).

Recombinant plasmid pBPCα104 was maintained within STM1 longer than the other plasmids, including pBPAGC21. The plasmid was present in 72% of colonies by the end of day 5 (approximately 100 generations).

5.3.4 Immunisation of BALB/c mice

The ability of STM1 harbouring the recombinant plasmids to induce specific immune responses targeted towards the α-toxin of *C. perfringens* was examined. Mice were orally vaccinated with STM1 harbouring each of the recombinant plasmids. Production of specific IgG targeted against the recombinant gene product (αInv or α104) expressed from STM1 was assessed using an ELISA coated with plc3 (α-toxin). Anti-α-toxin IgG responses were quite low and specific titres did not reach an OD_{450} of 0.2 (generally the end-point of the assay). Therefore the absorbance of the wells following sulphuric acid addition was measured at a dilution of 1 in 20 (Figure 5.11). No significant immune responses against α-toxin were observed in any of the vaccines tested when compared to control strain STM1(pBPAGC21).

Cytokine profiles of the same groups of mice were examined using the ELIspot assay. Capture antibodies against interferon-gamma (IFN-γ) and interleukin-4 (IL-4) were coated onto 96-well nitrocellulose backed plates. Following the addition of homogenised splenocytes and stimulation of cytokine production with α-toxin (plc3), cytokine producing cells were probed with biotin-labelled anti-cytokine antibodies. The IFN-γ or IL-4 producing cells were then detected following EXTRAvidin binding, and substrate addition. The number of IFN-γ producing cells was very low in all vaccines examined (Figure 5.12).

STM1(pBHAαInv) produced, on average 4 cells secreting IFN-γ out of 10^6 cells in total. The groups of mice vaccinated i.p with toxoid emulsified in FIA also induced only a few IFN-γ secreting cells. Concanavalin A stimulated-cells induced the secretion of IFN-γ from so many splenocytes that it was impossible to count individual cells (over 600).

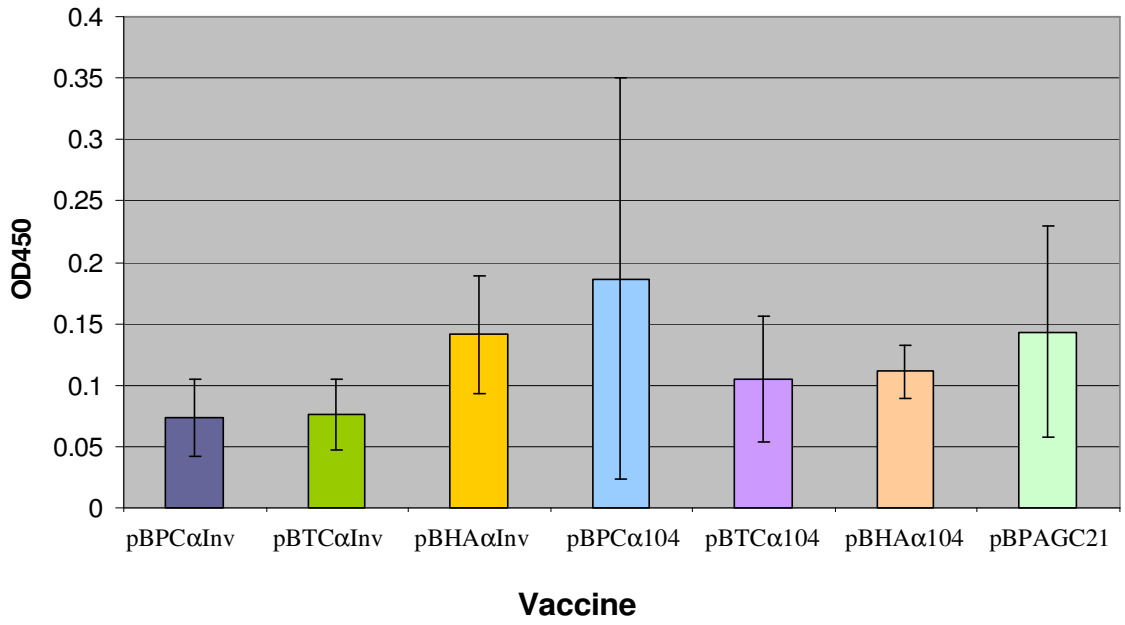


Figure 5.11: Anti-α-toxin IgG response of mice immunised with STM1 harbouring expression plasmids for the expression of α-toxin truncated genes. Serum was obtained 20 days following the final vaccination and assayed for anti-α-toxin IgG against purified plc3 (α-toxin) by an indirect ELISA. Each column represents the average absorbance at OD_{450} of 5 mice at a dilution of 1 in 20 (+-/ standard deviation). No significant differences were observed between groups of mice.

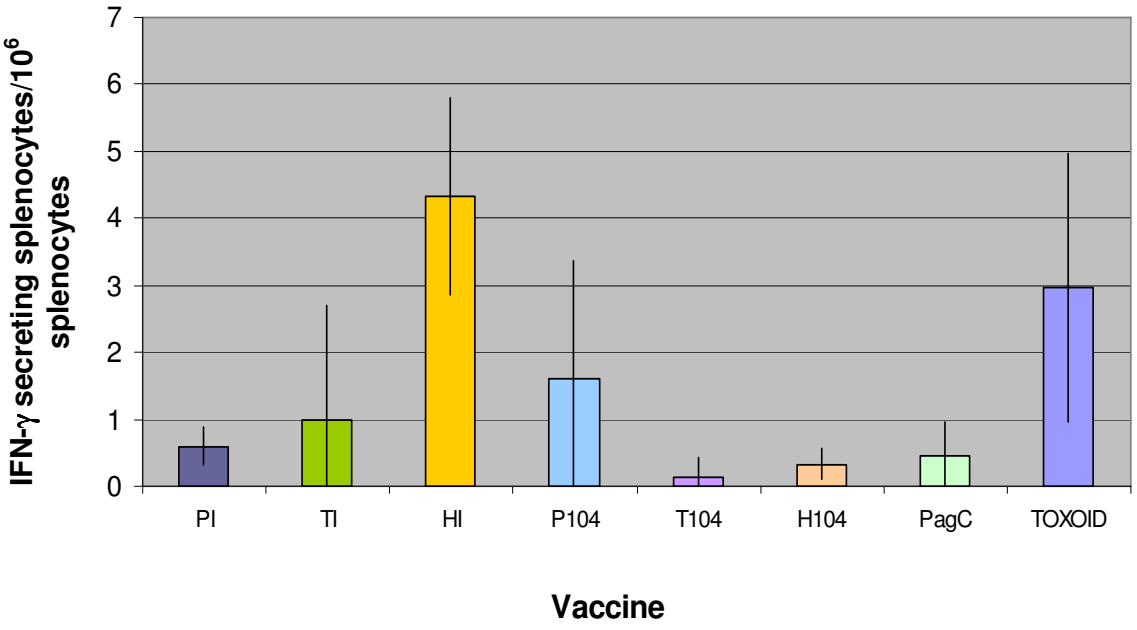


Figure 5.12: The induction of an interferon gamma response of splenocytes isolated from STM1 (harbouring expression plasmids) vaccinated mice via stimulation by α-toxin (plc3). Each column is representative of the average cell count of 5 mice vaccinated with STMI harbouring plasmids (+/- standard deviation): pBPCαInv (PI), pBTCαInv (TI), pBHAαInv (HI), pBPCα104 (P104), pBTCα104 (T104), pBHAα104 (H104), the negative control pBPAGC21 (PagC) or toxoid.

A much larger population of cells secreted IL-4 following stimulation by α-toxin (Figure 5.13). In fact all vaccines carrying the α-toxin truncated genes stimulated enhanced IL-4 secretion from spleen cells ($p<0.05$) when compared to the control group pBPAGC21, the group lacking an α-toxin truncate gene.

Vaccination with STM1(pBHAα104) and STM1(pBPCαInv) resulted in the highest number of splenocytes secreting IL-4 and were significantly higher than groups STM1(pBHAαInv) and STM1(pBPCα104) ($p<0.05$).

Toxoid immunised mice also resulted in IL-4 secreting splenocytes. On average, this vaccine group stimulated the production and secretion of IL-4 from about 220 splenocyte per 10^6 spleen cells. A significant difference between toxoid immunised mice and STM1(plasmid) immunised mice was not observed due to the large variation in IL-4 secretion from the toxoid immunised mice. Concanavalin-A also stimulated the production of IL-4 from spleen cells, and again there were too many cells too count (over $600/10^6$ cells).

5.3.5 Challenge of mice immunised with STMI harbouring recombinant α-toxin expression plasmids

Mice were orally vaccinated with STM1 harbouring the recombinant vaccine plasmids and prior to challenge with *C. perfringens* 60, serum from mice was collected for IgG detection (Figure 5.14). Antibody levels were quite low, with only 3 of the vaccines (pBTCαInv, pBHAαInv and pBPCα104) reaching an average absorbance (OD_{450}) above 0.2 when sera was examined at a dilution of 1/20.

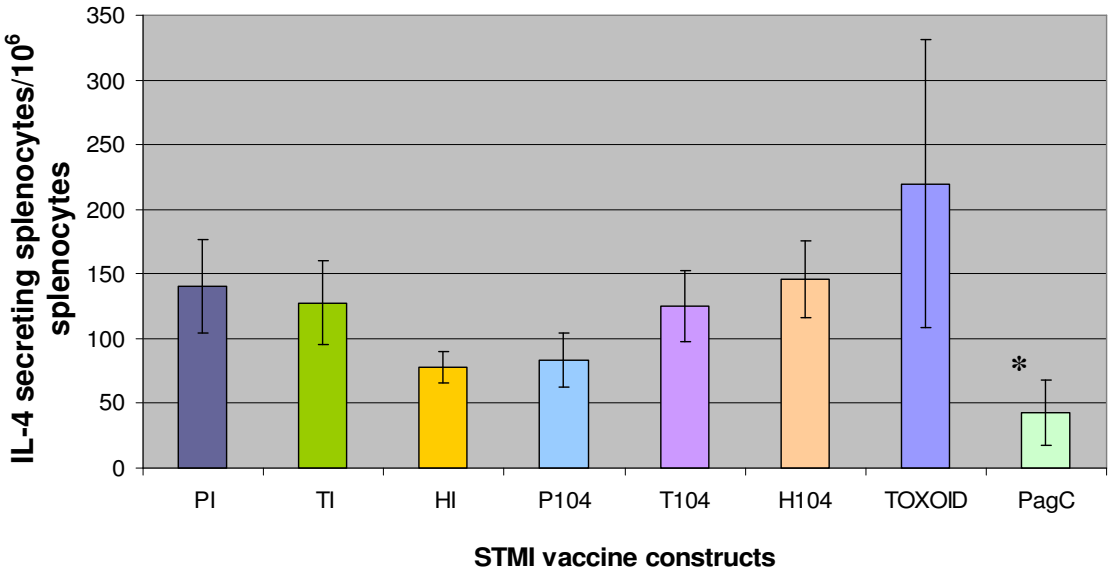


Figure 5.13: The induction of an interleukin-4 response of splenocytes isolated from STM1 (harbouring expression plasmids) vaccinated mice via stimulation by α-toxin (plc3). Each column is representative of the average cell count of 5 mice (+/- standard deviation) vaccinated with STMI harbouring plasmids: pBPCαInv (PI), pBTCαInv (TI), pBHAαInv (HI), pBPCα104 (P104), pBTCα104 (T104), pBHAα104 (H104), the negative control pBPAGC21 (PagC) and toxoid.

*Splenocytes from all STM1 constructs harbouring α-toxin gene truncates produced a significant amount of IL-4 when stimulated with α-toxin *in vitro* ($p<0.05$) when compared to the control STM1(pBPAGC21).

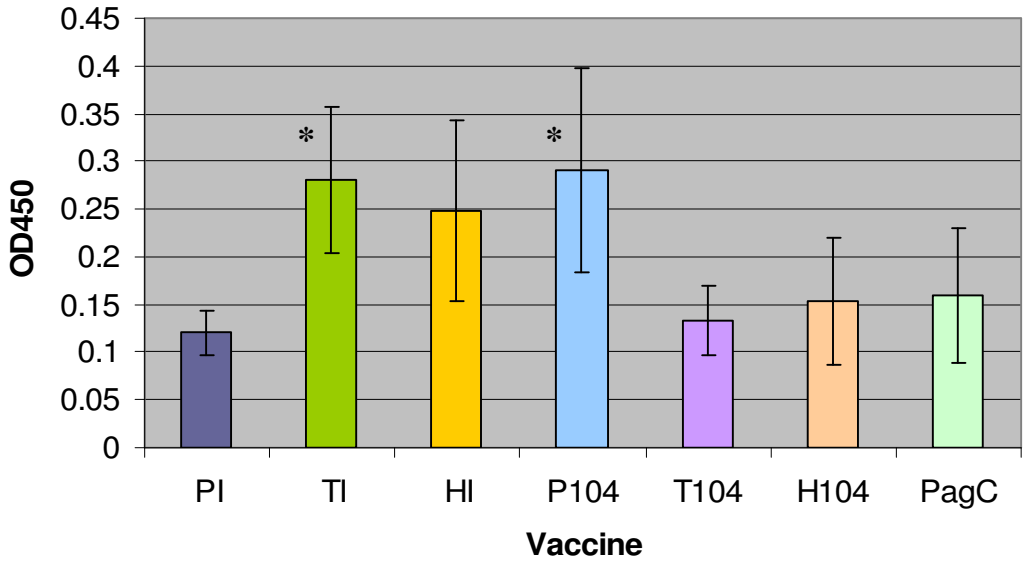


Figure 5.14: Serum anti-α-toxin IgG responses of mice vaccinated with STM1 harbouring plasmids for the expression of αInv and α104 prior to challenge with *C. perfringens*. Serum obtained from mice 11 days following the final (third) vaccination was analysed using an ELISA for the presence of anti-α-toxin-IgG. Each column is representative of the average absorbance at OD_{450} of 6 mice when serum was examined at a diluted of 1/20 (+/- standard deviation).

*TI and P104 are significantly different from PagC ($p<0.05$) according to the 2-tailed independent *t*-tests.

Plasmid abbreviations: pBPCαInv (PI), pBTCαInv (TI), pBHAαInv (HI), pBPCα104 (P104), pBTCα104 (T104), pBHAα104, or pBPAGC21 (PagC).

Pooled sera were examined for the presence of anti-*S.* Typhimurium IgG (Figure 5.15). It is quite clear that STM1 cells harbouring the control expressionless plasmid pBPAGC21 induced the production of a much higher IgG response towards *S.* Typhimurium antigens in mice when compared to STM1 harbouring expressed α-toxin gene truncates. Antibodies from the toxoid immunised mice also recognised some *S.* Typhimurium antigen, but this was most likely due to the presence of minute quantities of *E. coli* proteins that may have been present following purification of α-toxin from TOP10F' cells prior to toxoiding and vaccination (Chapter 3).

The percentage of the cumulative score over the maximum score achievable was used to graph the development of gas-gangrene following the *C. perfringens* challenge (Figure 5.16). Most mice developed clinical signs of gas-gangrene and even one toxoid vaccinated mouse succumbed to the challenge, with gangrene encompassing the challenged thigh. The percentage of mice affected by the challenge at the end of the scoring period is shown in Table 5.5.

The vaccine groups with lower overall gangrenous signs included STM1(pBHAα104), STM1(pBTCαInv), and STM1(pBPCαInv).

These groups exhibited a reduction and latency in the development of gas-gangrene and were also the groups with lower percentages of overall infection (Table 5.5). Although the results of vaccine group STM(pBHAα104) may be skewed as 2 mice from this group died during the vaccine trial prior to challenge (from unrelated causes) therefore reducing the power of the results. Following termination of the experiment, swabs (from 1-2 mice per group) of the infected and uninfected thigh, liver and spleen were taken and cultured for the presence of *C. perfringens* (incubated both aerobically and anaerobically on SBA and EYA).

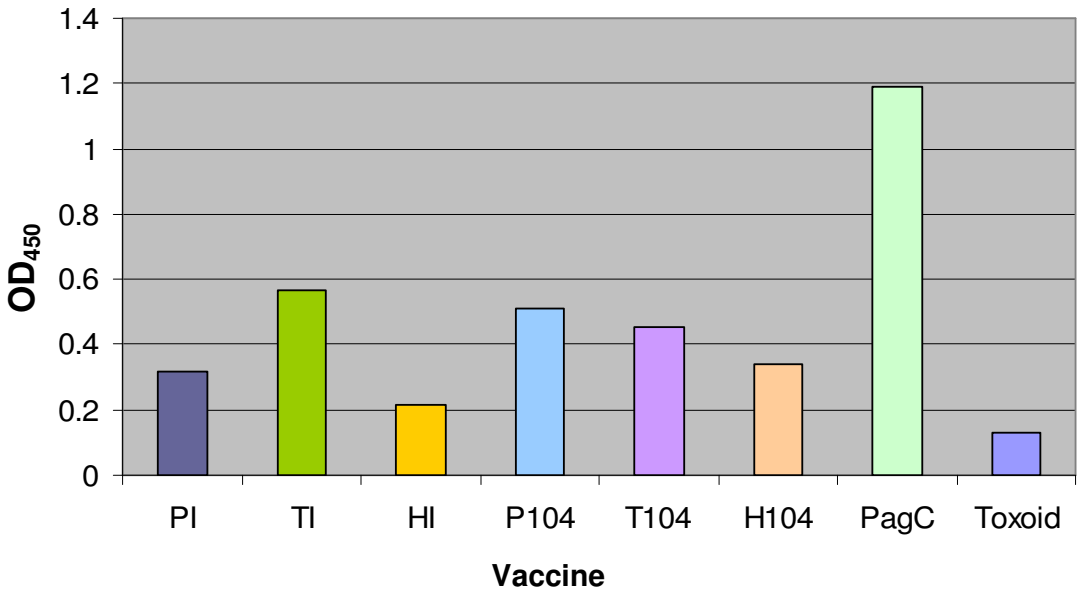


Figure 5.15: Serum anti-*S*. Typhimurium IgG immune response of mice vaccinated with STMI harbouring plasmids for the expression of αInv and α104 prior to challenge with *C. perfringens*. Serum obtained from mice 11 days following the final vaccination was assayed for anti-*Salmonella* IgG against *S*. Typhimurium whole cell antigens by ELISA. Each bar on the graph represents the OD_{450} value of pooled sera when examined at a dilution of 1/640.

Plasmid abbreviations: pBPCαInv (PI), pBTCαInv (TI), pBHAαInv, pBPCα104 (P104), pBTCα104 (T104), pBHAα104, or pBPAGC21 (PagC).

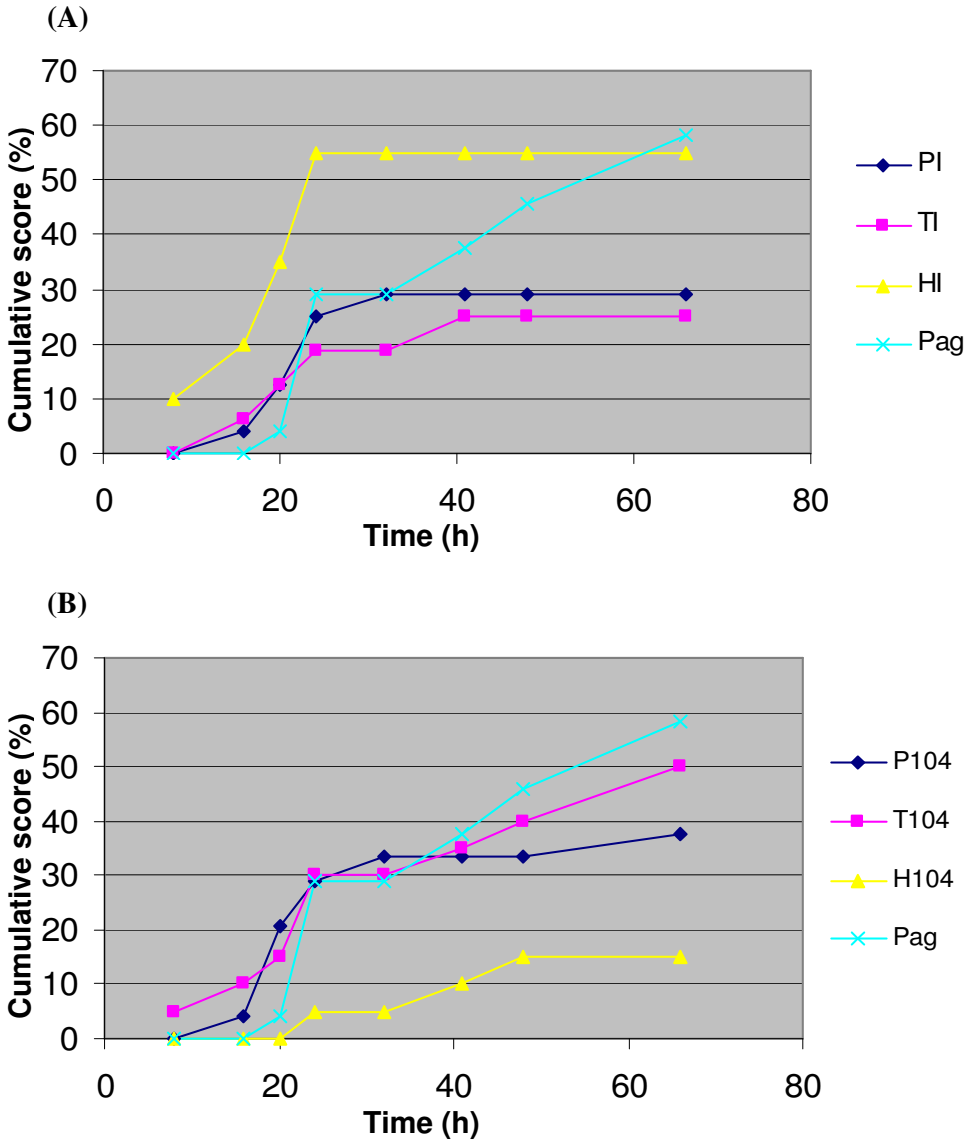


Figure 5.16: Gross pathology of mice infected with 5 x 10^6 *C. perfringens* cells following oral vaccination with STM1 harbouring α-toxin truncate expression plasmids. (A) pBPCαInv (PI), pBTCαInv (TI), pBHAαInv (HI) or **(B)** pBPCα104 (P104), pBTCα104 (T104), or pBHAα104 (H104). pBPAGC21 (Pag) was used as the control in the experiments and consisted of an expressionless plasmid. A 50 µL dose of *C. perfringens* was administered to the left thigh of BALB/c mice. Mice were then observed over a 66 hour period for the overt signs of disease (Table 4.3). The data reflects the % cumulative score of disease progression over 66 h.

Table 5.5: Percentage of mice within each vaccine group afflicted with signs of gas-gangrene (at 66 h).

Vaccine	Percentage of mice affected
pBPCαInv	66.6
pBHAαInv	80
pBTCαInv	75
pBHAα104	40
pBPCα104	83
pBTCα104	80
Toxoid	16.6
pBPAGC21	100

All mice examined-including the toxoid vaccinated group, showed the presence of *C. perfringens* in the infected thigh. The infection was localised, but bacteria survived.

The Pearson product-moment correlation (Zar, 1999) indicated a strong negative association between the number of IL-4 secreting cells and the level of gangrene development in the different vaccine groups (r^2=0.63, $p<0.05$) (Figure 5.17). STM1 vaccines resulting in a reduced infection in mice were also the same vaccines which resulted in higher IL-4 producing splenocytes. This included the vaccines STM1(pBPCαInv), STM1(pBTCαInv) and STM1(pBHAα104).

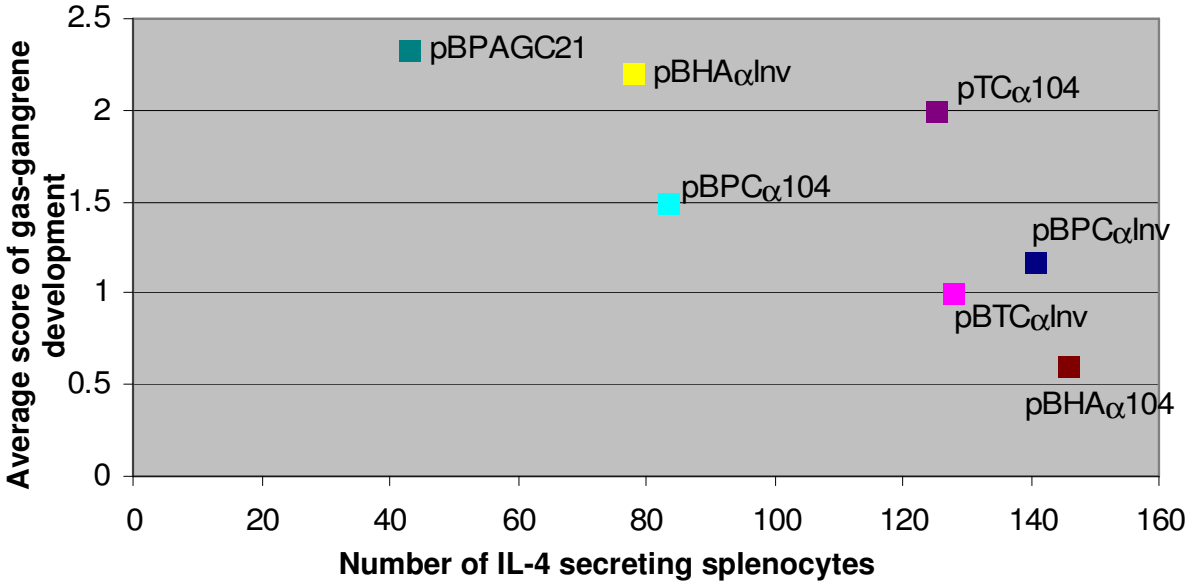


Figure 5.17: Correlation between the induction of IL-4 release by stimulated splenocytes and levels of gas-gangrene development in groups of vaccinated mice. Data represent the average score of disease and the average number of IL-4 producing cells per vaccine group. The Pearson product-moment correlation indicated a strong negative association between the number of IL-4 secreting cells and the level of gangrene development in the different vaccine groups (r^2=0.63, $p<0.05$).

5.4 Discussion

STM1, an attenuated *S.* Typhimurium (Δ*aro*A), is a registered vaccine currently used to immunise poultry to effectively eliminate or reduce the numbers of *S.* Typhimurium within their gastrointestinal tract (Alderton *et al.*, 1991; Coloe *et al.*, 1995; Jackson and Underwood, 2006). STM1 has the potential to be used as a combined vaccine by the incorporation of a heterologous antigenic gene carried on a plasmid, in effect stimulating cell-mediated and humoral responses to itself and the carrier protein (Khan *et al.*, 1994; Roberts *et al.*, 1998; Wang *et al.*, 1999b). The oral immunisation of mice with attenuated *S.* Typhimurium expressing heterologous antigens, is also able to elicit secretory IgA against the heterologous antigen within the intestinal mucosa of the host (Maskell *et al.*, 1987; Paton *et al.*, 1993; Ward *et al.*, 1999; Wang *et al.*, 1999b; Allen *et al.*, 2000). Necrotic enteritis of chickens is an enteric disease and vaccination with antigenic determinants delivered by a vector such as STM1 capable of stimulating intestinal secretory responses, along with other cell-mediated and humoral responses, may have the potential of protecting chickens from the disease.

The α-toxin of *C. perfringens* is a known virulence factor important in the pathogenicity of necrotic enteritis in chickens and other agriculturally important animals, and gas-gangrene in humans (Al-Sheikhly and Truscott, 1977b; Fukata *et al.*, 1988; Awad *et al.*, 1995; Songer, 1996; Awad *et al.*, 2001). Mutants of the α-toxin have been developed (Chapter 3) and immune responses induced following vaccination with the mutated α-toxin proteins have shown to provide protection against a *C. perfringens* challenge (Chapter 4). The current study was aimed at determining the potential of using STM1 as a delivery vehicle to direct the delivery and expression of the α-toxin truncated proteins αInv and α104 within mice.

Expression of recombinant antigens *in vitro* from commercial vectors is usually controlled by chemicals such as IPTG. In this study, a number of recombinant plasmids were created for the specific induction of protein expression under environmentally controlled conditions. These conditions would induce the expression of proteins on entry of STM1 into macrophages and therefore reduce the risks of overloading the metabolic machinery due to constitutively expressed heterologous proteins from high copy vectors.

The *pag*C is part of a locus within the *S.* Typhimurium genome controlled by a two component regulatory system, the PhoQ/PhoP system (Garcia Vescovi *et al.*, 1996; Vescovi *et al.*, 1997). The sensor, PhoQ represses transcription of the PhoP regulated genes in the presence of elevated $MgCl_2$ levels. When present in the extracellular environment of the eukaryote host, the PhoP/PhoQ system is switched off, repressed by the presence of high (>1mM) levels of $MgCl_2$. Once inside the membrane-bound *Salmonella*-containing vacuoles of macrophages, where $MgCl_2$ levels are very low (<50 μM), the system is switched on and activation of PhoP by PhoQ results in the expression of numerous virulence genes including *pag*C (Garcia Vescovi *et al.*, 1996; Vescovi *et al.*, 1997).

Expression of heterologous proteins from P_{pagC} have previously shown to induce a high level of antibody towards heterologous proteins, therefore this system was chosen to determine its ability to express, and elicit immune responses targeted to α-toxin truncated proteins (Dunstan *et al.*, 1999; Bullifent *et al.*, 2000; Chen and Schifferli, 2001).

The vector pBPCαInv containing P_{pagC} and *αInv* as shown in Figure 5.9A induces high levels of αInv expression at low levels of $MgCl_2$. Although levels up to 10 mM did not completely repress the expression, it did result in reduced expression. At a level of 100 mM $MgCl_2$ αInv expression was not detected by immunoblots indicating the repression of P_{pagC}.

The *htr*A is a stress response gene in *S*. Typhimurium (Johnson *et al.*, 1990; Johnson *et al.*, 1991). It is a virulence gene required for the survival of bacteria within macrophages during induction of an oxidative burst (e.g. release of NADPH oxidase) which occurs within the *Salmonella*-containing vacuoles of macrophages (Johnson *et al.*, 1990; Johnson *et al.*, 1991; Mutunga *et al.*, 2004). Like P_{pagC}, P_{htrA} has also been used for the expression of heterologous antigens within attenuated *S*. Typhimurium, and when administered to mice has resulted in the induction of strong immune response towards the heterologous antigen, and protection against challenge (Roberts *et al.*, 1998; Foynes *et al.*, 2003).

Oxidative stress can be mimicked *in vitro* by the addition of H_2O_2 to the media. The *htr*A promoter (P_{htrA}) used in this study was activated in the presence of H_2O_2 (Figure 5.9B). Other authors have documented the induction of protein expression from P_{htrA} in the presence of elevated temperatures (42ºC) (Roberts *et al.*, 1998; Foynes *et al.*, 2003). This was also tested but no αInv or α104 expression was evident at 42ºC. The discrepancies cannot be explained at present, although the *htr*A of *E. coli* (also known as *deg*P) has been shown to be expressed at both elevated temperatures and during oxidative stress (Pogliano *et al.*, 1997; Skorko-Glonek *et al.*, 1999).

The tac promoter (P_{tac}) is a hybrid promoter composed of the -10 region of *lac*UV5 and the -35 region of the *trp* promoter making it a high level inducible promoter (Amann *et al.*, 1983; de Boer *et al.*, 1983). Normally this promoter is repressed by the product of *lac*I. Due to the lack of machinery required to ferment lactose and hence the absence of the repressor lacI within *S*. Typhimurium, P_{tac} results in constitutive expression of the downstream gene within STM1 (Sellitti *et al.*, 1987; Chatfield *et al.*, 1992a). This promoter was chosen as a comparison with the *in vivo* inducible promoters for the expression of αInv and α104.

Protein αInv was constitutively expressed from STM1 carrying P_{tac} (pBTCαInv) as expected (Figure 5.9C).

Whilst αInv was expressed from all expression vectors, expression of α104 from each vector was unsuccessful under all inducive conditions examined. The α104 gene was amplified from pRplc104, a pRSETA expression vector containing the C-terminal domain of the α-toxin of *C. perfringens*. Expression from this vector resulted in a stable product containing an affinity N-His tag which included a total of 42 aa's prior to the start of the plc104 sequence. Amplification and cloning of this gene into STM1 expression plasmids omitted the initial 42 aa coding region for the N-His tag creating α104. This 42 aa N-His tag coding region may have been essential for the stable expression of the small protein α104. Other proteins such as the diphtheria toxin of *Corynebacterium diphtheriae* and the glutathione-S-transferase (GST) of *Schistosoma mansoni* when expressed alone in *S.* Typhimurium or *E. coli* expression systems results in poor expression of the protein. Ligation to the fragment C of the tetanus toxin gene stabilises the expression, with vaccination inducing antibodies against both fragments (Gomez-Duarte *et al.*, 1995; Barry *et al.*, 1996; Lee *et al.*, 2000). Ligation of *α104* to stabilising antigens such as the fragment C portion of the tetanus toxin gene or recloning of the 42 aa N-His tag coding region upstream of *α104* could possibly improve expression of *α104* within STM1 (Gomez-Duarte *et al.*, 1995). Transcription and translation analysis via mRNA and pulse chase experiments could be used to determine the fate of α104, whether or not lack of detectable expression is related to mRNA secondary structure formation or protein instability (Schmidt *et al.*, 1999).

Even though α104 was not detected in immunoblots, it was expressed in amounts high enough to induce an immune response. Splenocytes isolated from mice vaccinated with STM1 α104-expressing vectors (pBPCα104, pBTCα104 and pHAα104) actively secreted IL-

4 when stimulated with α-toxin (Figure 5.13). This IL-4 response was absent in the expressionless plasmid pBPAGC21, indicating that T-helper cells recognised the epitopes on α-toxin due to previous encounters with α104.

High copy number plasmids were used in this study in order to maximise the level of antigen expression following vaccination of mice (Cardenas and Clements, 1993). To counter-act the effects that high-copy number plasmids have within their hosts (Su *et al.*, 1992; Turner *et al.*, 1993; Dunstan *et al.*, 2003), that is, increased instability and reduced growth rates, *in vivo* inducible promoters were cloned upstream of the α-toxin truncated genes to prevent their constitutive expression which would otherwise result in further destabilisation of the vector.

Incorporation of the *in vivo* inducible promoters P_{htrA} and P_{pagC} did little to maintain plasmid stability *in vitro* as shown by the graphs in Figure 5.10. Most STM1 cells harbouring plasmid lost their plasmid within one to two days. Even in the absence of selection for plasmid encoded functions (the vector itself without insert), plasmid carriage can reduce host fitness (Abromaitis *et al.*, 2005). This was evident with plasmid pBPAGC21. The expressionless plasmid was lost over a 5 day period in the absence of the selective pressure ampicillin. However, the loss of this plasmid was gradual, with a 20% loss over 2 days, which was not as dramatic as the loss of plasmids expressing antigenic genes.

The only other stable vector within STM1 was pBPCα104. The two main differences between this vector and pBPCαInv is their size (pBPCαInv has an extra 822 bp) and the lack of protein expression of α104 *in vitro* from pBPCα104. Therefore the size difference and the expression levels of the two could have influenced the stability of the high-copy number vector within STM1.

Protein expression from P_{htrA} is minimal in the absence of H_2O_2 (Figure 5.9B) yet plasmids pBHAαInv and pBHAα104 were both very unstable in STM1 with their elimination occurring within the first day (Figure 5.11E-F). Transformation of STM1 with pBHAαInv also constantly resulted in a very low number of transformants (1-4 colonies) with reduced growth rates *in vitro*. This cannot be explained by the size of the plasmid (as it wasn't the largest of the plasmids) nor by the metabolic load caused by expression (as expression was reduced in the absence of H_2O_2), but perhaps regulation of the promoter required an increased energy expenditure, applying a metabolic burden on the host. It is possible that the presence of the high number of copies of P_{htrA} was titrating regulatory proteins away from the genome of STM1 (Garmory *et al.*, 2005) in turn affecting the growth and survival of the bacterium. Characterisation of the few positive transformants may help elucidate the nature of the reduced growth rate and reduced plasmid maintenance.

Immunogenicity of the vaccines was assessed by cytokine profiling using the ELIspot assay. Humoral responses were also examined using an ELISA assay.

When T-lymphocytes in a spleen cell population of vaccinated mice were incubated with α-toxin, they were activated and produced detectable amounts of IL-4 (Figure 5.13). Although this indicated a humoral response, a very low level of IgG was detected from mouse serum samples (Figures 5.11 and 5.14) and this level was not significantly different from the control vaccine group STMI(pBPAGC21). The presence of some IgG binding in the ELISA assays prompted the use of an immunoblot consisting of α-toxin probed with pooled mouse sera to confirm whether this low level of IgG was specific to the α-toxin. Unfortunately the immunoblot was negative for the detection of anti-α-toxin IgG. Perhaps employing a more sensitive assay such as an ELIspot for the detection of antibody secreting cells (by sampling cells from the bone marrow, spleen and Peyer's patches) in future could be used to determine

if a serum and mucosal antibody response is developed (Allen *et al.*, 2000). Serum was not collected for re-examination from mice following challenge. Strugnell *et al*., (1992) detected an increase in the humoral response following *Bordetella pertussis* aerosol challenge of mice vaccinated with attenuated *S*. Typhimurium expressing the p69 antigen. Had sera been re-examined in this study, a boost in the humoral response may have been detected, as challenged mice did show signs of reduced infection (Figure 5.16).

An effective immune response against infection by *S*. Typhimurium relies on the induction of cell mediated immunity (CMI) also referred to as a TH1 response, and is usually detected by the presence of IFN-γ secreting T-helper lymphocytes (Pashine *et al.*, 1999; Raupach and Kaufmann, 2001b; Norimatsu *et al.*, 2004). Due to the adjuvant effect of attenuated S. Typhimurium, delivery of heterologous antigens that do not generally produce a TH1 response are capable of inducing such responses when delivered within attenuated *S*. Typhimurium (Brown *et al.*, 1987; Strugnell *et al.*, 1992; Foynes *et al.*, 2003).

Although, the response of an STM1 delivered protein antigen is not entirely determined by the delivery vector but also influenced by inherent properties of the heterologous antigen. Differences in affinity and duration of stimulation between the antigen presenting cell and T-cell determine the final pattern of cytokine response secreted by the T-cells (Harokopakis *et al.*, 1997; Santana and Rosenstein, 2003). Therefore whilst STM1 generally elicits a TH1 response (Bachtiar *et al.*, 2003), the delivery and expression of recombinant α-toxin from STM1 primes a TH2 response. In this case the properties of the α-toxin truncated proteins αInv and α104 influenced the pattern of cytokine secretion more so than the delivery vector itself. Cytokine profiling at different time points would have to be conducted in order to confirm the TH2 pattern.

The strong correlation between IL-4 secretion and *C. perfringens* challenge data of vaccinated mice (Figure 5.17) indicate that IL-4 is necessary for protection against the lethal effects of *C. perfringens*. Vaccination of mice with the toxoid emulsified in FIA induced a large number of IL-4 secreting cells, a lack of IFN-γ secreting cells, a high level of anti-α-toxin IgG production, and the complete protection against challenge by *C. perfringens*. Although all STM1 harbouring the α-toxin recombinant expression vectors developed significant amounts of IL-4, a significant IgG response towards the α-toxin was undetected. This data suggests that protection from a gas-gangrene infection caused by a *C. perfringens* challenge is mediated by a TH2-cell response with a requirement for high levels of anti-α-toxin antibody production.

The level of antigen expressed affects the ability of mice to mount a humoral response (Fairweather *et al.*, 1990; Wick *et al.*, 1994) with the induction of protective antibodies requiring the presence of a threshold amount of antigen (Redman *et al.*, 1995). Such a threshold amount of antigen may not have been expressed in this study due to the instability of the plasmids expressing both αInv and α104, and the lack of detectable expression of α104. The number of STM1 harbouring plasmids is dramatically decreased within 20 generations (Figure 5.10). It is possible that *in vivo* the plasmid is lost even quicker than in culture, therefore reducing the potential immunogenicity of the constructs (Dunstan *et al.*, 2003; Foynes *et al.*, 2003).

Indirect evidence also exists indicating that the presence of the expression plasmids reduces the viability of the STM1 when within the mice. Higher anti-*Salmonella* IgG responses were observed with the control expressionless vaccine STM1(pBPAGC21) (Figure 5.15) indicating that this expressionless vaccine persisted longer in the host and was therefore able to stimulate a much stronger IgG response against *S.* Typhimurium whole cell antigens. On the other

hand, all vaccines expressing the α-toxin truncates resulted in poor IgG responses against *S*. Typhimurium indicating poor survival within the host. Plasmids expressing αInv and α104 were larger and more complex than pBPAGC21, and the presence of these high-copy number vectors actively expressing antigen may have lead to an increased metabolic burden on STM1 which compromised its colonising abilities, growth rate and survival (Su *et al.*, 1992; Coulson *et al.*, 1994a; Coulson *et al.*, 1994b; Dunstan *et al.*, 1998; Dunstan *et al.*, 2003).

Alternatives to the use of high copy number vectors relying on antibiotic selection includes the integration of antigenic genes into the genome of STM1 with the use of strong promoters to constitutively express the antigens at high levels, the use of balanced lethal plasmid systems, and the use of lower-copy number vectors (Curtiss *et al.*, 1989; Curtiss *et al.*, 1990; Chatfield *et al.*, 1992a; Chatfield *et al.*, 1992b; Hohmann *et al.*, 1995; Bullifent *et al.*, 2000; Chen and Schifferli, 2001; Dunstan *et al.*, 2003; Foynes *et al.*, 2003; Garmory *et al.*, 2003; Wyszynska *et al.*, 2004). These may be future approaches for the current work.

The timing and location of expression of the truncated α-toxin proteins αInv and α104 could have also lead to the lack of efficient antibody responses against the α-toxin. The high expression of antigens during the initial phase of invasion are crucial for the development of appropriate humoral responses (Wick *et al.*, 1994; Covone *et al.*, 1998).

The induction of immune responses from the macrophage inducible promoters would require invasion of Peyer's patches, phagocytosis into macrophages, induction of recombinant protein expression, processing of STMI and the associated recombinant protein within vacuoles, and presentation on the macrophage surface via MHC class II (Gahring *et al.*, 1990; Jones *et al.*, 1994; Lindgren *et al.*, 1996; Dunstan *et al.*, 1998). Bacterial viability was low within mice (as assessed by anti-*S*. Typhimurium IgG responses) and plasmid instability high, suggesting that

the number of viable STM1 cells retaining expression-plasmids and successfully reaching the macrophages may have been much lower than anticipated. This inturn resulting in only low levels of αInv or α104 expression. The metabolic load placed on STM1 by carriage of expression plasmids may have also reduced their ability to survive within the macrophages. For the induction of a humoral response, it may be better to use a system whereby protein is constantly expressed, that way increasing the chances of processing within all areas of the body involved in antigen sampling.

Expression of αInv and α104 from STM1 harbouring expression plasmids also lead to intracellular localisation of αInv and α104. Perhaps the constitutive expression of αInv and α104 leading to extracellular presentation, via secretion or surface expression, may hold more future promise in eliciting stronger humoral immune responses . The constitutive expression (timing), and extracellular presentation (location) of the recombinant proteins would ensure their immediate and constant delivery into the extracellular environment, increasing their chances of presentation to all APC's (Newton *et al.*, 1989; Su *et al.*, 1992; Hess *et al.*, 1997; Titball *et al.*, 1997; Chen and Schifferli, 2000; Ruiz-Perez *et al.*, 2002; Garmory *et al.*, 2003; Kang and Curtiss, 2003). This method may be a more plausible approach for induction of humoral responses and was evidenced with the toxoid immunised mice. Intraperitoneal administration of toxoid enabled its immediate presentation to APC's including B-cells, within the extracellular fluids (Janeway *et al.*, 2005). The depot effect provided by administration with FIA allowed the toxoid to remain within the host for a considerable length of time in order for the induction of an adequate humoral response (Aucouturier *et al.*, 2001; Billiau and Matthys, 2001) and ultimately protection from challenge.

Other options that could be examined to improve the humoral response of mice vaccinated with STM1 harbouring plasmids for the expression of αInv and α104 include the

augmentation of the immune response by the co-expression of auxiliary molecules known to enhance cytokines and receptors for induction of the TH2 response. Such molecules include the non-toxic subunit of pertussis toxin, fragment C of tetanus toxin and the non-toxic component of cholera toxin (Harokopakis *et al.*, 1997; Ryan *et al.*, 1998; Guillobel *et al.*, 2000; Lee *et al.*, 2000). The co-expression of other immuno-modulatory proteins such as IL-6 could also be examined to assist in the initiation of the TH2 response (Li *et al.*, 2003).

A comparison of the different αInv expression systems showed that IL-4 responses were not significantly different between the three promoters examined. Following challenge with *C. perfringens*, mice vaccinated with STM1(pBPCαInv) or STM1(pBTCαInv) resulted in lower levels of gas-gangrene. As mentioned earlier, pBHAαInv was not efficiently transformed into STM1 and the plasmid was very unstable *in vitro* which may have accounted for its reduced protective efficacy. Therefore both the constitutively expressed vector pBTCαInv, and the macrophage-inducible expression vector pBPCαInv should be used in future trials, perhaps placed into lower copy number vectors with greater long-term stability, to further assess their vaccine potential.

It is difficult to draw any conclusion from plasmids expressing α104 as detectable levels of expression were absent *in vitro*. Furthermore, vaccine pBHAα104 which resulted in some level of protection when challenged with *C. perfringens* only had four mice in the group, which may have biased the results.

Some clostridial antigens successfully expressed from attenuated *S.* Typhimurium include the fragment C of tetanus toxin, the toxin A fragment of *C. difficile* and a recombinant botulinum toxin, with vaccination inducing antibody production and protection against challenge (Fairweather *et al.*, 1990; Ward *et al.*, 1999; Foynes *et al.*, 2003).

This study demonstrated the feasibility of using STMI as a carrier for the *in vivo* expression of the *C. perfringens* α-toxin recombinant proteins αInv and α104. It is the first study to express *C. perfringens* antigens within an attenuated strain of *S.* Typhimurium, STM1. Activation of the TH2 arm of the immune system was observed, but it seems at this time, the level of activated IL-4 cells stimulated in response to vaccination with STM1 expressing the α-toxin truncated proteins αInv and α104 was not efficient enough to induce a detectable humoral response or to completely protect mice from a *C. perfringens* infection.

Future studies are focussing on chromosomal integration of αInv and α104 within STM1 downstream of strong promoters. The use of the hlyA secretion system of *E. coli* for the secretion of αInv and α104 is currently being examined within medium copy number vectors to improve α-toxin recombinant delivery from STM1.

Chapter 6

General discussion

Necrotic enteritis is an insidious infection of poultry caused by the proliferation of *C. perfringens* and its associated toxins (Al-Sheikhly and Truscott, 1977a; Al-Sheikhly and Truscott, 1977b; Al-Sheikhly and Truscott, 1977c; Fukata *et al.*, 1988). NE in chickens is often left untreated due to the difficulties in its detection with most diagnoses occurring retrospectively via on-farm necropsy (Parish, 1961a; van der Sluis, 2000). This can lead to significant economic losses due to unprecedented death of chickens, and an increased food conversion ratio in chickens with sub-clinical NE (Kaldhusdal and Hofshagen, 1992; Lovland and Kaldhusdal, 1999; van der Sluis, 2000). The only currently successful and commercially available preventative measure against NE is via antibiotic treatment (Watkins *et al.*, 1997; APVMA, 2006), but the pressure to reduce the use of antibiotics in food animals due to risks of antibiotic resistant bacteria developing, suggests the need for alternative methods of prophylaxis (Simonsen *et al.*, 1998; Molbak, 2004; Shea, 2004).

Vaccination of chickens is a possible option. One major contributing factor in the development of NE is the action of α-toxin expressed from *C. perfringens* (Al-Sheikhly and Truscott, 1977a; Fukata *et al.*, 1988; Heier *et al.*, 2001; Lovland *et al.*, 2004). Vaccines raising antibodies targeted towards the α-toxin of *C. perfringens* may be successful in preventing the development of NE.

This study involved the development of inactive truncated versions of the α-toxin as potential vaccine candidates for the prevention of NE. The first two proteins developed were based on truncation of the α-toxin from its α-helical N-terminal domain. The resulting proteins expressed from *E. coli* were quite insoluble and upon resolubilisation in chaotropic agents and

refolding in Tris buffer, began to re-aggregate over time. Amino acids at the extremity of the N-terminal domain of α-toxin are hidden within a hydrophobic pocket (Nagahama *et al.*, 1994; Guillouard *et al.*, 1996), and deletion of the N-terminal α-helices resulted in exposure of this hydrophobic region leading to misfolding of the protein. Immunisation of mice with both these proteins resulted in lower anti-α-toxin antibodies most likely due to the loss of essential conformational epitopes.

The α-toxin is a zinc metalloenzyme with the requirement of three zinc molecules in the active site for effective catalytic activity, and three calcium molecules for the successful docking to biological phospholipid membranes (Krug and Kent, 1984; Nagahama *et al.*, 1995; Guillouard *et al.*, 1997; Naylor *et al.*, 1999). Development of a third inactivated α-toxin protein involved the deletion of a region known to bind two of the zinc molecules. The deletion resulted in the removal of the catalytic activity of the α-toxin without affecting its phospholipid membrane binding properties. This newly developed protein, named plcInv3 was also capable of eliciting an antibody response within mice following intraperitoneal vaccination in FIA. These antibodies led to protection against a live intramuscular challenge with *C. perfringens* demonstrating the presence of essential epitopes within plcInv3 which led to the development of a protective immune response.

The C-terminal domain ($cpa_{247-370}$) has previously been shown to induce the production of protective antibodies within mice leading to protection against challenge with α-toxin and *C. perfringens* (Williamson and Titball, 1993). The study by Williamson and Titball (1993) prompted the author to examine the effects of further deleting this C-terminal region and examining its vaccine potential. Protein plc104 developed in this study was composed of seven of the eight β-sheets located within the C-terminus. The first β-sheet located at the N-terminus of $cpa_{247-370}$ was deleted. Protein plc104 lost its ability to bind phospholipid

membranes but retained some epitopes necessary for the development of antibodies and protective immune responses. However some breakthrough in protection was observed with some of the mice indicating that one or more conformational epitopes may be located in the deleted area, and their absence reduced the overall protectiveness of the plc104 antigen.

Delivery of the successful test vaccines plcInv3 and plc104 within mice was accomplished via the intraperitoneal administration within an emulsion of FIA. Even though this resulted in protective immune responses, it is not a feasible delivery system for domestic livestock, as FIA can cause localised inflammation (Aucouturier *et al.*, 2001), and parenteral delivery would be too laborious for animals such as chickens which are maintained at high stocking densities.

One approach to eliminate the side-effects of chemical adjuvants whilst reducing the labour required in administration of vaccines is the use of live vectored vaccines for the delivery of heterologous proteins. The attenuated *S*. Typhimurium *aro*A$^-$ mutants are one such approach (Alderton *et al.*, 1991; Wang *et al.*, 1999b). Attenuated *S*. Typhimurium has been shown to elicit cell mediated and humoral responses to both the heterologous protein and itself (Wang *et al.*, 1999b; Bachtiar *et al.*, 2003). An added advantage for use of *S*. Typhimurium mutants such as STM1, in the delivery of heterologous antigens, is the ability to stimulate an intestinal mucosal response to the foreign antigen when delivered orally to mice (Paton *et al.*, 1993; Ward *et al.*, 1999; Wang *et al.*, 1999b; Allen *et al.*, 2000).

As NE is an enteric disease, intestinal mucosal responses would be pivotal to the protection against this infection (Holmgren *et al.*, 1992). Therefore proteins plcInv3 and plc104 were examined for their immune-stimulating abilities when administered orally within the *S*. Typhimurium vaccine strain, STM1. The region coding for the affinity His tag of plcInv3

and plc104 was removed prior to cloning into STM1 and hence forth the proteins were known as αInv and α104 respectively.

One major drawback of using live vectored vaccines for the delivery of antigens to the host is the increased instability of the expression plasmids used in the absence of selective pressure, particularly when high levels of antigen are expressed (Strugnell *et al.*, 1992; Coulson and Titball, 1993; Coulson *et al.*, 1994a; Garmory *et al.*, 2003). One solution to overcome this problem is the use of plasmids containing *in vivo* inducible promoters (Dunstan *et al.*, 1999). The *in vivo* induction of antigen expression has also been shown to stimulate better immune responses over constitutively expressed antigens (Dunstan *et al.*, 1999).

The plasmids developed in this study were based on two *in vivo* inducible systems and one constitutive expression system. They included the use of the macrophage inducible promoters *pag*C (P_{pagC}) and *htr*A (P_{htrA}) and the strong constitutive tac promoter (P_{tac}). Unfortunately, unlike other results reported (Chatfield *et al.*, 1992a; Roberts *et al.*, 1998; Dunstan *et al.*, 1999; Bullifent *et al.*, 2000) these plasmids containing genes for the expression of αInv and α104 were not very stable *in vitro* (Figure 5.10), and there is a high possibility that the plasmids were lost even quicker *in vivo*, therefore reducing the potential immunogenicity of the constructs (Dunstan *et al.*, 2003; Foynes *et al.*, 2003).

The instability of the plasmids would have also affected the metabolic processes of STM1, resulting in loss of cell viability within the mouse host (Su *et al.*, 1992; Coulson *et al.*, 1994b; Dunstan *et al.*, 2003). This was indirectly observed by the examination of anti-*Salmonella*-IgG responses following vaccination of mice. STM1 harbouring protein-expressing plasmids produced much lower anti-*Salmonella* titers than the expressionless plasmid pBPAGC21. The metabolic load of the bacterium was compromised when expression plasmids were used,

and this negatively impacted on the growth of STM1, resulting in shorter survival time within mice, and the lack of induction of detectable levels of anti-α-toxin antibodies.

Although humoral responses were not observed directly through the detection of specific antibodies targeting the α-toxin, cytokine profiling with INF-γ and IL-4 revealed the stimulation of a TH2 response. Unfortunately, this response was not sufficient enough to completely protect mice from a challenge with a strain of *C. perfringens* producing the α-toxin. Protection was seen with mice immunised via the intraperitoneal route with toxoid in FIA suggesting a considerable amount of antibodies needs to be present for protection against *C. perfringens* to occur.

The partial protection of mice immunised with vaccines composed of STM1 expressing αInv and α104 indicate there is potential for this vectored vaccine system to be used in the protection of *C. perfringens* diseases caused by the α-toxin, although the system needs to be optimised for the maximal production of a humoral immune response. Some options for future work include the secretion of the α-toxin truncates from STM1 using the *hly*A *E. coli* secretory system (Garmory *et al.*, 2003), the use of lower copy number vectors to aid in the stability of plasmids (Coulson *et al.*, 1994a; Coulson *et al.*, 1994b; Dunstan *et al.*, 2003), or the integration of the α-toxin truncated genes within the STM1 chromosome under the control of strong promoters (Strugnell *et al.*, 1990; Hohmann *et al.*, 1995; Garmory *et al.*, 2003). Different attenuating mutations within *S.* Typhimurium strains can also dramatically affect the induction of immune responses to the expression of foreign antigens in bacteria (Schodel *et al.*, 1990; Karem *et al.*, 1995; Chabalgoity *et al.*, 1996; Dunstan *et al.*, 1998; Raupach and Kaufmann, 2001a). Therefore the use of other attenuated *S.* Typhimurium strains to deliver αInv and α104 may also provide induction of stronger humoral responses.

The development of an orally delivered vaccine targeted against the α-toxin of *C. perfringens* for the prevention of NE in chickens would be beneficial as it would reduce the use of antibiotics and reduce the losses incurred by the presence of undetectable sub-clinical forms of the disease.

An orally based vaccine is preferred to a parenteral administered vaccine due to the relative ease of manufacture, the ease of delivery to the chicken, and the benefit of local mucosal immune responses. Optimisation of the STM1 αInv and α104 expression systems is underway and focussed on chromosomal integration, and the use of the *Hly*A secretory system for the extracellular secretion of αInv and α104.

References

Aarestrup, F. M. 2002. Presented at the Beyond antimicrobial growth promoters in food animal production, Foulum, Denmark.

Aarestrup, F. M., H. Kruse, E. Tast, A. M. Hammerum, and L. B. Jensen. 2000. Associations between the use of antimicrobial agents for growth promotion and the occurrence of resistance among *Enterococcus faecium* from broilers and pigs in Denmark, Finland, and Norway. Microb. Drug. Resist. **6:**63-70.

Abraham, L. J., and J. I. Rood. 1985. Molecular analysis of transferable tetracycline resistance plasmids from *Clostridium perfringens*. J. Bacteriol. **161:**636-40.

Abromaitis, S., S. Faucher, M. Beland, R. Curtiss, 3rd, and F. Daigle. 2005. The presence of the *tet* gene from cloning vectors impairs *Salmonella* survival in macrophages. FEMS Microbiol. Lett. **242:**305-12.

Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts, and P. Walter (ed.). 2002. The Cell, 4th ed. Garland Science, New York.

Alderton, M. R., K. J. Fahey, and P. J. Coloe. 1991. Humoral responses and salmonellosis protection in chickens given a vitamin-dependent *Salmonella typhimurium* mutant. Avian Dis. **35:**435-42.

Allen, J. S., G. Dougan, and R. A. Strugnell. 2000. Kinetics of the mucosal antibody secreting cell response and evidence of specific lymphocyte migration to the lung after oral immunisation with attenuated *S. enterica* var. Typhimurium. FEMS Immunol. Med. Microbiol. **27:**275-81.

Allen, P. C., and R. H. Fetterer. 2002. Recent advances in biology and immunobiology of *Eimeria* species and in diagnosis and control of infection with these coccidian parasites of poultry. Clin. Microbiol. Rev. **15:**58-65.

Allen, S. D. 1985. *Clostridium*, p. 434-437. *In* E. H. Lynnette, A. Ballows, W. J. Hausler Jr, and H. J. Shadomy (ed.), Manual of clinical microbiology. American Society for Microbiology, Washington, D. C.

Al-Sheikhly, F., and R. B. Truscott. 1977a. The interaction of *Clostridium perfringens* and its toxins in the production of necrotic enteritis of chickens. Avian Dis. **21:**256-63.

Al-Sheikhly, F., and R. B. Truscott. 1977b. The pathology of necrotic enteritis of chickens following infusion of broth cultures of *Clostridium perfringens* into the duodenum. Avian Dis. **21:**230-40.

Al-Sheikhly, F., and R. B. Truscott. 1977c. The pathology of necrotic enteritis of chickens following infusion of crude toxins of *Clostridium perfringens* into the duodenum. Avian Dis. **21:**241-55.

Amann, E., J. Brosius, and M. Ptashne. 1983. Vectors bearing a hybrid trp-lac promoter useful for regulated expression of cloned genes in *Escherichia coli*. Gene **25:**167-78.

Anderson, E. L., T. Bowers, C. M. Mink, D. J. Kennedy, R. B. Belshe, H. Harakeh, L. Pais, P. Holder, and G. M. Carlone. 1994. Safety and immunogenicity of meningococcal A and C polysaccharide conjugate vaccine in adults. Infect. Immun. **62:**3391-5.

Angelakopoulos, H., K. Loock, D. M. Sisul, E. R. Jensen, J. F. Miller, and E. L. Hohmann. 2002. Safety and shedding of an attenuated strain of *Listeria monocytogenes* with a deletion of *act*A/*plc*B in adult volunteers: a dose escalation study of oral inoculation. Infect. Immun. **70:**3592-601.

APVMA 2006, posting date. PUBCRIS Registered Product Search Engine. [Online.] http://services.apvma.gov.au/PubcrisWebClient/welcome.do. Australian Pesticides and Veterinary Medicines Authority.

APVMA 2001, posting date. Special review of Avoparcin. [Online.] http://www.apvma.gov.au/chemrev/sravoparchin.shtmlAustralian. Pesticides and Veterinary Medicines Authority.

Aucouturier, J., L. Dupuis, and V. Ganne. 2001. Adjuvants designed for veterinary and human vaccines. Vaccine **19:**2666-72.

Ausubel, F., R. Brent, R. E. Kingston, M. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1994. Current protocols in molecular biology. John Wiley & Sons, New York.

Avigad, L. S., and A. W. Bernheimer. 1976. Inhibition by zinc of hemolysis induced by bacterial and other cytolytic agents. Infect. Immun. **13:**1378-81.

AVMA 2005, posting date. American Association of Avian Pathologists guidelines to judicious therapeutic use of antimicrobials in poultry. [Online.] http://www.avma.org/issues/policy/jtua_poultry.asp. Americal Veterinary Medical Association.

Awad, M. M., A. E. Bryant, D. L. Stevens, and J. I. Rood. 1995. Virulence studies on chromosomal alpha-toxin and theta-toxin mutants constructed by allelic exchange provide genetic evidence for the essential role of alpha-toxin in *Clostridium perfringens*-mediated gas gangrene. Mol. Microbiol. **15:**191-202.

Awad, M. M., D. M. Ellemor, R. L. Boyd, J. J. Emmins, and J. I. Rood. 2001. Synergistic effects of alpha-toxin and perfringolysin O in *Clostridium perfringens*-mediated gas gangrene. Infect. Immun. **69:**7904-10.

Awad, M. M., D. M. Ellemor, A. E. Bryant, O. Matsushita, R. L. Boyd, D. L. Stevens, J. J. Emmins, and J. I. Rood. 2000. Construction and virulence testing of a collagenase mutant of *Clostridium perfringens*. Microb. Pathog. **28:**107-17.

Baba, E., A. L. Fuller, J. M. Gilbert, S. G. Thayer, and L. R. McDougald. 1992. Effects of *Eimeria brunetti* infection and dietary zinc on experimental induction of necrotic enteritis in broiler chickens. Avian Dis. **36:**59-62.

Bacciarini, L. N., P. Boerlin, R. Straub, J. Frey, and A. Grone. 2003. Immunohistochemical localization of *Clostridium perfringens* beta2-toxin in the gastrointestinal tract of horses. Vet. Pathol. **40:**376-81.

Bachtiar, E. W., K. C. Sheng, T. Fifis, A. Gamvrellis, M. Plebanski, P. J. Coloe, and P. M. Smooker. 2003. Delivery of a heterologous antigen by a registered *Salmonella* vaccine (STM1). FEMS Microbiol. Lett. **227:**211-7.

Barry, E. M., Z. Altboum, G. Losonsky, and M. M. Levine. 2003. Immune responses elicited against multiple enterotoxigenic *Escherichia coli* fimbriae and mutant LT expressed in attenuated *Shigella* vaccine strains. Vaccine **21:**333-40.

Barry, E. M., O. Gomez-Duarte, S. Chatfield, R. Rappuoli, M. Pizza, G. Losonsky, J. Galen, and M. M. Levine. 1996. Expression and immunogenicity of pertussis toxin S1 subunit-tetanus toxin fragment C fusions in *Salmonella typhi* vaccine strain CVD 908. Infect. Immun. **64:**4172-81.

Barzaghi, G., C. Cerletti, and G. de Gaetano. 1988. Phospholipase C from *Clostridium perfringens* induces human platelet aggregation in plasma. Thromb. Haemost. **59:**236-9.

Ba-Thein, W., M. Lyristis, K. Ohtani, I. T. Nisbet, H. Hayashi, J. I. Rood, and T. Shimizu. 1996. The *vir*R/*vir*S locus regulates the transcription of genes encoding extracellular toxin production in *Clostridium perfringens*. J. Bacteriol. **178:**2514-20.

Beal, R. K., C. Powers, P. Wigley, P. A. Barrow, and A. L. Smith. 2004. Temporal dynamics of the cellular, humoral and cytokine responses in chickens during primary and secondary infection with Salmonella enterica serovar Typhimurium. Avian Pathol **33:**25-33.

Beuvery, E. C., F. van Rossum, and J. Nagel. 1982. Comparison of the induction of immunoglobulin M and G antibodies in mice with purified pneumococcal type 3 and meningococcal group C polysaccharides and their protein conjugates. Infect. Immun. **37:**15-22.

Biberstein, E. L. 1990. The *Clostridia*, p. 295-310. *In* E. L. Biberstein and Y. C. Zee (ed.), Review of Veterinary Microbiology. Blackwell Scientific Publications, Inc., London.

Billiau, A., and P. Matthys. 2001. Modes of action of Freund's adjuvants in experimental models of autoimmune diseases. J. Leukoc. Biol. **70:**849-60.

Boel, J., and S. Anderson. 2002. Presented at the Beyond antimicrobial growth promoters in food animal production., Foulum, Denmark.

Bollag, D. M., and S. J. Edelstein. 1991. Protein Methods. Wiley-Liss, New York.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248-254.

Branton, S. L., B. D. Lott, J. W. Deaton, W. R. Maslin, F. W. Austin, L. M. Pote, R. W. Keirs, M. A. Latour, and E. J. Day. 1997. The effect of added complex carbohydrates or added dietary fiber on necrotic enteritis lesions in broiler chickens. Poult. Sci. **76:**24-8.

Brett, S. J., L. Dunlop, F. Y. Liew, and J. P. Tite. 1993. Influence of the antigen delivery system on immunoglobulin isotype selection and cytokine production in response to influenza A nucleoprotein. Immunology **80:**306-12.

Brock, T. D., M. T. Madigan, J. M. Martinko, and J. Parker. 1994. Biology of microorganisms, 7th ed. Prentice-Hall Inc., New Jersey.

Brossier, F., M. Mock, and J. C. Sirard. 1999. Antigen delivery by attenuated *Bacillus anthracis*: new prospects in veterinary vaccines. J. Appl. Microbiol. **87:**298-302.

Brown, A., C. E. Hormaeche, R. Demarco de Hormaeche, M. Winther, G. Dougan, D. J. Maskell, and B. A. Stocker. 1987. An attenuated *aro*A *Salmonella typhimurium* vaccine elicits humoral and cellular immunity to cloned beta-galactosidase in mice. J. Infect. Dis. **155:**86-92.

Bryant, A. E., C. R. Bayer, S. M. Hayes-Schroer, and D. L. Stevens. 2003. Activation of platelet gpIIbIIIa by phospholipase C from *Clostridium perfringens* involves store-operated calcium entry. J. Infect. Dis. **187:**408-17.

Bryant, A. E., R. Y. Chen, Y. Nagata, Y. Wang, C. H. Lee, S. Finegold, P. H. Guth, and D. L. Stevens. 2000a. Clostridial gas gangrene. I. Cellular and molecular mechanisms of

microvascular dysfunction induced by exotoxins of *Clostridium perfringens*. J. Infect. Dis. **182:**799-807.

Bryant, A. E., R. Y. Chen, Y. Nagata, Y. Wang, C. H. Lee, S. Finegold, P. H. Guth, and D. L. Stevens. 2000b. Clostridial gas gangrene. II. Phospholipase C-induced activation of platelet gpIIbIIIa mediates vascular occlusion and myonecrosis in *Clostridium perfringens* gas gangrene. J. Infect. Dis. **182:**808-15.

Bryant, A. E., and D. L. Stevens. 1996. Phospholipase C and perfringolysin O from *Clostridium perfringens* upregulate endothelial cell-leukocyte adherence molecule 1 and intercellular leukocyte adherence molecule 1 expression and induce interleukin-8 synthesis in cultured human umbilical vein endothelial cells. Infect. Immun. **64:**358-62.

Bueschel, D., R. Walker, L. Woods, J. Kokai-Kun, B. McClane, and J. G. Songer. 1998. Enterotoxigenic *Clostridium perfringens* type A necrotic enteritis in a foal. J. Am. Vet. Med. Assoc. **213:**1305-7, 1280.

Bueschel, D. M., B. H. Jost, S. J. Billington, H. T. Trinh, and J. G. Songer. 2003. Prevalence of cpb2, encoding beta2 toxin, in *Clostridium perfringens* field isolates: correlation of genotype with phenotype. Vet. Microbiol. **94:**121-9.

Bullifent, H., A. Moir, M. M. Awad, P. T. Scott, J. I. Rood, and R. W. Titball. 1996. The level of expression of alpha-toxin by different strains of *Clostridium perfringens* is dependant on differences in promoter structure and genetic background. Anaerobe **2:**365-371.

Bullifent, H. L., K. F. Griffin, S. M. Jones, A. Yates, L. Harrington, and R. W. Titball. 2000. Antibody responses to *Yersinia pestis* F1-antigen expressed in *Salmonella typhimurium aro*A from *in vivo*-inducible promoters. Vaccine **18:**2668-76.

Bunting, M., D. E. Lorant, A. E. Bryant, G. A. Zimmerman, T. M. McIntyre, D. L. Stevens, and S. M. Prescott. 1997. Alpha toxin from *Clostridium perfringens* induces proinflammatory changes in endothelial cells. J. Clin. Invest. **100:**565-74.

Butterton, J. R., D. T. Beattie, C. L. Gardel, P. A. Carroll, T. Hyman, K. P. Killeen, J. J. Mekalanos, and S. B. Calderwood. 1995. Heterologous antigen expression in *Vibrio cholerae* vector strains. Infect. Immun. **63:**2689-96.

Canard, B., B. Saint-Joanis, and S. T. Cole. 1992. Genomic diversity and organization of virulence genes in the pathogenic anaerobe *Clostridium perfringens*. Mol. Microbiol. **6:**1421-9.

Cardenas, L., and J. D. Clements. 1992. Oral immunization using live attenuated *Salmonella* spp. as carriers of foreign antigens. Clin. Microbiol. Rev. **5:**328-42.

Cardenas, L., and J. D. Clements. 1993. Stability, immunogenicity and expression of foreign antigens in bacterial vaccine vectors. Vaccine **11:**126-35.

Casewell, M., C. Friis, E. Marco, P. McMullin, and I. Phillips. 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother. **52:**159-61.

Chabalgoity, J. A., C. M. A. Khan, A. A. Nash, and C. E. Hormaeche. 1996. A *Salmonella typhimurium htr*A live vaccine expressing multiple copies of a peptide comprising amino acids 8-23 of herpes simplex virus glycoprotein D as a genetic fusion to tetanus toxin fragment C protects mice from herpes simplex virus infection. Mol. Microbiol. **19:**791-801.

Chakraborty, T., F. Ebel, J. Wehland, J. Dufrenne, and S. Notermans. 1994. Naturally occurring virulence-attenuated isolates of *Listeria monocytogenes* capable of inducing long term protection against infection by virulent strains of homologous and heterologous serotypes. FEMS Immunol. Med. Microbiol. **10:**1-9.

Chatfield, S., M. Roberts, P. Londono, I. Cropley, G. Douce, and G. Dougan. 1993. The development of oral vaccines based on live attenuated *Salmonella* strains. FEMS Immunol. Med. Microbiol. **7:**1-7.

Chatfield, S. N., I. G. Charles, A. J. Makoff, M. D. Oxer, G. Dougan, D. Pickard, D. Slater, and N. F. Fairweather. 1992a. Use of the *nir*B promoter to direct the stable

expression of heterologous antigens in *Salmonella* oral vaccine strains: development of a single-dose oral tetanus vaccine. Biotechnology (N Y) **10:**888-92.

Chatfield, S. N., N. Fairweather, I. Charles, D. Pickard, M. Levine, D. Hone, M. Posada, R. A. Strugnell, and G. Dougan. 1992b. Construction of a genetically defined *Salmonella typhi* Ty2 *aro*A, *aro*C mutant for the engineering of a candidate oral typhoid-tetanus vaccine. Vaccine **10:**53-60.

Chen, H., and D. M. Schifferli. 2001. Enhanced immune responses to viral epitopes by combining macrophage-inducible expression with multimeric display on a *Salmonella* vector. Vaccine **19:**3009-18.

Chen, H., and D. M. Schifferli. 2000. Mucosal and systemic immune responses to chimeric fimbriae expressed by *Salmonella enterica* serovar Typhimurium vaccine strains. Infect. Immun. **68:**3129-39.

Cherayil, B. J., and D. Antos. 2001. Inducible nitric oxide synthase and *Salmonella* infection. Microbes Infect. **3:**771-6.

Chong, C., K. L. Bost, and J. D. Clements. 1996. Differential production of interleukin-12 mRNA by murine macrophages in response to viable or killed *Salmonella* spp. Infect. Immun. **64:**1154-60.

Clark, G. C., D. C. Briggs, T. Karasawa, X. Wang, A. R. Cole, T. Maegawa, P. N. Jayasekera, C. E. Naylor, J. Miller, D. S. Moss, S. Nakamura, A. K. Basak, and R. W. Titball. 2003. *Clostridium absonum* α-toxin: New insights into clostridial phospholipase C substrate binding and specificity. J. Mol. Biol. **333:**759-769.

Collie, R. E., and B. A. McClane. 1998. Evidence that the enterotoxin gene can be episomal in *Clostridium perfringens* isolates associated with non-food-borne human gastrointestinal diseases. J. Clin. Microbiol. **36:**30-6.

Coloe, P. J., M. R. Alderton, N. L. Gerraty, W. Christopher, and S. C. Smith. 1995. Aromatic vitamin-dependent *Salmonellae* as vaccines in food animals: efficacy and persistence. Dev. Biol. Stand. **84:**263-7.

Cornillot, E., B. Saint-Joanis, G. Daube, S. Katayama, P. E. Granum, B. Canard, and S. T. Cole. 1995. The enterotoxin gene (*cpe*) of *Clostridium perfringens* can be chromosomal or plasmid-borne. Mol. Microbiol. **15:**639-47.

Coulson, M. C., and R. W. Titball. 1993. Expression of *Bacillus anthracis* protective antigen in *Salmonella* typhimurium, p. 419-424. *In* F. Cabello (ed.), Biology of *Salmonella*. Plenum Press, New York.

Coulson, N. M., M. Fulop, and R. W. Titball. 1994a. *Bacillus anthracis* protective antigen, expressed in *Salmonella typhimurium* SL 3261, affords protection against anthrax spore challenge. Vaccine **12:**1395-401.

Coulson, N. M., M. Fulop, and R. W. Titball. 1994b. Effect of different plasmids on colonization of mouse tissues by the aromatic amino acid dependent *Salmonella typhimurium* SL 3261. Microb. Pathog. **16:**305-311.

Covone, M. G., M. Brocchi, E. Palla, W. Dias da Silveira, R. Rappuoli, and C. L. Galeotti. 1998. Levels of expression and immunogenicity of attenuated Salmonella enterica serovar typhimurium strains expressing Escherichia coli mutant heat-labile enterotoxin. Infect. Immun. **66:**224-31.

Cowen, B. S., L. D. Schwartz, R. A. Wilson, and S. I. Ambrus. 1987. Experimentally induced necrotic enteritis in chickens. Avian Dis. **31:**904-6.

Craven, S. E. 2000. Colonization of the intestinal tract by *Clostridium perfringens* and fecal shedding in diet-stressed and unstressed broiler chickens. Poult. Sci. **79:**843-9.

Craven, S. E., N. J. Stern, N. A. Cox, J. S. Bailey, and M. Berrang. 1999. Cecal carriage of *Clostridium perfringens* in broiler chickens given Mucosal Starter Culture. Avian Dis. **43:**484-90.

Curtiss, R., 3rd, J. E. Galan, K. Nakayama, and S. M. Kelly. 1990. Stabilization of recombinant avirulent vaccine strains *in vivo*. Res. Microbiol. **141:**797-805.

Curtiss, R., 3rd, K. Nakayama, and S. M. Kelly. 1989. Recombinant avirulent *Salmonella* vaccine strains with stable maintenance and high level expression of cloned genes *in vivo*. Immunol. Invest. **18:**583-96.

Czeczulin, J. R., R. E. Collie, and B. A. McClane. 1996. Regulated expression of *Clostridium perfringens* enterotoxin in naturally cpe-negative type A, B, and C isolates of *C. perfringens*. Infect. Immun. **64:**3301-9.

Czeczulin, J. R., P. C. Hanna, and B. A. McClane. 1993. Cloning, nucleotide sequencing, and expression of the *Clostridium perfringens* enterotoxin gene in *Escherichia coli*. Infect. Immun. **61:**3429-39.

Dalla Pozza, T., H. Yan, D. Meek, C. A. Guzman, and M. J. Walker. 1998. Construction and characterisation of *Salmonella typhimurium aro*A simultaneously expressing the five pertussis toxin subunits. Vaccine **16:**522-9.

Das, B. C., G. N. Dutta, G. Daube, and A. Phukan. 1997a. Characterization of *Clostridium perfringens* isolated from necrotic enteritis of fowls. Indian J. Anim. Sci. **67:**35-36.

Das, B. C., G. N. Dutta, L. A. Devriese, and A. Phukan. 1997b. Antimicrobial susceptibility of *Clostridium perfringens* isolated from necrotic enteritis cases of poultry. Indian Vet. J. **74:**553-555.

Daube, G., P. Simon, B. Limbourg, C. Manteca, J. Mainil, and A. Kaeckenbeeck. 1996. Hybridization of 2,659 *Clostridium perfringens* isolates with gene probes for seven toxins (alpha, beta, epsilon, iota, theta, mu, and enterotoxin) and for sialidase. Am. J. Vet. Res. **57:**496-501.

Davis, M. W. 1984. More on necrotizing enterocolitis: pigbel in Papua New Guinea. N. Engl. J. Med. **311:**1126-7.

de Boer, H. A., L. J. Comstock, and M. Vasser. 1983. The tac promoter: a functional hybrid derived from the trp and lac promoters. Proc. Natl. Acad. Sci. USA **80:**21-5.

de Jong, R., F. Altare, I. A. Haagen, D. G. Elferink, T. Boer, P. J. van Breda Vriesman, P. J. Kabel, J. M. Draaisma, J. T. van Dissel, F. P. Kroon, J. L. Casanova, and T. H. Ottenhoff. 1998. Severe mycobacterial and *Salmonella* infections in interleukin-12 receptor-deficient patients. Science **280:**1435-8.

Devriese, L. A., G. Daube, J. Hommez, and F. Haesebrouck. 1993. In vitro susceptibility of *Clostridium perfringens* isolated from farm animals to growth-enhancing antibiotics. J. Appl. Bacteriol. **75:**55-7.

Dhillon, A. S., P. Roy, L. Lauerman, D. Schaberg, S. Weber, D. Bandli, and F. Wier. 2004. High mortality in egg layers as a result of necrotic enteritis. Avian Dis. **48:**675-80.

Dougan, G., S. Chatfield, D. Pickard, J. Bester, D. O'Callaghan, and D. Maskell. 1988. Construction and characterization of vaccine strains of *Salmonella* harboring mutations in two different *aro* genes. J. Infect. Dis. **158:**1329-35.

Dougan, G., C. E. Hormaeche, and D. J. Maskell. 1987. Live oral *Salmonella* vaccines: potential use of attenuated strains as carriers of heterologous antigens to the immune system. Parasite. Immunol. **9:**151-60.

Dougan, G., L. Smith, and F. Heffron. 1989. Live bacterial vaccines and their application as carriers for foreign antigens. Adv. Vet. Sci. Comp. Med. **33:**271-300.

Drasar, B. S., and A. K. Roberts. 1990. Control of the large bowel microflora, p. 95-100. *In* M. J. Hill and P. D. Marsh (ed.), Human microbial ecology. CRC Press Inc., Boca Raton.

Droual, R., H. L. Shivaprasad, and R. P. Chin. 1994. Coccidiosis and necrotic enteritis in turkeys. Avian Dis. **38:**177-83.

Dunstan, S. J., C. P. Simmons, and R. A. Strugnell. 1998. Comparison of the abilities of different attenuated *Salmonella typhimurium* strains to elicit humoral immune responses against a heterologous antigen. Infect. Immun. **66:**732-40.

Dunstan, S. J., C. P. Simmons, and R. A. Strugnell. 2003. *In vitro* and *in vivo* stability of recombinant plasmids in a vaccine strain of *Salmonella enterica* var. Typhimurium. FEMS Immunol. Med. Microbiol. **37:**111-9.

Dunstan, S. J., C. P. Simmons, and R. A. Strugnell. 1999. Use of *in vivo*-regulated promoters to deliver antigens from attenuated *Salmonella enterica* var. Typhimurium. Infect. Immun. **67:**5133-41.

Dytham, C. 2003. Choosing and using statistics: A biologist's guide, 2nd ed. Blackwell Publishing, Oxford.

Eaton, J. T., C. E. Naylor, A. M. Howells, D. S. Moss, R. W. Titball, and A. K. Basak. 2002. Crystal structure of the *Clostridium perfringens* alpha-toxin with the active site closed by a flexible loop region. J. Mol. Biol. **319:**275-81.

Ebert, E., V. Oppling, E. Werner, and K. Cussler. 1999. Development and prevalidation of two different ELISA systems for the potency testing of *Clostridium perfringens* beta and epsilon-toxoid containing veterinary vaccines. FEMS Immunol. Med. Microbiol. **24:**299-311.

el Idrissi, A. H., and G. E. Ward. 1992. Development of double sandwich ELISA for *Clostridium perfringens* beta and epsilon toxins. Vet. Microbiol. **31:**89-99.

Ellemor, D. M., R. N. Baird, M. M. Awad, R. L. Boyd, J. I. Rood, and J. J. Emmins. 1999. Use of genetically manipulated strains of *Clostridium perfringens* reveals that both alpha-toxin and theta-toxin are required for vascular leukostasis to occur in experimental gas gangrene. Infect. Immun. **67:**4902-7.

Ellis, R. J. 1997. Molecular chaperones: avoiding the crowd. Curr. Biol. **7:**R531-3.

Elwinger, K., E. Berndtson, B. Engstrom, O. Fossum, and L. Waldenstedt. 1998. Effect of antibiotic growth promoters and anticoccidials on growth of *Clostridium perfringens* in the caeca and on performance of broiler chickens. Acta. Vet. Scand. **39:**433-41.

Elwinger, K., C. Schneitz, E. Berndtson, O. Fossum, B. Teglof, and B. Engstom. 1992. Factors affecting the incidence of necrotic enteritis, caecal carriage of *Clostridium perfringens* and bird performance in broiler chicks. Acta. Vet. Scand. **33:**369-78.

Engstrom, B. E., C. Fermer, A. Lindberg, E. Saarinen, V. Baverud, and A. Gunnarsson. 2003. Molecular typing of isolates of *Clostridium perfringens* from healthy and diseased poultry. Vet. Microbiol. **94:**225-35.

Fach, P., and M. R. Popoff. 1997. Detection of enterotoxigenic *Clostridium perfringens* in food and fecal samples with a duplex PCR and the slide latex agglutination test. Appl. Environ. Microbiol. **63:**4232-6.

Fahnert, B., H. Lilie, and P. Neubauer. 2004. Inclusion bodies: formation and utilisation. Adv. Biochem. Eng. Biotechnol. **89:**93-142.

Fairweather, N. F., S. N. Chatfield, A. J. Makoff, R. A. Strugnell, J. Bester, D. J. Maskell, and G. Dougan. 1990. Oral vaccination of mice against tetanus by use of a live attenuated *Salmonella* carrier. Infect. Immun. **58:**1323-6.

Finnie, J. W., P. C. Blumbergs, and J. Manavis. 1999. Neuronal damage produced in rat brains by *Clostridium perfringens* type D epsilon toxin. J. Comp. Pathol. **120:**415-20.

Foynes, S., J. L. Holley, H. S. Garmory, R. W. Titball, and N. F. Fairweather. 2003. Vaccination against type F botulinum toxin using attenuated *Salmonella enterica* var Typhimurium strains expressing the BoNT/F H(C) fragment. Vaccine **21:**1052-9.

Fujii, Y., S. Nomura, Y. Oshita, and J. Sakurai. 1986. Excitatory effect of *Clostridium perfringens* alpha toxin on the rat isolated aorta. Br. J. Pharmacol. **88:**531-9.

Fukata, T., Y. Hadate, E. Baba, T. Uemura, and A. Arakawa. 1988. Influence of *Clostridium perfringens* and its toxin in germ-free chickens. Res. Vet. Sci. **44:**68-70.

Gahring, L. C., F. Heffron, B. B. Finlay, and S. Falkow. 1990. Invasion and replication of *Salmonella* typhimurium in animal cells. Infect. Immun. **58:**443-8.

Garcia Vescovi, E., F. C. Soncini, and E. A. Groisman. 1996. Mg2+ as an extracellular signal: environmental regulation of *Salmonella* virulence. Cell **84:**165-74.

Garmory, H. S., N. Chanter, N. P. French, D. Bueschel, J. G. Songer, and R. W. Titball. 2000. Occurrence of *Clostridium perfringens* beta2-toxin amongst animals, determined using genotyping and subtyping PCR assays. Epidemiol. Infect. **124:**61-7.

Garmory, H. S., M. W. Leckenby, K. F. Griffin, S. J. Elvin, R. R. Taylor, M. G. Hartley, J. A. Hanak, E. D. Williamson, and R. M. Cranenburgh. 2005. Antibiotic-free plasmid stabilization by operator-repressor titration for vaccine delivery by using live *Salmonella enterica* Serovar Typhimurium. Infect. Immun. **73:**2005-11.

Garmory, H. S., R. W. Titball, K. F. Griffin, U. Hahn, R. Bohm, and W. Beyer. 2003. *Salmonella enterica* serovar Typhimurium expressing a chromosomally integrated copy of the *Bacillus anthracis* protective antigen gene protects mice against an anthrax spore challenge. Infect. Immun. **71:**3831-6.

Gazdzinski, P., and R. J. Julian. 1992. Necrotic enteritis in turkeys. Avian Dis. **36:**792-8.

Gentschev, I., G. Dietrich, S. Spreng, A. Kolb-Maurer, V. Brinkmann, L. Grode, J. Hess, S. H. Kaufmann, and W. Goebel. 2001. Recombinant attenuated bacteria for the delivery of subunit vaccines. Vaccine **19:**2621-8.

George, W. L., and S. M. Finegold. 1985. Clostridia in the human gastrointestinal flora, p. 1-37. *In* S. P. Borriello (ed.), Clostridia in gastrointestinal disease. CRC Press Inc., Boca Raton.

Germanier, R., and E. Fuer. 1975. Isolation and characterization of Gal E mutant Ty 21a of *Salmonella typhi*: a candidate strain for a live, oral typhoid vaccine. J. Infect. Dis. **131:**553-8.

Gibert, M., C. Jolivet-Reynaud, M. R. Popoff, and C. Jolivet-Renaud. 1997. Beta2 toxin, a novel toxin produced by *Clostridium perfringens*. Gene **203:**65-73.

Gibert, M., L. Petit, S. Raffestin, A. Okabe, and M. R. Popoff. 2000. *Clostridium perfringens* iota-toxin requires activation of both binding and enzymatic components for cytopathic activity. Infect. Immun. **68:**3848-53.

Ginter, A., E. D. Williamson, F. Dessy, P. Coppe, H. Bullifent, A. Howells, and R. W. Titball. 1996. Molecular variation between the alpha-toxins from the type strain (NCTC 8237) and clinical isolates of *Clostridium perfringens* associated with disease in man and animals. Microbiology **142 (Pt 1):**191-8.

Gomez-Duarte, O. G., J. Galen, S. N. Chatfield, R. Rappuoli, L. Eidels, and M. M. Levine. 1995. Expression of fragment C of tetanus toxin fused to a carboxyl-terminal fragment of diphtheria toxin in *Salmonella typhi* CVD 908 vaccine strain. Vaccine **13:**1596-602.

Gomez-Ortiz, M., F. X. Gomis-Ruth, R. Huber, and F. X. Aviles. 1997. Inhibition of carboxypeptidase A by excess zinc: analysis of the structural determinants by X-ray crystallography. FEBS Lett. **400:**336-40.

Goossens, P. L., and G. Milon. 1992. Induction of protective CD8+ T lymphocytes by an attenuated *Listeria monocytogenes act*A mutant. Int. Immunol. **4:**1413-8.

Goossens, P. L., G. Milon, P. Cossart, and M. F. Saron. 1995. Attenuated *Listeria monocytogenes* as a live vector for induction of CD8+ T cells *in vivo*: a study with the nucleoprotein of the lymphocytic choriomeningitis virus. Int. Immunol. **7:**797-805.

Grave, K., M. C. Kaldhusdal, H. Kruse, L. M. Harr, and K. Flatlandsmo. 2004. What has happened in norway after the ban of avoparcin? Consumption of antimicrobials by poultry. Prev. Vet. Med. **62:**59-72.

Guillobel, H. C., J. I. Carinhanha, L. Cardenas, J. D. Clements, D. F. de Almeida, and L. C. Ferreira. 2000. Adjuvant activity of a nontoxic mutant of *Escherichia coli* heat-labile enterotoxin on systemic and mucosal immune responses elicited against a heterologous antigen carried by a live *Salmonella enterica* serovar Typhimurium vaccine strain. Infect. Immun. **68:**4349-53.

Guillouard, I., P. M. Alzari, B. Saliou, and S. T. Cole. 1997. The carboxy-terminal C_2-like domain of the α-toxin from *Clostridium perfringens* mediates calcium-dependent membrane recognition. Mol. Microbiol. **26:**867-876.

Guillouard, I., T. Garnier, and S. T. Cole. 1996. Use of site-directed mutagenesis to probe structure-function relationships of alpha-toxin from *Clostridium perfringens*. Infect. Immun. **64:**2440-4.

Gustafson, C., and C. Tagesson. 1989. Phospholipase activation and arachidonic acid release in cultured intestinal epithelial cells (INT 407). Scand. J. Gastroenterol. **24:**475-84.

Guzman, C. A., R. M. Brownlie, J. Kadurugamuwa, M. J. Walker, and K. N. Timmis. 1991. Antibody responses in the lungs of mice following oral immunization with *Salmonella typhimurium aro*A and invasive *Escherichia coli* strains expressing the filamentous hemagglutinin of *Bordetella pertussis*. Infect. Immun. **59:**4391-7.

Hackett, J. 1990. *Salmonella*-based vaccines. Vaccine **8:**5-11.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557-80.

Hanna, P. C., E. U. Wieckowski, T. A. Mietzner, and B. A. McClane. 1992. Mapping of functional regions of *Clostridium perfringens* type A enterotoxin. Infect. Immun. **60:**2110-4.

Harokopakis, E., G. Hajishengallis, T. E. Greenway, M. W. Russell, and S. M. Michalek. 1997. Mucosal immunogenicity of a recombinant *Salmonella typhimurium*-cloned heterologous antigen in the absence or presence of coexpressed cholera toxin A2 and B subunits. Infect. Immun. **65:**1445-54.

Hatheway, C. L. 1990. Toxigenic clostridia. Clin. Microbiol. Rev. **3:**66-98.

Hauer, P. J., T. J. Yeary, and R. F. Rosenbusch. 2004. Cloning and molecular characterization of the beta toxin (phospholipase C) gene of *Clostridium haemolyticum*. Anaerobe **10:**243-254.

Heier, B. T., A. Lovland, K. B. Soleim, M. Kaldhusdal, and J. Jarp. 2001. A field study of naturally occurring specific antibodies against *Clostridium perfringens* alpha toxin in Norwegian broiler flocks. Avian Dis. **45:**724-32.

Helmboldt, C. F., and E. S. Bryant. 1971. The pathology of necrotic enteritis in domestic fowl. Avian Dis. **15:**775-80.

Henderson, S. C., D. I. Bounous, and M. D. Lee. 1999. Early events in the pathogenesis of avian salmonellosis. Infect. Immun. **67:**3580-6.

Herholz, C., R. Miserez, J. Nicolet, J. Frey, M. Popoff, M. Gibert, H. Gerber, and R. Straub. 1999. Prevalence of beta2-toxigenic *Clostridium perfringens* in horses with intestinal disorders. J. Clin. Microbiol. **37:**358-61.

Hess, J., G. Dietrich, I. Gentschev, D. Miko, W. Goebel, and S. H. Kaufmann. 1997. Protection against murine listeriosis by an attenuated recombinant *Salmonella typhimurium* vaccine strain that secretes the naturally somatic antigen superoxide dismutase. Infect. Immun. **65:**1286-92.

Hess, J., C. Ladel, D. Miko, and S. H. Kaufmann. 1996. *Salmonella typhimurium aro*A-infection in gene-targeted immunodeficient mice: major role of CD4+ TCR-alpha beta cells and IFN-gamma in bacterial clearance independent of intracellular location. J. Immunol. **156:**3321-6.

Heuer, O. E., K. Pederson, J. S. Andersen, L. B. Jensen, J. E. Olsen, and M. Madsen. 2002. Presented at the Beyond Antimicrobial Growth Promoters in Food Animal Production, Foulum, Denmark.

Hilleman, M. R. 2000. Vaccines in historic evolution and perspective: a narrative of vaccine discoveries. Vaccine **18:**1436-47.

Hofacre, C. L., R. Froyman, B. Gautrias, B. George, M. A. Goodwin, and J. Brown. 1998a. Use of Aviguard and other intestinal bioproducts in experimental *Clostridium perfringens*-associated necrotizing enteritis in broiler chickens. Avian Dis. **42:**579-84.

Hofacre, C. L., R. Froyman, B. George, M. A. Goodwin, and J. Brown. 1998b. Use of Aviguard, virginiamycin, or bacitracin MD against *Clostridium perfringens*-associated necrotizing enteritis. J. Appl. Poultry Res. **7:**412-418.

Hoffmann, F., and U. Rinas. 2004. Stress induced by recombinant protein production in *Escherichia coli*. Adv. Biochem. Eng. Biotechnol. **89:**73-92.

Hofshagen, M., and M. Kaldhusdal. 1992. Barley inclusion and avoparcin supplementation in broiler diets. 1. Effect on small intestinal bacterial flora and performance. Poult Sci. **71:**959-69.

Hohmann, E. L., C. A. Oletta, W. P. Loomis, and S. I. Miller. 1995. Macrophage-inducible expression of a model antigen in *Salmonella typhimurium* enhances immunogenicity. Proc. Natl. Acad. Sci. USA **92:**2904-8.

Hoiseth, S. K., and B. A. Stocker. 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature **291:**238-9.

Holmgren, J., C. Czerkinsky, N. Lycke, and A. M. Svennerholm. 1992. Mucosal immunity: implications for vaccine development. Immunobiology **184:**157-79.

Holmquist, B., and B. L. Vallee. 1974. Metal substitutions and inhibition of thermolysin: spectra of the cobalt enzyme. J. Biol. Chem. **249:**4601-7.

Hopkins, S., J. P. Kraehenbuhl, F. Schodel, A. Potts, D. Peterson, P. de Grandi, and D. Nardelli-Haefliger. 1995. A recombinant *Salmonella typhimurium* vaccine induces local immunity by four different routes of immunization. Infect. Immun. **63:**3279-86.

Hubener, K., W. Vahjen, and O. Simon. 2002. Bacterial responses to different dietary cereal types and xylanase supplementation in the intestine of broiler chicken. Arch. Tierernahr. **56:**167-87.

Hunter, S. E., J. E. Brown, P. C. Oyston, J. Sakurai, and R. W. Titball. 1993. Molecular genetic analysis of beta-toxin of *Clostridium perfringens* reveals sequence homology with

alpha-toxin, gamma-toxin, and leukocidin of *Staphylococcus aureus*. Infect. Immun. **61:**3958-65.

Hunter, S. E., I. N. Clarke, D. C. Kelly, and R. W. Titball. 1992. Cloning and nucleotide sequencing of the *Clostridium perfringens* epsilon-toxin gene and its expression in *Escherichia coli*. Infect. Immun. **60:**102-10.

Ito, A. 1970. Alpha toxoid of *Clostridium perfringens*. II. Immunogenicity of the toxoid. Jpn. J. Med. Sci. Biol. **23:**21-30.

Izhar, M., L. DeSilva, H. S. Joysey, and C. E. Hormaeche. 1990. Moderate immunodeficiency does not increase susceptibility to *Salmonella typhimurium aro*A live vaccines in mice. Infect. Immun. **58:**2258-61.

Jackson, C., and G. Underwood 2006, posting date. Development of *Salmonella* vaccination strategies for the australian poultry industry. [Online.] http://www.bioproperties.com.au/vaccines/documents/SalmonellaVaccinesv1.pdf. Bioproperties.

Janeway, C. A., P. Travers, M. Walport, and M. J. Shlomchik (ed.). 2005. Immunobiology: The immune system in health and disease, 6th ed. Garland Science Publishing, New York.

Jensen, L. B., A. M. Hammerum, H. Hasman, and F. M. Aarestrup. 2002. Presented at the Beyond Antimicrobial Growth Promoters in Food Animal Production, Foulum, Denmark.

Jepson, M., H. L. Bullifent, D. Crane, M. Flores-Diaz, A. Alape-Giron, P. Jayasekeera, B. Lingard, D. Moss, and R. W. Titball. 2001. Tyrosine 331 and phenylalanine 334 in *Clostridium perfringens* alpha-toxin are essential for cytotoxic activity. FEBS Lett. **495:**172-7.

Jepson, M., A. Howells, H. L. Bullifent, B. Bolgiano, D. Crane, J. Miller, J. Holley, P. Jayasekera, and R. W. Titball. 1999. Differences in the carboxy-terminal (putative phospholipid binding) domains of *Clostridium perfringens* and *Clostridium bifermentans*

phospholipases C influence the hemolytic and lethal properties of these enzymes. Infect. Immun. **67:**3297-301.

Johansson, A., C. Greko, B. E. Engstrom, and M. Karlsson. 2004. Antimicrobial susceptibility of Swedish, Norwegian and Danish isolates of *Clostridium perfringens* from poultry, and distribution of tetracycline resistance genes. Vet. Microbiol. **99:**251-7.

John, B., D. Rajagopal, A. Pashine, S. Rath, A. George, and V. Bal. 2002. Role of IL-12-independent and IL-12-dependent pathways in regulating generation of the IFN-gamma component of T cell responses to *Salmonella typhimurium*. J. Immunol. **169:**2545-52.

Johnson, C. C. 1989. *Clostridium perfringens* food poisoning, p. 629-638. *In* S. M. Finegold and W. L. George (ed.), Anaerobic infections in humans. Academic Press, London.

Johnson, K., I. Charles, G. Dougan, D. Pickard, P. O'Gaora, G. Costa, T. Ali, I. Miller, and C. Hormaeche. 1991. The role of a stress-response protein in *Salmonella typhimurium* virulence. Mol Microbiol **5:**401-7.

Johnson, K. S., I. G. Charles, G. Dougan, I. A. Miller, D. Pickard, P. O'Goara, G. Costa, T. Ali, and C. E. Hormaeche. 1990. The role of a stress-response protein in bacterial virulence. Res. Microbiol. **141:**823-5.

Jones, B. D., N. Ghori, and S. Falkow. 1994. *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. J. Exp. Med. **180:**15-23.

Jost, B. H., S. J. Billington, H. T. Trinh, D. M. Bueschel, and J. G. Songer. 2005. Atypical *cpb*2 genes, encoding beta2-toxin in *Clostridium perfringens* isolates of nonporcine origin. Infect. Immun. **73:**652-6.

Kadra, B., J. P. Guillou, M. Popoff, and P. Bourlioux. 1999. Typing of sheep clinical isolates and identification of enterotoxigenic *Clostridium perfringens* strains by classical methods and by polymerase chain reaction (PCR). FEMS Immunol. Med. Microbiol. **24:**259-66.

Kaldhusdal, M., and M. Hofshagen. 1992. Barley inclusion and avoparcin supplementation in broiler diets. 2. Clinical, pathological, and bacteriological findings in a mild form of necrotic enteritis. Poult. Sci. **71:**1145-53.

Kaldhusdal, M., M. Hofshagen, A. Lovland, H. Langstrand, and K. Redhead. 1999. Necrotic enteritis challenge models with broiler chickens raised on litter: evaluation of preconditions, *Clostridium perfringens* strains and outcome variables. FEMS Immunol. Med. Microbiol. **24:**337-43.

Kanakaraj, R., D. L. Harris, J. G. Songer, and B. Bosworth. 1998. Multiplex PCR assay for detection of *Clostridium perfringens* in feces and intestinal contents of pigs and in swine feed. Vet. Microbiol. **63:**29-38.

Kang, H. Y., and R. Curtiss, 3rd. 2003. Immune responses dependent on antigen location in recombinant attenuated *Salmonella typhimurium* vaccines following oral immunization. FEMS Immunol. Med. Microbiol. **37:**99-104.

Karasawa, T., X. Wang, T. Maegawa, Y. Michiwa, H. Kita, K. Miwa, and S. Nakamura. 2003. *Clostridium sordellii* phospholipase C: gene cloning and comparison of enzymatic and biological activities with those of *Clostridium perfringens* and *Clostridium bifermentans* phospholipase C. Infect. Immun. **71:**641-6.

Karem, K. L., J. Bowen, N. Kuklin, and B. T. Rouse. 1997. Protective immunity against herpes simplex virus (HSV) type 1 following oral administration of recombinant *Salmonella typhimurium* vaccine strains expressing HSV antigens. J. Gen. Virol. **78 (Pt 2):**427-34.

Karem, K. L., S. Chatfield, N. Kuklin, and B. T. Rouse. 1995. Differential induction of carrier antigen-specific immunity by *Salmonella typhimurium* live-vaccine strains after single mucosal or intravenous immunization of BALB/c mice. Infect. Immun. **63:**4557-63.

Katahira, J., N. Inoue, Y. Horiguchi, M. Matsuda, and N. Sugimoto. 1997a. Molecular cloning and functional characterization of the receptor for *Clostridium perfringens* enterotoxin. J. Cell. Biol. **136:**1239-47.

Katahira, J., H. Sugiyama, N. Inoue, Y. Horiguchi, M. Matsuda, and N. Sugimoto. 1997b. *Clostridium perfringens* enterotoxin utilizes two structurally related membrane proteins as functional receptors *in vivo*. J. Biol. Chem. **272:**26652-8.

Katayama, S., B. Dupuy, G. Daube, B. China, and S. T. Cole. 1996. Genome mapping of *Clostridium perfringens* strains with I-*Ceu*I shows many virulence genes to be plasmid-borne. Mol. Gen. Genet. **251:**720-6.

Katayama, S., O. Matsushita, J. Minami, S. Mizobuchi, and A. Okabe. 1993. Comparison of the alpha-toxin genes of *Clostridium perfringens* type A and C strains: evidence for extragenic regulation of transcription. Infect. Immun. **61:**457-63.

Kelneric, Z., T. Naglic, and I. Udovicic. 1996. Prevention of necrotic enteritis in piglets by vaccination of pregnant gilts with a *Clostridium perfringens* type C and D bacterin-toxoid. Vet. Med. (Praha) **41:**335-8.

Kerr, M. A., and A. J. Kenny. 1974. The molecular weight and properties of a neutral metallo-endopeptidase from rabbit kidney brush border. Biochem. J. **137:**489-95.

Khan, C. M., B. Villarreal-Ramos, R. J. Pierce, G. Riveau, R. Demarco de Hormaeche, H. McNeill, T. Ali, N. Fairweather, S. Chatfield, and A. Capron. 1994. Construction, expression, and immunogenicity of the *Schistosoma mansoni* P28 glutathione S-transferase as a genetic fusion to tetanus toxin fragment C in a live aro attenuated vaccine strain of *Salmonella*. Proc. Natl. Acad. Sci. USA **91:**11261-5.

Klaasen, H. L., M. J. Molkenboer, J. Bakker, R. Miserez, H. Hani, J. Frey, M. R. Popoff, and J. F. van den Bosch. 1999. Detection of the beta2 toxin gene of *Clostridium perfringens* in diarrhoeic piglets in The Netherlands and Switzerland. FEMS Immunol. Med. Microbiol. **24:**325-32.

Kochi, S. K., K. P. Killeen, and U. S. Ryan. 2003. Advances in the development of bacterial vector technology. Expert Rev. Vaccines **2:**31-43.

Kokai-Kun, J. F., and B. A. McClane. 1997. Deletion analysis of the *Clostridium perfringens* enterotoxin. Infect. Immun. **65:**1014-22.

Kondo, F. 1988. *In vitro* lecithinase activity and sensitivity to 22 antimicrobial agents of *Clostridium perfringens* isolated from necrotic enteritis of broiler chickens. Res. Vet. Sci. **45:**337-40.

Kondoh, M., A. Masuyama, A. Takahashi, N. Asano, H. Mizuguchi, N. Koizumi, M. Fujii, T. Hayakawa, Y. Horiguchi, and Y. Watanbe. 2005. A novel strategy for the enhancement of drug absorption using a claudin modulator. Mol. Pharmacol. **67:**749-56.

Koprowski, H., 2nd, M. M. Levine, R. J. Anderson, G. Losonsky, M. Pizza, and E. M. Barry. 2000. Attenuated *Shigella flexner*i 2a vaccine strain CVD 1204 expressing colonization factor antigen I and mutant heat-labile enterotoxin of enterotoxigenic *Escherichia coli*. Infect. Immun. **68:**4884-92.

Krieg, N. R. (ed.). 1984. Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, Baltimore.

Krug, E. L., and C. Kent. 1984. Phospholipase C from *Clostridium perfringens*: preparation and characterization of homogeneous enzyme. Arch. Biochem. Biophys. **231:**400-10.

Kurioka, S., and M. Matsuda. 1976. Phospholipase C assay using p-nitrophenylphosphorylcholine together with sorbitol and its application to studying the metal and detergent requirement of the enzyme. Anal. Biochem. **75:**281-9.

La Ragione, R. M., A. Narbad, M. J. Gasson, and M. J. Woodward. 2004. *In vivo* characterization of *Lactobacillus johnsonii* FI9785 for use as a defined competitive exclusion agent against bacterial pathogens in poultry. Lett. Appl. Microbiol. **38:**197-205.

La Ragione, R. M., and M. J. Woodward. 2003. Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype Enteritidis and *Clostridium perfringens* in young chickens. Vet. Microbiol. **94:**245-56.

Labbe, R. G. 1991. *Clostridium perfringens.* J. Assoc. Off. Anal. Chem. **74:**711-714.

Lattemann, C. T., Z. X. Yan, A. Matzen, T. F. Meyer, and H. Apfel. 1999. Immunogenicity of the extracellular copper/zinc superoxide dismutase of the filarial parasite *Acanthocheilonema viteae* delivered by a two-phase vaccine strain of *Salmonella typhimurium*. Parasite Immunol. **21:**219-24.

Lawrence, G. W., D. Lehmann, G. Anian, C. A. Coakley, G. Saleu, M. J. Barker, and M. W. Davis. 1990. Impact of active immunisation against enteritis necroticans in Papua New Guinea. Lancet **336:**1165-7.

Lee, J. J., K. A. Sinha, J. A. Harrison, R. D. de Hormaeche, G. Riveau, R. J. Pierce, A. Capron, R. A. Wilson, and C. M. Khan. 2000. Tetanus toxin fragment C expressed in live *Salmonella* vaccines enhances antibody responses to its fusion partner *Schistosoma haematobium* glutathione S-transferase. Infect. Immun. **68:**2503-12.

Leslie, D., N. Fairweather, D. Pickard, G. Dougan, and M. Kehoe. 1989. Phospholipase C and haemolytic activities of *Clostridium perfringens* alpha-toxin cloned in *Escherichia coli*: sequence and homology with a *Bacillus cereus* phospholipase C. Mol. Microbiol. **3:**383-92.

Levy, S. B. 1978. Emergence of antibiotic-resistant bacteria in the intestinal flora of farm inhabitants. J. Infect. Dis. **137:**689-90.

Li, Y., K. Reichenstein, R. Ullrich, T. Danner, B. U. von Specht, and H. P. Hahn. 2003. Effect of in situ expression of human interleukin-6 on antibody responses against *Salmonella typhimurium* antigens. FEMS Immunol. Med. Microbiol. **37:**135-45.

Lindgren, S. W., I. Stojiljkovic, and F. Heffron. 1996. Macrophage killing is an essential virulence mechanism of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **93:**4197-201.

Linton, A. H., K. Howe, P. M. Bennett, M. H. Richmond, and E. J. Whiteside. 1977. The colonization of the human gut by antibiotic resistant *Escherichia coli* from chickens. J. Appl. Bacteriol. **43:**465-9.

Lister, S. A. 1996, posting date. Coccidiosis - A Continuing challenge? [Online.] http://www.antecint.co.uk/main/coccidio.htm. DuPont Animal Health Solutions.

Lo, W. F., H. Ong, E. S. Metcalf, and M. J. Soloski. 1999. T cell responses to Gram-negative intracellular bacterial pathogens: a role for CD8+ T cells in immunity to *Salmonella* infection and the involvement of MHC class Ib molecules. J. Immunol. **162:**5398-406.

Logan, A. J., E. D. Williamson, R. W. Titball, D. A. Percival, A. D. Shuttleworth, J. W. Conlan, and D. C. Kelly. 1991. Epitope mapping of the alpha-toxin of *Clostridium perfringens*. Infect. Immun. **59:**4338-42.

Londono, L. P., S. Chatfield, R. W. Tindle, K. Herd, X. M. Gao, I. Frazer, and G. Dougan. 1996. Immunisation of mice using *Salmonella typhimurium* expressing human papillomavirus type 16 E7 epitopes inserted into hepatitis B virus core antigen. Vaccine **14:**545-52.

Long, J. R., J. R. Pettit, and D. A. Barnum. 1974. Necrotic enteritis in broiler chickens. II. Pathology and proposed pathogenesis. Can. J. Comp. Med. **38:**467-74.

Lovland, A., and M. Kaldhusdal. 1999. Liver lesions seen at slaughter as an indicator of necrotic enteritis in broiler flocks. FEMS Immunol. Med. Microbiol. **24:**345-51.

Lovland, A., and M. Kaldhusdal. 2001. Severely impaired production performance in broiler flocks with high incidence of *Clostridium perfringens*-associated hepatitis. Avian Pathol. **30:**73-81.

Lovland, A., M. Kaldhusdal, K. Redhead, E. Skjerve, and A. Lillehaug. 2004. Maternal vaccination against subclinical necrotic enteritis in broilers. Avian Pathol. **33:**83-92.

Lovland, A., M. Kaldhusdal, and L. J. Reitan. 2003. Diagnosing *Clostridium perfringens*-associated necrotic enteritis in broiler flocks by an immunoglobulin G anti-alpha-toxin enzyme-linked immunosorbent assay. Avian Pathol. **32:**527-34.

Lucey, B. P., and G. M. Hutchins. 2004. William H. Welch, MD, and the discovery of Bacillus welchii. Arch Pathol Lab Med **128:**1193-5.

Luh, K.-T., P.-R. Hsueh, L.-J. Teng, H.-J. Pan, Y.-C. Chen, J.-J. Lu, J.-J. Wu, and S.-W. Ho. 2000. Quinupristin-dalfopristin resistance among Gram-positive bacteria in Taiwan. Antimicrob. Agents Chemother. **44:**3374-3380.

Lyons, R. W., C. L. Samples, H. N. DeSilva, K. A. Ross, E. M. Julian, and P. J. Checko. 1980. An epidemic of resistant *Salmonella* in a nursery. Animal-to-human spread. JAMA **243:**546-7.

Lyristis, M., A. E. Bryant, J. Sloan, M. M. Awad, I. T. Nisbet, D. L. Stevens, and J. I. Rood. 1994. Identification and molecular analysis of a locus that regulates extracellular toxin production in *Clostridium perfringens*. Mol. Microbiol. **12:**761-77.

MacFarlane, M. G., and B. C. J. G. Knight. 1941. The biochemistry of bacterial toxins. I. The lecithinase activity of *Cl. welchii* toxins. Biochem. J. **35:**884-902.

Makela, P. H. 2000. Vaccines, coming of age after 200 years. FEMS Microbiol. Rev. **24:**9-20.

Mallya, S. K., and H. E. Van Wart. 1989. Mechanism of inhibition of human neutrophil collagenase by gold(I) chrysotherapeutic compounds. Interaction at a heavy metal binding site. J. Biol. Chem. **264:**1594-601.

Mann, H. B., and D. R. Whitney. 1947. On a test on whether one of two random variables is stochastically larger than the other. Ann. Math. Statist. **18:**50-60.

Manteca, C., G. Daube, T. Jauniaux, A. Linden, V. Pirson, J. Detilleux, A. Ginter, P. Coppe, A. Kaeckenbeeck, and J. G. Mainil. 2002. A role for the *Clostridium perfringens* beta2 toxin in bovine enterotoxaemia? Vet. Microbiol. **86:**191-202.

Mantis, N. J. 2005. Vaccines against the category B toxins: Staphylococcal enterotoxin B, epsilon toxin and ricin. Adv. Drug. Deliv. Rev. **57:**1424-39.

Martel, A., L. A. Devriese, K. Cauwerts, K. De Gussem, A. Decostere, and F. Haesebrouck. 2004. Susceptibility of *Clostridium perfringens* strains from broiler chickens to antibiotics and anticoccidials. Avian Pathol. **33:**3-7.

Marvaud, J. C., B. G. Stiles, A. Chenal, D. Gillet, M. Gibert, L. A. Smith, and M. R. Popoff. 2002. *Clostridium perfringens* iota toxin. Mapping of the Ia domain involved in docking with Ib and cellular internalization. J. Biol. Chem. **277:**43659-66.

Maskell, D. J., K. J. Sweeney, D. O'Callaghan, C. E. Hormaeche, F. Y. Liew, and G. Dougan. 1987. *Salmonella typhimurium aro*A mutants as carriers of the *Escherichia coli* heat-labile enterotoxin B subunit to the murine secretory and systemic immune systems. Microb. Pathog. **2:**211-21.

Mastroeni, P., and N. Menager. 2003. Development of acquired immunity to *Salmonella*. J. Med. Microbiol. **52:**453-9.

Mastroeni, P., C. Simmons, R. Fowler, C. E. Hormaeche, and G. Dougan. 2000. Igh-6(-/-) (B-cell-deficient) mice fail to mount solid acquired resistance to oral challenge with virulent *Salmonella enterica* serovar Typhimurium and show impaired Th1 T-cell responses to *Salmonella* antigens. Infect. Immun. **68:**46-53.

Mastroeni, P., B. Villarreal-Ramos, and C. E. Hormaeche. 1993. Adoptive transfer of immunity to oral challenge with virulent salmonellae in innately susceptible BALB/c mice requires both immune serum and T cells. Infect. Immun. **61:**3981-4.

Mastroeni, P., B. Villarreal-Ramos, and C. E. Hormaeche. 1992. Role of T cells, TNF alpha and IFN gamma in recall of immunity to oral challenge with virulent salmonellae in mice vaccinated with live attenuated aro- salmonella vaccines. Microb. Pathog. **13:**477-91.

Matsumoto, S., H. Yukitake, H. Kanbara, and T. Yamada. 1998. Recombinant *Mycobacterium bovis bacillus* Calmette-Guerin secreting merozoite surface protein 1 (MSP1) induces protection against rodent malaria parasite infection depending on MSP1-stimulated interferon gamma and parasite-specific antibodies. J. Exp. Med. **188:**845-54.

Matsushita, C., O. Matsushita, S. Katayama, J. Minami, K. Takai, and A. Okabe. 1996. An upstream activating sequence containing curved DNA involved in activation of the *Clostridium perfringens plc* promoter. Microbiology **142 (Pt 9):**2561-6.

McClane, B. A., A. P. Wnek, K. I. Hulkower, and P. C. Hanna. 1988. Divalent cation involvement in the action of *Clostridium perfringens* type A enterotoxin. Early events in enterotoxin action are divalent cation-independent. J. Biol. Chem. **263:**2423-35.

McCourt, M. T., D. A. Finlay, C. Laird, J. A. Smyth, C. Bell, and H. J. Ball. 2005. Sandwich ELISA detection of *Clostridium perfringens* cells and alpha-toxin from field cases of necrotic enteritis of poultry. Vet. Microbiol. **106:**259-64.

McDonel, J. L. 1980. *Clostridium perfringens* toxins (type A, B, C, D, E). Pharmacol. Ther. **10:**617-55.

Mead, G. C. 2000. Prospects for 'competitive exclusion' treatment to control salmonellas and other foodborne pathogens in poultry. Vet. J. **159:**111-23.

Medina, E., C. A. Guzman, L. H. Staendner, M. P. Colombo, and P. Paglia. 1999. *Salmonella* vaccine carrier strains: effective delivery system to trigger anti-tumor immunity by oral route. Eur. J. Immunol. **29:**693-9.

Meer, R. R., and J. G. Songer. 1997. Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. Am. J. Vet. Res. **58:**702-5.

Miller, M. B., and B. L. Bassler. 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. **55:**165-99.

Minami, J., S. Katayama, O. Matsushita, C. Matsushita, and A. Okabe. 1997. Lambda-toxin of *Clostridium perfringens* activates the precursor of epsilon-toxin by releasing its N- and C-terminal peptides. Microbiol. Immunol. **41:**527-35.

Mitov, I., V. Denchev, and K. Linde. 1992. Humoral and cell-mediated immunity in mice after immunization with live oral vaccines of *Salmonella typhimurium*: auxotrophic mutants with two attenuating markers. Vaccine **10:**61-6.

Miyamoto, O., J. Minami, T. Toyoshima, T. Nakamura, T. Masada, S. Nagao, T. Negi, T. Itano, and A. Okabe. 1998. Neurotoxicity of *Clostridium perfringens* epsilon-toxin for the rat hippocampus via the glutamatergic system. Infect. Immun. **66:**2501-8.

Miyamoto, O., J. Minami, T. Toyoshima, T. Nakamura, T. Masada, S. Nagao, T. Negi, T. Itano, and A. Okabe. 1998. Neurotoxicity of Clostridium perfringens epsilon-toxin for the rat hippocampus via the glutamatergic system. Infect Immun **66:**2501-8.

Miyata, S., J. Minami, E. Tamai, O. Matsushita, S. Shimamoto, and A. Okabe. 2002. *Clostridium perfringens* epsilon-toxin forms a heptameric pore within the detergent-insoluble microdomains of Madin-Darby canine kidney cells and rat synaptosomes. J. Biol. Chem. **277:**39463-8.

Molbak, K. 2004. Spread of resistant bacteria and resistance genes from animals to humans - The public health consequences. J. Vet. Med. B **51:**364-369.

Molina, N. C., and C. D. Parker. 1990. Murine antibody response to oral infection with live aroA recombinant *Salmonella dublin* vaccine strains expressing filamentous hemagglutinin antigen from *Bordetella pertussis*. Infect. Immun. **58:**2523-8.

Mollby, R., and T. Holme. 1976. Production of phospholipase C (alpha-toxin), haemolysins and lethal toxins by *Clostridium perfringens* types A to D. J. Gen. Microbiol. **96:**137-44.

Mollby, R., C. E. Nord, and T. Wadstrom. 1973. Biological activities contaminating preparations of phospholipase C (α-toxin) from *Clostridium perfringens*. Toxicon **11:**139-47.

Moller, K., and P. Ahrens. 1996. Comparison of toxicity neutralization, ELISA and PCR tests for typing of *Clostridium perfringens* and detection of the enterotoxin gene by PCR. Anaerobe **2:**103-110.

Monack, D. M., B. Raupach, A. E. Hromockyj, and S. Falkow. 1996. *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. Proc. Natl. Acad. Sci. USA **93:**9833-8.

Morita, K., M. Furuse, K. Fujimoto, and S. Tsukita. 1999. Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc. Natl. Acad. Sci. USA **96:**511-6.

Moser, I., A. Hohmann, G. Schmidt, and D. Rowley. 1980. Salmonellosis in mice: studies on oral immunization with live avirulent vaccines. Med. Microbiol. Immunol. (Berl) **168:**119-28.

Moutafis, G. 2002. Ph.D. Doctor of Philosophy. RMIT University, Melbourne. Utilisation of an *aro-Salmonella* Enteritidis as a live vaccine and as an expression system for cloned antigens.

Moxley, R. A., and G. E. Duhamel. 1999. Comparative pathology of bacterial enteric diseases of swine, p. 83-101. *In* P. S. Paul and D. H. Francis (ed.), Mechanisms in the pathogenesis of enteric diseases, vol. 2. Kluwer Academic, New York.

Mutunga, M., S. Graham, R. D. De Hormaeche, J. A. Musson, J. H. Robinson, P. Mastroeni, C. M. Khan, and C. E. Hormaeche. 2004. Attenuated *Salmonella typhimurium htr*A mutants cause fatal infections in mice deficient in NADPH oxidase and destroy NADPH oxidase-deficient macrophage monolayers. Vaccine **22:**4124-31.

Nagahama, M., S. Hayashi, S. Morimitsu, and J. Sakurai. 2003. Biological activities and pore formation of *Clostridium perfringens* beta toxin in HL 60 cells. J. Biol. Chem. **278:**36934-41.

Nagahama, M., H. Iida, E. Nishioka, K. Okamoto, and J. Sakurai. 1994. Roles of the carboxy-terminal region of *Clostridium perfringens* alpha toxin. FEMS Microbiol. Lett. **120:**297-301.

Nagahama, M., K. Michiue, M. Mukai, S. Ochi, and J. Sakurai. 1998. Mechanism of membrane damage by *Clostridium perfringens* alpha-toxin. Microbiol. Immunol. **42:**533-8.

Nagahama, M., K. Michiue, and J. Sakurai. 1996. Membrane-damaging action of *Clostridium perfringens* alpha-toxin on phospholipid liposomes. Biochim. Biophys. Acta. **1280:**120-6.

Nagahama, M., M. Mukai, S. Ochi, and J. Sakurai. 2000. Role of tryptophan-1 in hemolytic and phospholipase C activities of *Clostridium perfringens* alpha-toxin. Microbiol. Immunol. **44:**585-9.

Nagahama, M., T. Nakayama, K. Michiue, and J. Sakurai. 1997. Site-specific mutagenesis of *Clostridium perfringens* alpha-toxin: replacement of Asp-56, Asp-130, or Glu-152 causes loss of enzymatic and hemolytic activities. Infect. Immun. **65:**3489-92.

Nagahama, M., Y. Okagawa, T. Nakayama, E. Nishioka, and J. Sakurai. 1995. Site-directed mutagenesis of histidine residues in *Clostridium perfringens* alpha-toxin. J. Bacteriol. **177:**1179-85.

Nagahama, M., A. Otsuka, and J. Sakurai. 2006. Role of tyrosine-57 and -65 in membrane-damaging and sphingomyelinase activities of *Clostridium perfringens* alpha-toxin. Biochim. Biophys. Acta. **1762:**110-4.

Nagahama, M., and J. Sakurai. 1991. Distribution of labeled *Clostridium perfringens* epsilon toxin in mice. Toxicon **29:**211-7.

Nagahama, M., and J. Sakurai. 1992. High-affinity binding of *Clostridium perfringens* epsilon-toxin to rat brain. Infect. Immun. **60:**1237-40.

Nagahama, M., and J. Sakurai. 1996. Threonine-74 is a key site for the activity of *Clostridium perfringens* alpha-toxin. Microbiol. Immunol. **40:**189-93.

Nagahama, M., T. Takahashi, and J. Sakurai. 1990. Effect of prior treatment with *Clostridium perfringens* epsilon toxin inactivated by various agents on lethal, pressor and contractile activities of the toxin. FEMS Microbiol. Lett. **60:**59-62.

Nakamoto, T. 2006. A unified view of the initiation of protein synthesis. Biochem. Biophys. Res. Commun. **341:**675-8.

Narayan, K. G. 1982. Food borne infection with *Clostridium perfringens* type A. Int. J. Zoonoses **9:**12-32.

Nauerby, B., K. Pedersen, and M. Madsen. 2003. Analysis by pulsed-field gel electrophoresis of the genetic diversity among *Clostridium perfringens* isolates from chickens. Vet. Microbiol. **94:**257-66.

Naylor, C. E., J. T. Eaton, A. Howells, N. Justin, D. S. Moss, R. W. Titball, and A. K. Basak. 1998. Structure of the key toxin in gas gangrene. Nat. Struct. Biol. **5:**738-46.

Naylor, C. E., M. Jepson, D. T. Crane, R. W. Titball, J. Miller, A. K. Basak, and B. Bolgiano. 1999. Characterisation of the calcium-binding C-terminal domain of *Clostridium perfringens* alpha-toxin. J. Mol. Biol. **294:**757-70.

Newton, A. C. 1995. Protein Kinase C: Seeing two domains. Curr. Biol. **5:**973-976.

Newton, S. M., C. O. Jacob, and B. A. Stocker. 1989. Immune response to cholera toxin epitope inserted in *Salmonella* flagellin. Science **244:**70-2.

Ninomiya, M., O. Matsushita, J. Minami, H. Sakamoto, M. Nakano, and A. Okabe. 1994. Role of alpha-toxin in *Clostridium perfringens* infection determined by using recombinants of *C. perfringens* and *Bacillus subtilis*. Infect. Immun. **62:**5032-9.

Nord, C. E., R. Mollby, C. Smyth, and T. Wadstrom. 1974. Formation of phospholipase C and theta-haemolysin in pre-reduced media in batch and continuous culture of *Clostridium perfringens* type A. J. Gen. Microbiol. **84:**117-27.

Norimatsu, M., V. Chance, G. Dougan, C. J. Howard, and B. Villarreal-Ramos. 2004. Live *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) elicit dendritic cell responses that differ from those induced by killed *S.* Typhimurium. Vet. Immunol. Immunopathol. **98:**193-201.

Nurmi, E., and M. Rantala. 1973. New aspects of *Salmonella* infection in broiler production. Nature **241:**210-1.

O'Brien, D. K., and S. B. Melville. 2004. Effects of *Clostridium perfringens* alpha-toxin (PLC) and perfringolysin O (PFO) on cytotoxicity to macrophages, on escape from the phagosomes of macrophages, and on persistence of *C. perfringens* in host tissues. Infect. Immun. **72:**5204-15.

O'Callaghan, D., D. Maskell, F. Y. Liew, C. S. Easmon, and G. Dougan. 1988. Characterization of aromatic- and purine-dependent *Salmonella typhimurium*: attention, persistence, and ability to induce protective immunity in BALB/c mice. Infect. Immun. **56:**419-23.

Ochi, S., K. Hashimoto, M. Nagahama, and J. Sakurai. 1996. Phospholipid metabolism induced by *Clostridium perfringens* alpha-toxin elicits a hot-cold type of hemolysis in rabbit erythrocytes. Infect. Immun. **64:**3930-3.

Ohtani, K., S. K. Bhowmik, H. Hayashi, and T. Shimizu. 2002a. Identification of a novel locus that regulates expression of toxin genes in *Clostridium perfringens*. FEMS Microbiol. Lett. **209:**113-8.

Ohtani, K., H. Hayashi, and T. Shimizu. 2002b. The *lux*S gene is involved in cell-cell signalling for toxin production in *Clostridium perfringens*. Mol. Microbiol. **44:**171-9.

Onderdonk, A. B., and S. D. Allen. 1995. *Clostridium*, p. 574-586. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover, and R. H. Yolken (ed.), Manual of clinical microbiology, 6th ed. American Society for Microbiology, Washington, D.C.

Pacheco, L. G., E. Zucconi, V. L. Mati, R. M. Garcia, A. Miyoshi, S. C. Oliveira, A. L. de Melo, and V. Azevedo. 2005. Oral administration of a live Aro attenuated *Salmonella* vaccine strain expressing 14-kDa *Schistosoma mansoni* fatty acid-binding protein induced partial protection against experimental schistosomiasis. Acta. Trop. **95:**132-42.

Parish, W. E. 1961a. Necrotic enteritis in the fowl (*Gallus gallus domesticus*). I. Histopathology of the disease and isolation of a strain of *Clostridium welchii*. J. Comp. Pathol. **71:**377-93.

Parish, W. E. 1961b. Necrotic enteritis in the fowl. II. Examination of the causal *Clostridium welchii*. J. Comp. Pathol. **71:**394-404.

Pashine, A., B. John, S. Rath, A. George, and V. Bal. 1999. Th1 dominance in the immune response to live *Salmonella typhimurium* requires bacterial invasiveness but not persistence. Int. Immunol. **11:**481-9.

Paton, J. C., J. K. Morona, S. Harrer, D. Hansman, and R. Morona. 1993. Immunization of mice with *Salmonella typhimurium* C5 *aro*A expressing a genetically toxoided derivative of the pneumococcal toxin pneumolysin. Microb. Pathog. **14:**95-102.

Patterson, J. A., and K. M. Burkholder. 2003. Application of prebiotics and probiotics in poultry production. Poult. Sci. **82:**627-31.

Payne, D., and P. Oyston. 1998. The *Clostridium perfringens* ε-toxin, p. 439-447. *In* J. I. Rood, B. A. McClane, J. G. Songer, and R. W. Titball (ed.), The *Clostridia*: Molecular biology and pathogenesis. Academic Press, San Diego.

Perelle, S., M. Domenighini, and M. R. Popoff. 1996. Evidence that Arg-295, Glu-378, and Glu-380 are active-site residues of the ADP-ribosyltransferase activity of iota toxin. FEBS Lett. **395:**191-4.

Perelle, S., M. Gibert, P. Boquet, and M. R. Popoff. 1993. Characterization of *Clostridium perfringens* iota-toxin genes and expression in *Escherichia coli*. Infect. Immun. **61:**5147-56.

Perelle, S., M. Gibert, P. Boquet, and M. R. Popoff. 1995. Characterization of *Clostridium perfringens* iota-toxin genes and expression in *Escherichia coli*. Infect. Immun. **63:**4967.

Petit, L., M. Gibert, D. Gillet, C. Laurent-Winter, P. Boquet, and M. R. Popoff. 1997. *Clostridium perfringens* epsilon-toxin acts on MDCK cells by forming a large membrane complex. J. Bacteriol. **179:**6480-7.

Pfeifer, J. D., M. J. Wick, R. L. Roberts, K. Findlay, S. J. Normark, and C. V. Harding. 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. Nature **361:**359-62.

Pfizer 2006, posting date. Clostridial diseases. [Online.] http://www.pfizerah.com. Pfizer.

Phalipon, A., and P. Sansonetti. 1995. Live attenuated *Shigella flexneri* mutants as vaccine candidates against shigellosis and vectors for antigen delivery. Biologicals **23:**125-34.

Pogliano, J., A. S. Lynch, D. Belin, E. C. Lin, and J. Beckwith. 1997. Regulation of *Escherichia coli* cell envelope proteins involved in protein folding and degradation by the Cpx two-component system. Genes Dev. **11:**1169-82.

Pogonka, T., C. Klotz, F. Kovacs, and R. Lucius. 2003. A single dose of recombinant *Salmonella typhimurium* induces specific humoral immune responses against heterologous *Eimeria tenella* antigens in chicken. Int. J. Parasitol. **33:**81-8.

Poirier, T. P., M. A. Kehoe, and E. H. Beachey. 1988. Protective immunity evoked by oral administration of attenuated *aro*A *Salmonella typhimurium* expressing cloned streptococcal M protein. J. Exp. Med. **168:**25-32.

Popoff, M. R., and P. Boquet. 1988a. *Clostridium spiroforme* toxin is a binary toxin which ADP-ribosylates cellular actin. Biochem. Biophys. Res. Commun. **152:**1361-8.

PoultrySite 2006, posting date. EU's antibiotic growth promoter ban only has short-term effect. [Online.] The Poultry Site http://www.thepoultrysite.com/LatestNews/Default.asp?AREA=LatestNews&Display=8830.

Quinn, P. J., W. J. Donnely, B. K. Markey, M. E. Carter, and F. C. Leonard. 2002. *Clostridium* species, p. 84-96. *In* P. J. Quinn, W. J. Donnely, B. K. Markey, M. E. Carter, and F. C. Leonard (ed.), Veterinary microbiology and microbial disease. Blackwell Science, Oxford.

Ranallo, R. T., C. P. Fonseka, F. Cassels, J. Srinivasan, and M. M. Venkatesan. 2005. Construction and characterization of bivalent *Shigella flexneri* 2a vaccine strains SC608(pCFAI) and SC608(pCFAI/LTB) that express antigens from enterotoxigenic *Escherichia coli*. Infect. Immun. **73:**258-67.

Raupach, B., and S. H. Kaufmann. 2001a. Bacterial virulence, proinflammatory cytokines and host immunity: How to choose the appropriate *Salmonella* vaccine strain? Microbes Infect. **3:**1261-9.

Raupach, B., and S. H. Kaufmann. 2001b. Immune responses to intracellular bacteria. Curr. Opin. Immunol. **13:**417-28.

Redman, T. K., C. C. Harmon, R. L. Lallone, and S. M. Michalek. 1995. Oral immunization with recombinant *Salmonella typhimurium* expressing surface protein antigen A of *Streptococcus sobrinus*: dose response and induction of protective humoral responses in rats. Infect. Immun. **63:**2004-11.

Rehman, H., W. A. Awad, I. Lindner, M. Hess, and J. Zentek. 2006. *Clostridium perfringens* alpha toxin affects electrophysiological properties of isolated jejunal mucosa of laying hens. Poult. Sci. **85:**1298-302.

Riddell, C., and X. M. Kong. 1992. The influence of diet on necrotic enteritis in broiler chickens. Avian Dis. **36:**499-503.

Rizo, J., and T. C. Sudhof. 1998. C2-domains, structure and function of a universal Ca^{2+}-binding domain. J. Biol. Chem. **273:**15879-15882.

Roberts, M., J. Li, A. Bacon, and S. Chatfield. 1998. Oral vaccination against tetanus: comparison of the immunogenicities of *Salmonella* strains expressing fragment C from the *nir*B and *htr*A promoters. Infect. Immun. **66:**3080-7.

Roit, I. 1994. Essential Immunology, 8th ed. Blackwell Scientific Publications, London.

Rood, J. I. 1998. Virulence genes of *Clostridium perfringens*. Annu. Rev. Microbiol. **52:**333-60.

Rood, J. I., and S. T. Cole. 1991. Molecular genetics and pathogenesis of *Clostridium perfringens*. Microbiol. Rev. **55:**621-48.

Rood, J. I., B. A. McClane, J. G. Songer, and R. W. Titball (ed.). 1997. The *Clostridia*: Molecular biology and pathogenesis. Academic Press, San Diego.

Rozkov, A., and S. O. Enfors. 2004. Analysis and control of proteolysis of recombinant proteins in *Escherichia coli*. Adv. Biochem. Eng. Biotechnol. **89:**163-95.

Ruiz-Perez, F., R. Leon-Kempis, A. Santiago-Machuca, G. Ortega-Pierres, E. Barry, M. Levine, and C. Gonzalez-Bonilla. 2002. Expression of the *Plasmodium falciparum* immunodominant epitope (NANP)(4) on the surface of *Salmonella enterica* using the autotransporter MisL. Infect. Immun. **70:**3611-20.

Ryan, E. T., J. R. Butterton, R. N. Smith, P. A. Carroll, T. I. Crean, and S. B. Calderwood. 1997. Protective immunity against *Clostridium difficile* toxin A induced by oral immunization with a live, attenuated *Vibrio cholerae* vector strain. Infect. Immun. **65:**2941-9.

Ryan, M., L. McCarthy, R. Rappuoli, B. P. Mahon, and K. H. Mills. 1998. Pertussis toxin potentiates Th1 and Th2 responses to co-injected antigen: adjuvant action is associated with enhanced regulatory cytokine production and expression of the co-stimulatory molecules B7-1, B7-2 and CD28. Int. Immunol. **10:**651-62.

Sadoff, J. C., W. R. Ballou, L. S. Baron, W. R. Majarian, R. N. Brey, W. T. Hockmeyer, J. F. Young, S. J. Cryz, J. Ou, G. H. Lowell, and et al. 1988. Oral *Salmonella typhimurium* vaccine expressing circumsporozoite protein protects against malaria. Science **240:**336-8.

Saint-Joanis, B., T. Garnier, and S. T. Cole. 1989. Gene cloning shows the alpha-toxin of *Clostridium perfringens* to contain both sphingomyelinase and lecithinase activities. Mol. Gen. Genet. **219:**453-60.

Sakurai, J., and C. L. Duncan. 1978. Some properties of beta-toxin produced by *Clostridium perfringens* type C. Infect. Immun. **21:**678-80.

Sakurai, J., and Y. Fujii. 1987. Purification and characterization of *Clostridium perfringens* beta toxin. Toxicon **25:**1301-10.

Sakurai, J., Y. Fujii, K. Dezaki, and K. Endo. 1984. Effect of *Clostridium perfringens* beta toxin on blood pressure of rats. Microbiol. Immunol. **28:**23-31.

Sakurai, J., Y. Fujii, M. Matsuura, and K. Endo. 1981. Pharmacological effect of beta toxin of *Clostridium perfringens* type C on rats. Microbiol. Immunol. **25:**423-32.

Sakurai, J., and K. Kobayashi. 1995. Lethal and dermonecrotic activities of *Clostridium perfringens* iota toxin: biological activities induced by cooperation of two nonlinked components. Microbiol. Immunol. **39:**249-53.

Sakurai, J., M. Nagahama, and Y. Fujii. 1983. Effect of *Clostridium perfringens* epsilon toxin on the cardiovascular system of rats. Infect. Immun. **42:**1183-6.

Sakurai, J., M. Nagahama, and T. Takahashi. 1989. Contraction induced by *Clostridium perfringens* epsilon toxin in the isolated rat ileum. FEMS Microbiol Lett **49:**269-72.

Sakurai, J., S. Ochi, and H. Tanaka. 1993. Evidence for coupling of *Clostridium perfringens* alpha-toxin-induced hemolysis to stimulated phosphatidic acid formation in rabbit erythrocytes. Infect. Immun. **61:**3711-8.

Sakurai, J., S. Ochi, and H. Tanaka. 1994. Regulation of *Clostridium perfringens* alpha-toxin-activated phospholipase C in rabbit erythrocyte membranes. Infect. Immun. **62:**717-21.

Sambrook, J., and D. W. Russell. 2001. Molecular cloning: A laboratory manual., 3rd ed. Cold Spring Harbor, New York.

Santana, M. A., and Y. Rosenstein. 2003. What it takes to become an effector T cell: the process, the cells involved, and the mechanisms. J. Cell. Physiol. **195:**392-401.

Sargeant, I. R., K. S. Cheng, P. V. Vincent, M. Davidson, R. Vicary, J. H. Wyllie, and M. M. Brett. 2000. An unusual case of 'firebowels'. J. Gastroenterol Hepatol. Nutr. **3:**23-24.

Sarker, M. R., R. J. Carman, and B. A. McClane. 1999. Inactivation of the gene (*cpe*) encoding *Clostridium perfringens* enterotoxin eliminates the ability of two cpe-positive *C. perfringens* type A human gastrointestinal disease isolates to affect rabbit ileal loops. Mol. Microbiol. **33:**946-58.

Sato, H., J. Chiba, and Y. Sato. 1989. Monoclonal antibodies against alpha toxin of *Clostridium perfringens*. FEMS Microbiol. Lett. **50:**173-6.

Schmidt, M., E. Viaplana, F. Hoffmann, S. Marten, A. Villaverde, and U. Rinas. 1999. Secretion-dependent proteolysis of heterologous protein by recombinant *Escherichia coli* is connected to an increased activity of the energy-generating dissimilatory pathway. Biotechnol. Bioeng. **66:**61-7.

Schneerson, R., O. Barrera, A. Sutton, and J. B. Robbins. 1980. Preparation, characterization, and immunogenicity of *Haemophilus influenzae* type b polysaccharide-protein conjugates. J. Exp. Med. **152:**361-76.

Schneitz, C. 2005. Competitive exclusion in poultry-30 years of research. Food Control 7th Karlsruhe Nutrition Congress on Food Safety **16:**657-667.

Schodel, F., D. R. Milich, and H. Will. 1990. Hepatitis B virus nucleocapsid/pre-S2 fusion proteins expressed in attenuated *Salmonella* for oral vaccination. J. Immunol. **145:**4317-21.

Schoepe, H., L. H. Wieler, R. Bauerfeind, T. Schlapp, H. Potschka, H. R. Hehnen, and G. Baljer. 1997. Neutralization of hemolytic and mouse lethal activities of *C. perfringens* alpha-toxin need simultaneous blockade of two epitopes by monoclonal antibodies. Microb. Pathog. **23:**1-10.

Schriefer, A., J. R. Maltez, N. Silva, M. Y. Stoeckle, M. Barral-Netto, and L. W. Riley. 1999. Expression of a pilin subunit *Bfp*A of the bundle-forming pilus of enteropathogenic *Escherichia coli* in an aroA live salmonella vaccine strain. Vaccine **17:**770-8.

Schwarz, S., C. Kehrenberg, and T. R. Walsh. 2001. Use of antimicrobial agents in veterinary medicine and food animal production. Int. J. Antimicrob. Agents. **17:**431-7.

Schweder, T., and M. Hecker. 2004. Monitoring of stress responses. Adv. Biochem. Eng. Biotechnol. **89:**47-71.

Sellitti, M. A., P. A. Pavco, and D. A. Steege. 1987. *lac* repressor blocks *in vivo* transcription of *lac* control region DNA. Proc. Natl. Acad. Sci. USA **84:**3199-203.

Shane, S. M., J. E. Gyimah, K. S. Harrington, and T. G. Snider, 3rd. 1985. Etiology and pathogenesis of necrotic enteritis. Vet. Res. Commun. **9:**269-87.

Shata, M. T., L. Stevceva, S. Agwale, G. K. Lewis, and D. M. Hone. 2000. Recent advances with recombinant bacterial vaccine vectors. Mol. Med. Today **6:**66-71.

Shatursky, O., R. Bayles, M. Rogers, B. H. Jost, J. G. Songer, and R. K. Tweten. 2000. *Clostridium perfringens* beta-toxin forms potential-dependent, cation-selective channels in lipid bilayers. Infect. Immun. **68:**5546-51.

Shea, K. 2004. Nontherapeutic use of antimicrobial agents in animal agriculture: Implications for pediatrics. Pediatrics **114:**862-868.

Shen, H., M. K. Slifka, M. Matloubian, E. R. Jensen, R. Ahmed, and J. F. Miller. 1995. Recombinant *Listeria monocytogenes* as a live vaccine vehicle for the induction of protective anti-viral cell-mediated immunity. Proc. Natl. Acad. Sci. USA **92:**3987-91.

Shimizu, T., K. Ohtani, H. Hirakawa, K. Ohshima, A. Yamashita, T. Shiba, N. Ogasawara, M. Hattori, S. Kuhara, and H. Hayashi. 2002a. Complete genome sequence of *Clostridium perfringens*, an anaerobic flesh-eater. Proc. Natl. Acad. Sci. USA **99:**996-1001.

Shimizu, T., K. Shima, K. Yoshino, K. Yonezawa, and H. Hayashi. 2002b. Proteome and transcriptome analysis of the virulence genes regulated by the VirR/VirS system in *Clostridium perfringens*. J. Bacteriol. **184:**2587-94.

Simonsen, G. S., H. Haaheim, K. H. Dahl, H. Kruse, A. Lovseth, O. Olsvik, and A. Sundsfjord. 1998. Transmission of *van*A-type vancomycin-resistant enterococci and *van*A resistance elements between chicken and humans at avoparcin-exposed farms. Microb. Drug Resist. **4:**313-8.

Simpson, L. L., B. G. Stiles, H. H. Zepeda, and T. D. Wilkins. 1987. Molecular basis for the pathological actions of *Clostridium perfringens* iota toxin. Infect. Immun. **55:**118-22.

Singh, U., L. L. Mitic, E. U. Wieckowski, J. M. Anderson, and B. A. McClane. 2001. Comparative biochemical and immunocytochemical studies reveal differences in the effects of *Clostridium perfringens* enterotoxin on polarized CaCo-2 cells versus Vero cells. J. Biol. Chem. **276:**33402-12.

Singh, U., C. M. Van Itallie, L. L. Mitic, J. M. Anderson, and B. A. McClane. 2000. CaCo-2 cells treated with *Clostridium perfringens* enterotoxin form multiple large complex species, one of which contains the tight junction protein occludin. J. Biol. Chem. **275:**18407-17.

Sirard, J. C., M. Weber, E. Duflot, M. R. Popoff, and M. Mock. 1997. A recombinant *Bacillus anthracis* strain producing the *Clostridium perfringens* Ib component induces protection against iota toxins. Infect. Immun. **65:**2029-33.

Skorko-Glonek, J., D. Zurawa, E. Kuczwara, M. Wozniak, Z. Wypych, and B. Lipinska. 1999. The *Escherichia coli* heat shock protease HtrA participates in defense against oxidative stress. Mol. Gen. Genet. **262:**342-50.

Smedley, J. G., 3rd, D. J. Fisher, S. Sayeed, G. Chakrabarti, and B. A. McClane. 2004. The enteric toxins of *Clostridium perfringens*. Rev. Physiol. Biochem. Pharmacol. **152:**183-204.

Smith, B. P., M. Reina-Guerra, B. A. Stocker, S. K. Hoiseth, and E. Johnson. 1984. Aromatic-dependent *Salmonella dublin* as a parenteral modified live vaccine for calves. Am. J. Vet. Res. **45:**2231-5.

Songer, J. G. 1997. Clostridial diseases of animals, p. 153-183. *In* J. I. Rood, J. McClane, J. G. Songer, and R. W. Titball (ed.), The *Clostridia*: Molecular biology and pathogenesis, 1st ed. Academic Press, San Diego.

Songer, J. G. 1998. Clostridial diseases of small ruminants. Vet. Res. **29:**219-32.

Songer, J. G. 1996. Clostridial enteric diseases of domestic animals. Clin. Microbiol. Rev. **9:**216-34.

Sonoda, N., M. Furuse, H. Sasaki, S. Yonemura, J. Katahira, Y. Horiguchi, and S. Tsukita. 1999. *Clostridium perfringens* enterotoxin fragment removes specific claudins from tight junction strands: Evidence for direct involvement of claudins in tight junction barrier. J. Cell. Biol. **147:**195-204.

Sorensen, H. P., and K. K. Mortensen. 2005. Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. J. Biotechnol. **115:**113-28.

Springer, S., and H. J. Selbitz. 1999. The control of necrotic enteritis in sucking piglets by means of a *Clostridium perfringens* toxoid vaccine. FEMS Immunol. Med. Microbiol. **24:**333-6.

Stabel, T. J., J. E. Mayfield, L. B. Tabatabai, and M. J. Wannemuehler. 1990. Oral immunization of mice with attenuated *Salmonella typhimurium* containing a recombinant plasmid which codes for production of a 31-kilodalton protein of *Brucella abortus*. Infect. Immun. **58:**2048-55.

Stabel, T. J., J. E. Mayfield, L. B. Tabatabai, and M. J. Wannemuehler. 1991. Swine immunity to an attenuated *Salmonella typhimurium* mutant containing a recombinant plasmid which codes for production of a 31-kilodalton protein of *Brucella abortus*. Infect. Immun. **59:**2941-7.

Steinthorsdottir, V., V. Fridriksdottir, E. Gunnarsson, and O. S. Andresson. 1998. Site-directed mutagenesis of *Clostridium perfringens* beta-toxin: expression of wild-type and mutant toxins in *Bacillus subtilis*. FEMS Microbiol. Lett. **158:**17-23.

Steinthorsdottir, V., H. Halldorsson, and O. S. Andresson. 2000. *Clostridium perfringens* beta-toxin forms multimeric transmembrane pores in human endothelial cells. Microb. Pathog. **28:**45-50.

Sterne, M., and I. Batty. 1975. Pathogenic clostridia. Butterworth & Co., London.

Stevens, D. L., and A. E. Bryant. 1997. Pathogenesis of *Clostridium perfringens* infection: mechanisms and mediators of shock. Clin. Infect. Dis. **25 Suppl 2:**S160-4.

Stevens, D. L., B. M. Laine, and J. E. Mitten. 1987. Comparison of single and combination antimicrobial agents for prevention of experimental gas gangrene caused by *Clostridium perfringens*. Antimicrob. Agents Chemother. **31:**312-6.

Stevens, D. L., R. W. Titball, M. Jepson, C. R. Bayer, S. M. Hayes-Schroer, and A. E. Bryant. 2004. Immunization with the C-domain of α-toxin prevents lethal infection, localizes tissue injury, and promotes host response to challenge with *Clostridium perfringens*. J. Infect. Dis. **190:**767-773.

Stiles, B. G., M. L. Hale, J. C. Marvaud, and M. R. Popoff. 2000. *Clostridium perfringens* iota toxin: binding studies and characterization of cell surface receptor by fluorescence-activated cytometry. Infect. Immun. **68:**3475-84.

Stiles, B. G., and T. D. Wilkins. 1986. Purification and characterization of *Clostridium perfringens* iota toxin: dependence on two nonlinked proteins for biological activity. Infect. Immun. **54:**683-8.

Strahan, K., S. N. Chatfield, J. Tite, G. Dougan, and C. E. Hormaeche. 1992. Impaired resistance to infection does not increase the virulence of *Salmonella htr*A live vaccines for mice. Microb. Pathog. **12:**311-7.

Strugnell, R., G. Dougan, S. Chatfield, I. Charles, N. Fairweather, J. Tite, J. L. Li, J. Beesley, and M. Roberts. 1992. Characterization of a *Salmonella typhimurium aro* vaccine strain expressing the P.69 antigen of *Bordetella pertussis*. Infect. Immun. **60:**3994-4002.

Strugnell, R. A., D. Maskell, N. Fairweather, D. Pickard, A. Cockayne, C. Penn, and G. Dougan. 1990. Stable expression of foreign antigens from the chromosome of *Salmonella typhimurium* vaccine strains. Gene **88:**57-63.

"STUDENT". 1908. The probable error of a mean. Biometrika **6:**1-25.

Stuve, G., M. Hofshagen, and G. Holt. 1992. Necrotizing lesions in the intestine, gizzard, and liver in captive capercaillies (*Tetrao urogallus*) associated with *Clostridium perfringens*. J. Wildl. Dis. **28:**598-602.

Su, G. F., H. N. Brahmbhatt, V. de Lorenzo, J. Wehland, and K. N. Timmis. 1992. Extracellular export of Shiga toxin B-subunit/haemolysin A (C-terminus) fusion protein expressed in *Salmonella typhimurium aro*A-mutant and stimulation of B-subunit specific antibody responses in mice. Microb. Pathog. **13:**465-76.

SVARM. 2002. Swedish Veterinary Antibiotics Resistance Monitoring. (http://www.sva.se/pdf/svarm2002.pdf [Online]. National Veterinary Institute, Uppsala, Sweden.

Tacket, C. O., M. B. Sztein, G. A. Losonsky, S. S. Wasserman, J. P. Nataro, R. Edelman, D. Pickard, G. Dougan, S. N. Chatfield, and M. M. Levine. 1997. Safety of live oral *Salmonella typhi* vaccine strains with deletions in *htr*A and *aro*C *aro*D and immune response in humans. Infect. Immun. **65:**452-6.

Takeda, Y., Y. Ogiso, and T. Miwatani. 1977. Effect of zinc ion on the hemolytic activity of thermostable direct hemolysin from *Vibrio parahaemolyticus*, streptolysin O, and Triton X-100. Infect. Immun. **17:**239-43.

Tamai, E., T. Ishida, S. Miyata, O. Matsushita, H. Suda, S. Kobayashi, H. Sonobe, and A. Okabe. 2003. Accumulation of *Clostridium perfringens* epsilon-toxin in the mouse kidney and its possible biological significance. Infect. Immun. **71:**5371-5.

Tannock, G. W. 1994. The acquisition of the normal microflora of the gastrointestinal tract, p. 1-17. *In* S. A. W. Gibson (ed.), Human health: the contribution of microorganisms. Springer-Verlag, London.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choisc. Nucleic Acids Res. **22:**4673-4680.

Timoney, J. F., J. H. Gillespie, F. W. Scott, and J. E. Barlough. 1988. Hagan and Bruner's microbiology and infectious diseases of domestic animals., 8 ed. Comstock Publishing Associates, Ithaca, N.Y.

Titball, R. W. 1997. Clostridial phospholipases, p. 223-242. *In* J. I. Rood, B. McClane, G. Songer, and R. W. Titball (ed.), The *Clostridia*: Molecular biology and pathogenesis. Academic Press, San Diego.

Titball, R. W., A. M. Fearn, and E. D. Williamson. 1993. Biochemical and immunological properties of the C-terminal domain of the alpha-toxin of *Clostridium perfringens*. FEMS Microbiol. Lett. **110:**45-50.

Titball, R. W., A. M. Howells, P. C. Oyston, and E. D. Williamson. 1997. Expression of the *Yersinia pestis* capsular antigen (F1 antigen) on the surface of an *aro*A mutant of *Salmonella typhimurium* induces high levels of protection against plague. Infect. Immun. **65:**1926-30.

Titball, R. W., S. E. Hunter, K. L. Martin, B. C. Morris, A. D. Shuttleworth, T. Rubidge, D. W. Anderson, and D. C. Kelly. 1989. Molecular cloning and nucleotide sequence of the alpha-toxin (phospholipase C) of *Clostridium perfringens*. Infect. Immun. **57:**367-76.

Titball, R. W., D. L. Leslie, S. Harvey, and D. Kelly. 1991. Hemolytic and sphingomyelinase activities of *Clostridium perfringens* alpha-toxin are dependent on a domain homologous to that of an enzyme from the human arachidonic acid pathway. Infect. Immun. **59:**1872-4.

Titball, R. W., C. E. Naylor, and A. K. Basak. 1999. The *Clostridium perfringens* α-toxin. Anaerobe **5:**51-64.

Titball, R. W., C. E. Naylor, J. Miller, D. S. Moss, and A. K. Basak. 2000. Opening of the active site of *Clostridium perfringens* α-toxin may be triggered by membrane binding. Int. J. Med. Microbiol. **290:**357-361.

Tornoe, N. 2002. Presented at the Beyond Antimicrobial Growth Promoters in Food Animal Production, Foulum, Denmark.

Traub, W. H., D. Bauer, and U. Wolf. 1991. Immunobiology of *Clostridium perfringens* type A: passive protection of NMRI mice. Western blot analysis of immunoreactive polypeptides. Chemotherapy **37:**318-26.

Truscott, R. B., and F. Al-Sheikhly. 1977. Reproduction and treatment of necrotic enteritis in broilers. Am. J. Vet. Res. **38:**857-61.

Tso, J. Y., and C. Siebel. 1989. Cloning and expression of the phospholipase C gene from *Clostridium perfringens* and *Clostridium bifermentans*. Infect. Immun. **57:**468-76.

Tsuge, H., M. Nagahama, H. Nishimura, J. Hisatsune, Y. Sakaguchi, Y. Itogawa, N. Katunuma, and J. Sakurai. 2003. Crystal structure and site-directed mutagenesis of enzymatic components from *Clostridium perfringens* iota-toxin. J. Mol. Biol. **325:**471-83.

Tsutsui, K., J. Minami, O. Matsushita, S. Katayama, Y. Taniguchi, S. Nakamura, M. Nishioka, and A. Okabe. 1995. Phylogenetic analysis of phospholipase C genes from *Clostridium perfringens* types A to E and *Clostridium novyi*. J. Bacteriol. **177:**7164-70.

Tukey, J. W. 1953. The problem of multiple comparisons. Princeton University, Department of Statistics. (unpublished).

Turner, S. J., F. R. Carbone, and R. A. Strugnell. 1993. *Salmonella typhimurium* delta *aro*A delta *aro*D mutants expressing a foreign recombinant protein induce specific major histocompatibility complex class I-restricted cytotoxic T lymphocytes in mice. Infect. Immun. **61:**5374-80.

Tweten, R. K. 2001. *Clostridium perfringens* beta toxin and *Clostridium septicum* alpha toxin: their mechanisms and possible role in pathogenesis. Vet. Microbiol. **82:**1-9.

USDA 1999, posting date. Antimicrobial resistance issues in animal agriculture. [Online.] http://www.aphis.usda.gov/vs/ceah/cei/taf/emerginganimalhealthissues_files/antiresist.entire.pdf. United States Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, Centers for Epidemiology and Animal Health, Center for Emerging Issues.

Uzal, F. A., and W. R. Kelly. 1997. Effects of the intravenous administration of *Clostridium perfringens* type D epsilon toxin on young goats and lambs. J. Comp. Pathol. **116:**63-71.

Vallee, B. L., and D. S. Auld. 1993b. New perspective on zinc biochemistry: cocatalytic sites in multi-zinc enzymes. Biochemistry **32:**6493-500.

van der Sluis, W. 2000. Necrotic enteritis (3): Clostridial enteritis is an often underestimated problem. World Poult. **16:**42-43.

Van Etten, W. J., and G. R. Janssen. 1998. An AUG initiation codon, not codon-anticodon complementarity, is required for the translation of unleadered mRNA in *Escherichia coli*. Mol. Microbiol. **27:**987-1001.

Vandekerckhove, J., B. Schering, M. Barmann, and K. Aktories. 1987. *Clostridium perfringens* iota toxin ADP-ribosylates skeletal muscle actin in Arg-177. FEBS Lett. **225:**48-52.

Vemulapalli, R., Y. He, S. M. Boyle, N. Sriranganathan, and G. G. Schurig. 2000. *Brucella abortus* strain RB51 as a vector for heterologous protein expression and induction of specific Th1 type immune responses. Infect. Immun. **68:**3290-6.

Vescovi, E. G., Y. M. Ayala, E. Di Cera, and E. A. Groisman. 1997. Characterization of the bacterial sensor protein PhoQ. Evidence for distinct binding sites for Mg^{2+} and Ca^{2+}. J. Biol. Chem. **272:**1440-3.

Viaplana, E., X. Rebordosa, J. Pinol, and A. Villaverde. 1997. Secretion-dependent proteolysis of recombinant proteins is associated with inhibition of cell growth in *Escherichia coli*. Biotechnol. Lett. **19:**373-377.

Vissiennon, T., S. Menger, and I. Langhof. 1996. Hepatic and renal ultrastructural lesions in experimental *Clostridium perfringens* type A enterotoxemia in chickens. Avian Dis. **40:**720-4.

Walker, M. J., M. Rohde, K. N. Timmis, and C. A. Guzman. 1992. Specific lung mucosal and systemic immune responses after oral immunization of mice with *Salmonella typhimurium aro*A, *Salmonella typhi* Ty21a, and invasive *Escherichia coli* expressing recombinant pertussis toxin S1 subunit. Infect. Immun. **60:**4260-8.

Walker, N., J. Holley, C. E. Naylor, M. Flores-Diaz, A. Alape-Giron, G. Carter, F. J. Carr, M. Thelestam, M. Keyte, D. S. Moss, A. K. Basak, J. Miller, and R. W. Titball. 2000. Identification of residues in the carboxy-terminal domain of *Clostridium perfringens*

alpha-toxin (phospholipase C) which are required for its biological activities. Arch. Biochem. Biophys. **384:**24-30.

Walker, P. D. 1992. Bacterial vaccines: old and new, veterinary and medical. Vaccine **10:**977-90.

Walker, P. D. 1991. Editorial. Vaccine **9:**S3.

Wang, J., V. Michel, C. Leclerc, M. Hofnung, and A. Charbit. 1999a. Immunogenicity of viral B-cell epitopes inserted into two surface loops of the *Escherichia coli* K12 LamB protein and expressed in an attenuated *aro*A strain of *Salmonell*a *typhimurium*. Vaccine **17:**1-12.

Wang, L., H. Curd, and P. R. Reeves. 1999b. Immunization of mice with live oral vaccine based on a *Salmonella enterica* (sv Typhimurium) *aro*A strain expressing the *Escherichia coli* O111 O antigen. Microb. Pathog. **27:**55-9.

Ward, S. J., G. Douce, D. Figueiredo, G. Dougan, and B. W. Wren. 1999. Immunogenicity of a *Salmonella typhimurium aro*A *aro*D vaccine expressing a nontoxic domain of *Clostridium difficile* toxin A. Infect. Immun. **67:**2145-52.

Waters, M., D. Raju, H. S. Garmory, M. R. Popoff, and M. R. Sarker. 2005. Regulated expression of the beta2-toxin gene (*cpb*2) in *Clostridium perfringens* type a isolates from horses with gastrointestinal diseases. J. Clin. Microbiol. **43:**4002-9.

Waters, M., A. Savoie, H. S. Garmory, D. Bueschel, M. R. Popoff, J. G. Songer, R. W. Titball, B. A. McClane, and M. R. Sarker. 2003. Genotyping and phenotyping of beta2-toxigenic *Clostridium perfringens* fecal isolates associated with gastrointestinal diseases in piglets. J. Clin. Microbiol. **41:**3584-91.

Watkins, K. L., T. R. Shryock, R. N. Dearth, and Y. M. Saif. 1997. *In vitro* antimicrobial susceptibility of *Clostridium perfringens* from commercial turkey and broiler chicken origin. Vet. Microbiol. **54:**195-200.

Watts, C. 1997. Capture and processing of exogenous antigens for presentation on MHC molecules. Annu. Rev. Immunol. **15:**821-50.

Wegner, A., and K. Aktories. 1988. ADP-ribosylated actin caps the barbed ends of actin filaments. J. Biol. Chem. **263:**13739-42.

WHO 2000, posting date. Overcoming antimicrobial resistance: World health report on infectious diseases. [Online.] http://www.who.int/infectious-disease-report/2000/. World Health Organisation.

Wick, M. J., C. V. Harding, S. J. Normark, and J. D. Pfeifer. 1994. Parameters that influence the efficiency of processing antigenic epitopes expressed in *Salmonella typhimurium*. Infect. Immun. **62:**4542-8.

Wieckowski, E. U., J. F. Kokai-Kun, and B. A. McClane. 1998. Characterization of membrane-associated *Clostridium perfringens* enterotoxin following pronase treatment. Infect. Immun. **66:**5897-905.

Wieckowski, E. U., A. P. Wnek, and B. A. McClane. 1994. Evidence that an approximately 50-kDa mammalian plasma membrane protein with receptor-like properties mediates the amphiphilicity of specifically bound *Clostridium perfringens* enterotoxin. J. Biol. Chem. **269:**10838-48.

Wierup, M. 2001. The Swedish experience of the 1986 year ban of antimicrobial growth promoters, with special reference to animal health, disease prevention, productivity, and usage of antimicrobials. Microb. Drug. Resist. **7:**183-90.

Wikstrom, P. M., L. K. Lind, D. E. Berg, and G. R. Bjork. 1992. Importance of mRNA folding and start codon accessibility in the expression of genes in a ribosomal protein operon of *Escherichia coli*. J. Mol. Biol. **224:**949-66.

Williams, R. B., W. W. Carlyle, D. R. Bond, and I. A. Brown. 1999. The efficacy and economic benefits of Paracox, a live attenuated anticoccidial vaccine, in commercial trials with standard broiler chickens in the United Kingdom. Int. J. Parasitol. **29:**341-55.

Williams, R. B., R. N. Marshall, R. M. La Ragione, and J. Catchpole. 2003. A new method for the experimental production of necrotic enteritis and its use for studies on the

relationships between necrotic enteritis, coccidiosis and anticoccidial vaccination of chickens. Parasitol. Res. **90:**19-26.

Williamson, E. D., and R. W. Titball. 1993. A genetically engineered vaccine against the alpha-toxin of *Clostridium perfringens* protects mice against experimental gas gangrene. Vaccine **11:**1253-8.

Withanage, G. S., P. Wigley, P. Kaiser, P. Mastroeni, H. Brooks, C. Powers, R. Beal, P. Barrow, D. Maskell, and I. McConnell. 2005. Cytokine and chemokine responses associated with clearance of a primary *Salmonella enterica* serovar Typhimurium infection in the chicken and in protective immunity to rechallenge. Infect. Immun. **73:**5173-82.

Wnek, A. P., and B. A. McClane. 1989. Preliminary evidence that *Clostridium perfringens* type A enterotoxin is present in a 160,000-Mr complex in mammalian membranes. Infect. Immun. **57:**574-81.

Worthington, R. W., and M. S. Mulders. 1975. Effect of *Clostridium perfringens* epsilon toxin on the blood brain barrier of mice. Onderstepoort J. Vet. Res. **42:**25-7.

Wyszynska, A., A. Raczko, M. Lis, and E. K. Jagusztyn-Krynicka. 2004. Oral immunization of chickens with avirulent *Salmonella* vaccine strain carrying *Campylobacter jejuni* 72Dz/92 *cja*A gene elicits specific humoral immune response associated with protection against challenge with wild-type *Campylobacter*. Vaccine **22:**1379-89.

Younan, M., W. Muller, and N. Borowy. 1995. Vaccination of grasscutters with a multicomponent clostridial vaccine. Zentralbl. Veterinarmed. B **42:**369-75.

Zar, J. H. 1999. Biostatistical analysis, 4th ed. Prentice-Hall Inc., New Jersey.

Appendix 1. Antigens delivered and expressed from attenuated *Salmonella* Typhimurium and the immune response(s) developed in the host

Antigen (from Organism)	*S.* Typhimurium attenuation	Protection/immune response	Reference
Viruses			
Immediate early protein ICP27 (Herpes simplex virus-HSV)	Δ*cya* (adenylate cyclate) Δ*crp* (cyclic AMP receptor protein) Δasd (aspartate b-semialdehyde dehydrogenase)	Protection (73%) CMI^ (no humoral)	(Karem *et al.*, 1997)
Glycoprotein D (HSV)	Δ*cya* Δ*crp* Δ*asd*	Low protection (31%) Humoral response (no CMI)	(Karem *et al.*, 1997)
E7 protein (Human papilloma virus- HPV)	Δ*aro*A Δ*aro*D	CMI-IgG2a and Humoral-IgA	(Londono *et al.*, 1996)
Nucleoprotein (Influenza virus)	Δ*aro*A	Humoral and CMI	(Brett *et al.*, 1993)
Core antigen/preS2-middle surface antigen (Hepatitis B virus)	Δ*aro*A	Humoral and CMI-IgG2a	(Schodel *et al.*, 1990)
Bacteria			
Fragment C of Tetanus toxin (*Clostridium tetani*)	Δ*aro*A	Protection, humoral (CMI ND*)	(Fairweather *et al.*, 1990)
Botulinum toxin type F (*Clostridium botulinum*)	Δ*aro*A	Protection, CMI (IgG2a) and humoral (IgG1)	(Foynes *et al.*, 2003)
Toxin A (C-terminal) (*Clostridium difficile*)	Δ*aro*A Δ*aro*D	Humoral (CMI ND)	(Ward *et al.*, 1999)

Appendix 1. Antigens delivered and expressed from *Salmonella* Typhimurium and the immune response(s) developed in the host

Antigen (from Organism)	***S*. Typhimurium attenuation**	**Protection/immune response**	**Reference**
P60 (*L. monocytogenes*)	Δ*aro*A	Protection CMI (humoral ND)	(Hess *et al.*, 1997)
Protective antigen (*Bacillus anthracis*)	Δ*aro*A	Protection, humoral-IgG1 and CMI-IgG2a	(Coulson *et al.*, 1994a; Garmory *et al.*, 2003)
F1 (capsular) antigen (*Yersinia pestis*)	Δ*aro*A	Protection, CMI and humoral	(Titball *et al.*, 1997)
Filamentous hemagglutinin (*Bordetella pertussis*)	Δ*aro*A	Humoral-IgG, IgA (CMI ND)	(Guzman *et al.*, 1991)
Pertussis toxin S1 subunit (*B. pertussis*)	Δ*aro*A	Humoral-IgG and IgA (CMI ND)	(Walker *et al.*, 1992)
P69 Antigen (*B. pertussis*)	Δ*aro*A Δ*aro*D	Protection, CMI (no humoral)	(Strugnell *et al.*, 1992)
Pertussis toxin subunits (*B. pertussis*)	Δ*aro*A	Humoral -not protective, (CMI ND)	(Dalla Pozza *et al.*, 1998)
BCSP31-31 kDa protein (*Brucella abortus*)	Δ*cya* Δ*crp*	Humoral, (No CMI)	(Stabel *et al.*, 1990; Stabel *et al.*, 1991)
Heat-labile enterotoxin B subunit (LT-B) *(Escherichia coli*)	Δ*aro*A	Humoral-IgA and IgG (CMI ND)	(Maskell *et al.*, 1987)
0111 O antigen (*E. coli*)	Δ*aro*A (STMI)	Humoral (CMI ND)	(Wang *et al.*, 1999b)
β-galactosidase (*E. coli*)	Δ*aro*A	Humoral and CMI	(Brown *et al.*, 1987)
Pilin subunit (BfpA) (Enteropathogenic *E. coli*)	Δ*aro*A	Humoral	(Schriefer *et al.*, 1999)
Type 5 M protein (*S. pyogenes*)	Δ*aro*A	Protection, Humoral-IgG IgA (CMI ND)	(Poirier *et al.*, 1988)
Superoxide dismutase (*Listeria monocytogenes*)	Δ*aro*A	Protection CMI (humoral ND)	(Hess *et al.*, 1997)

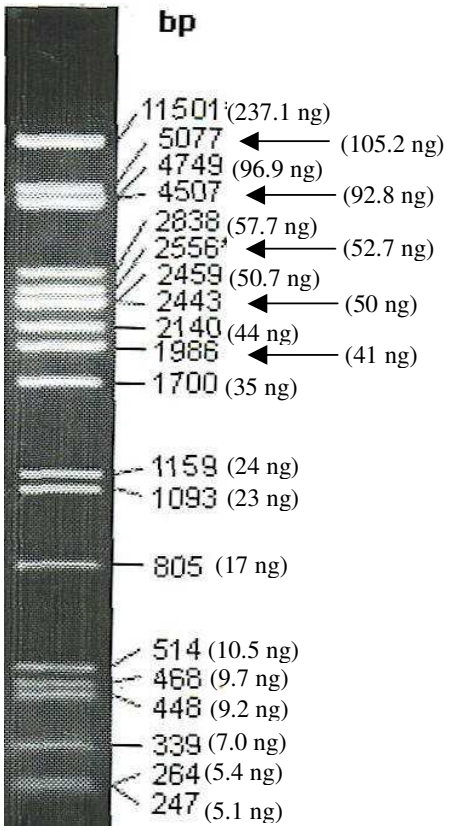
Antigen (from Organism)	***S.* Typhimurium attenuation**	**Protection/immune response**	**Reference**
SBR (salivary binding region of AGI/II surface protein antigen) (*Streptococcus mutans*)	Δ*aro*A Δ*aro*D	Humoral and CMI	(Harokopakis *et al.*, 1997)
Parasites			
Sm-14 (*Schistosoma mansoni* antigen) S. mansoni cercaria (partial protection achieved-as good as parenterally admin alone	Δ*aro*A	Partial protection, humoral (IgG) (CMI ND)	(Pacheco *et al.*, 2005)
Schistosoma mansoni Glutathione-S-transferase (P28)	Δ*aro*A	Humoral (CMI ND)	(Khan *et al.*, 1994)
Circumsporozoite protein of *Plasmodium berghei* Sporozoite challenge (partial protection 70%)	WR4017 (previously M206-unable to multiply in macrophages)	Protection, CMI (no humoral)	(Sadoff *et al.*, 1988)
Copper/zinc superoxide dismutase (*Acanthocheilonema viteae*)	Δ*aro*A	Humoral, partial protection, (CMI ND)	(Lattemann *et al.*, 1999)
β-galactosidase antigen (against a b-gal tumour developed in mouse model)	Δ*aro*A	Humoral-IgG, IgM CMI-$CD8^+$ CTL, depletion in tumour size, prevention of tumour growth	(Medina *et al.*, 1999)

^CMI: Cell mediated immunity

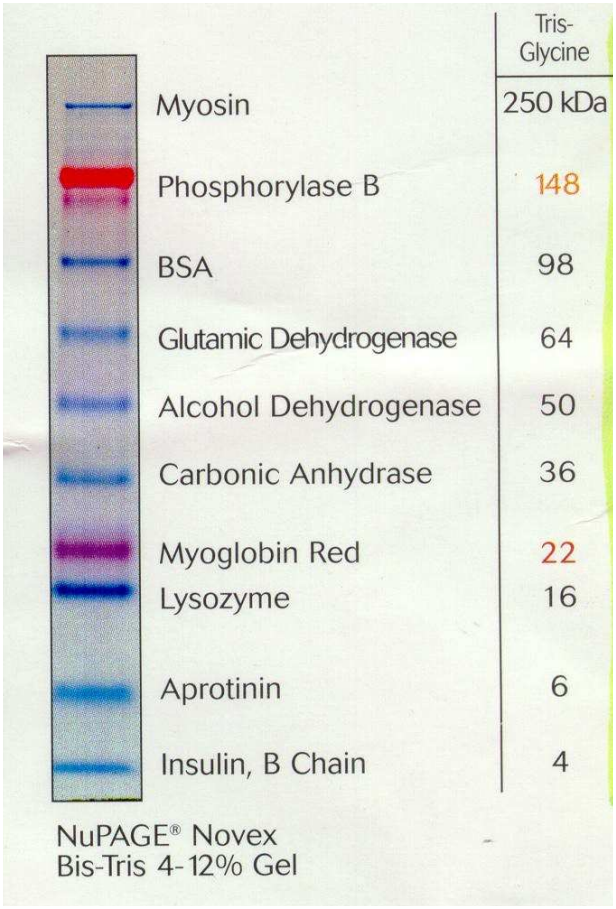
* ND= Not determined

Appendix 2. Lambda DNA digested with *Pst*I

Numbers in brackets indicate approximate DNA quantity of each fragment when 1µg of *Pst*I digested λ-DNA is separated by agarose gel electrophoresis



Appendix 3. SeeBlue protein marker



Appendix 4. DNA and predicted amino acid sequence of *plc* from *C. perfringens* type A strain 61.

DNA (black letters) and predicted amino acid sequence (green letters). The added 6 x CAT and its corresponding amino acid sequence is coloured purple.

```
  1  ATG AAA AGA AAG ATT TGT AAG GCG CTT ATT TGT GCT GCG CTA GCA ACT
       M   K   R   K   I   C   K   A   L   I   C   A   A   L   A   T

 49  AGC CTA TGG GCT GGG GCA TCA ACT AAA GTC TAC GCT TGG GAT GGA AAA
       S   L   W   A   G   A   S   T   K   V   Y   A   W   D   G   K

 97  ATT GAT GGA ACA GGA ACT CAT GCT ATG ATT GTA ACT CAA GGG GTT TCA
       I   D   G   T   G   T   H   A   M   I   V   T   Q   G   V   S

145  ATC TTA GAA AAT GAT CTG TCT AAA AAT GAA CCA GAA AGT GTA AGA AAA
       I   L   E   N   D   L   S   K   N   E   P   E   S   V   R   K

193  AAC TTA GAG ATT TTA AAA GAG AAC ATG CAT GAG CTT CAA TTA GGT TCT
       N   L   E   I   L   K   E   N   M   H   E   L   Q   L   G   S

241  ACT TAT CCA GAT TAT GAT AAG AAC GCC TAT GAT CTA TAT CAA GAT CAT
       T   Y   P   D   Y   D   K   N   A   Y   D   L   Y   Q   D   H

289  TTC TGG GAT CCT GAT ACA GAT AAT AAT TTC TCA AAG GAT AAT AGT TGG
       F   W   D   P   D   T   D   N   N   F   S   K   D   N   S   W

337  TAT TTA GCT TAT TCT ATA CCT GAC ACA GGG GAA TCA CAA ATA AGA AAA
       Y   L   A   Y   S   I   P   D   T   G   E   S   Q   I   R   K

385  TTT TCA GCA TTA GCT AGA TAT GAA TGG CAA AGA GGA AAC TAT AAA CAA
       F   S   A   L   A   R   Y   E   W   Q   R   G   N   Y   K   Q

433  GCT ACA TTC TAT CTT GGA GAG GCT ATG CAC TAT TTT GGA GAT ATA GAT
       A   T   F   Y   L   G   E   A   M   H   Y   F   G   D   I   D

481  ACT CCA TAT CAT CCT GCT AAT GTT ACT GCC GTT GAT AGC GCA GGA CAT
       T   P   Y   H   P   A   N   V   T   A   V   D   S   A   G   H

529  GTT AAG TTT GAG ACT TTT GCA GAG GAA AGA AAA GAA CAG TAT AAA ATA
       V   K   F   E   T   F   A   E   E   R   K   E   Q   Y   K   I

577  AAC ACA GCA GGT TGC AAA ACT AAT GAG GAT TTT TAT GCT GAT ATC TTA
       N   T   A   G   C   K   T   N   E   D   F   Y   A   D   I   L

625  AAA AAC AAA GAT TTT AAT GCA TGG TCA AAA GAA TAT GCA AGA GGT TTT
       K   N   K   D   F   N   A   W   S   K   E   Y   A   R   G   F

673  GCT AAA ACA GGA AAA TCA ATA TAC TAT AGT CAT GCT AGC ATG AGT CAT
       A   K   T   G   K   S   I   Y   Y   S   H   A   S   M   S   H
```

```
 721  AGT TGG GAT GAT TGG GAT TAT GCA GCA AAG GTA ACT TTA GCT AAC TCT
        S   W   D   D   W   D   Y   A   A   K   V   T   L   A   N   S

 769  CAA AAA GGA ACA GCA GGA TAT ATT TAT AGA TTC TTA CAC GAT GTA TCA
        Q   K   G   T   A   G   Y   I   Y   R   F   L   H   D   V   S

 817  GAG GGT AAT GAT CCA TCA GTT GGA AAG AAT GTA AAA GAA CTA GTA GCT
        E   G   N   D   P   S   V   G   K   N   V   K   E   L   V   A

 865  TAC ATA TCA ACT AGT GGT GAA AAA GAT GCT GGA ACA GAT GAC TAC ATG
        Y   I   S   T   S   G   E   K   D   A   G   T   D   D   Y   M

 913  TAT TTT GGA ATC AAA ACA AAG GAT GGA AAA ACT CAA GAA TGG GAA ATG
        Y   F   G   I   K   T   K   D   G   K   T   Q   E   W   E   M

 961  GAC AAC CCA GGA AAT GAT TTT ATG ACT GGA AGT AAA GAC ACT TAT ACT
        D   N   P   G   N   D   F   M   T   G   S   K   D   T   Y   T

1009  TTC AAA TTA AAA GAT GAA AAT CTA AAA ATT GAT GAT ATA CAA AAT ATG
        F   K   L   K   D   E   N   L   K   I   D   D   I   Q   N   M

1057  TGG ATT AGA AAA AGA AAA TAT ACA GCA TTC CCA GAT GCT TAT AAG CCA
        W   I   R   K   R   K   Y   T   A   F   P   D   A   Y   K   P

1105  GAA AAC ATA AAG ATA ATA GCA AAT GGA AAA GTT GTA GTA GAC AAA GAT
        E   N   I   K   I   I   A   N   G   K   V   V   V   D   K   D

1153  ATA AAT GAG TGG ATT TCA GGA AAT TCA ACT TAT AAT ATA AAA CAT CAT
        I   N   E   W   I   S   G   N   S   T   Y   N   I   K   H   H

1200  CAT CAT CAT CAT TAA
        H   H   H   H   -
```

Appendix 5. Sequence of region upstream from the *plc* start site (ATG) of vector pCplc3.

The *lac*Z ribosome binding site is boxed, the *lac*Z ATG site is highlighted green and plc3 ATG start site is highlighted purple. No RBS is present 8-12 bp upstream of the plc3 ATG.

```
1    ACA GGA AAC AGC TAT GAC CAT GAT TAC GCC AAG CTT GGT

40   ACC GAG CTC GGA TCC ACT AGT AAC GGC CGC CAG TGT GCT

79   GGA ATT CGG CTT TAA CGC TGC AGA TAA AAA ATG AAA AGA 118

AAG ATT TGT AAG GCG CTT ATT TGT GCT
```