University of Southern Queensland

THE DEVELOPMENT AND EVALUATION OF DNA VACCINES AGAINST WHOOPING COUGH USING A MURINE RESPIRATORY MODEL OF INFECTION

A dissertation submitted by

Scott Robert Fry, BSc (Hons)

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Abstract

Bordetella pertussis is the aetiological agent of whooping cough, a respiratory disease of humans that causes severe and potentially fatal manifestations in children. Whooping cough is endemic world-wide accounting for around 50 million cases and 300,000 deaths every year, with epidemics occurring every two to five years (Kerr & Matthews 2000). Although the current whole-cell and acellular vaccines are effective in conferring protection from clinical pertussis, they have been associated with serious systemic and local side reactions in up to a maximum of 18 percent of paediatric vaccinees after repeated boosters (Gold et al. 2003).

In this study, a suite of single antigen DNA vaccines, combination DNA vaccines and dual modality vaccines, were developed and evaluated for their potential to induce humoral and cell-mediated immune responses, and protective efficacy against *B. pertussis*, using the mouse respiratory challenge model. This study was based on the reported claims that DNA vaccines are capable of generating potent humoral and cell-mediated responses, and protection against numerous viral, parasitic and bacterial pathogens in small animal models. Four protective antigens, three of which are included in the currently-marketed acellular pertussis vaccine [aP], were evaluated as single antigen DNA vaccines, namely filamentous hemagglutinin (FHAB), pertactin (PRN), a genetically toxoided S1 subunit of pertussis toxin (PTS1.13L.129G) and genetically toxoided adenylate cyclase-hemolysin (CYAAL58), delivered either by the intramuscular (IM) route or by the oral route via attenuated *Salmonella typhimurium* strain SL3261. The immunogenicity and protective efficacy of these DNA vaccines was compared to that imparted by the DTaP (Infantrix™), a placebo-immunised group, and a vector-immunised group of mice. Two DNA vaccines encoding truncated FHA antigens, representing the entire immunodominant region (pcDNA3.1D/*fhaB1*) and dominant B cell epitopes (pcDNA3.1D/*fhaB2*), induced a predominantly Th1 response with high levels of IFN-γ and IL-2 produced by stimulated splenocytes *in vitro*. An antigen-specific IgG response was detected in the serum of mice but this was negated by an equivalent or larger IgG response to FHA in the serum of vector-immunised mice. Two AC-Hly DNA vaccines, encoding a genetically inactivated CyaA protoxin either alone (pcDNA3.1D/*cyaAL58*) or in combination with the accessory protein cyaC

(pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC*), both induced a potent Th1 cytokine response. However, the serum IgG response was not significant due to the presence of cross-reactive antibodies in the sera of vector-immunised and sham-immunised mice (placebo).

A DNA vaccine encoding a genetically inactivated S1 subunit of pertussis toxin (pcDNA3.1D/*pts1.13L.129G*), induced a purely Th1 response with high levels of IFN-γ. No antigen-specific or cross-reactive IgG was detected in the sera of mice immunised with pcDNA3.1D/*pts1.13L.129G* or vector only respectively. In contrast to the other antigens tested, the pertactin DNA vaccine induced a Th2-type response as indicated by a significant serum IgG response, the majority of which was IgG1, and lower levels of IFN-γ and IL-2. Mice immunised with each of the single antigen DNA vaccines showed a significantly improved rates of clearance compared to mice that received the vector only or placebo. Overall, their protective efficacy was inferior to that of the DTaP. It has been well established that effective immunisation against such a complex pathogen as *B. pertussis* requires multiple antigenic priming, with multi-component acellular vaccines often providing an improved level of protection compared to mono-component and two-component acellular vaccines. In order to induce a broad-spectrum immune response and thereby assess the true protective potential of DNA vaccination, a five-gene combination DNA vaccine was tested by direct IM injection of naked DNA (without any added adjuvant). The IM five-gene combination DNA vaccine generated strong Th1 responses to FHA, the inactivated S1 subunit of PT and inactivated CYAA, and a moderate serum IgG response to PRN. Importantly, the response to each antigen was equivalent or better than the respective single antigen DNA vaccines, which indicated that there was no antigenic competition. In fact, co-administration of the five genes resulted in an enhanced response to each antigen. The portal of entry for *B. pertussis*, like many other human pathogens, is via the mucosa and in the case of pertussis this is limited to the respiratory tract with no systemic dissemination in otherwise healthy individuals. Whilst the whole cell and acellular vaccines induce a potent serological response that can protect against *B. pertussis* infection, it is likely that the additional priming of a secretory response at the site of colonisation would inhibit colonisation, improve clearance of the pathogen from the lungs, and limit transmission. Hence, a five-gene combination DNA vaccine was delivered via the oral route using attenuated *Salmonella typhimurium* as the delivery vector, with the aim of stimulating a

mucosal response in the respiratory tract via the common mucosal pathway. The oral combination DNA vaccine generated a strong systemic response but a poor mucosal antibody response to recombinant or native *B. pertussis* antigens. Mice immunised with the oral combination DNA vaccine cleared an experimental infection at a significantly faster rate than mice immunised with the oral vector (*S. typhimurium* harbouring the pcDNA3.1D vector), but the clearance data showed that it was not nearly as effective as the IM combination DNA vaccine or DTaP.

In an attempt to induce a dichotomous humoral and cell-mediated immune response, two DNA vaccine prime-acellular vaccine boost regimens were evaluated: a parenteral-parenteral strategy referred to as the parenteral dual modality vaccine and an oral-parenteral strategy referred to as the oral dual modality vaccine. Priming involved administration of a five-gene combination DNA vaccine via either IM injection or oral gavage, and the boosters consisted of a laboratory constituted three-component acellular vaccine given via SC injection. Dual modality vaccination successfully induced a dichotomous Th1/Th2 response that conferred a degree of protection that was equivalent to that obtained with the commercial DTaP. Interestingly, a mucosal IgG response was also detected in the lung washes of mice immunised with the laboratory constituted acellular vaccine (as part of the dual modality vaccines) or Infanrix™ DTaP that was considered to be due to transudation of antibodies from serum. This study has been the first to demonstrate that immunisation with a five-gene combination DNA vaccine can elicit a protective immune response that approaches the level of protection conferred by DTaP, as judged by the number of days required for clearance of the pathogen from the lungs of vaccinated mice. In addition, both the parental and the oral dual modality vaccines were found to provide an equivalent or better protection against challenge with virulent *B pertussis* than that imparted by the commercial DTaP. This was despite the fact that the oral DNA combination vaccine was essentially non-protective. The induction of humoral and CMI responses, particularly by the parenteral dual modality vaccine, are highly encouraging and warrant further investigations on the safety and characterisation of anamnestic immune responses induced by the inclusion of different types of adjuvants in the vaccine formulations.

Declaration

I declare that all experimental work, results and analyses reported in this dissertation are entirely my own effort, except where otherwise acknowledged. I also certify that the work is original, has not been previously submitted for any other award and was conducted during my enrolment as a Doctor of Philosophy candidate at the University of Southern Queensland. Animal experiments were approved by the University of Southern Queensland Animal Ethics Committee (Approval no. 03STU194).

Candidate:

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Scott Robert Fry Date

Endorsement

Supervisor: Associate Supervisor:

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A/Prof T.K.S. Mukkur A/Prof Grant Daggard

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Position Position

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Date Date

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

AC	adenylate cyclase
AC-Hly	adenylate cyclase-hemolysin
ACT	adenylate cyclase toxin
ADP	adenosine diphosphate
ADRAC	Australian Adverse Drug Reaction Assessment Committee
AGRF	Australian Genome Research Facility
ANGIS	Australian National Genome Information Service
AP	alkaline phosphatase
APC	antigen presenting cell
ATP	adenosine triphosphate
BAL	bronchoalveolar lavage
BG	Bordet Gengou media
BSA	bovine serum albumin
bp	base pair
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
cDMEM	complete Dulbecco's Modified Eagle Media
CFU	colony forming units
CHO	Chinese Hamster Ovary cell line
CI	clearance index
CMI	cell-mediated immunity
CMV	cytomegalovirus
CNS	central nervous system
ConA	concanavalin A
CpG	cytosine-phosphate-guanosine
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Media
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNT	dermonecrotic toxin

dNTP	deoxynucleotide triphosphate
DTaP	Diptheria-Tetanus-acellular Pertussis vaccine
DTP	Diptheria-Tetanus-whole cell Pertussis vaccine
DTT	dithiothretol
EDAC	1-ethyl-3(3-dimethylaminopropyl) carbodiimide
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
FAE	follicle-associated epithelium
FBS	fetal bovine serum
FcR	receptor for the Fc portion of immunoglobulin
FFST	formalin-fixed *Salmonella typhimurium*
FHA	filamentous hemagglutinin
FIM	fimbriae
GALT	gut-associated lymphoid tissue
GFP	green fluorescent protein
GKO	gene knock-out
GSK	GlaxoSmithKline
HCl	hydrochloric acid
His	histidine
HKBP	heat-killed *Bordetella pertussis*
HRP	horse-radish peroxidase
IFN-γ	interferon-gamma
IC	intracerebral
IL	interleukin
IM	intramuscular
IN	intranasal
IP	intraperitoneal
IPTG	isopropyl β-D-thiogalactoside
IV	intravenous
kb	kilobase pair
kDa	kilo dalton
KO	knock-out

LB	Luria-Betani media
LBA	Luria-Betani media with ampicillin
LBAC	Luria-Betani media with ampicillin & chloramphenicol
LBAG	Luria-Betani media with ampicillin & glucose
LD_{50}	lethal dose which causes 50% death of a group of test animals
LF2000	lipofectamine 2000 reagent
LN	lymph nodes
LPS	lipopolysaccharide
LRT	lower respiratory tract
LRTI	lower respiratory tract infection
MALT	mucosal-associated lymphoid tissue
MHC	major histocompatibility complex
min	minute
$MgSO_4$	magnesium sulphate
$MgCl_2$	magnesium chloride
MSS	modified Stainer-Scholte media
MV	modified Verwey media
NALT	nasal-associated lymphoid tissue
NCBI	National Centre for Biotechnology Information
NK	natural killer cells
OD	optical density
O/N	overnight
OPD	*o*-phenylene diamine
ORF	open reading frame
P69	69 kDa outer membrane protein of *B. pertussis*
Pa	acellular pertussis vaccine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween-20
PCR	polymerase chain reaction
PMNL	polymorphonuclear leukocytes
PMSF	phenylmethylsulfonyl fluoride
PP	Peyer's patches
PRN	pertactin

PT	pertussis toxin
PVDF	polyvinylidene fluoride
Pw	whole-cell pertussis vaccine
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
S1	S1 subunit of pertussis toxin
SC	subcutaneous
SDS	sodium dodecyl sulfate
sec	second
SLID	sub-lethal infectious dose
SS	Stainer-Scholte media
TAE	tris acetate EDTA
TB	terrific broth
Tc	CD8+ cytotoxic T lymphocyte
TcR	T-cell receptor
TCT	tracheal cytotoxin
TGF-β	tumour growth factor-beta
TNF	tumour necrosis factor
Tween-20	polyoxyethylenesorbitan monolaurate
URT	upper respiratory tract
URTI	upper respiratory tract infection
USQ	University of Southern Queensland
vag	virulence-associated gene
vir	virulence
*vr*g	virulence-repressed gene
VLP	virus-like protein
WCV	whole-cell pertussis vaccine
WHO	World Health Organisation
wt	wild-type
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside

Chapter 1 Literature Review

1.1 The Whooping Cough Syndrome

Whooping cough was recognized as an acute respiratory disease of humans in the 16th century and the aetiological agent, *Bordetella pertussis*, was isolated and identified by Bordet and Gengou in 1906 (Cherry 1999a). Although all age groups are susceptible to infection, the disease is pronounced in infants due to the severe systemic complications that can accompany the characteristic paroxysmal cough (Poland 1996). The severe and potentially fatal complications include convulsions, bronchopneumonia, and encephalopathy (Mills 2001). Protection, albeit limited in duration, can be conferred by vaccination. As the recommended vaccination schedule does not commence until 2 months of age and the immune system remains immature throughout the first year of an infant's life (Nairn & Helbert 2002), children less than 1 year old are the highest risk group (Andrews, Herceg & Roberts 1997).

1.1.1 Clinical Disease

Typical pertussis, as defined by the World Health Organization (WHO) consists of the catarrhal, paroxysmal and convalescent phases. The catarrhal phase manifests as a cold-like illness with coryza and a mild cough with the onset of these symptoms usually 7 to 10 days after exposure. Whooping cough is highly contagious during the catarrhal phase. Proliferation of *B. pertussis* and their subsequent abundance in nasopharyngeal secretions provides a means of aerosol transmission via droplet spread or direct contact (Weiss & Hewlett 1986). Progression from a mild asymptomatic cough to convulsive or violent attacks (paroxysmal cough) indicates onset of the paroxysmal phase. Excessive mucous production, vomiting and lymphocytosis are additional manifestations that are commonly observed during this period of infection. A feature of the paroxysmal cough is the characteristic 'whoop' sound that occurs as a result of inhalation through a narrow glottis. Coughing attacks can occur frequently and can be so severe that exhaustion and apnoea result. This stage lasts for up to 4 weeks, after which time the majority of sufferers enter a convalescent phase that is characterised by a gradual recovery. However, the severe cough can re-emerge in the presence of an opportunistic infection (Olson 1975).

Apart from the classical pertussis syndrome, there are several forms of an atypical infection. Although the cough is the defining symptom of pertussis, it has been reported that culture-positive neonates and infants have presented with symptoms such as apnoea and seizures in the absence of paroxysms (Heininger et al. 1997). Adolescents and adults do not exhibit the characteristic cough and infection can be either asymptomatic or manifested as a mild but persistent cold-like illness (Cromer et al. 1993; Birkebaek et al. 1999). *B. parapertussis* can also infect humans but the disease state is often milder and far less common than the *B. pertussis* aetiology, whereas the remaining *Bordetella* spp, *B. bronchiseptica*, *B. holmesii*, *B. avium*, *B. hinzii* and *B. trematum* rarely cause infections in humans (Cherry 1999a).

1.1.2 Epidemiology

The incidence of pertussis was clearly influenced by the advent of effective prophylactic and therapeutic intervention in the mid 1940's. During the pre-vaccine era, pertussis was highly endemic with cyclic epidemics that occurred every 2 to 5 years (Cherry 1999a). In the USA alone the reported yearly attack rate was around 200-250/100,000 population from 1922 to 1945 (Figure 1.1) but due to under-reporting the true estimate was calculated at 872/100,000. Pertussis had such an impact that experimental whole-killed cell vaccines were tested on humans only 30 years after the organism was first isolated and upon favourable trials were rapidly approved for clinical use (Cherry 1999a).

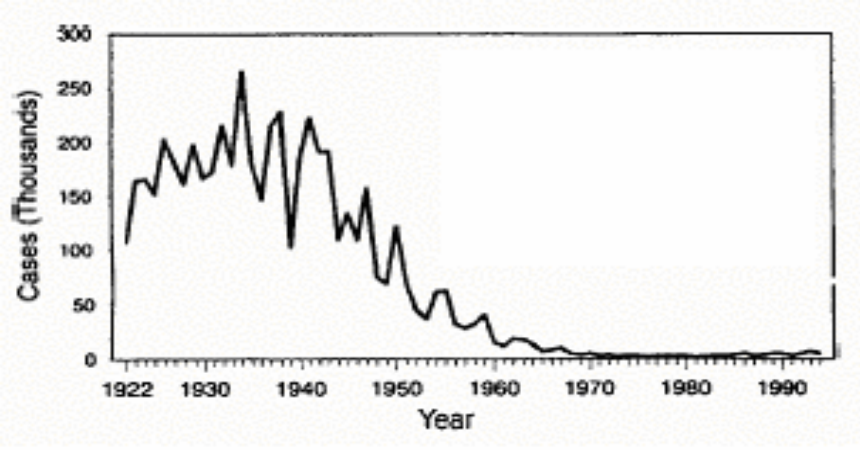

Figure 1.1: Reported incidence of pertussis from 1922 to 1994. The figure indicates the average notifications in the United States of America over this period (Cherry, 1999a)

Although the introduction and wide-spread use of pertussis vaccines caused a dramatic reduction in the incidence of pertussis (see Figure 1.1) this has not corresponded with a control in the circulation of pertussis (Cherry 1999b). It has recently been reported that the incidence of whooping cough has risen despite high vaccine coverage in developed countries such as Australia, Canada, the United States and the Netherlands (Mooi, van Loo & King 2001). For example Figure 1.2 shows that notifications in the Netherlands increased markedly from 1995 to 1997 and has continued to remain at this higher level even with an immunisation rate of 96% (de Melker et al. 1997). Similarly, in Australia pertussis has been epidemic since 1993 with notifications rising from 2/100,000 population in 1991 to a peak of 30.5/100,000 in 1994, despite a three-dose immunisation rate approaching 90% (Andrews, Herceg & Roberts 1997).

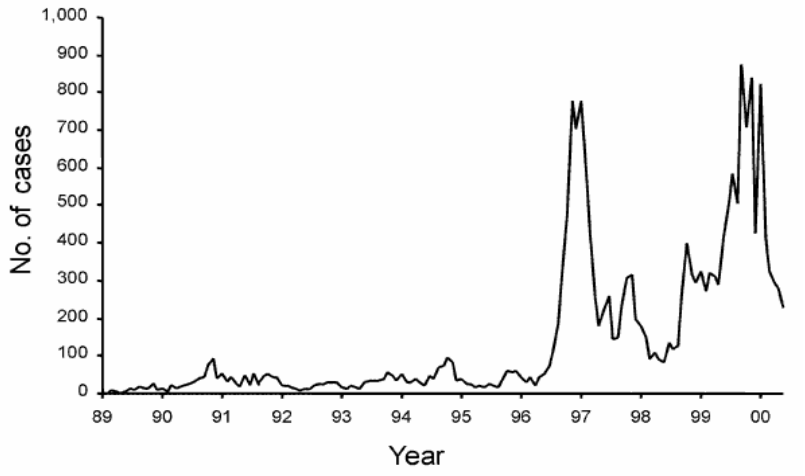

Figure 1.2: Recent increases in the reported cases of pertussis in countries with high vaccine coverage. This graph shows data obtained from The Netherlands since 1989 (Mooi, van Loo & King 2001).

There have been numerous suggestions as to the reasons for the recent and wide-spread re-emergence of pertussis. Although a number of factors such as increased public awareness, improvement in notification accuracy, decreased vaccine coverage and a reduction in vaccine quality could be applicable for developing countries, these possibilities have been largely excluded for the industrialised countries (de Melker et al. 1997). The adaptation of *B. pertussis* to vaccination has been suggested as a reason for the rise in developed countries. Mooi, van Loo & King (2001) DNA fingerprinted

Dutch strains collected from 1949 to 1996 and found notable changes between the pre-vaccine and post-vaccine populations of *B. pertussis*. This divergence was observed as polymorphisms within the pertussis toxin and pertactin antigens, both of which are dominant immunogens and components of acellular vaccines (Weber et al. 2001).

Currently the WHO has estimated that globally there are 50 million cases of pertussis reported each year with approximately 300,000 deaths, and at present it is one of the ten most common causes of death from infectious disease (Kerr & Matthews 2000). As is the case with the many vaccine preventable diseases, the majority of the morbidity and mortality associated with pertussis occurs in the developing countries in which adequate vaccination, diagnosis and treatment are not provided (Kerr & Matthews 2000). Of further concern is the belief that the incidence figures may be inaccurate, in that the rates of both classical pertussis in infants and atypical pertussis in adults are under-reported (Kerr & Matthews 2000).

The incidence of pertussis in adolescents and adults has become an area of increasing interest over the last decade. The obvious consequence of undetected and untreated pertussis in the mature age groups is the potential for transmission to other adults and more importantly to highly susceptible infants. The recent deaths attributed to pertussis in Australia have been restricted to infants under two months of age (Andrews, Herceg & Roberts 1997). Although these infants were too young to be vaccinated it was concluded that a reduction in the level of pertussis within the community, particularly from reservoirs such as asymptomatic adults would have diminished the risk of exposure. In view of the current situation, it is apparent that the immunisation of the adolescent and adult population is one aspect that will need to be addressed to control the spread of pertussis. The higher risk of side effects, excessive cost of expanded vaccination and the perceived weakness of the disease in adults are factors that have hindered the notion of vaccination beyond infancy (Cherry 1999b; Roduit et al. 2002).

1.1.3 Treatment

Erythromycin and the newer generation azithromycin and clarithromycin are the current drugs of choice for the treatment of pertussis, with a recommended course of at least 7 days (Halperin et al. 1997). As a member of the macrolide family of antibiotics these compounds disrupt bacterial protein synthesis by binding to the 50S ribosomal subunit and preventing the initiation of translation. Resistance to erythromycin has been identified (Lewis et al. 1995; Korgenski & Daly 1997). Although currently limited to several strains there are concerns that screening for antibiotic resistance in *B. pertussis* isolates has not been a routine test in clinical laboratories (Wright 1998). The mechanism of resistance has not yet been identified but it has been shown not to involve plasmid-encoded methylation of ribosomal RNA, which has been the mode used by resistant streptococcal and staphylococcal species (Lewis et al. 1995). As is the case with all other infectious diseases, the continual emergence of antibiotic resistance poses a limitation on the long-term future of chemotherapy and emphasises the need for the continual improvement in vaccine development.

1.2 *Bordetella pertussis*

Bordetella pertussis, the aetiological agent of whooping cough is a gram-negative, aerobic coccobacillus (Holt 1993). A strict human pathogen, *B. pertussis* produces an abundance of virulence factors during infection (Parton 1999). Adhesins such as filamentous hemagglutinin (FHA), pertactin (PRN), and fimbriae (FIM) are involved in colonisation of the respiratory mucosa via attachment to ciliated epithelia (Hazenbos et al. 1995a; Bassinet et al. 2000). By selective attachment to these ciliated epithelial cells the pathogen avoids the mucociliary escalator, an innate clearance mechanism in which inhaled bacteria are trapped in mucus and then expelled from the upper respiratory tract. As the bacteria proliferate in the respiratory tract a multitude of toxins, namely pertussis toxin, adenylate cyclase hemolysin, dermonecrotic toxin and tracheal cytotoxin are released (Weiss & Hewlett 1986). These toxins elicit both local effects such as local tissue damage and necrosis as well as systemic effects that include lymphocytosis, histamine sensitisation and increased insulin secretion.

1.2.1 Key Virulence Factors

Filamentous hemagglutinin (FHA), pertactin (P69), pertussis toxin (PTX) and adenylate cyclase toxin (AC-Hly) have been identified as the four key virulence factors of *B. pertussis* (Mills 2001).

1.2.1.1 *Filamentous Hemagglutinin*

Filamentous hemagglutinin (FHA) is a large 220-kDa outer membrane associated protein that performs a crucial function in pertussis pathogenesis. The diminished ability of an FHA-deficient mutant to adhere *in vivo* or to *in vitro* cultured epithelial cells, and the ability of anti-FHA antibodies to inhibit the attachment of virulent micro-organisms demonstrated that FHA has a central role in adhesion of *B. pertussis* to the ciliated epithelia (Tuomanen & Weiss 1985; Urisu, Cowell & Manclark 1985). Subsequent cloning and sequencing of the FHA structural gene (*fhaB*) by Relman et al. (1989) led to the identification of an Arg-Gly-Asp (RGD) tripeptide, a eukaryotic cell recognition signal, which suggested that FHA also played a role in attachment to leukocytes. FHA is a highly immunogenic protein. High titers of anti-FHA antibodies have been detected in human convalescent sera (Simondon et al. 1998) and are elicited to protective levels following immunisation with commercial pertussis vaccines (Mills et al. 1998). Similarly, antibodies generated against purified FHA, as a component of an acellular pertussis vaccine have been shown to be protective in animal models (Sato, Kimura & Fukumi 1984) and in human clinical trials (Edwards et al. 1995). In addition, Guzman et al. (1991a) demonstrated that recombinant *S. typhimurium* expressing FHA generated both systemic and mucosal antibodies following oral immunisation of mice.

1.2.1.2 *Pertactin (P.69)*

Brennan et al. (1988) identified a 69-kDa membrane-associated protein (P.69 or pertactin) using a library of monoclonal antibodies generated against the surface antigens of *B. pertussis*. Unlike FHA-deficient mutants, *B. pertussis* mutants deficient in pertactin were not inhibited in attachment to ciliated respiratory epithelial cells (Roberts et al. 1991). However, the identification of an Arg-Gly-Asp (RGD) tripeptide, which is present in other bacterial adhesins, including FHA, indicated that pertactin may also have a role in attachment to eukaryotic cells (Leininger et al. 1991). In the same study, it was demonstrated that purified P69 promoted attachment to Chinese hamster ovary (CHO) cells in an *in vitro* adhesion assay. It was thus suggested that in addition

to a possible role in the initial binding of *B. pertussis* to the respiratory epithelium, pertactin may also be a factor in either the attachment to phagocytes or other target cells (Section 1.4.6.1). The detection of highly conserved outer membrane associated proteins of similar molecular weight to that of pertactin in the other Bordetella spp. provides further support the notion that pertactin is an essential element in the pathogenesis of whooping cough (Li et al. 1992). As with FHA, pertactin is also a potent immunogen of *B. pertussis*. Both circulating and secretory anti-P69 antibodies have been detected in the serum of patients recovering from infection, whilst high titers of serum antibodies have been observed following immunisation with the whole-cell vaccine (Pw) (Thomas, Redhead & Lambert 1989). In addition, De Magistris et al. (1988) demonstrated that P69 was recognised by *B. pertussis*-specific T cell clones isolated from a convalescent donor.

1.2.1.3 *Pertussis Toxin*

Regarded as the central virulence factor during infection, pertussis toxin elicits a number of deleterious effects including leukocytosis, splenomegaly, histamine sensitisation, hypoglycaemia and hypoproteinemia (Hewlett 1999). Pertussis toxin (PT) is an exotoxin composed of five subunits (S1- S5) arranged in a manner that is consistent with the A-B family of toxins (Locht & Antoine 1995). Subunits S2, S3, S4 and S5 present in a 1:1:2:1 ratio forms the B oligomer, which has been demonstrated to provide two functions: receptor-mediated binding to the surface of target cells and translocation of the catalytic domain across the plasma membrane (Burnette et al. 1988). ADP ribosylation of the mammalian G regulatory proteins by the S1 subunit or A promoter of the toxin causes interference in communication between the receptors and effector proteins of the host cell, which results in the observed clinical manifestations (Locht & Antoine 1995). The central role of pertussis toxin in whooping cough was the basis for its evaluation as a potentially protective antigen. *B. pertussis* is the only member of the genus that produces the ADP-ribosylating exotoxin and emphasises the importance of pertussis toxin considering that infection with *B. parapertussis* often results in a milder or atypical whooping cough syndrome (Linnemann & Perry 1977). The presence of anti-PT antibodies in convalescent sera and the detection of high titers following immunisation with the Pw vaccine provided an indication of the protective potential of PT (Trollfors et al. 1999). The subsequent development and assessment of acellular pertussis vaccines has provided further insight into the immune response and

protective efficacy of PT. Phase 1 and 2 clinical trials of a mono-component pertussis toxoid vaccine revealed a statistically significant correlation between toxin neutralising antibodies and protection against pertussis (Taranger et al. 2001).

Nicosia & Rappuoli (1987) cloned the entire *ptx* operon into *E. coli* and revealed that although each individual subunit was expressed, the holotoxin was not assembled and was therefore non-functional. This indicated that *B. pertussis* possessed cytoplasmic accessory proteins involved in the post-translation modification and assembly of the holotoxin. Considering the inability of other prokaryotes such as *E. coli* to assemble the holotoxin, it seems highly unlikely that mammalian cells would have the cytoplasmic machinery required for expression of the entire PT. Although this appears to be a limitation in the development of a PT-based DNA vaccine, it has been proven that immunisation with the S1 subunit alone is effective in generating a protective immune response in mice (Walker et al 1992; Kamachi et al. 2003; 2004).

1.2.1.4 *Adenylate Cyclase-Hemolysin Toxin*

Glaser et al. (1988) identified an 8.7 kb DNA fragment from a *B. pertussis* recombinant gene library, which encoded a calmodulin-sensitive adenylate cyclase. Sequence analysis of the cloned DNA revealed an open reading frame consisting of five genes arranged in an operon, denoted as *cya*. The *cyaA* gene encodes an inactive protoxin that exhibits AC activity but is devoid of haemolytic or cell-invasive activity (Sebo et al. 1991). An alcyltransferase encoded by an upstream *cyaC* gene activates the protoxin by palmitolyation of the Lysine 983 residue, which confers the invasive and haemolytic activities that are absent in the precursor (Sebo et al. 1991). Considering the absence of an N-terminal signal sequence in the protoxin, the three accessory genes downstream of *cyaA* (*cyaB*, *cyaD* and *cyaE*) have a postulated role in secretion of the active toxin (Hanski 1989).

The adenylate cyclase-hemolysin toxin (AC-Hly) performs a crucial role in lung colonisation and the establishment of infection. The C-terminal haemolytic domain penetrates mammalian phagocytes (neutrophils, monocytes, NK cells and macrophages) and facilitates translocation of the N-terminal adenylate cyclase across the plasma membrane, which subsequently catalyses the conversion of the energy-deficient cyclic adenosine monophosphate from the high-energy compound adenosine triphosphate

(Weiss & Hewlett 1986). Although mammalian cells possess an adenylate cyclase enzyme that intrinsically catalyses this conversion, this process is self-limiting to prevent an unregulated loss of ATP (Lehninger, Nelson & Cox 1993). A unique feature of AC-Hly is its interaction with the eukaryotic regulatory protein calmodulin, which increases the catalytic activity of the toxin up to a thousand-fold (Weiss & Hewlett 1986). The dramatic reduction of ATP within the infected phagocyte causes a severe impairment of normal cellular functions, arsing from an inadequate supply of cellular energy (Sebo et al. 1991). The resulting inhibition of a phagocyte response is an essential factor in the establishment of infection considering that mutants defective in AC-Hly production have been shown to be avirulent, unable to proliferate, and are rapidly cleared from the lungs (Weiss et al. 1984).

Phagocytosis and killing of *B. pertussis* by neutrophils is an effective innate defence mechanism against infection. Lenz et al. (2000) used *B. pertussis* labelled with green fluorescent protein (GFP) to demonstrate that up to 99 % of the bacteria phagocytosed by neutrophils are killed following internalisation. In view of the inhibitory effect of AC-Hly on neutrophils, the generation of toxin neutralising antibodies would enhance phagocytosis and thus the clearance of *B. pertussis*. Weingart et al. (2000) used an *in vitro* phagocytosis assay to assess the value of AC-Hly neutralising antibodies in the prevention of *B. pertussis* colonisation. It was shown that human immune serum supplemented with neutralising monoclonal antibodies enhanced phagocytosis of *B. pertussis*, however monoclonal antibodies that merely recognised the toxin but failed neutralise activity were ineffective. High titers of anti-AC-Hly antibodies have been detected in both the sera of convalescent patients and in adults and children immunised with the whole cell vaccine (Farfel et al. 1990). Although it was not determined if the antibodies present were capable of neutralising toxin *in vitro*, the protective humoral response elicited by immunisation of mice with native and recombinant AC-Hly (Hormozi, Parton & Coote 1999) suggests that neutralising polyclonal antibodies generated *in vivo* are equally effective in enhancing phagocytosis as the *in vitro* assay using monoclonal antibodies.

1.2.2 Additional Virulence Factors

1.2.2.1 *Fimbriae*

B. pertussis can express two fimbrial proteins that have been shown to mediate the binding of non-opsonized *B. pertussis* to host cells. Fim2 and Fim3 are composed of distinctive major subunit (22.5 and 22 kDa, respectively) and a common minor subunit known as FimD. Geuijen et al. (1997) demonstrated that Fim binds to sulphated sugars, which are ubiquitous on the surface of respiratory cells and secretions. FimD has also been shown to bind to the very late antigen-5 receptor of monocytes and to indirectly activate complement receptor type 3 (Hazenbos et al. 1995b). Although the complete role of fimbriae in pathogenesis remains unclear, deletion mutants are less persistent than the wild-type (Mooi et al. 1992). Fim2 and Fim3 have been incorporated into some licensed acellular vaccines; however their inclusion does not appear to augment the protective efficacy of DTaP (Mills et al. 1998). In contrast, an early clinical trial of a pertussis WCV in the UK found a correlation between serum agglutinin titers and protection against whooping cough in infants (Medical Research Council 1956). Of these serum agglutinin titers, antibodies to FIM have been found to be main contributors, along with antibodies to pertactin (van den Berg et al. 1999).

1.2.2.2 *Dermonecrotic Toxin*

Identified by Bordet and Gengou in 1909, dermonecrotic toxin (DNT) was one of the first known virulence factors of *B. pertussis*. DNT is a heat-labile toxin that when injected intradermally causes necrotic lesions in animals and has been observed to be lethal in mice when given intravenously (Livey & Wardlaw 1984). It is a single polypeptide of 140 kDa that appears to be cytoplasmic rather than secreted (Horiguchi, Sugimoto & Matsuda 1994). It is believed to cause inflammation, vasoconstriction and lesions around the areas of colonisation but the precise mode of action is not well understood. In fibroblastic cell lines, DNT has been reported to inhibit both alkaline phosphatase activity and expression of type-I collagen and to also stimulate DNA and protein synthesis in the absence of cell division, resulting in polynucleation (Horiguchi, Sugimoto & Matsuda 1994). Unlike other virulence factors, a deficiency of DNT in a mutant strain did not adversely affect its virulence or pathogenicity compared to a wild-type (Weiss & Goodwin 1989). This lack of a perceived effect on *B. pertussis* virulence and the limited understanding of its action were the reasons for exclusion of DNT as a candidate antigen for DNA vaccination.

1.2.2.3 *BrkA*

BrkA (*Bordetella* resistance to killing) has been demonstrated to provide a role in resistance to serum killing by the antibody-dependent classical pathway of complement activation. The *brk* locus was identified as a source of resistance following the characterisation of a Tn5-induced mutant that showed a dramatic increase in sensitivity to immune serum (Weiss et al. 1983). The increased serum sensitivity was also observed with a previously identified Bvg mutant, but was absent with mutants of other Bvg-regulated virulence factors (Weiss et al. 1983). Subsequent cloning and sequencing of the locus revealed two open reading frames (Fernandez & Weiss 1998). The *brkA* ORF encodes a 103-kDa precursor that is processed at the C-terminal domain to yield the mature 30-kDa BrkA, a putative outer membrane protein (Fernandez & Weiss 1998). The current evidence that supports the role of BrkA as a mediator of resistance to the classical complement pathway has been outlined in section 1.2.4.2, which pertains to the mechanisms of immune system subversion by *B. pertussis*.

In addition to serum resistance, BrkA has been suggested as an additional mediator of attachment to host cells due to a 29% sequence homology to pertactin (Fernandez & Weiss 1998). The importance of BrkA to pathogenesis has been demonstrated by two investigations. Weiss & Goodwin (1989) found that a *brkA*-depleted mutant had significantly decreased virulence in mice compared to a wild type strain. Later, Oliver & Fernandez (2001) demonstrated that neutralising antibody to BrkA enhanced the bactericidal activity of *B. pertussis* anti-serum. Although BrkA was not chosen as a candidate for this study, the evidence suggests that its inclusion in future vaccine research is warranted.

1.2.2.4 *Tracheal Cytotoxin*

Tracheal cytotoxin (TCT) is believed to perform a central role in the destruction of ciliated tracheal epithelial cells, which is an early and significant feature in the onset of whooping cough (Parton 1999). Although the precise mechanisms of epithelial damage have not yet been revealed, the cytopathic effect of *B. pertussis* infection has been mimicked *in vitro* by the addition of purified TCT to cultured tracheal epithelial cells (Cookson et al. 1989). Characterisation of TCT revealed that it is a soluble fragment of cell wall peptidoglycan (Rosenthal et al. 1987). The structure was later identified as *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-(L)-alanyl-γ-(D)-glutamyl-*meso*-

diaminopimelyl-(D)-alanine, a small 921 Da disaccharide-tetrapeptide monomer (Cookson et al. 1989). Of interest is the range of biological activities that these muramyl peptides exhibit in addition to cytotoxicity, such as adjuvanticity, pyrogenicity, arthritogenicity and modulation of cell-mediated immune responses (Adam & Lederer 1984).

1.2.2.5 *Lipopolysaccharide*

As with the endotoxin of other gram-negative bacteria, the lipopolysaccharide (LPS) of *B. pertussis* is pyrogenic, mitogenic and toxic to host cells (Watanabe et al. 1990). *B. pertussis* LPS is composed of a lipid A portion linked to a branched oligosaccharide core and a trisaccharide and is structurally unique from other *Bordetella* spp. in that it lacks a repetitive O antigen (Peppler 1984). Allen and Maskell (1996) identified the 12-gene *wlb* locus that was required for the biosynthesis of the trisaccharide component. Mutants with targeted disruptions to these *wlb* genes were shown to be defective in colonisation of the respiratory tract compared to a wild-type strain (Harvill et al. 2000). These mutants were also significantly more sensitive to antibody-mediated killing *in vitro*, which indicated that LPS and in particular the trisaccharide component may provide the organism with an additional means (other than BrkA) for resistance to the classical complement pathway (Harvill et al. 2000). As a potent T cell mitogen, LPS can stimulate the release of inflammatory cytokines such as TNF-α and IL-12 from macrophages (Remoue et al. 1997; McNeela & Mills 2001). High levels of LPS-specific IgA and IgM has also been detected in the serum of infected or convalescent children (Novotny et al. 1991). Although not practical for use as a DNA vaccine LPS has an important role in both pathogenesis and immunity to pertussis.

1.2.3 Regulation of Virulence-Associated Gene Expression

B. pertussis possesses a two-component signal transduction system, known as BvgA/S that controls virulence factor expression. The Bvg system co-ordinately regulates the expression of two types of genes, the virulence-associated genes (*vag*) and the virulence-repressed genes (*vrg*). The control mechanism for this virulence regulation was found to originate from a single locus following studies of a Tn5-induced avirulent-phase mutant (Weiss et al. 1983; Weiss & Falkow 1984). Sequence analysis of this *vir* locus revealed similarities to the signal transduction systems of other bacterial pathogens such as OmpR and EnvZ from *E. coli* (Arico et al. 1989). The subsequently

re-named *bvg* locus was demonstrated to have two open reading frames that encoded a 135-kDa transmembrane histidine kinase (BvgS) with a sensory and transmissive role and a 23-kDa cytoplasmic regulator (BvgA) that bound to DNA upon activation (Scarlato et al. 1990).

In a cascade of events that is relatively complex compared to other two-component systems, *vag* activation is initiated by the auto-phosphorylation of BvgS. Phosphorylated BvgS then activates the BvgA receiver/regulator by a second phosphorylation. Activated BvgA binds directly upstream to the promoter regions of the *fha*, *bvgA* and *act* genes that encode FHA, BvgA and Act and initiates transcription. Over-expression of BvgA and Act results in the sequential activation of other *vag*'s. Hence, the *bvg* locus is pleiotropic and controls the expression of upwards of 20 virulence factors that include the FHA, pertactin, PTX, AC-Hly, DNT, FIM, and BrkA. Although all of these genes are under direct control of BvgA/S, their promoters have subtle differences that alter the time-course of expression. Under virulence permissive conditions promoters of the *vag* are activated in a systematic order but in presence of certain environmental stimuli or mutation, the BvgA/S system becomes inactivated.

These two mechanisms identified as the triggers for BvgA/S inactivation are phenotypic modulation and phase variation.

1.2.3.1 Phenotypic Modulation

In a number of studies, growth in the presence of high concentrations of $MgSO_4$ and nicotinic acid, or at a reduced temperature of 25°C was observed to cause a decrease or elimination in the expression of virulence factors (Lacey 1960; McPheat, Wardlaw & Novotny 1983). Importantly, it was also demonstrated that a return to permissive conditions resulted in a shift back to the virulent phase (Idigbe, Parton & Wardlaw 1981). Later, Melton & Weiss (1993) determined that in the presence of these environmental cues BvgS cannot auto-phosphorylate and therefore BvgA cannot be activated. An example of phenotypic modulation by the BvgA/S system has been outlined in Figure 1.3.

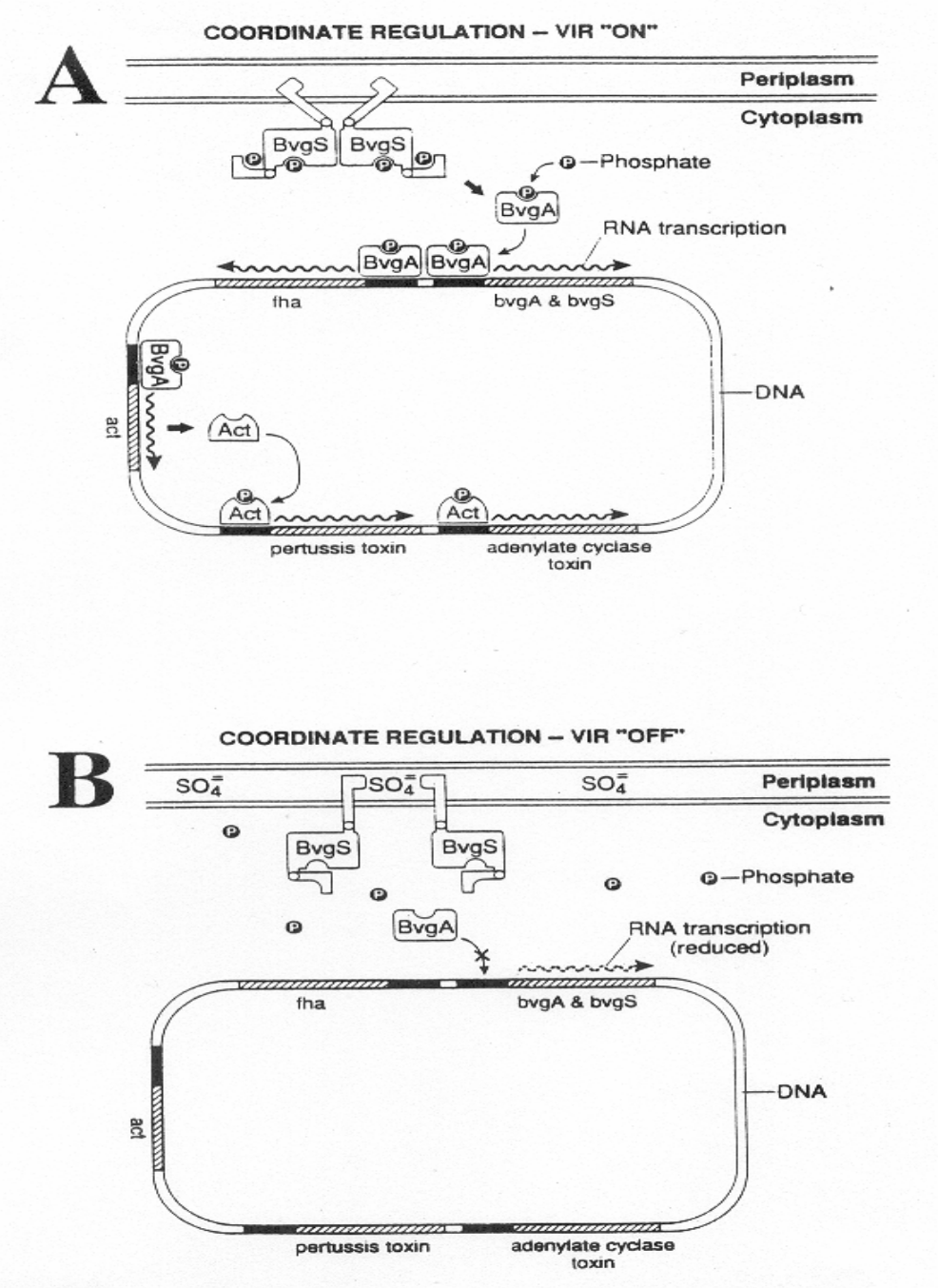

Figure 1.3: Model of BvgA/S regulation of vag expression by phenotypic modulation. (A) Under favourable conditions (in the absence of environmental cues) BvgA/S is active and virulence factors are sequentially expressed. (B) During growth at 25°C or in the presence of elevated nicotinic acid or $MgSO_4$ (shown), BvgS cannot auto-phosphorylate and the complex becomes inactive. Virulence - associated gene (*vag*) expression stops and an alternative phenotype arising from virulence-repressed gene (*vrg*) expression becomes uninhibited. Source: Melton & Weiss (1993), p 808.

1.2.3.2 Phase Variation

Phase variation involves a frame shift mutation within the *bvgS* gene (Stibitz et al. 1989). Stibitz et al. (1989) compared *Sal*I fragments from a vir^+ and vir^- strain and found that the frame shift occurred from the insertion of a cytidine base within a run of six cytidines at positions 829 to 834 of the gene sequence. This form of regulation has also been shown to be reversible. Weiss & Falkow (1984) reported that spontaneous avirulent (vir^-) mutants were detected at a frequency of 10^{-3} to 10^{-6} within virulent (vir^+) cultures of *B. pertussis*, and following isolation a similar ratio reverted back to the virulent phenotype. The benefit of this regulated virulence in *B. pertussis* has not yet been identified. It has been proposed that the down-regulation of virulence factors may coincide with transmission, immune response subversion or intracellular persistence (Kinnear, Marques & Carbonetti 2001). Currently, the specific number, nature and function of the *vrg*'s are unknown. A future understanding of their products will no doubt provide the answer. Regardless, *vag* expression has been shown to be an absolute requirement for infection in both the *B. pertussis* mouse and *B. bronchiseptica* rabbit models of infection (Cotter & Miller 1994; Merkel & Stibitz 1995).

1.3 History of Whooping Cough Prophylaxis

Whooping cough was responsible for widespread morbidity and mortality during the pre-immunisation era (Keitel 1999). In response to the high incidence and severity of the disease, whole-killed *B. pertussis* (Pw) was included as a component of a multivalent vaccine known as DTP (diphtheria toxoid, tetanus toxoid and whole killed *B. pertussis*). Although the incidence of pertussis quickly diminished following introduction of the DTP vaccine, a gradual reduction in Pw vaccine compliance and coverage due to the perceived risk of severe side effects resulted in the subsequent re-emergence of whooping cough (Donnelly et al. 2001). Observed side effects, attributed solely to the Pw component of the DTP vaccine included encephalopathy, febrile seizures, hypotonic–hypo-responsive episodes, swelling around the site of injection and sudden infant death syndrome (Poland 1996; Mills 2001). LPS has been implicated as a possible cause of the Pw-mediated reactogenicity (Griffith 1978; Kurokawa 1984). In response to public uncertainty surrounding the Pw vaccine, research was focussed on the development of an alternative and safer prophylactic. Various acellular pertussis vaccines (Pa) that consisted of a combination of the immunogenic antigens filamentous hemagglutinin, pertussis toxoid, pertactin and fimbriae were tested (van den Berg et al.

2000). Favourable results from human clinical trials led to the acceptance of the Pa vaccine combined with the established tetanus and diphtheria toxoids to produce the contemporary triple antigen vaccine, referred to as DTaP.

Since the introduction of DTaP numerous studies have evaluated and compared the efficacy and safety of the acellular versus the whole cell vaccine (Boughton 1996; Gustafsson et al. 1996; Keitel 1999; van den Berg et al. 2000). The consensus was that Pa vaccines conferred a level of protective immunity that was comparable to Pw vaccines but with a decrease in the incidence and severity of adverse side reactions. This view of improved safety has been somewhat contradicted by a recent report in which local side reactions are common in infants following multiple booster doses of the Pa vaccine (Rennels et al. 2000). These effects such as extensive swelling around the site of injection were believed to be caused by IgE-mediated hypersensitivity directed against the pertussis toxoid. Gold et al. (2003) also assessed the rate of side-reactions to DTaP in South Australia from 1997 to 2000. Importantly, they found that the side-reactions were so serious in 22% of vaccinated children that the Australian Adverse Drug Reaction Assessment Committee (ADRAC) had to be notified. Both studies indicate that side effects continue to plague current methods of immunisation against pertussis. Immunisation by mucosal routes such as oral or IN could alleviate the local and systemic reactogenicity arising from Pa and Pw vaccination.

Disregarding the issue of reactogenicity, double-blind studies have demonstrated that both the Pw and Pa vaccines induce a variable level of immunity, with figures ranging from 29% to 85% efficacy (Trollfors et al. 1995; Gustafsson et al. 1996; Storsaeter et al. 1998). Furthermore, it has been reported that *B. pertussis* is capable of infecting individuals previously vaccinated with either the Pw or Pa. Interestingly, Storsaeter et al. (1998) reported that 33% of individuals that received the licensed five-component acellular vaccine had evidence of post-immunisation infection following exposure to *B. pertussis*. In the same study, 82% of individuals immunised with Pw developed infection. DNA vaccines may provide a means of generating complete and long-lasting immunity against *B. pertussis*, because of their potential to generate both antibody and cell-mediated responses (Donnelly et al. 1997), with the latter being considered to be essential for long-term protection against whooping cough (Mills et al. 1993).

1.4 Immunity to *B. pertussis*

1.4.1 Principles of Immunology

The vertebrate immune system is divided into two broad categories: innate immunity that provides a non-specific first line of defence against all foreign substances, and acquired immunity that specifically targets and eliminates the foreign substances not eliminated by innate defences (Delves & Roitt 2000). Although innate immune mechanisms such as physical barriers, chemical barriers and cellular defences are an important aspect of protection from infectious disease, this review will concentrate on the generation of acquired immunity and the relevant innate immune defences that contribute to the acquired response. There are two reasons for this focus. Innate mechanisms of the upper and lower airways alone do not provide sufficient protection against *B. pertussis*. Secondly, the scope of this study is to evaluate alternative strategies for pertussis immunisation and therefore the enhanced priming of an acquired immune response.

1.4.1.1 *Acquired Immunity*

For the induction of an acquired immune response there are three portals of entry for a pathogen or antigen into the body, namely the bloodstream, lodgement in epidermal, dermal or subcutaneous tissues and via the mucosal surfaces such as the digestive, respiratory and genitourinary tracts (Basset et al. 2003). Upon direct entry of a pathogen or antigen into the circulation, it is carried to the spleen where it interacts with professional antigen-presenting cells (APC), T cells and B cells for the generation of a specific response (Delves & Roitt 2000). In relation to peripheral tissues, the antigen (free or trapped by professional APC) is transported via afferent lymph vessels to regional draining lymph nodes where as with the spleen, a specific response is induced. Following antigen presentation and immune induction in the spleen or lymph nodes (LN) a systemic response is generated in which effectors such as antibody or activated lymphocytes are released into circulation and migrate to the site of infection or inflammation (Basset et al. 2003). In comparison, a feature of antigen contact at the mucosal surfaces is the induction of both a local and systemic response (Staats et al. 1994). As the majority of pathogens including *B. pertussis* are encountered at the mucosal surfaces, the induction of local immune responses at these sites are important and has been discussed in further detail in section 1.5.1.3.

1.4.1.2 *T Cell Control of an Acquired Immune Response*

CD4+ T helper cells play a pivotal role in the generation of an acquired immune response. Upon leaving the thymus, CD4+ T cells have a naïve phenotype (Th0) and only differentiate into effector cells that are capable of stimulating either a humoral or CMI response depending on the appropriate activation signals. These activation signals or cytokine profiles are thought to be provided by local innate immune cells. Depending on the nature of cytokines present, CD4+ Th0 cells differentiate into two distinct subgroups referred to as Th1 or Th2.

In the presence of interleukin 12 (IL-12) released by macrophages, naïve CD4+ cells differentiate into Th1 effector cells (McKnight et al. 1994). When stimulated by antigen, the activated Th1 cells release cytokines such as interferon-γ (IFN-γ), interleukin-2 (IL-2) and tumour necrosis factor-β (TNF-β) that stimulate the proliferation and activation of CD8+ cytotoxic T cells and macrophages (Gurunathan, Klinman & Seder 2000). These activated cells seek and destroy host cells harbouring intracellular pathogens (viral, bacterial or parasitic). Th1 CD4+ cells also incite delayed-type hypersensitivity reactions. IFN-γ released from Th1 cells promotes the production of IgG2a antibodies, a sub-class of IgG which aids in the response to intracellular pathogens by complement activation, opsonisation and antibody-dependant cell mediated cytotoxicity of infected host cells (Markine-Goriaynoff & Coutelier 2002).

Conversely in the presence of IL-4 released by basophils or mast cells, naïve CD4+ cells differentiate into Th2 effector cells (McKnight et al. 1994). Upon antigenic stimulation, activated Th2 CD4+ cells release cytokines such as IL-4, IL-6 and IL-10 that stimulate the proliferation of antigen-specific B cells. Th2 cells also produce IL-5, which moderates class switching of IgM committed plasma cells to either IgG1 or IgA, during circulation or at a local site of infection (Gurunathan, Klinman & Seder 2000). Furthermore, eosinophils are also primed to act against extracellular parasites by cytokines released from Th2 lymphocytes. Hence as a generalisation, a Th2 and B cell immune response is induced to eliminate extracellular pathogens, whilst Th1 and cytotoxic T cells mediate a cell-mediated response against intracellular pathogens that avoid clearance from circulating antibody and polymorphonuclear leukocytes (PMNL's).

1.4.1.3 *Humoral or Cell-mediated Effectors*

The means by which an antigen is presented to T lymphocytes influences the type of effector response elicited. Exogenous antigen phagocytosed by professional APC such as macrophages and dendritic cells is processed and presented by a major histocompatibility complex (MHC) class II molecule for interaction with the T cell receptor complex (TcR) of CD4+ T cells. This interaction results in the induction of a humoral immune response that is mediated by memory B cells and antibody producing plasma cells (Delves & Roitt 2000).

Hence, antibodies are the effectors of the humoral immune response. Based on structural and functional characteristics antibodies are divided into five isotypes: G, M, A, D and E. Immunoglobulin G (IgG) is the predominant antibody in the body constituting about 80% of total Ig, and is equally distributed throughout intravascular and extravascular compartments (Ramanarayanan 1985). The four human subclasses of monomeric IgG (IgG1, IgG2, IgG3 and IgG4) each have distinct strengths and weaknesses over a range of functions that include: agglutination and precipitation of particulate and soluble antigens to facilitate capture by professional phagocytes; opsonisation which is mediated through binding to the Fc receptor of PMNL and APC; antibody-dependant cytotoxicity following interaction with natural killer (NK) cells; activation of the classical complement pathway; neutralisation of toxins and viruses; and placental passage for passive immunisation of the foetus (Ramanarayanan 1985). As discussed in section 1.5.1.4.3, the two IgA subclasses (IgA1 and IgA2) are the main effectors of mucosal secretions and primarily act by preventing attachment of pathogens to mucosal surfaces (Tomasi 1983). IgM is the first isotype synthesised in the primary response to T-dependent antigens and is also the main antibody generated after exposure to T-independent antigens (Sharon et al. 1975). Although not as multi-purposeful as IgG, IgM is highly efficient at agglutination and complement activation due to its pentametric form (Ramanarayanan 1985). The least abundant of all antibodies, IgE has an important role in hypersensitivity reactions due to high affinity Fc receptors on the surface of basophils and mast cells and has also been claimed to be important in protection against some parasitic infections (Gurish et al. 2004). Upon recognition of antigen by Fc bound IgE, the mast cell or basophil releases their granular contents such as histamine and heparin (Ramanarayanan 1985). Lastly, the role of IgD in the immune response is not well understood. As with other subclasses, it is expressed on the surface

of immature B cells as the B cell receptor, but unlike the remaining isotypes it does not appear to be secreted from terminally differentiated plasma cells. Rather it is released from the B cell surface which explains its relatively low concentration in serum (Ramanarayanan 1985).

On the other hand, endogenous antigen expressed within infected professional APC or somatic host cells are processed and presented in association with MHC class I molecules (Claman 1989). Expressions of an MHC class I/antigen complex on the host cell surface is recognised by the TcR complex of CD8+ cytotoxic T cells (Tc). Although the precise mode of activation remains unclear, upon interaction between the MHC class I/antigen complex and the CD8/TcR, activated Tc cells proliferate and differentiate to maturity in the presence of cytokines such as IL-2 released by CD4+ Th1 cells (Basset et al. 2003). The mature Tc cell may then kill the infected host cell using one or more antigen-dependent pathways. Firstly, perforins are released from granules and form transmembrane channels that increase membrane permeability in a similar manner to the membrane attack complex of complement (Wong & Pamer 2003). Tc cells also secrete IFN-γ and tumour necrosis factors that have antimicrobial properties such as the stimulation of nitric oxide and superoxide production, as well as chemokines that attract inflammatory cells (van Ginkel, Nguyen & McGhee 2000). Thirdly, it has been suggested that Tc can induce apoptosis of the target cell by releasing molecules that deregulate apoptotic caspases leading to programmed cell death (Zychlinsky & Sansonetti 1997).

In addition to the segregation of humoral and cellular immune responses, an additional feature of the mammalian immune system is compartmentalization into systemic and mucosal immunity (Clare, Huett & Dougan 2002). However, it is important to note that this compartmentalization is partial and there is interaction between the two systems (McCaughan, Adams & Basten 1984).

1.4.1.4 *Mucosal Immunity*

The mucosal surfaces of the digestive, respiratory and urogenital tracts are the main portals of entry for pathogenic microbes. Considering these sites, particularly the respiratory and digestive tracts have a large epithelial surface area which is frequently exposed to foreign agents and particles, a local response is necessary in the prevention and control of mucosal-acquired infections. Consistent with the collective view of the immune system, surveillance and protection of the mucosal tracts involves both innate and acquired mechanisms. Although physical and chemical barriers are effective in preventing colonization, certain highly adapted pathogens such as *Streptococcus pneumoniae*, *Hemophilus influenzae*, and importantly *Bordetella pertussis* have developed virulence factors that facilitate attachment, damage or invasion of respiratory epithelium (Daniele 1990). In cases of exposure to such pathogens the innate mechanisms are assisted mainly by the induction of secretory antibody. At the core of the acquired mucosal response is the distribution of numerous lymphoid masses along the mucous membranes referred to as mucosal-associated lymphoid tissue (Tomasi 1983).

1.4.1.4.1 *Mucosal-associated Lymphoid Tissue (MALT)*

At strategic positions along the mucosal surfaces are secondary lymphoid organs that are responsible for antigen processing and the induction of an antigen specific response (Daniele 1990). The collective MALTs are separated into the nasal-associated structures of the nasopharynx, tonsils and salivary glands (NALT), the bronchial-associated lymphoid tissue of the proximal airways (BALT) and gut-associated lymphoid tissue (GALT). Located in the sub-mucosa, the lymphoid structures or aggregates are highly organised into four distinct zones: a unique epithelial monolayer, a sub-epithelial dome, follicles and parafollicular areas. At the luminal surface of the lymphoid structures are specialized epithelia known as follicle-associated epithelium (FAE). Unlike conventional epithelia that are columnar, FAE are cuboidal and contain little or no mucous secreting goblet cells (MacDonald 2003). Embedded throughout the FAE are specialised microfold (M) cells, which are flattened cells that possess microvilli instead of the cilia present on normal mucosal epithelia. M cells have the unique ability to sample both soluble and particulate antigens directly from the lumen and then transport the antigens to the sub-epithelial dome by pinocytosis (Hobson et al. 2003). The dome contains a mixed population of plasma cells, naïve T and B cells and

APCs. The presence of dendritic cells in close proximity to naive lymphocytes indicates that antigen presentation and priming are the primary functions of this area (Daniele 1990). The surrounding follicles are enriched with B cells committed to IgA production, however unlike the germinal centres of the spleen and lymph nodes there is no terminal differentiation into plasma cells. Finally, the parafollicular areas are analogous to the follicles, but are T cell-dependant zones. Within this zone, only 35-38% are mature T cells and of which CD4+ helper and CD8+ cytotoxic T cells constitute 60 and 20%, respectively. Although dominated by with lymphocytes, a small percentage of functionally active macrophages (5-9%) have been observed (Spalding et al. 1983).

1.4.1.4.2 *Induction of an Acquired Mucosal Response*

Apart from being highly organized, MALT is also well vascularised. Naïve T and B cells from the thymus or bone marrow enter lymphoid structures via high endothelial venules (HEV). Upon release of antigens at the basolateral surface of the dome, professional antigen presenting cells such as DC engulf and process the antigen for presentation to B and T cells which are primed to become either memory or effector cell types and elicit an inductive or suppressive response (Brandtzaeg 2003). These primed cells then leave the inductive site and migrate to regional LN via lymphatic vessels (Daniele 1990). Within the LN, specific antigenic re-stimulation provided by circulating DC or free antigen transported from afferent lymphatic channels stimulates committed lymphocytes to differentiate and proliferate within the germinal centres (Brandtzaeg 2003). Under the influence of specific homing mechanisms and signals (discussed in further detail in section 1.4.1.4.5) activated lymphocytes re-locate via lymph or peripheral blood to various mucosal effector sites. In terms of antibody production, B cells settle in the lamina propria and complete terminal differentiation into plasma cells upon stimulation with IL-5 and IL-6 released by surrounding Th2 cells (Husband 2002). Returning helper, cytotoxic or suppressor T cells are distributed throughout the lamina propria or within the epithelial monolayer (Brandtzaeg 2003).

Dendritic cells also possess an intra-epithelial niche, from which they capture pathogens and antigens from lumen and either present directly to local B cells for stimulation of a T cell-independent IgA response or migrate to draining lymph nodes for induction of a T cell-dependant response (Fagarasan & Honjo 2003).

1.4.1.4.3 *Secretory Antibodies*

The majority of activated (Ig-producing) plasma cells are distributed along the sub-mucosal tissue or exocrine glands, which service epithelial surfaces (Brandtzaeg 2003). Of these cells 70-90% are committed to the production of IgA (Brandtzaeg et al. 1999). Hence, the induction of a secretory IgA (S-IgA) response has long been considered to be the most important means of defence against mucosal pathogens. IgA has numerous functions; however its main role involves immune exclusion (non-inflammatory) rather than elimination (pro-inflammatory). Firstly, it inhibits the attachment of bacteria to the mucosal cell surface by promoting agglutination and also the neutralisation of adhesins and other virulence factors. Secondly, the coating of viruses by IgA prevents both invasion of epithelial cells and certain mucosal carrier states by neutralisation of intraepithelial viruses. Thirdly, IgA is mucophilic (conferred by the secretory component) and enhances the entrapment of pathogens and antigens in mucous, thereby facilitating clearance by the innate mechanisms (Brandtzaeg 2003). It has also been suggested that in the presence of co-factors such as lysozyme and complement, IgA may promote phagocytosis of coated micro-organisms and foreign particles (Daniele 1990). Whilst there is evidence that IgA cannot effectively fix complement via the classical pathway and mature alveolar macrophages lack an Fc receptor for IgA (Reynolds 1986; Mota et al. 1988), it has been demonstrated that polymorphonuclear leukocytes, monocytes and early macrophages possess the relevant receptor (FcαR1) and upon recruitment to a site of inflammation these cells can effectively phagocytose IgA-coated complexes (Morton, van Egmond & van de Winkel 1996). Thus it appears that in the absence of an inflammatory infiltrate opsonisation by IgA is limited to triggering of the alternative pathway, and would be far less efficient than an IgG-mediated process.

The synthesis of secretory IgA is a multi-step process. Within the lamina propria, two IgA monomers covalently linked by a J-chain glycoprotein are secreted by committed plasma cells. The IgA dimers bind to an 80 kDa secretory component expressed as a receptor on the basolateral membrane of adjacent epithelial cells (Brandtzaeg 1983). The IgA-secretory component complex is then transported across the epithelial cytoplasm within a cytoplasmic vesicle and then released at the lumen surface by exocytosis. Following secretion, resistance to proteolytic degradation is an important feature of IgA. Whilst the secretory component protects both the IgA1 and IgA2 subclasses from endogenous proteases common in the mucosal tracts, certain bacterial

pathogens such as *Streptococcus pneumoniae* and *Hemophilus influenzae* produce proteases to which only IgA2 is resistant. A difference in the amino acid composition within the J-chain is the basis for the susceptibility of IgA1. Importantly, resistant IgA2 constitutes only ⅓ of total IgA in secretions, and IgA1 is the most common subclass in the URT (Daniele 1990). Whether *B. pertussis* produces an IgA-protease has not yet been reported.

Although often overlooked in favour of an S-IgA response, the presence of IgG within secretions may play an important role in mucosal defence, particularly in the lower respiratory tract. In fact, in the bronchoalveolar lavage (BAL) fluid of healthy humans IgG is the predominant class and constitutes approximately 18 – 20% of total protein, whereas IgA makes up only 9-11% (Daniele 1990). A more comprehensive study of antibody distribution within the respiratory system revealed that the relative proportions of IgG and IgA are different at various depths along the respiratory tract (Kaltreider & Chan 1976). This study found that there is a gradual decline in the concentration of IgA and an inversely proportionate rise in the concentration of IgG with increasing depth of the tract, such that salivary secretions contained mostly IgA and bronchial washes largely IgG. Unlike the URT in which exclusion by S-IgA and innate mechanisms are the main defences, protection of the lower airways is dependant on a large distribution of alveolar macrophages and therefore elimination. As all macrophages possess Fc receptors for IgG, it is not surprising that it is the most common antibody in the lower RT considering that opsonisation is one of the main attributes of IgG. Lacking a secretory factor, the mode of entry for IgG into the mucosal secretions is unclear. It has been proposed that plasma cells within the lamina propria secrete IgG into the interstitial space, from which it crosses the mucosa either by passive diffusion or at points of epithelial damage (Bienenstock & Befus 1980). Alternatively, it has been suggested that IgG can enter the mucosal secretions via transudation from systemic circulation (Scicchitano, Sheldrake & Husband 1986; Spiekermann et al. 2002).

1.4.1.4.4 *Local T-cell Responses*

To date, the main focus of a specific mucosal immune response has surrounded the induction of the humoral component, and specifically secretory IgA. Nevertheless, within the secondary lymphoid structures are parafollicular or dedicated T cell-dependant areas and it is also apparent that like B cells, T lymphocytes undergo a process of local stimulation, migration to lymph nodes or the spleen for proliferation and then homing back to various mucosal sites (Bienenstock et al. 1983).

1.4.1.4.5 *Common Mucosal Immune System – Lymphocyte Migration*

Rudzik et al. (1975) demonstrated that when B cells isolated from either the Peyer's patches or BALT of a donor rabbit were transferred to lethally irradiated recipients, re-population occurred at both the bronchial and intestinal lamina propria. These findings inspired the concept of a common mucosal immune system, in which immune cells primed at a mucosal site not only home back to the site of induction but also to distant mucosal effector sites (McDermott & Bienenstock 1979).

There have been numerous recent reports that confirm the existence of the common mucosal system. In mice, nasal administration of a human papilloma virus VLP (virus-like protein) generated neutralizing antibodies in the genital secretions (Balmelli et al. 1998). Similarly, specific IgA was detected in both nasal and vaginal secretions following the intranasal immunisation of monkeys with a bacterial antigen coupled to the B subunit of cholera toxin (Russell et al. 1996). Preliminary evidence has shown that the homing of lymphocytes to mucosal effector of the gut involves interaction between the α4β7 integrin and the mucosal addressin-1 (MAdCAM-1) molecule. Alternatively, homing to the NALT was mainly the result of interaction between the L-selectin of lymphocytes and peripheral node addressin (PNAd) (Berlin et al. 1993). There have also been suggestions of a chemokine-receptor network that is unique to each mucosal effector site (Zuercher 2003). Aside from the specific mechanisms of homing, random factors such as route of mucosal delivery, mode of vaccination and the antigen itself may also have an effect on the distribution and intensity of a mucosal immune response.

The only potential limitation with the application of a cross-immunisation approach is the evidence that committed lymphocytes preferentially home to the area of initial antigen contact and that only a smaller proportion migrate to distant effector sites (Zuercher 2003). The current view of specific integrin-addressin interactions at different mucosal sites supports this notion, as does the recent observation that the specific homing properties are conferred to proliferating T cells in the regional LN (Campbell & Butcher 2002). Therefore, lymphocytes primed in the PP with a primarily α4β7+ phenotype would lead to proliferation of lymphocytes in the mesenteric LNs with the same integrins and would therefore have a tendency to home to the endothelial venules of the gut that express the gut-specific MAdCAM-1. For example, Quiding-Jarbrink (1997) found that oral immunization with a protein antigen and adjuvant elicited a strong local response in the small intestine, a much weaker response in the mammary and salivary glands, but no antibody at other distant sites such as the large intestine, lower respiratory or genital tracts.

In spite of these observations, oral immunization using attenuated gut pathogens or biodegradable microparticles has been shown to be an effective means of generating a specific response in the lower respiratory tract (Forrest et al. 1991; Challacombe, Rahman & O'Hagan 1997; Allen, Dougan & Strugnell 2000). For instance, a specific secretory IgA and IgG response has been detected in respiratory secretions following oral immunisation of attenuated *S. typhimurium* expressed the FHA antigen of *B. pertussis* (Guzman et al. 1991b). On the basis of these results and other instances that support the common mucosal pathway, the oral delivery of DNA vaccines using the proven SL3261 carrier will be evaluated for the generation of a strong systemic and mucosal antibody response. Other examples of successful mucosal cross-immunisation with *Salmonella* and other intestinal pathogens have been mentioned in section 1.5.2.3.

1.4.2 Response to Whole-cell Versus Acellular Immunisation

Both whole-cell and acellular pertussis vaccines confer protection against virulent *B. pertussis* following immunisation (Ad Hoc Group 1988; Gustafsson et al. 1996). Nevertheless, it has long been regarded Pw and Pa vaccines elicit different immune response profiles in humans and animals (Ryan et al. 1997; Mills et al 1998). Samples tested from Phase 3 clinical trials were used to demonstrate that natural infection and vaccination with the Pw vaccine elicits a predominantly Th1-type cell-mediated

response, whereas Pa vaccines typically induce a strong humoral response but a comparably weak CMI response (Mills et al. 1998). However, several studies have contradicted this generalisation with reports of induction of potent CMI in children given acellular vaccines, as determined by proliferation of peripheral blood mononuclear cells in response to pertussis antigens (Cassone et al. 1997; Ausiello et al. 1998). In the Cassone et al (1997) study it was also shown that these two acellular vaccines were in fact better inducers of CMI than a whole-cell vaccine.

1.4.3 Importance of a Humoral Response against *B. pertussis*

Although a large emphasis has been placed on the induction of a potent antibody response by traditional pertussis vaccines there have been contradictory views surrounding the true value of a humoral response against *B. pertussis*. In some clinical trials of DTaP a direct correlation between a serological antibody response and protective immunity could not be identified (Ad Hoc Group 1988; Cherry et al. 1998), whereas in other studies the importance of a humoral immune response has been clearly demonstrated (Sato & Sato 1984; Shahin et al. 1990; Bruss & Siber 1999).

Transient protection has been demonstrated against *B. pertussis* following passive immunisation with both monoclonal and polyclonal antibody. Early studies revealed that protection, albeit variable, could be conferred against an aerosol or intracerebral challenge of mice by administration of monoclonal antibodies directed against the either the S1 subunit of pertussis toxin (Sato & Sato 1984) or pertactin (Shahin et al. 1990). Interestingly, anti-FHA antibodies were found to be significantly less potent for passive immunisation in comparison to anti-pertussis toxin or anti-pertactin formulations (Sato & Sato 1984). Bruss & Siber (1999) later demonstrated that the intravenous administration of a polyclonal anti-pertussis toxin IgG preparation could passively protect mice against an aerosol challenge.

The efficacy of therapeutic antibody preparations has also been investigated. Granstrom et al. (1991) tested an alternative antibody therapy on infants hospitalized with culture-confirmed whooping cough and reported a significant reduction of whoops in groups that received a hyperimmune serum preparation (raised against a two component acellular vaccine) compared to a placebo. Importantly, this study highlighted a limitation of immunoglobulin treatments, in that manifestations in infants or protective

efficacy in mice were only improved if the antibodies were administered within 7 days of exposure (Sato & Sato 1984; Granstrom et al. 1991). Mukkur et al. (2005) reported that clearance of virulent *B. pertussis* was significantly greater in previously infected mice when the hyperimmune serum used was raised against Pw than Pa, pertussis toxoid or FHA.

Aside from passive immunization studies, research involving transgenic mice models has also indicated an essential role for antibodies. It was demonstrated that unlike wild-type mice, B cell and antibody deficient ($Ig^{-/-}$) mice developed a chronic infection and failed to clear bacteria from the lungs after experimental infection (Mahon et al. 1996; Mills et al. 1998). Furthermore, immunisation of $Ig^{-/-}$ mice with either whole-cell or acellular pertussis vaccines failed to confer protection against an experimental challenge but protection was restored following the transfer of primed B cells. These observations were supported by Leef et al. (2000) who reported that intranasal immunisation of $Ig^{-/-}$ mice with formalin-fixed *B. pertussis* (FFBP) only provided partial protection against an aerosol challenge compared to complete protection in FFBP-immunised wild-type mice. Once again full protection was re-affirmed following transfer of immune B cells.

The effectiveness of the acellular vaccine provides a strong indication of the importance of humoral immunity. It is well documented that parenteral immunisation with DTaP induces a Th2-biased response characterised by high titers of antibody with little or no induction of a cell-mediated response (Redhead et al. 1993; Barnard et al. 1996). Furthermore, the Th2 response following DTaP immunisation was demonstrated to be clinically efficacious (Greco et al. 1996; Gustafsson et al. 1996; Olin et al. 1997). Nevertheless a theoretical shortcoming of priming with a polar Th2 immunisation regime would be the inability to clear intracellular pathogens. In the absence of a memory Th1 response, an infection would have the potential to become persistent despite the absence of acute symptoms in humans or an apparent clearance of bacteria from the respiratory tract of animals. This has been supported by an observation that the co-administration of IL-12 with an acellular vaccine, which induced a shift in bias from a Th2 to Th1 response, enhanced the rate of clearance following a bacterial challenge (Mahon et al. 1996).

1.4.4 Importance of Cell-Mediated Response against *B. pertussis*

The lack of correlation between protection and antibody levels observed following clinical trials of a two-component acellular pertussis vaccine suggested a lack of insight into the true nature of immunity to *B. pertussis* (Ad hoc group 1988). A subsequent study of convalescent humans identified CD4+ Th cells that proliferated in response to either whole-inactivated *B. pertussis* or individual antigens (De Magistris et al. 1988). Numerous reports have since used naïve and transgenic mouse models to establish the importance of CMI against *B. pertussis* (Mills et al. 1993; Redhead et al. 1993; Barbic et al. 1997). Redhead et al. (1993) observed variable T cell proliferative responses in mice following convalescent infection or immunisation with whole-cell or acellular vaccines. High levels of IFN-γ and IL-2 but no IL-4 or IL-5 were produced from the splenocytes of convalescent mice. The low or undetectable antibody response that accompanied these cytokines indicated a purely CD4+ Th1 response. Conversely, after two doses of an acellular vaccine a CD4+ Th2 response characterised by elevated IL-5 secretion from splenocytes and high titers of anti-*B. pertussis* IgG was observed. Immunisation of mice with the WCV induced a dichotomous response with evidence of both IFN-γ/IL-2 secretion and serum antibody and importantly the clearance of bacteria following an aerosol challenge was found to be enhanced by the Th1 type response.

Mills et al. (1993) demonstrated that non-immune wild-type mice developed a prolonged infection that cleared after roughly 35 days, whilst athymic or T-cell deficient mice failed to clear bacteria from the lungs, following aerosol challenge. Transfer of primed T cells to either athymic or irradiated naïve mice restored the capacity for clearance; however transfer of serum from convalescent mice could not restore the ability to completely eradicate pathogens from the respiratory tract. Thus, although there was evidence that CD4+ Th2 and B lymphocytes contributed to bacterial clearance, CD4+ Th1 lymphocytes were identified as the predominant cell type involved in a protective response to respiratory infection. Barbic et al. (1997) later observed that nude mice (T cell-depleted) or SCID mice (lymphocyte-depleted) mice failed to survive an aerosol challenge dose that was controlled after 8 weeks by naïve mice. Also, unlike antibody responses which have been shown to rapidly decline after the cessation of boosting, CMI responses can be maintained for several years in immunised children (Ausiello et al. 1998).

Upon confirmation that a CD4+ Th1 response was an important protective force against natural infection, the role of interferon-γ, a central Th1 cytokine, was evaluated. Barbic et al. (1997) showed that neutralisation of IFN-γ by regular administration of anti-IFN-γ monoclonal antibodies hindered clearance, whereas IL-4 inhibition had no deleterious effect with respect to clearance. Likewise, IFN-γ gene knockout mice (GKO strain) also exhibited a similarly impeded response to the control of an aerosol challenge compared to the wild type. In addition, Mahon et al. (1997) observed an uncharacteristic disseminated infection in IFN-γR$^{-/-}$ mice that was accompanied with a high mortality rate, in contrast to wild-type mice that contained the infection to the respiratory tract with no deaths.

1.4.5 Mucosal Immunity against *B. pertussis*

The high degree of protection afforded by strong systemic responses from parenteral delivered whole-cell or acellular vaccines seems to have resulted in limited research into the role or importance of specific mucosal effectors. This is somewhat surprising considering that *B. pertussis* is a strictly mucosal pathogen (no systemic dissemination) and many of its virulence factors act locally (Mahon et al. 1997).

An influx of CD4+ T cells, macrophages, neutrophils and to lesser extent CD8+ T cells has been demonstrated following *B. pertussis* infection of naive mice (Khelef et al. 1994; McGuirk et al. 1998). This infiltration of neutrophils is important with respect to the action of *B. pertussis*–specific IgA. Hellwig et al. (2001b) demonstrated that the phagocytosis of *B. pertussis* by human polymorphonuclear leukocytes (PMNL) was enhanced through coating with IgA. This study also used FcαR1-transgenic mice to confirm that the opsonisation was mediated by the FcαR1 receptor of the PMNL. Cross-linking of this receptor has previously been shown to induce phagocytosis, degranulation and the release of pro-inflammatory cytokines by PMNL (Van Spriel & van de Winkel 2001).

Consistent with the lack of knowledge regarding the induction of a specific CMI response at mucosal effector sites (section 1.5.1.4.3); there has been limited research into the local cellular response to *B. pertussis*. Nevertheless, numerous studies have proven that a systemic Th1 response is capable of clearing an experimental challenge of *B. pertussis* in mice (Mills et al. 1993; Redhead et al. 1993). This indicates a direct

correlation between the detection of a systemic CMI response against *B. pertussis* and as yet uncharacterised potential of specific cellular effectors at the mucosal surfaces.

1.4.6 Mechanisms of Immune System Subversion

It is clearly advantageous and necessary for pathogens to have developed measures or mechanisms that either suppress or permit evasion of the immune system in order to prolong infection and transmission. Consistent with this theme, *B. pertussis* has evolved a vast array of potent virulence factors that provide a degree of immune subversion.

1.4.6.1 *Invasion and Survival within Host Cells*

The first evidence that *B. pertussis* was capable of intracellular survival within mammalian host cells was provided by Crawford & Fishel (1959), who reported that organisms were successfully recovered from mammalian tissue culture following antibiotic treatment. Cheers & Gray (1969) also described this invasive phenomenon *in vivo* after it was apparent that *B. pertussis* survived within alveolar macrophages during an active immune response. In a later investigation of the intracerebral challenge model, Hopewell et al. (1972) witnessed seemingly viable bacteria within the cytoplasm of ependymal cells following transmission electron microscopy. Despite these preliminary observations, whooping cough was widely regarded as a toxin-mediated disease and *B. pertussis* as having a purely extracellular existence. It was not until 1989 and onwards that the invasiveness of *B. pertussis* was re-examined in further detail. Ewanowich et al. (1989b) found that *B. parapertussis* invaded and survived in both epithelial-like HeLa 229 cells and primary cultures of human respiratory epithelium. Similarly, *B. pertussis* was also shown to invade and survive within HeLa cells (Ewanowich et al. 1989a). Later, it was observed that *B. pertussis* and *B. bronchiseptica* were capable of survival within professional APCs (Friedman et al. 1992; Guzman et al. 1994).

Friedman et al. (1992) also showed that both spontaneous and Tn*5*-induced avirulent strains were significantly less invasive and did not survive within these cells. This observation underscores the view that a Bvg^- phase or the down-regulation of virulence factor expression was required for intracellular persistence (Gueirard et al. 1998; Kinnear, Marques & Carbonetti 2001). The invasiveness of numerous *B. pertussis*

deletion mutants has since revealed the probable involvement of FHA, PT, Pertactin and AC-Hly. Strains devoid of FHA, PT and two other unidentified virulence-associated factors had significantly reduced invasion compared to a wild-type (Ewanowich et al. 1989a). These mutants with disruption of individual *vag*'s were clearly less inhibited than a *bvg*$^-$ strain and suggested that multiple virulence factors act co-operatively to mediate the invasion process. Interestingly, the invasion of AC-Hly$^-$ mutants was significantly enhanced compared to the parental strain. Considering that the action of AC-Hly impedes the phagocytosis of *B. pertussis* by neutrophils (Weingart & Weiss 2000), the toxin may have a similar inhibitory effect on endocytosis by epithelia and APC. The binding of FHA and pertactin to host cells via interaction between the RGD sequence and integrins such as CR3 on macrophages (Ishibashi, Claus & Relman 1994) and α5β1 on respiratory epithelia (Ishibashi, Relman & Nishikawa 2001) may provide the a trigger for endocytosis. Similarly, van't Wout et al. (1992) found carbohydrate recognition domains within the S2 and S3 subunits of PT that have an affinity for the CR3 integrin of human macrophages.

There are important implications from the discovery of an intracellular niche. An intracellular phase may explain the persistent infection commonly associated with pertussis and the inability to detect a carrier state using conventional bacteriologic surveys (Krantz et al. 1986). Apart from the initial report of Crawford & Fishel (1959) there has been no further evidence of intracellular replication. This suggests that *B. pertussis* is not adapted for an intracellular existence but it is more like a transient phase to avoid immune detection and serum factors.

Host cell invasion could be viewed as a "double-edged sword" with evidence that the generation of opsonising antibodies against the adhesins necessary for internalisation, such as FHA, pertactin and FIM also contribute to enhanced phagocytosis and killing by neutrophils (Weingart & Weiss 2000). Although the bacteria are capable of survival within macrophages and epithelial cells, neutrophils remain effective killers of phagocytosed *B. pertussis*, with only about 1% survival (Lenz, Weingart & Weiss 2000). Therefore, there appears to be a balancing act between the induction of endocytosis in epithelia and APC by *B. pertussis* adhesins and the inhibition of neutrophil phagocytosis by AC-Hly.

1.4.6.2 *Serum Resistance*

Several studies have provided evidence that *B. pertussis* may mediate resistance to the classical pathway of killing through the products of the Bvg-regulated *brk* locus (see Section 1.2.2.3). Although the precise mechanisms surrounding the resistance to antibody-dependent complement remain unknown, BrkA has been shown to be a contributing factor.

Fernandez & Weiss (1998) assessed the resistance of numerous *B. pertussis* mutants and clinical isolates following a 1-hour exposure to 20% human immune serum. Under *in vitro* conditions independent BvgS and BrkA-depleted mutants displayed a significantly increased sensitivity compared with a wild-type strain, whilst a mutant with a duplicate copy of *brk* had a 2 to 5-fold increase in resistance compared with the wild-type strain. In contrast, mutant strains deficient of other Bvg-regulated virulence factors, namely PTX, FHA and DNT had no significant alteration in serum sensitivity (Fernandez & Weiss 1998). In the same study, clinical isolates of *B. pertussis* (27 isolated from 1989 to 1993) and a *B. holmesii* strain were found to be equally resistant as the wild type. However, there was significant variation observed for an alternative wild-type strain and its derivatives. It was concluded that although the precise mechanisms of serum resistance are not understood, BrkA mediates resistance to the classical or antibody-dependent pathway of killing and that this process was Bvg-dependent (Fernandez & Weiss 1998).

Recently Pishko et al. (2003) demonstrated that *Bordetella* spp also acquired resistance to the alternative pathway of complement activation by a process that was independent of Bvg and BrkA. A derivative of the wild type Tohama I recovered from infected mice was observed to be almost completely resistant to a saturated environment (90%) of naïve rabbit, rat or mouse serum that lacked specific antibodies. In stark contrast, broth cultured bacteria were completely susceptible to the naïve serum. In a second experiment, broth-cultured *B. pertussis* was killed but *in vivo*-recovered *B. pertussis* were viable following treatment with serum supplemented with EGTA-$MgCl_2$ a chelating agent that inhibits the classical but not the alternative pathway. The O antigen of LPS has been implicated as the contributor for *B. parapertussis* and *B. bronchiseptica* resistance in the absence of antibody (Burns et al. 2003). However as *B.*

pertussis LPS lacks the O antigen, resistance must be provided by an alternative but as yet unknown means.

1.4.6.3 *Manipulation of Immune Cells*

Of relevance to this project has been the recent evidence that the adhesins FHA and pertactin stimulate the proliferation of a different CD4+ subtype, known as Tr1 or Th3 cells during infection (McGuirk, McCann & Mills 2002). Upon stimulation, the regulatory Tr1 cells secrete interleukin-10 (IL-10) and tumour growth factor-β (TGF-β) that suppress both Th1 and Th2 responses respectively (Groux et al. 1997). The study by McGuirk and colleagues also revealed that FHA inhibited IL-12 and promoted IL-10 production in dendritic cells. In addition to the suppression of Th2 cell proliferation that this cytokine profile directed the expansion of the Tr1 subtype from naïve T cell precursors. The reported inhibition of Th1 by FHA and possibly pertactin are a concern for the development of DNA vaccines that encompass these antigens, based on the drive of this method towards the priming of a cell-mediated response. In this project the entire recombinant pertactin antigen will be expressed but only truncated forms of FHA with the dominant epitopes will be used (see section 4.1). Considering the epitopes that stimulate the Tr1 subset have not yet been identified (McGuirk, McCann & Mills 2002), it is possible that they reside within the N-terminal portion of FHA omitted from the DNA vaccines used in this study (section 4.1).

1.4.6.4 *Antigenic Variation*

The re-emergence of whooping cough in developed countries with highly vaccinated populations led to several investigations of antigenic variation in two key virulence factors of *B. pertussis*. Pertactin and PT were the focus of sequencing comparisons between vaccine strains, currently circulating strains and clinical isolates collected since the commencement of immunisation, on the basis that serum antibodies to these two antigens have been correlated with protection in infants (Cherry et al. 1998; Storsaeter et al. 1998). Within Dutch strains Mooi et al. (1998) identified variations at three amino acid positions that spanned two regions of the S1 subunit of PT, and importantly these amino acid substitutions occurred within previously identified T and B cell epitopes. It was also discovered that pertactin had one polymorphic region. These polymorphisms consisted of variations in the frequency of GGAVP and GGFGP repeats immediately downstream from the RGD site, which has been shown to be an important mediator in

attachment to mammalian cells (Mooi et al. 1998). In follow-up studies, Mooi et al. (1999) and Fry et al. (2001) found similar polymorphisms in *B. pertussis* isolates from Finland and the United Kingdom, respectively.

These identified polymorphisms have occurred over decades with an increasing divergence away from vaccine strains and it has been suggested that the cause of this antigenic shift has been selection pressure from efficacious whole-cell vaccination (Mooi et al. 1998). Antigenic variation needs to be an obvious consideration in the development of future pertussis vaccination strategies including DNA vaccination; however, it is beyond the scope of this study to assess the impact of these polymorphisms on DNA vaccine efficacy.

1.4.7 Summary of Immunity to *B. pertussis*

B. pertussis is a complex and highly adapted respiratory pathogen. They produce an array of toxins, outer membrane proteins and other virulence determinants which contribute immune protection and immune system subversion in addition to pathogenesis (Mills 2001). Of these virulence factors, FHA, PT, Pertactin and AC-Hly are responsible for the majority of these antagonistic effects, and hence effective priming of the immune system against these four antigens is a important for protection.

The mechanisms of acquired immunity against *B. pertussis* infection following natural infection or vaccination are not yet fully understood (Carter et al. 2004). It is well recognised that in general whole-cell vaccines elicit a dichotomous Th1/Th2 response that resembles the natural immune response, whereas acellular vaccines typically induce a strong Th2 humoral response with little or no CMI (Mills et al. 1998). To date, much of the focus has been centred on the role of antibodies. There is little doubt that antibodies are required for protection, albiet most likely in the early phase of infection. As with the majority of other infectious diseases antibodies have a multidimensional role in immunity to *B. pertussis*, from the prevention of colonisation to clearance of the pathogen via opsonisation and complement fixation, neutralisation of toxins and antibody-directed cytotoxicity (van den Berg et al. 2001). However, the lack of a definitive serological correlate(s) of protection and the discovery that *B. pertussis* can also survive and persist within macrophages suggests that cell-mediated immunity is central to immunity, but this role is likely to exend beyond T-helper mediated

responses. For instance, production of nitric oxide by activated macrophages has been reported to contribute to the elimination of intracellular bacteria (Mills et al 2001). It has been shown that nitric oxide synthase knockout mice are more susceptible to *B. pertussis* challenge than wild-type mice (Carter et al. 2004). Furthermore, a longer duration of immunity is often associated with CMI rather than humoral responses (Mills et al. 1993).

The somewhat contradictory evidence surrounding effective acquired immunity to *B. pertussis* also poses problems with the design of a novel and improved vaccine formulation. Recent studies by Mills et al. (1998) and Leef et al. (2000) have provided a comprehensive assessment of the mechanisms of immunity to *B. pertussis*. Intranasal immunisation of both wild-type and an array of transgenic mice such as TCR KO ($Tcr\beta^-$ $Tcr\delta^-$), $CD4^-$ KO, CD8 KO (β2-$microglobulion^-$), and B cell KO ($Igh6^-$) with formalin-fixed *B. pertussis* demonstrated that cellular responses, from both CD4+ T cells and B cells, were the main contributor to protection from aerosol infection (Leef et al. 2000). Interestingly, although the authors agreed that an antibody response was advantageous, they argued that the beneficial B cell involvement was not related to antibody production but rather antigen presentation, cytokine production and interaction with NK cells and macrophages. Mills et al. (1998) also assessed the immune responses and bacterial clearance in convalescent or immunised wild-type and knockout mice following aerosol infection. The results confirmed the important role of IFN-γ in recovery from natural infection and in response to Pw immunisation. However, in contrast to Leef et al. (2000) aerosol challenge of Pa and Pw-immunised mice with disruption of the μ immunoglobulin heavy-chain gene ($Ig^{-/-}$) identified an absolute need for antibody in successful clearance. Finally, in an all-inclusive review of immunity to *B. pertussis*, Mills (2001) argued in favour of complementary roles for humoral and cell-mediated components.

1.5 DNA Vaccines

1.5.1 Background of DNA Vaccine Technology

DNA vaccines consist of a plasmid DNA backbone encompassing a strong eukaryotic or viral promoter and polyadenylation signal that facilitates expression from a recombinant antigen-encoding gene within mammalian host cells (Mor & Elisa 1999). Although the precise mechanisms are not known, DNA vaccines are believed to mimic

the processing of viral antigens, in that somatic host cells are transfected with plasmid DNA, after which a foreign antigen can be endogenously expressed, processed, and then presented by the MHC class I pathway (Lowrie 1998). Endogenously expressed recombinant protein may also be secreted into the extracellular compartment and taken up by circulating professional APCs for activation of CD4+ cells via presentation with MHC class II molecules, in a process referred to as cross-priming (Sharma & Khuller 2001). Alternatively, APCs can be the primary targets for DNA transduction and direct priming of CD4+ and CD8+ T cells (Gurunathan, Klinman & Seder 2000). Hence the *in vivo* expression of an antigen is a novel method for the generation of both humoral and cell-mediated immune responses. Examples of humoral responses to DNA-encoded antigens include: HIV envelope protein (Wang et al. 1993), Hepatitis B surface antigen (Davis, Michel & Whalen 1993), *Leishmania major* gp63 (Xu & Liew 1995), *Mycobacterium tuberculosis* Ag85 (Huygen et al. 1996) and recently the S1 subunit of *B. pertussis* (Kamachi, Konda & Arakawa 2003). Similarly, examples of cytotoxic and helper T cell responses have included HIV envelope protein (Shiver et al. 1995), Hepatitis B surface antigen (Schirmbeck et al. 1995) and Herpes Simplex Virus (HSV) (Manickan et al. 1995).

The most important feature of any vaccine is the ability to confer a protective immune response to the recipient. Ulmer et al. (1993) were first to demonstrate that protective immunity could be conferred following the injection of plasmid DNA. The generation of both antigen-specific antibodies and Tc against the nucleoprotein of influenza A cross-protected mice against challenge from a circulating strain that was antigenically divergent from the vaccine strain. Since this initial finding the field of DNA-based vaccinology has rapidly expanded and DNA vaccines have now been proven effective against a number of parasitic, viral and bacterial infections in pre-clinical models (Vanderzanden et al. 1998; Wang et al. 1998; Delogu et al. 2000).

The main advantage of DNA vaccines is the ease and low cost of production. With a high copy origin of replication, large yields of plasmid DNA can be purified from bacteria (typically *E. coli*) and suspended in a saline solution ready for delivery. Importantly, DNA vaccine vectors do not have a eukaryotic origin of replication which prevents replication within the host cell and prevents integration into chromosomal DNA (Donnelly et al. 1997). Some other potential advantages of DNA vaccines over

conventional approaches include: no risk of reversion to a pathogenic state; potential priming of both humoral and cell-mediated immune responses; direct transfection of professional APC and somatic cells; improved stability and long-lasting immunity through the induction of memory T cells (Whalen 1996). The use of inducible promoters, immunoregulatory genes, or gene fusions that enhance or modulate the host's immune response against the target antigen also demonstrates the versatility and limitless potential of DNA vaccines.

1.5.2 Proof of Principle

Following construction of a DNA vaccine, an important issue that must be addressed is the method by which the plasmid construct will be delivered to the cells of the recipient. To date there are three modes of DNA vaccine delivery: direct intramuscular (IM) injection of purified plasmid DNA, biolistic particle bombardment (gene gun) and to mucosal inductive sites via carrier molecules or attenuated microbes (Tuting et al. 1999).

1.5.2.1 *IM DNA Vaccination*

With the discovery that myocytes were up to 1000 times more permissive than other tissues with respect to transfection of naked DNA, direct injection into the quadriceps or tibialis anterior muscle has been the most commonly used method of DNA immunisation (Fynan et al. 1993).

Nyika et al. (1998) found survival rates of up to 88% against a lethal challenge of the intracellular rickettsia *Cowdria ruminatium* after two IM doses of a DNA vaccine compared to nil survival in sham-vaccinated mice. This two dose regime of a DNA vaccine that encoded the major antigenic protein 1 (MAP1) generated a clear Th1 response characterised by elevated IFN-γ and IL-2 with only background levels of IL-4, IL-5, IL-6 and IL-10. Similarly, Velikovsky et al. (2002) demonstrated that a Th1 response induced by IM vaccination against the lumazine synthase antigen of *Brucella abortus* significantly reduced bacterial burden after challenge, but a humoral response generated by a recombinant subunit preparation could not. Of particular relevance was the success obtained with the use of the pcDNA3 eukaryotic expression vector, the same plasmid DNA backbone used for this work.

Whilst highly efficient in the uptake of DNA, a limitation with this site is that unlike the skin and mucosal surfaces which require constant immune surveillance muscle tissue has a lower distribution of professional APCs. This predominance for MHC class I-restricted presentation of antigens may explain the strong bias towards Th1 type responses that are frequently observed with IM DNA vaccination and also the relatively large dosage required.

1.5.2.2 *Particle Bombardment or Gene Gun Administration*

Gene-gun immunisation involves firing DNA-coated gold particles into the epidermis. Fynan et al. (1993) tested the efficacy of an influenza virus hemagglutinin DNA vaccine in mice across all routes of administration. They found that although parenteral routes such as IM and IV as well as intranasal immunisation provided excellent protection, gene-gun delivered DNA was by far the most efficient. As little as 0.4μg of DNA provided an equivalent 95% level of protection to 300μg of naked DNA administered by IM injection. The other important distinction between the gene gun and direct injection approaches is the polarisation of immune responses generated by each mode. Unlike the largely Th1 type response elicited by IM injection, gene gun responses are dominated by a Th2 response resulting in the induction of humoral rather than cell mediated effectors (Feltquate et al. 1997). For example, a Th2 response characterised by a high IgG1:IgG2a ratio completely protected mice from a LD_{50} challenge dose of anthrax toxin after gene gun immunisation with plasmids that encoded the protective antigen (PA) or lethal factor (LF) fragments of the toxin (Price et al. 2001). Scheiblhofer et al. (2003) also demonstrated the induction of a specific antibody response dominated by IgG1 following gene-gun immunisation against *Borrelia burgdorferi*, the aetiological agent of Lyme disease. This Th2 response protected 90% of mice from an intraperitoneal (IP) challenge of 10^4 spirochetes.

1.5.2.3 *Mucosal Delivery*

Oral delivery of DNA vaccines provides an added potential for priming of the common mucosal immune system, and the potential for stimulating mucosal antibody production at both local and distant effector sites in addition to a systemic response (Darji et al. 1997). This is important in so far as little or no secretory antibodies have been observed after IM or gene-gun immunisation (Donnelly et al. 1997).

Attenuated enteric bacteria such as *S. typhimurium* have been used as carriers for the targeted delivery of DNA vaccines to the inductive sites of the digestive mucosa (Sizemore, Branstrom & Sadoff 1995). Through a typical pattern of infection, attenuated *S. typhimurium* harbouring the DNA vaccine penetrate the epithelial barrier of the PP through M cells where they are phagocytosed by dendritic cells and macrophages that subsequently migrate to mesenteric LN and into circulation (Sirard, Niedergang & Kraehenbuhl 1999). Unlike virulent strains that can survive phagocytosis, the attenuated carriers are rapidly eliminated and processed for antigen presentation. It is presumed that at this point the plasmids are liberated leading to the endogenous expression of the encoded antigen by an as yet unknown mechanism.

Highlighting the promise of salmonella-mediated delivery, Flo et al. (2001) immunised mice with three oral doses of an *aroA S. typhimurium* DNA vaccine encoding the glycoprotein D gene of HSV and detected a strong mucosal and systemic cellular response that fully protected against an intravaginal challenge. In fact no disease symptoms were observed and there was less viral shedding compared to IM DNA vaccination. More recently, Karpenko et al. (2004) demonstrated that rectal immunisation with attenuated *S. typhimurium* harbouring a HIV polyepitope DNA vaccine induced a potent CTL and antibody response that was superior to IM injection of either naked DNA or plasmid encapsulated in a VLP.

Some alternative approaches to mucosal delivery of DNA that have provided similarly encouraging results include: (a) other attenuated bacteria such as *Shigella* and *Listeria monocytogenes* (Shata and Hone 2001; Vecino et al. 2002), (b) attenuated viruses and virus-like proteins (Wang et al. 2003), and (c) encapsulation with cationic lipids, liposomes or biodegradable beads (Hobson et al. 2003). As *B. pertussis* infections are initiated and limited to the respiratory mucosa, the priming of a specific mucosal immune response would be beneficial in limiting colonisation of *B. pertussis* at the site of entry.

1.5.3 Adjuvants for DNA Vaccination

With the establishment of DNA vaccination as a robust means of immunisation, much of the focus has now shifted to enhancing their immunogenicity, particularly for larger animals and humans, through the use of adjuvants. Trials with conventional adjuvants such as Freund's or aluminium salts have been limited in most part due to the concomitant advances in molecular and recombinant techniques that have seen the advent of genetic adjuvants. However Periwal et al. (2003) demonstrated that a modified cholera toxin, known to be a potent mucosal adjuvant, was successful in boosting the systemic and mucosal immune response to a Norwalk virus VLP. Genetic adjuvants are expression vectors of cytokines or other molecules that modulate the immune response when co-administered with the vaccine antigen (Sasaki et al. 2003). Predictably, the application of Th2 cytokines improved IgG1 responses and down-regulated cellular effectors, whereas the use of Th1 cytokines augments CMI and at the same time was antagonistic to Th2 responses such as decreasing the IgG1/IgG2a ratio (Boyaka & McGhee 2001a; Scheerlinck 2001). For instance, when an IL-12-encoding plasmid was co-administered with a hepatitis B DNA vaccine, mice showed an elevated Tc and IgG2a response which corresponded to improved protective efficacy (Chow et al. 1998).

Finally, the bacterial-derived DNA vaccine vector itself has been shown to possess adjuvant properties. Among several other differences, bacterial DNA has been shown to have a 16-20 fold higher frequency of cytosine-phosphate-guanosine (CpG) dinucleotides compared to mammalian DNA (Sato et al. 1996; McCluskie et al. 2001). Furthermore, in eukaryotes these dinucleotides are normally methylated as opposed to being unmethylated in prokaryotes. These bacterial CpG motifs have been demonstrated to activate macrophages, dendritic cells, NK cells to secrete pro-inflammatory cytokines such as TNF-α, interferons, IL-12 and IL-18 that stimulate the differentiation of naïve T cells into Th1 cells (Hemmi et al. 2000; de Jong et al. 2002). This further imparts the capacity for strong Th1 responses to DNA vaccination.

1.6 Experimental Vaccines

Apart from DNA vaccination, there are other novel pertussis vaccines at various stages of development, such as live attenuated mutants and pertussis antigens encapsulated in biodegradable particles, which represent potential alternatives to Pw and Pa vaccines.

In parallel with the development of DNA vaccines against pertussis, our laboratory is currently in the process of evaluating an *aro*Q mutant of *B. pertussis* as a live attenuated vaccine, delivered via the intranasal route. Preliminary experiments have shown that the metabolite-deficient strain was safe as a vaccine in mice and survived long enough to induce a significant IgA and IgG response in both the serum and respiratory secretions (Cornford-Nairn et al. 2005; Mukkur et al. 2005). The mutant also induced a systemic cell-mediated response as indicated by the production of IFN-γ and IL-12. Mice immunised with the aroQ mutant were protected against challenge with a sub-lethal infectious dose, clearing the pathogen within 6 days post-challenge. Similarly, Roberts et al. (1990) developed an auxotrophic *B. pertussis* mutant by insertion of a kanamycin resistance cassette into the *aro*A gene. Mice immunised with the *aro*A mutant via inhalation of aerosols induced an IgM, IgA and IgG response that conferred protection against a subsequent aerosol challenge with the virulent parental strain. A pertussis toxin-deficient mutant expressing the glutathione S-transferase of *Schistosoma mansoni* as an FHA fusion protein was found to be effective as both a live attenuated vaccine and as well as a delivery vehicle for heterologous antigens (Mielcarek et al. 1998). When given as a single intranasal dose, the mutant protected mice from challenge with virulent *B. pertussis* and also the *Schistosoma mansoni* parasite (Mielcarek et al. 1998).

The main advantage of live attenuated vaccines is that they offer a non-invasive means of vaccinating infants and children, which closely resembles the natural infectious process and thereby could provide long-lasting immunity (Locht et al. 2004). However, the current limitation with this mode of vaccination surrounds its safety, with concerns that include the risk of reversion to a pathogenic form, the presence of toxins including LPS, pertussis toxin and adenylate cyclase-hemolysin, as well as the presence of antibiotic resistance markers used for gene inactivation.

An alternative to live attenuated mutants for mucosal immunisation is the use of inert carriers such as liposomes and biodegradable particles for oral or IN delivery of antigens (Guzman et al. 1993; Conway et al. 2001). Both oral and parenteral delivery of purified pertussis toxoid and filamentous hemagglutinin encapsulated in poly-lactide-co-glycolide polymers was demonstrated to protect mice from an aerosol-induced *B. pertussis* infection (Conway et al. 2001). However, several factors were found have a critical effect on the immunogenicity of the biodegradable formulations, specifically the size of the particles used and the route of delivery. Immunisation with larger microparticles generated a largely Th1 response. On the other hand, immunisation with the smaller nanoparticles elicited a largely Th2 response irrespective of whether the oral or parenteral routes were employed. Interestingly, a protective response was observed after a single parenteral dose of the pertussis microparticles, whereas three oral doses of the pertussis nanoparticles were required to confer the same degree of protection (Conway et al. 2001).

1.7 Objective of this Research Project

The continued prevalence of whooping cough coupled with an increasing incidence of serious post-immunisation side reactions, particularly with booster vaccination, indicates that conventional vaccines are not completely satisfactory and necessitates the development of alternative vaccine(s) that are safe and are capable of providing long-term protection. DNA vaccination is a novel and powerful method of immunisation that has yet to be evaluated as an alternative to Pw and Pa vaccines; however its protective potential against other infectious agents combined with the many potential advantages suggested that this approach was worth investigating. After extensive studies of *B. pertussis* and its many virulence factors, filamentous hemagglutinin, pertactin, pertussis toxin and adenylate cyclase-hemolysin have been identified as the four major protective antigens using animal models and in human studies. Therefore, the objective of this study was to evaluate each of the above-mentioned key protective antigens as DNA vaccine candidates using the mouse respiratory model as an immunisation-challenge system. Since the current evidence indicates a role for both humoral and cell-mediated immunity in protection against whooping cough, the second objective was to develop a regimen that elicited both Th1 and Th2 responses against multiple *B. pertussis* antigens.

Chapter 2 Methods

A list of the materials used in this study including chemicals, buffers, media and the composition of reagents is provided as Appendix A.

2.1 Bacterial Strains and Culture Conditions

Bacterial strains used in this study are listed in Table 2.1

2.1.1 *Bordetella pertussis*

Bordetella pertussis strains Tohama I and BP338 were kindly donated by Professor Allison Weiss (University of Cincinnati, Ohio). Tohama I is a virulent (vir^+) or Phase I strain that was recovered from a patient during the 1950's epidemic in Japan (Kasuga et al. 1954). BP338 is a virulent, nalidixic acid-resistant derivative of Tohama I (Weiss & Goodwin 1989).

2.1.2 *Escherichia coli*

2.1.2.1 *TOP10*

The initial host of the plasmid constructs was *E. coli* strain TOP10. TOP10 are provided as a component of both the pTrcHis2-TOPO and pcDNA3.1/V5-His-TOPO cloning kits (Invitrogen), and has been designed to provide optimal transformation efficiency following a cloning procedure.

2.1.2.2 *XL10-Gold*

XL10-Gold is an *E. coli* strain that was provided as a component of the Quick-Change™ XL Site-Directed Mutagenesis Kit (Stratagene). XL10-Gold was derived from the *E. coli* K12 strain XL1-Blue (Stratagene) and has been engineered to efficiently repair nicked plasmid DNA.

2.1.2.3 *BL21 Codon Plus-RP*

BL21 CodonPlus (DE3)-RP *E. coli* (Stratagene) were used for optimum expression of *B. pertussis* antigens for use in the purification of recombinant antigens. This strain possessed a pACYC-based plasmid that encodes extra copies of the *argU* and *proL* tRNA genes in addition to conferring chloramphenicol resistance.

Table 2.1: Bacterial strains used in this study.

Strain	Genotype/Phenotype	Source or Reference
Escherichia coli		
TOP10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
XL10-Gold	Tet^{r}Δ (*mcr*A) 183 Δ (*mcrCB-hsd*SMR-*mrr*) 173 *end*A1 *sup*E44 *thi*-1 *recA1 gyrA96 relA1 lac* Hte	Stratagene
BL21-CodonPlusRP	B F$^-$ *ompT hsdS*(r_B^- m_B^-) *dcm*+ Tet^r *gal* λ (DE3) *endA* Hte [*argU proL* Cam^r]	Stratagene
Salmonella typhimurium		
P9121	F$^-$ lam$^-$ *leu hsd*L(r^-m^+) *trp*D2 *rps*L120 *ilv*452 *met*E551 *met*A22 *hsd*A(r^- m^+) *hsd*B(r^-m^+)	Ref: Bullas & Ryu (1983) Reeves, P.R. [University of Sydney]
SL3261	Attenuated *aro*A derivative of virulent strain SL3201	Ref: Hoiseth & Stocker (1981) Stocker, B.A.D. [Stanford University]
Bordetella pertussis		
Tohama I	Virulent (Phase I) Wild-type strain.	Weiss, A [University of Cincinnati]
BP338	Nalidixic acid resistant derivative of Tohama I	Weiss, A [University of Cincinnati]

2.1.3 *Salmonella typhimurium*

2.1.3.1 *P9121*

Salmonella typhimurium LT2 strain P9121 (also known as LB5000) is a restriction minus/modification positive (r^-m^+) mutant that was used as an intermediate host strain for DNA vaccine plasmids (Bullas & Ryu 1983). Each of the *E. coli*-based plasmid constructs were passaged through the r^-m^+ P9121 prior to transformation into the (r^+m^+) *S. typhimurium* vaccine strain SL3261. P9121 was kindly donated by Professor Peter Reeves, Department of Microbiology, University of Sydney.

2.1.3.2 *SL3261*

S. typhimurium strain SL3261, an *aro*A mutant (Hoiseth & Stocker 1981) was used as the live vaccine strain for oral delivery of the DNA vaccine constructs. SL3261 was provided by Professor Bruce Stocker, Stanford University.

2.1.4 Routine Culture and Preservation of Bacterial Strains

B. pertussis was cultured on Bordet-Gengou (BG) agar, an enriched blood-based media. Modified Stainer-Scholte (MSS) broth or Modified Verwey (MV) broth were used for cultivation of B. pertussis for the preparation of challenge suspensions and the extraction of genomic DNA respectively. MSS was a modification of the commonly used and chemically-defined Stainer-Scholte broth, in which the base media was supplemented with heptakis(2,6-*O*-Dimethyl)β-Cyclodextrin (Stainer & Scholte 1970; Imaizumi et al. 1983). Similarly, MV was Verwey broth with the addition of heptakis(2,6-*O*-Dimethyl)β-Cyclodextrin (Verwey et al. 1949, D. Farrell unpublished). BG plates inoculated with *B. pertussis* stocks or lung homogenates were incubated at 37°C in a humidified atmosphere for 48 to 72 hours, whereas broth cultures were incubated at 37°C and 150rpm for 24 to 48 hours.

Luria-Bertani (LB) broth and LB agar were the media of choice for culture of *E. coli* and *S. typhimurium*. Media was supplemented with ampicillin (100μg/ml) and chloramphenicol (170μg/ml) when selection pressure was required for the maintenance of strains transformed plasmid DNA. During the induction of recombinant protein expression, glucose was included at a concentration of 0.5% (v/v) in the primary cultures and agar plates to inhibit constitutive (leaky) expression of recombinant proteins.

During attempts to optimise certain experiments, LB broth was substituted with terrific broth (TB), an enriched media that promotes increased growth rates and cell numbers (Sambrook, Fritsch & Maniatis 1989). *S. typhimurium* and *E. coli* were routinely grown for 14 hours at 37°C and with shaking at 200rpm for broth culture.

All bacterial strains were stored at -70°C for long-term preservation using glycerol stocks and cryogenic beads. For the preparation of glycerol stocks, glycerol was added to mid-log phase broth culture at a final concentration of 20% (v/v), with 1ml aliquots stored in cryogenic vials. For preservation using the Microbank bead system (Pro-Lab Diagnostics), three colonies were individually emulsified in the cryo-preservative fluid. Vials were inverted to mix and the fluid removed prior to storage.

2.2 Mammalian Cell Lines

The CHO-K1 cell line was obtained through the courtesy of Professor Peter Timms (Queensland University of Technology) and is a subclone of the Chinese Hamster Ovary (CHO) parental line (Puck, Cieciura & Robinson 1958). CHO-K1 was used for the pertussis toxin and adenylate cyclase toxicity assays (Section 2.13). COS-7 was donated by Dr Graeme Walker (Queensland Institute of Medical Research) and is an African Green Monkey Kidney cell derived from the CV-1 line (Gluzman 1981). This cell line was used to confirm the mammalian expression of recombinant antigens following transient transfection with the DNA vaccine constructs.

2.2.1 Mammalian Cell Culture

COS-7 were maintained in DMEM media supplemented with 10% foetal bovine serum (FBS), 25mM HEPES, 100 Units/ml penicillin and 100µg/ml of streptomycin (Invitrogen). The CHO-K1 cells were maintained in Ham's F12 media supplemented with 10% FBS, 25mM HEPES, 100 Units/ml penicillin and 100µg/ml streptomycin (Invitrogen). Frozen ampoules were removed from liquid N_2 storage, thawed in a 37°C water bath and then transferred to 9 ml of pre-warmed growth media in a 15ml centrifuge tube. The suspensions were mixed by inversion and centrifuged for 10 minutes at 150×g. Supernatants were removed and the pellets resuspended in 1ml of pre-warmed media. The 1ml suspensions were added to a 25cm^2 flask that contained 5ml of pre-warmed media and incubated in a humidified environment at 37°C with 5% CO_2.

2.2.1.1 *Sub-Culture of Cell Lines*

All cell lines were routinely sub-cultured or split into 25cm^2 flasks unless otherwise stated. Once cell density reached 90-100% confluence, the existing growth media was removed and adherent cells were dislodged by adding 1ml of Trypsin-EDTA (Invitrogen) with incubation for 3-5 minutes at 37°C. Cells were gently but thoroughly resuspended after addition of 1 ml pre-warmed media. A 20µl aliquot of the cell suspension was removed and counted using a haemocytometer (see Appendix B). Cells were seeded to a density of 5×10^5 in 6ml of pre-warmed media and cultured at 37°C with 5% CO_2 and high humidity.

2.2.1.2 *Preservation of Cell Lines*

Cells to be frozen were cultured in 75 cm^2 flasks until 80% confluent. Adherent cells were dislodged by trypsinisation as per Section 2.2.1.1. Trypsinised cells were removed and added to a centrifuge tube that contained 10ml of pre-warmed media. A 20µl aliquot of the suspension was removed for counting and the remainder centrifuged at 150×g for 10 minutes. The supernatant was removed and the pellet resuspended to a density of 1×10^6 cells/ml in complete media supplemented with 10% DMSO (storage media). One ml aliquots of the cell suspension were transferred to cryovials for storage in liquid N_2.

2.3 Plasmid Vectors

The plasmids used or constructed in this study are shown in Table 2.2. Commercial vectors were selected for the prokaryotic and eukaryotic expression of cloned *B. pertussis* gene sequences. The DNA vaccine vector, pcDNA3.1D/V5-His-TOPO (Invitrogen), was used for expression of recombinant antigens within transfected mammalian cells via a human cytomegalovirus (CMV) promoter and a bovine growth hormone (BGH) polyadenylation signal. Recombinant proteins expressed within transfected cells were chimeric V5/His fusions that enabled detection with a either a V5 or His tag-specific antibody. The respective gene sequences were simultaneously cloned into the pTrcHis2-TOPO vector (Invitrogen) for purification of the recombinant antigens from *E. coli*, for use in downstream applications such as western blotting, ELISA and the re-stimulation of *in vitro* cultured splenocytes. Maps and details of both vectors have been included in Appendix C.

Plasmids pcDNA3.1D/*lacZ* and pTrcHis2/*lacZ* (Invitrogen) encode a β-galactosidase-fusion protein and were used as positive controls for expression experiments in mammalian cell lines and *E. coli* respectively. As introduced in Section 2.1.2.3, pACYC-RP (Stratagene) was a specialised construct that encoded the tRNA genes *argU* and *proL*. This plasmid was stably transformed within the *E. coli* BL21 Codon Plus strain and was used to enhance the expression of the recombinant proteins. Plasmid pUC18 was used as a positive control for all bacterial transformation experiments.

2.4 Isolation of Genomic DNA

Genomic DNA was extracted from *B. pertussis* using the Aqua Pure Genomic DNA Isolation Kit (BioRad). *B. pertussis* strain Tohama I was plated onto BG agar from glycerol stocks to obtain isolated colonies. After 72 hours incubation a single colony was selected and used to seed 10ml of Verwey broth. The broth culture was then incubated until the OD_{625} reached 1.0. A 500µl aliquot of the cell suspension was pelleted by centrifugation at 13,000×g for 2 minutes and the supernatant decanted. Genomic DNA was then extracted according to the manufacturer's instructions. A 5µl sample of the DNA sample was analysed using a 0.8% TAE agarose gel to determine approximate yield.

2.5 Amplification of Target Gene Sequences from Genomic DNA

2.5.1 Oligonucleotides

The oligonucleotides used for amplification in this project are listed in Table 2.3. Oligonucleotides were designed based on the published Genbank (NCBI) sequences of the *fhaB*, *prn*, *ptxS1*, *cyaA* and *cyaC* genes of *B. pertussis*, with the accession numbers M60351.1, AJ006158, M13223, A14850 and M57286 respectively. Each primer pair was analysed and rated using the NetPrimer primer analysis program (http://www.premierbiosoft.com/netprimer/netprimer.html) to access the possible secondary structure formations and dimers that could have interfered with the PCR efficiency. Oligonucleotides were synthesised by Geneworks (Adelaide) or Invitrogen (N.Z.), with the lyophilised stocks reconstituted to 2µg/ml and the working solutions resuspended to 25pmol/µl using RNase/DNase-free water (Invitrogen). Primer stocks and working solutions were stored at -20°C.

Table 2.2: Plasmids used and constructed in this study.

Plasmid	Description	Source
General Plasmids		
pcDNA3.1D	DNA vaccine vector for eukaryotic expression via CMV promoter. Amp^r.	Invitrogen
pcDNA3.1/*lacZ*	pcDNA3.1D/V5-His-TOPO encompassing *lacZ* gene for expression control.	Invitrogen
pTrcHis2	Cloning vector for expression of recombinant proteins in *E.coli* via hybrid *trp*/*lac* promoter. Amp^r.	Invitrogen
pTrcHis2	pTrcHis2-TOPO encompassing *lacZ* gene for expression control	Invitrogen
pUC18	General purpose vector encompassing *lacZ* gene used as transformation control.	Amersham
pACYC-RP	Vector encoding rare tRNAs for enhanced expression of proteins from GC rich templates. $Chloramp^r$.	Stratagene
Constructed Plasmids		
pcDNA3.1D/*fhaB1*	pcDNA3.1D/V5-His-TOPO encompassing a large fragment of the *fhaB* gene from Tohama I.	This study
pcDNA3.1D/*fhaB2*	pcDNA3.1D/V5-His-TOPO encompassing a small fragment of the *fhaB* gene from Tohama I.	This study
pcDNA3.1D/*prn*	pcDNA3.1D/V5-His-TOPO encompassing a fragment of the *prn* gene from Tohama I.	This study
pcDNA3.1D/*pts1*	pcDNA3.1D/V5-His-TOPO encompassing the *S1* gene of the *ptx* operon from Tohama I.	This study
pcDNA3.1D/*pts1.13L*	pcDNA3.1D/V5-His-TOPO encompassing the *ptxS1*gene of Tohama I with two point mutations for conversion of arginine to leucine @ aa 13.	This study
pcDNA3.1D/*pts1.13L.129G*	pcDNA3.1D/V5-His-TOPO encompassing the *ptxS1*gene of Tohama I with four point mutations for conversion of arginine to leucine @ aa 13 and glutamic acid to glycine @ aa 129.	This study
pcDNA3.1D/*cyaA*	pcDNA3.1D/V5-His-TOPO encompassing the *cyaA* gene of Tohama I.	This study
pcDNA3.1D/*cyaAL58*	pcDNA3.1D/V5-His-TOPO encompassing the *cyaA* gene of Tohama I with two point mutations to convert lysine to methionine @ aa 58.	This study
pcDNA3.1D/*cyaC*	pcDNA3.1D/V5-His-TOPO encompassing the *cyaC* gene of Tohama I.	This study

pTrcHis2/*fhaB1*	pTrcHis2-TOPO encompassing a large fragment of the *fhaB* gene from Tohama I.	This study
pTrcHis2/*fhaB2*	pTrcHis2-TOPO encompassing a small fragment of the *fhaB* gene from Tohama I.	This study
pTrcHis2/*prn*	pTrcHis2-TOPO encompassing a fragment of the *prn* gene from Tohama I.	This study
pTrcHis2/*pts1*	pTrcHis2-TOPO encompassing the *S1* gene of the *ptx* operon from Tohama I.	This study
pTrcHis2/*pts1.13L*	pTrcHis2-TOPO encompassing the *ptxS1*gene from Tohama I with two point mutations for conversion of lysine to leucine @ aa 13.	This study
pTrcHis2/*pts1.13L.129G*	pTrcHis2-TOPO encompassing the *ptxS1*gene from Tohama I with four point mutations for conversion of lysine to leucine @ aa 13 and glycine to glutamic acid @ aa 129.	This study
pTrcHis2/*cyaA*	pTrcHis2-TOPO encompassing the *cyaA* gene from Tohama I.	This study
pTrcHis2/*cyaAL58*	pTrcHis2-TOPO encompassing the *cyaA* gene from Tohama I with a single point mutation to convert leucine to methionine @ aa 58.	This Study
pTrcHis2/*cyaC*	pTrcHis2-TOPO encompassing the *cyaC* gene from Tohama I.	This Study
pcDNA3.1	Self-ligated pcDNA3.1/V5-His-TOPO used as a vaccine control.	Dr Austen Chen (USQ)

Table 2.3: Oligonucleotides used in this study.

Note 1: Separate sense PCR amplification primers were required for cloning into the pcDNA3.1D/V5-His-TOPO and pTrcHis-TOPO vectors due to the requirement for a kozak leader sequence (CACCATGG) for the pcDNA3.1D/V5-His-TOPO PCR products (see Appendix B).

Note 2: Underlined bases in the mutagenesis oligonucleotide sequence indicate the point mutations.

Oligonucleotide	Description	Sequence (5' – 3')
PCR Amplification		
FHAB3577DNAF	Sense primer for amplification of *fhaB* gene downstream from base 3577 of Genbank sequence (Accession # M60351.1) - for cloning into pcDNA3.1D/V5-His-TOPO.	CACCATGGACATCATCA TGGATGCGAAG
FHAB5740DNAF	Sense primer for amplification of *fhaB* gene downstream from base 5740 of Genbank sequence, for cloning into pcDNA3.1D/V5-His-TOPO.	CACCATGGGCTATGTCA ACTACTGG
FHAB3259TRCF	Sense primer for amplification of *fhaB* gene downstream from base 3259, for cloning into pTrcHis-TOPO.	GTTAAGAGCGATGGCG GCCTTCAG
FHAB5737TRCF	Sense primer for amplification of *fhaB* gene downstream from base 5737, for cloning into pTrcHis-TOPO.	ATCGGCTATGTCAACTA CTGGTTG
FHAB6966R	Anti-sense primer for amplification of *fhaB* gene upstream of base 6966.	CGTGAAAATATGCGGAT TCACGAGCAC
FHAB6750R	Anti-sense primer for amplification of *fhaB* gene upstream of base 6750.	CAGCACGGTTTGTTCCT TGGGATAGAA
PRN198DNAF	Sense primer for amplification of *prn* gene downstream from base 198 of Genbank sequence (Accession # AJ006158), for cloning into pcDNA3.1D/V5-His-TOPO.	CACCATGGCGCATGCCG ACTGGAACAACCAG
PRN198TRCF	Sense primer for amplification of *prn* gene downstream from base 198, for cloning into pTrcHis-TOPO.	GCGCATGCCGACTGGAA CAACCAGTCCATC
PRN2273R	Anti-sense primer for amplification of *prn* gene upstream of base 2273.	TGTGGCATAGCCCCCGA CATGCACGCTGTC
PTX507DNAF	Sense primer for amplification of *ptxS1* gene downstream from base 507 of Genbank sequence (Accession # M13223), for cloning into pcDNA3.1D/V5-His-TOPO.	CACCATGCGTTGCACTC GGGCAATTCGC
PTX507TRCF	Sense primer for amplification of *ptxS1* gene downstream from base 507, for cloning into pTrcHis-TOPO.	ATGCGTTGCACTCGGGC AATTCGC
PTX1313DNAR	Anti-sense primer for amplification of *ptxS1* gene upstream of base 1313, for cloning into pcDNA3.1D/V5-His-TOPO.	GAACGAATACGCGATG CTTTCGTAGTACAC
PTX1313TRCR	Anti-sense primer for amplification of *ptxS1*gene upstream of base 1313, for cloning into pTrcHis2-TOPO.	GAACGAATACGCGATG CTTTCGTAGTA

CYAA883DNAF	Sense primer for amplification of *cyaA* gene downstream from base 883 of Genbank sequence (Accession # A14850), for cloning into pcDNA3.1D/V5-His-TOPO.	CACCATGGATGTTTGGT TCTTGCAGAAGGATGAG
CYAA883TRCF	Sense primer for amplification of *cyaA* gene downstream from base 883, for cloning into pTrcHis-TOPO.	GATGTTTGGTTCTTGCA GAAGGATGAGGTTCTG
CYAA6096R	Anti-sense primer for amplification of *cyaA* gene upstream of base 6096.	GCGCCAGTTGACAGCCA GGGACTGCAT
CYAC111DNAF	Sense primer for amplification of *cyaC* gene downstream from base 111 of Genbank sequence (Accession # M57286), for cloning into pcDNA3.1D/V5-His-TOPO.	CACCATGGCGCACCCAA CACGG
CYAC135TRCF	Sense primer for amplification of *cyaC* gene downstream from base 135, for cloning into pTrcHis-TOPO.	CCCGCTCCACCATGCAC CGATGTC
CYAC713R	Anti-sense primer for amplification of *cyaC* gene upstream of base 713.	GGCGGTGCCCCGGCCTC G
Sequencing		
SFPCSEQF	Sequencing downstream from 5' ligation site of pcDNA3.1D/V5-His-TOPO.	TAATACGACTCACTATA GGG
SFPCSEQR	Sequencing upstream from 3' ligation site of pcDNA3.1/V5-His-TOPO.	TAGAAGGCACAGTCGA GG
SFTRCSEQF	Sequencing downstream from 5' ligation site of pTrcHis2-TOPO.	GAGGTATATATTAATGT ATCG
SFTRCSEQR	Sequencing upstream from 3' ligation site of pTrcHis2-TOPO.	GATTTAATCTGTATCAG G
SEQFHAB3760	Sense primer for sequencing of *fhaB* insert downstream from base 3760.	GTCGCGAAGAAGCTGTT TCTC
SEQFHAB4264	Sense primer for sequencing of *fhaB* insert downstream from base 4264.	ACCAAAGGCGAGATGC AGATC
SEQFHAB4741	Sense primer for sequencing of *fhaB* insert downstream from base 4741.	AACGCGGGCAAGATGC AGGTC
SEQFHAB5197	Sense primer for sequencing of *fhaB* insert downstream from base 5197.	GATTTCACCAACACGGG ATCC
SEQFHAB5737	Sense primer for sequencing of *fhaB* insert downstream from base 5737.	ATCGGCTATGTCAACTA CTGGTTG
SEQFHAB6286	Sense primer for sequencing of *fhaB* insert downstream from base 6286.	AAGATCTTTGGCGAGTA CAAGAAGCTG
SEQFHAB6724	Sense primer for sequencing of *fhaB* insert downstream from base 6742.	TTCTATCCCAAGGAACA AACCGTG

SEQPRN498	Sense primer for sequencing of *prn* insert downstream from base 498.	GACACCTGGGACGACG AC
SEQPRN924	Sense primer for sequencing of *prn* insert downstream from base 924.	GTTCCCGGTGGTGCGGT T
SEQPRN1398	Sense primer for sequencing of *prn* insert downstream from base 1398.	TCGCTGTCCATCGACAA C
SEQPTX801	Sense primer for sequencing of *ptxS1* insert downstream from base 801.	GAACATCGCATGCAGG AA
SEQPTX1167	Sense primer for sequencing of *ptxS1* insert downstream from base 1167.	ATCGTCGGCACATTGGT G
SEQCYAA1267	Sense primer for sequencing of *cyaA* insert downstream from base 1267.	GACAACGACGTCAACA GC
SEQCYAA1684	Sense primer for sequencing of *cyaA* insert downstream from base 1684.	GAACGCATCGACTTGTT G
SEQCYAA2041	Sense primer for sequencing of *cyaA* insert downstream from base 2041.	AAAAGCCTGTTCGACGA T
SEQCYAA2524	Sense primer for sequencing of *cyaA* insert downstream from base 2524.	ACCGTGAGCGGTTTTTT C
SEQCYAA2863	Sense primer for sequencing of *cyaA* insert downstream from base 2863.	CAATCGCACTATGCGGA T
SEQCYAA3220	Sense primer for sequencing of *cyaA* insert downstream from base 3220.	GAACAACTGGCCAATTC G
SEQCYAA3688	Sense primer for sequencing of *cyaA* insert downstream from base 3688.	AAGCACAGCATCAAACT GGAT
SEQCYAA4036	Sense primer for sequencing of *cyaA* insert downstream from base 4036.	GATTCGATCACCGGCAA T
SEQCYAA4450	Sense primer for sequencing of *cyaA* insert downstream from base 4450.	GATGGCAACGACACGA TA
SEQCYAA4882	Sense primer for sequencing of *cyaA* insert downstream from base 4882.	AACGACATGCTGTATGG C
SEQCYAA5161	Sense primer for sequencing of *cyaA* insert downstream from base 5161.	GTTTCCGGTATCGAGAA C
SEQCYAA5757	Sense primer for sequencing of *cyaA* insert downstream from base 5757.	GGCGATGATACTTATCT GTTC

Mutagenesis

PTXS1-13LF	Sense primer for two point mutations at bases 646 and 647 of *ptxS1* insert	TACCGCTATGACTCCCT GCCGCCGGAGGAC

PTXS1-13LR	Anti-sense primer for two point mutations at bases 646 and 647 of *ptxS1* insert.	GAAAACGTCCTCCGGCG GCAGGGAGTCATAGCG GTA
PTXS1-129GF	Sense primer for two point mutations at bases 994 and 995 of *ptxS1* insert.	GCCACCTACCAGAGCGG CTATCTGGCACACCGG
PTXS1-129GR	Anti-sense primer for two point mutations at bases 994 and 995 of *ptxS1* insert.	CCGGTGTGCCAGATAGC CGCTCTGGTAGGTGGC
CYAA-58LF	Sense primer for two point mutations at bases 1151 and 1152 of *cyaA* insert.	GAAGGGGTGGCCACCA TGGGATTGGGCGTGCAC
CYAA-58LR	Anti-sense primer for two point mutations at bases 1151 and 1152 of *cyaA* insert.	GTGCACGCCCAATCCCA TGGTGGCCACCCCTTC

2.5.2 Polymerase Chain Reaction (PCR)

Targeted gene sequences from *B. pertussis* genomic DNA were amplified by PCR (Saiki et al. 1988). The constituents for all genomic PCR reactions were purchased from Invitrogen unless otherwise stated. The nature of the reaction mixture and cycling parameters used was dependent on the target sequence and whether blunt-ended or A-tailed products were required.

For unidirectional cloning into the pcDNA3.1D/V5-His-TOPO vector, a blunt-ended PCR product with a 5' Kozak leader sequence was used. The inclusion of the CACCATGG kozak sequence has been reported to enhance the expression of open reading frames in eukaryotic cells by improving the efficiency of ribosomal assembly on mRNA transcripts (Kozak 1987). For this purpose, a reaction was used that consisted of 1×Platinum *Pfx* buffer, 0.3mM dNTP's, 1mM $MgSO_4$, 25pmol of sense and anti-sense primer, 1-2.5ng of gDNA or 20ng of plasmid DNA template, 2×PCR Enhancer Solution (denaturant for GC-rich templates), 1 – 1.5 Units of Platinum *Pfx* DNA polymerase and Ultra pure water (Millipore) up to 50µl.

Alternatively, cloning into the pTrcHis2-TOPO vector required an A-tailed PCR product for efficient ligation into the T-tailed vector, in the commonly used TA cloning method. To obtain the A-tailed PCR products, a reaction mixture that consisted of 1×Platinum *Taw* buffer, 0.2mM dNTP's, 2mM $MgSO_4$, 25pmol of sense and anti-sense primer, 1-2.5ng of gDNA or 20ng of plasmid DNA template, 2×PCR Enhancer Solution, 1 – 1.5 Units of Platinum *Taq* High Fidelity DNA polymerase and Ultra pure water (Millipore) up to volume of 50µl was used. To avoid confusion, the forward primers for amplification products for pcDNA3.1D/V5-His-TOPO cloning had the DNA abbreviation incorporated in the nomenclature, whereas the sense primers for products for cloning into pTrcHis2-TOPO had the TRC abbreviation. These abbreviations were not necessary for the shared reverse primers.

Temperature cycling was performed with a PCR Express Gradient thermal cycler (ThermoHybaid). The cycling parameters used for the amplification of each target sequence are outlined in Tables 2.4 to 2.8. Four µl of each PCR product was analysed by agarose gel electrophoresis upon completion of the thermo-cycling.

Table 2.4: Optimum cycling conditions for amplification of *fhaB*.

Oligonucleotides	FHAB3577DNAF and FHAB6966R (set #1) or FHAB3259TRCF and FHAB6966R (set #2)	
Product length (bp)	3389 (set #1) or 3707 (set #2) respectively	
Oligonucleotides	FHAB5740DNAF and FHAB6750R (set #3) or FHAB5737TRCF and FHAB6750R (set #4)	
Product length (bp)	1010 (set #3) and 1013 (set #4) respectively	
Cycling parameters		
Stage 1	1 cycle	95°C for 5 min
Stage 2	33 cycles	95°C for 30 sec
		51°C for 30 sec
		68°C for 4 min or 1min*
Stage 3	1 cycle	68°C for 10 min
		11°C hold

Note: Two distinct truncated fragments of the *fhaB* gene were amplified for cloning into the pcDNA3.1D/V5-His-TOPO and pTrcHis2-TOPO vectors. * Depending on product length.

Table 2.5: Optimum cycling conditions for amplification of *prn*.

Oligonucleotides	PRN198DNAF and PRN273R (set #5) or PRN198TRCF and PRN273R (set #6)	
Product length (bp)	2075	
Cycling parameters		
Stage 1	1 cycle	95°C for 5 min
Stage 2	32 cycles	95°C for 30 sec
		58°C for 30 sec
		68°C for 2 min
Stage 3	1 cycle	68°C for 10 min
		11°C hold

Table 2.6: Optimum cycling conditions for amplification of *ptS1*.

Oligonucleotides	PTX507DNAF and PTX1313DNAR (set #7) or PTX507TRCF and PTX1313TRCR (set #8)	
Product length (bp)	806	
Cycling parameters		
Stage 1	1 cycle	95°C for 5 min
Stage 2	35 cycles	95°C for 30 sec
		59°C for 30 sec
		68°C for 1 min
Stage 3	1 cycle	68°C for 10 min
		11°C hold

Table 2.7: Optimum cycling conditions for amplification of *cyaA*.

Oligonucleotides	CYAA883DNAF and CYAA6096R (set #9) or CYAA883TRCF and CYAA6096R (set #10)	
Product length (bp)	5213	
Cycling parameters		
Stage 1	1 cycle	95°C for 5 min
Stage 2	33 cycles	95°C for 1 min
		60°C for 1 min
		68°C for 6 min
Stage 3	1 cycle	68°C for 10 min
		11°C hold

Table 2.8: Optimum cycling conditions for amplification of *cyaC*.

Oligonucleotides	CYAC111DNAF and CYAC713R (set #11) or CYAC135TRCF and CYAC713R (set #12)	
Product length (bp)	602 or 578	
Cycling parameters		
Stage 1	1 cycle	95°C for 5 min
Stage 2	33 cycles	95°C for 30 sec
		55°C for 30 sec
		68°C for 1 min
Stage 3	1 cycle	68°C for 10 min
		11°C hold

2.5.3 Purification of PCR Products

The High Pure PCR Product Purification Kit (Roche) was used to clean-up selected PCR products prior to cloning. PCR products were either purified directly from the reaction tube or from agarose gel according to the manufacturers' instructions. The method of PCR product purification was dependent on the purity of the target band following agarose gel electrophoresis. Purified PCR products were re-run on an agarose gel to determine yield against the Low Mass DNA marker (Invitrogen).

2.6 Cloning into pcDNA3.1D/V5-His-TOPO and pTrcHis2-TOPO Vectors

A feature of the commercial pcDNA3.1D/V5-His-TOPO or pTrcHis2-TOPO vectors was the incorporation of TOPO cloning technology, in which Topoisomerase I was covalently bound to the 3' ends of the vector. The use of this technology provided a simple and rapid means of PCR product ligation without the need for a DNA Ligase reaction. Depending on the concentration, between 0.5 and 4µl of purified PCR product was added to 1µl of Salt Solution, 1µl of vector and DNase/RNase-free dH_20 up to 5µl for the pcDNA3.1D/V5-His-TOPO cloning reaction. The pTrcHis2-TOPO cloning reaction was identical, except the Salt Solution that was omitted. The reaction was mixed and incubated for 5 minutes at room temperature for ligation. All reagents listed were supplied with the respective cloning kit.

2.6.1 Transformation of TOP10 *E. coli*

Immediately following ligation, 2µl of the cloning reaction was added to a vial of competent TOP10 *E. coli*, gently mixed, and incubated on ice for 30 minutes. Cells were heat-shocked for 30 seconds at 42°C and incubated on ice for 2 minutes. 250µl of pre-warmed SOC media was added to the cells followed by incubation for 1 hour at 37°C and 200rpm. A 200µl aliquot was spread onto LB agar plates supplemented with glucose and ampicillin, and incubated for 14 hours at 37°C. Each transformation reaction included both a positive and negative control. For the positive control TOP10 cells were transformed with 10pg of pUC18, whereas the negative control involved a mock transformation of TOP10 in the absence of plasmid DNA.

Five colonies from each transformation were randomly selected for a screening procedure that involved overnight culture for plasmid DNA isolation and automated sequencing. This process was used to confirm that the insert was in the correct orientation and the base sequence was error-free.

2.6.2 Isolation of plasmid DNA

Plasmid DNA required for DNA sequencing was isolated using the Qiaprep Miniprep Spin Kit (Qiagen) or the Nucleospin Plasmid Miniprep Kit (Macherey Nagel). Both methods are based on the absorption of DNA onto a silica membrane, released after alkaline lysis of bacterial cells. Ten ml of LB (with antibiotics) was inoculated with a single colony and incubated for 14 hours at 37°C and 200rpm. A 4 ml aliquot was pelleted at 14,100×g for 1 minute and DNA isolated according to the manufacturer's protocol. Plasmid DNA was eluted from the columns using 50µl DNase/RNase-free dH_20 with centrifugation at 14,100×g for 1 minute.

2.7 DNA Sequencing

Inserts and insert flanking regions of the vector (ligation points) from each of the selected plasmid clones were sequenced using the ABI PRISM BigDye Terminator version3.1 Ready Reaction Kit (Applied Biosystems). The reaction and cycling conditions used were based on the manufacturer's recommendations, except the sequencing of pTrcHis2-TOPO constructs, which required optimisation due to low yields of plasmid DNA template.

2.7.1 Oligonucleotides

Oligonucleotides used for sequencing are listed in Table 2.3. The sequences of the oligonucleotides required for sequencing the insert flanking regions of the vector were supplied with the respective pcDNA3.1D/V5-His-TOPO or pTrcHis2-TOPO cloning kits (Invitrogen). The design of oligonucleotides for sequencing of the larger inserts was based on published Genbank (NCBI) sequences (see Appendix D for accession numbers and primer annealing sites). A gap of approximately 400 bp was allowed between each sequencing primer to ensure for overlap in the resulting outputs (chromatograms). Oligonucleotides were synthesised by Geneworks (Adelaide), with the lyophilised stocks reconstituted to 0.5µg/ml and the working solutions resuspended to 3.2pmol/µl using RNase/DNase-free dH_20 (Invitrogen).

2.7.2 Big Dye Terminator Reaction

For sequencing of pcDNA3.1D/V5-His-TOPO constructs, a 12µl reaction containing 0.5×BigDye v3.1 Ready Reaction Mix, 300-400ng plasmid DNA, 3.2pmol oligonucleotide and RNase/DNase free dH20 was used. Alternatively, for the sequencing of pTrcHis2-TOPO constructs (low yield of plasmid DNA) a reaction containing 0.5×BigDye v3.1 Ready Reaction Mix, 3.2pmol oligonucleotide and up to 15.5µl with plasmid DNA (300 – 400ng) was used. The cycling parameters for each sequencing reaction were identical with 1 cycle of 95°C for 5 min followed by 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min.

2.7.3 Clean-up of Sequencing Reaction Extension Products

Extension products from BigDye Terminator reactions were purified by isopropanol precipitation. The entire volume of the reaction was transferred to a 1.7 ml microcentrifuge tube. Isopropanol and DNase/RNase-free dH_20 was added to obtain a final isopropanol concentration of 60±5%. Tubes were vortexed, incubated at room temperature for 15 minutes and then centrifuged at 15,000×g for 20 minutes. Supernatants were removed and 250µl of 75% isopropanol added to the pellet.

Following centrifugation at 15,000×g for 5 minutes, the supernatants were removed and the pellet air dried. DNA pellets were sent for automated sequencing (gel separation) at the Australian Genome Research Facility (AGRF, Brisbane).

2.8 Site-Directed Mutagenesis of Toxin Inserts

Site-directed mutagenesis of the cloned *ptS1 and cyaA* inserts was performed using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturers' instructions. Inactivation of the recombinant S1 subunit of pertussis toxin required four point mutations of the cloned *pts1* inserts at bases 646, 647, 994 and 995 (Pizza et al. 1989). Hence, two mutagenesis procedures were performed to change Arginine to Leucine and then Glutamic acid to Glycine at amino acid positions 13 and 129 respectively.

Inactivation of the recombinant adenylate cyclase-hemolysin involved two point mutations at bases 1150 and 1151 of the cloned *cyaA* inserts, such that the Lysine was changed to Methionine at amino acid position 58 (Au, Masure & Storm 1989).

Point mutation of a 4.5kb pWhitescript plasmid (Stratagene) was used as a positive control for all mutagenesis procedures. The *lacZ* insert of the pWhitescript plasmid had an in-frame TAA stop codon, which interfered with β-galactosidase expression. Subsequent point mutation of the thymidine residue in the stop codon to a cytidine residue using the control oligonucleotides generated a glutamine codon switch. Transformation of *E. coli* with the mutated pWhitescript conveyed a blue phenotype on selective LB agar supplemented with IPTG and X-Gal, by virtue of an unimpeded β-galactosidase expression in the mutants.

2.8.1 Oligonucleotides

The oligonucleotides used for the mutagenesis PCR reactions are listed in Table 2.3. Mutagenesis oligonucleotides were designed from published Genbank (NCBI) sequences of the *ptxS1*, and *cyaA* genes (accession # M13223 and A14850 respectively). Both sense and anti-sense oligonucleotides were designed with a melting temperature (T_m) $\geq$ 78°C with the desired point mutation in the median base positions. Oligonucleotides were synthesised by Geneworks (Adelaide). Lyophilised stocks were reconstituted to 1μg/μl and the working solutions 100ng/μl with RNase/DNase-free dH_20.

2.8.2 Site-Directed Mutagenesis PCR

All of the necessary components of the mutagenesis PCR reaction were supplied with the exception of the custom mutagenesis oligonucleotides (Geneworks) and Ultra pure dH_20 (Millipore). Each 50µl control or test reaction consisted of 1×reaction buffer, 10ng plasmid DNA template, 125ng sense primer, 125ng anti-sense primer, 1×dNTP mix, 3×QuikSolution, 2.5 Units of *PfuTurbo* DNA polymerase and RNase/DNase free dH_20 up to 50µl. The cycling conditions used for the mutagenesis of the *ptxS1* and *cyaA* inserts are shown in Tables 2.9 and 2.10 respectively.

Table 2.9: Optimum cycling conditions for site-directed mutagenesis of *ptxS1* insert.

Oligonucleotides	PTXS1-13LF and PTXS1-13LR or PTXS1-129GF and PTXS1-129GR	
Cycling parameters		
Stage 1	1 cycle	95°C for 1 min
Stage 2	18 cycles	95°C for 50 sec
		60°C for 50 sec
		68°C for 12 min
Stage 3	1 cycle	68°C for 7 min
		11°C hold

Table 2.10: Optimum cycling conditions for site-directed mutagenesis of *cyaA* insert.

Oligonucleotides	CYAA-58LF and CYAA-58LR	
Cycling parameters		
Stage 1	1 cycle	95°C for 1 min
Stage 2	18 cycles	95°C for 50 sec
		60°C for 50 sec
		68°C for 22 min
Stage 3	1 cycle	68°C for 7 min
		11°C hold

A 10µl aliquot of each control or test reaction was analysed by agarose gel electrophoresis to determine the outcome of the PCR. Provided that the reactions were successful, parental (non-mutated and methylated) supercoiled dsDNA was digested with *Dpn* I restriction enzyme prior to transformation into XL10-Gold *E. coli*. One unit of *Dpn* I (10U/µl) was added to the remaining 40µl of each reaction tube, mixed, and incubated at 37°C for 1.5 hours.

2.8.3 Transformation of XL10-Gold *E. coli*

Heat shock transformation of competent XL10-Gold cells (Stratagene) was performed according to the manufacturers' recommendations. A 250µl volume of the mutagenesis transformations and 50µl of transformation controls were spread onto LB agar plates with or without ampicillin and incubated overnight at 37°C.

Five colonies from each mutagenesis experiment were selected for screening by overnight culture (Section 2.1.4) followed by isolation of plasmid DNA and sequencing. Plasmid DNA was isolated using the Macherey Nagel Nucleospin Kit (Section 2.6.2). Automated sequencing was performed on the targeted mutation site of the selected plasmid DNA clones that had an appropriate molecular size, as determined by agarose gel electrophoresis. Following identification of a successful mutation, the remaining insert was sequenced to confirm the integrity of the coding region.

2.9 Transient Expression of Recombinant Protein in Mammalian Cell Lines

Once cloning of antigenic genes and fragments into the pcDNA3.1D/V5-His-TOPO vector was completed, the *in vitro* expression levels of the recombinant proteins in a mammalian system was confirmed by transfection of COS-7 cells.

2.9.1 Isolation of Ultra Pure plasmid DNA

The Nucleobond AX100 Plasmid DNA Isolation Kit (Macherey-Nagel) was used to obtain up to 100µg of transfection-grade plasmid DNA. All components were supplied with the kit unless otherwise stated. For each DNA isolation, 100ml of overnight culture was pelleted by centrifugation for 15 minutes at 6000×g and 4°C. Plasmid DNA was then isolated according to the manufacturer's instructions.

Eluted DNA was precipitated by adding 0.7 volumes (12.5ml) of 100% isopropanol followed by centrifugation for 40 minutes at 15,500×g and 4°C. The supernatant was removed and the pellet washed with 70% ethanol (equilibrated to 4°C) followed by centrifugation for 15 minutes at 15,500×g and 4°C. The supernatant was again removed and the pellet air-dried prior to resuspension in 1ml of DNase/RNase-free dH_20 (Invitrogen). The concentration and purity was then determined by agarose gel electrophoresis and spectrophotometry respectively (see Section 2.15.2.1).

2.9.2 Transfection

Transfection, or the delivery of nucleic acids into eukaryotic cells, was performed using the Lipofectamine 2000 (LF2000) reagent (Invitrogen). The day before transfection, cells from two 90-100% confluent flasks were trypsinised, counted and seeded into a 24-well plate at 2×10^5 cells/well in 0.5 ml of complete DMEM without antibiotics. The plate was incubated at 37°C with 5% CO_2 until 90-95% confluent (12-24 hours). The plasmid DNA required for each well was prepared by diluting 0.5 to 1.5µg DNA into 50µl of OPTI-MEM serum-free media (Invitrogen). Simultaneously, for each test well 2 to 4µl of LF2000 reagent was diluted into 50µl of OPTI-MEM and incubated at room temperature for 5 minutes. The DNA and LF2000 dilutions were then combined and incubated at room temperature for a further 20 minutes. The DNA/LF2000 complexes were added drop-wise to the respective wells and mixed gently. Transfected cells were incubated for 24 to 48 hours at 37°C with 5% CO_2. Following the incubation, cells from each well were scraped and pelleted by centrifugation at 14,000×g for 1 minute. Pellets were washed with 1×PBS and resuspended in 40µl 1×Sample Buffer in preparation for PAGE and western blotting (Sections 2.10 and 2.11).

For each transfection procedure, expression of β-galactosidase from the plasmid pcDNA3.1D/*lacZ* (Invitrogen) was used as the positive control. The negative controls involved transfection with plasmid DNA and no LF2000, LF2000 without plasmid DNA, and the absence of both DNA and LF2000.

2.10 PAGE

Discontinuous SDS-PAGE was performed according to the method of Laemmli (1970). The Mini Protein 3 electrophoresis system (BioRad) was used for all PAGE procedures. The composition of the 4%/12% discontinuous gels used has been outlined in Appendix A. Briefly, gels were prepared and inserted into the mini protein 3 cassette. The inner chamber was completely filled, and the electrode exposed to the outer chamber covered with 1×Tris-Glycine Running Buffer. Samples were prepared in 1×Sample Buffer, heated for 5 minutes at 95°C and loaded into the submerged wells. Electrophoresis was performed at 200V until the desired level of migration was achieved. The broad range BenchMark® ladder (Invitrogen) was used to determine the molecular mass of the recombinant proteins. Gels were removed and protein stained with coomassie blue (Appendix B).

2.11 Western Blotting

Resolved protein(s) from unstained SDS-PAGE gels were transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech) using a Trans-Blot apparatus (BioRad). For each transfer, a piece of PVDF with equivalent dimensions to the gel was soaked in Methanol for 1 minute. The PVDF, 2 pieces of blotting paper and 2 fibre pads were then equilibrated in Transfer buffer. A transfer sandwich was assembled under transfer buffer in the following order: fibre pad, blotting paper, gel, PVDF, blotting paper and fibre pad. The transfer sandwich was inserted into a transfer cassette, which was in-turn placed into the tank. A frozen cooling cell was inserted and the tank filled with Transfer buffer. Depending on the size of the target protein(s), transfer was run at 100V for 1-2 hours.

Following transfer, the target recombinant protein was detected using either the Western Breeze Kit (Invitrogen) according to the manufacturer's instructions or an in-house protocol outlined in Appendix B. For the detection of recombinant protein expressed from *E. coli*, anti-histidine alkaline phosphatase conjugated antibody (Invitrogen) was used at a 1:5000 dilution. Alternatively recombinant proteins expressed from transfected mammalian cells were detected using a 1:2000 dilution of anti-V5 alkaline phosphatase conjugated antibody (Invitrogen).

For detection of IgG in serum samples or AC-Hly monoclonal antibodies, membranes were incubation with a 1:2000 dilution of an anti-mouse IgG HRP (Pierce) conjugate. For molecular weight determinations the BenchMark® ladder (Invitrogen) was transferred onto PVDF and detected using the anti-His antibodies, whereas the pre-stained version was transferred onto the membrane for western blots using the anti-V5 antibody, serum or ascites.

2.12 Purification of Recombinant Proteins

2.12.1 Induction of Recombinant Protein Expression

Optimal conditions for the induction of recombinant protein expression were determined empirically by a pilot experiment. The appropriate *E. coli* strains were plated on LB agar (with ampicillin and chloramphenicol as required) from glycerol stocks and incubated overnight to obtain isolated colonies. Ten ml of LB broth with ampicillin was inoculated with a single isolated colony and incubated for 14 hours. The primary culture was diluted 1:200 into 600ml of LBA broth (no glucose) and incubated until the OD_{600} reached 0.6. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1mM, and the culture incubated for 4 hours at 37°C and 200rpm to induce recombinant protein expression. Cells were pelleted at 12,500×g for 5 minutes and the pellet stored at -70°C until required.

2.12.2 Transformation of BL21 CodonPlus(RP) *E. coli*

Heat shock transformation of BL21 CodonPlus(RP) competent cells (Stratagene) was performed according to the manufacturers' recommendations. A 200µl aliquot of each transformation reaction was spread onto LB agar with or without selective antibiotics. Plates were incubated at 37°C for 16 hours. The positive control for the BL21 transformations was pUC18, whereas the negative control consisted of competent cells without plasmid DNA.

Five colonies from each BL21 transformation were screened for expression of the respective recombinant proteins by induction with IPTG followed by PAGE and western blotting.

2.12.3 Affinity Chromatography

Ni-NTA agarose (Qiagen) was used for the purification of recombinant 6×His-tagged proteins from induced *E. coli* lysates, in a batch format. The composition of reagents used for the purification procedures are included in Appendix A. It was determined in a pilot expression experiment that most of the target recombinant proteins were either poorly soluble or insoluble in a native buffer. Therefore recombinant proteins were purified under denaturing conditions.

A 100ml pellet of induced cells was resuspended in 10ml Buffer A (pH 8.0), and incubated for 1 hour at RT with shaking at 60rpm. To enhance lysis, the suspension was sonicated in an ice bath for 5 cycles of 30 seconds on/60 seconds off at 60% duty and maximum output using a Branson Sonifier. The lysate was centrifuged at 10,000×g for 25 minutes and 1ml Ni-NTA agarose added to the supernatant. The slurry was incubated for 40 minutes at RT with shaking at 1rev/sec to allow resin/His-tagged protein interaction. The slurry was then loaded into a disposable 4ml column and flowthrough collected by gravity flow. The matrix was washed with 8 column volumes (CV) of Buffer C (pH 6.3) followed by elution of recombinant protein with four 0.5 CV of Buffer D (pH 5.9) and a further four 0.5 CV of Buffer E (pH 4.5). A 30µl aliquot of the wash and each eluate was analysed by PAGE. Appropriate eluates were pooled and concentrated using vivaspin20 columns (Appendix C) for use in ELISA, western blot and splenocyte stimulation.

2.12.4 Quantitation of Protein

The concentration of purified recombinant protein was determined using a modified Bradford assay. The Coomassie Plus Protein Assay Reagent (Pierce) was used according to the manufacturer's instructions. A 2mg/ml BSA standard (Pierce) was used for the preparation of standards ranging from 2.5 – 25µg/ml in ultra pure dH_20. Unknown samples were diluted between 1:5 and 1:20 with dH_20. A 150µl volume of each standard and unknown preparation was added to appropriate wells of a 96-well microplate in duplicate. A further 150µl of Coomassie Plus Reagent was added to each well and gently mixed with the samples. Absorbance and concentration was then measured at 595nm using a Model 550 Microplate Reader (BioRad) after a 30 second incubation at room temperature.

2.13 Toxicity Assays

It was necessary to confirm that the genetic inactivation of the recombinant adenylate cyclase and pertussis toxins by site-directed mutagenesis was successful, prior to their use in experiments involving the use of animals. This was tested using two *in vitro* cell assays.

2.13.1 CHO Cell Assay for Determination of Pertussis Toxin Activity

The ADP-ribosyltransferase, NAD glycohydrolase, leukocytosis and histamine sensitization assays are all well established *in vivo* methods for testing the activity of pertussis toxin (Kimura et al. 1990). However, the CHO cell assay has been shown to be an acceptable *in vitro* method for the determination of pertussis toxin activity (Hewlett et al. 1983). This assay is based on the observation that CHO cells exhibit a characteristic rounded and clustered morphology in response to the action of PTX. Although Hewlett et al. (1983) developed this assay as a method to assess the activity of native and inactivated toxin molecules, the use of pertussis toxin as a DNA vaccine antigen was limited to S1 subunit alone (see Section 5.1). As the B oligomer or remaining subunits of the toxin are required for translocation of the S1 subunit across the cell membrane, the modified method of Castro et al. (2001) was used. As such, native or genetically-inactivated recombinant S1 subunits were endogenously expressed within CHO-K1 cells following transfection with the respective eukaryotic expression plasmids.

CHO-K1 cells were seeded into two 6-well plates at 5×10^5 cells/well in 2ml of complete F12 without antibiotics. Plates were incubated at 37°C with 5% CO_2 until cells were 80% confluent. At this point cells were transfected with 2.5-5µg of pcDNA3.1D/*ptS1*, pcDNA3.1D/*ptS1.13L* or pcDNA3.1D/*ptS1.13L.129G* using the Lipofectamine 2000 method (see Section 2.9.2). As a positive control CHO-K1 cells were exposed to 80ng, 800ng, 8µg or 40µg of commercially purified pertussis toxin (List Biologicals) in 0.5ml OPTI-MEM. Negative controls included transfection of CHO-K1 with pcDNA3.1D/*ptS1* in the absence of LF2000 and treatment of CHO-K1 with LF2000 only. Plates were incubated at 37°C with 5% CO_2 for 24 hours with regular observation for evidence of the clustered morphology in the test and positive control wells. The morphology of the cells in each well was documented as a digital image after 12 hours of incubation.

2.13.2 Cyclic AMP Assay for the Determining the Activity of Adenylate Cyclase-Hemolysin

Adenylate cyclase activity has traditionally been assayed *in vitro* by measuring the production of cyclic AMP from radioactively labelled ATP [α-^{32}P] (Salomon, Londos & Rodbell 1974; Hanoune et al. 1977). Although a reliable and proven method, this radioimmunoassay requires ~10-250µg/ml of pure recombinant antigen for each reaction, a requirement that could not be achieved in this project. Hence, an alternative approach for the assay of cAMP was devised to quantitatively assess the toxicity of the recombinant adenylate cyclase-haemolysin antigen. The assay was a three-step procedure that involved: (i) transfection or treatment of CHO-K1 cells with selected eukaryotic expression plasmids or purified antigen, (ii) lysis of CHO-K1 cells following incubation, and (iii) analysis of cAMP levels in lysates using a cAMP immunoassay.

2.13.2.1 *Transfection of CHO-K1 Cells*

Four 6-well plates were seeded with 5×10^5 cells/well in 4ml of complete F12 (without antibiotics) and incubated until 90% confluent. Transfections were performed using the LF2000 reagent as previously described. Briefly, an appropriate amount of plasmid for each well (see Table 2.11) was diluted up to 250µl with OPTI-MEM. Similarly, the respective amount of LF2000 required for each well was also diluted up to 250µl with Opti-MEM, and incubated at RT for 5 minutes. LF2000 dilutions were added to the appropriate plasmid dilution tube, mixed and incubated at RT for 20 minutes to allow the formation of complexes. During this incubation period the media in each well was replaced with 4ml of fresh complete F12 without antibiotics. As an assay control, CHO cells were treated with various concentrations of a commercial adenylate cyclase toxin (List Biologicals) ranging from 40ng/ml to 40µg/ml were prepared in 500µl of OPTI-MEM (Table 2.11). DNA-LF2000 complexes and purified AC-Hly preparations were added drop-wise to their respective wells and incubated for 24 hours at 37°C with 5% CO_2.

2.13.2.2 *Lysis of Treated CHO-K1 Cells*

The manufacturer's instructions for the cAMP EIA failed to provide sufficient detail regarding effective lysis of treated mammalian cells, and simply recommended the use of 0.1M HCl. As this was an unpublished method for assessing the toxicity of recombinant adenylate cyclase, the optimal lysis conditions were determined (see Appendix B for details). As a result of this lysis experiment, CHO-K1 cells were treated with 2ml of 0.1% Triton X100 and incubated for 30 minutes at room temperature. Lysates were collected, divided into 1ml aliquots and the supernatants collected by centrifugation at 14,000×g for 1 minute. Concentrated HCl (37%) was added to each 1ml supernatant to a final concentration of 0.1M to prevent degradation of cAMP by endogenous phosphodiesterases. Supernatants were stored at -20°C until required for the cAMP immunoassay.

2.13.2.3 *cAMP Immunoassay*

A cAMP immunoassay (Biomol Research Laboratories Inc.) was used according to the manufacturer's instructions. All reagents were supplied with the kit unless otherwise stated. The kit is a competitive immunoassay for the quantitative determination of cAMP in samples treated with HCl. Non-acetylated cAMP standards or samples were bound to immobilised anti-cAMP antibodies in competition with AP-conjugated cAMP. Hence, absorbance measured at 405nm was inversely proportional to the cAMP concentration.

Briefly, 50µl of Neutralising reagent was dispensed into each well with the exception of the Blank and Total Activity (TA) wells. A 100µl volume of 0.1M HCl/0.1% Triton X100 was then added to the non-specific binding (NSB) and zero standard (Bo) wells. Cyclic AMP standards #1 through #5 (200, 50, 12.5, 3.12, 0.78pmol/ml) were prepared by dilution of the 2000pmol/ml stock with 0.1M HCl/0.1% Triton X100. One hundred µl was added to the appropriate wells in duplicate. A 100µl volume of each undiluted test sample was added to the appropriate well followed by 50µl of 0.1M HCl/0.1% Triton X100 to the NSB wells. Fifty µl of cAMP-AP conjugate was added to all wells except Blank and TA. A further 50µl of rabbit anti-cAMP antibody solution was added to all wells except Blank, TA, and NSB. The plate was incubated for 2.5 hours at RT with shaking at 200rpm. Each well was washed three times with 1×Wash Buffer and tapped dry. Five µl of cAMP-AP conjugate was added to the TA wells before 200µl of

the p-nitrophenyl phosphate (pNPP) substrate was added to each well. The plate was incubated for 1 hour at RT without light or shaking. The reactions were stopped with 50µl of stop solution and the absorbance read at 405nm with correction at 595nm.

Table 2.11: Outline of Transfection Component of cAMP Assay.

Plate #	Well #	Treatment	Amount of plasmid DNA or protein	Vol. (µl) of Lipofectamine2000
A	1	pcDNA3.1D/*cyaAL58* (test)	2.5µg pDNA	9
	2	pcDNA3.1D/*cyaAL58* (test)	5µg pDNA	17.5
	3	pcDNA3.1D/*cyaAL58* (test)	7.5µg pDNA	26
	4	pcDNA3.1D/*cyaA* (control)	2.5µg pDNA	9
	5	pcDNA3.1D/*cyaA* (control)	5µg pDNA	17.5
	6	pcDNA3.1D/*cyaA* (control)	7.5µg pDNA	26
B	1	pcDNA3.1D/*fhaB1* (control)	2.5µg pDNA	9
	2	pcDNA3.1D/*fhaB1* (control)	5µg pDNA	17.5
	3	pcDNA3.1D/*fhaB1* (control)	7.5µg pDNA	26
	4	No pDNA (control)	Nd	9
	5	No pDNA (control)	Nd	17.5
	6	No pDNA (control)	Nd	26
C	1	AC-Hly toxin (control)	40µg/ml protein	Nd
	2	AC-Hly toxin (control)	4µg/ml protein	Nd
	3	AC-Hly toxin (control)	400ng/ml protein	Nd
	4	AC-Hly toxin (control)	40ng/ml protein	Nd
	5	Cells only (control)	Nd	Nd
	6	Cells only (control)	Nd	Nd

Key: pcDNA3.1D/*cyaAL58* - DNA vaccine construct encoding mutated adenylate cyclase Ag
pcDNA3.1D/*cyaA* - DNA vaccine construct encoding non-mutated adenylate cyclase Ag
pcDNA3.1D/*fhaB1* - DNA vaccine construct encoding Filamentous hemagglutinin Ag
AC-Hly toxin - Commercially purified Adenylate Cyclase Toxin
LF2000 – Lipofectamine 2000 transfection reagent
Nd – Not determined

Average net optical density (OD) was calculated for each standard and sample by subtracting the average NSB OD from the average bound OD. The binding of each standard pair was then calculated as a percentage of the maximum binding by dividing the average net OD by the net Bo OD. The standard curve was generated by plotting percent bound (B/Bo) versus cAMP concentration and the equation to the curve was used to calculate the cAMP concentration (pmol/ml) in each test sample.

2.14 Transformation of *S. typhimurium*

Upon the successful completion of the toxicity assays, the *S. typhimurium* vaccine strain SL3261 was transformed with the eukaryotic expression plasmids (pcDNA3.1D/*fhaB1*, pcDNA3.1D/*prn*, pcDNA3.1D/*cyaAL58*, pcDNA3.1D/*cyaC* and pcDNA3.1D/*pts1.13L.129G*) in preparation for the second mouse experiment. It was determined that an optimum transformation efficiency of *S. typhimurium* strain P9121 was obtained following heat shock with competent cells prepared with a proprietary SUPER-COMP media and RbCl/$CaCl_2$ salt solution (Qbiogene). For unknown reasons, the transformation of SL3261 could only be achieved by electroporation. Prior to transformation, SL3261 was cultured on M9 minimal media and M9 + aromatic amino acid mix (see Appendix A for compostion), to confirm its dependence on an exogenous source of the aromatic compounds including 2,3-dihydroxybenzoic acid (DHBA), para-hydroxybenzoic acid (pHBA), para-aminobenzoic acid (pABA), tryptophan, phenylalanine and tyrosine.

2.14.1 Preparation of RbCl/$CaCl_2$ Competent Cells

RbCl/$CaCl_2$ competent cells were prepared using a Super-Comp media and RbCl/$CaCl_2$ salt solution (Qbiogene), as outlined in Appendix B.

2.14.2 Heat Shock Transformation

A 50µl aliquot of frozen competent cells was thawed on ice and transferred to a chilled Falcon 2059 tube (BectonDickinson). Plasmid DNA (20-30ng) was added to the competent P9121 cells, gently mixed, and incubated on ice for 30 minutes. Cells were heat shocked for 30 seconds at 42°C and then incubated on ice for a further 2 minutes. A 250µl aliquot of pre-warmed SOC media was added to the cells followed by incubation for 1 hour at 37°C and 200rpm. A 200µl aliquot of the cell suspension was spread onto LB agar plates (with or without antibiotics).

Plates were incubated for 14 hours at 37°C. Five colonies were selected for a screening procedure that involved plasmid DNA isolation followed by transfection of COS-7. The molecular weight of the recombinant protein expressed from the plasmid isolated from the selected P9121 transformants was compared to the protein expressed from the original *E. coli*-based plasmids. For all P9121 transformations, 10pg of pUC18 was used as the positive control. A reaction with competent cells and no plasmid DNA was carried out as the negative control for all transformation experiments.

2.14.3 Electroporation

The electroporation of *aro*A *S. typhimurium* strain SL3261 was performed using the BioRad Gene Pulser® apparatus according the protocol of Casjens et al. (1991).

2.14.3.1 *Preparation of Electrocompetent SL3261*

The reagents and procedure used for the preparation of electrocompetent *S. typhimurium* are presented in Appendices A and B respectively.

2.14.3.2 *Pulse Transformation*

Aliquots of electrocompetent cells were thawed on ice for 5 minutes and then 50ng of methylated plasmid, isolated from the respective r^-m^+ *S. typhimurium* P9121 transformant, was added with gentle mixing The DNA-cell suspension was transferred to a pre-chilled 2mm gap cuvette (BTX), and pulsed for 9 to 13 msec at 2.4kV, 25μF capacitance with 400Ω resistance. Immediately after the pulse, cells were suspended in 0.5 ml of SOC and incubated at 37°C and 225 rpm for 1 hour. A 250 μl aliquot of the transformation reaction was spread plated onto LB agar supplemented with 100μg/ml of ampicillin and incubated at 37°C for 14 hours. As a positive control 20ng of plasmid DNA previously isolated from a transformed r^+m^+ strain of *S. typhimurium* was used. A mock transformation (no DNA) was included as a negative control for each transformation.

Five colonies representing the transformed SL3261 were screened by plasmid DNA isolation and recombinant protein expression as described in Section 2.14.2.

2.15 Assessment of the Immunogenicity and Protective Potential of the Candidate Vaccines in Mice

The immunogenicity and protective efficacy of the different vaccine candidates delivered via the intramuscular or oral routes were determined in two mouse experiments. The schedule for dosing, challenge and sampling of mice in both experiments is represented in Figure 2.1. Briefly, each experimental group of 17 mice were given either three test or control doses at three week intervals (see Sections 2.15.2 and 2.15.3 for details). Two weeks after the final booster 5 mice per group were sacrificed to determine the nature of the immune response generated by each vaccine. The remaining 12 mice per group were challenged with virulent *B. pertussis* to assess the protective efficacy of the different vaccine candidates (see Section 2.15.4 for details of the aerosol challenge protocol).

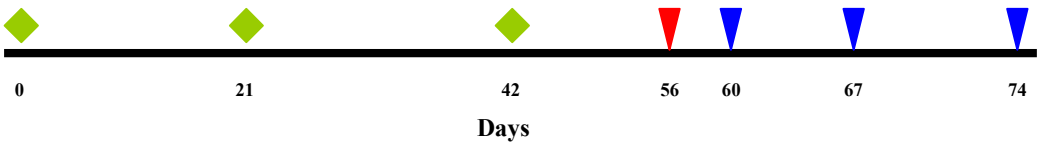

Figure 2.1: Schedule for Vaccination, Challenge and Sampling of Mice to Study Immunogenicity and Protective efficacy of DNA Vaccination. Administration of candidate vaccines or controls (◆), collection of serum and organs samples from 5 mice per group or aerosol challenge for remaining 12 mice per group (▼) and collection of lung samples from challenged mice (▼).

2.15.1 General Maintenance of Mice

Six-week old female Balb/C mice were obtained from the Animal Resource Centre (Canning Vale, Western Australia). The specific pathogen free mice were randomly segregated into cages and housed within an Isolation Cabinet fitted with charcoal and HEPA filters (TechnoPlas). Mice were provided with pellet food and water *ad libitum* and were allowed 7 days to acclimatise prior to the commencement of experiments.

2.15.2 First DNA Vaccine Trial

Given the paucity of data on the protective potential of DNA vaccines against whooping cough, the exception being a recent study with the pertussis toxin S1 subunit (Kamachi, Honda & Arakawa 2003), the goal of this initial animal experiment was to assess the nature of the immune response and protective efficacy afforded by each of four selected pertussis antigens. To achieve this objective the individual eukaryotic expression plasmids were delivered parenterally via a direct IM injection of naked DNA. Since it has been reported that vaccination with a single antigen does not provide a sufficient protective immune response against *B. pertussis* (Mills 2001), an additional group of mice were vaccinated with a combination of all five DNA vaccine plasmids.

2.15.2.1 *Large-Scale Isolation of Plasmid DNA for IM Delivery*

Endotoxin-free plasmid DNA required for IM delivery of the DNA vaccines was obtained using the Endo-free Plasmid Giga Prep Kit (Qiagen) according to the manufacturer's instructions. Following isopropanol precipitation of the eluted plasmid DNA a 0.5µl sample was analysed for purity and molecular weight by TAE agarose gel electrophoresis using a 1.0% gel. The plasmid DNA isolated from each preparation was analysed by UV spectrophotometry to determine concentration (260 nm) and purity (260/280nm). One hundred µl from each plasmid DNA sample was centrifuged for 1 minute at 14,000×g to clarify the solution. Ten-fold serial dilutions ranging from 10^{-1} to 10^{-3} were prepared using DNase/RNase-free H_20 (Invitrogen). The absorbance of each dilution was read at 260nm and then 280nm with DNase/RNase-free H_20 as the blank. Quantitation of plasmid DNA was based on the premise that an absorbance reading of 1.0 at 260nm corresponded to 50µg/ml for double-stranded DNA (Sambrook, Fritsch & Maniatis 1989). Alternatively, the 260/280nm ratio was used to assess the purity of plasmid DNA, in which a value of 1.8 - 2.0 corresponded to a pure sample that was free from protein or phenol contaminants (Sambrook, Fritsch & Maniatis 1989).

Table 2.12: Outline of First DNA Vaccine Trial.

Group	Dose	Route of Administration	Dosage	Mice	Description
1	pcDNA3.1D/*fhaB1* pcDNA3.1D/*fhaB1* + Challenge	IM IM + Aerosol	3×100µg 3×100µg + SLID	5 12	Test
2	pcDNA3.1D/*fhaB2* pcDNA3.1D/*fhaB2* + Challenge	IM IM + Aerosol	3×100µg 3×100µg + SLID	5 12	Test
3	pcDNA3.1D/*prn* pcDNA3.1D/*prn* + Challenge	IM IM + Aerosol	3×100µg 3×100µg + SLID	5 12	Test
4	pcDNA3.1D/*ptxM* pcDNA3.1D/*ptxM* + Challenge	IM IM + Aerosol	3×100µg 3×100µg + SLID	5 12	Test
5	pcDNA3.1D/*cyaAM* pcDNA3.1D/*cyaAM* + Challenge	IM IM + Aerosol	3×100µg 3×100µg + SLID	5 12	Test
6	pcDNA3.1D/*cyaAM* + pcDNA3.1D/*cyaC* pcDNA3.1D/*cyaAM* + pcDNA3.1D/*cyaC* + Challenge	IM IM + Aerosol	3×100µg 3×100µg + SLID	5 12	Test
7	DNA Vaccine Combination DNA Vaccine Combination + Challenge	IM IM + Aerosol	3×100µg 3×100µg + SLID	5 12	Test
8	Vector only Vector only + Challenge	IM IM + Aerosol	3×100µg 3×100µg + SLID	5 12	negative control
9	Saline Saline + Challenge	IM IM + Aerosol	0.1 ml 0.1 ml + SLID	5 12	placebo
10	DTaP (Infanrix™) DTaP (Infanrix™) + Challenge	SC SC + Aerosol	0.2 SHD 0.2 SHD + SLID	5 12	positive control
Total				170	

Key: SLID - Sub-lethal Infectious dose.
0.2 SHD - 20% of the standard human dose.

2.15.2.2 *Intramuscular Vaccination Regime*

All mice received three doses of a test vaccine or control (Table 2.12) at three-week intervals. DNA vaccine test doses consisted of 100µg plasmid DNA in 100µl of 1×PBS (50µl injected into each quadricep). The self-ligated pcDNA3.1 vector with no antigenic gene insert (constructed by Dr Austen Chen, USQ) was used as a DNA vaccine control. A 100µl dose of 1×PBS and a 0.2 standard human dose (SHD) of DTaP (Infanrix™) were used as the respective placebo and positive controls. Each dose was administered via the IM route with a 29 gauge needle with the exception DTaP, which was administered via the SC route. Prior to IM injection, mice were anaesthetised with an IP dose of 80mg/Kg and 16mg/Kg respectively of ketamine and xylazine (Blackshaw & Allan 1995). Anaesthesia was confirmed by the absence of a footpad-pinch response.

2.15.3 Second DNA Vaccine Trial

Upon evaluation of the response to the individual and combination IM DNA vaccines, the efficacy of an oral combination DNA vaccine and various dual modality vaccines (DNA vaccine prime-protein boost) was determined in a second mouse trial.

An in-house acellular vaccine (aP) rather than DTaP (Infanrix™) was used as the booster doses for the dual modality vaccines. This internal vaccine was prepared according to the formula of the pertussis component of the Infanrix™ DTaP (GSK) (Section 2.15.3.2). Although it would have been desirable to use the commercial DTaP for boosting, a clause in the contract with GSK for supply of the product required that the sample not be modified. Although the sample itself was not to be altered, the typical dosing regime was and to avoid potential contractual conflict the subunit vaccine formulation was prepared in-house. To avoid confusion, from this point onwards the in-house acellular vaccine will be referred to as aP, and the Infanrix™ vaccine as DTaP.

Consistent with the first DNA vaccine trial, mice received three doses of a test or control vaccine (Table 2.13) at three-week intervals. Naked plasmid DNA was injected into the quadriceps whereas the DTaP or aP was injected subcutaneously as described in Section 2.15.2.2. *S. typhimurium* DNA vaccines and controls were delivered by oral gavage using a catheter tip (tubing only) attached to a 1ml syringe (see Section 2.15.3.1).

Each dose of the *S. typhimurium* vaccine consisted of 0.1ml of a $1x10^{10}$ CFU/ml suspension (in bicarbonate buffered saline). Group 1 mice were given $1x10^{9}$ CFU of *S. typhimurium* harbouring the pcDNA3.1D/V5-His-TOPO vector. Group 2 mice received a combination oral DNA Vaccine, in which $2x10^{8}$ CFU of five recombinant *S. typhimurium* SL3261 strains (SL3261-pcDNA3.1D/*fhaB1*, SL3261-pcDNA3.1D/*prn*, SL3261-pcDNA3.1D/*cyaAL58*, SL3261-pcDNA3.1D/*cyaC* and SL3261-pcDNA3.1D/*ptS1.13L.129G*) were pooled for a combined dose of $1x10^{9}$ CFU. Groups 3 and 4 were administered two dual modality vaccines, in which either an oral or IM DNA vaccine priming dose was boosted with aP. Group 5 mice were primed with an IM dose of pcDNA3.1D/V5-His-TOPO vector and then boosted twice with aP to determine the impact of a single priming dose of the oral or IM DNA vaccine. Groups 6 and 7 were given the respective placebo and DTaP controls.

2.15.3.1 *Culture of S. typhimurium Vaccine Strains for Oral Delivery*

Preparation of the recombinant *S. typhimurium* suspensions required the generation of growth curves for each strain based on the Miles and Misra technique (Miles & Misra 1938). Briefly, an overnight primary culture was diluted 1:200 into 50ml of LB broth (with ampicillin) and incubated at 37°C and 200rpm. Serial ten-fold dilutions were prepared from 1ml samples taken at various OD_{600} readings from 0.2 and above. Triplicate 50µl drops from the 10^{-3} to 10^{-8} dilutions were spotted on a divided LB plate (with ampicillin) and incubated overnight at 37°C. An average CFU/ml from each OD_{600} reading was calculated from the triplicate counts of the appropriate dilution. Growth curves of each SL3261 vaccine strain are shown in Appendix D. To ensure optimum cell viability, each oral vaccine dose was prepared from fresh cultures. Each recombinant strain of *S. typhimurium* SL3261 was plated on LB agar (with ampicillin) from glycerol stocks and incubated overnight at 37°C. For each strain, an 8ml primary culture of LB broth (with 100µg/ml ampicillin) was inoculated with an isolated colony and incubated overnight at 37°C and 200rpm. A 250ml secondary culture was then inoculated with a 1:100 dilution of the primary culture and incubated at 37°C and 200rpm until the target OD_{600} was reached (varied for each strain as determined from the growth curves). The required volume of culture was centrifuged at 2500×g for 10 minutes and the pellets were gently resuspended in 3% $NaHCO_3$ in PBS.

2.15.3.2 *Preparation of aP (In-house Pertussis Acellular Vaccine)*

For each dose, 5µg of Pertussis toxoid (Section 2.15.3.3) was combined with 5µg of FHA (Siekaguku) and 1.6µg of rPRN (Section 5.2.3) in 0.1ml of DTaP suspension buffer (SUV buffer) for an equivalent 0.2 standard human dose (SHD). For consistency, the SUV buffer was saline with 5mg/ml 2-Phenoxyethanol as a preservative and 1mg/ml $AlOH_3$ as an adjuvant.

2.15.3.3 Chemical Inactivation of Pertussis Toxin

For the preparation of the custom subunit vaccine, 600µg of PTX (Siekaguku) was chemically inactivated with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDAC) according to the method of Christodoulides, Parton & Stewart (1989). Briefly, to obtain the recommended 80:1 EDAC to PTX ratio, PTX and EDAC were resuspended to 50µg/ml and 4mg/ml respectively in protein suspension (PS) buffer. The suspension was incubated at 37°C for 24 hours and then dialysed against PS buffer using a vivaspin20 column (Vivaflow), to remove the EDAC. Details of the dialysis procedure are described in Appendix B. The integrity and concentration of the toxoid preparation was confirmed by PAGE and the modified Bradford assay.

2.15.4 Aerosol Challenge

In both DNA vaccine trials 12 of 17 mice per group were challenged with a pre-determined sub-lethal infectious dose (SLID) of virulent Tohama I two weeks after the final booster dose (Chapter 3). Details relating to the SLID and induction of Tohama I virulence via repeated passage through mice are described in Section 3.2.2.

2.15.4.1 *Preparation of Bordetella pertussis for Aerosol Challenge*

A BG plate was inoculated with passaged Tohama I (from glycerol stocks) and incubated for 3 to 4 days at 37°C to obtain isolated colonies. Ten ml of MSS broth was inoculated with 4 to 6 individual colonies and incubated at 37°C and 150 rpm for 48 hours. The primary culture was diluted 1:150 into 500ml of MSS and incubated under the same conditions until the OD_{625} reached 0.5. Based on a pre-determined growth curve of Tohama I in MSS (OD_{625} of 0.5 = 1.17×10^9 CFU/ml), an appropriate amount of culture was pelleted at 2500×g for 10 minutes to obtain a pellet of 5×10^{11} CFU. The pellet was gently resuspended in 50ml of 1% casein for a final concentration of 1×10^{10} CFU/ml.

Table 2.13: Outline of Second DNA Vaccine Trial.

Group	Dose	Route	Mice	Dosage/Comments
1	SL3261 - pcDNA3.1 vector	3×Oral	5	1×10^9 CFU/dose as a negative control for group 2
	SL3261 - pcDNA3.1 vector + Challenge	3×Oral + SLID	12	
2	Combination SL3261 DNA Vaccine	3×Oral	5	Five different SL3261 transformants at 2×10^8 CFU each for total of 1×10^9 CFU/dose.
	Combination SL3261 DNA Vaccine + Challenge	3×Oral + SLID	12	
3	Parenteral Dual Modality: IM Combination DNA Vaccine prime and 2 × aP boosters	1×IM prime, 2×SC boost	5	IM DNA prime followed by boosting with two doses of an in-house subunit vaccine to determine whether a dichotomous Th1/Th2 systemic response could be induced and the potential for enhanced clearance of SLID compared to purely Th2 response to DTaP.
	IM Combination DNA Vaccine prime and 2 × aP boosters + Challenge	1×IM prime, 2×SC boost+ SLID	12	
4	Oral Dual Modality: Oral Combination DNA Vaccine primer and 2 × aP boosters	1×Oral prime, 2×SC boost	5	Oral DNA prime followed by boosting with two doses of an in-house subunit vaccine to determine whether a mucosal antibody response could be induced in conjunction with a potent systemic immune response and the potential for enhanced clearance of SLID compared to DTaP.
	Oral Combination DNA Vaccine primer and 2 × aP boosters + Challenge	1×Oral prime, 2×SC boost + SLID	12	
5	Vector Dual Modality: IM Vector primer and 2 × aP boosters	1×IM prime 2×SC boost	5	Control for groups 3 and 4.
	IM Vector primer and 2 × aP boosters + Challenge	3×SC + SLID	12	
6	Placebo	1×Oral, 1×IM, 1×SC	5	Negative control
	Placebo + Challenge	1×Oral, 1×IM, 1×SC + SLID	12	
7	DTaP (Infanrix™)	3×SC	5	0.2 SHD as positive control
	DTaP (Infanrix™) + Challenge	3×SC + SLID	12	
	Total		119	

Key: SLID - Sub-lethal Infectious dose.

0.2 SHD - 20% of the standard human dose.

2.15.4.2 *Exposure of Mice to B. pertussis Tohama I Aerosols*

Prior to challenge, the chamber and restraints (Sections 3.2.1) were sterilised by UV exposure for 30 minutes, followed by a 70% ethanol rinse. The 12 mice per group were challenged simultaneously. Mice were placed in the restraints and inserted into the ports of the chamber. The six nebulisers were filled with 8ml of the challenge preparation. The chamber was activated and run for 10 minutes, which was deemed to be the exposure time required for a SLID of 5×10^5 CFU/mouse lung (see Section 3.3.2). Aerosols were allowed to settle for 20 minutes, after which time mice were removed from the restraints and returned to cages.

2.15.5 Sampling

For both DNA vaccination experiments, blood samples and spleens were collected from 5 mice per group two weeks after the final booster dose. For the remaining 12 challenged mice, blood and lungs were collected at three distinct time points after exposure to the SLID. Four mice were sacrificed at days 4, 7 and 14 for the determination of bacterial counts in the lungs. The only variation between the sampling procedures of the first and second DNA vaccine trials was the additional removal of BAL fluid from the mice in the second *in vivo* experiment, due to the mucosal delivery of certain vaccines.

2.15.5.1 *Serum*

Prior to removal of blood and organs, mice were anaesthetised with an intraperitoneal (IP) injection of ketamine and xylazine (Section 2.15.2.2). Sedated mice were exsanguinated via cardiac puncture using a 26-guage needle attached to a 1ml syringe. Following collection, serum was separated in a paediatric serum collection tube (Starstedt). Briefly, blood was transferred into individual tubes, mixed by inversion, and incubated at 4°C for 1-2 hours to allow for clotting. Tubes were centrifuged for 2 minutes at 6,100×g and the serum aspirated from the gel interface. Aliquots were stored at -20°C until required.

2.15.5.2 *Lung Washing for Collection of BAL Fluid*

Bronchoalveolar lavage (BAL) fluid was collected by pertracheal cannulation as described elsewhere (Guzman et al. 1991; Dalla Pozza et al. 1998). After exsanguination, a ventral incision was made to expose the thorax and throat. Connective tissue was cleared from around the trachea and a catheter was inserted approximately 10mm. Lungs and bronchi were gently washed with 0.7ml of chilled 1×PBS supplemented with a protease-inhibitor cocktail (Roche). Lung washes were centrifuged at 3000×g for 5 minutes to remove particulate matter, with the supernatants stored at -20°C until assayed for the presence of antigen-specific IgA and IgG by ELISA.

2.16 Immunological Determinations

2.16.1 Stimulation of Splenocytes Cultured *in vitro*

Immediately following cervical cordotomy, spleens from each mouse per group were dissected and pooled in ice-cold 5ml of Dulbecco's Modified Eagle Media for splenocytes (sDMEM) without foetal bovine serum (FBS). Tissue was ground through a 70μM cell strainer (BectonDickinson) into a 50ml tube with a final rinse with 3ml of sDMEM without FBS. Cells were pelleted for 10 minutes at 1000×g and the supernatant was decanted. Although red blood cells can be removed at this point by centrifugation using a Ficoll gradient, this step was not performed in an effort to ensure maximal viability of the splenocytes. The cell pellet was thoroughly resuspended in 10ml sDMEM without FBS and a 10μl aliquot taken for counting with a haemocytometer in which splencyte viability was checked by staining with trypan blue (Section 2.2.1). After a further 10 minute centrifugation at 1000×g the pellet was resuspended with sDMEM to a concentration of 1×10^7 splenocytes/ml. One ml of complete sDMEM (with 10% FBS) was added to the required number of wells of a 24 well tissue culture plate (TPP), followed by 1 ml of the cell suspension for a final concentration of 5×10^6 splenocytes/well. The cells were incubated at 37°C with 5% CO_2 after selected pertussis antigens and mitogens were added into duplicate wells (Table 2.14). The supernatants were removed after 24 hours incubation for quantitation of IL-2 production and after 72 hours for the measurement of IFN-γ and IL-4 production (Mills et al. 1993). Splenocyte supernatants were stored in aliquots at -20°C or -70°C until required.

2.16.1.1 *Preparation of Splenocyte Stimulants*

For the preparation of a heat-killed *B. pertussis* (HKBP) lysate, 200ml of MSS was inoculated with 2ml of an overnight primary culture of Tohama I and incubated until the OD_{625} reached 0.5 (~ 1.17×10^9 cells/ml as previously determined from Tohama I growth curve). The cells were centrifuged at 2500×g for 10 minutes and the pellet resuspended in 1×PBS. Cells were heated to 56°C for 30 minutes with occasional stirring, pelleted at 4000×g for 60 minutes (4°C) and resuspended in 200ml of 1×PBS. The suspension was sonicated for 5 cycles of 30 seconds on/60 seconds off at 60% duty and stored at -20°C and then the total protein concentration was estimated using the modified Bradford assay described in Section 2.12.4. The HKBP lysate was added to the respective wells at a concentration of 5µg/ml.

To prepare the formalin-fixed *S. typhimurium* (FFST), 20ml of LB was inoculated with a single colony of SL3261 and incubated until the OD_{600} reached 0.5 (~ 4×10^8 cells/ml as determined from the SL3261 growth curve, Appendix B). Formaldehyde was added to the culture at a concentration of 1% and incubated at 37°C for 4 hours. The suspension was centrifuged at 2000×g for 10 minutes and the pellet resuspended in 20ml of bicarbonate buffer. The suspension was sonicated for 5 cycles of 30 seconds on/60 seconds off at 60% duty and then the total protein concentration was estimated (as described in Section 2.12.4). The FFST lysate was again added to the respective wells at a concentration of 5µg/ml.

For the remaining wells, DTaP or purified recombinant protein were added at a high dose of 5µg/ml or a low dose of 1µg/ml. A 0.5ml dose of DTaP contained 25µg of Pertussis toxoid, 25µg of FHA and 8µg of pertactin. Hence for stimulation with DTaP, 5µg of Pertussis toxoid, 5µg of FHA and 1.6µg of pertactin were used by adding 100µl of the Infanrix™ preparation to the well. As DTaP contains alum, a control well was included in which splenocytes were treated with SUV buffer (10mg/ml 2-phenoxyethanol, 2mg/ml $AlOH_3$ in saline) to: (i) assess whether there was an adverse effect on the viability of splenocytes; and (ii) be a blank for DTaP-stimulated splenocytes. There was no observable loss in cell viability between splenocyes treated with alum-containing SUV buffer and untreated splenocytes. The T cell mitogen concanavalinA (conA) was added at 2.5µg/ml as a positive control, and 100µl of denaturing protein (DP) buffer was added to splenocytes as a negative control well.

Table 2.14: Stimulants Used for Each Pooled Splenocyte Population[a].

Stimulant	Conc./Amt	First DNA Vaccine Trial Group No.[b]	Second DNA Vaccine Trial Group No.[c]
rFHAB1 (High)	5µg/ml	1, 2, 7, 8, 9, 10	1, 2, 3, 4, 5, 6, 7
rFHAB1 (Low)	1µg/ml	1, 2	Nd
rFHAB2 (High)	5µg/ml	1, 2, 7, 8, 9, 10	1, 2, 3, 4, 5, 6, 7
rFHAB2 (Low)	1µg/ml	1, 2	Nd
rPRN (High)	5µg/ml	3, 7, 8, 9, 10	1, 2, 3, 4, 5, 6, 7
rPRN (Low)	1µg/ml	3	Nd
rPTS1.13L.129G (High)	5µg/ml	4, 7, 8, 9, 10	1, 2, 3, 4, 5, 6, 7
rPTS1.13L.129G (Low)	1µg/ml	4	Nd
rCYAAL58 (High)	5µg/ml	5, 6, 7, 8, 9, 10	1, 2, 3, 4, 5, 6, 7
rCYAAL58 (Low)	1µg/ml	5, 6	Nd
rCYAAL58 + rCYAC (High)	5µg/ml	5, 6, 7, 8, 9, 10	1, 2, 3, 4, 5, 6, 7
rCYAAL58 + rCYAC (Low)	1µg/ml	5, 6	Nd
DTaP (High)	5µg/ml	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	1, 2, 3, 4, 5, 6, 7
DTaP (Low)	1µg/ml	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	1, 2, 3, 4, 5, 6, 7
Heat-Killed *B. pertussis*	5µg/ml	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	1, 2, 3, 4, 5, 6, 7
Formalin-Fixed *S. typhimurium*	5µg/ml	Nd	1, 2, 3, 4, 5, 6, 7
Denaturing Protein Buffer (negative control)	100 µl	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	1, 2, 3, 4, 5, 6, 7
DTaP Buffer (negative control)	100 µl	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	1, 2, 3, 4, 5, 6, 7
Concanavalin A (positive control)	2.5µg/ml	1, 2, 3, 4, 5, 6, 7, 8, 9, 10	1, 2, 3, 4, 5, 6, 7

[a] - 5×10^6 splenocytes cultured from the pooled spleens of five mice per group

[b] - Experimental groups from preliminary DNA Vaccine trial (see Table 2.14)

[c] - Experimental groups from supplementary DNA Vaccine trial (see Table 2.15)

Nd – Not determined.

2.16.1.2 *Interferon-γ ELISA*

Interferon-γ (IFN-γ) levels in the 72-hour culture supernatants were quantitated using a Mouse IFN-γ Sandwich ELISA (Pierce) according to the manufacturer's instructions.

2.16.1.3 *Interleukin-2 ELISA*

Interleukin-2 (IL-2) levels in the 24-hour culture supernatants were quantitated using a Mouse IL-2 Sandwich ELISA (Pierce) according to the manufacturer's instructions.

2.16.1.4 *Interleukin-4 ELISA*

Interleukin-4 (IL-4) levels in the 72-hour culture supernatants were quantitated using a Mouse IL-4 Sandwich ELISA (Pierce) according to the manufacturer's instructions.

2.16.2 Enzyme-linked Immunosorbent Assay (ELISA) of Serum and BAL Fluid

Antigen-specific antibody isotypes in serum and BAL fluid of individual mice was quantitated using an indirect ELISA. Maxisorp 96-well plates (Nunc) were coated overnight at 4°C with 50µl of 5µg/ml of antigen in coating buffer. Recombinant protein (Section 2.10.5), commercial antigen (List or Sekaguku) or FFST (Section 2.16.1.1) were used for the coating of plates. As commercially purified pertactin was not available, antibodies were only measured against recombinant pertactin. For all assays, a pool of serum from placebo-immunised mice was included for the generation of a base line for determination of end-point titers. In addition to a set of blanks, a positive or plate control that consisted of a 1:200 dilution of in-house murine DTaP hyperimmune serum was included to standardise absorbance values between multiple plates.

Following overnight coating, plates were washed 3 times with PBST buffer and tapped dry. Remaining unbound absorption sites were blocked with 100µl of phosphate-buffered saline with tween20 (PBST) and 5% skim milk. After 1 hour at 37°C, blocking buffer was removed and 50µl of 2-fold serial dilutions of test or negative control serum in PBST+5% skim milk were added to either antigen-coated or uncoated background wells in duplicate. Plates were incubated for 2 hours at 37°C, washed 3 times with PBST and tapped dry. A 100µl aliquot of a 1:2000 dilution of either goat anti-mouse IgG-HRP (Pierce), goat anti-mouse IgG1-HRP (Zymed), goat anti-mouse IgG2a-HRP (Zymed), or goat anti-mouse IgA-HRP conjugates (Pierce) was added to each well except the zero blanks and incubated for 2 hours at 37°C. Plates were washed

5 times with PBST and tapped dry. OPD substrate (Sigma) was added to each well and developed for 30 minutes at RT in the dark. Absorbances were read at 450 nm using the Model 550 Plate Reader (BioRad). Titration curves were generated by plotting the specific absorbance (absorbance value minus background) versus serum dilution. End-point titers were determined as the reciprocal of the highest dilution at a cut-off point 3 standard deviations above the mean absorbance of sera from placebo-immunised mice. In the event the highest dilution of test serum exceeded the cut-off value, regression was used to approximate the end-point.

2.17.3 Clearance of *B. pertussis* from Lungs

At 4 days, 7 days and 14 days post-challenge the lungs from 4 mice were removed and placed in sterilised collection tubes (chilled on ice) that contained a 1ml volume of glass beads (1mm diameter) emersed in 1ml of 1% Casein. Lung tissue was then homogenised for 1 minute at 4600rpm with a mini bead-beater (Sigma). Ten-fold serial dilutions of each homogenate were prepared in 1% Casein using a 96-well plate. Quadruplicate 25µl aliquots from the 10^0 to 10^{-5} dilutions were added drop-wise onto BG agar plates and incubated at 37°C for 4 - 5 days. Bacterial counts from the appropriate dilution were adjusted to CFU/lung value. Means of the $\log_{10}$ CFU/ml and standard errors (SE) were calculated and plotted for each time point. A clearance index (CI) was calculated using the method of van den Berg et al. (2001), in which CI = mean log10 number of *B. pertussis*/ml × days ± SE. The limit of detection was approximately 100 CFU/lung (Xing et al. 1999).

2.18 Statistical Analysis

Significant differences in the cAMP EIA, antibody titers and clearance index (CI) values were determined using a T test for paired comparison or ANOVA for multiple comparisons (SPSS program, version 11.5). Differences between groups were considered significant at P values < 0.05.

Chapter 3 Development of an Aerosol Challenge Protocol

3.1 Introduction

The availability of an effective challenge model is important in the development of vaccines against human infectious diseases. It not only permits assessment of the efficacy of the vaccine candidates but can also provide valuable insight into the mechanisms of protective immunity to the target pathogen. Although *B. pertussis* is a strictly human pathogen, the bacterium is capable of colonising both the respiratory tract and cerebral tissues of rodents and primates, when administered in high doses (Sato et al. 1980). From this early observation, three different animal models of *B. pertussis* infection have been developed: the intracerebral (IC), intranasal (IN) and aerosol challenge methods.

Since the introduction of the *B. pertussis* bacterin in the mid 1940's the IC mouse protection test, referred to as the Kendrick test, has been the gold standard for determining the potency of whole-cell vaccines (Kendrick et al. 1947). The impetus for its adoption arose from a strong correlation between mouse protection and clinical efficacy in children (Medical Research Council 1959). However, it is not a suitable model for determining the efficacy of acellular pertussis vaccines as the results of potency testing in mice do not correlate with the level of protection in infants (Mills et al. 1998; Xing et al. 1999). A modified ic test has since been developed in Japan and endorsed throughout Asia after it was shown to be a more accurate indicator of the potency of modern acellular vaccines (Watanabe & Nagai 2005). Nevertheless, the traditional and modified ic methods require a high level of experience with the technique and a degree of surgical skill for it to be a reliable and ethical test according to guidelines set out by the WHO. Also, the ic test does not reproduce any features of the human infection and therefore may only account for the systemic response generated by a candidate vaccine. It was for these reasons that the Kendrick test was not selected as the model for testing the efficacy of the DNA vaccines against *B. pertussis*, and a more user-friendly model was sought that more closely represents the process of natural infection.

The intranasal or aerosol challenge of mice induces a respiratory infection that replicates many of the human manifestations such as leukocytosis, hypoglycaemia, histamine sensitisation and confinement to the respiratory tract. However, Sato *et al.* (1980) dismissed the intranasal method on the basis that pilot experiments failed to provide a uniform or reproducible infection. In contrast, Guiso *et al.* (1999) directly compared the aerosol and intranasal models in relation to their ability to differentiate between a bi-component vaccine with a low clinical efficacy of 42% and a tri-component preparation with 89% efficacy. They found that whilst the intranasal challenge resembled clinical performance and allowed discrimination in the rates of clearance, the aerosol model showed no significant difference between the two Pa formulations. On the downside, previous intranasal challenge experiments performed in our laboratory have resulted in a high degree of lung contamination from normal flora (R. Cornford Thesis). The presence of contamination is not surprising considering that inhalation of a relatively large volume of inoculum would gather normal flora as it passed through the upper respiratory tract and into the lungs. Further, it has also been necessary to lightly anaesthetise mice prior to administration of the challenge suspension (Guiso *et al.* 1999). In order to reduce the risk of lung contamination, avoid the need for sedation, and mimic the natural mode of exposure it was decided to adopt the aerosol challenge model.

A great deal more information relating to the mechanisms of protection against *B. pertussis* has been obtained in the last decade using the aerosol challenge model. For instance, pertussis was once regarded as a single toxin disease and that immunity was only associated with a strong systemic antibody response. It has now been established using the murine respiratory model of infection that: (i) *B. pertussis* is a complex pathogen with many virulence determinants that influence pathogenicity and immune subversion; (ii) *B. pertussis* has the capacity for both extracellular colonisation of the respiratory mucosa and intracellular survival within ciliated respiratory epithelia or macrophages; and (iii) CMI complements the humoral immune response and enhances elimination of the pathogen (Mills 2001; Mills et al. 1998). Despite certain shortcomings, such as the absence of coughing and the development of pneumonia rather than an URTI, the aerosol challenge model has proven to deliver a consistent and reproducible infection which can discriminate between vaccines of high and low efficacy (Mills et al. 1998). It is now widely regarded to be the most accurate

representation of natural infection (Sato et al. 1980; Weiss & Goodwin 1989; Xing et al. 1999; Canthaboo et al. 2000). In light of its selection as the method of challenge, the objectives were to:

1. Construct an aerosol chamber and develop a challenge protocol for the mass delivery of *B. pertussis* aerosols to mice, based on successful reference designs
2. Confirm the virulence of the *B. pertussis* Tohama I challenge strain
3. Determine the optimal sub-lethal infectious dose as a function of exposure time
4. Assess the performance of the sub-lethal aerosol challenge protocol and its capacity for induction of an infection.

3.2 Materials and Methods

3.2.1 Chamber

The aerosol chamber used in the challenge experiments is shown in Figure 3.1. It was based on a design originally developed at the National Institute for Biological Standards and Control (NIBSC), Hertfordshire, United Kingdom as part of a bioassay to test the potency of pertussis vaccines (Xing et al. 1999; Canthaboo et al. 2000). Detailed plans of the reference chamber and the associated bioassay protocol were kindly provided by Dr Dorothy Xing (NIBSC).

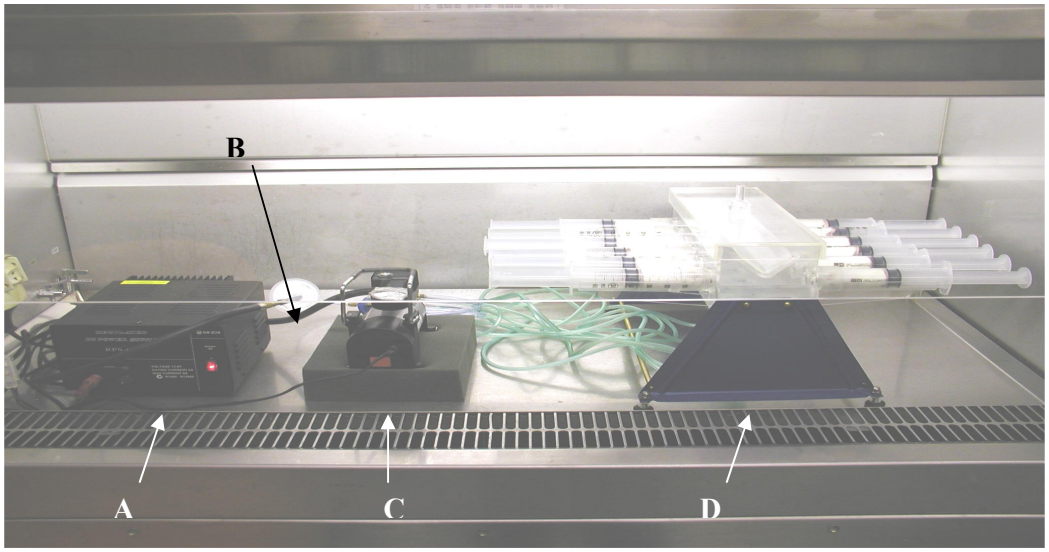

Figure 3.1: Purpose-built aerosol chamber for respiratory challenge of non-immunised and immunised mice. A – power unit, B – 0.45 µm air filter, C – air compressor, and D – Perspex chamber.

3.2.2 Materials for Chamber Construction

- Power unit and air compressor
- Perspex and sheet metal
- 0.45 μm air filter (Sartorius)
- Brass air flow divider (custom-made by O. Kinder)
- Disposable nebuliser bowls and connective tubing (Becton-Dickinson)
- 50ml concentric tip syringe (Becton-Dickinson)
- Aerosol trap containing 70% Ethanol (not shown in Figure 3.1)[a]

[a] The aerosol trap was attached to the outlet on the lid of the chamber, and consisted of glass tubing submerged in 70% Ethanol within a stoppered cylindrical flask.

3.2.3 Chamber Design

The aerosol chamber used to infect mice was built by Mr Oliver Kinder (University of Southern Queensland). Air was pumped by positive pressure alone through a 0.45μm filter to a brass divider, which separated airflow into six equal lengths of tubing. Tubing was connected to one of six plastic nebulisers that were distributed along the base of the chamber. Situated above the nebuliser openings, a deflector plate directed aerosols towards twelve ports, from which the nose of the restrained mice protruded. Mice were restrained and positioned using an open-ended 50ml disposable syringe. The entire unit was housed within a Class II biohazard cabinet during operation for personal safety and to maintain sterility.

3.2.4 Preparation of *Bordetella pertussis* Tohama I for Use in Challenge Studies

A feature of *B. pertussis* is its ability to alter its phase of virulence depending on certain environment cues. Growth at low temperature or in the presence of magnesium ions or nicotinic acid has been shown to induce a reversible shift from a virulent phase I to a non-virulent phase IV state (Melton & Weiss 1993). Long-term storage or repeated *in vitro* sub-culture has also been suggested as a trigger for phase variation (Goldman, Hanski & Fish 1985). To ensure that the challenge strain was in phase I, stocks of *B. pertussis* were passaged twice through Balb/C mice (see Table 3.1). Each passage involved execution of the following procedures: (i) the preparation of a challenge dose, (ii) exposure of mice to aerosols, (iii) removal of lungs 24 hours exposure, (iv) culture of lung isolates on BG agar, and (v) storage of the isolates at -70°C after each passage.

Table 3.1: Protocol for passage of *B. pertussis* Tohama I for use in challenge experiments.

Group[a]	Dose[b] (CFU/ml)	Duration of Exposure(min.)	No. of Mice	Description
1	1×10^{10}	15	12	Initial passage
2	1×10^{10}	15	12	Second passage

[a] Two groups of twelve mice were exposed to aerosols for 15minutes. [b] The challenge dose for the first passage (Group 1) was prepared from -70°C stocks of *B. pertussis* Tohama I. The second passage (Group 2) involved challenging mice with an isolate derived from the first passage (Group 1).

An 8ml suspension of *B. pertussis* was required for each of the 6 nebulisers, with 48ml required in total (Sato et al. 1980; Mills et al. 1998; Xing et al. 1999; Guiso et al. 1999; Canthaboo et al. 2000). The optimal challenge suspension for aerosol infection of mice from a consensus of these reference studies was 1×10^{10} CFU/ml in 1% casein, and as such a 50ml suspension of Tohama I at 1×10^{10} CFU/ml was prepared for each run. To prepare this suspension, 10ml of MSS broth was inoculated with isolated colonies of *B. pertussis* Tohama I and incubated for 48 hours at 37°C with shaking at 150rpm. A 500ml MSS broth was inoculated with 2ml of the primary culture (1:250 dilution) and incubated at 37°C/150rpm until the OD_{625} reached 0.5. It had been previously determined using a Tohama I growth curve that an OD_{625} value of 0.5 corresponded to 1.17×10^{9} CFU/ml. The volume of the batch culture required to obtain 5×10^{11} CFU was centrifuged at 2500×g for 10 minutes. Culture supernatant was removed and the pellet resuspended in 50ml of 1% casein.

For the first passage, twelve mice were placed into restraints which were then inserted into the ports of the chamber. Each nebuliser was loaded with 8ml of the *B. pertussis* suspension. The chamber was activated and mice were exposed to aerosols for 15 minutes. After a 24-hour incubation period to allow for colonisation, the lungs of infected mice were removed and individually placed in 500µl of 1% Casein. Lungs were homogenised using a mini bead-beater (see Section 2.17.3) and 200µl of each homogenate was spread plated onto BG agar plates. After 4 days incubation at 37°C in humidified atmosphere three pure isolated colonies were selected for culture and storage at -70°C as described in Section 2.1.4. This procedure was repeated for the second passage using *B. pertussis* Tohama I isolated from the first passage, cultured in MSS broth.

3.2.5 Confirmation of Phase I Status of Passaged *B. pertussis*

The virulence status of the passaged *B. pertussis* Tohama I strain used for challenge was determined by the presence of haemolysis on BG agar and subsequently confirmed by detection of the AC-Hly antigen, the virulence factor considered to be responsible for the haemolytic activity, by western blotting.

3.2.5.1 *Preparation of B. pertussis Lysates for Western Blotting*

Six different *B. pertussis* lysates were prepared for SDS-PAGE. Five of the six lysates were Tohama I with the sixth one being strain BP338, a nalidixic acid resistant derivative of Tohama I (Table 2.1). The five Tohama I isolates represented the original stock, an isolate from the first mouse passage, and three selected isolates from the second passage. Each isolate was plated onto BG agar from glycerol cultures and incubated for 4 days at 37°C in a humidified atmosphere. Growth was removed using a sterile Dacron swab and resuspended in 1% casein to an OD_{625} of 0.5. A 20µl aliquot of each suspension was lysed in 40µl of 1×SDS PAGE sample buffer and heated at 98°C for 5 minutes (Sambrook, Fritsch & Maniatis 1989).

3.2.5.2 *Western Blotting for Detection of AC-Hly Antigen*

A 20µl sample of each lysate was loaded on a PAGE gel and the resolved according to procedure outlined in Section 2.10. Fractionated proteins were then transferred to PVDF membrane as described in Section 2.11. After blocking, membranes were incubated with a 1:1000 dilution of 3D1 adenylate cyclase toxin monoclonal antibody (List Biologicals). For detection of bound IgG, membranes were incubated with an anti-mouse IgG HRP conjugate (Pierce) and visualised using the OPTI-4CN substrate (BioRad).

3.2.6 Optimisation of Challenge Exposure Time

An experiment was performed to determine the exposure time required to obtain a sub-lethal infectious dose (SLID) of 5×10^5 CFU/lung from 1×10^{10} CFU/ml suspension of *B. pertussis* for use as the challenge dose in the two DNA vaccine trials. The consensus SLID used for *B. pertussis* respiratory challenge of mice has been reported to be 5×10^5 CFU/lung (Sato et al. 1980; Guiso et al. 1999; Xing et al. 1999). As the chamber used in this study was slightly modified from the NIBSC design, it was deemed necessary to redefine the exposure time required to achieve this dose. Groups of between 4 to 8 mice

were exposed to aerosols generated from a 1×10^{10} CFU/ml suspension of *B. pertussis* Tohama I for set time periods ranging from 0 to 30 minutes (Table 3.2). Four hours after exposure, mice were sacrificed and the lungs removed. Bacterial loads (CFU/lung) were determined for each exposure by culturing serial dilutions of lung homogenates on BG plates (see Section 2.17.3). Plates were incubated for 4 days at 37°C in a humid atmosphere (see Section 2.1.4).

Table 3.2: Determination of SLID of *B. pertussis* Tohama I aerosol challenge.

Group	Suspension (CFU/ml)	Exposure (min)	Mice
1	0	0	8
2	1×10^{10}	5	8
3	1×10^{10}	10	8
4	1×10^{10}	20	8
5	1×10^{10}	30	8
Total			**40**

3.2.7 Performance of the Aerosol Challenge Method

Groups of 12 unimmunised mice were exposed the pre-determined SLID (section 3.2.4) over three separate experiments to assess the chamber performance. The bacterial burden in the lungs of infected mice was measured at 4, 7 and 14 days post-challenge. Lungs were removed and homogenised using a mini-bead beater as described in Section 2.17.3. Ten-fold serial dilutions ranging from 10^0 to 10^{-5} of each homogenate was prepared in 1% Casein and 25µl aliquots were added drop-wise onto BG agar plates in quadruplicate according to the Miles and Misra method (Miles & Misra 1938). Plates were incubated at 37°C for 4 days in a humidified atmosphere and the CFU/lung calculated. Means of the log10 CFU/lung ± SE were calculated were plotted for each time point to determine the clearance curves of non-immunised mice after challenge with phase I *B. pertussis* Tohama I.

3.3 Results

3.3.1 Confirmation of *B. pertussis* Virulence

Both stock and passaged *B. pertussis* showed characteristic growth on the selective BG agar with the appearance of small, pearly grey colonies and an amorphous zone of β-hemolysis as previously described (Holt 1993; Kerr & Matthews 2000). Western blotting of both stock and passaged *B. pertussis* lysates with the 3D1 adenylate cyclase

toxin monoclonal antibody revealed the presence of a single band with a molecular weight over 190 kDa that was considered to represent expression of the AC-Hly antigen. Purified AC-Hly from *B. pertussis* (List Biologicals) was included as a control. However, there was an excessive amount of background or smearing observed with the commercial AC-Hly antigen. Clearly, the commerical antigen was not purified to homogeniety and the smearing is most likely due to cross-reactivity between the contaminating proteins present in this preparation and the 3D1 monoclonal antibody.

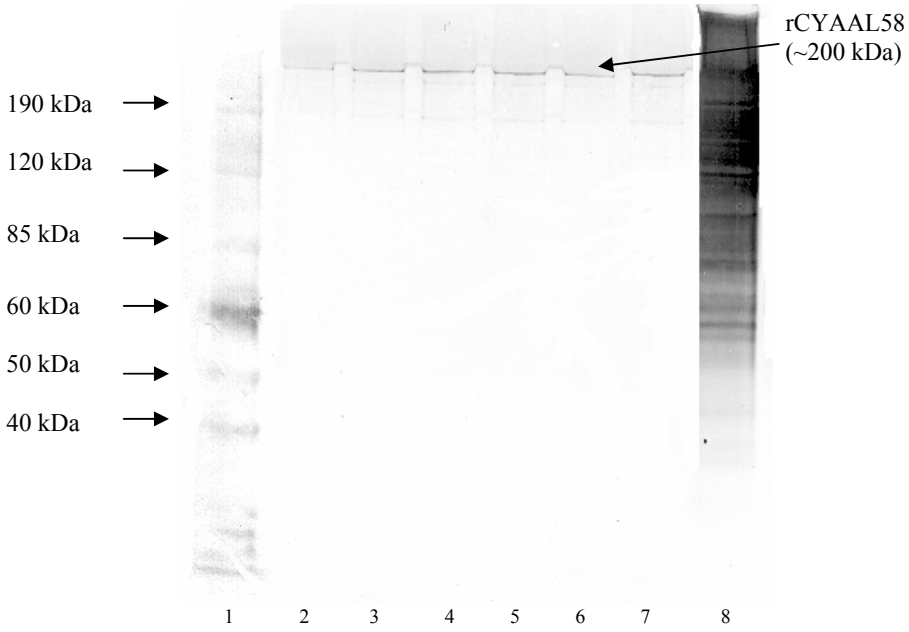

Figure 3.2: Detection of adenylate cyclase toxin expression in *B. pertussis* Tohama I. Detection of AC-Hly with 3D1 monoclonal IgG1 antibody in lysate of: original *B. pertussis* Tohama I stock (lane 2), single passaged Tohama I (lane 3), double passaged Tohama I (lane 4), second passage of *B. pertussis* Tohama I (lanes 5, 6 and 7) and purified AC-Hly (List) (lane 8). Lane 1 - Pre-stained BenchMark protein ladder (190kDa to 10kDa - Invitrogen)

3.3.2 Optimum Exposure Time of Mice to *B. pertussis* Aerosols

In order to determine the duration of exposure required to obtain a SLID of approximately 5×10^5 CFU/lung, naïve mice were exposed to aerosols of virulent *B. pertussis* Tohama I for various periods of time that ranged from 5 to 30 minutes. The data from the SLID experiment demonstrated a predictable dose-response relationship between length of exposure and the subsequent bacterial load in the lungs (Figure 3.3). No bacteria were detected in the lungs of the non-exposed group, which indicated that prior to challenge the lungs were free from *B. pertussis* or other contaminants. After a 5 minute exposure to aerosols, the mean count was 2.36×10^5 ($\pm$ 3.49×10^4) CFU/lung,

whereas a 10 minute exposure resulted in a roughly two-fold increase to 5.71×10^5 ($\pm$ 1.28×10^5) CFU/lung. Following 20 and 30 minutes of contact with the aerosols the mean lung counts increased to 1.03×10^6 ($\pm$ 1.15×10^5) and 1.13×10^6 ($\pm$ 1.29×10^5) CFU respectively, both of which were beyond the targeted SLID. From this data, a 10 minute exposure to aerosols from a 1×10^{10} CFU/ml suspension of *B. pertussis*, was deemed to be the time required to obtain a SLID of 5×10^5 CFU/mouse lung

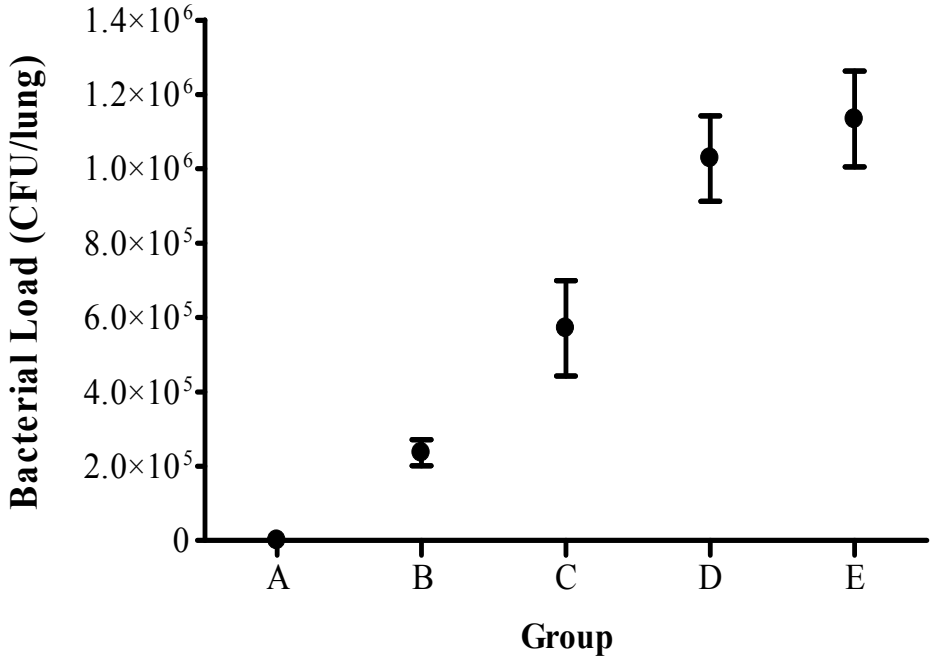

Figure 3.3: Bacterial loads in the lungs after various periods of exposure to *B. pertussis* Tohama I aerosols. Balb/C mice were exposed to aerosols from a 1×10^{10} CFU/ml suspension of Tohama I for: A – 0 minutes/negative control (n = 4); B – 5 minutes (n = 6); C – 10 minutes (n = 6); D – 20 minutes (n = 7) and E – 30 minutes (n = 8). Points represent mean count ± SE.

3.3.3 Assessment of the Aerosol Challenge Protocol

The capacity of the sub-lethal aerosol challenge protocol to successfully induce an infection was demonstrated by recording the numbers of *B. pertussis* in the lungs of unimmunised mice over a two week post-challenge period. Reproducibility of the challenge protocol was also confirmed by comparing the colonisation of lungs across three separate experiments (Figure 3.4). Experiment 1 showed that after an initial colonisation of 5×10^5 CFU/lung, *B. pertussis* proliferated to a mean of 1.31×10^6 CFU/lung after 4 days and to a maximum of 3.12×10^6 at day 7. Bacterial counts at day

14 showed a 4-fold reduction to a level that approached the initial colonisation rate. A similar pattern of lung colonisation was observed in the second and third experiments. Statistical comparison of the three experiments indicated the challenge method was reliable and reproducible. No significant differences were observed between the counts at day 4, day 7 or day 14. In each experiment, there was low mouse to mouse variation in the lung counts at each time-point as evident by the low standard error values.

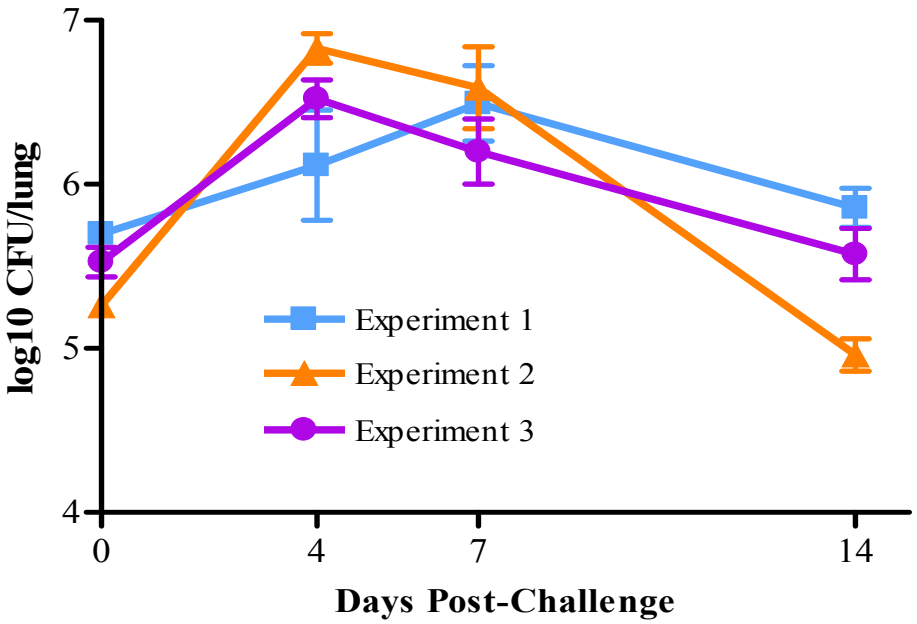

Figure 3.4: Reliability and reproducibility of the in-house aerosol challenge model. Points represent the mean ± SE bacterial counts in the lungs of unimmunised mice in three separate experiments after exposure to a SLID of *B. pertussis*.

3.4 Discussion

The Kendrick test has long been considered the "gold-standard" for determining the potency of whole-cell vaccines. However, for as yet unknown reasons the Kendrick test fails to predict the potency of modern acellular vaccines (Mills et al. 1998; Xing et al. 1999). With the exception of Japan, the introduction of acellular vaccines and their gradual replacement of the WCV have led to a shift towards the use of respiratory challenge models with mounting evidence questioning the validity of the IC protection test. These concerns surround the lack of parallels with natural infection, different host-pathogen interactions at the intracerebral versus respiratory sites of colonisation, an absence of mucosal immune effector mechanisms and inconsistencies among more

recent clinical trials (Standfast 1958; Preston & Evans 1963; Greco et al. 1996; Simondon et al. 1998; Dagg, Xing & Walker 1999). Sato et al. (1980) were the first to describe aerosol challenge as a viable alternative to the Kendrick test. Although *B. pertussis* is not a natural pathogen of mice, Sato and colleagues demonstrated that inhalation of high numbers of the virulent Tohama I strain produced a uniform and reproducible infection in the lower respiratory tract of mice. Their results showed that aerosol challenge produced an infection that was dose-dependant. Higher doses such as exposure to 1×10^{11} CFU/ml suspensions for 30 minutes were lethal, whereas the lower dose of 1×10^{9} CFU/ml for 30 minutes was sub-lethal and were characterised by a progression from colonisation to proliferation and an eventual convalescence. Sato et al. (1980) also observed a strong association between *B. pertussis* and ciliated epithelial cells using scanning electron microscopy and physiological indicators of infection such as a reduction in body weight, pulmonary oedema and leukocytosis, all of which are consistent with the human infection. Another similarity found between infection in mice and humans was the higher severity of infection with decreasing age (Xing et al. 1999).

Following construction of the chamber, the initial goal was to confirm the virulence of the challenge strain. *B. pertussis* is sensitive to environmental cues and can respond by reversibly shifting from a state of virulence to avirulence. Phase variation is known to occur spontaneously at a frequency of between one per 10^{3} to 10^{6} organisms or in response to growth at low temperatures or in the presence of a high concentration of $MgSO_4$ or nicotinic acid (Weiss & Hewlett 1986). The mechanism of the phase shift is a two-component signal transduction system, known as BvgA/S that directly up or down-regulates the expression of most *B. pertussis* virulence factors. In order to ensure that *B. pertussis* Tohama I cultured from glycerol broths stored at -70°C were virulent, two rounds of *in vivo* mouse passages were carried out. The virulence of the challenge strain was confirmed after passage by the presence of hemolysis upon culture of the pathogen from the lung on BG agar, and the expression of AC-Hly.

The next stage of development of an aerosol challenge method was the determination of an optimum SLID. By using a suspension of 1×10^{10} CFU/ml, it was found that a 10 minute exposure to aerosols provided a colonisation rate of around 5×10^{5} CFU/lung, which has been identified as an ideal sub-lethal dose from a consensus of previous

aerosol challenge studies (Sato et al. 1980; Guiso et al. 1999; Xing et al. 1999). Challenge in chambers based on the Sato design required a 30 minute exposure time to obtain an equivalent infectious dose. According to the WHO Expert Committee on Biological Standardisation a period not exceeding 2.5 hours was required between preparation of the *B. pertussis* suspension and administration to the last mouse in pertussis challenge experiments (WHO 1975). Therefore in order to challenge a large population of mice (i.e. 120 mice in the first DNA vaccine trial and 84 mice in the second DNA vaccine trial) with a single batch culture of *B. pertussis*, it was necessary to reduce the period of exposure required to obtain the 5×10^5 CFU/lung SLID, especially when taking into account the lag period that is necessary to allow for complete removal of aerosols from the chamber. The main advantages of a shortened exposure time with the in-house chamber design were optimum viability of the challenge suspensions and reduced holding stress to mice. Xing et al. (1999) achieved a SLID of 1×10^5 CFU/lung within 5 minutes exposure after using a 1×10^{12} CFU/ml suspension. Although it is apparent that the period of exposure could have been reduced even further with a more concentrated suspension of *B. pertussis*, it was decided to persist with a 2 log less concentrated challenge suspension from a workplace health and safety perspective. The performance indicators of the in-house aerosol challenge protocol were also favourable. In the first of three experiments, a colonisation rate of 5×10^5 CFU/lung was detected several hours after infection and was followed by a gradual proliferation of *B. pertussis*. A peak in bacterial numbers was recorded at 7 days post-challenge and from this point onwards a gradual reduction in lung bacterial counts was observed. The observed colonisation profile was comparable with other aerosol challenge studies (Xing et al. 1999; McGuirk & Mills 2000; Carter et al. 2004). For instance, Mills et al. (1998) found that aerosol infection of placebo-immunised mice was consistent across numerous experiments with the greatest lung counts observed between 4 and 7 days post-challenge followed by a gradual reduction in numbers. The infection obtained with our aerosol challenge protocol was similar in two follow-up experiments. Although it would have been desirable to perform more than three experiments to validate the aerosol protocol, time constraints prevented further testing. The level of reproducibility with these chambers has been more extensively evaluated by others. Xing et al. (1999) found only slight differences in the level of colonisation across 17 separate experiments and concluded that these variations were not significant in context to the large CFU/lung scores. Canthaboo et al. (2000)

performed a further 7 experiments using an identical chamber design and found no significant variation and in light of the similar kinetics of infection between these key references studies and our in-house design, it was deemed unnecessary to perform additional reproducibility experiments.

The only concern regarding the aerosol challenge model surrounds the lack of several key clinical features. Sato et al. (1980) observed that the murine respiratory infection lacked two main clinical manifestations seen in infected infants, namely the cough and therefore any form of transmission. Later, Woods et al. (1989) showed that intra-tracheal inoculation of rats with *B. pertussis* encased in agar beads induced a respiratory infection that featured the paroxysmal coughing attacks and transmission to unchallenged cohorts. Histopathology of infected rat lungs was also found to be consistent with infant autopsy reports by way of a mild lymphocytic bronchial infiltrate associated with peribronchial hyperplasia, which progressed to necrosis and inflammation of the respiratory mucosa (Woods et al. 1989). Despite a much closer association with human pertussis, the obvious limitation with the rat model is that the mode of inoculation would not only be time-consuming but require a high level of surgical skill, notwithstanding the risk of introducing a nosocomial infection. Also, encasing micro-organisms in agar beads for challenge infection represents an artificial and unnatural means of inducing pathophysiology that has no relationship to the natural infection process induced by *B. pertussis* in humans.

In summary, the main features of an ideal challenge model are: similarity with the natural infection process, correlation with clinical efficacy data and reproducibility. The aerosol challenge model used in this project not only satisfies the aforementioned criteria but can also reflect the influence of both mucosal and systemic responses to immunisation. Although the in-house aerosol chamber was based on a well established design (Xing et al. 1999), it had several modifications that necessitated a brief evaluation of its performance and the duration of exposure required to obtain an optimum SLID. It was observed that a SLID of 5×10^5 CFU/lung could be delivered in a short period, and like its contemporaries, the in-house chamber induced a reproducible infection that enabled evaluation of the protective efficacy of the various DNA vaccines developed in this study.

Chapter 4 Filamentous Hemagglutinin DNA Vaccines: Development and Characterisation of the Immune Response in Mice Vaccinated via the IM Route

4.1 Introduction

Filamentous hemagglutinin (FHA) is an outer membrane-associated protein (OMP) which is expressed by *B. pertussis* during the initial stages of infection. It has hemagglutinating activity and mediates attachment of the pathogen to a wide range of host cells and surfaces including: ciliated and non-ciliated epithelia, macrophages and the mucous membrane of the upper and lower respiratory tracts (Relman et al. 1989). Three distinct adhesin domains have been identified within the FHA polypeptide. A carbohydrate-binding domain or lectin-like domain is believed to initiate binding to ciliated epithelial cells (Prasad et al. 1993). Once bound to ciliated epithelial cells, the pathogen releases a suite of toxins that cause local tissue damage in addition to numerous systemic effects. Local damage to the respiratory epithelium enables FHA to bind to non-ciliated epithelial cells and extracellular matrices such as the sulphated glycoconjugates of respiratory mucous, via a second heparin-binding domain (Hannah et al. 1994). FHA also has an RGD tripeptide, a motif known to mediate adhesion to macrophages through interaction with the leukocyte integrin CR3 (Relman et al. 1989; Villard et al. 2005). RGD sequences are common to other bacterial adhesins such as pertactin, viral adhesins like the VP1 protein of the foot-and-mouth disease virus and also mammalian cell adhesins such as fibronectin (Baranowski et al. 2001).

Apart from its important role in pathogenesis, FHA is highly immunogenic. Protective antibodies have been detected in animals infected with *B. pertussis* (Kimura et al. 1990; Amsbaugh, Li & Shahin 1993). Active immunisation with purified FHA or passive immunisation with anti-FHA monoclonal or polyclonal antibody improves the clearance of bacteria from the lungs of challenged mice (Edwards et al. 1995). In humans, high titers of anti-FHA antibodies have been detected in convalescent sera or following immunisation with Pw and Pa vaccines (Mills et al. 1998; Simondon et al. 1998). Mucosal routes of immunisation with FHA have also proven to be effective in animal models. Oral immunisation with *S. typhimurium* expressing FHA has elicited both a systemic and mucosal antibody response (Guzman et al. 1991), whereas intranasal

vaccination with purified FHA or oral immunisation with FHA encapsulated in biodegradable particles (in combination with other pertussis antigens) elicits a predominately secretory response that protects animals from respiratory challenge with *B. pertussis* (Roberts et al. 1993; Conway et al. 2001).

FHA is a large and complex antigen. Synthesised as a 367-kDa precursor, it undergoes extensive post-translational modification with processing at the N-terminus and cleavage of a large portion of the C-terminus (Locht et al. 1993). The 220 kDa mature FHA is transported to the periplasm and exported through the outer membrane with the aid of a number of accessory proteins and chaperones, encoded by the *fhaA*, *fhaC*, *fhaD* and *fhaE* genes (Renauld-Mongenie et al. 1996). Whilst the complexity of FHA favours the generation of a robust immune response it highlights one of the limitations of DNA vaccine technology – the potential for a low-level of heterologous expression of large bacterial antigens within transfected mammalian cells (Ertl & Thomsen 2003; Garmory, Brown & Titball 2003). An obvious strategy to alleviate this concern was to use truncated fragments that would allow an increased level of heterologous expression and at the same time maintain a high level of immunogenicity.

4.1.1 The Immunodominant Region of FHA as a Candidate for DNA Vaccination

The identification of FHA as a potent immunogen and its inclusion as a component of acellular pertussis vaccines lead to the identification of specific B and T cell epitopes within a broad immunodominant region. Delisse-Gathoye et al. (1990) were the first to describe the immunodominant region, located between the central RGD sequence and the C-terminus of the mature antigen (Figure 4.1). Further analysis of FHA with monoclonal and polyclonal antibodies has confirmed that the majority of B-cell epitopes are located in this domain (Leininger et al. 1997; Wilson et al. 1998; Piatti 1999). Although not as well characterised, a number of T cell determinants have also been identified within this region. Di Tommaso et al. (1991) screened various recombinant and proteolytic fragments of FHA with 16 human T cell clones from two adult donors, one of whom had recovered from a childhood infection and the other received the Pw vaccine as a child. It was found that 7 of the 16 clones recognised epitopes within the immunodominant region, more specifically the C-terminal end from residues 1653 to 2347. Some of the remaining clones were shown to be reactive against the N-terminal portion of mature FHA, outside the immunodominant region (Di Tommaso et al. 1991).

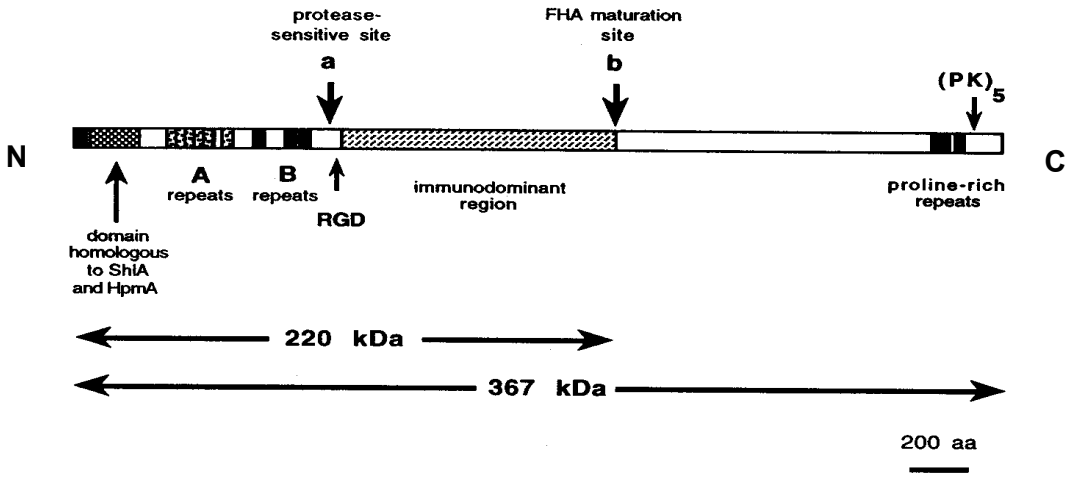

Figure 4.1: Important domains and regions of the precursor and mature FHAB antigen. A schematic representation of the amino acid sequence of FHA including domains with a high degree of homology to the hemolysins of *Serratia marcescens* (ShlA) and *Proteas mirabilis* (HpmA), and an Arg-Gly-Asp (RGD) tripeptide. The black box at the N terminus represents the signal peptide.
Source: Locht et al. (1993), p. 654.

4.1.2 Selection of a Second FHA-Based DNA Vaccine Encoding Dominant B cell Epitopes

Leininger et al. (1997) identified specific B cell epitopes within the immunodominant region by screening truncated fragments FHA with a series of monoclonal antibodies. This method identified a concentration of epitopes at the COOH terminus referred to as the type I domain, and another collection of epitopes near the NH_2-terminus referred to as the type II domain. Although sera from convalescent patients and Pa-immunised infants recognised both type I and type II domains, the majority (78%) of the monoclonal antibodies tested were specific for epitopes within the type I domain, which suggested that this region possessed the majority of immunologically important epitopes (Leininger et al. 1997). Wilson et al. (1998) performed a more detailed analysis of the type I domain using a phage display library with polyclonal antibodies and also found that in general the highly reactive B cell epitopes were at the COOH-terminus (Figure 4.2). Piatti (1999) confirmed the location of these B cell determinants by screening twelve truncated FHA-fusion proteins with a panel of human convalescent sera. The dominant epitopes were shown to reside within a 1-kb region that spanned positions 5781-6800 of the published Genbank sequence (accession # M60351.1).

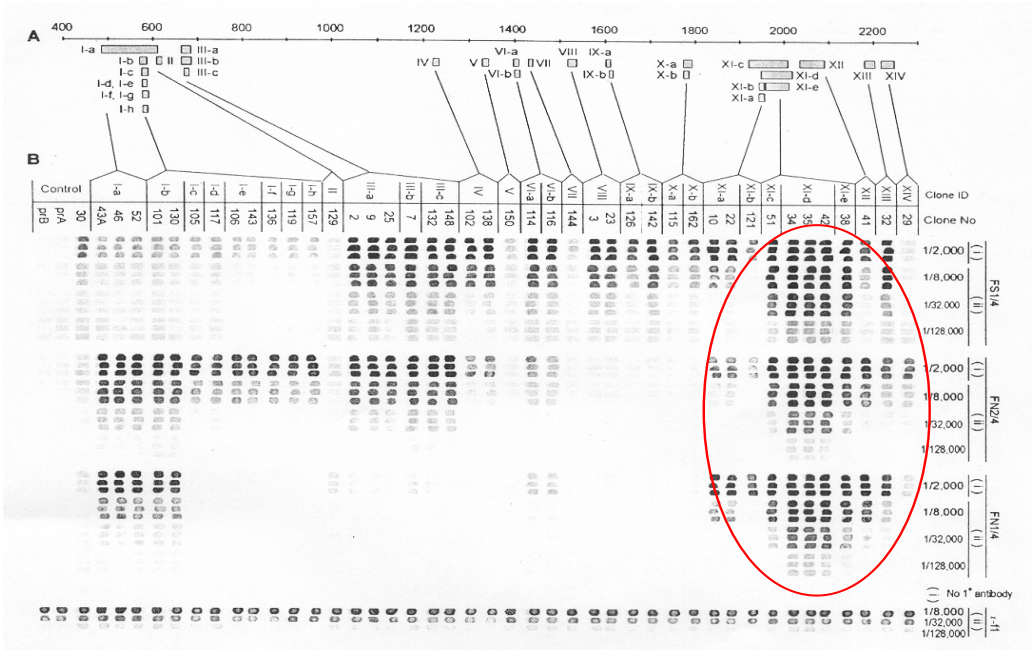

Figure 4.2: Antigenic analysis of mature FHAB with a phage display library (Wilson et al. 1998). Encircled area of the mapped B cell epitopes represents the region of FHAB antigen selected as the coding region of the second FHA-based DNA vaccine. A: relative positions within FhaB of antibody-reactive clones assayed by means of dot blotting with anti-FHA antibodies and serum. B: dot blot assay of 30 unique clones.

The results from these epitope mapping studies were combined to formulate two FHA-based DNA vaccines: a fragment encoding the entire immunodominant region **(*fhaB1*)**, and a smaller 1010 bp fragment from bases 5740 to 6750 encoding the dominant B cell epitopes of the type I domain **(*fhaB2*)**. The specific aims of work embodied in this study were to:

1. Develop two FHA eukaryotic expression constructs which encoded a broader but poorly-defined immunodominant region and a smaller well-defined fragment encompassing dominant B cell epitopes;
2. Develop corresponding prokaryotic expression constructs for small-scale purification of the respective recombinant proteins from *E. coli*;
3. Examine transient expression of recombinant FHA antigens within mammalian cell lines *in vitro*;
4. Evaluate the immunogenicity of the FHA-based DNA vaccines following immunisation of mice by the IM route, and
5. Compare the protective efficacy of the mono-component DNA vaccines versus a commercial DTaP using an aerosol challenge model of *B. pertussis* infection.

4.2 Methodology

This section provides an outline of the methods used, with specific details for each procedure described in Chapter 2.

4.2.1 Culture of *B. pertussis* Tohama I and Extraction of Genomic DNA

B. pertussis Tohama I was cultured as described in Chapter 2, Section 2.1.4. Genomic DNA was isolated from *B. pertussis* using the Aqua Pure Genomic DNA Isolation Kit (BioRad) according to the manufacturer's instructions (see Chapter 2, Section 2.4).

4.2.2 Construction of Recombinant Plasmids

4.2.2.1 PCR Amplification of fhaB Gene from Genomic DNA

Custom-made primers (Geneworks) were designed to amplify two different fragments of the *fhaB* gene based on the published DNA sequence (Genbank accession no. M60351.1, Appendix D.2). Separate sense PCR amplification primers were required for cloning into the pcDNA3.1D/V5-His-TOPO and pTrcHis2-TOPO vectors due to the requirement for a kozak leader sequence (CACCATGG) for unidirectional cloning into pcDNA3.1D/V5-His-TOPO and optimal expression of heterologous proteins in transfected mammalian cell lines (see Appendix B).

The PCR of *fhaB1* and *fhaB2* was performed according to the parameters outlined in Chapter 2, Section 2.5.2. Briefly, Platinum Pfx DNA polymerase was used to generate blunt-ended products for cloning into pcDNA3.1D/V5-His-TOPO, whereas Platinum Taq High Fidelity DNA polymerase was used to generate products with a single deoxyadenosine added to the 3' ends for cloning into pTrcHis2-TOPO.

The larger of the two *fhaB* fragments (*fhaB1*), encoding the entire immunodominant region, was amplified with the following sets of primer sequences:

For cloning into pcDNA3.1D/V5-His-TOPO
FHAB3577DNAF – 5' CACCATGGACATCATCATGGATGCGAAG 3'
FHAB6966R – 5' CGTGAAAATATGCGGATTCACGAGCAC 3'
For cloning into pTrcHis2-TOPO
FHAB3259TRCF – 5' GTTAAGAGCGATGGCGGCCTTCAG 3'
FHAB6966R – 5' CGTGAAAATATGCGGATTCACGAGCAC 3'

The smaller of *fhaB* fragment (*fhaB2*), encoding dominant B cell epitopes, was amplified with the following sets of primer sequences:

For cloning into pcDNA3.1D/V5-His-TOPO
FHAB5740DNAF – 5' CACCATGGGCTATGTCAACTACTGG 3'
FHAB6750R – 5' CAGCACGGTTTGTTCCTTGGGATAGAA 3'
For cloning into pTrcHis2-TOPO
FHAB5737TRCF – 5' ATCGGCTATGTCAACTACTGGTTG
FHAB6750R – 5' CAGCACGGTTTGTTCCTTGGGATAGAA 3'

PCR products were purified as described in Chapter 2, Section 2.5.3.

4.2.2.2 Cloning of PCR products into pTrcHis2-TOPO and pcDNA3.1D/V5-His-TOPO Expression Vectors

The pTrcHis2-TOPO TA Expression Kit (Invitrogen) was used for cloning and transformation, according to the manufacturer's instructions. Purified PCR products were cloned into the pTrcHis2-TOPO vector and transformed into *E. coli* TOP10 cells as described in Chapter 2, Section 2.6. Inserts cloned into the pTrcHis2 vector were flanked by a c-myc epitope and a hexahistidine sequence at the 3' end. Similarly, the pcDNA3.1D/V5-His TOPO Cloning Kit (Invitrogen) was used for cloning and transformation, according to the manufacturer's instructions (Chapter 2, Section 2.6). Inserts cloned into the pcDNA3.1D/V5-His-TOPO vector were flanked by a V5 epitope and a hexahistidine tag. The maps of pcDNA3.1D/V5-His-TOPO and pTrcHis2-TOPO vectors are shown in Appendix C.1 and C.2. Plasmid maps of pcDNA3.1D/*fhaB1* and pcDNA3.1D/*fhaB2* constructs are shown in Figures 4.3 and 4.4 respectively.

4.2.2.3 Screening of Plasmid Clones

Plasmid DNA from selected TOP10 transformants was isolated by Miniprep and analysed on a 1% TAE agarose gel (Chapter 2, Section 2.6.2). The insert sequences of the recombinant plasmids with a molecular weight equivalent to the expected size were verified by DNA sequencing as described in Chapter 2, Section 2.7. Primer sequences used for sequencing are listed in Table 2.3. Small fragments obtained from sequencing reactions were assembled into contiguous sequences (contigs) and aligned against the Published *fhaB* gene sequence using the ClustalW program provided by ANGIS (Australian National Genomic Information Service).

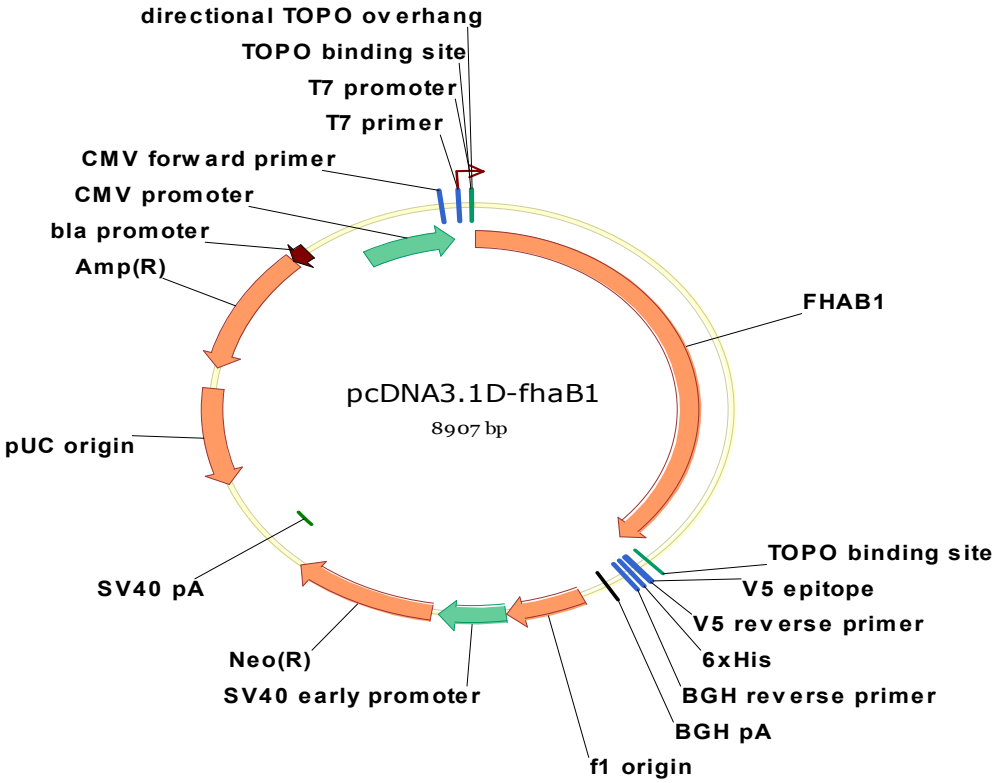

Figure 4.3: Map of pcDNA3.1D/*fhaB1* generated using Vector NTI (Invitrogen). A description of each of the vector features is outlined in Appendix C.1.

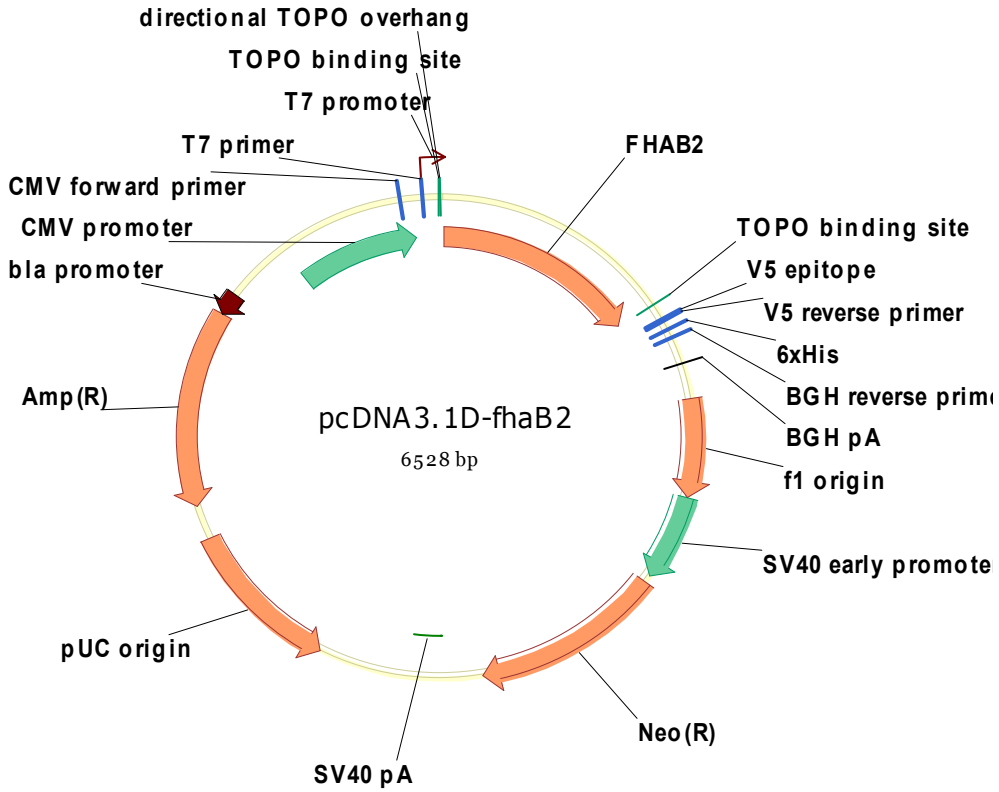

Figure 4.4: Map of pcDNA3.1D/*fhaB2* generated using Vector NTI (Invitrogen).

4.2.3 Expression of Recombinant FHAB Fusion Proteins in *E. coli*

Pilot expression of recombinant FHAB1 and rFHAB2 fusion proteins, encoded by pTrcHis2/*fhaB1* and pTrcHis2/*fhaB2* respectively, in TOP10 *E. coli* was performed according to the protocol outlined in Chapter 2, Section 2.12.1. Expression of rFHAB1 and rFHAB2 was detected by Western blotting with a HRP conjugated anti-His (C-term) antibody (Chapter 2, Section 2.11). To enhance expression levels from the GC-rich *B. pertussis* inserts, pTrcHis2/*fhaB1* and pTrcHis2/*fhaB2* plasmids were transformed into BL21 CodonPlus(RP) *E. coli* as described in Chapter 2, Section 2.12.2.

4.2.4 Purification of Recombinant FHAB Fusion Proteins

Recombinant FHAB1 and rFHAB2 fusion proteins were purified from BL21 CodonPlus(RP) *E. coli* by Ni-NTA affinity chromatography under denaturing conditions as previously described (Chapter 2, Section 2.12.3). Purified protein was checked by SDS-PAGE and Western blot (Chapter 2, Sections 2.10 and 2.11). Eluates were concentrated by ultra-centrifugation (Appendix C.6) and the yield estimated with a modified Bradford assay (Chapter 2, Section 2.12.4) for use in ELISA and *in vitro* re-stimulation of splenocytes.

4.2.5 Transient Transfection of COS-7 Mammalian Cells and Expression

The mammalian cell line COS-7 was transfected with pcDNA3.1D/*fhaB1* and pcDNA3.1D/*fhaB2* as previously described (Chapter 2, Section 2.9). Constitutive expression of rFHAB1 and rFHAB1 was detected by Western blot using an AP-conjugated anti-V5 antibody (Chapter 2, Section 2.11).

4.2.6 Transformation of *S. typhimurium* with pcDNA3.1D/*fhaB1* and pcDNA3.1D/*fhaB2*

The *aro*A *Salmonella typhimurium* strain SL3261 was transformed with the pcDNA3.1D/*fhaB1* and pcDNA3.1D/*fhaB2* plasmids for the oral delivery of the DNA vaccine constructs (Chapter 8). To successfully transform the *E. coli* methylated plasmids into SL3261 (intact restriction system), an initial passage through the r^-m^+ *S. typhimurium* strain P9121 was required. *S. typhimurium* P9121 were transformed using the heat shock method (Chapter 2, Section 2.14.2), whereas *S. typhimurium* SL3261 were transformed by electroporation (Chapter 2, Section 2.14.3).

4.2.7 Immunisation of Mice with pcDNA3.1D/*fhaB1* and pcDNA3.1D/*fhaB2* via the IM Route

A component of the first DNA vaccine efficacy trial involved immunisation of mice with the two FHA DNA vaccines as described in Chapter 2, Section 2.15.2. Immunisation involved direct injection of naked DNA into the quadriceps. To obtain sufficient plasmid for a 3 dose regime, large scale isolation was performed from 2.5 L cultures of TOP10-pcDNA3.1D/*fhaB1* and TOP10-pcDNA3.1D/*fhaB2* (Chapter 2, Section 2.15.2.1). Of the 17 mice per group vaccinated, 5 were sampled for serum and organs two weeks after the third and final dose.

4.2.8 Determination of Immune Response and Protective Efficacy to DNA Vaccination

Serum samples were analysed for the presence of Ag-specific IgG, IgG1 and IgG2a by ELISA (Chapter 2, Section 2.16.2) and western blot (Appendix B.1), whereas the cytokine profiles of re-stimulated splenocytes was used to evaluate the CMI response (Chapter 2, Section 2.16.1). The remaining 12 mice/group were challenged with a predetermined sub-lethal infectious dose (SLID) of virulent *B. pertussis* Tohama I as previously described (Chapter 2, Section 2.15.4). The lungs and serum of challenged mice were sampled at three time points with 4 mice sacrificed at each point. For direct comparison of DNA vaccine efficacy, a group of mice vaccinated with the pcDNA3.1D/V5-His-TOPO vector was included along with the placebo and DTaP (Infanrix™) vaccinated groups.

4.3 Results

4.3.1 Isolation of Tohama I Genomic DNA

Genomic DNA (gDNA) was isolated from Tohama I *B. pertussis* using a commercial kit. As the potential yield from a *B. pertussis* extraction was not known, gDNA was extracted from the lysates of cells pelleted from 0.5ml, 1.0ml, 2.0ml and 3.0ml broth cultures (Figure 4.5; lanes 2 to 5 respectively). Using the Low DNA Mass Ladder (Invitrogen) as a guide, there was no difference in the yield between the various lysate volumes with each of the eluates having an estimated concentration of 1µg/µl. For all PCR reactions gDNA was diluted to a working concentration of 1ng/µl.

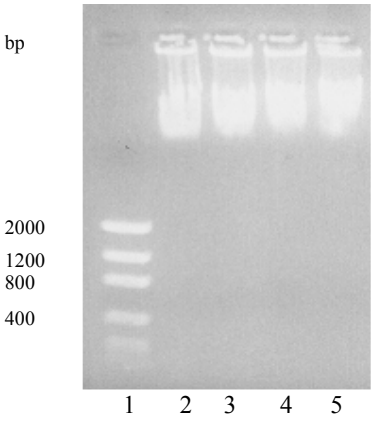

Figure 4.5: Genomic DNA extracted from *B. pertussis* Tohama I. Genomic DNA extracted from 0.5ml (lane 2), 1.0ml (lane 3), 2.0ml (lane 4) and 3.0ml (lane 5) of Tohama I broth culture. Lane 1: Low DNA Mass ladder (2000bp to 100bp - Invitrogen).

4.3.2 PCR Amplification of *fhaB1* Fragments

For cloning into pcDNA3.1D/V5-His-TOPO, a 3389 bp *fhaB1* target was amplified from gDNA using the FHAB3577DNAF and FHAB6966R primers with Platinum *Pfx* DNA polymerase (Figure 4.6; A). For cloning into pTrcHis2-TOPO, a 3707 bp *fhaB1* fragment was amplified using the FHAB3259TRCF and FHAB6966R primers with Platinum *Taq* DNA polymerase (Figure 4.6; B).

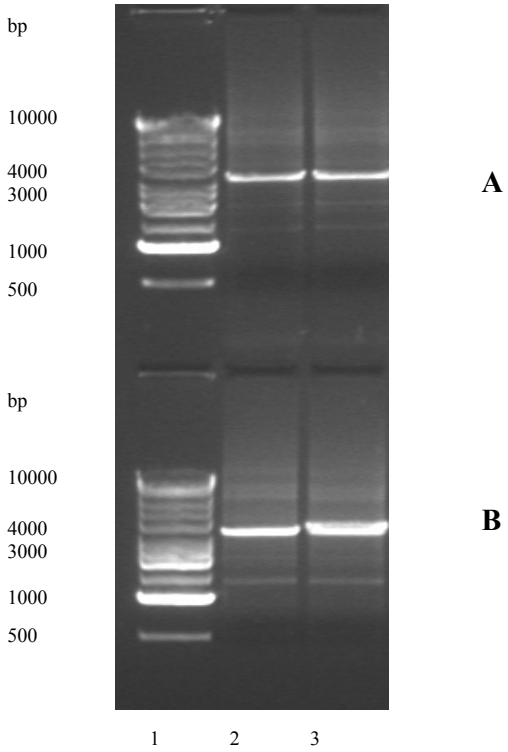

Figure 4.6: PCR of *fhaB1* fragment. (A) PCR of *fhaB1* with FHAB3577DNAF/FHAB6966R primers. Lane 1 – Kilobase Marker (10kb to 0.5kb - Amersham), lanes 2 and 3 – *fhaB1* from Tohama I gDNA. (B) PCR of *fhaB1* with FHAB3259TRCF/FHAB6966R primers. Lane 1 – Kilobase Marker (Amersham), lanes 2 and 3 – *fhaB1* target from Tohama I gDNA.

4.3.3 Generation of pcDNA3.1D/*fhaB1* and pTrcHis2/*fhaB1* Plasmids

The blunt-ended 3389 bp fragment was purified and cloned into pcDNA3.1D/V5-His-TOPO for eukaryotic expression. At the same time, the 3707 bp A-tailed fragment was cloned into the pTrcHis2-TOPO vector for inducible expression in *E. coli.* To screen for positive clones, the pcDNA3.1D/*fhaB1* and pTrcHis2/*fhaB1* plasmids were first isolated from TOP10 and checked for correct molecular weight on a 1% agarose gel. Putative pcDNA3.1D/*fhaB1* and pTrcHis2/*fhaB1* plasmids with a molecular weight of just over 8 Kb and 8.9 Kb respectively (Figure 4.7; lanes 3 and 5) were sequenced to confirm that the inserts were error-free and in the correct orientation. DNA sequences of the cloned *fhaB1* and *fhaB2* inserts were compared against the published Genbank sequence of *fhaB* (accession # M60351.1) by alignment using the ClustalW program (WebAngis). No base mismatches detected (Appendix D.3).

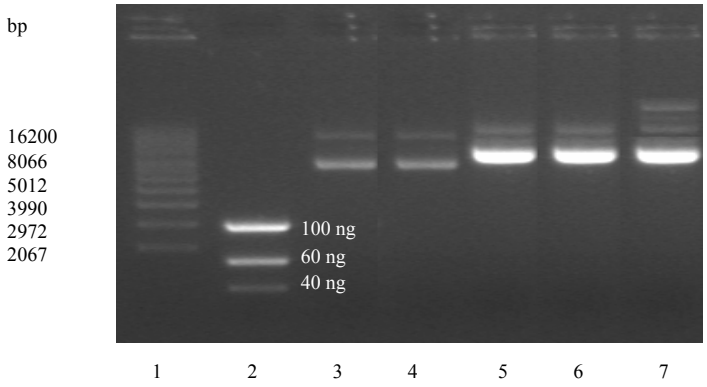

Figure 4.7: pTrcHis2/*fhaB1* and pcDNA3.1D/*fhaB1* plasmids. Lane 1 – Supercoiled DNA ladder (16,210bp to 2,067bp - Invitrogen); lane 2 – Low DNA Mass ladder (Invitrogen); lane 3 – pTrcHis2/*fhaB1* from TOP10; lane 4 – pTrcHis2/*fhaB1* from BL21 Codon Plus; lane 5 - pcDNA3.1D/*fhaB1* from TOP10; lane 6 - pcDNA3.1D/*fhaB1* from P9121; lane 7 - pcDNA3.1D/*fhaB1* from SL3261.

4.3.4 Amplification of the *fhaB2* Fragment by PCR

For cloning into pcDNA3.1D/V5-His-TOPO, a 1010 bp *fhaB2* target was amplified using FHAB5740DNAF and FHAB6750R primers with Platinum *Pfx* DNA polymerase (Figure 4.8; lanes 3 to 5). For cloning into pTrcHis2-TOPO, a 1013 bp *fhaB2* fragment was amplified using the FHAB5737TRCF and FHAB6750R primers with Platinum *Taq* DNA polymerase (Figure 4.8; lanes 6 to 8).

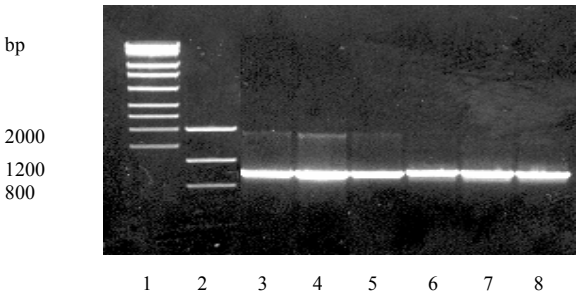

Figure 4.8: PCR of *fhaB2* fragment. Lane 1 - Kilobase Marker (10kb to 0.5kb - Amersham); lane 2 - Low DNA Mass ladder (Invitrogen); lanes 3 to 5 – amplification of *fhaB2* target with Platinum *Taq* High Fidelity DNA polymerase and FHAB5737TRCF/FHAB6750R oligonucleotides; lanes 6 to 8 – amplification of *fhaB* target with Platinum *Pfx* DNA polymerase and FHAB5740DNAF/FHAB6750R oligonucleotides.

4.3.5 Generation of pcDNA3.1D/*fhaB2* and pTrcHis2/*fhaB2* Plasmids

The blunt-ended 1010 bp fragment amplified with Platinum *Pfx* DNA polymerase was purified and cloned into pcDNA3.1D/V5-His-TOPO. Alternatively, the 1013 bp A-tailed fragment amplified with Platinum *Taq* DNA polymerase was cloned into the pTrcHis2-TOPO vector for expression in *E. coli.* To screening for positive clones, pcDNA3.1D/*fhaB2* and pTrcHis2/*fhaB2* plasmids were isolated from TOP10 *E. coli* and checked on a 1% agarose gel. Putative pTrcHis2/*fhaB2* and pcDNA3.1D/*fhaB2* and plasmids with a molecular weight equivalent to 5394 bp and 6528 bp respectively (Figure 4.9, lanes 3 and 5) were verified by sequencing. DNA sequences of the cloned *fhaB2* inserts were compared against the published Genbank sequence of *fhaB* (accession # M60351.1) by alignment using the ClustalW program (WebAngis). No base mismatches were observed (Appendix D.3).

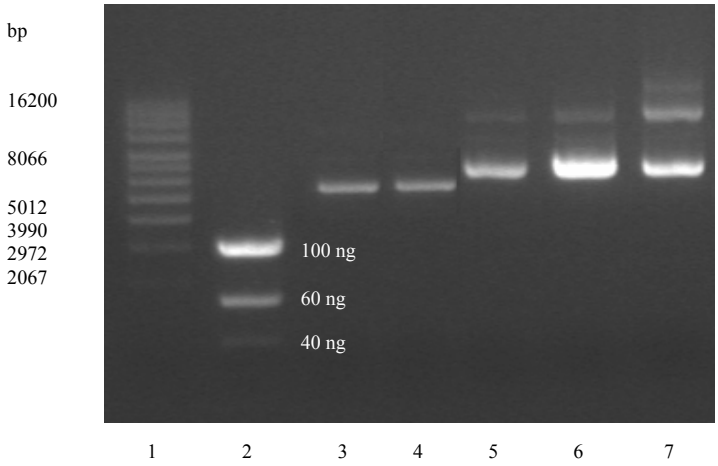

Figure 4.9: Isolation of pTrcHis2/*fhaB2* and pcDNA3.1D/*fhaB2* plasmids. Lane 1 – Supercoiled DNA ladder (16,210bp to 2,067bp - Invitrogen); lane 2 – Low DNA Mass ladder (Invitrogen); lane 3 – pTrcHis2/*fhaB2* from TOP10; lane 4 – pTrcHis2/*fhaB2* from BL21 Codon Plus; lane 5 - pcDNA3.1D/*fhaB2* from TOP10; lane 6 - pcDNA3.1D/*fhaB2* from P9121; lane 7 - pcDNA3.1D/*fhaB2* from SL3261.

For evaluation as an oral DNA vaccine, pcDNA3.1D/*fhaB1* and pcDNA3.1D/*fhaB2* were transformed into *aro*A *S. typhimurium* strain SL3261 (see Chapter 8). In order to successfully transform SL3261, the sequence confirmed pcDNA3.1D/*fhaB1* and pcDNA3.1D/*fhaB2* plasmids were passaged through the r^-m^+ *S. typhimurium* strain P9121. The plasmid DNA isolated from P9121 and SL3261 was identical in size to the pcDNA3.1D/*fhaB1* and pcDNA3.1D/*fhaB2* isolated from *E. coli* (Figures 4.7 and 4.9).

4.3.6 Expression of rFHAB1 and rFHAB2 in TOP10 *E. coli*

Low but detectable expression of rFHAB1 was observed following induction of TOP10-pTrcHis2/*fhaB1* with 1mM IPTG (Figure 4.10). In a pilot experiment, the duration of IPTG induction had little effect on the level of rFHAB1 expression with no observable increase between 1 and 5 hours of incubation (Figure 4.10, lanes 2 to 6). Using the BenchMark protein standard as a guide, the size of the band was ~ 130 kDa and corresponded to the predicted molecular weight of 119 kDa for rFHAB1 when the additional fusion components i.e. 6×His tag were taken into account. Although detected by western blot, the rFHAB1 band could not be distinguished in the SDS-PAGE gel of TOP10-pTrcHis2/*fhaB1* lysates when compared to the lysate of TOP10 without plasmid DNA.

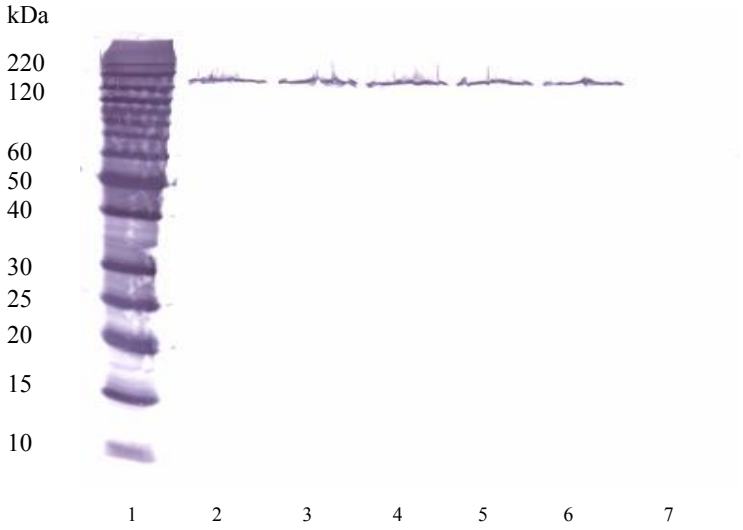

Figure 4.10: Western blotting of rFHAB1 expression in TOP10 *E. coli*. Lane 1 - BenchMark protein ladder (220kDa to 10kDa - Invitrogen); lanes 2 to 6 – rFHAB1 expression following 1, 2, 3, 4, or 5 hours of IPTG induction; and lane 7 - TOP10 (negative control) induced for 4 hours under identical conditions.

As with rFHAB1 there was a low level of expression of rFHAB2 in TOP10 *E. coli* following induction with IPTG. Again rFHAB2 could not be distinguished from native TOP10 proteins after SDS-PAGE analysis and could only be observed after western blot with an anti-His antibody (Figure 4.11). The size of rFHAB2 was estimated to be between 45 and 50 kDa compared to the calculated size of 37 kDa (Translate GCG program, WebAngis). The additional molecular weight was most likely due to additional fusion components of the vector such as the myc epitope and histidine tag (see Appendix C.2).

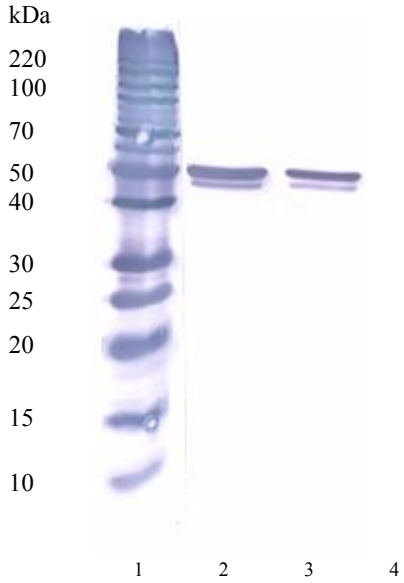

Figure 4.11: Western blotting of rFHAB2 expression in *E. coli*. Lane 1 - BenchMark protein ladder (220kDa to 10kDa - Invitrogen); Lanes 2 and 3 – rFHAB2 expression from TOP10 following 3 and 4 hours of induction. Lane 4 - Native TOP10 (negative control) induced for 4 hours under identical conditions.

4.3.7 Purification of rFHAB1 and rFHAB2 from BL21 *E. coli*

Early attempts at purification of rFHAB1 and rFHAB2 from induced TOP10 lysates by Ni-NTA affinity chromatography were unsuccessful (results not shown). The low level of recombinant protein expression was thought to be the main contributing factor and more specifically because of variations in codon usage between the GC-rich inserts from *B. pertussis* and the TOP10 *E. coli* host. Comparison of codon usage tables for both *B. pertussis* Tohama I and *E. coli* K12 revealed a coding GC of 68.10% versus 51.8% and obvious differences in codon preferences, in particular CGC, GCC, CCC and GGC (Appendix B.2). To overcome the codon usage issue, pTrcHis2/*fhaB1* and pTrcHis2/*fhaB2* were transformed into *E. coli* strain BL21 CodonPlus(DE3)-RP, a specialised strain engineered to provide enhanced expression of recombinant proteins from GC-rich templates. The level of rFHAB1 and rFHAB2 expression was enhanced in the BL21 strain following induction with IPTG (results not shown). This improved level of expression allowed for successful purification of rFHAB1 (Figure 4.12) and rFHAB2 (Figure 4.13) under denaturing conditions. The recombinant protein eluates were of reasonable purity (50 to 80%) with a low level of contaminating protein, which probably represented degraded rFHAB1 peptides.

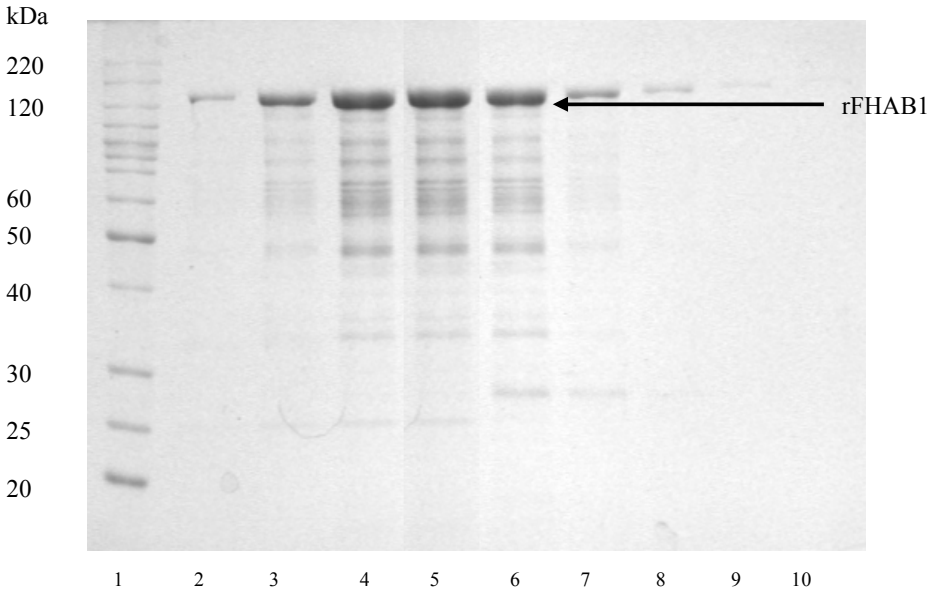

Figure 4.12: PAGE of affinity purified rFHAB1 from BL21 CodonPlus *E. coli*. Lane 1: BenchMark Protein ladder (220kDa to 10kDa - Invitrogen); lanes 2 to 5 – elution from 0.5 ml flow-throughs of Buffer D pH 5.9; lanes 6 to 10 – elution from 0.5 ml flow-throughs of Buffer E pH 4.5.

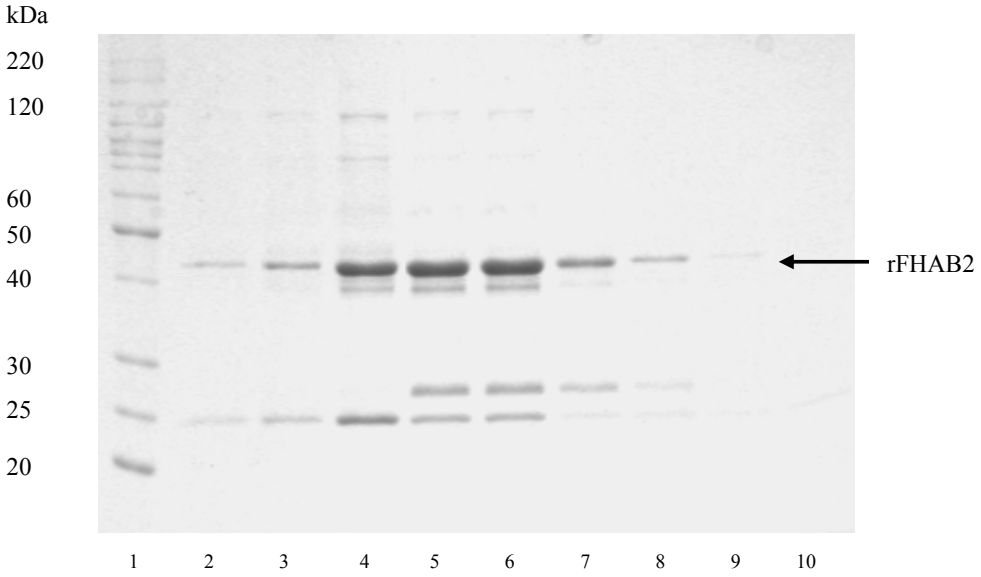

Figure 4.13: PAGE of affinity purified rFHAB2 from BL21 CodonPlus *E. coli*. Lane 1: BenchMark Protein ladder (220kDa to 10kDa - Invitrogen); lanes 2 to 5 – elution from 0.5 ml flow-throughs of Buffer D pH 5.9; lanes 6 to 10 – elution from 0.5 ml flow-throughs of Buffer E pH 4.5.

4.3.8 Transient Expression of rFHAB1 and rFHAB2 in COS-7

To determine if rFHAB1 and rFHAB2 could be constitutively expressed within a mammalian cell system, COS 7 cells were transfected with pcDNA3.1D/*fhaB1* or pcDNA3.1D/*fhaB2* plasmids. Expression of rFHAB1 and rFHAB2 was observed by western blotting with an anti-V5 epitope antibody (Figure 4.14). The sizes of rFHAB1 and rFHAB2 were similar to that of expression in *E. coli* and as an additional check, COS 7 cells were transfected with pcDNA3.1D/*fhaB1* or pcDNA3.1D/*fhaB2* isolated from *S. typhimurium* strains P9121 and SL3261 (see Chapter 8). No differences in size or level of expression were observed from the different sources of pDNA. No bands corresponding to either rFHAB1 or rFHAB2 were observed in the respective negative controls (COS-7 cells transfected with self-ligated pcDNA3.1-V5-TOPO) (not shown).

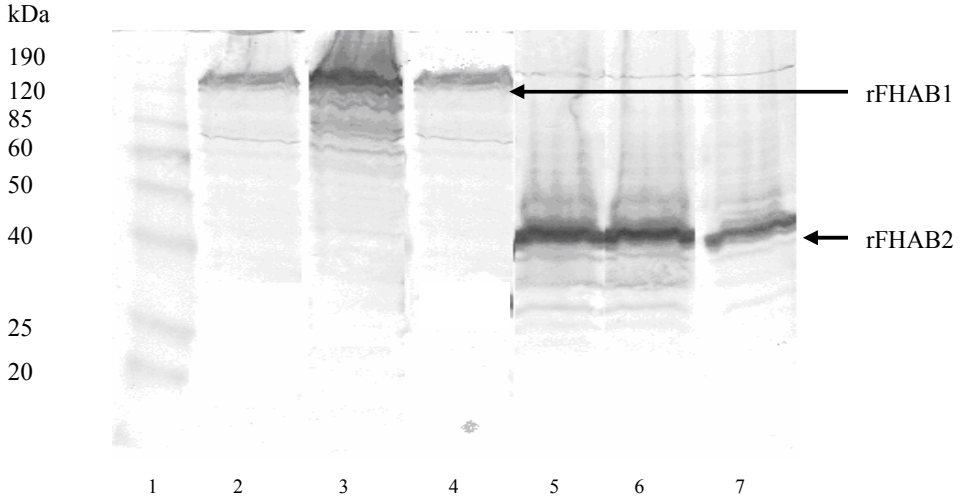

Figure 4.14: Western blotting of rFHAB1 and rFHAB2 expression in COS-7 cells. Lane 1 - Pre-stained BenchMark protein ladder (190kDa to 10kDa - Invitrogen). Expression of rFHAB1 following transfection of COS-7 with pcDNA3.1D/*fhaB1* isolated from TOP10 (lane 2), P9121 (lane 3) and SL3261 (lane 4). Expression of rFHAB2 following transfection of COS-7 with pcDNA3.1D/*fhaB2* isolated from TOP10 (lane 5), P9121 (lane 6) and SL3261 (lane 7).

4.3.9 Immunogenicity of pcDNA3.1D/*fhaB1* and pcDNA3.1D/*fhaB2* DNA Vaccines

BALB/c mice were immunised with three doses of pcDNA3.1D/*fhaB1* or pcDNA3.1D/*fhaB2* via the IM route as described in section 2.15.2. Two weeks after the second and final booster, five mice from each group were killed and samples taken for immunological assays. Splenocytes isolated from pooled spleens from each group of five mice were cultured in the presence of antigens for 24 to 72 hours (see section 2.16.1). Supernatants from the 24 hour cultures were tested for IL-2, whereas IFN-γ and IL-4 production was determined from the supernatants of 72 hour cultures.

4.3.9.1 *Measurement of IFN-γ Production by Antigen-Stimulated Splenocytes*

Splenocytes of mice immunised with pcDNA3.1D/*fhaB1* produced a very high level of IFN-γ when stimulated with either rFHAB1 (8490 pg/ml) or ConA (6000 pg/ml), a natural T cell mitogen (Figure 4.15). In contrast, splenocytes from vector and DTaP-immunised mice produced low levels of IFN-γ when cultured with rFHAB1. Interestingly, when the splenocytes from pcDNA3.1D/*fhaB1*-immunised mice were stimulated with either DTaP or a heat-killed *B. pertussis* lysate much lower levels of IFN-γ was produced compared to stimulation with the recombinant antigen with only 227 and 428pg/ml respectively (Appendix D.2).

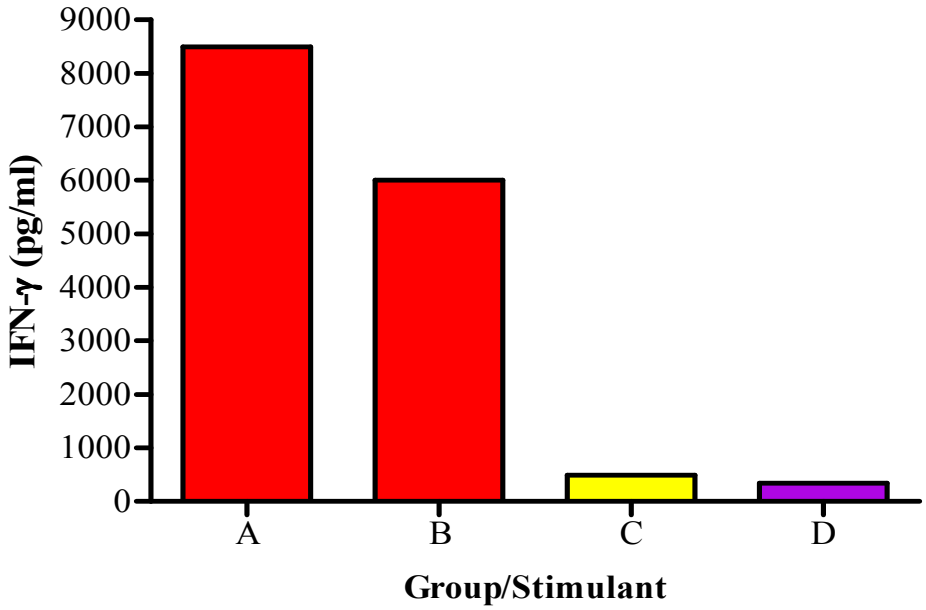

Figure 4.15 IFN-γ production from rFHAB1-stimulated splenocytes. Splenocytes from pcDNA3.1D/*fhaB1*-immunised mice stimulated with 5μg/ml rFHAB1 (A) and 2.5μg/ml ConA (B). Splenocytes from vector-immunised mice stimulated with 5μg/ml rFHAB1 (C). Splenocytes from DTaP-immunised mice stimulated with 5μg/ml rFHAB1 (D).

Splenocytes from pcDNA3.1D/*fhaB2*-immunised mice produced only a moderate level of IFN-γ when stimulated with rFHAB2 (2051 pg/ml) with a more than four-fold reduction in IFN-γ levels compared with the pcDNA3.1D/*fhaB1* group. When stimulated with ConA these splenocytes produced a high level of IFN-γ (6367 pg/ml). Again, a much lower level of IFN-γ was produced when these cells were stimulated with DTaP (199 pg/ml) or a heat-killed *B. pertussis* lysate (176 pg/ml) rather than recombinant antigen (Appendix D.2).

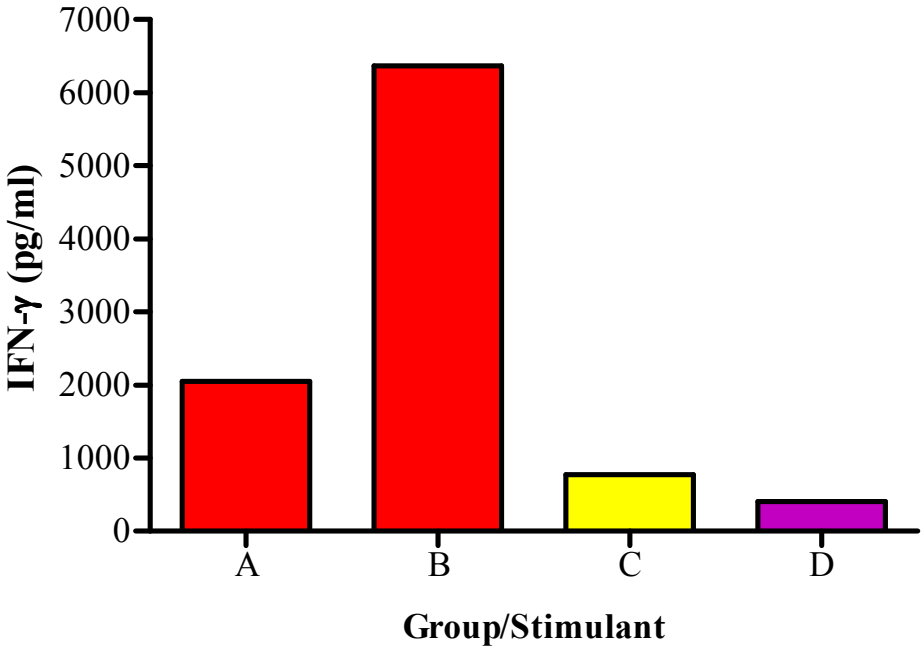

Figure 4.16: IFN-γ production from rFHAB2-stimulated splenocytes. Splenocytes from pcDNA3.1D/*fhaB2*-immunised mice stimulated with 5μg/ml rFHAB2 (A) and 2.5μg/ml ConA (B). Splenocytes from vector-immunised mice stimulated with 5μg/ml rFHAB2 (C). Splenocytes from DTaP-immunised mice stimulated with 5μg/ml rFHAB2 (D).

4.3.9.2 *Measurement of IL-2 Production by Antigen-Stimulated Splenocytes*

Overall, the IL-2 profiles for both pcDNA3.1D/*fhaB1* and pcDNA3.1D/*fhaB2*-immunised mice were not as clear cut as the IFN-γ readings.

Splenocytes from pcDNA3.1D/*fhaB1*, vector or DTaP-immunised mice all produced low levels of IL-2 upon stimulation with rFHAB1, viz. 169, 47.9 and 46.2pg/ml respectively (Figure 4.17). In contrast, a very large amount of IL-2 was produced when splenocytes were stimulated with ConA (10780 pg/ml).

There was even less of a difference in IL-2 production between pcDNA3.1D/*fhaB2*, vector and DTaP-immunised mice when stimulated with rFHAB2, viz. 77, 39 and 31pg/ml respectively (Figure 4.18). Again there were very high levels of IL-2 produced (11345 pg/ml) from the ConA stimulated splenocytes.

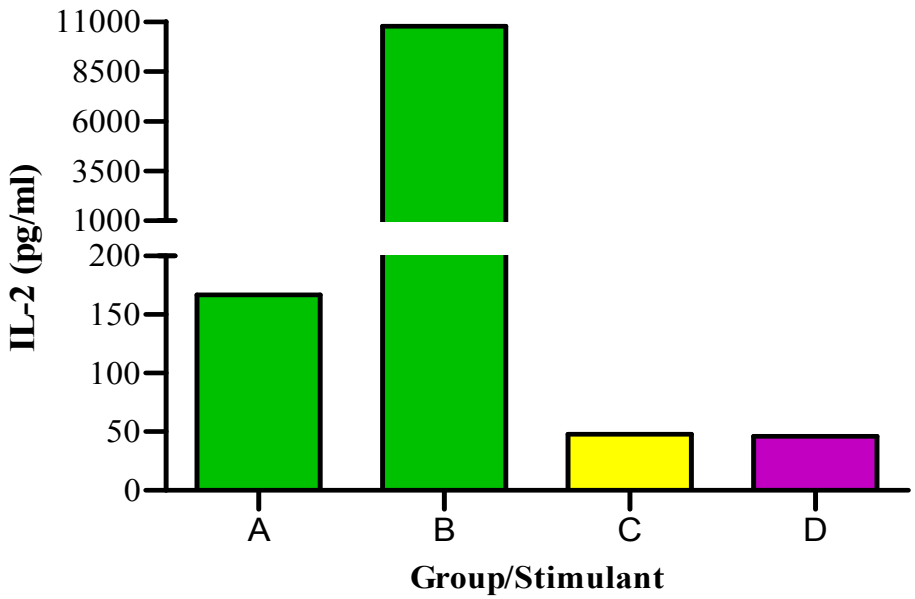

Figure 4.17: IL-2 production from rFHAB1-stimulated splenocytes. Splenocytes from pcDNA3.1D/*fhaB1*-immunised mice stimulated with 5µg/ml rFHAB1 (A) and 2.5µg/ml ConA (B). Splenocytes from vector-immunised mice stimulated with 5µg/ml rFHAB1 (C). Splenocytes from DTaP-immunised mice stimulated with 5µg/ml rFHAB1 (D).

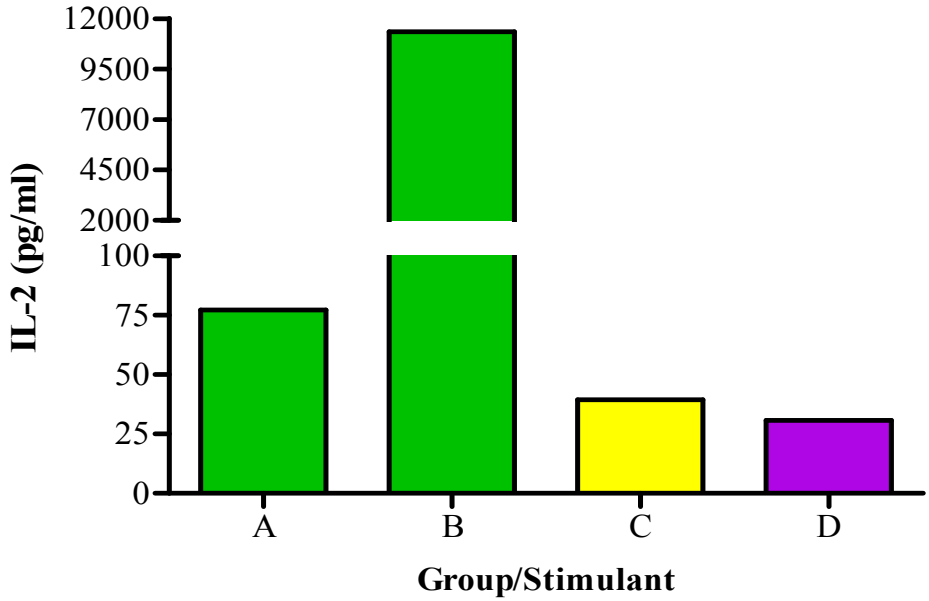

Figure 4.18: IL-2 production from rFHAB2-stimulated splenocytes. Splenocytes from pcDNA3.1D/*fhaB2*-immunised mice stimulated with 5µg/ml rFHAB2 (A) and 2.5µg/ml ConA (B). Splenocytes from vector-immunised mice stimulated with 5µg/ml rFHAB2 (C). Splenocytes from DTaP-immunised mice stimulated with 5µg/ml rFHAB2 (D).

4.3.9.3 *Measurement of IL-4 Production by Antigen-Stimulated Splenocytes*

The highest level of IL-4 was detected in the culture supernatants of splenocytes isolated from DTaP-immunised mice (Table 4.1). Mice immunised with pcDNA3.1D/*fhaB1* produced very low levels of IL-4 when stimulated with rFHAB1. No IL-4 was produced from the cells of pcDNA3.1D/*fhaB2* or vector-immunised mice. There were consistently high levels of IL-4 produced when splenocytes were stimulated with ConA (Table 4.1).

Table 4.1: IL-4 production from rFHAB1 and rFHAB2-stimulated splenocytes.

Stimulant	µg/ml	IL-4 (pg/ml)			
		pcDNA3.1D/*fhaB1*	pcDNA3.1D/*fhaB2*	pcDNA3.1 vector	DTaP
rFHAB1	5	39.7	0	0	75.9
rFHAB2	5	7.5	0	0	72.9
ConA	2.5	462.8	462.8	511.3	1081.9

4.3.9.4 *Serum Antibody Response Following Vaccination with pcDNA3.1D/fhaB1 or pcDNA3.1D/fhaB2 by ELISA via the IM Route*

Two weeks after the second booster dose, blood samples were taken from mice to assess the endpoint IgG titers to rFHAB1, rFHAB2 and native FHA. Analysis of the serum IgG responses revealed that mice immunised with DTaP developed substantially higher anti-rFHAB1 titers when compared to mice immunised with pcDNA3.1D/*fhaB1* (Figure 4.19) and pcDNA3.1D/*fhaB2* (Figure 4.20). This difference was not found to be statistically significant due to large variability across each of the five mice in the DTaP group (Appendix D).

The serum IgG titers specific for native FHA showed a similar trend. Mice vaccinated with DTaP generated significantly higher titers than mice immunised with pcDNA3.1D/*fhaB1* ($P < 0.001$) or the vector only ($P < 0.05$) (Figure 4.19). Surprisingly, mice immunised with the pcDNA3.1 vector also had higher titers of rFHAB1 and FHA-specific IgG than mice immunised with pcDNA3.1D/*fhaB1*, although there was no significant difference.

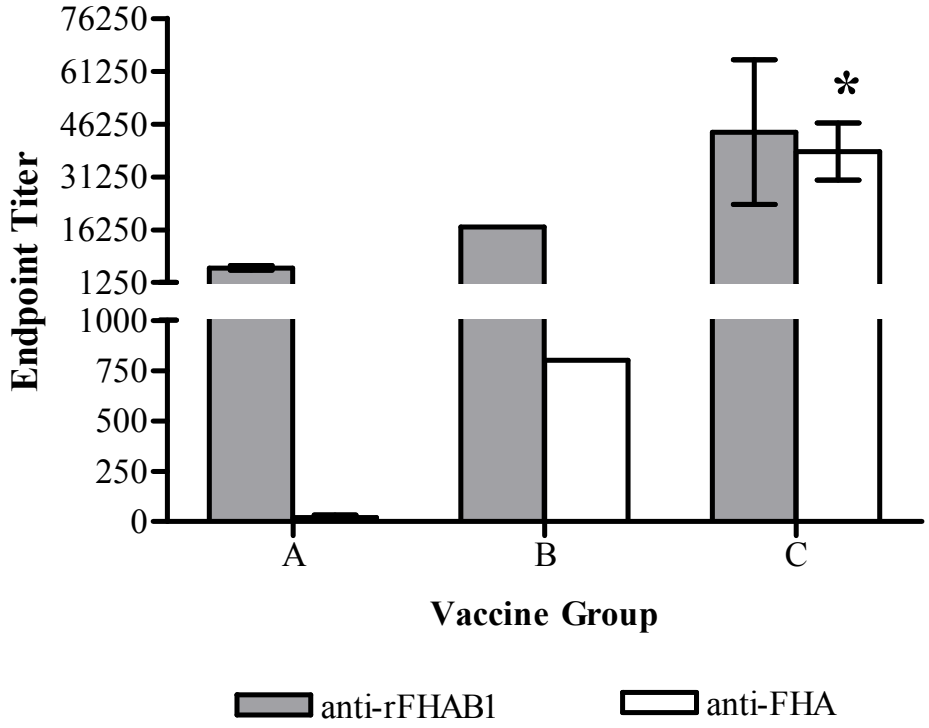

Figure 4.19: Endpoint titers of anti-filamentous hemagglutinin IgG in the sera of mice vaccinated with pcDNA3.1D/*fhaB1*. Mean +/- SE from five mice vaccinated with pcDNA3.1D/*fhaB1* (A); pooled serum from five mice vaccinated with pcDNA3.1 vector (B); and mean +/- SE from five mice vaccinated with DTaP (C). * Significantly different from pcDNA3.1D/*fhaB1* ($P < 0.001$) and vector ($P < 0.05$).

Overall the IgG response to the pcDNA3.1D/*fhaB2* DNA vaccine was lower than pcDNA3.1D/*fhaB1* (Figure 4.20). Again, the anti-rFHAB2 titer was much higher in mice vaccinated with the vector only compared with mice vaccinated with pcDNA3.1D/*fhaB2*. This trend was also evident for the anti-FHA response, with endpoint titers of 35 for pcDNA3.1D/*fhaB2* versus 6400 for the vector only group. The anti-FHA titers in mice vaccinated with DTaP were significantly greater than in the serum of mice vaccinated with pcDNA3.1D/*fhaB2* ($P < 0.01$) or the vector only ($P < 0.05$). DTaP-immunised mice also generated higher IgG titers specific for rFHAB2 than pcDNA3.1D/*fhaB2*-immunised mice, but the difference was not significant (Figure 4.20).

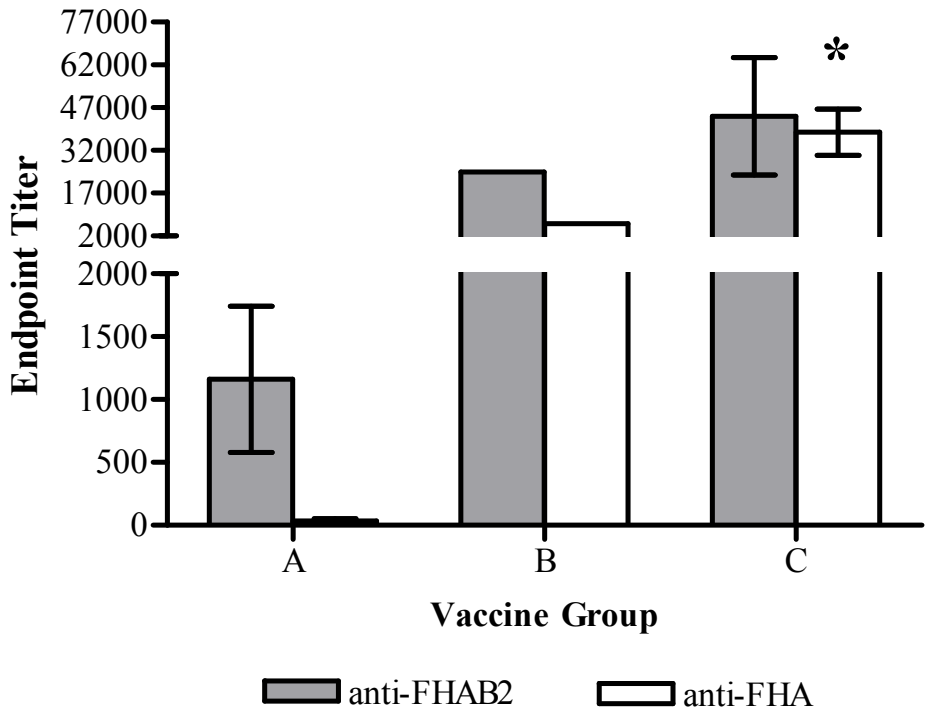

Figure 4.20: Endpoint titers of anti-filamentous hemagglutinin IgG in the sera of mice vaccinated with pcDNA3.1D/*fhaB2*. A - mean +/- SE from five mice immunised with pcDNA3.1D/*fhaB2*, B –pooled serum from five mice vaccinated with pcDNA3.1 vector and C - mean +/- SE from five mice immunised with DTaP. * Significantly different from pcDNA3.1D/*fhaB2* ($P < 0.01$) and vector ($P < 0.05$).

4.3.10 Protective Efficacy of pcDNA3.1D/*fhaB1* and pcDNA3.1D/*fhaB2* Vaccination

To assess the protective efficacy of the FHA-based DNA vaccines, immunised mice were challenged with a pre-determined SLID of Tohama I two weeks after the second booster. The bacterial load in the lungs of test and control mice was then determined at three time points over a 14 day period in order to compare the rate of clearance. Mice vaccinated with pcDNA3.1D/*fhaB1* showed an enhanced rate of clearance compared to the placebo or pcDNA3.1D vector groups (Figure 4.21). At 4 days post-challenge, all three groups had a slight two to four-fold increase in bacterial numbers. However, whereas the negative control mice failed to reduce the bacterial burden beyond the initial colonisation level pcDNA3.1D/*fhaB1*-immunised mice showed a 7-fold reduction in numbers by day 7, which progressed to a roughly 750-fold reduction after two weeks.

The kinetics of clearance from pcDNA3.1D/*fhaB2*-immunised mice showed a trend that was similar but less pronounced to that of mice immunised with pcDNA3.1D/*fhaB1*. A slight increase after day 4 was followed by 7-fold decrease in CFU/lung by day 7 and a 60-fold decrease at by day 14 (Figure 4.22).

Mice immunised with DTaP completely cleared *B. pertussis* from the lungs within 7 days. Unlike the other three groups there was no initial increase in bacterial numbers, but rather a slight decrease was observed after 4 days. From day 4 there was a rapid clearance with no bacteria were detected in lung homogenates in day 7 or day 14. Overall, the commercial vaccine was clearly more efficacious than either of the mono-component DNA vaccines.

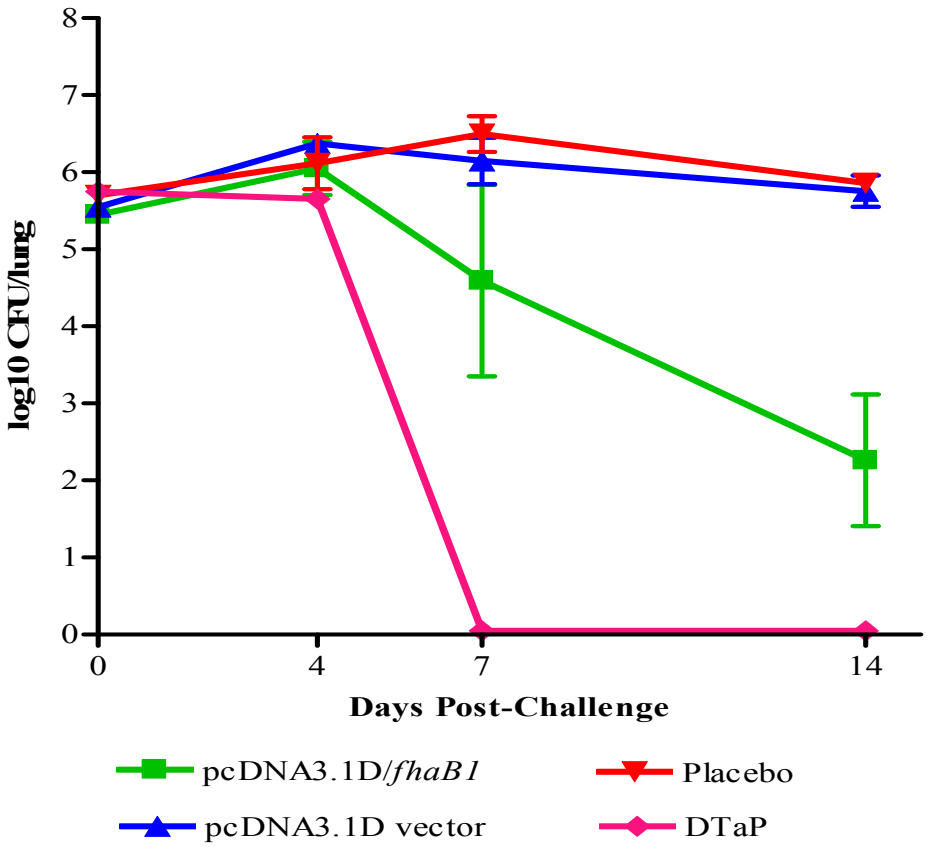

Figure 4.21: Clearance of *B. pertussis* from the lungs of mice vaccinated with pcDNA3.1D/*fhaB1* following aerosol challenge. Data points represent mean count of CFU/lung from 5 mice +/- SE.

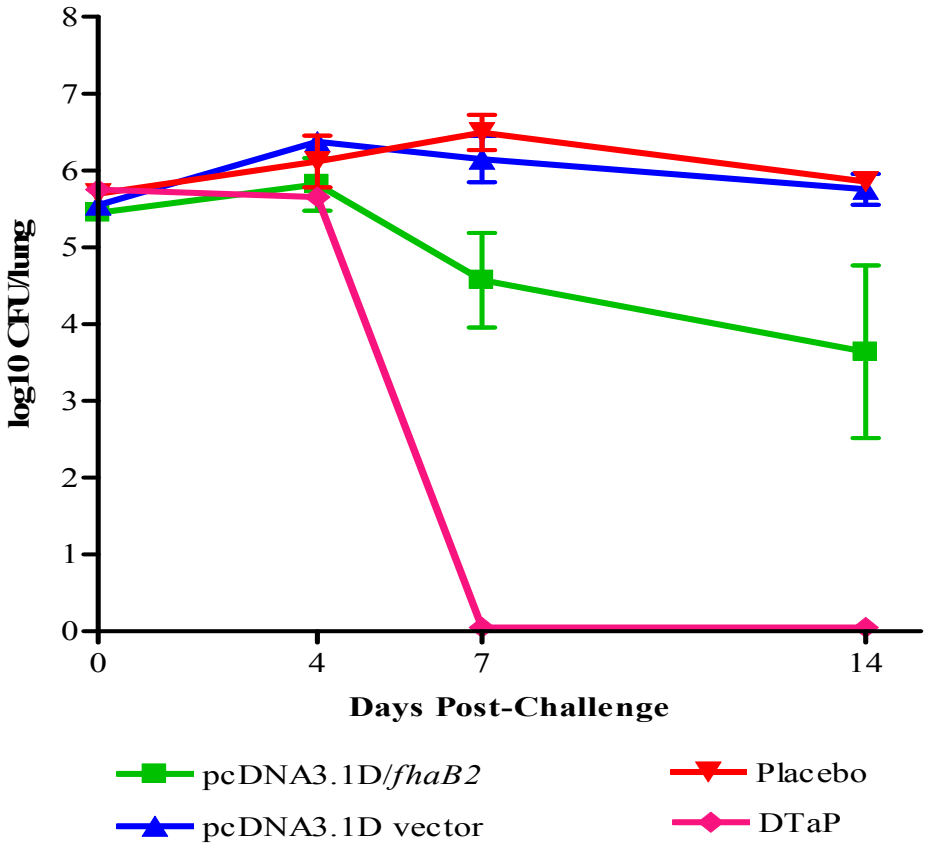

Figure 4.22: Clearance of *B. pertussis* from the lungs of mice vaccinated with pcDNA3.1D/*fhaB2* following aerosol challenge. Data points represent mean count of CFU/lung from 5 mice +/- SE.

When the clearance data from each group of mice was expressed as a percentage of the unimmunised mice (placebo group) the bacterial load in mice immunised with pcDNA3.1D/*fhaB1* was reduced to 0.09% of the untreated controls by day 14 post-challenge (Table 4.2). Similarly, *B. pertussis* counts in the pcDNA3.1D/*fhaB2* group were reduced to 1.08%. The calculation of a clearance index (CI) indicated that mice vaccinated with either DTaP or the FHA-based DNA vaccines had a significantly improved rate of clearance of *B. pertussis* than mice vaccinated with the placebo or vector only (Table 4.2).

Table 4.2: Post-challenge clearance data from mice vaccinated with pcDNA3.1D/*fhaB1* and pcDNA3.1D/*fhaB2*.

Vaccine Group	% of Untreated Control[a]			CI[b]
	Day 4	Day 7	Day 14	
Placebo	n/a	n/a	n/a	151.75 ± 1.65
Vector	255.7	63.5	115.3	151.78 ± 4.68
pcDNA3.1D/*fhaB1*	152.7	2.23	0.09	49.55 ± 11.65 *
pcDNA3.1D/*fhaB2*	89.3	2.12	1.08	67.24 ± 16.91 **
DTaP	30.5	0	0	22.43 ± 0.05 ***

[a] Quantitated as percentage of bacteria cleared from the lungs relative to the untreated group of mice.

[b] Clearance Index ± SE = mean log10 of *B. pertussis*/ml × days (van den Berg et al. 2001).

* Significantly different from placebo ($P < 0.005$) and vector ($P < 0.001$).

** Significantly different from placebo ($P < 0.05$) and vector ($P < 0.01$).

*** Significantly different from placebo and vector ($P < 0.001$).

4.4 Discussion

The vast majority of DNA vaccine studies to date have focussed on viral pathogens, which is logical considering that DNA vaccination mirrors the natural process of viral infection i.e. transfection of host cells and endogenous expression of antigen. However, the spectrum of DNA vaccine studies has recently expanded to include intracellular bacteria such as *Chlamydia pneumoniae*, *Mycobacterium tuberculosis*, *Listeria monocytogenes* and even extracellular pathogens like *Bacillus anthracis* and *Borrelia burgdorferi* (Zhong et al. 1997; Cornell et al. 1999; Delogu et al. 2000; Price et al. 2001; Scheiblhofer et al. 2003). Whilst many of the DNA vaccines directed against these bacterial pathogens have shown promise, with the exception of a recent study by Kamachi et al. (2003), it has not yet been explored as an alternative means of immunisation against pertussis. Kamachi et al. (2003) reported that three gene-gun injections of a pertussis toxin S1 DNA vaccine induced a specific antibody response that protected against an IC challenge. In contrast, the IM route of delivery and an aerosol challenge were chosen for this investigation and the rationale for this selection was three-fold. Firstly, it was necessary to establish a proof of principle for each of the target antigens. Muscles are highly efficient in the uptake of naked DNA and in the short history of nucleic acid vaccination it has been the most commonly used mode of

delivery. The IM route has been shown to be a simple and reliable means of generating an efficacious immune response with many examples of protection from an experimental challenge (Johnson et al. 2000; Miyaji et al. 2002; Senna et al. 2003). Secondly, recent studies of immunity to pertussis have highlighted the importance of CMI in the rapid clearance of bacteria and for long-term protection (Mills et al. 1993; Redhead et al. 1993), and when delivered via the IM route DNA vaccines are known to be potent inducers of a Th1-type response (Cornell et al. 1999; Rodriguez et al. 2001; Scheiblhofer et al. 2003). Last, the benefits of aerosol exposure over the intracerebral challenge include greater reproducibility, a better correlation with clinical trial data of whole cell and acellular pertussis vaccines, and a more accurate representation of the natural infectious process in humans. The basis for the selection of an aerosol challenge method has been discussed in more detail in Chapter 3 (Sections 3.1 and 3.4).

The immunogenic potential of the two FHA DNA vaccines was assessed by direct IM injection. Results showed that the DNA vaccines induced a predominantly cell-mediated immune response with elevated production of the Th1 cytokines IFN-γ and IL-2 and low levels of serum IgG. It was observed that the potent CMI response generated in DNA-vaccinated mice significantly improved the rate of clearance of *B. pertussis* after aerosol challenge compared with sham-immunised mice. Our findings support those of Redhead et al. (1993), in which a murine respiratory infection model was used to demonstrate that CMI plays an important role in protection from *B. pertussis*. They found that natural infection and immunisation with a Pw vaccine stimulated the production of IFN-γ and IL-2 from splenocytes but no IL-4 or IL-5, whereas immunisation with an acellular vaccine induced IL-5 production and the generation of high serum IgG titers. Interestingly, the rate of clearance following an aerosol challenge was enhanced in the Th1 responsive mice (Redhead et al 1993). In a different study by the same group of researchers, it was found that T cell-deficient mice developed a persistent infection that could not be cleared following an aerosol challenge (Mills et al. 1993). Transfer of immune CD4+ Th1 cells to athymic mice restored the potential for clearance of an experimental challenge within 21 days, whereas passive immunisation with immune sera only showed a slight improvement (Mills et al. 1993). The results from this preliminary DNA vaccine study have confirmed that an FHA-specific CMI response contributes to protection against *B. pertussis* in the absence of high titers of antibodies.

The FHA-specific Th1 cell response to DNA vaccination did not appear to be mitigated by using the larger of the two truncated antigens, as seen by the high levels of IFN-γ and IL-2 produced in the spleens of mice immunised with a sequence encoding the immunodominant region of FHA (pcDNA3.1D/*fhaB1*). Di Tommaso et al. (1991) has provided some insight into the location of T cell determinants with the FHA polypeptide after mapping the recognition of recombinant or proteolytic fragments with human T cell clones. Seven of the sixteen clones recognised the immunodominant region encoded by pcDNA3.1D/*fhaB1*, whereas the remaining clones either recognised a 98 kDa N-terminal fragment of did not recognise any of the subregions tested (Di Tommaso et al. 1991). Although Di Tommaso et al. (1991) identified some T cell clones that reacted specifically to the very C-terminus of mature FHA, the reduced Th1 response to the pcDNA3.1D/*fhaB2* vaccine suggests that important T cell epitopes are not closely associated with the dominant B cell epitopes and are located towards the N-terminal end of the immunodominant region. In addition to inducing lower levels of the Th1 cytokines IFN-γ and IL-2, the pcDNA3.1D/*fhaB2* vaccine also generated lower IgG titers, and a slower rate of clearance of the aerosol challenge than pcDNA3.1D/*fhaB1*. Our data has shown that reducing the size FHA to a small and well-defined group of B cell epitopes compromised its overall immunogenicity and should be taken into consideration during the development of future FHA-based vaccines.

The humoral response to the FHA DNA vaccines was compared with an acellular pertussis vaccine using an indirect ELISA, with either recombinant protein or native FHA as the capture antigen. Serum ELISA results showed that the two FHA DNA vaccines induced a poor antibody response with significantly lower titers of serum IgG than in DTaP-immunised mice. In contrast to our immunogenicity data, FHA is often a potent inducer of serum and mucosal antibodies. For instance, high titers of anti-FHA antibodies have been detected in humans that have recovered from infection (Simondon et al. 1998). FHA antibodies are also elicited to protective levels following immunisation with the whole cell and acellular pertussis vaccines (Mills et al. 1998). Intranasal immunisation of mice with FHA induces both systemic IgG as well as a strong antibody response in the lungs after repeated boosting (Roberts et al. 1993). A likely reason for the relatively poor humoral response to the FHA DNA vaccines was the tendency for IM-administered DNA vaccines to elicit a Th1-type response (Johnson et al. 2000; Epstein et al. 2002; Velikovsky et al. 2002). It has been well documented

that parenteral and in particular IM administration of naked DNA stimulates CMI, whereas dermal or mucosal delivery elicits an antibody or dichotomous response (Scheiblhofer et al. 2003). Although the precise mechanism of action of DNA vaccines has not yet been determined, they appear to mimic viral infection with endogenous expression, MHC class I presentation and activation of CD4+ Th1 cells. Furthermore, CpG motifs present at a higher frequency in the bacterial-derived plasmid backbones are known to have immunomodulatory properties, in that they act as an adjuvant for augmenting cell-mediated immune responses (Klinman et al. 1997; Jakob et al. 1998).

The structure of the truncated antigen itself could have also been a factor in the poor humoral response. Both truncated fragments were derived from the C-terminal domain. As protein folding commences at the N-terminus and is guided by the formation of secondary structures that are governed by energy minimisation, the folding of the rFHAB1 and rFHAB2 would not have been influenced by the native N-terminal residues. Hence, the structure of these truncated proteins may have been different to the native antigen, which would prevent the generation of antibodies that recognise native conformational epitopes. The results from ELISA using rFHAB1 versus native FHA as the capture antigen tended to support this notion. The antibodies generated by DNA vaccination were specific for the recombinant antigen but showed a much lower affinity for the native antigen. In contrast, IgG in the serum of mice vaccinated with DTaP and the cross-reactive IgG in the serum of vector-immunised mice recognised both forms of the antigen with equal affinity.

An unexpected outcome from the first DNA vaccine trial was the detection of cross-reactive anti-FHA antibodies in the serum of vector-immunised mice. In fact, the titers of cross-reactive IgG to both native and recombinant FHA were substantially greater in mice that received the vector only than in either of the pcDNA3.1D/*fhaB1* or pcDNA3.1D/*fhaB2*-immunised groups. A low background titer in the serum of placebo mice, which were used to generate the ELISA cut-off value, indicated that the cross-reactivity was most likely a vector-mediated effect. As the neomycin phosphotransferase gene (neomycin resistance) was the only other gene sequence under the control of a eukaryotic promoter, a cross-reaction may have occurred between the antibiotic resistance marker and FHA. Whilst it is probable that FHA shares some B cell epitopes with neomycin phosphotransferase, a BlastN search of both nucleotide

sequences or a BlastP search of protein sequences failed to show up any identity. Similar responses to vector DNA have been reported for BHV, influenza and HIV (Cox et al. 1993; Fynan et al. 1993; Wang et al. 1993). The low background in the placebo group also eliminated the possibility that the out-sourced population of specific pathogen-free mice had been exposed to infectious elements with homology to FHA. Furthermore, a similar cross-reactivity was observed with adenylate cyclase-hemolysin in the vector-immunised mice and against these same two antigens in the second mouse experiment, as discussed in Chapters 7 and 8. Cytokine profiles indicated that the cross-reactivity was limited to a humoral response as the splenocytes of the vector-immunised mice produced very low levels of IFN-γ which were equivalent to the placebo group upon stimulation with rFHAB1, rFHAB2 or heat-killed *B. pertussis*. The impact of the vector-induced cross-reactivity towards FHA was shown to be minimal, with no difference in the rates of clearance between the vector and placebo-immunised mice following aerosol challenge. Conversely, mice vaccinated with DNA encoding either rFHAB1 or rFHAB2 showed significantly improved levels of protection compared to both negative control groups.

In the event of future work with FHA-based DNA vaccines, the exact source of the cross-reactivity and a possible solution would need to be determined. For example, replacement of the pcDNA3.1D/V5-His-TOPO eukaryotic expression vector with a specifically designed minimalistic vector could alleviate the cross-reactivity. In addition, antibiotic resistance genes (ampicillin resistance gene of the pcDNA3.1D/V5-His-TOPO vector) have been identified as a safety risk and unsuitable for inclusion in human or livestock vaccines (Centre for Disease Control, and Food & Drug Administration, United States of America). Moreno et al. (2004) have developed a range of immunogenically-defined gene expression vectors that are limited to an antigen expression cassette and lack the superfluous and potentially dangerous plasmid backbone sequences. These minimalisitic vectors were found to be equally effective as conventional eukaryotic expression vectors in terms of *in vivo* antigen expression.

DTaP was included as a standard to indicate the true value of DNA vaccination with FHA. Mice immunised with DTaP showed a high degree of seroconversion with strong IgG titers detected against either recombinant or native FHA. This predominantly Th2 response was accompanied by a weak cellular response with only low levels of IFN-γ and IL-2 compared to the moderate to high levels observed following DNA vaccination. Interestingly, there was also a low level of IL-4 (a Th2 cytokine) produced from splenocytes of DTaP-immunised mice. Although this result was unexpected considering acellular vaccines have been reported to induce a strong CD4+ Th2-type response, Carter et al. (2004) observed similarly low levels of IL-4 expression in Pa-immunised mice after an aerosol challenge, albeit determined through a lack of mRNA in the lungs rather than cytokine production from *in vitro*-stimulated splenocytes.

A feature of DTaP immunisation was the complete clearance of *B. pertussis* within 7 days. However, there seems to be a certain degree of variation between the clearance data of DTaP from our study and other reported observations. Carter et al. (2004) observed that an aerosol-induced infection persisted for up to 20 days post-challenge in mice vaccinated with Pa with only a slight reduction in bacterial counts after 7 days. Mills et al. (1998) evaluated the protective efficacy of the same acellular vaccine (in terms of dosage and composition) in mice and found that *B. pertussis* were still present in lung homogenates up to day 14 following an aerosol challenge. Factors that may have contributed to the increased protective efficacy observed with the acellular vaccine used in this investigation are the: quantity of antigen and method of detoxification used in the vaccine preparation, size and frequency of dosing, aerosol exposure, virulence of the challenge strain, and species of mice used. For instance, Carter et al. (2004) vaccinated NIH mice with a two-component acellular pertussis vaccine via the IP route whereas Balb/C mice were vaccinated a three-component acellular vaccine via the SC route for this investigation. The challenge doses were also variable across the three studies. Overall, the results from chapter have reiterated that the potent Th2-restricted response of DTaP affords a high degree of protection but also that an FHA-specific CMI response elicited by DNA vaccination can also effectively control an experimental *B. pertussis* infection.

Although important differences between the DTaP and DNA dosing regimes meant that an acellular vaccine was not the most ideal positive control, conclusions can still be drawn from the clearance curves, albeit with a certain degree of caution. Firstly, the response to DNA vaccination with FHA alone had a positive effect on the rate of *B. pertussis* clearance and was achieved in this absence of additional antigens or an external adjuvant, which suggested that DNA vaccination with multiple antigens and/or adjuvants would be highly efficacious. Secondly, it was demonstrated that either a largely Th1-type immune response (from DNA vaccination) or predominately Th2-type response (from DTaP) alone was sufficient to confer protection from an experimental challenge. Some of the key differences that prevented any ironclad comparison between DTaP and the two FHA DNA vaccines were in relation to the different composition (protein versus DNA), dosage (multi-component DTaP versus mono-component DNA vaccine), the route of administration (SC versus IM) and the inclusion of adjuvant in DTaP versus naked DNA.

An important aspect of DNA vaccine efficacy relates to the level of endogenous antigen expression. As with the expression of recombinant proteins in commonly used systems such as *E. coli*, yeast and insect cell lines, the level of *in vivo* expression of a recombinant protein can be influenced by numerous factors at the DNA level including codon usage and promoter region; at the protein level such as molecular weight and cytotoxicity; and at the tissue level relating to the precision and efficiency of delivery and transfection. An alternative approach that may improve the expression of a large prokaryotic antigen such as FHA in a mammalian system, other than the reduction to its minimal essential immunogenic components, would be the incorporation of an intron. Codon optimisation through the use of synthetic genes may be yet another means of improving the level of endogenous expression. Increased *in vivo* transfection efficiency would also improve expression levels and lead to enhanced MHC class I presentation. Several methods of boosting transfection efficacy currently exist such as through the use of liposomes, *in vivo* electroporation, or encapsulation in microparticles (Widera et al. 2000; O'Hagan et al. 2001; Gregoriadis et al. 2002). Pre-administration of destructive compounds that promote tissue regeneration or repair such as cardiotoxin, bupivacaine, or hypertonic solutions have also been found to enhance the uptake of DNA by muscle cells (Coney et al. 1994; Davis et al. 1994).

In summary, mice immunised with pcDNA3.1D/*fhaB1* and pcDNA3.1D/*fhaB2* elicited a Th1-type immune response. Whilst both FHA-based DNA vaccines had significantly improved rates of clearance compared to the negative control mice, the mice vaccinated with pcDNA3.1D/*fhaB1* showed a more rapid reduction of bacteria from the lungs. In contrast, DTaP-immunised mice generated very high titers of serum IgG but low levels of the Th1 cytokines IFN-γ and IL-2 and completely cleared *B. pertussis* from the lungs by day 7 post-challenge. Both the immunogenicity and challenge data indicated that the acellular vaccine was superior to the FHA DNA vaccines. However, DTaP was a multi-component formulation compared with the mono-component DNA vaccines and considering only naked DNA was administered with no adjuvant; the performance of the DNA vaccines was encouraging.

Chapter 5 Pertactin DNA Vaccine: Development and Characterisation of the Immune Response in Mice Vaccinated via the IM Route

5.1 Introduction

Pertactin (PRN) is a non-fimbrial outer membrane protein (OMP) expressed by virulent *B. pertussis* that belongs to the autotransporter family of proteins. The antigen is expressed as a 93 kDa precursor, which is then processed at both its N and C-termini to yield a mature 69 kDa (P.69) protein (Charles et al. 1993). The post-translational modification involves cleavage of a 34 amino acid residue signal peptide and a 30 kDa C-terminal domain (P.30). Synonymous with other autotransporter proteins, the P.30 domain is believed to assist in translocation of the mature protein onto the surface of *B. pertussis* (Charles et al. 1994).

Three important motifs have been identified within the mature P.69 polypeptide sequence: an Arg-Gly-Asp (RGD) tripeptide, a Gly-Gly-X-X-Pro (GGXXP) repeat region and a Pro-Gln-Pro (PQP) repeat region, with the former involved in attachment to the integrin family of eukaryotic receptors and the latter two being highly immunogenic and polymorphic (Relman et al. 1989). The crystal structure of PRN has been solved and confirmed that both the RGD sequence and GGXXP repeats are exposed on the surface of the antigen (Emsley et al. 1996). Roberts et al. (1991) found that a P.69/P30 depleted mutant of *B. pertussis* had a reduced ability to interact with mammalian cells *in vitro* and comparative adhesion and invasion assays with the $P.69^{-}$ mutant and wild-type strains indicated that PRN was involved in the binding to HEp2 cells, a human epithelial cell line. Unlike the other the adhesins of *B. pertussis* such as FHA and fimbriae, which are among the first antigens to be expressed during infection, PRN is expressed at a later stage and it may have a supportive role in attachment to ciliated epithelial cells by increasing the avidity of binding between the pathogen and host cell. The RGD sequence suggests that PRN is involved in attachment to macrophages, which *B. pertussis* cells are known to invade as a means evading the host's immune system (Ewanowich et al. 1989a; Friedman et al. 1992; Guzman et al. 1994).

PRN is a highly polymorphic antigen with 13 variants identified thus far. Of these 13 variants, the most common are Prn1-3 (Mooi et al. 1998; 1999). Variation is largely confined to two regions, known as region 1 and region 2. Located at the N-terminus of the P.69 polypeptide, region 1 is composed of the GGXXP repeats and has the majority of polymorphic residues. Region 2 is towards the C-terminus, includes the PQP repeats, and has fewer amino acid variations. Compared to Prn1 and Prn3, Prn2 has an additional GGXXP repeat that is thought to alter the secondary structure such that antibodies that recognise this region 1 are type specific, whereas the only difference between Prn1 and Prn3 is two amino acid substitutions within region 1 (He et al. 2003). The effect of these polymorphisms on immunogenicity and protection has not yet been fully characterised but preliminary studies have shown that it can reduce the efficacy of whole-cell and tri-component acellular vaccines (Fry et al. 2001; King et al. 2001, He et al. 2003). For instance, whole-cells pertussis vaccines prepared from strains with Prn1 have shown to have reduced efficacy against circulating strains that express Prn2 or Prn3 (King et al. 2001).

Numerous studies have shown that PRN is a protective antigen in both animals and humans (Shahin et al. 1990). Circulating and secretory antibodies have been detected in the serum of patients recovering from infection and high titers of serum IgG has been observed following immunisation with Pw and Pa vaccines; the presence of which has been shown to correlate with protection (Cherry et al. 1998; Storsaeter et al. 1998). Passive immunisation with specific monoclonal antibodies has also been shown to protect mice from respiratory challenge (Thomas, Redhead & Lambert 1989; Shahin et al. 1990). Immunisation of mice with purified P.69 via the IP route elicits a strong serum antibody response that is protective against a lethal respiratory challenge, a test that correlates with protection in humans (Shahin et al. 1990). Intranasal immunisation with P.69 has also been shown to enhance the clearance of *B. pertussis* following an aerosol challenge (Roberts et al. 1993). Moreover, when included in modern acellular vaccines, pertactin was found to enhance their overall efficacy compared to the two-component (FHA and PT) acellular vaccines (Gustafsson et al. 1996; Mooi et al. 1998).

Recombinant PRN has been expressed in a variety of heterologous systems such as *E. coli*, *S. typhimurium*, the yeast *Pichia pastoris* and the insect cell line *Spodoptera frugiperda* (Romanos et al. 1991; Strugnell et al. 1992; Charles et al. 1993). As yet there have been no reports of expression of pertactin in mammalian cell lines, which were the targets of DNA vaccination. Furthermore, with the exception of a recent study by Hijnen et al. (2004), which identified specific antibody binding sites within the GGXXP repeat region, relatively little is known about the location of important B and T cell epitopes. Hence, it was decided to immunise with a truncated *prn* gene that encoded the mature P.69 polypeptide, which would allow for presentation of the full range of epitopes.

The aims of the research embodied in this study were as follows:

1. Develop a DNA vaccine encoding the mature pertactin antigen
2. Purify recombinant pertactin from *E. coli* for use in immunological assays
3. Demonstrate constitutive expression of rPRN within a mammalian cell line
4. Evaluate the immunogenicity of the pertactin DNA vaccines in mice and compare the protective efficacy against a tri-component acellular vaccine using an established aerosol challenge model

5.2 Methodology

Specific details of the methods used are outlined in Chapter 2.

5.2.1 Construction of Recombinant Plasmids

5.2.1.1 PCR Amplification of prn Gene from Genomic DNA

PCR of the *prn* gene from Tohama I genomic DNA was performed according to the parameters outlined in Chapter 2, Section 2.5.2. Platinum *Pfx* DNA polymerase was used to generate blunt-ended products for cloning into pcDNA3.1D/V5-His-TOPO and Platinum *Taq* High Fidelity DNA polymerase was used to generate A-tailed products for cloning into pTrcHis2-TOPO. Custom-made primers (Geneworks) were designed to amplify the *prn* gene based on the published DNA sequence (Genbank accession no. AJ006158, Appendix D.3). The sequences of the primers used were:

For cloning into pcDNA3.1D/V5-His-TOPO
PRN198DNAF – 5' CACCATGGCGCATGCCGACTGGAACAACCAG 3'
PRN2273R – 5' TGTGGCATAGCCCCCGACATGCACGCTGTC 3'
For cloning into pTrcHis2-TOPO
PRN198TRCF – 5' GCGCATGCCGACTGGAACAACCAGTCCATC 3'
PRN2273R – 5' TGTGGCATAGCCCCCGACATGCACGCTGTC 3'

PCR products were purified as described in Chapter 2, Section 2.5.3.

5.2.1.2 Cloning of PCR products into pTrcHis2-TOPO and pcDNA3.1D/V5-His-TOPO Expression Vectors

The pTrcHis2-TOPO TA Expression Kit (Invitrogen) was used for cloning and transformation, according to the manufacturer's instructions. Purified PCR products were cloned into the pTrcHis2-TOPO vector and transformed into *E. coli* TOP10 cells as described in Chapter 2, Section 2.6. The pcDNA3.1D/V5-His TOPO Cloning Kit (Invitrogen) was used for cloning and transformation, according to the manufacturer's instructions (Chapter 2, Section 2.6). The plasmid map of pcDNA3.1D/*prn* is shown as Figure 5.1.

5.2.1.3 Screening of Plasmid Clones

Plasmid DNA from selected TOP10 transformants was isolated by Miniprep and analysed on a 1% TAE agarose gel (Chapter 2, Section 2.6.2). Insert sequences of the recombinant plasmids with a molecular weight equivalent to the expected size were verified by DNA sequencing as described in Chapter 2, Section 2.7. Primer sequences used for sequencing are listed in Table 2.3. Small fragments obtained from sequencing reactions were assembled into contigs and aligned against the Published *prn* gene sequence using the ClustalW program provided by ANGIS.

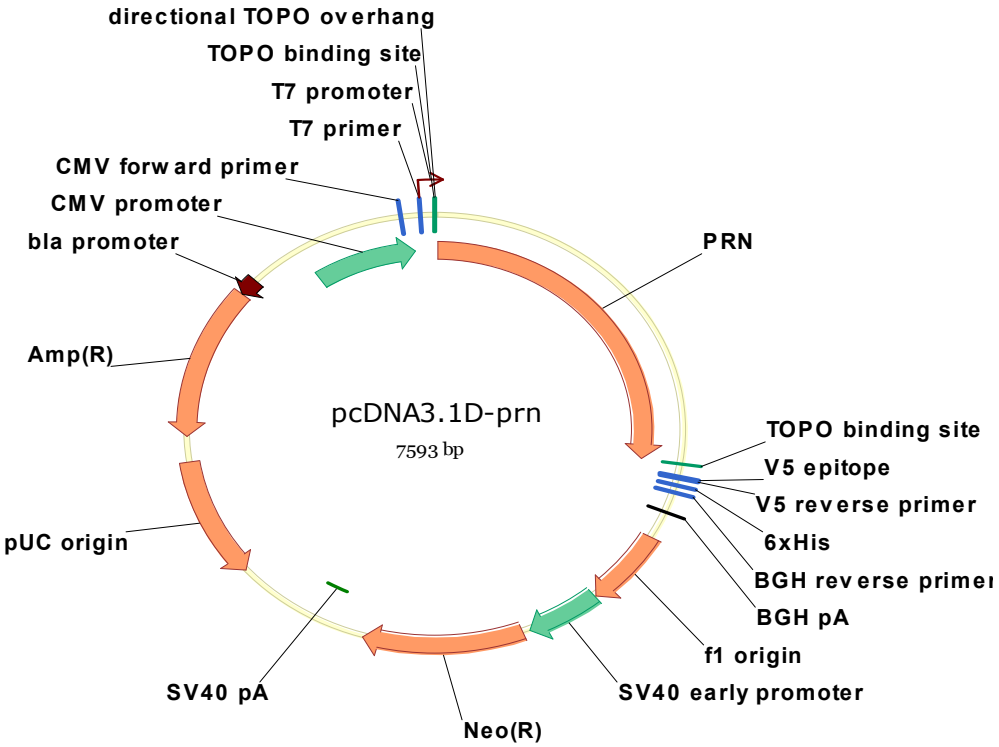

Figure 5.1: Map of pcDNA3.1D/*prn* generated using Vector NTI (Invitrogen).

5.2.2 Expression of Recombinant PRN in *E. coli*

Pilot expression of rPRN in TOP10 *E. coli* was performed according to the protocol outlined in Chapter 2, Section 2.12.1. Recombinant protein expression was detected by Western blotting with a HRP conjugated anti-His (C-term) antibody (Chapter 2, Section 2.11). The pTrcHis2/*prn* plasmid was transformed into BL21 CodonPlus(RP) *E. coli* as described in Chapter 2, Section 2.12.2, to enhance expression levels from the GC-rich *B. pertussis* insert sequence.

5.2.3 Purification of Recombinant PRN

Recombinant PRN was purified from BL21 CodonPlus(RP) *E. coli* by Ni-NTA affinity chromatography under denaturing conditions as previously described (Chapter 2, Section 2.12.3). Purified protein was checked by SDS-PAGE and Western blot (Chapter 2, Sections 2.10 and 2.11). Protein samples were concentrated by ultra-centrifugation (Appendix C.6) with the yield estimated by modified Bradford assay (Chapter 2, Section 2.12.4).

5.2.4 Transient Transfection of COS-7 Mammalian Cells and Expression

The mammalian cell line COS-7 was transfected with pcDNA3.1D/*prn* as previously described (Chapter 2, Section 2.9). Expression of rPRN was detected by Western blot using an AP-conjugated anti-V5 antibody (Chapter 2, Section 2.11).

5.2.5 Transformation of *S. typhimurium* with pcDNA3.1D/*prn*

The *aro*A *Salmonella typhimurium* strain SL3261 was transformed with the pcDNA3.1D/*prn* plasmid for mucosal delivery via the oral route (Chapter 8). Initial passage through the r^-m^+ *S. typhimurium* strain P9121 was required to successfully transform the *E. coli*-methylated plasmids into SL3261, which possesses an intact DNA restriction system. *S. typhimurium* P9121 were transformed using the heat shock method (Chapter 2, Section 2.14.2), whereas *S. typhimurium* SL3261 were transformed by electroporation (Chapter 2, Section 2.14.3).

5.2.6 Immunisation of Mice with pcDNA3.1D/*prn* via the IM Route

A component of the first DNA vaccine efficacy trial involved immunisation of mice with the PRN DNA vaccine as described in Chapter 2, Section 2.15.2. Immunisation involved direct injection of naked DNA into the quadriceps. To obtain sufficient plasmid for a 3 dose regime, large scale isolation was performed from 2.5 L cultures of TOP10-pcDNA3.1D/*prn* (Chapter 2, Section 2.15.2.1).

5.2.7 Determination of Immune Response and Protective Efficacy to DNA Vaccination

Of the 17 mice per group vaccinated, 5 were sampled for serum and organs two weeks after the third and final dose. Serum samples were analysed for the presence of Ag-specific IgG, IgG1 and IgG2a by ELISA (Chapter 2, Section 2.16.2) and Western blot (Appendix B.1), whereas the cytokine profiles of re-stimulated splenocytes was used to indicate CMI (Chapter 2, Section 2.16.1). The remaining 12 mice/group were challenged with a predetermined sub-lethal infectious dose (SLID) of virulent *B. pertussis* Tohama I as previously described (Chapter 2, Section 2.15.4). Mice vaccinated with pcDNA3.1D/V5-His-TOPO vector only or a placebo was used as negative controls and mice immunised with a 0.2 SHD of DTaP were included as a positive control.

5.3 Results

5.3.1 Preparation of pcDNA3.1D/*prn* and pTrcHis2/*prn* Constructs

A 2079 bp fragment of the *prn* gene was successfully amplified from genomic DNA using either Platinum *Pfx* DNA polymerase to obtain a blunt-ended product or Platinum *Taq* High Fidelity DNA polymerase for A-tailed product (Figure 5.2). After clean-up, blunt-ended were cloned into the pcDNA3.1D/V5-His-TOPO vector and A-tailed PCR products into the pTrcHis2-TOPO vector. Plasmid DNA was isolated from TOP10 transformants and checked for size on an agarose gel (Figure 5.3). Putative pcDNA3.1D/*prn* constructs with the appropriate size of ~ 7.6 kb and pTrcHis2/*prn* clones with a size of ~ 6.5 kb were sequence confirmed. The yield of pTrcHis2/*prn* (15ng/µl) was much lower than pcDNA3.1D/*prn* (150ng/µl) and the sequencing reaction needed to be optimised to obtain suitable chromatograms (see Section 2.7 for details).

Correct orientation and integrity of the *prn* inserts sequences from pcDNA3.1D/*prn* and pTrcHis2/*prn* clones was established by comparison against the published Genbank sequence using the WebAngis ClustalW program (Appendix D.3).

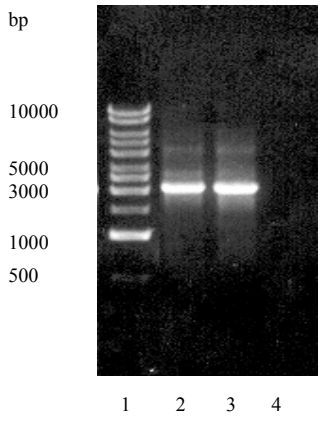

Figure 5.2: PCR of *prn* gene. Lane 1 - Kilobase Marker (10kb to 0.5kb - Amersham); lane 2 - amplification with Platinum *Taq* DNA polymerase and PRN198TRCF/PRN2273R oligonucleotides; lane 3 – amplification with Platinum *Pfx* DNA polymerase and PRN198DNAF/PRN2273R oligonucleotides; lane 4 - negative control.

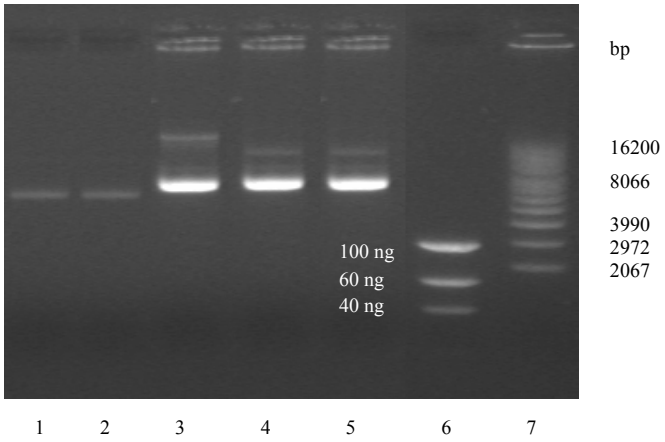

Figure 5.3: Isolation of pTrcHis2/*prn* and pcDNA3.1D/*prn*. Lane 1 – pTrcHis2/*prn* from TOP10; lane 2 – pTrcHis2/*prn* from BL21 Codon Plus; lane 3 - pcDNA3.1D/*prn* from TOP10; lane 4 - pcDNA3.1D/*prn* from P9121 *S. typhimurium*; lane 5 - pcDNA3.1D/*prn* from SL3261 *S. typhimurium*; lane 6 – Low DNA Mass ladder (Invitrogen); lane 7 – Supercoiled DNA Ladder (16,210bp to 2,067bp - Invitrogen).

Upon verification of the pcDNA3.1D/*prn* construct, the r^-m^+ *S. typhimurium* strain P9121 was successfully transformed with the *E. coli*–derived plasmid DNA. Plasmid DNA isolated from transformed P9121 was found to be of the same size (~ 7.6 kb) as the plasmid DNA isolated from *E. coli* TOP10 (Figure 5.3). The attenuated *S. typhimurium* strain SL3261 to be used for oral DNA vaccination (Chapter 8) was transformed with pcDNA3.1D/*prn* isolated from P9121 and revealed to be equivalent in size to the plasmid DNA isolated from TOP10 or P9121 (Figure 5.3).

5.3.2 Expression and Purification of rPRN from *E. coli*

Following IPTG induction of the selected TOP10-pTrcHis2/*prn* clone, expression of the rPRN fusion protein was detected by western blotting using a HRP-conjugated anti-His antibody (Figure 5.4). A dominant band was observed with an apparent molecular weight of 90 kDa, using the Benchmark protein ladder as a reference. The size of rPRN was substantially larger than expected in so far as when the pertactin antigen of *B. pertussis* was initially characterised it migrated as a 69 kDa protein when fractionated by SDS-PAGE (Brennan et al. 1988). The molecular weight of the rPRN amino acid sequence was also calculated to be around 70 kDa using the Translate GCG program (WebAngis).

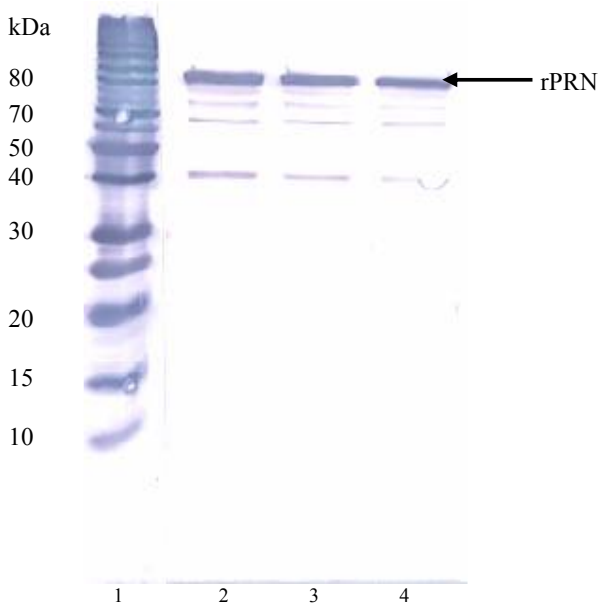

Figure 5.4: Western blotting of rPRN expression in TOP10 *E. coli* after IPTG induction. Lane 1 - BenchMark ladder (220kDa to 10kDa - Invitrogen); lanes 2 to 4 – rPRN expression after 3, 4 and 5 hours of induction.

There are two likely explanations for the larger than expected molecular weight of recombinant PRN. Firstly, additional vector encoded fusion components such as the C-terminal histidine tag and myc epitope would account for a slight increase in size. Secondly, pertactin has a high proline content and recombinant PRN expressed in BL21 CodonPlus *E. coli* has previously been shown to migrate at a slower than expected rate on SDS-PAGE (MacArthur & Thornton 1991; Hijnen et al. 2005). Four bands of a lower molecular weight were detected with the anti-His antibody and these most likely represented degradation products. Whilst rPRN expression was detected by western blotting, the IPTG-induced protein could no be distinguished from the native proteins of TOP10 *E. coli* in SDS-PAGE (not shown). This indicated a low level of expression and affinity purification of rPRN was unsuccessful from this strain (Figure not shown). Following transformation of the BL21 CodonPlus strain of *E. coli* with the pTrcHis2/*prn* plasmid, the level of rPRN expression was enhanced to a level that enabled successful purification under denaturing conditions (Figure 5.5). As a final check, purified protein was detected by western blot with anti-His-HRP antibody (not shown).

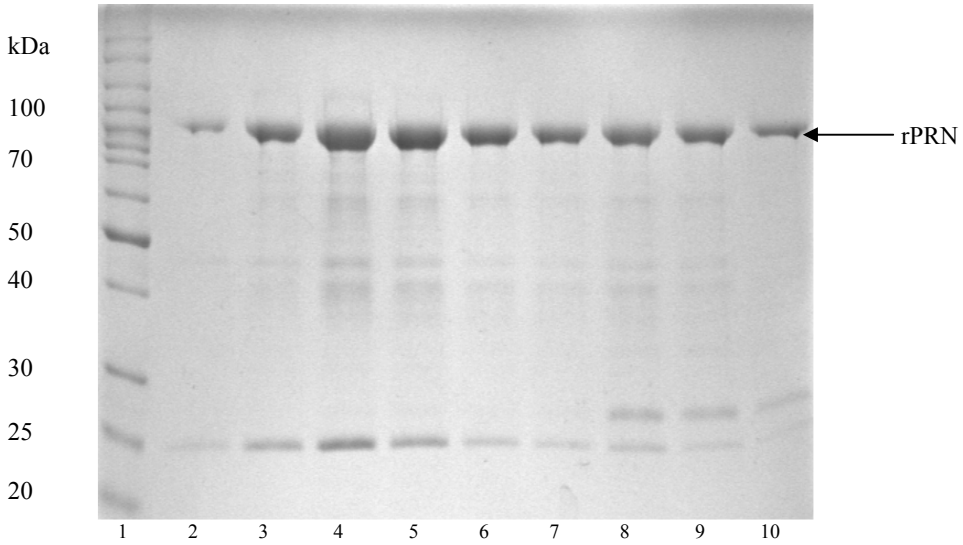

Figure 5.5: PAGE of affinity purified rPRN from BL21 CodonPlus *E. coli*. Lane 1: BenchMark Protein ladder (220kDa to 10kDa - Invitrogen); Lanes 2 to 5 – elution from 0.5 ml flow-throughs of buffer D pH 5.9; Lanes 6 to 10 – elution from 0.5 ml flow-throughs of buffer E pH 4.5.

5.3.3 Transient Expression of rPRN in COS-7 Cells

To evaluate the *in vitro* expression of rPRN within mammalian cells, COS 7 cells were transfected with pcDNA3.1D/*prn* isolated from TOP10 *E. coli*. Constitutive expression of rPRN was detected by western blotting using an anti-V5 epitope AP conjugated antibody (Figure 5.6). The apparent molecular weight of rPRN expressed in COS 7 cells was 85 kDa and roughly equivalent to that observed for rPRN expressed in *E. coli*. This indicated that no significant post-translational modification such as glycosylation had occurred that may have impacted on the immunogenicity. In addition, COS 7 cells were also transfected with pcDNA3.1D/*prn* plasmid isolated from P9121 or SL3261 *S. typhimurium*. No differences in size or the level of expression of rPRN were apparent following transfection with DNA isolated from the different bacterial strains (Figure 5.6). No band corresponding to rPRN was observed in the negative control or COS-7 cells transfected with self-ligated pcDNA3.1-V5-TOPO (not shown).

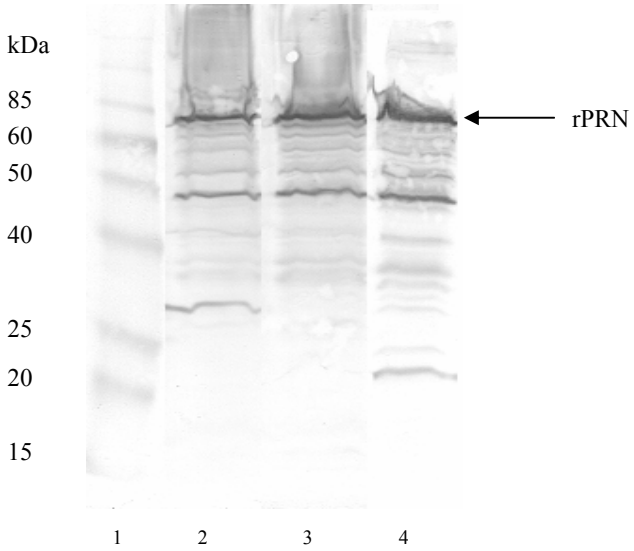

Figure 5.6: Expression of rPRN in COS-7 cells. Lane 1 - Pre-stained BenchMark protein ladder (190kDa to- 10kDa - Invitrogen). Expression of rPRN following transfection of COS-7 with pcDNA3.1D/*prn* isolated from: lane 2 - TOP10 *E. coli*; lane 3 - P9121 *S. typhimurium*; and lane 4 - SL3261 *S. typhimurium*.

5.3.4 Immunogenicity and Protective efficacy of pcDNA3.1D/*prn*

BALB/c mice were immunised with 3 IM doses of pcDNA3.1D/*prn* as per Section 2.15.2. Two weeks after the second and final booster, a small subgroup of 5 mice were sacrificed and sampled for the measurement of antibody and cytokine levels. Also at two weeks after the second booster a larger subgroup of 12 vaccinated mice were challenged with a predetermined SLID of virulent *B. pertussis*. The clearance of the SLID from the lungs was monitored over a 14 day period.

5.3.4.1 Cytokine Profiles Following IM DNA Vaccination with pcDNA3.1D/prn

Splenocytes from immunised mice were cultured in the presence of various stimulants for either 24 or 72 hours. Supernatants from the 24 hour cultures were tested for IL-2 production, whereas the IFN-γ and IL-4 production was determined from the 72 hour supernatants (see Section 2.16.1 for details). It was found that when splenocytes of mice vaccinated with pcDNA3.1D/*prn* were stimulated with rPRN, a moderate level of IFN-γ was produced compared to lower IFN-γ from the splenocytes of mice vaccinated with the pcDNA3.1 vector and the 0.2 SHD of DTaP (Figure 5.7). It was also shown that the splenocytes from mice immunised with pcDNA3.1D/*prn* produced a high level of IFN-γ when stimulated with ConA, a natural T cell mitogen.

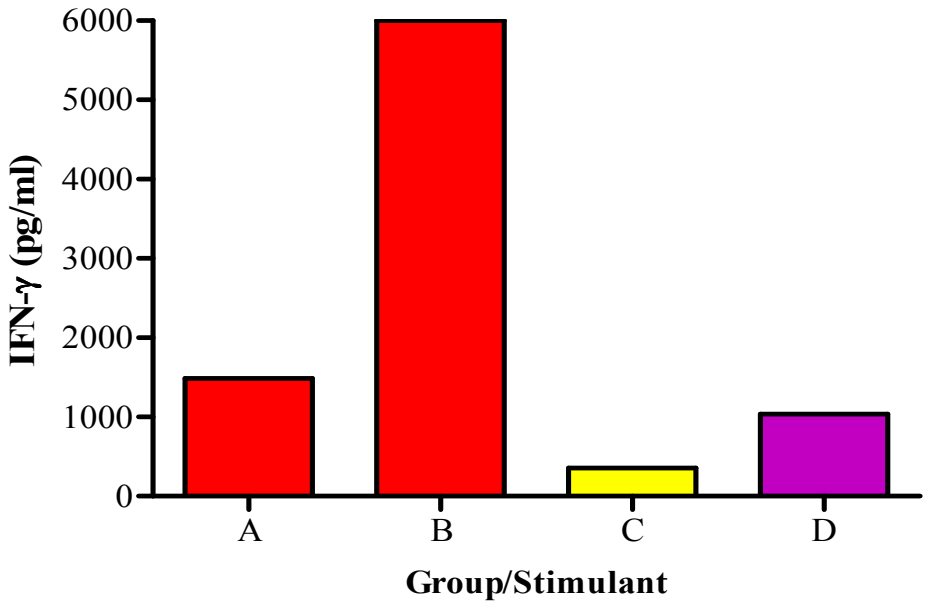

Figure 5.7: IFN-γ production from rPRN-stimulated splenocytes. Splenocytes from pcDNA3.1D/*prn*-immunised mice stimulated with 5µg/ml rPRN (A) and 2.5µg/ml ConA (B). Splenocytes from vector-immunised mice stimulated with 5µg/ml rPRN (C). Splenocytes from DTaP-immunised mice stimulated with 5µg/ml rPRN (D).

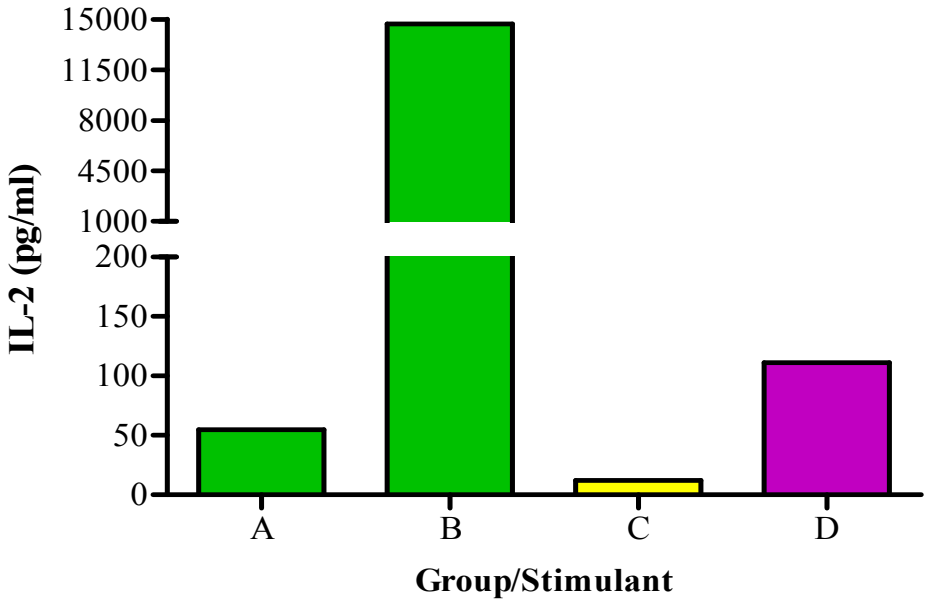

Figure 5.8: IL-2 production from rPRN-stimulated splenocytes. Splenocytes from pcDNA3.1D/*prn*-immunised mice stimulated with 5µg/ml rPRN (A) and 2.5µg/ml ConA (B). Splenocytes from vector-immunised mice stimulated with 5µg/ml rPRN (C). Splenocytes from DTaP-immunised mice stimulated with 5µg/ml rPRN (D).

The level of IL-2 production from splenocytes stimulated with rPRN was very low compared to stimulation with ConA (Figure 5.8). It was observed that mice vaccinated with DTaP had a two-fold increase in the level of IL-2 compared to mice given the pcDNA3.1D/*prn* DNA vaccine, and 10 times more IL-2 than the vector-immunised group (Figure 5.8).

With respect to IL-4 production, the greatest amount of IL-4 was detected in the splenocyte supernatants of mice vaccinated with DTaP (Table 5.1). There was 3.5 times less IL-4 produced from the splenocytes of mice vaccinated with pcDNA3.1D vector-after stimulation with rPRN and no IL-4 detected for the mice vaccinated with pcDNA3.1D/*prn* upon stimulation with rPRN (Table 5.1). A high level of IL-4 was produced when the splenocytes from each group was stimulated with ConA.

Table 5.1: IL-4 production from rPRN-stimulated splenocytes.

Stimulant	µg/ml	IL-4 (pg/ml)		
		pcDNA3.1D/*prn*	pcDNA3.1 vector	DTaP
rPRN	5	0	16.8	58.9
ConA	2	426.8	511.3	1081.9

5.3.4.2 Serum Antibody Response Following Intramuscular Vaccination with pcDNA3.1D/prn

Serum from mice vaccinated with pcDNA3.1D/*prn*, pcDNA3.1 vector and DTaP were tested for levels of anti-rPRN IgG by ELISA. Mice immunised with the Infanrix™ DTaP induced a significantly greater anti-rPRN response than mice immunised with pcDNA3.1D/*prn* ($P < 0.005$) or pcDNA3.1D vector only ($P < 0.001$). Nevertheless, a strong IgG titer was generated following three doses of the pcDNA3.1D/*prn* DNA vaccine that far exceeded the low anti-rPRN titer in the sera from vector-immunised mice (Figure 5.9). Each of the five mice vaccinated with pcDNA3.1D/*prn* showed induction of rPRN-specific antibodies (seroconversion), however there was a substantial amount of variation in the levels of the systemic IgG produced in each individual mouse with titers ranging from a high of 84337 to a low of 10472 (Figure 5.10).

Sera from mice immunised with pcDNA3.1D/*prn*, pcDNA3.1D vector and DTaP was analysed to determine the isotype specificity of the anti-rPRN IgG response. Mice immunised with pcDNA3.1D/*prn* had an IgG1 to IgG2a ratio of 4:1 with a clear bias towards the induction of IgG1 (Table 5.2). IgG1 also dominated the serum antibody response to DTaP, but unlike immunisation with the DNA vaccine no trace of IgG2a was detected (Table 5.2).

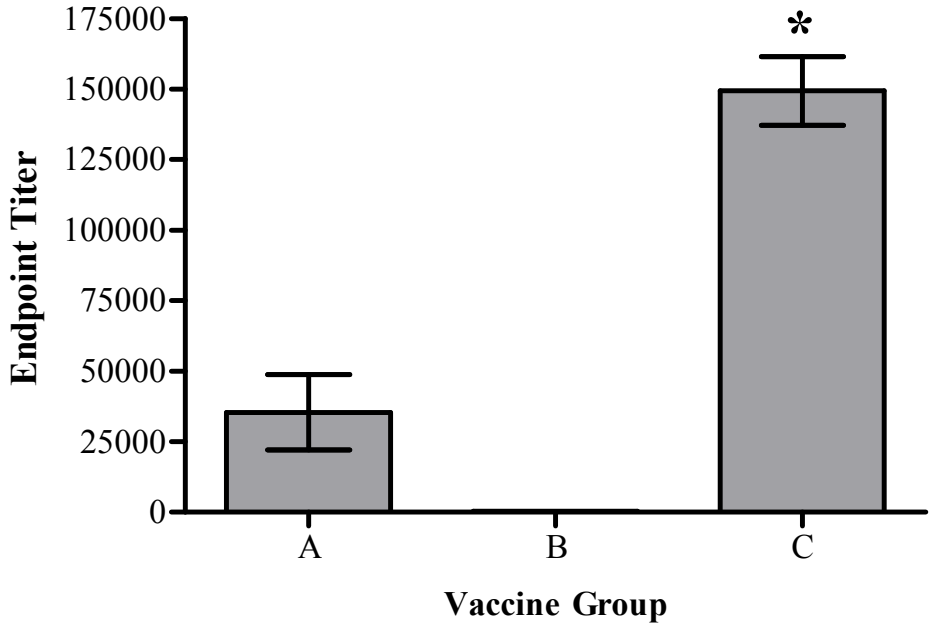

Figure 5.9: Anti-rPRN IgG endpoint titers in sera of mice vaccinated with pcDNA3.1D/*prn*. A – pcDNA3.1D/*prn*, B – pcDNA3.1 vector, C – DTaP. Bars represent mean scores from five mice +/- SE. * Significantly different from pcDNA3.1D/*prn* ($P < 0.005$) and vector ($P < 0.001$).

Table 5.2: Serum IgG1 and IgG2a in mice vaccinated with pcDNA3.1D/*prn*. Figures represent titers from pooled serum.

Antigen	pcDNA3.1D/*prn*		Vector		DTaP	
	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a
rPRN	6400	1600	200	0	88087	0

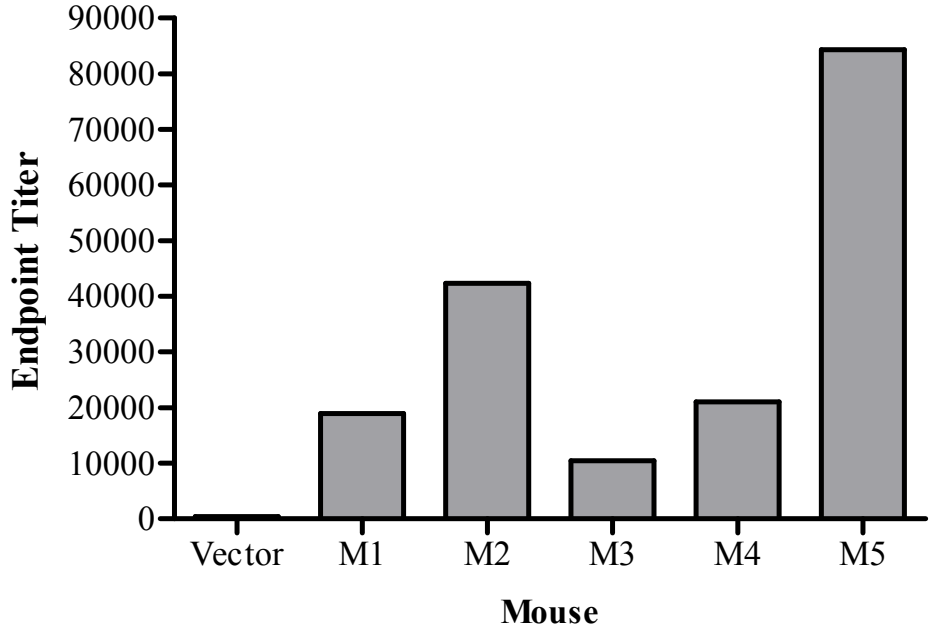

Figure 5.10: Anti-rPRN IgG endpoint titers in sera from individual mice vaccinated with pcDNA3.1/*prn*.

5.3.4.3 Protective Efficacy of pcDNA3.1D/prn Vaccination

Mice given three doses of pcDNA3.1D/*prn* or the respective controls were infected with a pre-determined SLID of Tohama I to determine the protective efficacy of the pertactin DNA vaccine. The bacterial loads in the lungs of challenged mice were measured at three time points over a 14 day period for comparison with the placebo, vector only and DTaP control groups. Mice vaccinated with pcDNA3.1D/*prn* showed a steady rate of clearance with a 60-fold reduction in the number of *B. pertussis* after two weeks compared to the apparently unimpaired colonisation of *B. pertussis* in the placebo and vector-immunised mice (Figure 5.11). Nevertheless, the sub-lethal experimental infection could not be totally cleared within 14 days by mice vaccinated with the pcDNA3.1D/*prn* DNA vaccine and was clearly less efficacious than mice vaccinated with DTaP, in which *B. pertussis* was completely cleared from the lungs within 7 days (Figure 5.11).

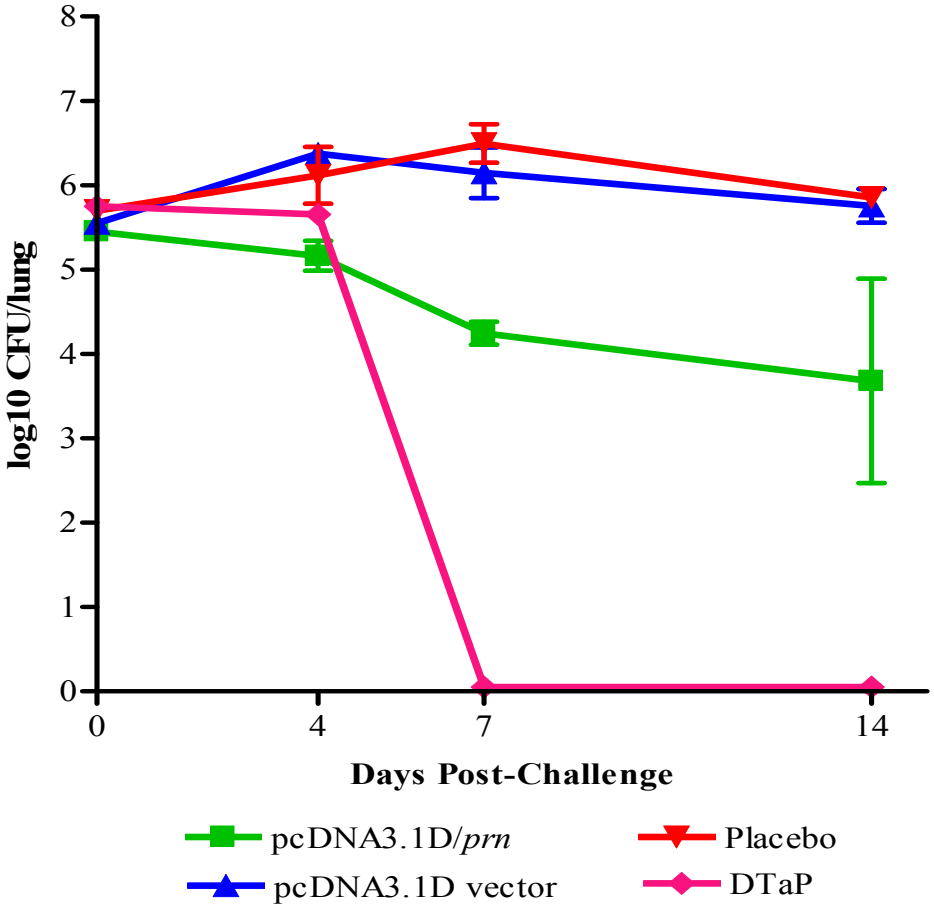

Figure 5.11: Bacterial loads in lungs of pcDNA3.1D/*prn*-vaccinated mice following aerosol challenge. Data points represent mean count of CFU/lung from 5 mice +/- SE.

The clearance data showed that the bacterial load in mice immunised with pcDNA3.1D/*prn* was reduced to 1.18% (8.5×10^3 CFU/lung) of the placebo controls (7.2×10^5 CFU/lung) by day 14 post-challenge (Table 5.3). The clearance index identified a significant increase in the rate of clearance for pcDNA3.1D/*prn*-immunised mice compared to the negative controls. Furthermore, this data also confirmed that DTaP mice had significantly improved elimination of the SLID to not only the placebo and vector groups but also the DNA vaccine (Table 5.3).

Table 5.3: Post-challenge clearance data from mice immunised with pcDNA3.1D/*prn*.

Vaccine Group	% of Untreated Control[a]			CI[b]
	Day			
	4	7	14	
Placebo	na	na	na	151.75 ± 1.65
Vector	255.7	63.5	115.3	151.78 ± 4.68
pcDNA3.1D/*prn*	19.77	1	1.18	82.24 ± 17.33 *
DTaP	30.5	0	0	22.43 ± 0.05 **

[a] Quantitated as percentage of bacteria cleared from the lungs relative to the untreated group of mice.

[b] Clearance Index ± SE = mean log10 number of *B. pertussis*/ml × days (van den Berg et al. 2001).

* Significantly different from placebo ($P < 0.05$) and vector ($P < 0.05$).

** Significantly different from placebo ($P < 0.001$), vector ($P < 0.001$) and pcDNA3.1D/*prn* ($P < 0.05$).

5.3.5 Recognition of rPRN with Mouse Immune Sera

High titers of anti-pertactin IgG was detected in the sera of mice immunised with pcDNA3.1D/*prn* using an indirect ELISA. Considering there was a high level of cross-reactive antibodies to FHA in the serum of mice vaccinated with the pcDNA3.1 vector only, the specificity of the response to pertactin in DNA or DTaP-immunised mice was confirmed by western blotting with AP-conjugated goat anti-mouse IgG, as described in Section 2.11. As a reference for the anti-sera blots, rPRN was detected with an AP-conjugated monoclonal anti-His antibody (1:2000). Pooled serum from mice immunised with pcDNA3.1D/*prn* was diluted 1:2000, whereas DTaP immune serum was diluted 1:3000. Pertactin was recognised by both DTaP and DNA vaccine immune sera (Figure 5.12). The multiple bands or smearing detected may be due to either degradation of the recombinant protein or non-specific binding. Recombinant PRN was not recognised by the sera from vector-immunised mice (data not shown).

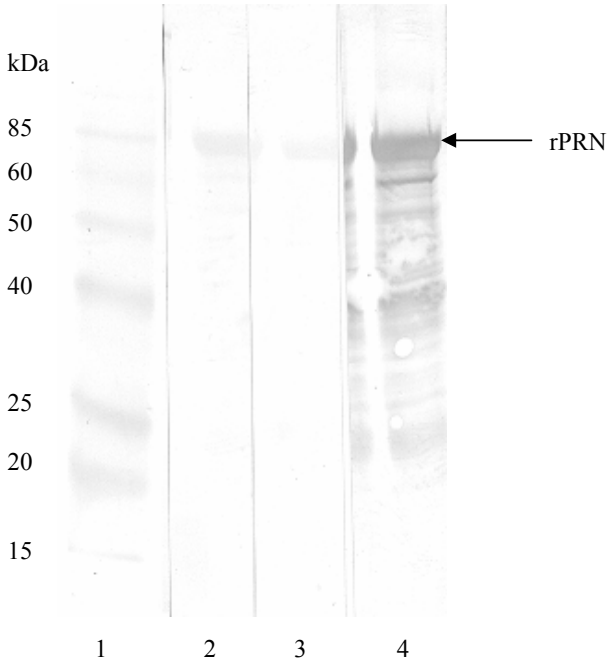

Figure 5.12: Recognition of rPRN by immune sera from mice. Western blotting of: lane 1 - Prestained Benchmark Protein ladder (190kDa to 10kDa – Invitrogen), lane 2 – rPRN with anti-His monoclonal, lane 3 – rPRN with pcDNA3.1D/*prn* anti-sera, lane 4 – rPRN with DTaP anti-sera.

5.4 Discussion

The response of mice to the pertactin DNA vaccine was interesting in that it was a more Th2 than Th1-mediated immune response. It was anticipated that IM injection of the pertactin DNA vaccine would elicit a potent cell-mediated response as observed with the two FHA DNA vaccines. Although direct intramuscular injection of DNA vaccines typically induces Th1-type immune responses (Johnson et al. 2000; Epstein et al. 2002; Velikovsky et al. 2002), in this study IM delivery of a pertactin DNA vaccine favoured the generation of serum antibodies. A high IgG1:IgG2a ratio and low IFN-γ levels confirmed that the response was Th2-driven. The basis for this unexpected result was not investigated but several factors such as the nature of the pertactin epitopes, cellular localisation, or the level of endogenous expression may have influenced the observed response.

Doria-Rose & Haigwood (2003) have suggested that the nature of the antigen itself, irrespective of the route of administration, may govern the type of immune response generated, and most of the immunological data on P.69 to date has pointed towards the induction of antibody rather than CMI. Shahin et al. (1990) reported that active immunisation of mice with native P.69 elicited a specific polyclonal antibody response that afforded protection against a lethal challenge. Anti-P.69 IgG was also detected in a panel of paired sera from 10 children that received three doses of a whole-cell vaccine followed by delayed boosting with a tri-component acellular pertussis vaccine. Roberts et al. (1993) found that IN immunisation of mice with P.69 effectively induced a specific local antibody response with the detection of IgA and IgG in lung washes as well as isolation of antibody-producing plasma cells from homogenised lung tissue. Similarly, immunisation of mice with an *aroA aroD* strain of *S. typhimurium* expressing high levels of P.69 generated a systemic IgG response, irrespective of whether the route of delivery was oral, intravenous or subcutaneous (Anderson, Dougan & Roberts 1996). Anderson, Dougan & Roberts (1996) reported that oral immunisation also induced a secretory and cellular response to pertactin. Hence, although IM injection with our pertactin DNA vaccine failed to induce a strong CMI response, mucosal delivery of a pertactin DNA vaccine by attenuated *S. typhimurium* may provide the means for stimulation of a dichotomous humoral and cellular response.

Cellular localisation of endogenously expressed antigen has also been reported to modulate the immune response to DNA vaccines (Boyle et al. 1997). It is widely accepted that to promote Th2 activation and antibody production, an antigen must be processed and presented via the MHC class II pathway. For endogenous antigen from a DNA vaccine vector to enter the MHC class II pathway the heterologous protein must either be secreted into the extracellular milieu and taken up by local antigen presenting cells, or defy convention and gain direct access to acid vesicles from the cytosol (Constant & Bottomly 1997). The design of the DNA vaccine constructs was such that neither *prn* nor any of the other antigenic inserts had signal peptides that could facilitate periplasmic localisation or secretion. However, as previously described pertactin belongs to the autotransporter family of proteins. Autotransporters are group of secreted proteins from gram-negative bacteria that commonly have three functional domains: an N-terminal signal peptide, the secreted (mature) protein, and a C-terminal pore-forming domain (Henderson, Navarro-Garcia & Nataro 1998). Although only the

sequence encoding the mature protein was targeted for DNA vaccination in this study, the apparent molecular weight of the recombinant PRN was greater than the reported 69 kDa, and it is possible that a portion of the pore-forming domain was included that supported its export from the transfected cell and uptake by local APC's for presentation by MHC class II molecules. Another possible explanation for the high level of antibody production may be that rPRN was more efficiently expressed *in vivo* than either rFHAB1 or rFHAB2, allowing for increased secretion from the transfected myofibers and again increased uptake by APC's.

As expected, mice vaccinated with DTaP induced high IgG ELISA titers against rPRN. Further analysis of serum samples showed that this IgG response consisted entirely of the IgG1 sub-class and although both DNA and DTaP induced a Th2 response, the serum IgG titers were significantly higher in mice vaccinated with DTaP. Disregarding the effect of the other vaccine antigens and adjuvant in the DTaP formulation, these significantly higher titers corresponded to an enhanced level of protection when compared to mice vaccinated with the DNA vaccine, placebo or vector only. Mice vaccinated with the pertactin DNA vaccine also had a significantly improved rate of clearance of *B. pertussis* when compared to the vector or placebo control groups. Two independent field trials indicated that there was a correlation between anti-P.69 antibodies and protection, but also that there was a lack of correlation with the other pertussis antigens (Cherry et al. 1998; Storsaeter et al. 1998). It is interesting however that of all the antigens associated with protective immunity, pertactin and pertussis toxin are the only two antigens in which polymorphism has been reported (King et al. 2001). The results of this study have re-iterated that a Th2 response to this antigen protects against an aerosol challenge with virulent *B. pertussis*.

Two highly immunogenic regions have been identified within mature PRN (Mooi et al. 1998). King et al. (2001) found that the majority of both mouse monoclonal and human polyclonal IgG are directed against a portion of P69 referred to as region 1. Moreover, immunisation of mice with peptides derived from region 1 was shown to reduce colonisation of the trachea and lungs following respiratory challenge. Passive immunisation with a monoclonal antibody directed at a linear epitope of this region also enhanced elimination of bacteria from the lungs. Region 1 is an 80 aa sequence that flanks the important RGD triplet and consists of multiple Gly-Gly-X-X-Pro (GGXXP)

repeats. Region 2 is a 277 residue sequence near the C-terminus with numerous repeats of the Pro-Glu-Pro (PQP) motif (King et al. 2001). Crystallisation studies have revealed that both regions are exposed at the surface of pertactin but more importantly they encompass all of the known sequence variation or polymorphism (Emsley et al. 1996). To date, three pertactin variants, namely Prn1, Prn2 and Prn3 have been identified (Fry et al. 2001). It has been determined that Pw vaccines expressing Prn1 were significantly less efficacious against infection with a Prn2 or Prn3 isolate (Mooi et al. 1999). Whilst pertactin DNA vaccine performed well, the template used for amplification and cloning of the *prn* gene was derived from the same strain used for challenge. Therefore the cross-protective potential of this pertactin DNA vaccine against challenge with *B. pertussis* strains expressing pertactin variants still needs to be determined and will constitute a truer test of the DNA vaccine efficacy.

He et al. (2003) assessed the specificity of antibodies generated against the repeats of region 1 among the three most common pertactin variants, namely Prn1, Prn2 and Prn3 and found that the response was type-specific with no apparent cross-reactivity. Prn1 and Prn3 differ by two amino acid substitutions within region 1, whereas Prn2 variants are the most structurally distinct with deletion of an entire Gly-Gly-X-X-Pro repeat unit. This prompted the suggestion that the hyper-variable regions are recognised as conformational rather than linear epitopes (He et al. 2003). In this proof-of-principle DNA vaccine study, mice were immunised and challenged with an identical pertactin variant and the effect of pertactin polymorphism was not determined. However, one of main advantages of DNA vaccination over the conventional whole-cell or subunit vaccines surrounds the flexibility of the DNA backbone and a suite of DNA vaccines with mutant *prn* alleles could easily be developed that would cater for the regional variation observed in circulating strains of *B. pertussis*.

Although PT has long been regarded as the dominant antigen of *B. pertussis*, antigenic variation combined with a strong correlation between pertactin antibodies and protection suggests that PRN may be of similar importance. Whilst it would have been preferable to determine the antibody response against both native and recombinant pertactin, the native form was not commercially available. Montaraz, Novotny & Ivanyi (1985) published a method for purification of P.69 from *B. pertussis* using a three-step procedure consisting of outer membrane protein extraction, anion-exchange

and affinity chromatography. Previous attempts at repeating this native purification in our laboratory have failed to provide sufficient yield or purity. Rather than performing a time-consuming optimisation of the native purification method it was decided to rely on rPRN purified by Ni-NTA affinity chromatography. It has been established that recombinant pertactin, either over-expressed in the yeast *Pichia pastoris* or insect cells, has similar structural and immunological properties to the native antigen and can induce an equivalent degree of protection (Romanos et al. 1991; Charles et al. 1993). Hijnen et al. (2005) used circular dichroism spectroscopy and monoclonal antibodies that a specific for conformational epitopes to verify that recombinant PRN expressed as inclusion bodies in *E. coli* has an identical secondary structure to native P.69 after refolding. In retrospect, it would have been worthwhile to use refolded rPRN in addition to the denatured protein in the ELISA's to account for antibodies directed against conformational epitopes. Nevertheless, the detection of very high antibody titers in the serum of mice vaccinated with DTaP demonstrated that denatured rPRN was a satisfactory ELISA antigen.

In summary, immunisation with a pertactin DNA vaccine enhanced clearance of *B. pertussis* from the lungs of mice following a sub-lethal respiratory challenge. The rate of clearance was significantly better than cohorts given a placebo or plasmid vector only, but the response to the pertactin DNA vaccine was not as protective as the response to DTaP, which conferred complete clearance of *B. pertussis* within 7 days of challenge. When administered via the IM route, the vaccine elicited a strong serum antibody response rather than the characteristic CMI response observed with many other DNA vaccines given parenterally. The major anti-PRN IgG subclass detected in mice immunised with the DNA vaccine or DTaP was IgG1, and confirmed the induction of a Th2-mediated response by both vaccines. It was determined by western blotting with the sera from the test and control groups that the antibody-mediated protection was due to the response to endogenous pertactin in the case of DNA vaccination or exogenous pertactin antigen in the case of DTaP, and not the result of cross-reactivity. This body of work has provided the first insight into the efficacy of a pertactin-based DNA vaccine against respiratory infection with *B. pertussis* and justifies the inclusion of pertactin in a combination DNA vaccine (Chapter 8).

Chapter 6 Pertussis Toxin DNA Vaccine: Development and Characterisation of the Immune Response of Mice Vaccinated via the IM Route

6.1 Introduction

Pertussis toxin (PT) is a 105 kDa exotoxin that is secreted into the extracellular milieu by virulent *B. pertussis*. The action of PT causes numerous systemic manifestations including histamine sensitisation, insulin secretion and lymphocytosis (Locht & Keith 1986). PT also has mitogenic and adjuvant properties, stimulates release of fatty acids from adipocytes, interferes with chemotaxis and increases vascular permeability (Munoz et al. 1981; Hewlett et al. 1983). It is an AB_5-type exotoxin with a hexameric structure consisting of five subunits (S1, S2, S3, S4 and S5) arranged in a 1:1:1:2:1 stoichiometry (Tamura et al. 1982). The catalytic A promoter or S1 subunit is an ADP-ribosyltransferase that disrupts signal transduction by modifying G_i, G_o and G_t proteins (Moss et al. 1983). The B oligomer (S2 - S5) binds to glycoconjugate receptors on the host cell surface and facilitates internalisation of the toxin (Brennan et al. 1988). The PT subunits are encoded by an operon of five genes and like other classical AB toxins, such as cholera toxin, the five subunits are individually expressed and translocated to the periplasm where holotoxin assembly occurs (Hirst & Holmgren 1987). Nicosia & Rappuoli (1986) cloned the entire *ptx* operon in *E. coli* and found that although each individual subunit was expressed the holotoxin was not assembled. Subsequent attempts at heterologous expression of complete PT have also failed (Nicosia et al. 1987). Considering the inability of other prokaryotes such as *E. coli* to assemble the toxin, it was presumed that mammalian cells would also lack the necessary cytoplasmic machinery (accessory proteins or chaperones) required for expression.

High titers of anti-PT antibodies have been detected in convalescent sera and are also reported to be generated following immunisation with Pw or Pa vaccines (Storsaeter et al. 1998; van den Berg et al. 2000). Passive immunisation with monoclonal antibodies has been shown to protect mice against an aerosol challenge, albeit for only a short duration (Sato & Sato 1984). Phase 1 and 2 clinical trials of a mono-component pertussis toxoid vaccine have revealed that there is a significant correlation between high titers of neutralising antibodies and protection (Taranger et al. 2001).

Although holotoxin assembly appeared to be a limitation in the development of a PT-based DNA vaccine, it has been proven that immunisation with the S1 subunit alone can effectively prime the immune system. For instance, Walker et al. (1992) detected a specific systemic and mucosal humoral response following the oral immunisation of mice with attenuated *S. typhimurium* that expressed recombinant S1. Lee et al. (2003) demonstrated that when fused to the B promoter of cholera toxin (CT), a chimeric S1/CT antigen induced a specific mucosal IgA and systemic IgG response following intranasal immunisation of mice. Other studies have also had success using S1-derived vaccines (Boucher et al. 1994; Barry et al. 1996; Lee et al. 1999; Nascimento et al. 2000; Kamachi et al. 2003; 2004).

Owing to its central role in both pathogenicity and immunogenicity and its inclusion in all modern acellular vaccines, the S1 subunit of pertussis toxin (PT) was an obvious candidate for evaluation as a DNA vaccine antigen. Hence, the objectives of the work presented in this chapter were to:

1. Construct a DNA vaccine that encodes the S1 subunit of the pertussis toxin antigen
2. Inactivate the S1 subunit using an established site-directed mutagenesis approach
3. Demonstrate inactivation of the mutagenised S1 subunit using a Chinese Hamster Ovary (CHO) Cell assay
4. Express and purify the recombinant S1 subunit from *E. coli* for use in immunological assays, and
5. Evaluate the immunogenicity of the S1-based DNA vaccine in mice, and compare the protective efficacy with that of a commercial DTaP, using an aerosol challenge model.

6.2 Overview of Methodology

This section provides an outline of the methods used, with specific details for each procedure described in Chapter 2.

6.2.1 Construction of Recombinant Plasmids

6.2.1.1 PCR Amplification of pts1 Gene from Genomic DNA

PCR of the *pts1* gene was performed according to the parameters outlined in Chapter 2, Section 2.5.2. Platinum *Pfx* DNA polymerase was used to generate blunt-ended products for cloning into pcDNA3.1D/V5-His-TOPO and Platinum *Taq* High Fidelity DNA polymerase was used to generate A-tailed products for cloning into pTrcHis2-TOPO. Custom-made primers (Geneworks) were designed to amplify the *pts1* gene based on the published DNA sequence of the *ptx* gene (Genbank accession no. M13223, Appendix D.4). The sequences of the primers used were:

For cloning into pcDNA3.1D/V5-His-TOPO
PTX507DNAF – 5' CACCATGCGTTGCACTCGGGCAATTCGC 3'
PTX1313DNAR – 5' GAACGAATACGCGATGCTTTCGTAGTACAC 3'
For cloning into pTrcHis2-TOPO
PTX507TRCF – 5' ATGCGTTGCACTCGGGCAATTCGC 3'
PTX1313TRCR – 5' GAACGAATACGCGATGCTTTCGTAGTA 3'

PCR products were purified as described in Chapter 2, Section 2.5.3.

6.2.1.2 Cloning of PCR products into pTrcHis2-TOPO and pcDNA3.1D/V5-His-TOPO Expression Vectors

The pTrcHis2-TOPO TA Expression Kit (Invitrogen) was used for cloning and transformation, according to the manufacturer's instructions. Purified PCR products were cloned into the pTrcHis2-TOPO vector and transformed into *E. coli* TOP10 cells as described in Chapter 2, Section 2.6. The pcDNA3.1D/V5-His TOPO Cloning Kit (Invitrogen) was used for cloning and transformation, according to the manufacturer's instructions (Chapter 2, Section 2.6). The plasmid map of pcDNA3.1D/*pts1* is shown as Figure 6.1.

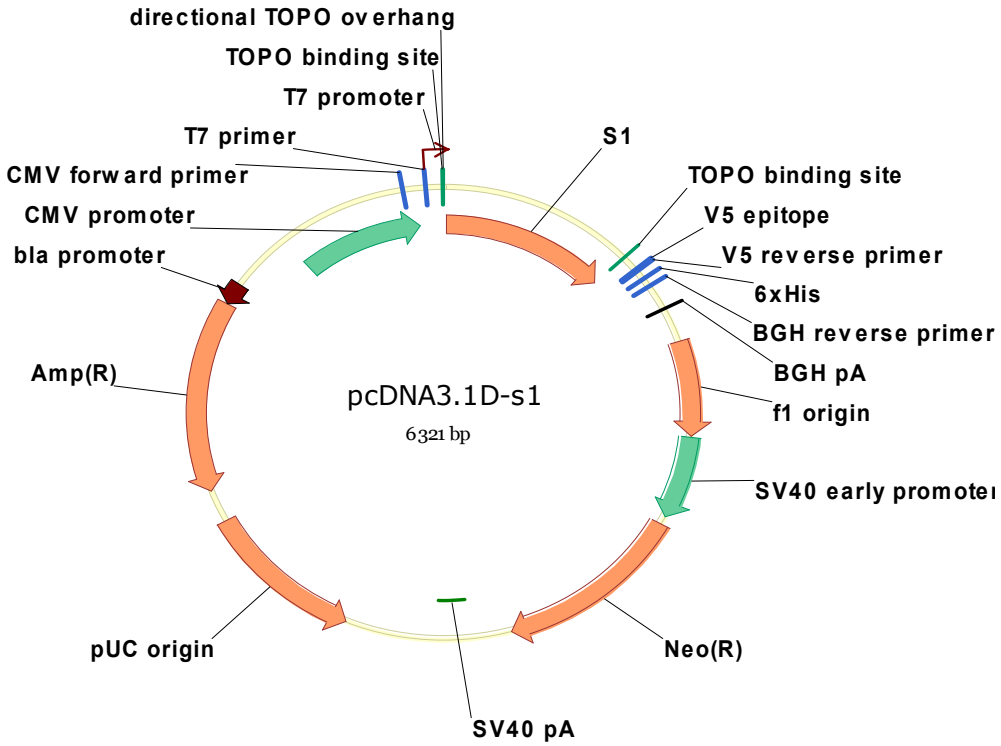

Figure 6.1: Map of pcDNA3.1D/*pts1* generated using Vector NTI (Invitrogen).

6.2.1.3 Screening of Plasmid Clones

Plasmid DNA from selected TOP10 transformants was isolated by Miniprep and analysed on a 1% TAE agarose gel (Chapter 2, Section 2.6.2). The insert sequences of the recombinant plasmids with a molecular weight equivalent to the expected size were verified by DNA sequencing as described in Chapter 2, Section 2.7. Primer sequences used for sequencing are listed in Table 2.3. Small fragments obtained from sequencing reactions were assembled into contigs and aligned against the published *ptx* gene sequence using the ClustalW program (ANGIS).

6.2.2 Site-Directed Mutagenesis

According to the method of Pizza et al. (1989), the S1 insert of pTrcHis2/*pts1* was inactivated using two amino acid substitutions in which the arginine at position 13 was replaced with leucine (pTrcHis2/*pts1.13L*) and then the glutamic acid at position 129 was changed to glycine (pTrcHis2/*pts1.13L.129G*). Mutagenesis was carried out using the Quick Change XL Site-Directed Mutagenesis Kit (Stratagene) as described in Chapter 2, Section 2.8. For each point mutation, PCR products were transformed into the XL10-Gold strain of *E. coli* followed by plasmid DNA isolation and sequencing.

Rather than repeating the mutagenesis procedure for pcDNA3.1D/*pts1*, the inserts of pTrcHis2/*pts1.13L* and pTrcHis2/*pts1.13L.129G* were amplified by PCR (Chapter 2, Section 2.5.2) and then ligated into the pcDNA3.1D/V5-His-TOPO vector (Chapter 2, Section 2.6). Inserts of pcDNA3.1D/*pts1.13L* and pcDNA3.1D/*pts1.13L.129G* plasmids were sequenced as previously described (Chapter 2, Section 2.7). Sequences of the mutagenic primers (mutant nucleotides shown as bold and underlined) were:

For incorporation of R13L mutation
PTXS1-13LF – 5' TACCGCTATGACTCCC**TG**CCGCCGGAGGAC 3'
PTXS1-13LR – 5' GAAAACGTCCTCCGGCGGC**AG**GGAGTCATAGCGGTA 3'
For incorporation of E129G mutation:
PTXS1-129GF – 5' GCCACCTACCAGAGCG**GC**TATCTGGCACACCGG 3'
PTXS1-129GR – 5' CCGGTGTGCCAGATA**GC**CGCTCTGGTAGGTGGC 3'

6.2.3 Chinese Hamster Ovary (CHO) Cell Assay

To verify inactivation of the mutant S1 subunit, a CHO cell assay was performed as described in Chapter 2, Section 2.13.1. CHO-K1 cells were transfected with the pcDNA3.1D/*pts1*, pcDNA3.1D/*pts1.13L* and pcDNA3.1D/*pts1.13L.129G* plasmid constructs or treated with a commercial pertussis toxin preparation.

6.2.4 Expression of rPTS1.13L.129G in *E. coli*

Pilot expression of rPTS1.13L.129G in TOP10 *E. coli* was performed according to the method outlined in Chapter 2, Section 2.12.1. Expression of rPTS1.13L.129G was detected by Western blotting with an anti-His (C-term) antibody (Chapter 2, Section 2.11).

6.2.5 Purification of rPTS1.13L.129G

Recombinant PTS1.13L.129G was purified from BL21 CodonPlus(RP) *E. coli* by Ni-NTA affinity chromatography under denaturing conditions as previously described (Chapter 2, Section 2.12.3). Purified protein was checked by SDS-PAGE and Western blot (Chapter 2, Sections 2.10 and 2.11). Protein samples were concentrated by ultra-centrifugation (Appendix C.6) with the yield estimated by modified Bradford assay (Chapter 2, Section 2.12.4). Recombinant PTS1.13L.129G was purified for use in ELISA and splenocyte stimulation.

6.2.6 Transient Transfection of COS-7 Mammalian Cells and Expression

The mammalian cell line COS-7 was transfected with pcDNA3.1D/*pts1.13L.129G* as previously described (Chapter 2, Section 2.9). Expression of rPTS1.13L.129G was detected by Western blotting using an AP-conjugated anti-V5 antibody (Chapter 2, Section 2.11).

6.2.7 Transformation of *S. typhimurium* with pcDNA3.1D/*pts1.13L.129G*

The *aro*A *Salmonella typhimurium* strain SL3261 was transformed with the pcDNA3.1D/*pts1.13L.129G* plasmid for mucosal delivery via the oral route (Chapter 8). Initial passage through the r^-m^+ *S. typhimurium* strain P9121 was required to methylate the *E. coli*-propagated plasmids for successful transformation into SL3261, which possesses an intact DNA restriction system. *S. typhimurium* P9121 were transformed using the heat shock method (Chapter 2, Section 2.14.2), whereas *S. typhimurium* SL3261 were transformed by electroporation (Chapter 2, Section 2.14.3).

6.2.8 Immunisation of Mice with pcDNA3.1D/*pts1.13L.129G* via the IM Route

A component of the first DNA vaccine efficacy trial involved immunisation of mice with the pcDNA3.1D/*pts1.13L.129G* DNA vaccine as described in Chapter 2, Section 2.15.2. Immunisation involved direct injection of naked DNA into the quadriceps. To obtain sufficient plasmid for a 3 dose regime, large scale isolation was performed from 2.5 L cultures of TOP10-pcDNA3.1D/*pts1.13L.129G* (Chapter 2, Section 2.15.2.1).

6.2.9 Determination of Immune Response and Protective Efficacy to DNA Vaccination

Of the 17 mice per group vaccinated, 5 were sampled for serum and organs two weeks after the third and final dose. Serum samples were analysed for the presence of Ag-specific IgG, IgG1 and IgG2a by ELISA (Chapter 2, Section 2.16.2) and Western blot (Appendix B.1), whereas the cytokine profiles of re-stimulated splenocytes was used to indicate CMI (Chapter 2, Section 2.16.1). The remaining 12 mice/group were challenged with a predetermined sub-lethal infectious dose (SLID) of virulent *B. pertussis* Tohama I as previously described (Chapter 2, Section 2.15.4). For direct comparison of DNA vaccine efficacy, a group vaccinated with the pcDNA3.1 vector only was included as a control along with placebo and DTaP.

6.3 Results

6.3.1 Construction of pcDNA3.1D/*pts1.13L.129G* and pTrcHis2/*pts1.13L.129G*

The *S1* gene of the *ptx* operon was successfully amplified from *B. pertussis* Tohama I genomic DNA. Figure 6.2 shows the 806 bp product generated with Platinum *Pfx* DNA polymerase and PTX507DNAF/PTX1313DNAR oligonucleotides. Similarly, Figure 6.3 shows the same 806 bp target amplified using the Platinum *Taq* High Fidelity DNA polymerase and PTX507TRCF/PTX1313TRCR oligonucleotides. PCR with Platinum *Taq* High Fidelity was efficient over a wide range of annealing temperatures (45.1 - 60.2 °C), whereas Platinum *Pfx* was only efficient at higher annealing temperatures i.e. 55 to 60°C.

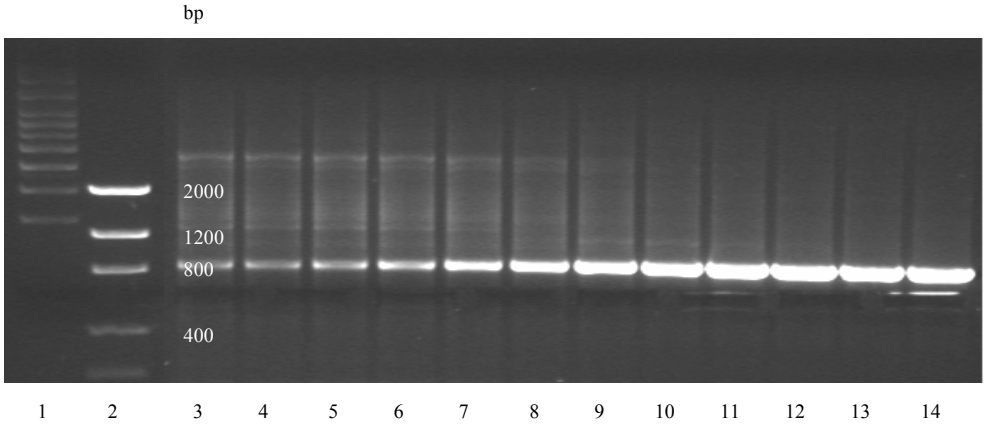

Figure 6.2: PCR of *pts1* gene using Platinum *Pfx* DNA polymerase. Lane 1 - Kilobase Marker (10kb to 0.5kb - Amersham); lane 2 - Low DNA Mass ladder (2000bp to 100bp - Invitrogen); lanes 3 to 14 - amplification of *pts1* with PTX507DNAF/PTX1313DNAR oligonucleotides and gradient annealing temp of 45 to 60°C.

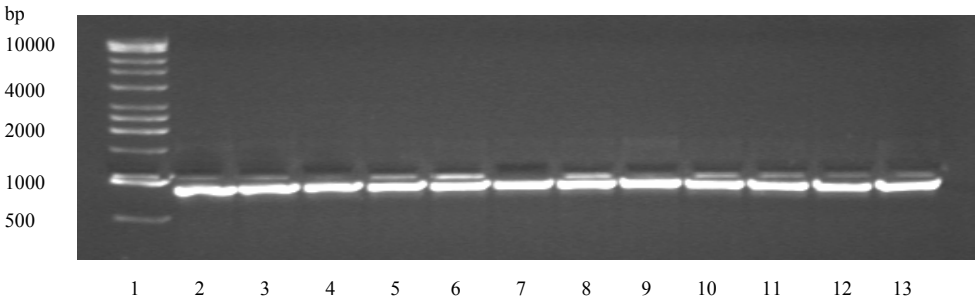

Figure 6.3: PCR of *pts1* gene using Platinum *Taq* High Fidelity polymerase. Lane 1 - Kilobase Marker (10kb to 0.5kb - Amersham); lanes 2 to 13 – amplification of *pts1* with PTX507TRCF/PTX1313TRCR oligonucleotides and gradient annealing temp of 45 to 60°C.

Blunt-ended (Platinum *Pfx*) and A-tailed (Platinum *Taq*) PCR products (see Section 6.2.1.1) were purified and cloned into the respective pcDNA3.1D/V5-His-TOPO and pTrcHis2-TOPO vectors (refer to section 2.5.2). Plasmid DNA from transformed TOP10 cells were isolated and checked against the expected size on a 1% agarose gel. The inserts of putative pcDNA3.1D/*pts1* clones with an appropriate size of 6.3 kb (Figure 6.4, lane 3) were sequenced and compared to the published Genbank sequence using the WebAngis ClustalW program (not shown). This alignment revealed the inserts were correctly orientated with no base mismatches. Likewise, the pTrcHis2-TOPO/*pts1* clones that were 5.2 kb in size (Figure 6.5, lane 3) were screened by DNA sequencing, with orientation and base composition shown to be correct by ClustalW alignment.

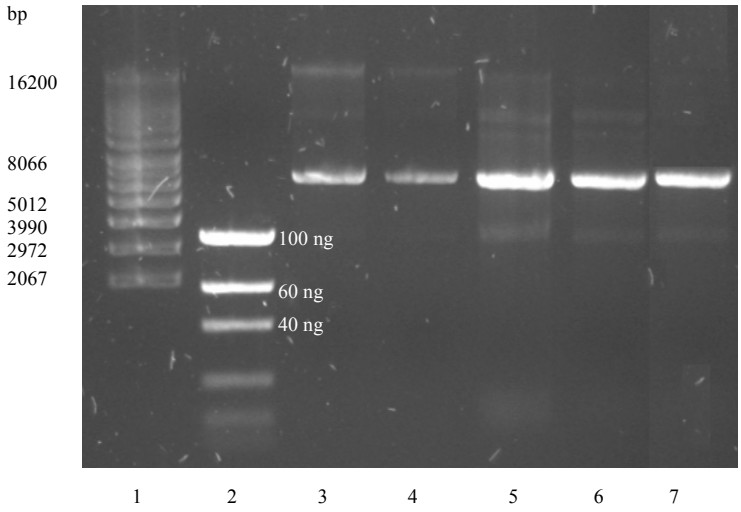

Figure 6.4: Isolation of pcDNA3.1D/*pts1*, pcDNA3.1D/*pts1.13L* and pcDNA3.1D/*pts1.13L.129G* from *E. coli* and *S. typhimurium*. Lane 1 – Supercoiled DNA ladder (16,210bp to 2,067bp - Invitrogen); lane 2 – Low DNA Mass ladder (2000bp to 100bp - Invitrogen); lane 3 - pcDNA3.1D/*pts1* from TOP10; lane 4 - pcDNA3.1D/*pts1.13L* from XL10-Gold; lane 5 - pcDNA3.1D/*pts1.13L.129G* from XL10-Gold; lane 6 - pcDNA3.1D/*pts1.13L.129G* from *S. typhimurium* P9121; lane 7 - pcDNA3.1D/*pts1.13L.129G* from *S. typhimurium* SL3261.

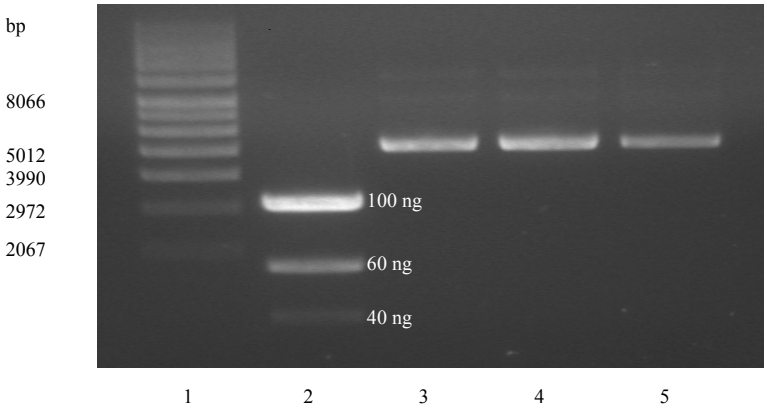

Figure 6.5: Isolation of pTrcHis2/*pts1*, pTrcHis2/*pts1.13L.19G* and pTrcHis2/*pts1.13L.129G* from *E. coli*. Lane 1 - Supercoiled DNA ladder (16,210bp to 2,067bp - Invitrogen); lane 2 - Low DNA Mass ladder (2000bp to 100bp - Invitrogen); lane 3 - pTrcHis2/*pts1* from TOP10; lane 4 - pTrcHis2/*pts1.13L.19G* from XL10-Gold; lane 5 - pTrcHis2/*pts1.13L.129G* from XL10-Gold *E. coli*.

The sequence-confirmed pTrcHis2/*pts1* plasmid was used as the template for site-directed mutagenesis of the *s1* insert. Amplification of the 5.2 kb target pre-empted a successful mutagenesis reaction (Figure 6.6). Colonies from the transformation of XL10-Gold *E. coli* with the mutagenesis PCR product were screened by isolation of plasmid DNA (Figure 6.5, lane 4) followed by sequencing of the insert to confirm successful mutation of the bases at positions 646 and 647. Importantly alignment with the Genbank sequence also revealed no unwanted mismatches (not shown). The mutagenesis procedure was repeated with the pTrcHis2/*pts1.13L* plasmid used as the template for mutation of bases 994 and 995. Sequencing was again used to confirm that the second mutation was successful.

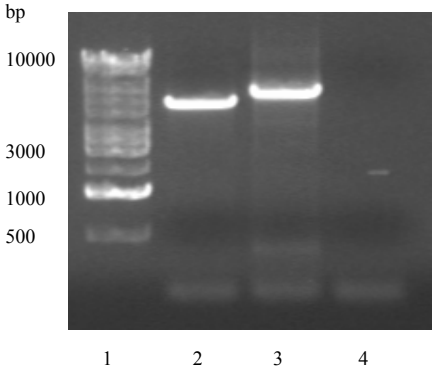

Figure 6.6: Site-directed mutagenesis PCR of pTrcHis2/*pts1*. Lane 1 – Kilobase Marker (10kb to 0.5kb - Amersham); lane 2 – positive control pWhiteScript; lane 3 – putative pTrcHis2/*pts1.13L.129G* reaction; lane 4 – negative control (no plasmid DNA template).

Rather than repeat the two mutagenesis procedures for pcDNA3.1D/*pts1*, the mutated inserts were amplified using the pTrcHis2/*pts1.13L* and pTrcHis2/*pts1.13L.129G* plasmids as a template. The 0.8 kb targets were successfully amplified using Platinum *Pfx* DNA polymerase (Figure 6.7) and cloned into pcDNA3.1D/V5-His-TOPO. Plasmid DNA was isolated from selected XL10-Gold transformants and those with an appropriate size (6320 bp)were sequenced (Figure 6.8).

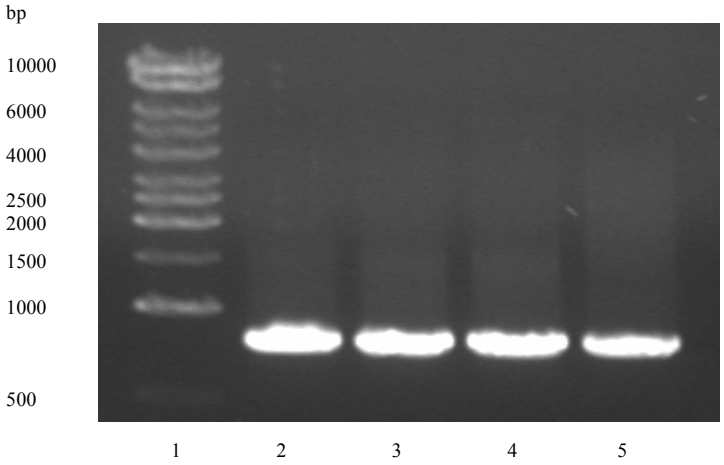

Figure 6.7: PCR of *pts1.13L* and *pts1.13L.129G* inserts. Lane 1 – Kilobase Marker (10kb to 0.5kb - Amersham); lanes 2 and 3 – amplification of *pts1.13L* from pTrcHis2/*pts1.13L* template with Platinum *Pfx* DNA polymerase and PTX507DNAF/PTX1313DNAR oligonucleotides; lanes 4 and 5 - amplification of *pts1.13L.129G* from pTrcHis2/*pts1.13L.129G* template with Platinum *Pfx* DNA polymerase and PTX507DNAF/PTX1313DNAR oligonucleotides.

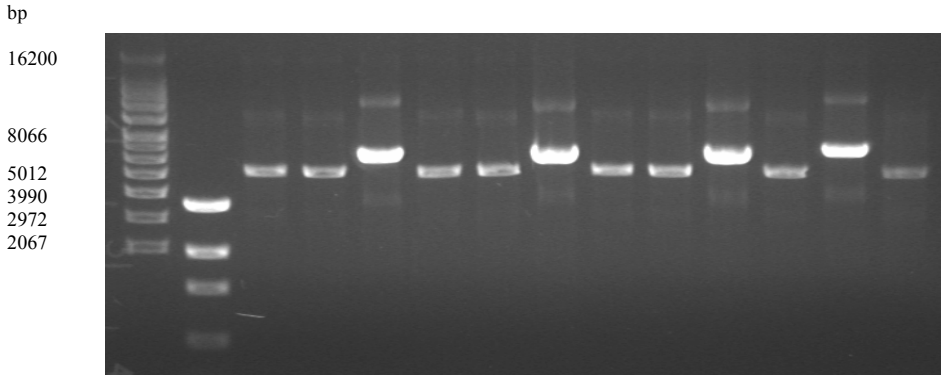

Figure 6.8: Isolation of pcDNA3.1D/*pts1.13L* and pcDNA3.1D/*pts1.13L.129G* from XL10-Gold *E. coli*. Lane 1 - Supercoiled DNA ladder (16,210bp to 2,067bp – Invitrogen); lane 2 – Low DNA Mass ladder (2000bp to 100bp - Invitrogen); lanes 3 to 8 – putative pcDNA3.1D/*pts1.13L* clones #1 to #6; lanes 9 to 14 – putative pcDNA3.1D/*pts1.13L.129G* clones #1 to #6.

Alignment of the pcDNA3.1D/*pts1.13L* and pcDNA3.1D/*pts1.13L.129G* insert sequences with that of the published *ptx* gene indicated that the desired mutations were present and that no additional base errors were incorporated (Appendix D.4).

6.3.2 Transient Expression of rPTS1.13L.129G in COS-7 Cells

COS 7 cell lines were transfected with pcDNA3.1D/*pts1.13L.129G* isolated from *E. coli* XL10 Gold to confirm constitutive expression of rPTS1.13L.129G. Using an AP-conjugated anti-V5 antibody, multiple bands were detected with a MW of between 25 and 40kDa by western blot (Figure 6.9). This double banding pattern has been observed by others (Castro, Mcnamara & Carbonetti 2001) and was speculated to be due to proteolytic degradation but may also be due to incomplete reduction of disulfide bonds within S1. Nevertheless, the size of these bands was equivalent to the expected 33 kDa MW of rPTS1.13L.129G as calculated using the Translate GCG program (WebAngis).

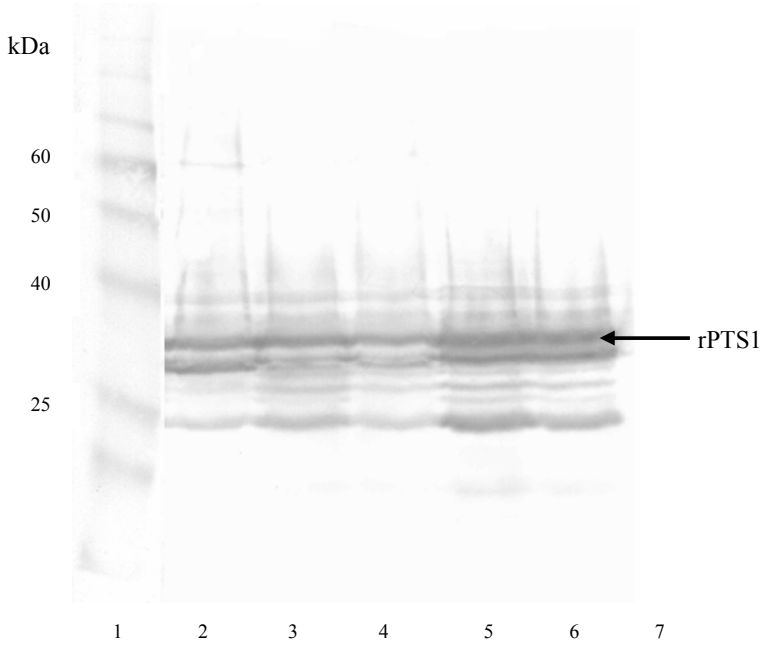

Figure 6.9: Western Blotting of rPTS1, rPTS1.13L and rPTS1.13L.129G expression in COS-7 cells. Lane 1 - BenchMark protein ladder (220kDa to 10kDa - Invitrogen); lane 2 – rPTS1 from pcDNA3.1D/*pts1* (plasmid isolated from *E. coli* TOP10), lane 3 – rPTS1.13L from pcDNA3.1D/*pts1.13L* (plasmid isolated from *E. coli* XL10-Gold), lane 4 – rPTS1.13L.129G from pcDNA3.1D/*pts1.13L.129G* (plasmid isolated from *E. coli* XL10-Gold), lane 5 – rPTS1.13L.129G from pcDNA3.1D/*pts1.13L.129G* (plasmid isolated from *S. typhimurium* P9121), lane 6 – rPTS1.13L.129G from pcDNA3.1D/*pts1.13L.129G* (plasmid isolated from *S. typhimurium* SL3261), and lane 7 – negative control (COS-7 lysate).

6.3.3 Cytotoxicity of Recombinant S1 Analogues

A CHO cell clustering assay was used to determine the relative toxicity of the mutant rPTS1.13L.129G and rPTS1.13L analogues compared to the non-mutated recombinant S1 protein and wild-type holotoxin. Since the observation that CHO cells exhibit an atypically rounded and clustered morphology in the presence of exogenous PT (Hewlett et al. 1983), the CHO cell assay has been refined and is now a standardised measure of ADP-ribosylase activity i.e. cytotoxicity of pertussis toxoids.

The suitability of this assay as a confirmation of S1 inactivity was evident by the clear difference between the morphologies observed in the test and positive control groups. The first positive control involved the transfection of CHO cells with pcDNA3.1D/*pts1* for the endogenous expression of the non-mutated or "wild-type" rPTS1 protein. Unlike the untreated cells (Figure 6.10E and F) that grew as a confluent monolayer, CHO-K1 cells transfected with pcDNA3.1D/*pts1* had a clearly clustered appearance after a 12 hour incubation period (Figure 6.10A). As expected this clustered morphology was also observed with the cells exposed to wild-type pertussis toxin (Figure 6.10D). Overall the clustering appeared to be slightly more pronounced and compact in this group than in the pcDNA3.1D/*pts1* control group.

Endogenous expression of rPTS1.13L.129G following transfection of CHO cells with pcDNA3.1D/*pts1.13L.129G*, had little or no effect on the cell morphology (Figure 6.10C). This can be seen by a similar morphology to that of the untreated cells. The cumulative effect of two point mutations versus a single amino acid substitution was tested by including a group of CHO cells transfected with pcDNA3.1D/*pts1.13L* for the endogenous expression of rPTS1.13L. Whilst there appeared to be a low level of clustering that was not present in the pcDNA3.1D/*pts1.13L.129G* treatment group; the clustering was less obvious than with the wild-type S1 sequence or the exogenous PT treatment (positive controls).

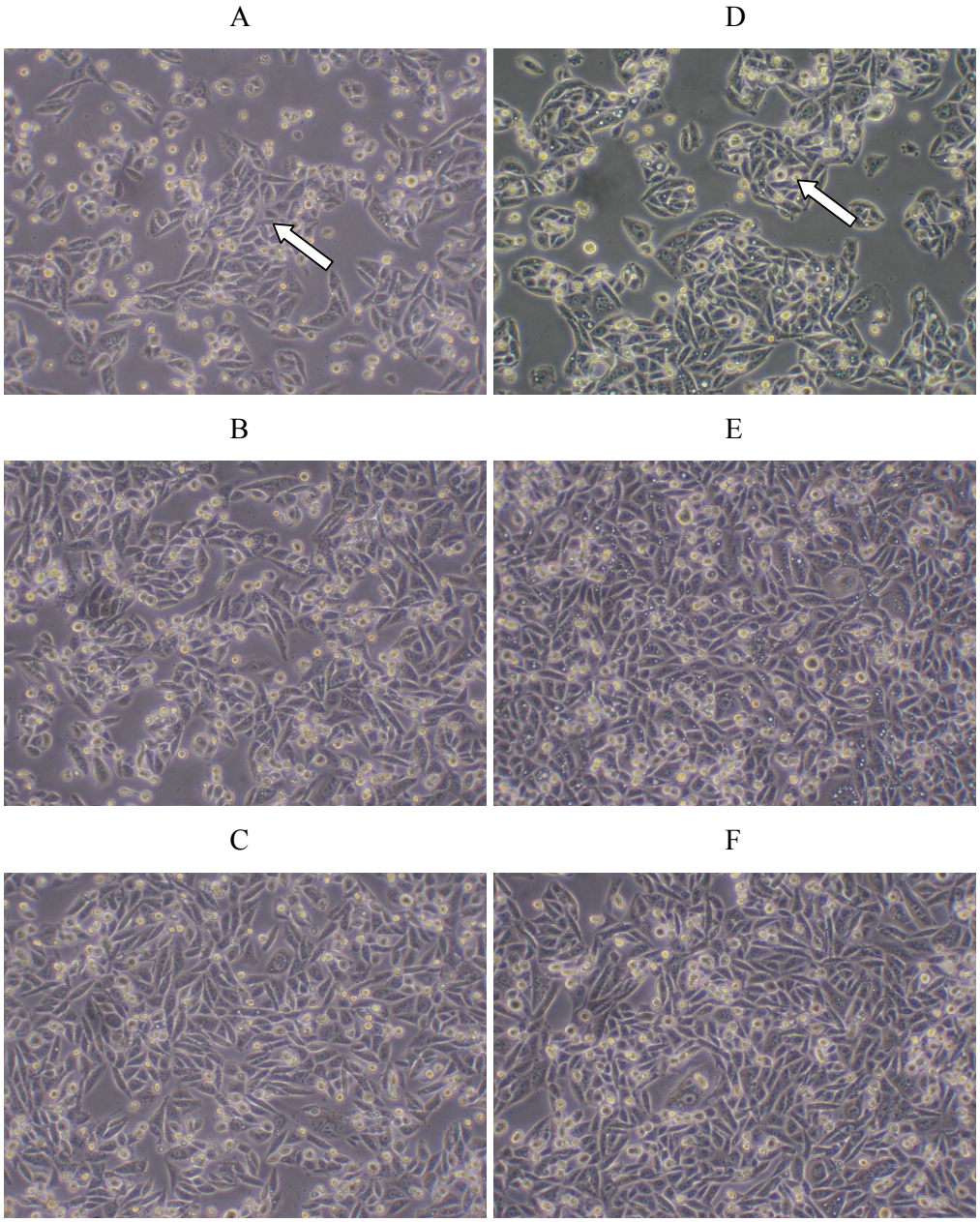

Figure 6.10: CHO-K1 cell morphology following exposure to rPTS1 and rPTS1.13L and rPTS1.13L.129G antigens. A representative section of adherent cells was recorded 20 hours after: A – transfection with 2.5 µg pcDNA3.1D/*pts1* (endogenous expression of rPTS1), B – transfection with 2.5 µg pcDNA3.1D/*pts1.13L* (rPTS1.13L), C – transfection with 2.5 µg pcDNA3.1D/*pts1.13L.129G* (rPTS1.13L.129G), D – treatment with 800ng Pertussis toxin (List Biologicals), E – addition of Lipofectamine 2000 reagent without pDNA (untreated), and F – addition of plasmid DNA without Lipofectamine 2000 reagent (untreated). Arrows indicate the characteristic clustered morphology of CHO-K1 following the action of the S1 subunit of PT.

6.3.4 Expression and Purification of rPTS1.13L.129G

Induced expression of the rPTS1.13L.129G fusion protein was detected in the lysate of XL10 Gold-pTrcHis2/*pts1.13L.129G* by western blotting with a HRP conjugated anti-His antibody (Figure 6.11). A dominant band with a MW of 30 kDa was observed and corresponded to the MW of rPTS1.13L.129G when expressed in COS-7 cells (Figure 6.9). The level of rPTS1.13L.129G expression in XL10 Gold was found to be sufficient for preparative purification by affinity chromatography (Figure 6.12) and the transformation of BL21 Codon Plus *E. coli* with pTrcHis2/*pts1.13L.129G* was not required.

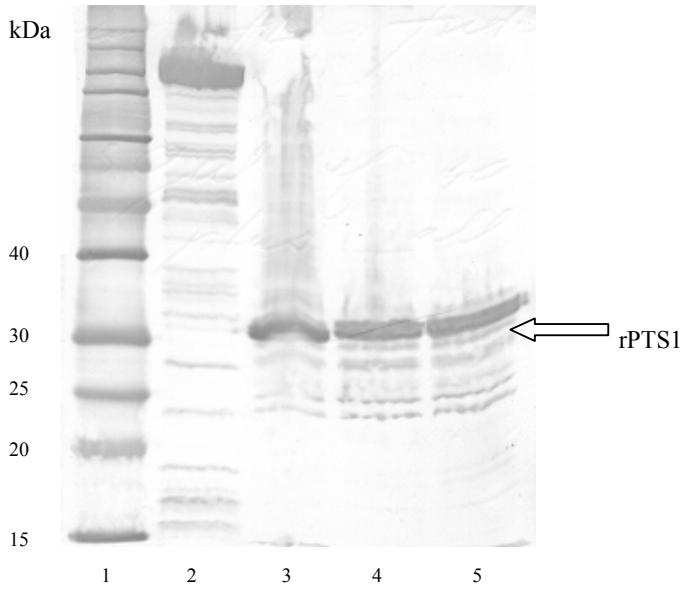

Figure 6.11: Western blotting of rPTS1 and rPTS1.13L/PTS1.13L.129G expression in *E. coli*. Lane 1 - BenchMark protein ladder (220kDa to 10kDa – Invitrogen); lane 2 - β-galactosidase-His fusion protein (expression control); lane 3 - rPTS1; lane 4 - rPTS1.13L and lane 5 - rPTS1.13L.129G detected with HRP-conjugated anti-His antibody.

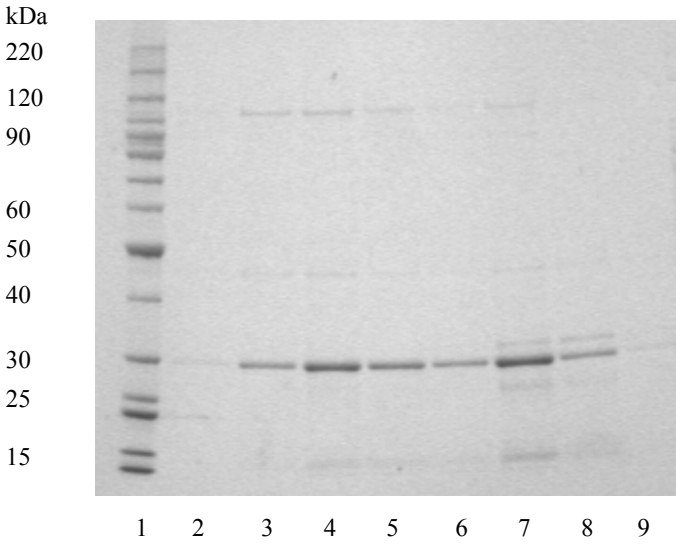

Figure 6.12: PAGE of affinity purified rPTS1.13L.129G. Lane 1: BenchMark Protein Ladder (220kDa to 10kDa – Invitrogen); lanes 2 to 5 – elution from 0.5 ml flow-throughs of buffer D pH 5.9; lanes 6 to 9 – elution from 0.5 ml flow-throughs of buffer E pH 4.5.

6.3.5 Immunogenicity and Protective efficacy of Vaccination with pcDNA3.1D/*pts1.13L.129G* by the IM Route

Balb/c mice were immunised with three IM doses of pcDNA3.1D/*pts1.13L.129G* as per section 2.15.2. Two weeks after the second and final booster, a small subgroup of 5 mice were sacrificed and sampled for the measurement of antibody and cytokine levels. Also at two weeks after the second booster a larger subgroup of 12 vaccinated mice were challenged with a predetermined SLID of virulent *B. pertussis*. The clearance of the SLID from the lungs was monitored over a 14 day period.

6.3.5.1 *Cytokine Profiles Following DNA Vaccination with pcDNA3.1D/pts1.13L.129G*

Splenocytes of mice immunised with pcDNA3.1D/*pts1.13L.129G* produced a very high level of IFN-γ when cultured in the presence of either rPTS1.13L.129G or ConA, with 5253 and 6000 pg/ml respectively (Figure 6.13). However, upon stimulation with either DTaP or a heat-killed *B. pertussis* lysate these cells produced much lower levels of IFN-γ with 450 and 1629 pg/ml respectively. Splenocytes from vector and DTaP-immunised mice produced an equivalent low level IFN-γ response when stimulated with rPTS1.13L.129G.

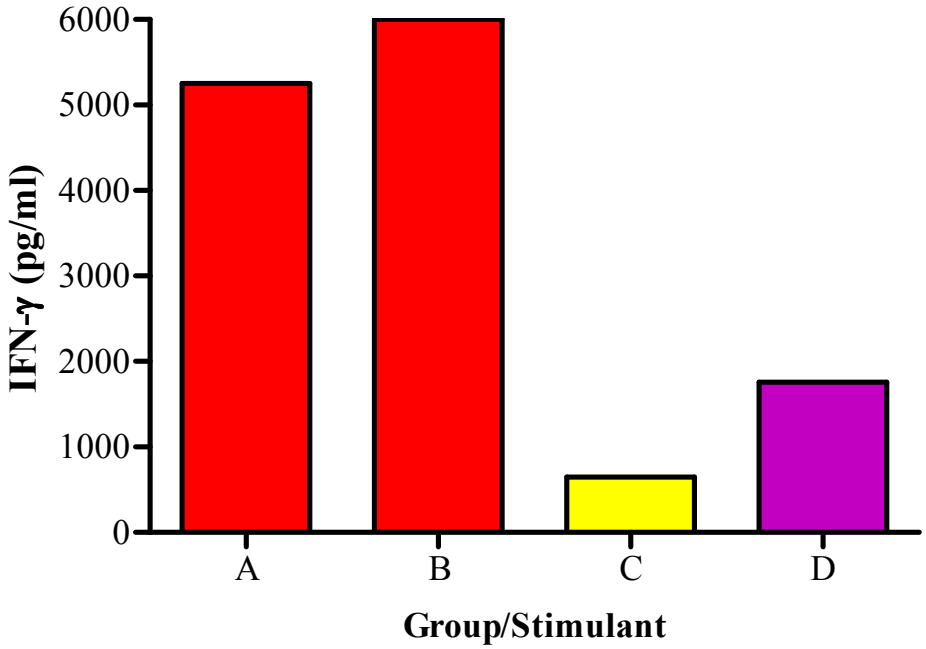

Figure 6.13: IFN-γ production from rPTS1.13L.129G-stimulated splenocytes. Splenocytes from pcDNA3.1D/*pts1.13L.129G*-immunised mice stimulated with 5µg/ml rPTS1.13L.129G (A) and 2.5µg/ml ConA (B). Splenocytes from vector-immunised mice stimulated with 5µg/ml rPTS1.13L.129G (C). Splenocytes from DTaP-immunised mice stimulated with 5µg/ml rPTS1.13L.129G (D).

There were no observable differences in IL-2 production between these groups of splenocytes with the obvious exception of the ConA-stimulated cells (Figure 6.14). Splenocytes from pcDNA3.1D/*pts1.13L.129G*, vector and DTaP-immunised mice all produced low levels of IL-2 when stimulated with rPTS1.13L.129G. There was virtually no IL-2 (5 pg/ml) detected when the pcDNA3.1D/*pts1.13L.129G*-primed splenocytes were stimulated with DTaP. In contrast, a very large amount of IL-2 (12,388 pg/ml) was produced when these same cells were stimulated with ConA (Figure 6.14). The greatest amount of IL-4, a Th2 cytokine, was produced in the supernatants of DTaP-immunised mice when stimulated with the commercial vaccine formulation (Table 6.1). In mice vaccinated with pcDNA3.1D/*pts1.13L.129G* there was virtually no IL-4 produced when stimulated with rPTS1.13L.129G or DTaP. Again, a relatively large amount of IL-4 was produced upon stimulation with ConA. Mice vaccinated with pcDNA3.1D vector produced equivalent levels of IL-4 with that of mice given the DNA vaccine.

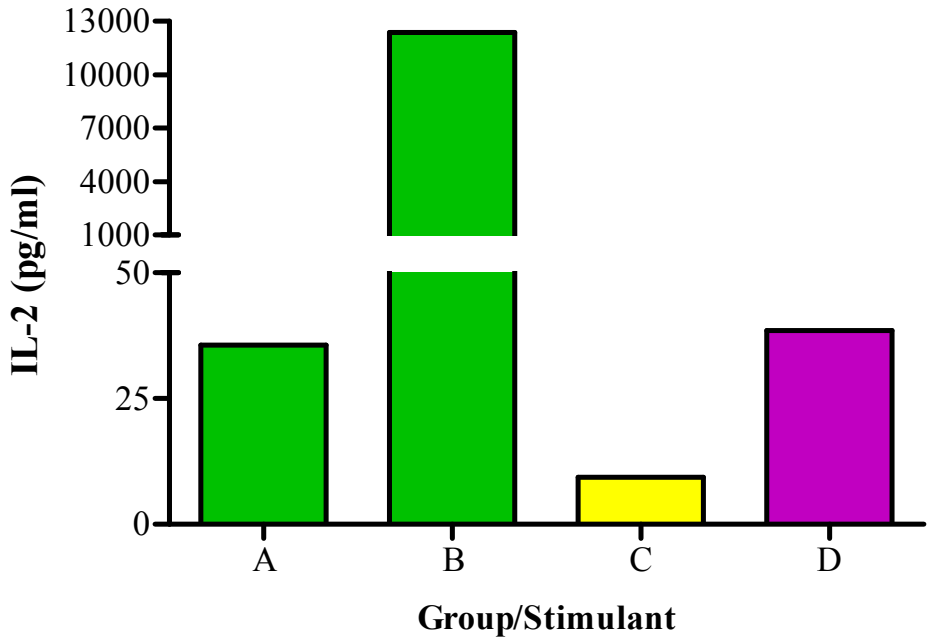

Figure 6.14: IL-2 production from rPTS1.13L.129G-stimulated splenocytes. Splenocytes from pcDNA3.1D/*pts1.13L.129G*-immunised mice stimulated with 5µg/ml rPTS1.13L.129G (A) and 2.5µg/ml ConA (B). Splenocytes from vector-immunised mice stimulated with 5µg/ml rPTS1.13L.129G (C). Splenocytes from DTaP-immunised mice stimulated with 5µg/ml rPTS1.13L.129G (D).

Table 6.1: IL-4 production from rPTS1.13L.129G-stimulated splenocytes. IL-4 secreted from $5x10^6$ splenocytes cultured for 72 hours in the presence of rPTS1.13L.129G, Infanrix™ DTaP and ConA.

Stimulant	**µg/ml**	**IL-4 (pg/ml)**		
		pcDNA3.1D/*pts1.13L.129G*	**pcDNA3.1 vector**	**DTaP**
rPTS1.13L.129G	5	15.3	12.8	5.3
DTaP	5	9.5	6.4	36.9
DTaP	10	2.3	6	69.9
ConA	2.5	468.2	511.3	1081.9

6.3.5.2 *Serum Antibody Response Following DNA Vaccination with pcDNA3.1D/pts1.13L.129G*

No serum IgG against the rPTS1.13L.129G fusion protein or wild-type PT was detected in mice vaccinated with the pcDNA3.1D/*pts1.13L.129G* or mice vaccinated with the pcDNA3.1D vector only (Table 6.2). In contrast, a rPTS1.13L.129G-specific IgG response was observed in the serum of mice immunised with DTaP, with a mean titer of 14080 (± 9308) but was not significantly greater than the DNA vaccine group of negative control groups. The IgG response to the entire PT (commercial supplier) antigen was significantly greater in mice vaccinated with DTaP compared with those given the DNA vaccine ($P < 0.01$). Further analysis of serum samples from mice immunised with DTaP showed that the vast majority of IgG present was of the IgG1 subclass (Table 6.3).

Table 6.2: Anti-Pertussis toxin IgG endpoint titers in sera of mice vaccinated with pcDNA3.1D/*pts1.13L.129G*. Figures represent mean scores from five mice +/- SE. * Significantly different from pcDNA3.1D/*pts1.13L.129G* ($P < 0.01$).

Group	**Anti-rPTS1.13L.129G**		**Anti-PTX**	
	Titer	**SE**	**Titer**	**SE**
pcDNA3.1D/*pts1.13L.129G*	0	0	0	0
pcDNA3.1D vector	0	0	0	0
DTaP	14080	9308	92369*	17753

Table 6.3: Serum IgG1 and IgG2a titers of mice vaccinated with pcDNA3.1D/*ptS1.13L.129G*. Bars represent scores from pooled serum. Nd - Not determined.

Antigen	**pcDNA3.1D/*pts1.13L.129G***		**pcDNA3.1D vector**		**DTaP**	
	IgG1	**IgG2a**	**IgG1**	**IgG2a**	**IgG1**	**IgG2a**
rPTS1.13L.129G	0	0	200	0	Nd	Nd
PTX	Nd	Nd	Nd	Nd	146689	100

6.3.5.3 *Protective Efficacy of pcDNA3.1D/pts1.13L.129G DNA Vaccine*

Each group of mice in the preliminary mouse experiment, including mice immunised with pcDNA3.1D/*pts1.13L.129G* as well as the negative and positive control groups, were infected with a pre-determined SLID of Tohama I. As a measure of protective efficacy, bacterial numbers from the lungs of challenged mice were recorded at three time points over a 14 day period to indicate the rate of clearance.

Results of the challenge experiment showed that the rate of clearance from pcDNA3.1D/*pts1.13L.129G*-immunised mice was superior to the placebo or vector only negative controls but inferior to the DTaP-immunised group (Figure 6.15). At day 4 post-challenge, mice vaccinated with either pcDNA3.1D/*pts1.13L.129G*, the placebo or vector only had a slight two to four-fold increase in bacterial numbers compared to day 0 counts. By day 7 the DNA vaccinated mice showed a 10-fold reduction in numbers followed by an over 6000-fold reduction after two weeks, whereas the negative control mice failed to show any reduction in CFU/lung counts. Despite controlling the experimental challenge, DNA-vaccinated mice failed to completely clear the *B. pertussis* infection within the 14 day period. On the other hand, mice immunised with DTaP demonstrated a more rapid and complete clearance with no bacteria detected in the lungs from day 7 post-challenge.

Further analysis of the clearance data showed that bacterial counts in mice immunised with pcDNA3.1D/*pts1.13L.129G* were reduced to 1.46% of the placebo controls at day 7 post-challenge and then to 0.001% at 14 days post-challenge (Table 6.4). The calculation of a clearance index (CI) indicated a significant increase in the rate of clearance for mice vaccinated with pcDNA3.1D/*pts1.13L.129G* compared to mice vaccinated with the placebo ($P < 0.001$) or plasmid vector only ($P < 0.001$) (Table 6.4). Moreover, this data also showed that immunisation of mice with DTaP conferred a significantly improved degree of protection from a SLID of *B. pertussis* than the S1 DNA vaccine ($P < 0.05$) (Table 6.4).

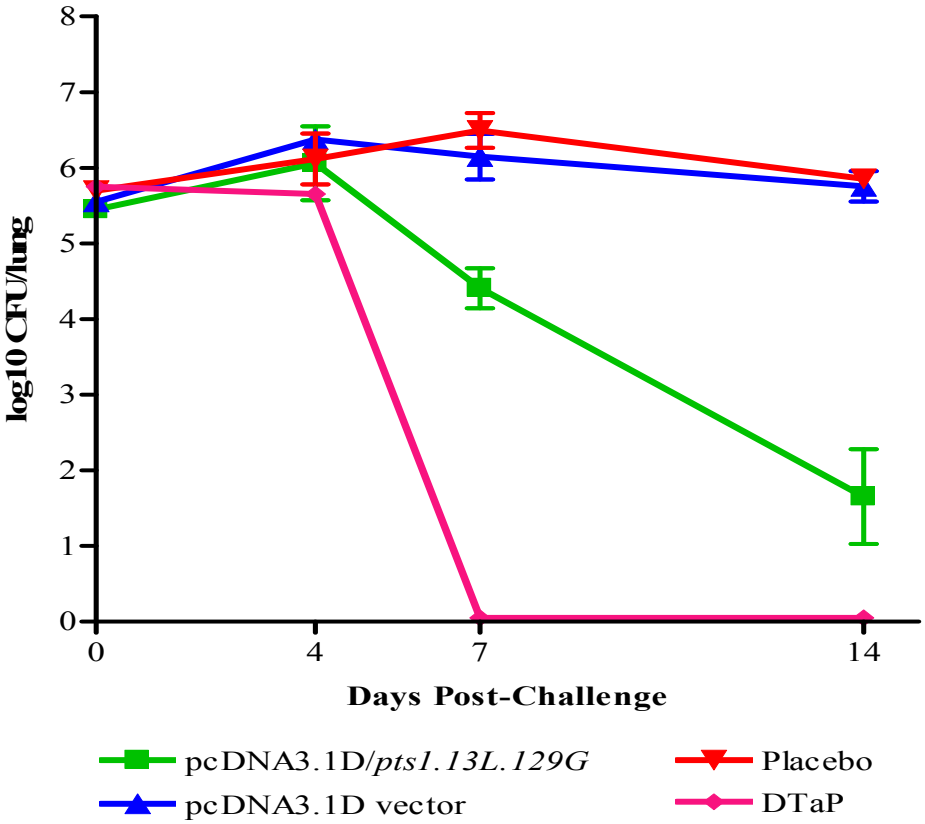

Figure 6.15: Bacterial loads in lungs of pcDNA3.1D/*pts1.13L.129G* vaccinated mice following aerosol challenge. Data points represent mean count of CFU/lung from 5 mice +/- SE.

Table 6.4: Post-challenge clearance data from mice immunised with pcDNA3.1D/*pts1.13L.129G*.

Vaccine Group	% of Untreated Control[a] Day			CI[b]
	4	7	14	
Placebo	n/a	n/a	n/a	151.75 ± 1.65
Vector	255.7	63.5	115.3	151.78 ± 4.68
pcDNA3.1D/*pts1.13L.129G*	156.5	1.46	0.001	61.9 ± 10.28 *
DTaP	30.5	0	0	22.43 ± 0.05 **

[a] Quantitated as percentage of bacteria cleared from the lungs relative to the untreated (placebo) mice.

[b] Clearance Index ± SE = mean log10 number of *B. pertussis*/ml × days (van den Berg et al. 2001).

* Significantly different from placebo ($P < 0.005$) and vector ($P < 0.005$).

** Significantly different from placebo ($P < 0.001$), vector ($P < 0.001$) and pcDNA3.1D/*pts1.13L.129G* ($P < 0.05$).

6.4 Discussion

PT was an obvious candidate for evaluation as a DNA vaccine antigen. PT is the major virulence factor of *B. pertussis* and as a toxoid is a component of all modern acellular vaccines. From a vaccine design stand-point it would have been preferable to prime animals with the entire toxin however the complexity of the *ptx* operon limited the construction of DNA vaccines to an individual subunit. Secretion of the oligomeric toxin is a complex process. Each of the five subunits has a separate signal peptide that enables transport to the periplasm for processing and assembly (Craig-Mylius, Stenson & Weiss 2000). Despite continued attempts it has not been possible to express complete pertussis toxin in any heterologous system, with the exception of the closely related *B. bronchiseptica* (Walker et al. 1991; Suarez et al. 1997). Holotoxin assembly has been attempted without success in *E. coli* and *S. typhimurium* and even replacement of wild-type signal peptides with β-lactamase leader sequences did not improve the yield of correctly assembled toxin in *E. coli* (Dalla Pozza, Yan & Walker 1997). Dalla Pozza et al. (1998) reported that recombinant *aro*A *S. typhimurium* expressing the five subunits could not protect mice from a virulent challenge, which was attributed to incomplete processing of the subunits and not the method of delivery. Attempts at *in vitro* assembly via the reconstitution of purified subunits have also proven to be ineffective (Yamakawa, Sato & Sato 1990; Burnette et al. 1992). The *ptl* (pertussis toxin liberation) operon is necessary for efficient secretion of the assembled holotoxin (Weiss, Johnson and Burns 1993), but it appears as though *B. pertussis* may also possess a unique set of chaperones or proteases that enable correct processing and assembly of the subunits prior to secretion. Expression of the *ptx* operon in mammalian cells have not been reported but considering the difficulty associated with holotoxin assembly in other gram negative bacteria it was decided to focus on the delivery of a single PT subunit.

Of the five subunits, S1 appears to be the immunodominant moiety. Monoclonal and polyclonal antibodies generated against the S1 subunit have been reported to neutralise the toxin *in vitro* and protect mice against a lethal aerosol or intracerebral challenge (Sato et al. 1984; Halperin, Issekutz & Kasina 1991; Sato, Sato & Ohishi 1991). De Magistris et al. (1989) isolated PT-specific T-cell clones from an immune (convalescent) donor and found that the majority (83%) were specific for the S1 subunit. Mapping of S1 with recombinant fragments and synthetic peptides identified

the dominant T-cell epitopes (De Magistris et al 1989). There is also strong evidence that immunisation with the S1 subunit alone can elicit a protective immune response. Passive immunisation with monoclonal antibody specific for the S1 subunit protected mice against an experimental infection (Sato & Sato 1990). When coupled with a diphtheria toxin cross-reactive mutant protein (CRM 197) or bovine serum albumin (BSA), two 17-mer S1 peptides induced high ELISA titers and provided protection against a lethal PT challenge of mice (Askelof et al. 1990). Lee et al. (1999) later reported that oral vaccination of mice with a *Streptococcus gordonii* mutant expressing a chimeric S1 fusion protein generated a specific antibody response that conferred resistance to the toxic effects of PT. A recombinant *Mycobacterium bovis* BCG strain expressing genetically detoxified S1 subunit as a β-lactamase fusion also protected mice from a *B. pertussis* infection, in this instance from an intracerebral challenge (Nascimento et al. 2000). Walker et al. (1992) detected PT-specific IgG in the serum as well as IgG and IgA specific for PT in lung washes following oral immunisation of mice with attenuated *S. typhimurium* or *E. coli* expressing the S1 subunit. The success of these toxin neutralising therapies and vaccines justified the inclusion of S1 in the suite of DNA vaccines tested as part of this investigation.

An important aspect in the development of an S1-based DNA vaccine was inactivation of the *S1* gene. The multiple systemic manifestations elicited by PT have seen the antigen implicated in the serious side effects observed following immunisation with whole cell vaccines (Sato, Kimura & Fukumi 1984), and the WHO has directed that the catalytic domain (S1 subunit) of PT must be inactivated in the development of any pertussis vaccine (Cherry 1986). Of particular relevance to this project was the report that endogenous expression of S1 in a CHO cells had a cytotoxic effect as determined by the clustering of tranfected cells (Castro, McNamara & Carbonetti 2001). Pizza et al. (1989) found that substitution of two amino acid residues within the S1 polypetide by site-directed mutagenesis yielded a mutant subunit that was devoid of ADP-ribosylase activity but which retained its immunogenicity. It was revealed that substitution of Glutamic acid^{129} with Glycine in combination with either Arginine^{9} to Lysine, Arginine^{13} to Leucine or Tryptophan^{26} to Isoleucine completely abolished enzymatic activity (Pizza et al. 1989). Nencioni et al. (1990) characterised the 9K/129G mutant PT molecule and showed that they had the same physical and immunological properties of wild-type PT including migration in PAGE, affinity for S1-specific

monoclonal antibodies, mitogenicity for T cells and hemagglutinating activity. It was also demonstrated that the 9K/129G toxoid induced high titers of neutralising antibodies and protected mice against an intracerebral challenge (Nencioni et al 1990). For this study we chose the 13L/129G double point mutation as it was reported to have marginally less leukocytosis-promoting activity, greater T cell recognition, and a higher survival rate following lethal challenge than 9K/129G toxoid (Pizza et al. 1989). To validate our selection, the toxicity of wild-type S1, a single-point mutant S1.13L and the double-point mutant S1.13L.129G was compared by transfection of CHO-K1 cells with the respective eukaryotic expression constructs. The results of the modified CHO cell assay were consistent with the observations of Castro, McNamara & Carbonetti (2001). Castro, McNamara & Carbonetti (2001) observed clustering in CHO cells that expressed the wild-type S1 polypeptide and a normal CHO cell morphology after endogenous expression of the 9K/129G mutant S1 subunit. ADP- ribosylation assays confirmed that endogenous expression of wild-type S1 yielded an active enzyme, whereas expression of the 9K/129G showed a profile that was similar to the untreated control (Castro, McNamara & Carbonetti 2001). From our results it was evident that rPTS1.13L.129G had no apparent ADP-ribosylase activity as the morphology of CHO cells transfected with the pcDNA3.1D/*pts1.13L.129G* plasmid was identical to morphology of untreated cells (negative control). In contrast, CHO cells transfected with the parental or non-mutated S1 plasmid construct (pcDNA3.1D/*pts1*) showed a rounded and clustered morphology that was comparable with exposure to wild-type PT. The appearance of the round and clustered morphology of transfected CHO cells was reduced following endogenous expression of rS1.13L and then completely eliminated with rS1.13L.129G, which highlighted the need for the double-point mutation.

The concept that DNA vaccination with the S1 subunit alone can provide protection from the systemic effects of either PT or virulent *B. pertussis* has recently been proven in a mouse protection model (Kamachi et al. 2003; 2004). Kamachi et al. (2003) found that three gene-gun injections with a pcDNA/S1 DNA vaccine induced a significant IgG1 titer that protected against both the toxic effects of PT and a lethal intranasal challenge with virulent *B. pertussis*. In a follow-up study, a suite of DNA vaccines encoding truncated fragments of the S1 subunit were tested for the induction of a protective response to the effects of pertussis toxin (Kamachi et al. 2004). It was reported that gene gun immunisation of mice with the N-terminal enzymatic domain

(residues 1 to 180) was more efficacious than immunisation with the entire S1 sequence and indicated that the C-terminal or G-protein-binding domain (residues 181 to 236) was not essential for the induction of a protective response to PT. Unlike the Kamachi et al. (2003), our approach to DNA vaccination focused on the delivery of genetically inactivated S1 (13L/129G) via the IM route rather than gene-gun administration of a functional subunit or truncated derivative. DNA vaccination of mice with pcDNA3.1D/*pts1.13L.129G* induced a potent CMI response as indicated by a high level of IFN-γ produced from splenocytes re-stimulated with rS1.13L.129G. In fact, levels of this cytokine approached that of concanavalin A - a natural and potent T cell mitogen. When splenocytes from placebo-immunised mice were stimulated with rPTS1.13L.129G, a low or basal level of IFN-γ was produced, which ruled out the possibility that the recombinant antigen itself was mitogenic. At first the lack of mitogenic activity of rPTS1.13L.129G was seen as contrary to earlier reports in which the 9K/129G PT mutant retained the mitogenic activity of wild-type PT for T cells (Nencioni et al. 1990). However, Nencioni et al. (1990) evaluated the mitogenicity of a mutant toxoid and not the mutant S1 subunit alone and it is likely that the mitogenic properties of PT are limited to the B oligomer, which is logical considering the function of the B oligomer is binding to glycoconjugate receptors on the surface of most mammalian cell types, including T cells (Locht & Antoine 1995).

There were no antigen-specific or cross-reactive antibodies to either rPTS1.13L.129G or wild-type PT detected in the serum of mice immunised with the S1 DNA vaccine or vector-immunised mice, respectively. The reported significant antibody levels following gene-gun immunisation (Kamachi et al. 2003; 2004) suggested that the lack of antibody induction was not antigen-dependant but rather dictated by the IM method of delivery. Although there has relatively little insight gained into the mechanisms of DNA uptake and processing of endogenously expressed antigen, it has become apparent that the mode of delivery appears to have a major influence on the generation of either a humoral or cellular response. Most DNA vaccines administered via the IM route induce a Th1-biased response with generally poor antibody titers (Swihart et al. 1995; Raz et al. 1996). The most likely reasons for this tendency towards CMI are that endogenous expression favours presentation to CD8+ Tc cells via the MHC class I pathway; and intrinsic CpG motifs are capable of stimulating a variety of proinflammatory cytokines (Klinman et al. 1997; Gurunathan, Klinman & Seder 2000). On the contrary, DNA

vaccines delivered intradermally by gene gun direct a Th2 response dominated by serum antibody (Pertmer et al. 1996; Prayaga et al. 1997; Kamachi et al 2003; 2004). Although the Th2 response elicited via gene-gun vaccination has been shown to confer a good degree of protection, in reality it would offer little or no advantage over the existing acellular vaccines and would be unlikely a yield a more potent antibody response than DTaP.

Mice vaccinated with DTaP showed a high degree of seroconversion with high IgG ELISA titers detected against the recombinant S1 and wild-type PT antigens. On average endpoint titers were 6 to 7 fold higher against the wild-type antigen compared to rPTS1.13L.129G, an outcome that was expected when the antibody response to the remaining four subunits are taken into account. As discussed in previous chapters (see Chapter 4, Section 4.4 and Chapter 5, Section 5.4), there was a weak cellular response to the acellular vaccine with only low levels of IFN-γ and IL-2 produced by splenocytes stimulated with either rS1.13L.129G or DTaP. Again, complete clearance of bacteria in mice vaccinated with DTaP by day 7 post-challenge was a feature of the acellular vaccine. Although acellular vaccines have been shown to induce protection via the induction of anti-PT antibodies, their potential to provide long-term protection, or lack thereof, has been questioned and the need to induce a complementary CMI response suggested (Peppoloni et al. 1991; Mills et al. 1993; Mills 2001).

It is widely believed that the protective mechanisms evoked against PT are toxin-neutralising IgG1 antibodies (anti-toxin) in the serum (Schneerson et al. 1996). Kamachi et al. (2003) found that gene-gun immunisation with an S1 DNA vaccine generated significant IgG1 titers that protected against both the toxic effects of PT and a lethal intranasal challenge with *B. pertussis*. Clearly, the Th2 type response elicited by DTaP and intradermal (gene-gun) DNA vaccination confers protection against *B. pertussis* infection. Despite the absence of a detectable serum IgG response, mice immunised with the S1 DNA vaccine showed a significantly improved reduction in bacterial counts compared to the negative control groups. Nevertheless the lack of toxin-neutralising IgG in the serum is a limitation of DNA vaccination via the IM route. It is likely that a composite vaccine, in which purified toxoid is either co-administered or used as a booster, would be required for widespread acceptance of this mode of immunisation.

In this study, the commercial acellular vaccine was far more efficacious than the mono-component S1 DNA vaccine. However, direct comparisons between DTaP and the S1 DNA vaccine must be made cautiously considering the effect of multiple antigenic priming and adjuvant with DTaP versus single antigenic priming and a lack of immune modulation with the S1 DNA vaccine. Redhead et al. (1993) found that high serum antibody levels resulted in an earlier decline in the numbers of *B. pertussis* recovered from the lungs of challenged mice, but that complete clearance was dependent on CMI. On the other hand, the dichotomous albeit slightly Th1-biased response to whole cell vaccines has been shown to produce a greater reduction in bacteria compared with the purely Th2 response induced by acellular vaccines (Mills et al. 1998). Irrespective of which arm of the immune system can more effectively clear the pathogen, there is no doubt that a combination of both humoral and cellular effectors would be optimal for long-term protection against whooping cough.

Chapter 7 Adenylate Cyclase-Hemolysin DNA Vaccines: Development and Characterisation of the Immune Response of Mice Vaccinated via the IM Route

7.1 Introduction

Adenylate cyclase-hemolysin (AC-Hly) is one of four toxins produced by *B. pertussis* during infection. AC-Hly belongs to the RTX (repeats in toxin) family of bacterial exotoxins, characterised by having between 30 and 38 repeating units of a Ca^{2+}-binding nonapeptide (Welch 2001). The toxin is synthesised and secreted as a 200 kDa monomer with two functional domains. The N-terminal or catalytic domain has AC activity whereas the C-terminal or hemolytic domain is involved in membrane attachment, pore-formation and translocation of the catalytic domain across the plasma membrane (Ladant & Ullmann 1999).

The 1300 amino residue hemolytic domain includes a hydrophobic region with four membrane-spanning α-helices that penetrate the target cell membrane and a glycine/aspartate-rich repeat region that has a high affinity for Ca^{2+} (Ladant & Ullmann 1999). Penetration of the catalytic domain has been shown to be a Ca^{2+}-dependent process with attachment and insertion of the hydrophobic region occurring at low Ca^{2+} concentrations, whereas the unfolding of the catalytic domain and its translocation through the channels created by pore formation requires a high concentration of Ca^{2+} (Ahuja et al. 2004). The repeat region may also serve as the ligand for attachment to the membranes of phagocytic cells, via interaction with the $\alpha_M\beta_2$ integrin, referred to as the CD11b/CD18 receptor (Guermonprez et al. 2001). However, AC-Hly has been shown to invade and intoxicate non-phagocytic cell types, which suggests that there may not be an absolute requirement for a specific cell surface receptor to initiate membrane penetration (Welch 2001; Martin et al. 2004). Although the main function of the haemolytic domain is to facilitate translocation of the catalytic domain a by-product of this pore-forming activity is cell lysis, often observed as hemolysis (Martin et al. 2004).

Once released into the cytosol, the activity of the N-terminal adenylate cyclase domain is enhanced up to 1000-fold by calmodulin, a sensory protein endogenous to most mammalian cell types (Ladant & Ullmann 1999). The activated adenylate cyclase then catalyses the conversion of cAMP from ATP to supraphysiologic levels, leading to an impairment of normal cellular functions or even cell death by apoptosis (Khelef & Guiso 1995). Expression of active adenylate cyclase-hemolysin is reliant on the *cya* operon of five genes; *cyaA*, *cyaB*, *cyaC*, *cyaD* and *cyaE*. Glaser et al. (1988) were the first to clone the *cyaA* gene, which encodes the structural protein or protoxin. Upon translation, the CyaA protoxin has full adenylate cyclase activity but lacks the haemolytic and cell invasive properties (Sebo et al. 1991). Activation of haemolytic domain requires post-translational palmitoylation of lysine residues at positions 860 and 983 by the *cyaC* acyltransferase (Barry et al. 1991). Transport and secretion of ACT appears to be dependent on the products of the *cyaB*, *cyaD* and *cyaE* genes. Although their precise roles in this process are still largely unknown it is believed to occur through a process similar to the type I mechanism of secretion of *E. coli* α hemolysin (Glaser et al. 1988).

AC-Hly is an important virulence factor in *B. pertussis* pathogenesis. The primary targets of AC-Hly intoxication are immune effector cells such as neutrophils, monocytes, NK cells and macrophages (Ahuja et al. 2004). Inhibition of their immune cell functions such as chemotaxis, phagocytosis and the production of superoxide radicals provides an obvious advantage for survival and colonisation of *B. pertussis* (Khelef, Zychlinsky & Guiso 1993). *B. pertussis* strains deficient in AC-Hly expression are less virulent than wild-type parental strains, with rapid clearance from the lungs of infected mice (Weiss et al. 1984; Goodwin & Weiss 1990). Using an intranasal model of infection, Carbonetti et al. (2005) also observed that an AC-Hly mutant (in-frame deletion within the cyaA gene) had a significantly lower rate of colonisation compared to a wild-type strain of *B. pertussis*.

The immune response to adenylate cyclase toxin has been determined in various animal models. Passive immunisation of mice with AC-Hly antibodies conferred protection against a respiratory challenge with virulent *B. pertussis* (Guiso et al. 1989). Guiso et al. (1989) also reported that immunisation with purified adenylate cyclase toxin induced an antibody response that protected mice from the lethal respiratory challenge. Betsou

et al. (1995) immunised mice with various truncated derivatives of the toxin and demonstrated that AC-Hly antibodies are predominantly directed against the repeat region of the hemolytic domain. On the contrary, immunisation with inactivated AC-Hly has been shown to effectively neutralise the AC activity of the toxin, through the generation of antibodies that recognise the catalytic domain (MacDonald-Fyall et al. 2004). These neutralising antibodies also promote phagocytosis by human neutrophils (Weingart et al. 2000). In humans, AC-Hly antibodies have been detected in the serum of patients infected with *B. pertussis*, those that have recovered from pertussis and children immunised with killed whole-cell vaccines (Farfel et al. 1990; Arciniega et al. 1991; Cherry et al. 2004). Despite its important role in pathogenesis and well established antigenicity, none of the currently licensed acellular pertussis vaccines have included an AC-Hly toxoid.

A further justification for the inclusion of an AC-Hly toxoid in future pertussis vaccines surrounds its immunomodulatory properties. MacDonald-Fyall et al. (2004) demonstrated that CyaA exhibits adjuvanticity towards other *B. pertussis* antigens. A mutant CyaA lacking adenylate cyclase activity was found to augment the serum IgG responses to the co-administered filamentous hemagglutinin, pertactin and pertussis toxoid antigens. Moreover, it was also found that splenocytes and macrophages produced higher levels of IFN-γ and nitric oxide respectively, in response to heat-killed *B. pertussis*, after co-immunisation with the CyaA toxoid compared to immunisation with FHA, PRN and PT alone (MacDonald-Fyall et al. 2004).

An important consideration in our AC-Hly DNA vaccine strategy is the apparent requirement for post-translational palmitoylation of the CyaA protoxin to generate a protective immune response. Betsou et al. (1995) reported that the protective activity of CyaA is dependent on the post-translational activation of the hemolytic domain by CyaC, as immunisation of mice with acylated CyaA conferred protection against an experimental challenge whereas immunisation with the protoxin could not. To account for differences in the immune response and protective efficacy of non-acylated versus acylated CyaA, two separate vaccine strategies were tested. The first group of mice were immunised with a single DNA vaccine that encoded the *cyaA* gene alone, whereas the second group were immunised with a combination DNA vaccine that delivered both *cyaA* and *cyaC* genes as an equimolar mixture of two plasmid constructs.

It was also necessary to inactivate the N-terminal AC activity of the protoxin to prevent any potentially cytotoxic effects to vaccinees. The ATP-binding site resides within residues 54 and 70 of the catalytic domain and site directed mutagenesis of lysine 58 has been shown to completely abolish enzymatic activity without affecting the immunogenicity or haemolytic activity (Au, Masure & Storm 1989).

The aims of the work outlined in this chapter was to determine whether a humoral and/or cellular immune response is generated following DNA vaccination of mice with an AC-Hly toxoid, and the subsequent level of protection conferred against an aerosol challenge. In order to accomplish these aims it was necessary to:

1. Develop eukaryotic expression constructs encoding a cyaA toxoid and the cyaC acyltransferase
2. Develop corresponding prokaryotic expression constructs
3. Determine the level of heterologous expression in both *E. coli* for small-scale recombinant protein purification and transfected mammalian cells as precursor to *in vivo* expression
4. Evaluate the immunogenicity of an AC-Hly DNA vaccine in mice, and
5. Compare the protective efficacy of the mono-component DNA vaccines versus a proprietary DTaP formulation using the aerosol challenge model

7.2 Methodology

This section provides an outline of the relevant methods used. For the specific details of each procedure refer to Chapter 2.

7.2.1 Construction of Recombinant Plasmids

7.2.1.1 PCR Amplification of cyaA Gene from Genomic DNA

PCR of the *cyaA* gene was performed as outlined in Chapter 2, Section 2.5.2. Platinum *Pfx* DNA polymerase was used to generate blunt-ended products for cloning into pcDNA3.1D/V5-His-TOPO and Platinum *Taq* High Fidelity DNA polymerase was used to generate A-tailed products for cloning into pTrcHis2-TOPO. Custom-made primers (Geneworks) were designed to amplify *cyaA* based on the published DNA sequence (Genbank accession no. A14850, Appendix D.5). Sequences of the primers used were:

For cloning into pcDNA3.1D/V5-His-TOPO
CYAA883DNAF – 5' CACCATGGATGTTTGGTTCTTGCAGAAGGATGAG 3'
CYAA6096R – 5' GCGCCAGTTGACAGCCAGGGACTGCAT 3'
For cloning into pTrcHis2-TOPO
CYAA883TRCF – 5' GATGTTTGGTTCTTGCAGAAGGATGAGGTTCTG 3'
CYAA6096R – 5' GCGCCAGTTGACAGCCAGGGACTGCAT 3'

7.2.1.2 PCR Amplification of cyaC Gene from Genomic DNA

PCR of the *cyaC* gene was performed as described in Chapter 2, Section 2.5.2. Blunt-ended products for cloning into pcDNA3.1D/V5-His-TOPO were amplified using Platinum *Pfx* DNA polymerase and A-tailed products for cloning into pTrcHis2-TOPO were amplified using Platinum *Taq* DNA polymerase. Primers (Geneworks) were designed to amplify the *cyaC* gene based on the published DNA sequence (Genbank accession no. M57286, Appendix D.5). The sequences of the primers used were:

For cloning into pcDNA3.1D/V5-His-TOPO
CYAC111DNAF – 5' CACCATGGCGCACCCAACACGG 3'
CYAC713R – 5' GGCGGTGCCCCGGCCTCG 3'
For cloning into pTrcHis2-TOPO
CYAC135TRCF – 5' CCCGCTCCACCATGCACCGATGTC 3'
CYAC713R – 5' GGCGGTGCCCCGGCCTCG 3'

PCR products were purified as described in Chapter 2, Section 2.5.3.

7.2.1.3 Cloning of PCR products into pTrcHis2-TOPO and pcDNA3.1D/V5-His-TOPO Expression Vectors

The pTrcHis2-TOPO TA Expression Kit (Invitrogen) was used for cloning and transformation, as per the manufacturer's instructions. Purified PCR products were cloned into the pTrcHis2-TOPO vector and transformed into *E. coli* TOP10 cells as described in Chapter 2, Section 2.6. The pcDNA3.1D/V5-His TOPO Cloning Kit (Invitrogen) was used for cloning and transformation, according to the manufacturer's instructions (Chapter 2, Section 2.6). The maps of pcDNA3.1D/V5-His-TOPO and pTrcHis2-TOPO vectors are shown in Appendix C.1 and C.2 respectively. Plasmid maps of pcDNA3.1D/*cyaA* and pcDNA3.1D/*cyaC* constructs are shown in Figures 7.1 and 7.2 respectively.

7.2.1.4 Screening of Plasmid Clones

Plasmid DNA from selected TOP10 transformants was isolated by Miniprep and analysed on a 1% TAE agarose gel (Chapter 2, Section 2.6.2). The insert sequences of the recombinant plasmids with a molecular weight equivalent to the expected size were verified by DNA sequencing as described in Chapter 2, Section 2.7. Primer sequences used for sequencing are listed in Table 2.3. Small fragments obtained from sequencing reactions were assembled into contiguous sequences (contigs) and aligned against the published *cyaA* or *cyaC* gene sequence using the ClustalW program provided by ANGIS (Australian National Genomic Information Service).

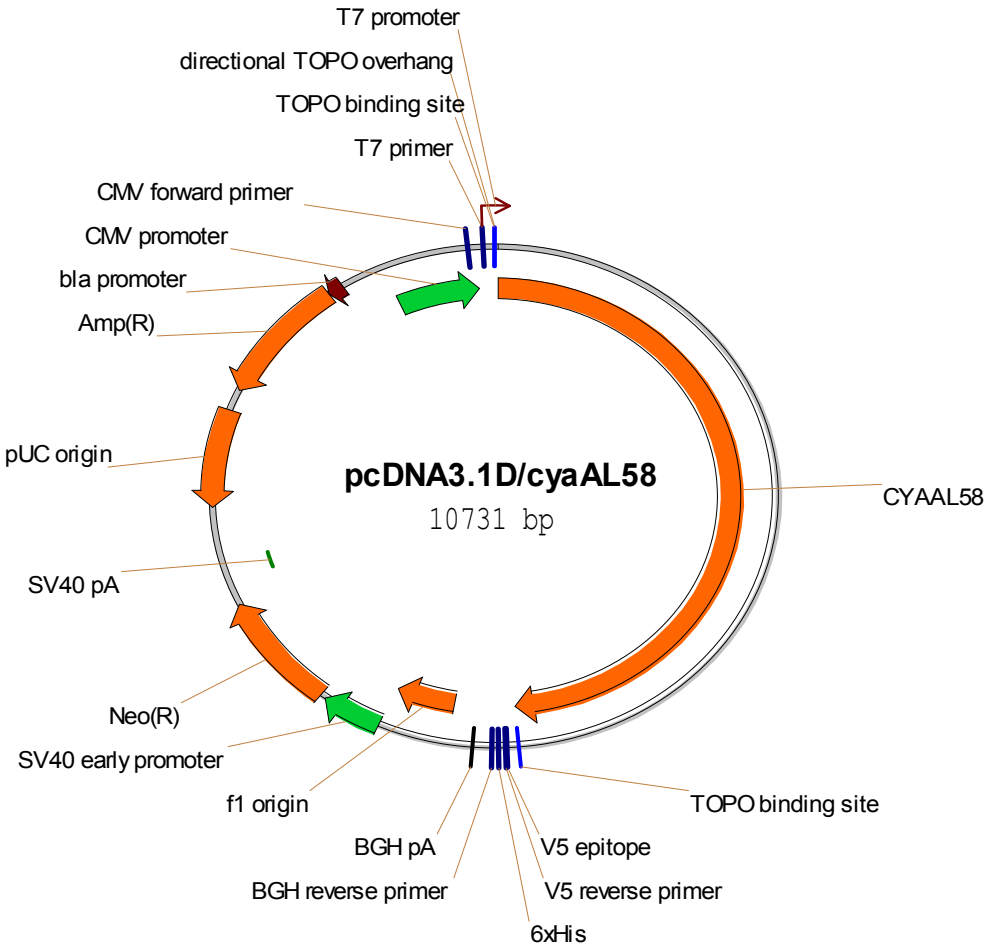

Figure 7.1: Map of pcDNA3.1D/*cyaAL58* generated using Vector NTI (Invitrogen).

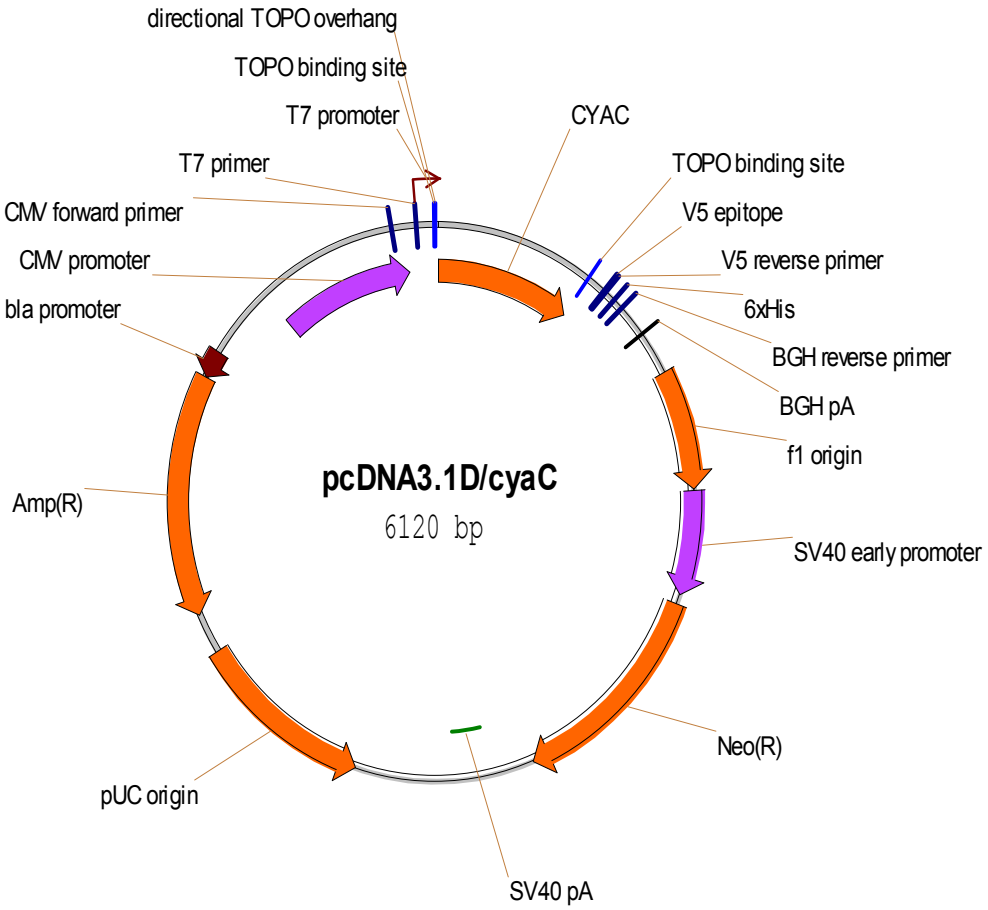

Figure 7.2: Map of pcDNA3.1D/*cyaC* generated using Vector NTI (Invitrogen).

7.2.2 Site-Directed Mutagenesisof *cyaA* Gene Inserts

The *cyaA* inserts of pTrcHis2/*cyaA* and pcDNA3.1D/*cyaA* were inactivated using a single amino acid mutation, in which the lysine at position 58 was replaced with methionine to generate pTrcHis2/*cyaAL58* and pcDNA3.1D/*cyaAL58* (Au, Masure & Storm 1989). Mutagenesis was carried out using the Quick Change XL Site-Directed Mutagenesis Kit (Stratagene) as described in Chapter 2, Section 2.8. For each point mutation, PCR products were transformed into the XL10-Gold strain of *E. coli* followed by plasmid DNA isolation and sequencing. Sequences of the mutagenic primers (mutant nucleotides shown as bold and underlined) were:

CYAA-58LF - 5' GAAGGGGTGGCCACCA**TG**GGATTGGGCGTGCAC 3'

CYAA-58LR – 5' GTGCACGCCCAATCC**CA**TGGTGGCCACCCCTTC 3'

7.2.3 Cyclic AMP Assay

To confirm that the point mutation had abolished enzymatic activity of CYAA, a modified CHO cell assay and cAMP assay were performed as described in Chapter 2, Section 2.13.2. The precise outline of the transfection/treatment of CHO-K1 cells has been summarised in Table 2.11. Briefly, CHO cells were transfected with pcDNA3.1D/*cyaA*, the mutant pcDNA3.1D/*cyaAL58* construct or treated with native adenylate cyclase toxin (List Biologicals) as a positive control. Treatment with LF2000 alone and untreated cells were included as negative controls. Transfection of CHO cells with pcDNA3.1D/*fhaB1* was included to assess the effect of endogenous expression of an unrelated antigen on the survival and morphology of CHO cells. The levels of cAMP produced in treated and non-treated cells was determined using an enzyme immunoassay as described in Chapter 2, Section 2.13.2.3.

7.2.4 Expression of rCYAAL58 in *E. coli*

Pilot expression of rCYAAL58 in XL10-Gold *E. coli* was performed according to the procedure outlined in Chapter 2, Section 2.12.1. Expression of rCYAAL58 was detected by Western blotting with a HRP conjugated anti-His (C-term) antibody (Chapter 2, Section 2.11).

7.2.5 Expression of rCYAC in *E. coli*

Pilot expression of rCYAC in *E. coli* was also performed according to the protocol outlined in Chapter 2, Section 2.12.1. Expression of rCYAC was detected by Western blotting with a HRP conjugated anti-His (C-term) antibody (Chapter 2, Section 2.11). To enhance the expression level of rCYAC, the pTrcHis2/*cyaC* plasmid was transformed into BL21 CodonPlus(RP) *E. coli* as described in Chapter 2, Section 2.12.2.

7.2.6 Purification of rCYAAL58 and rCYAC Fusion Proteins

Recombinant CYAAL58 and rCYAC fusion proteins were purified from XL10-Gold or BL21 CodonPlus(RP) *E. coli* respectively by Ni-NTA affinity chromatography under denaturing conditions (Chapter 2, Section 2.12.3). Purified protein was checked by SDS-PAGE and Western blot (Chapter 2, Sections 2.10 and 2.11). Eluates were concentrated by ultra-centrifugation (Appendix C.6) and the yield estimated using a modified Bradford assay (Chapter 2, Section 2.12.4) for use in immunological assays.

7.2.7 Transient Transfection of COS-7 Mammalian Cells and Expression

The mammalian cell line COS-7 was transfected with pcDNA3.1D/*cyaAL58* and pcDNA3.1D/*cyaC* as previously described (Chapter 2, Section 2.9). Constitutive expression of rCYAAL58 and rCYAC was detected by Western blot using an AP-conjugated anti-V5 antibody (Chapter 2, Section 2.11).

7.2.8 Transformation of *S. typhimurium* with pcDNA3.1D/*cyaAL58* and pcDNA3.1D/*cyaC*

The *aro*A *Salmonella typhimurium* strain SL3261 was transformed with the pcDNA3.1D/*cyaAL58* and pcDNA3.1D/*cyaC* plasmids for mucosal delivery via the oral route (Chapter 8). Plasmids were initially passaged through the r^-m^+ *S. typhimurium* strain P9121 prior to transformation of the *aro*A SL3261 strain, which possesses an intact restriction system. *S. typhimurium* P9121 were transformed using the heat shock method (Chapter 2, Section 2.14.2), whereas *S. typhimurium* SL3261 were transformed by electroporation (Chapter 2, Section 2.14.3).

7.2.9 Immunisation of Mice with pcDNA3.1D/*cyaAL58* Alone or in Combination with pcDNA3.1D/*cyaC* via the IM Route

A component of the first DNA vaccine efficacy trial involved immunisation of mice with either: 3 doses of pcDNA3.1D/*cyaAL58* alone or pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC* in combination as outlined in Chapter 2, Section 2.15.2. Immunisation involved direct injection of naked DNA into the quadriceps. To obtain sufficient plasmid for a 3 dose regime, large scale isolation was performed from 2.5 L cultures of TOP10-pcDNA3.1D/*cyaAL58* and TOP10-pcDNA3.1D/*cyaC* (Chapter 2, Section 2.15.2.1).

7.2.10 Determination of Immune Response and Protective Efficacy to DNA Vaccination

Of the 17 mice per group vaccinated, 5 were sampled for serum and organs two weeks after the third and final dose. Serum samples were analysed for the presence of IgG, IgG1 and IgG2a specific for rCYAAL58 and commercial adenylate cyclase toxin (AC-Hly) by ELISA (Chapter 2, Section 2.16.2) and western blot (Appendix B.1). Cytokine profiles of re-stimulated splenocytes were used to evaluate the CMI response (Chapter 2, Section 2.16.1). The remaining 12 mice/group were challenged with a pre-

determined sub-lethal infectious dose (SLID) of virulent *B. pertussis* Tohama I as described in Chapter 2, Section 2.15.4. The lungs and serum of challenged mice were sampled at three time points with 4 mice sacrificed at each point. For direct comparison of DNA vaccine efficacy, a group of mice vaccinated with the pcDNA3.1D/V5-His-TOPO vector was included along with the placebo and DTaP (Infanrix™) vaccinated groups.

7.3 Results

7.3.1 PCR Amplification of the *cyaA* Gene

For cloning into pcDNA3.1D/V5-His-TOPO, a 5217 bp sequence was amplified using the CYAA883DNAF and CYAA6096R primers with Platinum *Pfx* DNA polymerase (Figure 7.3). For cloning into pTrcHis2-TOPO, an identical 5217 bp fragment was amplified using the CYAA883TRCF and CYAA6096R primers with Platinum *Taq* DNA polymerase (Figure 7.3).

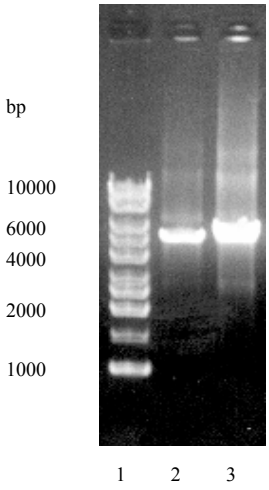

Figure 7.3: PCR of *cyaA* gene. Lane 1 - Kilobase Marker (10kb to 0.5kb - Amersham); lane 2 - amplification with Platinum *Taq* DNA polymerase and CYAA883TRCF/CYAA6096R oligonucleotides; lane 3 - amplification with Platinum *Pfx* DNA polymerase and CYAA883DNAF/CYAA6096R oligonucleotides.

7.3.2 Generation of pcDNA3.1D/*cyaA* and pTrcHis2/*cyaA* Plasmids

The 5217 bp blunt-ended PCR product was purified and cloned into pcDNA3.1D/V5-His-TOPO for eukaryotic expression. Alternatively, the A-tailed PCR fragment was cloned into the pTrcHis2-TOPO vector for inducible expression in *E. coli.* Putative pcDNA3.1D/*cyaA* and pTrcHis2/*cyaA* plasmids were isolated following transformation of TOP10 *E. coli* and checked against their expected sizes (vector plus insert) on a 1% agarose gel (Figures 7.4 and 7.5). The pcDNA3.1D/*cyaA* and pTrcHis2/*cyaA* clones which were equivalent in size to the expected 10731 and 9598 bp respectively were sequenced and aligned against the published Genbank sequence of *cyaA* (accession # 14850) using the WebAngis ClustalW program. The alignment revealed clones with inserts that were correctly orientated with no base errors (Appendix D.5).

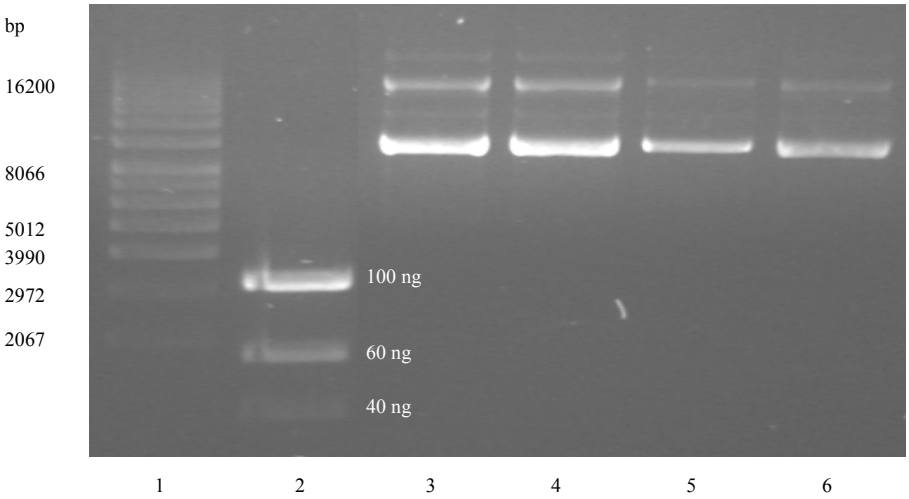

Figure 7.4: Isolation of pcDNA3.1D/*cyaA* and pcDNA3.1D/*cyaAL58* from *E. coli* and *S. typhimurium*. Lane 1 – Supercoiled DNA ladder (16,210bp to 2,067bp - Invitrogen); lane 2 – Low DNA Mass Ladder (2000bp to 100bp - Invitrogen); lane 3 - pcDNA3.1D/*cyaA* from TOP10; lane 4 - pcDNA3.1D/*cyaAL58* from XL10-Gold; lane 5 - pcDNA3.1D/*cyaAL58* from P9121 *S. typhimurium*; lane 6 - pcDNA3.1D/*cyaAL58* from SL3261 *S. typhimurium*.

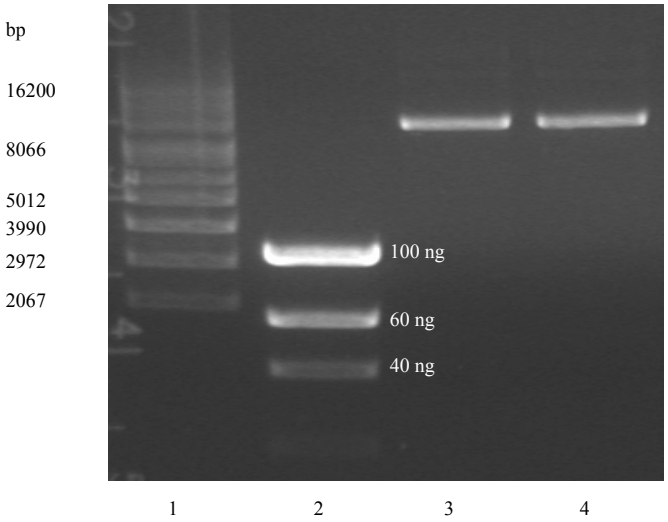

Figure 7.5: Isolation of pTrcHis2/*cyaA* and pTrcHis2/*cyaAL58* from *E. coli*. Lane 1 - Supercoiled DNA ladder (16,210bp to 2,067bp - Invitrogen); lane 2 - Low DNA Mass ladder (2000bp to 100bp - Invitrogen); lane 3 - pTrcHis2/*cyaA* from TOP10 and lane 4 - pTrcHis2/*cyaAL58* from XL10-Gold following site-directed mutagenesis.

7.3.3 PCR Amplification of the *cyaC* Gene

For cloning into pcDNA3.1D/V5-His-TOPO, a 602 bp product was amplified using CYAC111DNAF/CYAC713R primers with Platinum *Pfx* DNA polymerase; whereas for cloning into pTrcHis2-TOPO, a 578 bp fragment was amplified using the CYAC135TRCF and CYAC713R primers and Platinum *Taq* DNA pol. (Figure 7.6).

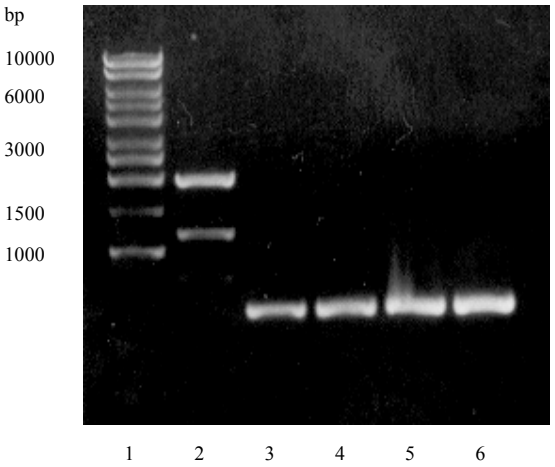

Figure 7.6: PCR of *cyaC* gene. Lane 1 - Kilobase Marker (10kb to 0.5kb - Amersham); lane 2 - Low DNA Mass ladder (2000bp to 100bp - Invitrogen). Amplification of *cyaC* with CYAC135TRCF/CYAC713R primers (lanes 3 and 4) and with CYAC111DNAF/CYAC713R primers (lanes 5 and 6).

7.3.4 Generation of pcDNA3.1D/*cyaC* and pTrcHis2/*cyaC* Plasmids

Putative pcDNA3.1D/*cyaC* and pTrcHis2/*cyaC* constructs were isolated from the selected TOP10 *E. coli* transformants and checked against their expected sizes on a 1% agarose gel. Putative pcDNA3.1D/*cyaC* and pTrcHis2/*cyaC* clones were sequenced for alignment against the published *cyaC* sequence (accession # M57286). Alignment revealed that the insert sequences were both error-free and in the correct orientation (Figure D.13).

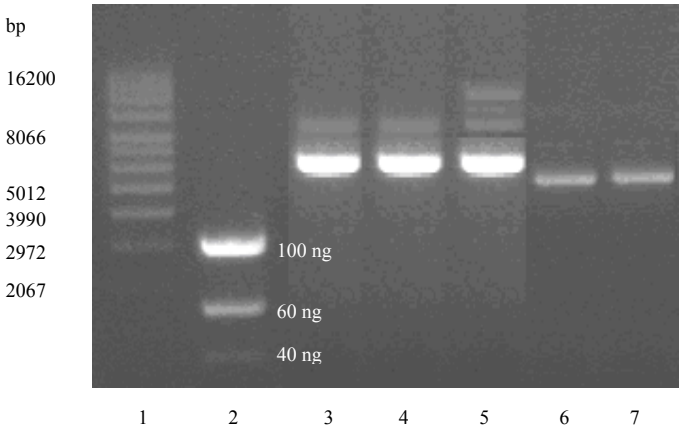

Figure 7.7: Isolation of pcDNA3.1D/*cyaC* and pTrcHis2/*cyaC* from *E. coli* and *S. typhimurium*. Lane 1 – Supercoiled DNA ladder (16,210bp to 2,067bp - Invitrogen); lane 2 – Low DNA Mass ladder (2000bp to 100bp - Invitrogen); lane 3 – pcDNA3.1/*cyaC* from TOP10; lane 4 – pcDNA/*cyaC* from P9121 *S. typhimurium*; lane 5 - pcDNA3.1D/*cyaC* from SL3261 *S. typhimurium*; lane 6 – pTrcHis2/*cyaC* from TOP10; lane 7 - pcDNA3.1D/*fhaB2* from BL21 Codon Plus.

7.3.5 Site-directed Mutagenesis of pcDNA3.1D/*cyaA* and pTrcHis2/*cyaA*

Sequence confirmed pcDNA3.1D/*cyaA* and pTrcHis2/*cyaA* plasmids were used as the templates for site-directed mutagenesis. Amplification of the respective 10.7 and 9.6 kb targets pre-empted a successful mutagenesis reaction (Figure 7.8). Colonies from the transformation of XL10-Gold *E. coli* were screened by isolation of plasmid DNA and sequencing. The size of the selected pcDNA3.1D/*cyaAL58* mutant was the same as the original pcDNA3.1D/*cyaA* (Figure 7.4, lane 4), as was pTrcHis2/*cyaAL58* when compared to pTrcHis2/*cyaA* (Figure 7.6, lane 4). Alignment with the Genbank sequence of *cyaA* revealed that the point mutations at positions 1150 and 1151 were successful and no other mismatches or errors were present within the insert sequence (Appendix D.5).

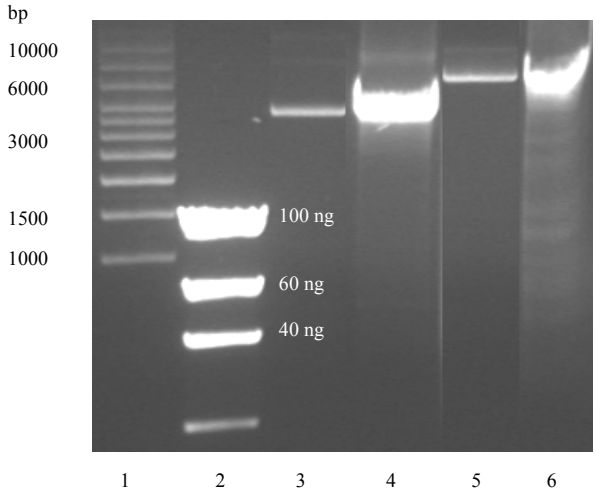

Figure 7.8: Site-directed mutagenesis PCR of pTrcHis2/*cyaA* and pcDNA3.1D/*cyaA*. Lane 1 – Kilobase Marker (10kb to 0.5kb - Amersham); lane 2 – Low DNA Mass ladder (Invitrogen); lane 3 – pTrcHis2/*cyaA* template; lane 4 – putative pTrcHis2/*cyaAL58*; lane 5 – pcDNA3.1D/*cyaA* template; lane 5 – putative pcDNA3.1D/*cyaAL58*.

7.3.6 Expression of rCYAAL58 and rCYAC in COS-7

COS-7 cell lines were transfected with pcDNA3.1D/*cyaAL58* to verify transient expression of the rCYAAL58 protein within mammalian cells. Using an anti-V5 epitope AP conjugated antibody a single band of approximately 200 kDa was detected by western blot (Figure 7.9). The non-mutated rCYAA protein could not be detected from the lysate of cells transfected with pcDNA3.1D/*cyaA* (lane 2). The MW of the single band detected in the blots was larger the calculated size of 181.3 kDa for rCYAAL58 (Translate GCG - WebAngis) but equivalent to its apparent size on SDS-PAGE (Rogel et al. 1989; Iwaki, Kamachi & Konda 2000). No bands corresponding to rCYAAL58 were detected in the lysate of the negative control of COS-7 cells transfected with self-ligated pcDNA3.1-V5-TOPO (figure not shown).

Likewise, the expression of rCYAC was confirmed in COS-7 cells following transfection with pcDNA3.1D/*cyaC*. A band approximately 30 kDa in size was detected in the test lysates but was absent in the corresponding negative controls after western blotting with the anti-V5 conjugated antibody (Figure 7.10). The MW of rCYAC was larger than the calculated 22.1 kDa (Translate GCG – WebAngis). Again, the additional vector-encoded fusion products such as the V5 epitope and Histidine tag would account for the increased size.

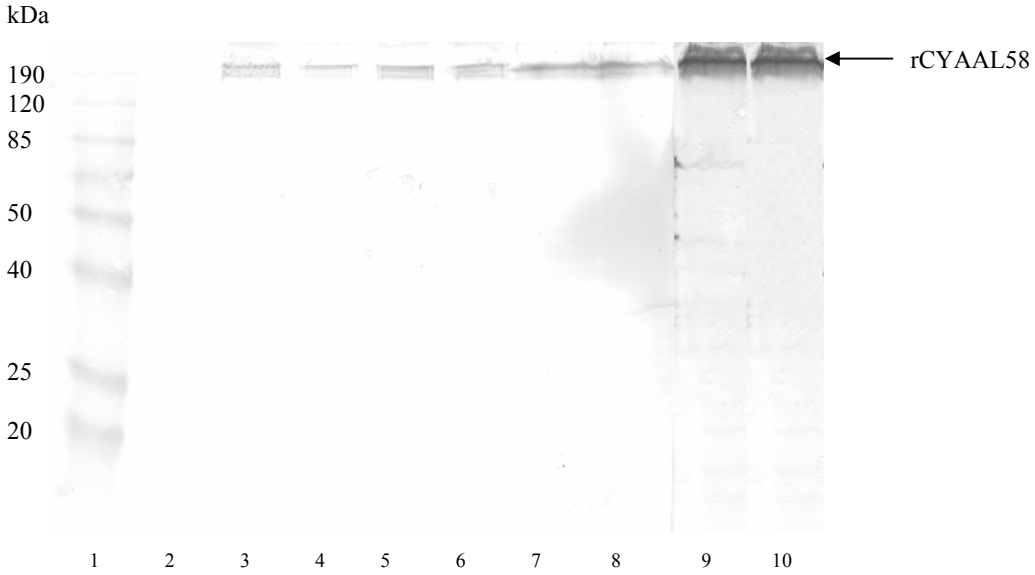

Figure 7.9: Western blotting of rCYAA and rCYAAL58 expression in COS-7 cells. Constitutive expression of rCYAA following transfection with pcDNA3.1D/*cyaA* (lane 2) and rCYAAL58 expression following transfection of COS-7 with pcDNA3.1D/*cyaAL58* isolated from TOP10 *E. coli* (lanes 3 to 8), P9121 *S. typhimurium* (lane 9), and SL3261 *S. typhimurium* (lane 10). Lane 1 - Pre-stained BenchMark protein ladder (190 to 10kDa - Invitrogen).

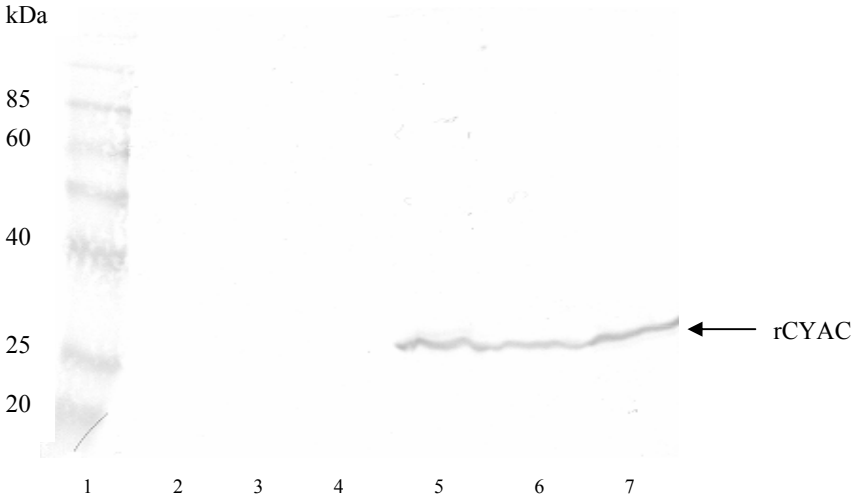

Figure 7.10: Western blotting of rCYAC expression in COS-7 cells. Lane 1 - Pre-stained BenchMark protein ladder (190kDa to 10kDa - Invitrogen). Lysates of COS-7 from transfection negative controls: lane 2 - no pcDNA3.1D/*cyaC* and no transfection reagent; lane 3 - 3µl transfection reagent and no pcDNA3.1D/*cyaC*; lane 4 - 1µg pcDNA3.1D/*cyaC* and no transfection reagent. Constitutive expression of rCYAC following transfection of COS-7 with pcDNA3.1D/*cyaC* isolated from: lane 5 - TOP10 *E. coli*; lane 6 - P9121 *S. typhimurium*; and lane 7 - SL3261 *S. typhimurium*.

7.3.7 Cytotoxicity of rCYAAL58

The level of cAMP production in transfected CHO-K1 cells and their morphology as a result of these treatments were the two indicators used to determine the relative toxicity of the mutant rCYAAL58 protein versus the unaltered rCYAA and native adenylate cyclase toxin. The results of the cAMP immunoassay revealed significant differences across the four test groups (Figure 7.11). Virtually undetectable levels of cAMP were detected in the lysates of CHO-K1 cells transfected with pcDNA3.1D/*cyaAL58* with a mean of 0.68±0.24pmol/ml. Likewise, untreated cells had a very low level of cAMP with 0.52±0.1pmol/ml that was not significantly different from the pcDNA3.1D/*cyaAL58* group. In contrast, CHO cells transfected with the unaltered pcDNA3.1D/*cyaA* plasmid or treated with native adenylate cyclase had a significantly higher levels of cAMP with 108.79±9.51 and 86.51±7.66pmol/ml respectively ($P < 0.001$).

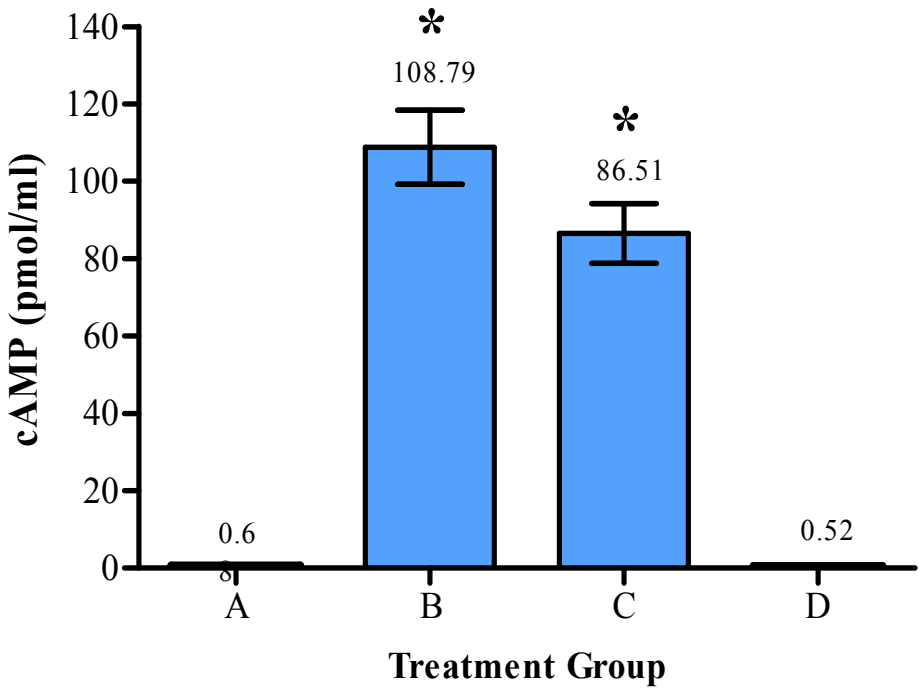

Figure 7.11: Cyclic AMP levels in CHO-K1 cells following exposure to rCYAA or rCYAAL58. A – CHO-K1 transfected with pcDNA3.1D/*cyaAL58* (endogenous expression of rCYAAL58), B – CHO-K1 transfected with pcDNA3.1D/*cyaA* (endogenous expression of rCYAA), C – CHO-K1 treated with AC-Hly toxin (List Biologicals), and D – untreated CHO-K1 cells. Bars represent the mean score of six replicate wells +/- SE. * Significantly different from pcDNA3.1D/*cyaAL58* ($P < 0.001$).

Consistent with the results of the cAMP assay, CHO cells exhibited an altered morphology in response to the four different treatments (Figure 7.12). CHO cells transfected with pcDNA3.1D/*cyaAL58* were observed to have a similar morphology with that of the untreated cells. These two groups of cells formed a compact monolayer and the cells were irregular in both shape and orientation (Figure 7.12B and D).

On the other hand, the CHO cells transfected with pcDNA3.1D/*cyaA* and treated with native toxin had a different morphology when compared to untreated cells. These cells were elongated and had a tendency to grow in parallel to each other (Figure 7.12A and C). This altered morphology was more pronounced in the cells cultured with the native toxin, whereas the cells transfected with pcDNA3.1D/*cyaA* were less confluent and appeared to be a certain degree of cell death.

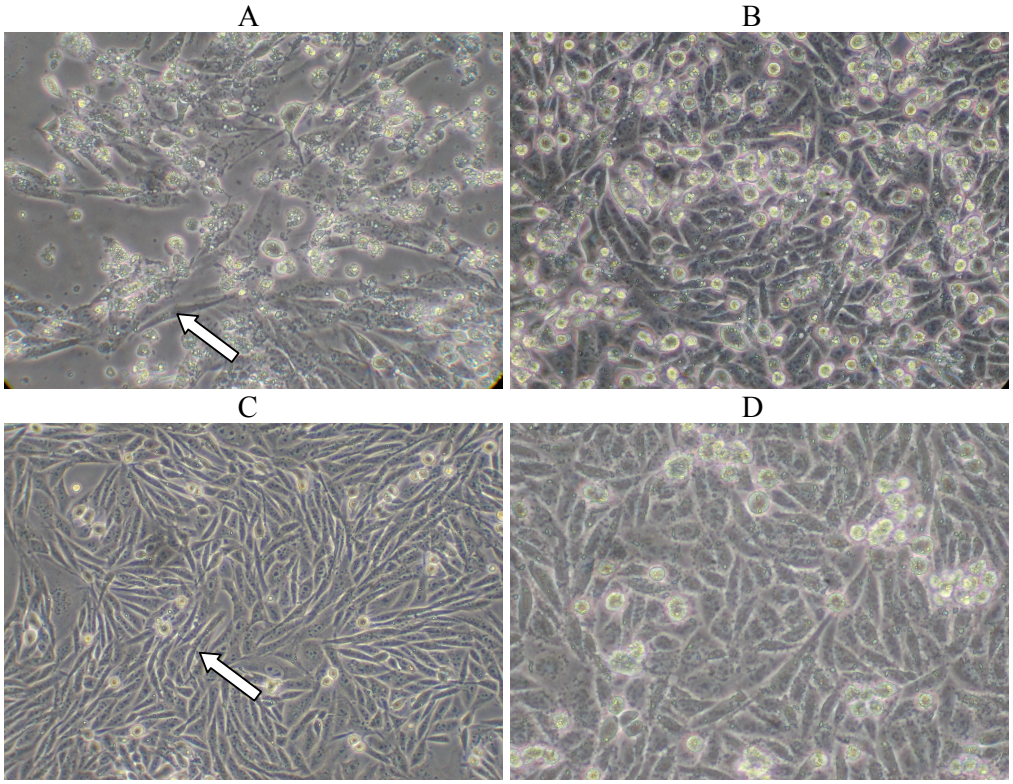

Figure 7.12: CHO-K1 cell morphology following exposure to rCYAA or rCYAAL58. A representative section of adherent cells was photographed 34 hours after: A – transfection with 5 µg pcDNA3.1D/*cyaA* (endogenous expression of rCYAA), B – transfection with 5 µg pcDNA3.1D/*cyaAL58* (endogenous expression of rCYAAL58), C – treatment with 80 µg AC-Hly toxin (List Biologicals), D – no additives (untreated). Arrows indicate the elongated morphology of CHO-K1 cells with supra-physiological levels of cAMP.

7.3.8 Expression and Purification of rCYAAL58 and rCYAC from *E. coli*

Purification of rCYAAL58 proved to be difficult. After induction with IPTG, rCYAA and rCYAAL58 were detected in the lysates of TOP10-pTrcHis2/*cyaA* and XL10 Gold-pTrcHis2/*cyaAL58* by western blot using an anti-His HRP conjugated antibody (Figure 7.13). A clear band with an apparent MW of ~200 kDa was detected, which corresponded to the calculated MW of rCYAAL58 (translate GCG program – WebAngis) and also the MW of rCYAAL58 detected in COS-7 cells. Despite detection in the western blot, the level of rCYAAL58 expression in *E. coli* XL10-Gold was very low i.e. when the lysates of XL10 Gold-pTrcHis2/*cyaAL58* and XL10-Gold were compared by PAGE, no unique 200 kDa band could be discerned after staining with coomassie blue (Figure not shown). Furthermore, the BL21 CodonPlus(RP) *E. coli* strain used to increase expression of rFHAB1, rFHAB2 and rPRN could not be successfully transformed with the pTrcHis2/*cyaAL58* construct. After an optimisation process, rCYAAL58 was adequately purified from the XL10-Gold strain using immobilised metal affinity chromatography (IMAC) (Figure 7.14).

Figure 7.13: Western Blotting of rCYAA and rCYAAL58 expression in *E. coli*. Lane 1 - BenchMark protein ladder (220kDa to 10kDa - Invitrogen); lanes 2 to 4 – rCYAAL58 expression from XL10-Gold following 2,3 or 4 hours of induction; lanes 5 to 8 – rCYAA expression from TOP10 following 2,3 or 4 hours of induction; lane 8 - native TOP10 (no plasmid) induced for 4 hours under identical conditions was also included as the negative control.

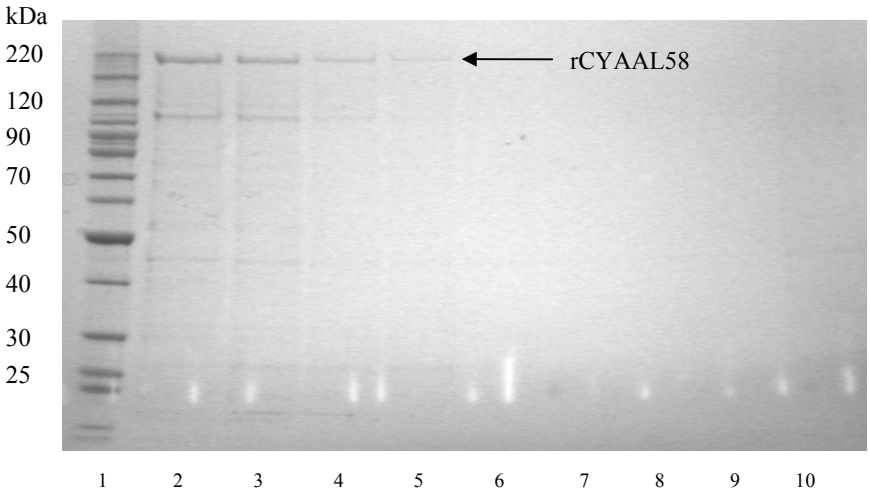

Figure 7.14. PAGE of affinity purified rCYAAL58 from *E. coli*. Lane 1: BenchMark Protein ladder (220kDa to 10kDa - Invitrogen); lanes 2 to 5 – elution from 0.5 ml flow-throughs of buffer D pH 5.9; lanes 6 to 10 – elution from 0.5 ml flow-throughs of buffer E pH 4.5.

Expression and purification of rCYAC was far less problematic. A 30 kDa protein was detected in a TOP10-pTrcHis2/*cyaC* lysate in the western blot (Figure 7.15). This band was equivalent in size to the fusion protein detected in transfected COS-7 cells. The BL21+ strain was successfully transformed with the pTrcHis2/*cyaC* construct and a high yield of rCYAC was subsequently purified by IMAC (Figure 7.16). Native or untransformed TOP10 *E. coli* was included as a negative control in this experiment and no banding was observed using the anti-His antibody (figure not shown).

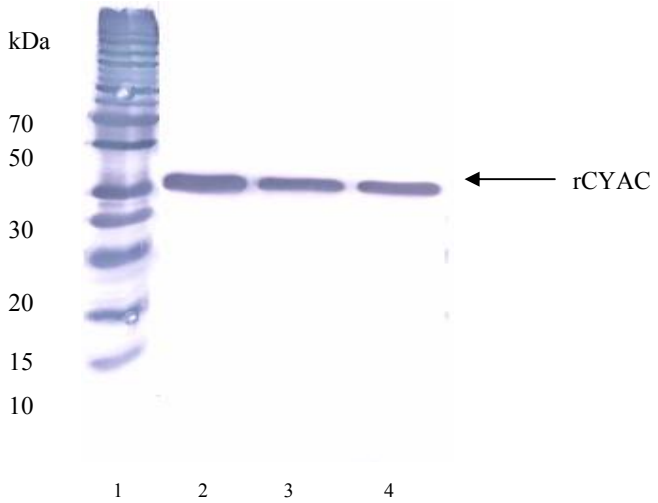

Figure 7.15: Western blotting of rCYAC expression in *E. coli*. Lane 1 - BenchMark protein ladder (220kDa to 10kDa - Invitrogen); lanes 2 to 4 – rCYAC expression in TOP10 following 2, 3 or 4 hours of induction.

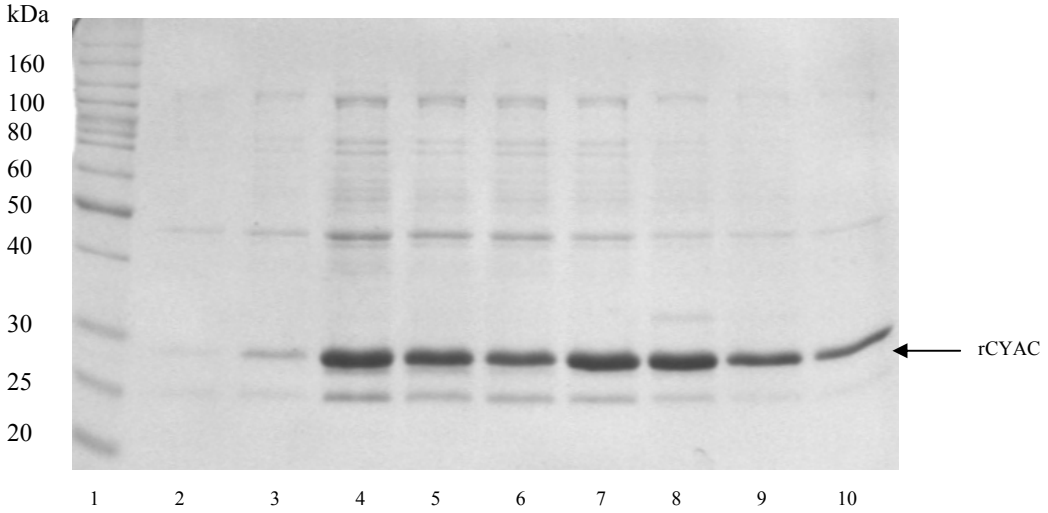

Figure 7.16: PAGE of affinity purified rCYAC from *E. coli*. Lane 1: BenchMark Protein ladder (220kDa to 10kDa - Invitrogen); lanes 2 to 5 – elution from 0.5 ml flow-throughs of buffer D pH 5.9; lanes 6 to 10 – elution from 0.5 ml flow-throughs of buffer E pH 4.5.

7.3.9 Immunogenicity and Protective efficacy of Vaccination with pcDNA3.1D/*cyaAL58* Alone or in Combination with pcDNA3.1D/*cyaC* by the IM Route

7.3.9.1 Cytokine Profiles Following DNA Vaccination with pcDNA3.1D/cyaAL58 Alone or in Combination with pcDNA3.1D/cyaC

Splenocytes from immunised mice were cultured in the presence of rCYAAL58, ConA or protein extraction buffer (DP buffer) for either 24 or 72 hours. Supernatants from the 24 hour cultures were tested for IL-2, whereas the IFN-γ and IL-4 production was determined from the 72 hour supernatants. Splenocytes of mice immunised with pcDNA3.1D/*cyaA* produced a large amount of IFN-γ when stimulated with rCYAAL58 or ConA at 4054 and 7044 pg/ml respectively (Figure 7.17). Pooled splenocytes from vector-immunised mice produced moderate amount of IFN-γ (1107 pg/ml) when cultured with the rCYAAL58 stimulant. This trend was the same for mice immunised with pcDNA3.1D/*cyaAL58* and pcDNA3.1D/*cyaC* in combination, however the IFN-γ produced by these splenocytes was much greater (7890 pg/ml) than immunisation with pcDNA3.1D/*cyaAL58* alone when stimulated with rCYAAL58 (Figure 7.18).

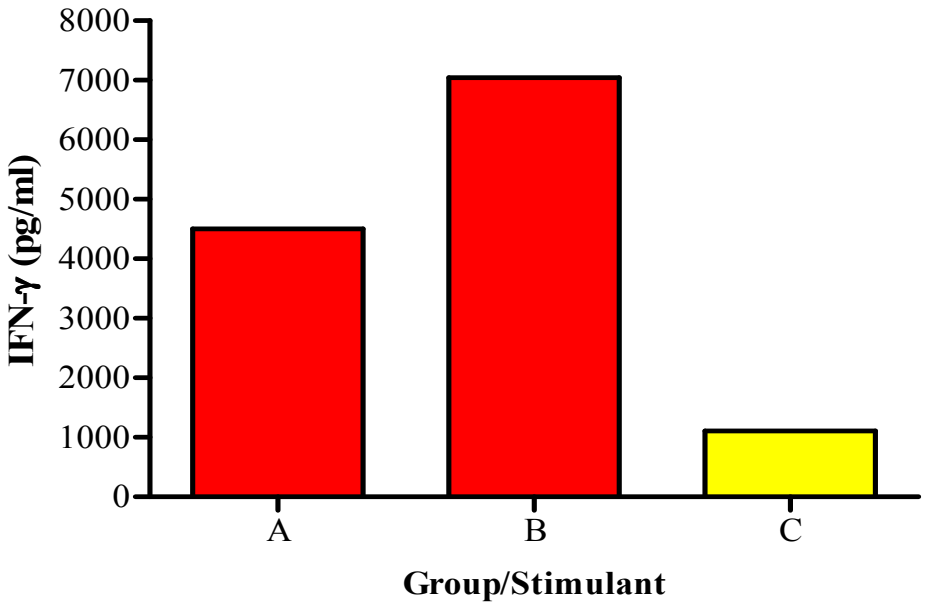

Figure 7.17: IFN-γ production from rCYAAL58-stimulated splenocytes. Splenocytes from pcDNA3.1D/*cyaAL58*-immunised mice stimulated with 5µg/ml rCYAAL58 (A) and 2.5µg/ml ConA (B). Splenocytes from vector-immunised mice stimulated with 5µg/ml rCYAAL58 (C).

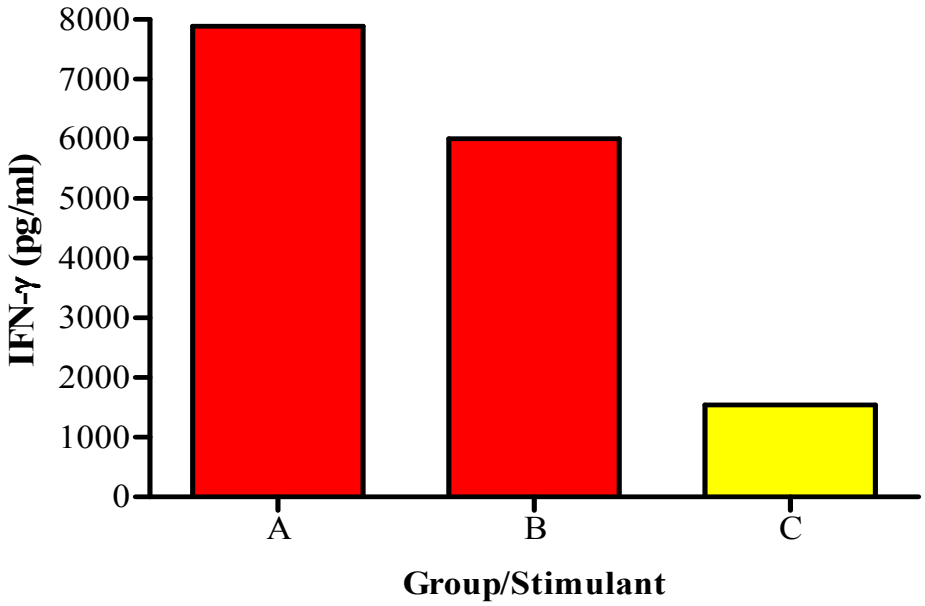

Figure 7.18: IFN-γ production from rCYAAL58-stimulated splenocytes. Splenocytes from mice vaccinated with pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC* stimulated with 5µg/ml rCYAAL58 (A) and 2.5µg/ml ConA (B). Splenocytes from vector-immunised mice stimulated with 5µg/ml rCYAAL58 (C).

When stimulated with rCYAAL58, splenocytes of mice immunised with pcDNA3.1D/*cyaAL58* produced two-fold more IL-2 than the splenocytes of vector-immunised mice, with 258.6 versus 135.2pg/ml (Figure 7.19). In contrast, high levels of IL-2 (15453 pg/ml) was detected in the supernatants of splenocytes stimulated with ConA. The splenocytes of mice immunised with pcDNA3.1D/*cyaAL58* and pcDNA3.1D/*cyaC* in combination yielded higher levels of IL-2 than the splenocytes of mice immunised with pcDNA3.1D/*cyaAL58* alone, when stimulated by rCYAAL58 (Figure 7.20).

There was either little or no IL-4 produced by the splenocytes of mice vaccinated with plasmid DNA (vaccines or vector) when stimulated with rCYAAL58 or DTaP (Table 7.1). For DTaP-immunised mice, splenocytes produced a low amount of IL-4 when stimulated with the homogenous vaccine preparation. ConA again stimulated a high degree of cytokine production in all groups.

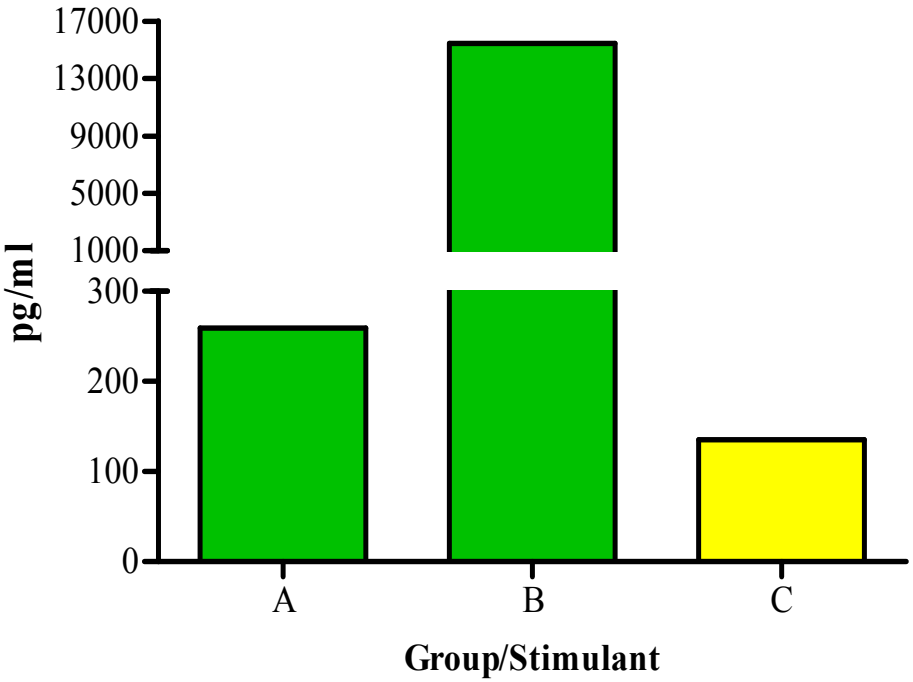

Figure 7.19: IL-2 production from rCYAAL58-stimulated splenocytes. Splenocytes from pcDNA3.1D/*cyaAL58*-immunised mice stimulated with 5µg/ml rCYAAL58 (A) and 2.5µg/ml ConA (B). Splenocytes from vector-immunised mice stimulated with 5µg/ml rCYAAL58 (C).

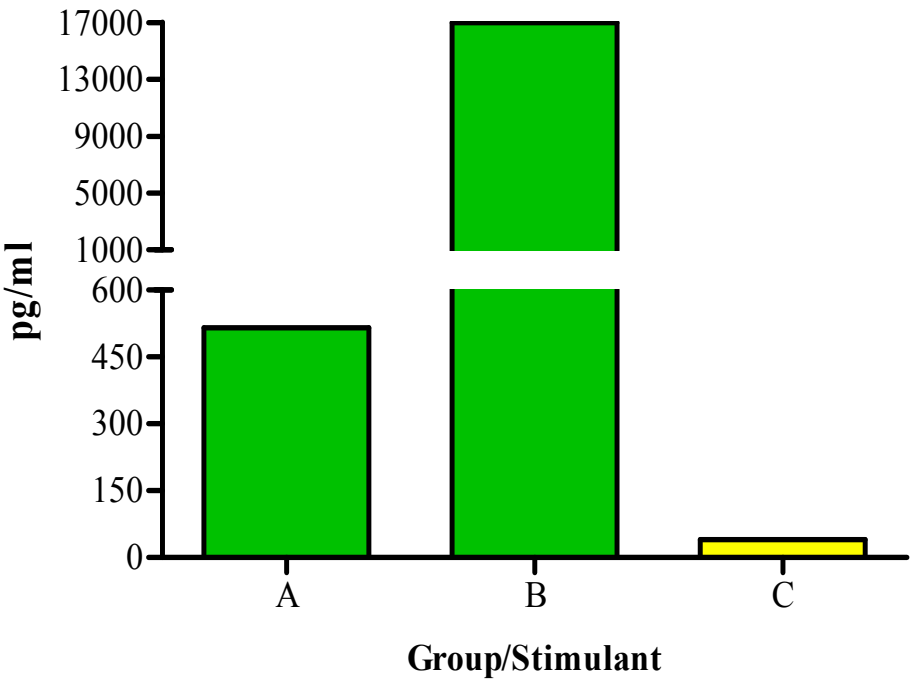

Figure 7.20: IL-2 production from rCYAAL58-stimulated splenocytes. Splenocytes from mice vaccinated with pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC* stimulated with 5µg/ml rCYAAL58 (A) and 2.5µg/ml ConA (B). Splenocytes from vector-immunised mice stimulated with 5µg/ml rCYAAL58 (C).

Table 7.1: IL-4 production from *in-vitro* re-stimulated splenocytes following vaccination of mice with pcDNA3.1D/*cyaAL58* with or without co-administration of pcDNA3.1D/*cyaC*. IL-4 secreted from $5x10^6$ splenocytes cultured for 72 hours in the presence of rCYAAL58, DTaP or ConA. Nd – not determined.

Stimulant	**µg/ml**	**IL-4 (pg/ml)**			
		pcDNA3.1D/*cyaAL58*	**pcDNA3.1D/*cyaAL58* + pcDNA3.1D/cyaC**	**pcDNA3.1 vector**	**DTaP**
rCYAAL58	5	0	5.7	19.4	Nd
DTaP	10	7.5	4	6.4	36.9
DTaP	5	0	9	6	69.9
ConA	2	462.8	462.8	511.3	1081.9

7.3.9.2 Serum Antibody Response Following Vaccination of Mice with pcDNA3.1D/cyaAL58 Alone or in Combination with pcDNA3.1D/cyaC

No rCYAAL58 or AC-Hly specific IgG was detected in the serum of mice immunised with pcDNA3.1D/*cyaAL58*. However, when pcDNA3.1D/*cyaC* was co-administered with pcDNA3.1D/*cyaAL58* an elevated IgG response was detected. The value of this IgG response was overshadowed by a comparable rCYAAL58 and ACT-specific response detected in the serum of vector-immunised mice (Figure 7.21). Due to a high degree of standard error there were no significant differences in mean titers across the three vaccine groups. There was however an apparent difference in the distribution of the IgG subclasses for combination and vector-immunised mice. Mice immunised with the DNA vaccine had a low but purely IgG1 response, whereas the vector group had only IgG2a antibodies (Table 7.2).

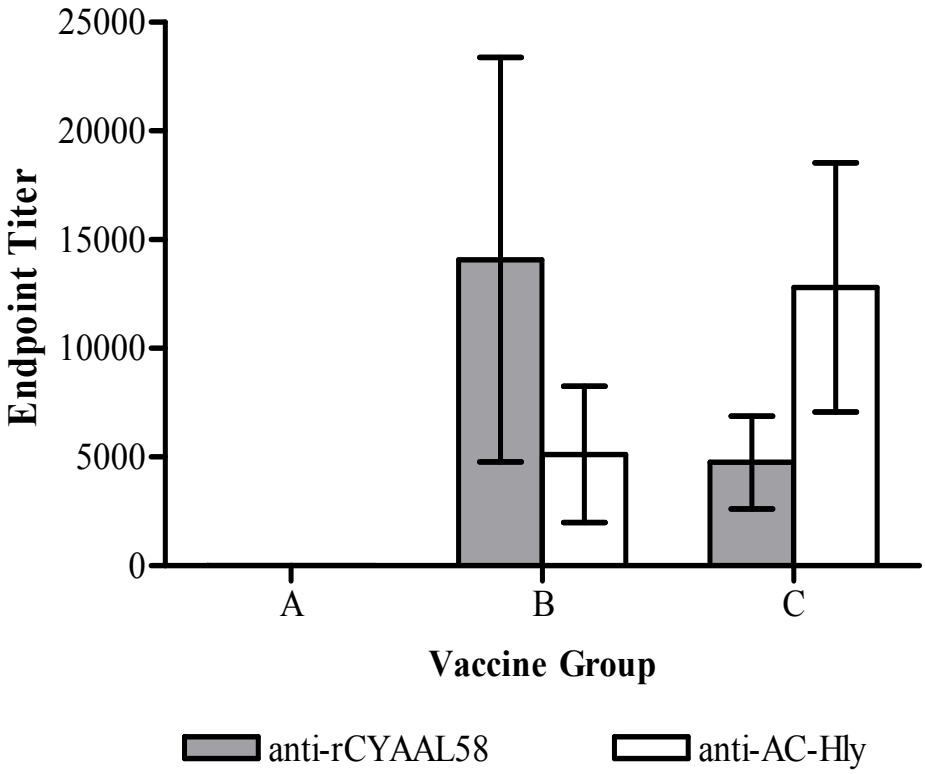

Figure 7.21: Anti-adenylate cyclase toxin IgG titers in the sera of mice vaccinated with pcDNA3.1D/*cyaAL58* or pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC* in combination. Bars represent mean scores from five mice +/- SE immunised with: A - pcDNA3.1/*cyaAL58*, B - pcDNA3.1/*cyaAL58* + pcDNA3.1/*cyaC* in combination and C - pcDNA3.1 vector.

Table 7.2: Serum IgG1 and IgG2a in mice vaccinated with pcDNA3.1D/*cyaAL58* with or without pcDNA3.1D/*cyaC*. Figures represent anti-rCYAAL58 titers from pooled serum.

Antigen	pcDNA3.1D/*cyaAL58*		pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC*		Vector	
	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a
rCYAAL58	0	0	100	0	0	3200

7.3.9.3 Protective Efficacy of Vaccination with pcDNA3.1D/cyaAL58 Alone or in Combination with pcDNA3.1D/cyaC by the IM Route

The protective efficacy of the monocomponent pcDNA3.1D/*cyaAL58* and dual component pcDNA3.1D/*cyaAL58* and pcDNA3.1D/*cyaC* vaccines was assessed by aerosol challenge with a Tohama I SLID. The bacterial counts in the lungs of test and control mice were evaluated over three time points.

Mice vaccinated with pcDNA3.1D/*cyaAL58* alone or in combination with pcDNA3.1D/*cyaC* showed an enhanced rate of clearance compared to the placebo or vector-immunised mice (Figure 7.22). At 4 days post-challenge, mean bacterial counts in all four groups were similar and showed slight two to six-fold increases in CFU/lung. Consistent with the efficacy of the other DNA vaccines tested, mice administered with the combination of pcDNA3.1D/*cyaAL58* and pcDNA3.1D/*cyaC* constructs had a 7-fold reduction in numbers by day 7, which progressed to a roughly 400-fold reduction after two weeks. In comparison, pcDNA3.1D/*cyaAL58*-immunised mice cleared the SLID at a much slower rate, with a 3 and 30-fold reduction after 7 and 14 days respectively. As indicated in the previous chapters the mice from negative control groups failed to control the experimental infection and bacterial counts were not reduced below the initial colonisation level. As reported previously, DTaP-immunised mice completely cleared *B. pertussis* from the lungs within 7 days. Unlike the other three groups there was no initial increase in bacterial numbers. A slight decrease in numbers observed after 4 days was proceeded by a rapid clearance as no bacteria were detected in lung homogenates from day 7 onwards.

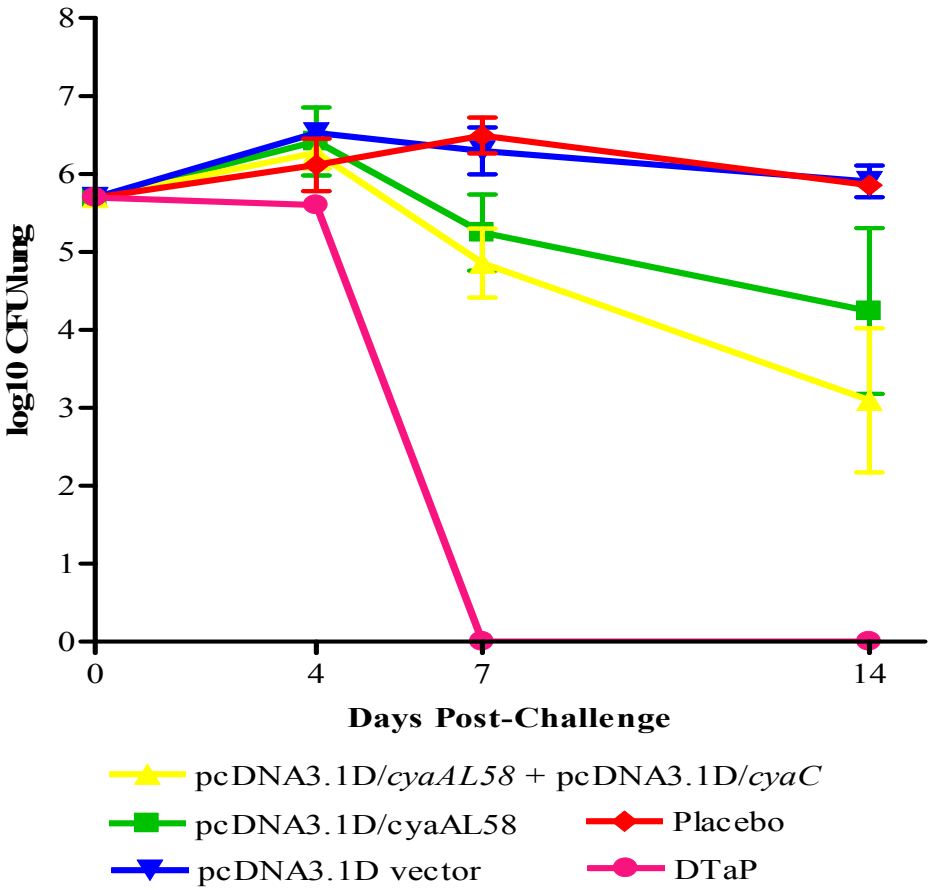

Figure 7.22: Clearance of *B. pertussis* from the lungs of mice vaccinated with pcDNA3.1/*cyaAL58* or pcDNA3.1/*cyaAL58* + pcDNA3.1/*cyaC* in combination following aerosol challenge. Data points represent mean count of CFU/lung from five mice +/- SE.

The clearance data showed that the bacterial load in mice immunised with pcDNA3.1D/*cyaAL58* alone was reduced to 2.4% (1.74×10^4 CFU/lung) of the untreated controls (7.2×10^5 CFU/lung), whereas the counts in mice that received both pcDNA3.1D/*cyaAL58* and pcDNA3.1D/*cyaC* was reduced to 0.17% (1.25×10^3) (Table 7.3). It was found that mice immunised with pcDNA3.1D/*cyaAL58* alone had significantly ($P < 0.05$) improved rates of clearance compared to the placebo-immunised mice (Table 4.3). Furthermore, the clearance rates of mice immunised with the pcDNA3.1D/*cyaAL58* and pcDNA3.1D/*cyaC* combination were also significantly better than both placebo ($P < 0.01$) and vector only ($P < 0.05$) controls. Mice immunised with DTaP were significantly more proficient at clearing *B. pertussis* than placebo-immunised mice ($P < 0.001$), vector-immunised mice ($P < 0.001$) and pcDNA3.1D/*cyaAL58*-immunised mice ($P < 0.05$).

Table 7.3: Post-challenge clearance data from mice immunised with pcDNA3.1D/*cyaAL58* and pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC* in combination.

Vaccine Group	% of Untreated Control[a] Day			CI[b]
	4	7	14	
Placebo	n/a	n/a	n/a	151.75 ± 1.65
Vector	255.7	63.5	115.3	151.78 ± 4.68
pcDNA3.1D/*cyaAL58*	200	5.6	2.4	99.06 ± 16.87 *
pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC*	142.7	2.3	0.17	67.03 ± 13.95 **
DTaP	30.5	0	0	22.43 ± 0.05 ***

[a] Quantitated as percentage of bacteria cleared from the lungs relative to the untreated group of mice.

[b] Clearance Index ± SE = mean log10 number of *B. pertussis*/ml × days (van den Berg et al. 2001).

* Significantly different from placebo ($P < 0.05$). ** Significantly different from placebo ($P < 0.01$) and vector ($P < 0.05$). *** Significantly different from placebo ($P < 0.001$), vector ($P < 0.001$) and pcDNA3.1D/*cyaAL58.13L.129G* ($P < 0.05$).

7.3.10 Recognition of rCYAAL58 and AC-Hly with Mouse Immune Sera

Cross-reactive IgG to rCYAAL58 was detected in the serum of vector-immunised mice by ELISA. Western blotting was performed to confirm that the cross-reactive antibodies were specific for rCYAAL58. Detection of rCYAAL58 with a monoclonal anti-His antibody (1:2000) was used as a positive control and as a size reference for comparison to the anti-sera blots, which was necessary as the higher molecular weight markers (190 and 120 kDa) were not successfully transferred onto the membrane. Blotting indicated that rCYAAL58 was recognised by mice injected with the DNA vaccines (Figure 7.23). However, it was also confirmed that vector-immunised mice had generated cross-reactive IgG antibodies that were specific for both rCYAAL58 and native AC-Hly. Some additional lower molecular weight bands were observed with: (i) rCYAAL58 probed with anti-sera from mice immunised with pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC* in combination; (ii) rCYAAL58 probed with anti-sera from mice immunised with vector only; and (iii) AC-Hly probed with anti-sera from mice immunised with vector (Figure 7.23, lanes 3, 4 and 5, respectively). These lower MW bands may be due to either degradation of rCYAAL58 and AC-Hly or non-specific interactions with native *E. coli* proteins, which contaminated the affinity purified rCYAAL58 eluates (see Figure 7.14).

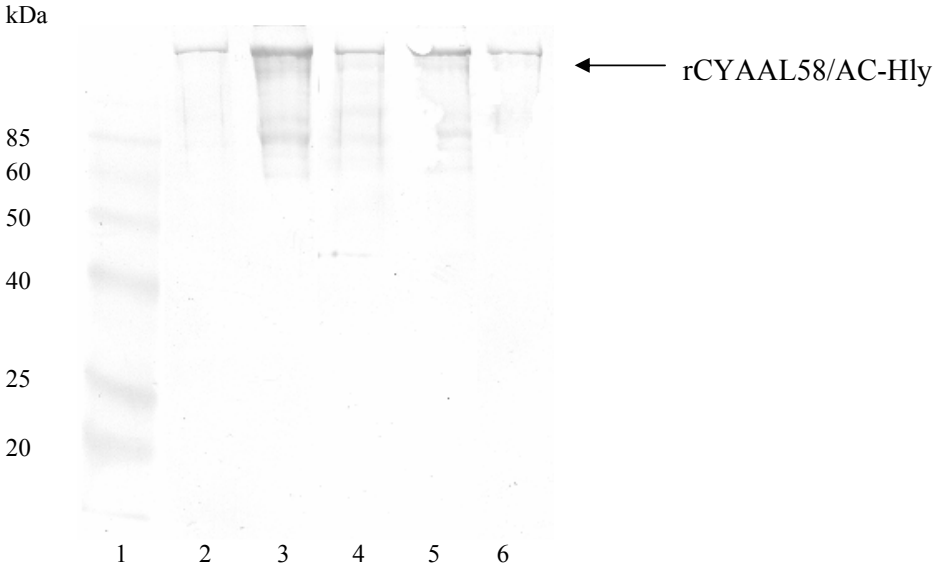

Figure 7.23: Recognition of rCYAAL58 and AC-Hly by sera from mice vaccinated with pcDNA3.1D/*cyaAL58*, pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC*, vector only and DTaP. Western blotting of rCYAAL58 with: pcDNA3.1D/*cyaAL58* anti-sera (lane 2), pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC* anti-sera (lane 3), vector only anti-sera (lane 4) and anti-His monoclonal antibody (lane 6). AC-Hly detected with vector only anti-sera (lane 5). Lane 1 - Prestained Benchmark Protein ladder (190kDa to 10kDa - Invitrogen)

7.4 Discussion

In the design of an AC-Hly DNA vaccine several key factors needed to be considered specifically its large size, post-translational activation and cytotoxicity. The large size and complexity of AC-Hly was predicted to result in a low level of heterologous expression, whether induced in *E. coli* or constitutively in mammalian cells. The concerns surrounding a low level of heterologous expression were realised when rCYAAL58 could not be easily purified from *E. coli*. Factors that may have limited the expression and purification of rCYAAL58 were differences in codon usage between *B. pertussis* and *E. coli*, the large size of the recombinant antigen, mild toxicity to *E .coli*, and partial masking of the histidine tag. Although a sufficient amount of protein was obtained for the immunological assays, the process required a lot of optimisation in terms of the induction and lysis conditions and chromatography. Whilst the truncation of FHA was logical and feasible, the results from previous studies have indicated it was not an option for development of an AC-Hly DNA vaccine (Barry et al. 1991; Betsou, Sebo & Guiso 1995).

For unknown reason(s), the BL21 CodonPlus strain used to successfully enhance expression levels of rFHAB1, rFHAB2 and rPRN could not be transformed with the pTrcHis2/*cyaAL58* plasmid. The pTrcHis2/*cyaAL58* plasmid was relatively large at 9.6 Kb and whilst this has been known to reduce transformation efficiency (Sheng, Mancino & Birren 1995), TOP10 and XL10 Gold *E. coli* strains were successfully transformed with the pTrcHis2/*cyaA* and pTrcHis2/*cyaAL58* plasmids, albeit at a much lower efficiency compared to the other smaller plasmid constructs generated in this study. Similarly, TOP10 and XL10 Gold along with the *S. typhimurium* strains were transformed with the larger pcDNA3.1D/*cyaAL58* construct. Toxicity of the recombinant protein can also have an impact on transformation efficiency as well as expression levels. In our case the detectable expression of wild-type rCYAA and mutant rCYAAL58 in *E. coli* effectively ruled out toxicity as a limiting factor. Rogel et al. (1989) had also shown expression of the *cyaA* gene in *E. coli* with no apparent toxic effects. Sebo et al. (1991) later reported that CyaC-activated adenylate cyclase toxin could be expressed at high levels within a laboratory strain of *E. coli*. Lastly, positive (pUC18) and negative controls (no DNA) were routinely included in all transformations and indicated that the competent cells were not only viable but the transformation protocol was sound.

An important feature of AC-Hly that was considered for design of a DNA vaccine was the two forms of post-translational activation of the toxin: fatty acylation of lysine residues 860 and 983 by the ancillary CyaC acyltransferase of *B. pertussis* prior to secretion; and enhanced AC activity through interaction with the human sensory protein calmodulin upon entry of the N-terminal domain into the target cell (Ladant 1988; Hackett et al. 1994). The action of calmodulin was not an issue as only a mutant devoid of AC activity was used to prevent any of the deleterious effects associated with administration of a functional N-terminal domain. On the other hand, it has been demonstrated that C-terminal fatty acylation was essential for toxic activity but importantly for protective immunity as well (Betsou, Sebo & Guiso 1995). These same authors earlier found that recombinant AC-Hly purified from *E. coli* was only a protective antigen after the addition of CyaC (Betsou, Sebo & Guiso 1993). The implications of acylation for DNA vaccine development are largely unknown i.e. whether delivery of *cyaAL58* and *cyaC* from separate plasmids would result in an equimolar uptake within a single host cell and if acylation of a foreign protein antigen

still occurred post-translationally in a mammalian system. Because of this uncertainty it was decided to pursue two strategies: DNA vaccination with inactivated CyaA alone and the co-administration of both CyaA with the accessory CyaC protein.

The issue of cytotoxicity was overcome with the use of an established point mutation proven to ablate adenylate cyclase activity and abolish virulence in mice (Au, Masure and Storm 1989). After obtaining relatively pure recombinant protein, the cytotoxicity of rCYAAL58 or lack thereof was tested *in vitro*. Although inactivity of this point mutant has long been established, the subtle difference of directed intracellular expression versus exogenous treatment meant that for safety and ethical reasons further confirmation was necessary. The gold standard for the measurement of AC activity has been the cAMP assay developed by Salomon, Londos & Rodbell (1974) and modified by Hanoune et al. (1977). Although a reliable and proven method, this radioimmunoassay required a relatively large amount of recombinant antigen, which could not be obtained for the reasons outlined above. Therefore an alternative cAMP assay was devised to quantitatively assess the endogenous toxicity of the native versus mutant antigens. The three-step assay involved transfection of CHO-K1 cells with selected eukaryotic expression plasmids or treatment with commercially purified antigen, followed by lysis of CHO-K1 cells after a 12-hour incubation, and then analysis of the cAMP levels in lysates using a commercial EIA. CHO cells have proven to be a valuable tool in toxicity testing as seen with the morphological changes in response to the pertussis toxin ADP-ribosylase (S1 subunit). It has been reported that CHO cells exhibit an elongated morphology in response to cholera toxin and *E. coli* enterotoxin, a change attributed solely to elevated cAMP levels (Guerrant et al. 1974). As AC-Hly has an identical mechanism of action the CHO cell assay was selected for the determination of AC activity via intracellular cAMP concentrations combined with morphological features. An added benefit of this cellular assay format was the relative simplicity over the conventional test. For instance, the assay did not require co-transfection of rCYAC, as this modification is only necessary to confer the invasive properties to the protoxin and CYAA alone has full catalytic activity (Rogel et al. 1989). The addition of calmodulin was also not required as this molecule is intrinsic to mammalian cells (Guermonprez et al. 2001). Overall, the cytotoxicity assay indicated that the "wild-type" rCYAA protein was functional after endogenous expression in a mammalian system and the point mutation successfully eliminated AC activity and was

safe to use *in vivo*. An advantage of applying a single point mutation rather than a large deletion was that a native structural conformation would be maintained. Betsou, Sebo & Guiso (1995) demonstrated that structural or conformational integrity is important factor in generating a protective anti-toxin response. Activation of the haemolytic domain by palmitoylation of residues 860 and 983 was also found to be necessary to generate an optimal immune response against AC-Hly (Betsou, Sebo & Guiso 1993).

In order to identify whether co-immunisation with CyaC had a positive effective *in vivo* it was decided to evaluate DNA immunisation by the IM route with either the inactivated *cyaAL58* gene alone or in combination with the accessory *cyaC* gene. Both the mono-component and two component DNA vaccines induced a strong CMI response as indicated by a high level of IFN-γ production by re-stimulated splenocytes. A comparably weak serum antibody response was detected in the test groups. The likely reasons for predominantly Th1 response to DNA vaccination have been discussed in chapters 4 and 6. Briefly, endogenous expression of antigens within myocytes often favours the generation of a Th1 or cellular response (Cornell et al. 1999; Rodriguez et al. 2001; Scheiblhofer et al. 2003). On occasions when IM delivery has produced a strong humoral response it has been attributed to cross priming. Cross priming involves the uptake of antigen or peptides by antigen presenting cells following secretion from transfected somatic cells (Gurunathan, Klinman & Seder 2000). The large size of rCYAAL58 may have led to a low level of expression and subsequently poor secretion. Alternatively, endogenous expression may have been sufficient but the central hydrophobic region of the antigen may have anchored it within the plasma membrane.

In many ways the immunogenicity of the adenylate cyclase-hemolysin DNA vaccines was consistent with the response to FHA. The low antigen-specific IgG titers detected in the serum of mice vaccinated with the *cyaAL58*/*cyaC* combination DNA vaccine was masked by an equivalent response in the vector only group. Considering the IgG response to rCYAAL58 and AC-Hly in vector-immunised mice was well above the placebo cut-off, cross-reactivity with neomycin phosphotransferase (neomycin resistance gene on the pcDNA3.1D/V5-His-TOPO vector) may have occurred. This type of vector cross-reactivity has not been uncommon with similar responses against BHV, influenza and HIV antigens and the presence of neomycin phosphotransferase cross-reactivity against two of the four antigens tested in our study re-iterates the need

to develop a minimalistic expression vector in future DNA vaccine trials (Cox, Zamb & Babiuk 1993; Fynan et al. 1993; Wang et al. 1993). Although AC-Hly could share some B cell epitopes with neomycin phosphotransferase a BlastN and BlastP searches of both nucleotide and amino acid sequences revealed no significant identity.

An important aspect of the cross-reactivity to AC-Hly was that, unlike FHA, a low level of IgG with specificity for rCYAAL58 and commercial AC-Hly was detected in the serum of the placebo group (Appendix D.5). This indicated that whilst FHA cross-reactivity was entirely vector-mediated, there were additional factors contributing to the strong background against rCYAAL58 and AC-Hly. Coincidently, AC-Hly is a large and complex antigen that has sequence similarities with many bacterial antigens such as the adenylate cyclase toxin of *Bacillus anthracis*, and in particular the other RTX toxins such as the leukotoxin from *pasteurella hemolytica* and the α-hemolysin of *E. coli* (Goyard et al. 1989). Therefore it is a possibility that the out-sourced population of specific pathogen free mice had been exposed to infectious elements with homology to AC-Hly. Another potential source of cross-reactivity may have been an autoantibody response to endogenous adenylate cyclases. Although a closely related epitope has been identified between mammalian adenylate cyclases and AC-Hly toxin (Goyard et al. 1989), it seems unlikely that auto-antibodies would be present at such high levels.

The protective efficacy the AC-Hly DNA vaccines were consistent with the other single antigen DNA vaccines. Clearance of the aerosol-induced infection was significantly improved in mice immunised with the DNA vaccines compared to those given the vector or placebo but was not as effective as DTaP. It was clear that immunisation with pcDNA3.1D/*cyaAL58* and pcDNA3.1D/*cyaC* in combination was superior to pcDNA3.1D/*cyaAL58* alone. IFN-γ production was more than four-fold greater in mice immunised with the *cyaAL58*/*cyaC* combination and disregarding the presence of vector cross-reactivity, the mean serum IgG titers were also higher than the pcDNA3.1D/*cyaAL58* group. Hormozi, Parton and Coote (1999) also observed that mice immunised with CyaC-activated CyaA survived a lethal IC challenge but this level of protection could not be conferred with the CyaA protoxin or a chimeric adenylate cyclase-leukotoxin.

Chapter 8 Immunisation of Mice with Five-Gene Combination DNA Vaccines and DNA/Protein Dual Modality Vaccines

8.1 Introduction

It is well established that *B. pertussis* expresses a vast array of virulence factors during infection that include: (i) adhesins (FHA, pertactin and fimbriae) which mediate attachment to both structural cells and immune cells of the host; and (ii) a myriad of toxins that cause tissue necrosis (DNT and TCT), interference of normal immune cell function (AC-Hly) as well as numerous systemic complications (PT). Moreover, analysis of the now complete genome sequence of *B. pertussis* has unearthed several new virulence-associated genes that may be included in future acellular vaccines or represent additional targets for the generation of improved attenuated vaccines (Locht et al. 2004). The introduction of antigenically-defined acellular vaccines has revealed that multi-component formulations typically confer an improved level of protection compared to monocomponent and two-component vaccines (Olin 1997; Plotkin & Cadoz 1997; Halperin 1999; Decker & Edwards 2000). Therefore, it could be argued that the generation of an optimal immune response against such a complex pathogen as *B. pertussis* requires multiple antigenic priming. On the basis of encouraging results for each of the single antigen DNA vaccines in terms of their immunogenicity and protective efficacy, five-gene combination DNA vaccines were tested with the aim of inducing a broad-spectrum immune response. It was decided to initially deliver the five-gene DNA vaccine by direct IM injection, to determine whether there would be a diminished, unaltered or enhanced effect on the immune response to each antigen.

As a generalisation, vaccines delivered via parenteral routes, irrespective of whether it involves DNA or protein, tends to elicit a poor or non-existent mucosal response (Dougan, Huett & Clare 2002; Rigano & Walmsley 2005). Therefore, it was decided to evaluate whether the combination DNA vaccine if delivered orally via attenuated *S. typhimurium* could induce a local secretory response in the respiratory mucosa. The basis for selection of oral immunisation over the other mucosal routes, such as intranasal or rectal, was three-fold. Firstly, attenuated Salmonella and Shigella spp have been reported to be effective vectors for oral delivery of heterologous antigen or DNA (Renauld-Mongenie et al. 1996; Ranallo et al. 2005). Although attenuated respiratory

pathogens such as *Listeria monocytogenes* and *Mycobacterium bovis* BCG (Bacille Calmette-Guerin strain) have been used in the past with some success, these models are not as well characterised (Gentschev et al. 2000; Nascimento et al. 2000). Secondly, specific mucosal and systemic antibodies have been generated by *aro*A *S. typhimurium* expressing *B. pertussis* antigens (Walker et al. 1992; Anderson, Dougan & Roberts 1996; Dalla Pozza et al. 1998). Thirdly, as a forward-looking measure in the event that mucosal immunisation was efficacious and a potential alternative to Pa or Pw, ingestion would conceivably be the most compliant method of delivery for infants and children. The *aro*A attenuated strains of *S. typhimurium* have been the most commonly used mutants for oral delivery of heterologous antigens. Oral delivery of recombinant *aro*A *S. typhimurium* has been reported to induce a secretory response at both local and distant mucosal surfaces, in addition to a potent systemic response (Oyston et al. 1995; Fagan et al. 2001; Koesling et al. 2001). The absence of certain aromatic compounds in mammals such as *p*-amino benzoic acid and 2,3 dihydroxybenzoic acid, which are essential for bacterial growth, sparked the initial interest in use of aromatic-dependent bacteria as vehicles for oral delivery of heterologous antigens (Dougan, Hormaeche & Maskell 1987). It was discovered that disruption of the *aro* biochemical pathway prevents synthesis of these essential aromatic compounds, and as mammalian cells do not possess this pathway and there are no alternative route for their production, *aro*A *S. typhimurium* mutants are avirulent in mice (Hoiseth & Stocker 1981).

As an extension of the combination DNA vaccine approach it was decided to test the efficacy of a DNA vaccine-prime/acellular vaccine-boost strategy, also referred to as dual modality vaccination. Acellular pertussis vaccines have been found to typically induce a strong Th2-driven antibody response but a relatively poor Th1-based cell-mediated response (Mills et al. 1998). Hence, this dual modality approach offered the promise of a dichotomous Th1/Th2 response and a more realistic improvement over the highly regarded acellular vaccines rather than an optimistic shift to DNA vaccination alone, a largely untried and untrusted platform in humans. For dual modality vaccination, mice were primed with either an IM or oral dose of a five-gene combination DNA vaccine followed by boosting with a laboratory constituted acellular vaccine (aP), via the SC route.

8.2 Overview of Methodology

The specific details of each procedure have been outlined in Chapter 2.

8.2.1 Transformation of SL3261

Heat-shock or electroporation were the two methods used to transform the *aro*A *S.typhimurium* strain SL3261 with the eukaryotic expression plasmids (see Sections 2.14.2 and 2.14.3 respectively). Prior to transformation of SL3261, the *E. coli*-based plasmids were passaged through the r^-m^+ *S. typhimurium* strain P9121 for methylation and protection from the intact restriction system of the *aro*A strain SL3261 (r^+m^+).

8.2.2 Preparation of Five-Gene Combination DNA Vaccines

Five of the six plasmid constructs evaluated as individual DNA vaccines were pooled as a combination DNA vaccine and administered via either direct IM injection (IM combination DNA vaccine) or orally via *aro*A *S. typhimurium* strain SL3261 (oral combination DNA vaccine). The less efficacious pcDNA3.1D/*fhaB2* construct was not included as a component of the combination DNA vaccines.

For IM delivery, 20µg of the plasmids pcDNA3.1D/*fhaB1*, pcDNA3.1D/*prn*, pcDNA3.1D/*pts1.13L.129G*, pcDNA3.1D/*cyaAL58* and pcDNA3.1D/*cyaC* were combined to constitute a dose of 100µg. As described in section 2.15.2.1, large scale plasmid DNA isolation from *E. coli* was performed using the endotoxin-free Plasmid Giga Prep Kit (Qiagen). Plasmid DNA was precipitated and resuspended in 1×PBS for IM injection. For oral delivery, 2×10^8 CFU of five recombinant *S. typhimurium* transformants (SL3261-pcDNA3.1D/*fhaB1*; SL3261-pcDNA3.1D/*prn*; SL3261-pcDNA3.1D/*pts1.13L.129G*; SL3261-pcDNA3.1D/*cyaAL58*; SL3261-pcDNA3.1D/*cyaC*) were combined for a total dose of 1×10^9 CFU in 3% $NaHCO_3$. Pre-determined growth curves for the recombinant *S. typhimurium* SL3261 were used to calculate the amount of culture required to pellet 2×10^8 CFU of each of the five vaccine strains (Chapter 2, Section 2.15.3.1). The five cell pellets were combined in 1ml of 3% $NaHCO_3$ as a 1×10^{10} CFU/ml suspension. Mice received three 0.1 ml doses of the 1×10^{10} CFU/ml suspension at three-week intervals via oral gavage. As a negative control for the oral combination DNA vaccine, a group of mice were given three 0.1ml doses of SL3261 transformed with the self-ligated pcDNA3.1/V5-His-TOPO vector at 1×10^{10} CFU/ml (oral vector group).

8.2.3 Preparation of In-House Acellular Pertussis Vaccine (aP)

Dual modality vaccination involved priming with a combination DNA vaccine followed by two boosters with an acellular vaccine. Under a licence, a limited amount of Infanrix™ DTaP was kindly donated by GlaxoSmithKline (Australia) for use as a control in this DNA vaccine study. This agreement did not cover its use as a booster for dual modality vaccination. As a means of overcoming this limitation, a three-component acellular pertussis vaccine (aP), modelled on the pertussis component of the Infanrix™ vaccine, was constituted in our laboratory and used as the acellular vaccine booster in the dual modality vaccines.

8.2.4 Immunisation of Mice with Five-Gene Combination DNA Vaccines and Dual Modality Vaccines

The IM combination DNA vaccine was evaluated along side the individual DNA vaccines in the first mouse trial, whereas the oral combination DNA vaccine and dual modality vaccines were evaluated in a second mouse trial (Chapter 2, Section 2.15).

8.3 Results

8.3.1 Efficacy of Five-Gene Combination DNA Vaccines

To evaluate the immunogenicity of the five-gene combination DNA vaccines, the levels of IFN-γ, IL-2 and IL-4 produced by *in vitro* stimulated splenocytes and the titers of serum IgG were determined by ELISA. In addition, lung washes were performed for mice immunised with the oral combination DNA vaccine, oral vector and DTaP to test for the presence of mucosal IgA and IgG responses.

8.3.1.1 Cytokine Profile following Five-Gene Combination DNA Vaccination

The determination of cytokine levels in the supernatants of antigen-stimulated splenocytes revealed clear differences in the production of IFN-γ between the combination DNA vaccines and the respective vector only controls (Figures 8.1 and 8.2). Pooled splenocytes from mice immunised with the IM combination DNA vaccine produced much higher levels of the Th1 cytokines (IFN-γ and IL-2) than the vector-immunised mice when stimulated with recombinant *B. pertussis* antigens (Figure 8.1). The splenocytes of mice immunised with the Infanrix™ DTaP produced much lower levels of IFN-γ in response to recombinant *B. pertussis* antigens compared to the IM combination DNA vaccine (Appendix D.6)

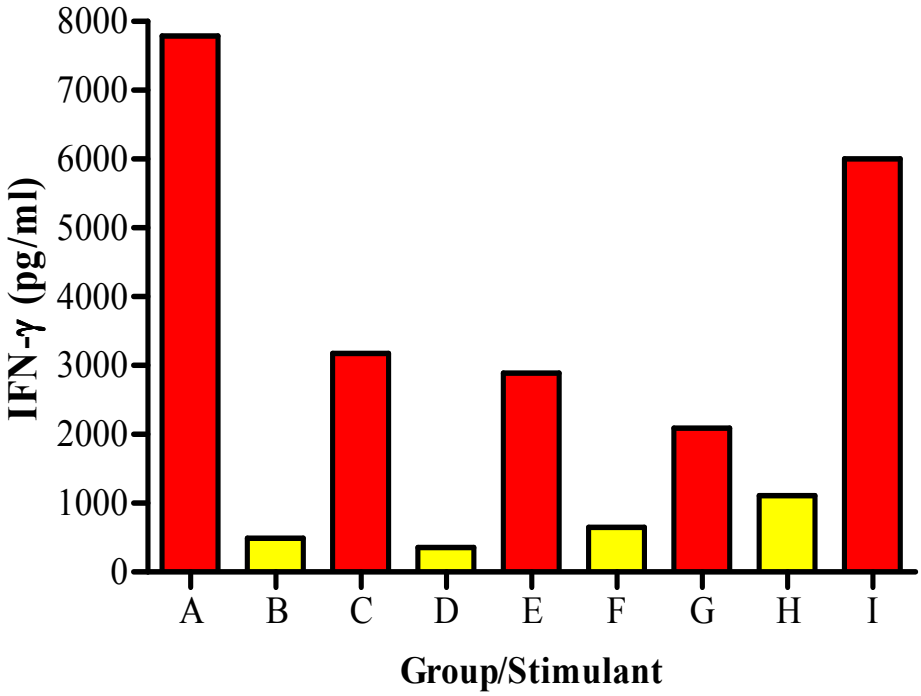

Figure 8.1: IFN-γ production from *in-vitro* re-stimulated splenocytes following immunisation with an IM combination DNA vaccine. IFN-γ secreted from 5×10^6 splenocytes cultured in the presence of 5µg/ml rFHAB1 (A and B), 5µg/ml rPRN (C and D), 5µg/ml rPTS1.13L.129G (E and F), 5µg/ml rCYAAL58 (G and H) and 2µg/ml ConA (I). Red filled columns represent mice vaccinated with IM combination DNA vaccine and yellow filled columns represent mice vaccinated with vector only.

Splenocytes of mice immunised with the oral combination DNA vaccine produced levels of IFN-γ that were more than 4-fold greater than the splenocytes of mice immunised with the oral vector, when stimulated with rFHAB1 (Figure 8.2). Similarly, when stimulated with rPRN and rPTS1.13L.129G, there was a respective 7-fold and 9-fold increase in IFN-γ compared to the oral vector-immunised mice. Splenocytes from mice immunised with DTaP produced low to undetectable levels of IFN-γ in response to the recombinant *B. pertussis* antigens but were more responsive to stimulation with native antigens in the form of the Infanrix™ acellular vaccine itself (Appendix D.6). A large amount of IFN-γ was produced when the splenocytes from mice that received the oral vaccines (oral combination DNA vaccine and oral vector) were stimulated with a *S. typhimurium* lysate (Appendix D.6).

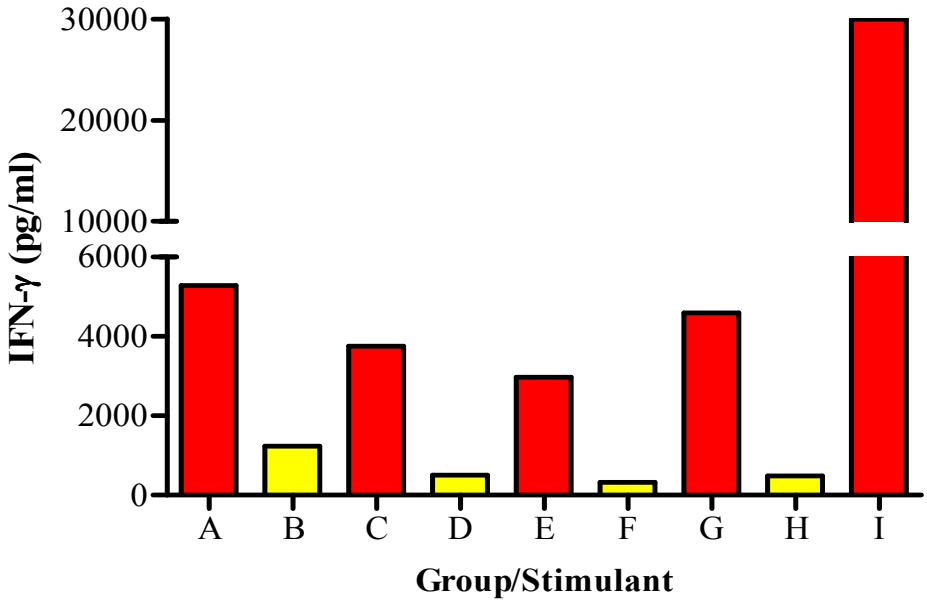

Figure 8.2: IFN-γ production from *in-vitro* re-stimulated splenocytes following immunisation with an oral combination DNA vaccine. IFN-γ secreted from 5×10^6 splenocytes cultured in the presence of 5µg/ml rFHAB1 (A and B), 5µg/ml rPRN (C and D), 5µg/ml rPTS1.13L.129G (E and F), 5µg/ml rCYAAL58 (G and H) and 2µg/ml ConA (I). Red filled columns represent mice vaccinated with oral combination DNA vaccine and yellow filled columns represent mice vaccinated with the oral vector.

Data from IL-2 assays showed that oral or IM combination DNA vaccination produced higher levels of the Th2 cytokine than either the vector only controls (oral vector and IM vector respectively) or DTaP (Table 8.1). Pooled splenocytes of mice immunised with the IM combination DNA vaccine produced between 5 and 7 times more IL-2 in response to rFHAB1, rPRN and rPTS1.13L.129G than the IM vector group. There was no difference in IL-2 production between the IM combination DNA vaccine and IM vector groups when splenocytes were stimulated with rCYAAL58 (Table 8.1).

With the exception of rPRN, the oral combination DNA vaccine stimulated higher levels of IL-2 than the mice immunised with the oral vector, which produced no IL-2 in response to the recombinant *B. pertussis* antigens (Table 8.1).

The splenocytes of DTaP-immunised mice also produced no IL-2 when stimulated with rFHAB1, rPRN, rPTS1.13L.129G or rCYAAL58 (Table 8.1).

Table 8.1: IL-2 production from *in-vitro* re-stimulated splenocytes following immunisation with the IM or oral five-gene combination DNA vaccine. FFST – formalin-fixed *S. typhimurium*. Nd – Not determined.

Stimulant	µg/ml	IL-2 (pg/ml)				
		Oral vector	Oral combination DNA vaccine	IM vector	IM combination DNA vaccine	DTaP
rFHAB1	5	0	11.4	47.9	249.5	0
rPRN	5	0	0	12	90.7	0
rPTS1.13L.129G	5	0	54.8	9.4	42.8	0
rCYAAL58	5	0	172.3	135.2	134.3	0
rCYAAL58+rCYAC	5	0	71.1	40.2	6.8	0
FFST	5	185.6	138.8	Nd	Nd	0
ConA	2	881	3760	25360.2	16134.2	973

Analysis of IL-4 production from splenocytes of mice immunised with the IM and oral five-gene combination DNA vaccines was inconclusive. No IL-4 was produced in the oral combination DNA vaccine and oral vector groups except upon stimulation with a *S. typhimurium* lysate or ConA, and low but variable levels of IL-4 were detected in the supernatants of the IM combination DNA vaccine and IM vector groups (Table 8.2).

Table 8.2: IL-4 production from *in-vitro* re-stimulated splenocytes following immunisation with the IM or oral five-gene combination DNA vaccine. FFST – formalin-fixed *S. typhimurium*. Nd – Not determined.

Stimulant	µg/ml	IL-4 (pg/ml)				
		Oral vector	Oral combination DNA vaccine	IM vector	IM combination DNA vaccine	DTaP
rFHAB1	5	0	0	0	22.5	0
rPRN	5	0	0	16.8	19.2	0.8
rPTS1.13L.129G	5	0	0	12.8	16.7	0
rCYAAL58	5	0	0	19.4	7.8	0
rCYAAL58 + rCYAC	5 each	0	0	10.5	2.5	0
FFST	5	11	6.3	Nd	Nd	0.7
ConA	2	932.5	540.8	509.4	481.3	837

8.3.1.2 Serum IgG Response to Five-Gene Combination DNA Vaccination

Levels of IgG specific for native *B. pertussis* antigens (FHA, PTX and AC-Hly) and recombinant antigens (rFHAB1, rPRN, rPTS1.13L.129G and rCYAAL58) generated by the combination DNA vaccines were determined by indirect ELISA. No anti-rFHAB1 or anti-FHA IgG response was detected in mice that received the oral combination DNA vaccine. Although a low mean titer of anti-rFHAB1 IgG was detected in the serum of mice immunised with the IM combination DNA vaccine, this response was overshadowed by a higher titer in the serum of vector-immunised mice (Table 8.3). DTaP-immunised mice generated significantly higher titers of anti-rFHAB1 and anti-FHA IgG than mice immunised with the IM combination DNA vaccine ($P < 0.05$), oral combination DNA vaccine ($P < 0.005$) and the vector-only controls ($P < 0.05$).

The IgG responses to rPRN, rPTS1.13L.129G and native PT were also significantly greater in mice immunised with DTaP compared to mice given the IM or oral combination DNA vaccines (Figure 8.3 and Table 8.4). However, consistent with the response to the pcDNA3.1D/*prn* DNA vaccine, a strong IgG response to rPRN was generated following immunisation with the IM and oral combination DNA vaccines (Figure 8.3). Low rPTS1.13L.129G-specific IgG titers were detected in the serum of mice that received the oral combination DNA vaccine but not in mice that were immunised with the IM combination DNA vaccine (Table 8.4).

Mice immunised with the IM combination DNA vaccine generated a large rCYAAL58-specific and AC-Hly-specific IgG response, however this was negated by an equivalent response in the mice immunised with the vector only (Table 8.5). High titers of anti-*S. typhimurium* IgG were generated following immunisation with the oral combination DNA vaccine or oral vector (Figure 8.4). There was an observable increase in the IgG response to *S. typhimurium* in the oral DNA vaccine group compared to the oral vector, but this increase was not statistically significant.

Table 8.3: Endpoint titers of anti-filamentous hemagglutinin IgG in sera of mice immunised with the five-gene combination DNA vaccines.

Group[a]	anti-rFHAB1		anti-FHA	
	Titer	SE	Titer	SE
First Mouse Experiment				
IM Vector	12800	0	800	0
IM Five-Gene Combination DNA Vaccine	4480	2395	Nd	Nd
DTaP (Infanrix™)	43980*	20573	38400**	8095
Second Mouse Experiment				
Oral Vector	0	0	0	0
Oral Five-Gene Combination DNA Vaccine	0	0	0	0
DTaP (Infanrix™)	2560***	392	960	640

[a] The five-gene combination DNA vaccines (IM or oral) were tested in a separate mouse experiments, hence the variation in the antibody titers in the DTaP group. * Significantly different from IM vector ($P < 0.05$) and IM combination DNA vaccine ($P < 0.05$). ** Significantly different from IM vector ($P < 0.005$). *** Significantly different from oral vector ($P < 0.005$) and oral combination DNA vaccine ($P < 0.005$). Nd – Not determined.

Table 8.4: Endpoint titers of anti-pertussis toxin IgG in sera of mice vaccinated with five-gene combination DNA vaccines.

Group[a]	anti-rPTS1.13L.129G		anti-PTX	
	Titer	SE	Titer	SE
First Mouse Experiment				
IM Vector	0	0	0	0
IM Five-Gene Combination DNA Vaccine	450	295	Nd	Nd
DTaP (Infanrix™)	14080*	9308	92369**	17753
Second Mouse Experiment				
Oral Vector	9600	4411	0	0
Oral Five-Gene Combination DNA Vaccine	34560	17363	12800	12800
DTaP (Infanrix™)	51200***	14022	97280****	30720

Antibodies to both rPTS1.13L.129G and commercial PT were measured by indirect ELISA. [a] The five-gene combination DNA vaccines (Oral and IM) and respective controls were assessed in a separate experiments as per Table 8.1. * Significantly different from IM combination DNA vaccine ($P < 0.01$). ** Significantly different from IM vector ($P < 0.01$). *** Significantly different from oral vector ($P < 0.05$). **** Significantly different from oral combination DNA vaccine ($P < 0.05$). Nd – Not determined.

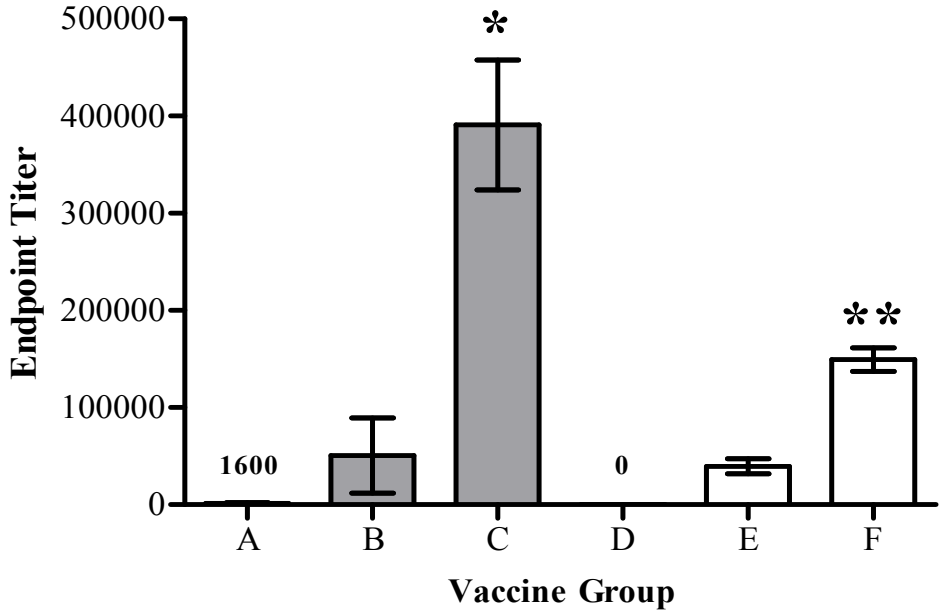

Figure 8.3: Endpoint titers of anti-rPRN IgG in sera of mice vaccinated with the combination DNA vaccines. Filled columns represent oral combination DNA vaccine: A – oral vector, B – oral combination DNA vaccine, C – DTaP (Infanrix™), and unfilled columns represent IM combination DNA vaccine: D – IM vector, E – IM combination DNA vaccine and F- DTaP (Infanrix™). Columns represent mean +/- SE. * Significantly different from oral vector ($P < 0.005$) and oral combination DNA vaccine ($P < 0.05$). ** Significantly different from IM vector ($P < 0.005$) and IM combination DNA vaccine ($P < 0.005$).

Table 8.5: Endpoint titers of anti-adenylate cyclase toxin IgG in sera of mice vaccinated with five-gene combination DNA vaccines.

Group	anti-rCYAAL58		anti-AC-Hly	
	Titer	SE	Titer	SE
First Mouse Experiment				
IM Vector	83246	0	12800	5724
IM Five-Gene Combination DNA Vaccine	87326	16797	Nd	Nd
DTaP (Infanrix™)	0	0	0	0
Second Mouse Experiment				
Oral Vector	405	230	599	367
Oral Five-Gene Combination DNA Vaccine	0	0	1127	373
DTaP (Infanrix™)	0	0	0	0

Antibodies to both recombinant CYAA and commercial Adenylate cyclase toxin were measured. Nd – Not determined.

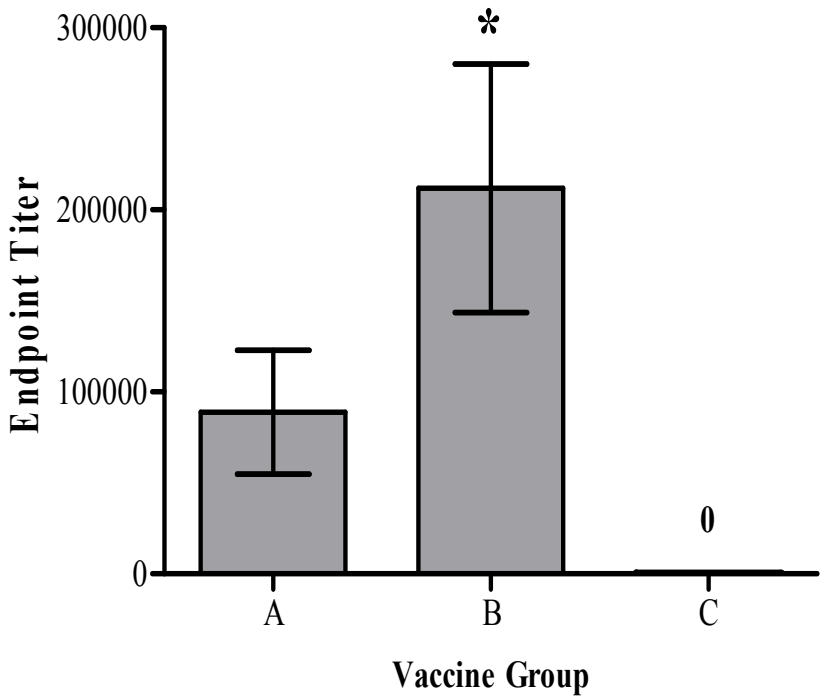

Figure 8.4: Endpoint titers of anti-*S. typhimurium* SL3261 IgG in sera of mice vaccinated with the oral five-gene combination DNA vaccine. A – oral vector, B – oral five-gene combination DNA vaccine, C – DTaP. Columns represent mean scores from five mice +/- SE. * Significantly different from DTaP ($P < 0.05$).

Overall, the serum IgG response to the IM combination DNA vaccine was poor in comparison to that of DTaP. After normalising the serum IgG data from mice immunised with the IM combination DNA vaccine against the serum IgG responses in vector-immunised mice, the only notable IgG response elicited was against rPRN (Figure 8.3). Unlike DTaP which induced an exclusive IgG1 response to rPRN, the IgG1:IgG2a ratio in mice that received the IM combination DNA vaccine was lower with 2:1 (Table 8.7).

The serum IgG response to the oral combination DNA vaccine was broader than the IM combination DNA vaccine with the induction of antibodies to both rPRN and rPTS1.13L.129G (Figure 8.3 and Table 8.4). For rPRN antibodies, there was an equivalent IgG1/IgG2a response with no apparent bias for either isotype (Table 8.6). However, the anti-rPTS1.13L.129G response to the oral combination DNA vaccine was dominated by IgG1 with a 16-fold higher titer than IgG2a (Table 8.6). Interestingly, no

total IgG specific for rFHAB1 was detected in the serum of mice using an anti-mouse IgG (Pierce), but a 4:1 ratio of IgG1 to IgG2a was detected using anti-mouse IgG1 and anti-mouse IgG2a antibodies (Xymed) for detection of the IgG isotypes. The serum IgG response to all *B. pertussis* antigens tested in DTaP-immunised mice showed a clear bias towards the IgG1 isotype, which indicated a Th2 response (Table 8.6).

Table 8.6: Serum IgG1 and IgG2a titers in mice vaccinated with an oral five-gene combination DNA vaccine. Figures represent pooled serum from five mice. FFST – formalin-fixed *S. typhimurium* lysate.

Antigen	**Oral combination DNA vaccine**		**Oral vector**		**DTaP**	
	IgG1	**IgG2a**	**IgG1**	**IgG2a**	**IgG1**	**IgG2a**
rFHAB1	3200	800	0	100	12800	0
rPRN	1600	1600	1600	400	516608	3200
rPTS1.13L.129G	102400	6400	25600	3200	203481	100
rCYAAL58	0	200	0	100	0	0
FHA	0	50	0	0	3200	400
PTX	0	0	0	0	502903	800
AC-Hly	0	50	0	100	0	0
FFST	460256	41168	204800	12800	6400	0

Table 8.7: Serum IgG1 and IgG2a titers in mice vaccinated with an IM five-gene combination DNA vaccine. Figures represent pooled serum from five mice. Nd – not determined.

Antigen	**IM combination DNA vaccine**		**IM vector**		**DTaP**	
	IgG1	**IgG2a**	**IgG1**	**IgG2a**	**IgG1**	**IgG2a**
rFHAB1	0	0	400	200	800	0
rPRN	3200	1600	200	0	88087	0
rPTS1.13L.129G	0	0	200	0	Nd	Nd
rCYAAL58	200	200	0	200	Nd	Nd
FHA	Nd	Nd	Nd	Nd	3200	400
PTX	Nd	Nd	Nd	Nd	146689	100

8.3.1.3 Mucosal Antibody Response to the Oral Combination DNA Vaccine

No *B. pertussis*-specific IgA was detected in pooled lung washes of mice immunised with the oral combination DNA vaccine or oral vector (Appendix D.6). However, very low titers of IgA specific for a *S. typhimurium* lysate were detected in the pooled BAL fluid of both the oral combination DNA vaccine and oral vector groups (Appendix D.6).

A mucosal IgG response to rFHAB1, FHA, rPRN and PTX was detected in the lung washes of mice immunised with DTaP via the SC route but not in the BAL fluid of mice that received the oral combination DNA vaccine or oral vector (Table 8.8). However, low titers of mucosal IgG specific for rPTS1.13L.129G and *S. typhimurium* lysate were detected in the BAL fluid of mice immunised with either the oral vaccine or oral vector (Table 8.9). Little or no IgG specific for rCYAAL58 or native AC-Hly were detected.

Table 8.8: Antigen-specific IgG in BAL fluid of mice immunised with the oral five-gene combination DNA vaccine.

Group	**Anti-rFHAB1**		**Anti-FHA**		**Anti-rPRN**		**Anti-PTX**	
	Titer	**SE**	**Titer**	**SE**	**Titer**	**SE**	**Titer**	**SE**
Oral Combination DNA Vaccine	0	0	0	0	0	0	0	0
Oral Vector	0	0	0	0	0	0	0	0
DTaP	180	66	360	183	1040	240	1280	480

Table 8.9: Antigen-specific IgG in BAL fluid of mice immunised with the oral five-gene combination DNA vaccine.

Group	**Anti-rPTS1.13L.129G**		**Anti-rCYAAL58**		**Anti-AC-Hly**		**Anti-SL3261**	
	Titer	**SE**	**Titer**	**SE**	**Titer**	**SE**	**Titer**	**SE**
Oral Combination DNA Vaccine	280	150	10	10	20	20	340	125
Oral Vector	820	614	0	0	0	0	340	189
DTaP	260	140	0	0	0	0	0	0

8.3.1.4 Protective Efficacy of Five-Gene Combination DNA Vaccination

The protective efficacy of the combination DNA vaccines was evaluated in the sub-lethal aerosol challenge model. Bacterial counts in the lungs of challenged mice were recorded at three time points (viz. 4, 7 and 14 days post-challenge).

Mice immunised with DTaP and the IM combination DNA vaccine completely cleared the experimental infection within 7 and 14 days respectively (Figure 8.5). On the contrary, the oral combination DNA vaccine group could not successfully eliminate *B. pertussis*, with a high mean bacterial count at two weeks post-challenge (Figure 8.6). Mice that received the oral vector, IM vector and placebo failed to control the SLID, and lung counts were either not reduced or only marginally reduced below the initial colonisation level.

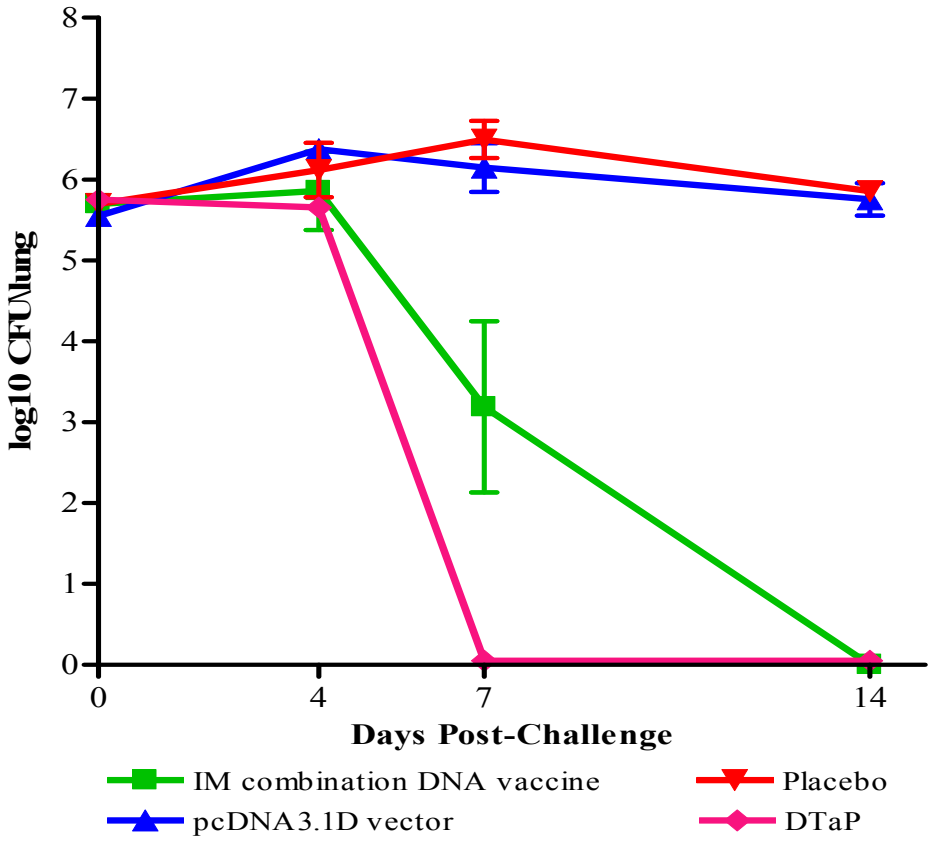

Figure 8.5: Clearance of *B. pertussis* challenge from the lungs of mice vaccinated with the IM five-gene combination DNA vaccine. Data points represent mean count of CFU/lung from 4 mice +/- SE.

The clearance data showed that the bacterial loads in mice immunised the IM combination DNA vaccine were effectively reduced to 55.2% and 0.05% of the untreated controls after 4 and 7 days post-challenge (Table 8.10). However, *B. pertussis* counts in the oral combination DNA vaccine group were only reduced to 37.8% (3.35×10^4 CFU) of the placebo mice (9.13×10^4 CFU) after two weeks (Table 8.11). CI (clearance index) values showed that the IM combination DNA vaccine group had a significantly improved rate of clearance compared to both placebo ($P < 0.005$) and vector-immunised mice ($P < 0.005$). Moreover, DTaP was also significantly more efficacious than the IM combination DNA vaccine ($P < 0.05$) (Table 8.10). Despite a poor clearance curve, mice-immunised with the oral DNA vaccine were found to be significantly better at eliminating *B. pertussis* than the oral vector ($P < 0.005$) and placebo ($P < 0.005$) groups (Table 8.11). Mice immunised with DTaP were significantly more effective in clearing the aerosol challenge from the lungs than the oral combination DNA vaccine ($P < 0.005$).

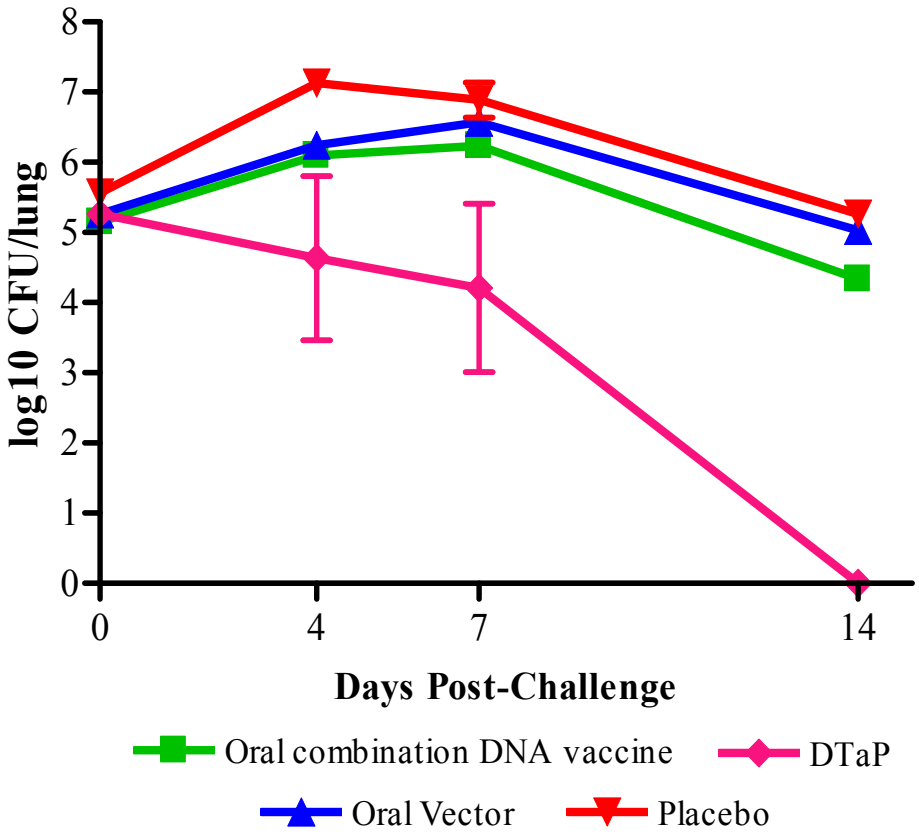

Figure 8.6: Clearance of *B. pertussis* challenge from the lungs of mice vaccinated with the oral five-gene combination DNA vaccine. Data points represent mean count of CFU/lung from 4 mice +/- SE.

Table 8.10: Post-challenge clearance data from mice immunised with the IM combination DNA vaccine

Vaccine Group	% of Untreated Control[a] Day			CI[b]
	4	7	14	
Placebo	na	na	na	151.75 ± 1.65
Vector	255.7	63.5	115.3	151.78 ± 4.68
IM Combination DNA Vaccine	55.2	0.05	0	45.5 ± 0.868 *
DTaP	30.5	0	0	22.43 ± 0.05 **

[a] Percentage of bacteria cleared from the lungs relative to the untreated group of mice.

[b] Clearance Index ± SE = mean log10 number of *B. pertussis*/ml × days (van den Berg et al. 2001).

* Significantly different from placebo ($P < 0.005$) and vector ($P < 0.005$).

** Significantly different from placebo ($P < 0.005$), vector ($P < 0.005$) and IM combination DNA vaccine ($P < 0.05$).

Table 8.11: Post-challenge clearance data from mice immunised with the oral combination DNA vaccine

Vaccine Group	% of Untreated Control[a] Day			CI[b]
	4	7	14	
Placebo	na	na	na	141.55 ± 1.92
Oral Vector	25.9	95.6	117.2	141 ± 0.75
Oral Combination DNA Vaccine	2.9	71.1	37.8	132.4 ± 1.43 *
DTaP	0.64	0.42	0	22.15 ± 10.14 **

[a] Percentage of bacteria cleared from the lungs relative to the untreated group of mice.

[b] Clearance Index ± SE - mean log10 number of *B. pertussis*/ml × days (van den Berg et al. 2001).

* Significantly different from placebo ($P < 0.005$) and vector ($P < 0.005$).

** Significantly different from placebo ($P < 0.005$), vector ($P < 0.005$) and Oral Combination DNA Vaccine ($P < 0.005$).

8.3.2 Dual Modality Vaccination: Priming with a Five-Gene Combination DNA Vaccine and Boosting with an In-House Acellular Vaccine

The efficacy of two different DNA vaccine-prime/acellular vaccine-boost regimens was determined by the measurement of: (i) serum IgG, (ii) mucosal antibodies in BAL fluid, (iii) cytokine production in primary splenocyte cultures, and (iv) the clearance of a sub-lethal aerosol challenge (SLID). The first of the dual modality vaccines involved priming with a five-gene combination DNA vaccine via the IM route followed by two boosters with an in-house acellular vaccine via the SC route. The second of the dual modality vaccines involved priming with a five-gene combination DNA vaccine via the oral route (recombinant *S. typhimurium*) and boosting with the in-house acellular vaccine via the SC route. From this point onwards the former will be referred to as the parenteral dual modality vaccine and the latter as the oral dual modality vaccine. Three doses of a 0.2 standard human dose (SHD) of Infanrix™ DTaP or a placebo were included as positive and negative controls. Moreover, to directly compare the benefits of dual modality vaccination versus immunisation with the acellular vaccine alone, a vector dual modality vaccine group was included. Mice in this group were primed with vector only via the IM route follwed by boosting with an in-house acellular vaccine.

8.3.3 Immunogenicity and Protective Efficacy of the Parenteral Dual Modality Vaccine in Mice

8.3.3.1 Cytokine Production Induced by the Parenteral Dual Modality Vaccine

Splenocytes from mice immunised with the parenteral dual modality vaccine produced very high levels of IFN-γ when stimulated with rFHAB1, rPRN, rPTS1.13L.129G and rCYAA (Figure 8.7). The splenocytes of mice immunised with the parenteral dual modality vaccination produced substantially higher levels of IFN-γ than mice immunised with the vector dual modality regimen or DTaP (Figure 8.7). When stimulated with rFHAB1, there was a more than 5-fold increase in IFN-γ produced from the splenocytes of parenteral dual modality group (9916 pg/ml) compared to the splenocytes of the vector dual modality group (1860 pg/ml) and a 13-fold increase compared to the splenocytes of DTaP-immunised mice (763 pg/ml). Stimulation with rPRN produced a more than 3-fold increase in the IFN-γ compared with the vector dual modality group and 7-fold higher levels than DTaP-immunised mice. Similarly, stimulation with rPTS1.13L.129G resulted in 3-fold and 5-fold increases in IFN-γ than the vector-dual modality and DTaP controls.

Interestingly, when compared to the IM combination DNA vaccine (Figure 8.1), the level of IFN-γ production was enhanced by priming with the IM five-gene combination DNA vaccine and boosting with an in-house acellular vaccine (Figure 8.7). When stimulated with rFHAB1, there was a 1.25-fold increase in IFN-γ produced from the splenocytes of parenteral dual modality group (9916 pg/ml) compared to the splenocytes of the IM five-gene combination DNA vaccine group (7790 pg/ml). Similarly, a more than 1.5-fold increase in IFN-γ was detected upon stimulation with rPRN and rPTS1.13L.129G. Stimulation with rCYAA produced a more than 2-fold increase in the IFN-γ compared with the splenocytes of mice immunised with the IM combination DNA vaccine.

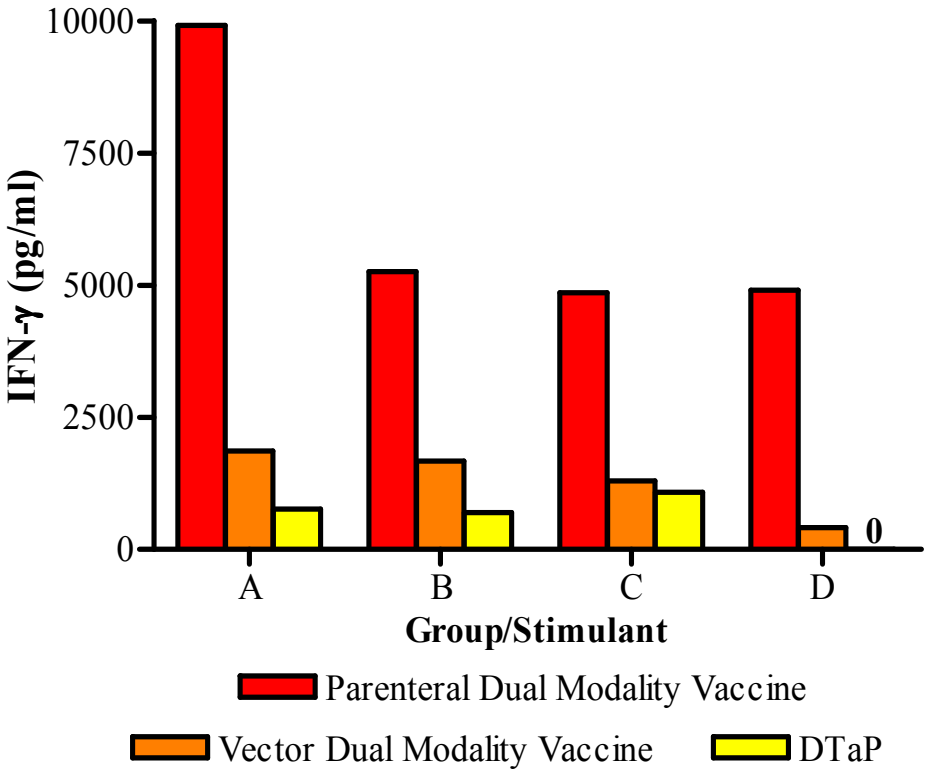

Figure 8.7: IFN-γ production from *in-vitro* re-stimulated splenocytes following immunisation with the parenteral dual modality vaccine. IFN-γ secreted from 5×10^6 splenocytes cultured for 72 hours in the presence of: A - 5µg/ml rFHAB1, B - 5µg/ml rPRN, C - 5µg/ml rPTS1.13L.129G, and D - 5µg/ml rCYAAL58.

Splenocytes from the parenteral dual modality vaccine group produced far greater levels of the IL-2 than the splenocytes of DTaP-immunised mice (Figure 8.8). Overall the parenteral dual modality vaccine elicited a marginally higher IL-2 response than the vector dual modality control group, with the exception of rFHAB1 stimulation, which resulted in a larger 3-fold increase in IL-2 production.

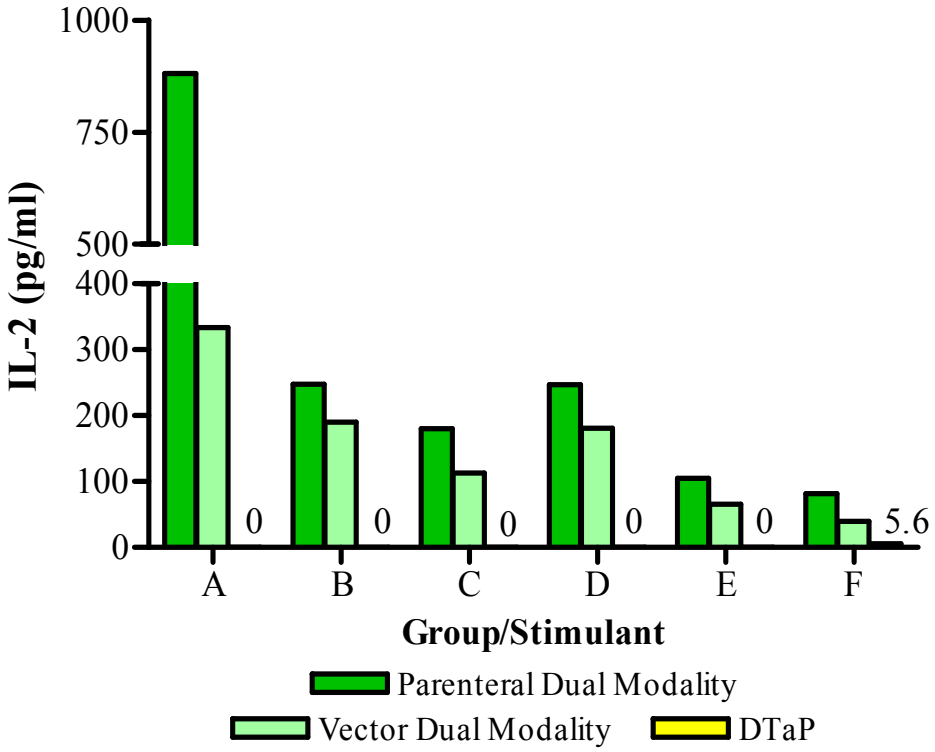

Figure 8.8: IL-2 production from *in-vitro* re-stimulated splenocytes following immunisation with a parenteral dual modality vaccine. IL-2 secreted from 5×10^6 splenocytes cultured for 24 hours in the presence of: A - 5µg/ml rFHAB1, B - 5µg/ml rPRN, C - 5µg/ml rPTS1.13L.129G, D - 5µg/ml rCYAAL58, E - *S. typhimurium* SL3261 lysate, F – *B. pertussis* Tohama I lysate.

With respect to the production of IL-4, the splenocytes of mice immunised with the parenteral dual modality vaccines or the vector dual modality produced low levels of the Th2 cytokine in response to stimulation with recombinant *B. pertussis* antigens (Table 8.12). Splenocytes from mice that received the parenteral dual modality vaccine produced very low IL-4 when stimulated with rFHAB1 and rPRN and no IL-4 upon stimulation with rPTS1.13L.129G or rCYAA. Splenocytes of DTaP-immunised produced no IL-4 when stimulated with the recombinant *B. pertussis* antigens.

Table 8.12: IL-4 production from *in-vitro* re-stimulated splenocytes following immunisation with the parenteral dual modality vaccine.

Stimulant	µg/ml	IL-4 (pg/ml)		
		Parenteral Dual Modality Vaccine	Vector Dual Modality	DTaP
rFHAB1	5	53.6	21.1	0
rPRN2	5	11.6	6	0.8
rPTS1.13L.129G	5	0	0	0
rCYAAL58	5	0	0	0
FFST	5	3	2.7	0.7
ConA	2	1000	824.3	837

IFN-γ secreted from $5x10^6$ splenocytes cultured for 72 hours in the presence of selected stimulants.

8.3.3.2 Serum Antibody Response to the Parenteral Dual Modality Vaccine

The parenteral dual modality vaccine generated a significantly higher titer of serum IgG to rFHAB1 than DTaP ($P < 0.05$) (Figure 8.9). Mice immunised with the parenteral dual modality vaccine also elicited a higher of anti-rFHAB1 IgG response than the vector dual modality regimen but the difference was not significant (Figure 8.9). When antibodies to native (commercially sourced) FHA were measured, the vector dual modality vaccine elicited a significantly higher response than the parenteral dual modality vaccine ($P < 0.05$) and DTaP ($P < 0.005$) (Figure 8.9). Considering that vector-immunised mice were found to induce high titers of antibodies that were cross-reactive with FHA in the first mouse trial, it is most likely that the elevated anti-FHA titers seen in mice immunized with the vector dual modality vaccine were also the result of cross-reactivity between FHA and neomycin phosphotranferase (encoded by the DNA vaccine vector).

Mice immunised with the Infanrix™ DTaP generated a significantly higher anti-rPRN IgG response than mice immunised with the parenteral dual modality vaccine ($P < 0.05$) or the vector dual modality regimen ($P < 0.05$) (Figure 8.10). The anti-rPRN IgG response was higher in the serum of mice immunised with the parenteral dual modality vaccine group compared to the vector dual modality group although it was not statistically significant (Figure 8.10).

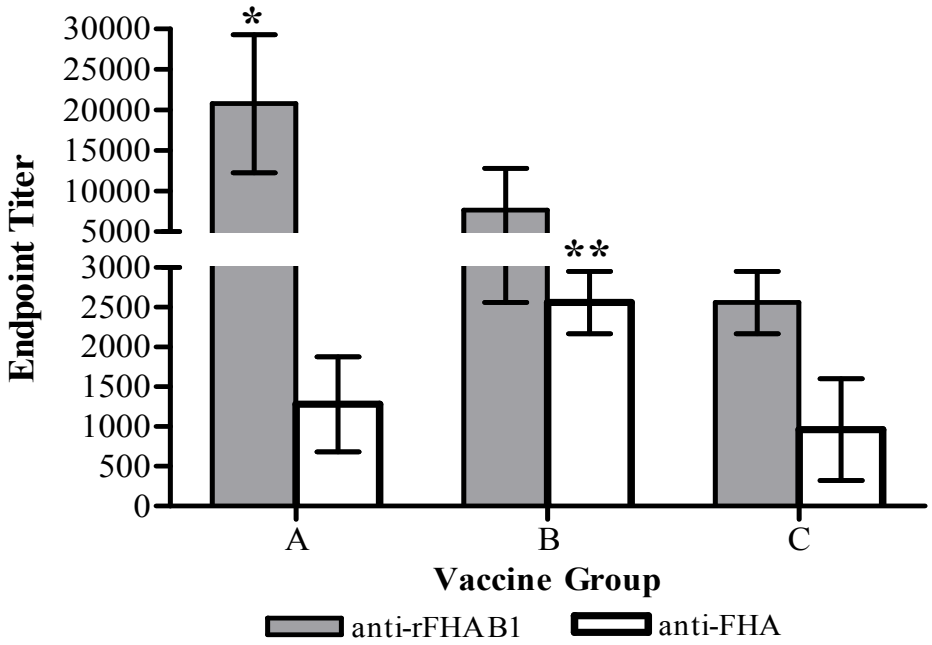

Figure 8.9: Endpoint titers of anti-rFHAB1 and anti-FHA IgG in sera of mice immunised with the parenteral dual modality vaccine. Columns represent mean scores from five mice +/- SE immunised with: A – parenteral dual modality vaccine, B - vector dual modality vaccine and C – DTaP. * Significantly different from DTaP ($P < 0.05$). ** Significantly different from parenteral dual modality vaccine ($P < 0.05$) and DTaP ($P < 0.005$).

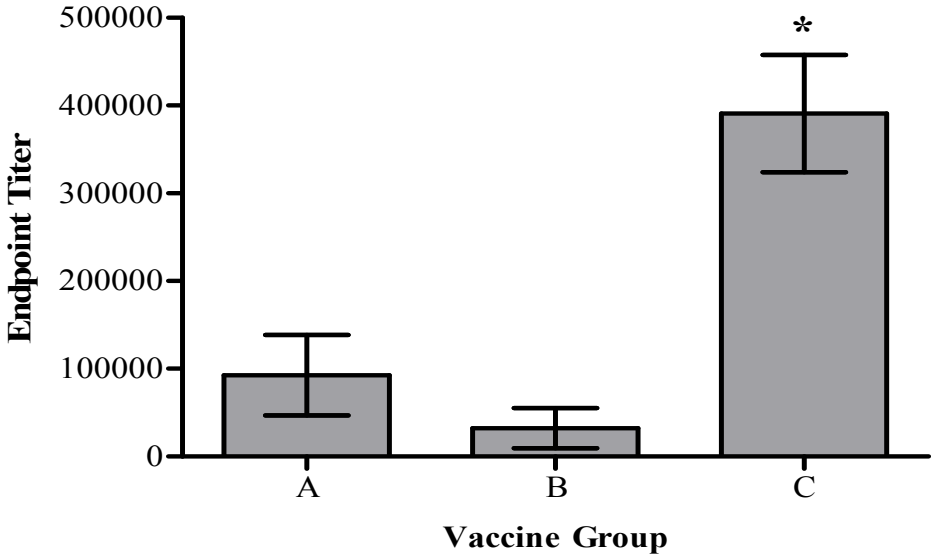

Figure 8.10: Endpoint titers of anti-rPRN IgG in sera of mice immunised with the parenteral dual modality vaccine. Columns represent mean scores from five mice +/- SE immunised with: A – parenteral dual modality vaccine, B - vector dual modality and C – DTaP. * Significantly different from parenteral dual modality vaccine ($P < 0.05$) and vector dual modality vaccine ($P < 0.05$).

In terms of the serum IgG response to native PT, the parenteral dual modality vaccine and vector dual modality regimen produced a significantly higher response than DTaP, with a roughly 4-fold increase in the endpoint titers (Figure 8.11). In contrast, there was no observable difference in the levels IgG against rPTS1.13L.129G across the three groups (Figure 8.11). Overall, the titers of serum IgG specific for the rPTS1.13L.129G recombinant protein were more than 7-fold lower than the response to the native PT holotoxin (commercially sourced) in each vaccine group. No IgG specific for the either the rCYAAL58 fusion protein or native adenylate cyclase toxin (AC-Hly) was detected.

Analysis of the serum IgG response showed a clear bias towards the generation of IgG1 rather than IgG2 (Table 8.13). With the exception of rCYAAL58 and native AC-Hly, very high titers of IgG1 were detected against all *B. pertussis* antigens, compared to a very low IgG2a response, in the sera of mice immunised with the parenteral dual modality vaccine, vector dual modality and DTaP (Table 8.13).

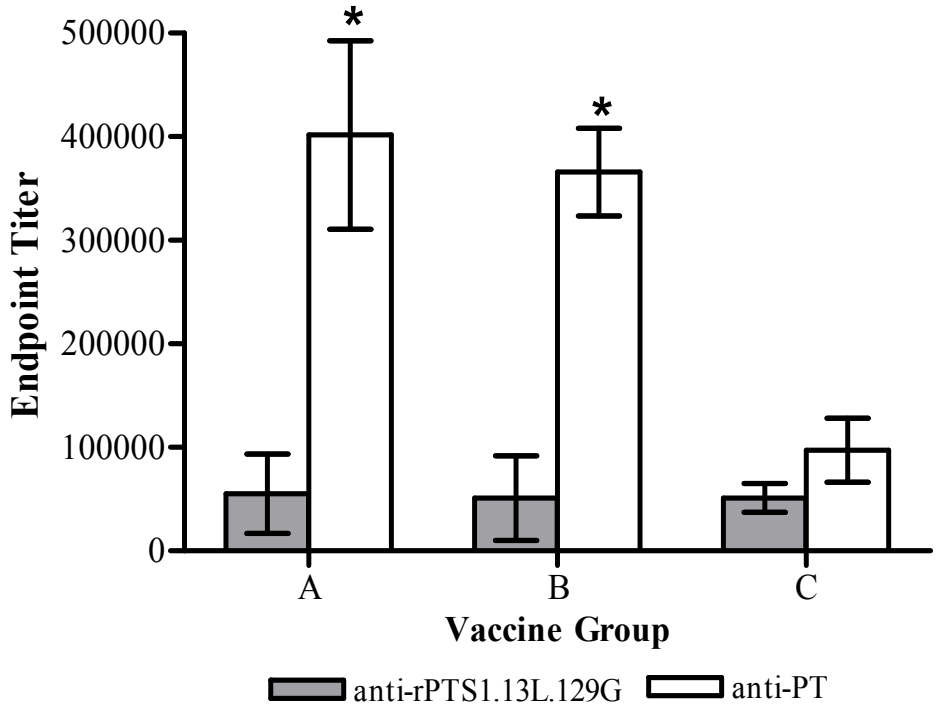

Figure 8.11: Endpoint titers of anti-pertussis toxin IgG in the sera of mice immunised with the parenteral dual modality vaccine. Columns represent mean scores from five mice +/- SE immunised with: A - parenteral dual modality vaccine, B - vector dual modality and C – DTaP. * Significantly different from DTaP from parenteral dual modality vaccine ($P < 0.05$) and vector dual modality ($P < 0.05$).

Table 8.13: Serum IgG1 and IgG2a titers in mice vaccinated with dual modality vaccines. Figures represent antibody titers in the pooled serum sample from five mice.

Antigen	Parenteral Dual Modality Vaccine		Vector Dual Modality		DTaP	
	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a
rFHAB1	51200	400	51200	200	12800	0
rPRN	228571	6400	204800	800	516608	3200
rPTS1.13L.129G	207352	100	51200	100	203481	100
rCYAAL58	0	0	0	0	0	0
FHA	12800	100	12800	400	3200	400
PT	831524	400	520556	800	502903	800
AC-Hly	400	0	0	0	0	0

8.3.3.3 Mucosal Antibody Response to the Parenteral Dual Modality Vaccine

No antigen-specific IgA was present in the BAL fluid of mice immunised with the parenteral dual modality vaccine, vector dual modality regimen or DTaP (Appendix D.6). Nevertheless, low titers of IgG specific for FHA, rPRN and PT were detected in these samples.

The endpoint titers of anti-rFHAB1 mucosal IgG was higher in mice that received the parenteral and vector dual modality vaccine compared to DTaP, whereas the DTaP generated higher titers of mucosal IgG specific for native FHA (Figure 8.12). DTaP-immunised mice generated significantly higher ($P < 0.05$) levels of rPRN-specific IgG than the parenteral dual modality vaccine (Figure 8.13). The mucosal IgG response to PT was significantly higher ($P<0.05$) in mice given the vector dual modality vaccine than that of the parenteral dual modality vaccine or DTaP groups (Table 8.14), for which the only plausible explanation was a combination of cross-reactivity and mouse to mouse variation. No IgG specific for either rCYAAL58 or native AC-Hly was detected in the BAL fluid of mice immunised with the dual modality vaccines or DTaP.

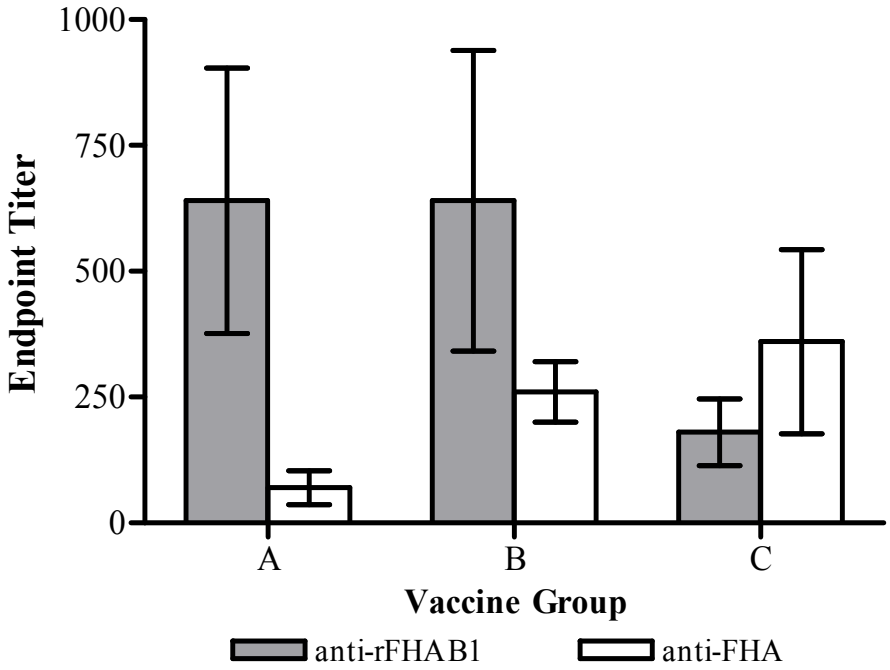

Figure 8.12: Anti-filamentous hemagglutinin IgG in the BAL fluid of mice immunised with the parenteral dual modality vaccine. Columns represent mean scores from five mice +/- SE immunised with: A - parenteral dual modality vaccine, B - vector dual modality and C – DTaP.

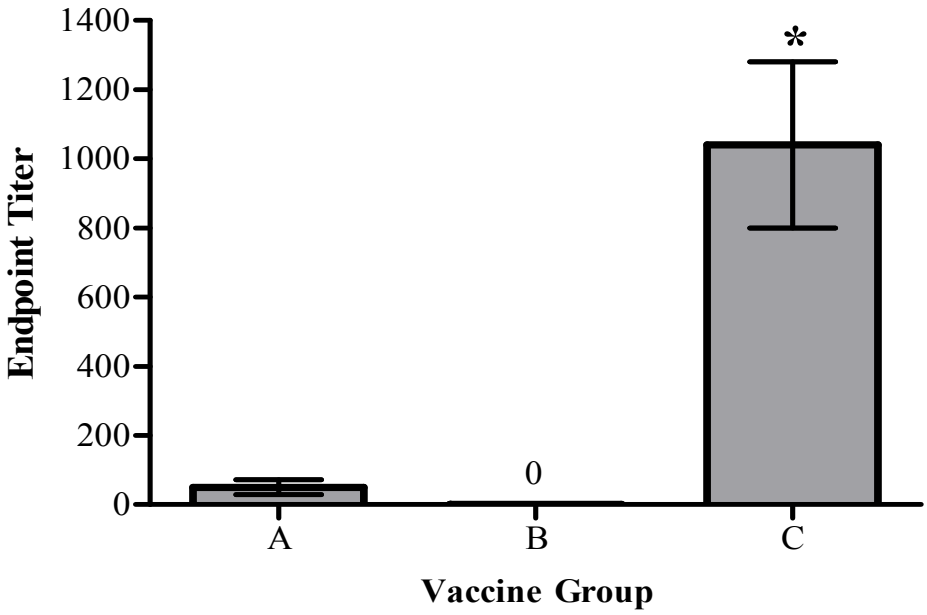

Figure 8.13: Anti-rPRN IgG in the BAL fluid of mice vaccinated with the parenteral dual modality vaccine. Columns represent mean scores from five mice +/- SE immunised with: A - parenteral dual modality vaccine, B – vector dual modality vaccine and C - DTaP. * Significantly different from parenteral dual modality vaccine ($P < 0.05$).

Table 8.14: Anti-pertussis toxin IgG in the BAL fluid of mice vaccinated with the parenteral dual modality vaccine.

Vaccine Group	Anti-rPTS1.13L.129G		Anti-PT	
	Titer	SE	Titer	SE
Parenteral Dual Modality Vaccine	50	22	720	80
Vector Dual Modality Vaccine	0	0	2400*	506
DTaP	260	140	1280**	480

* Significantly different from parenteral dual modality vaccine ($P < 0.05$) and DTaP ($P < 0.05$). ** Significantly different from parenteral dual modality vaccine ($P < 0.05$).

8.3.3.4 Clearance of SLID following Immunisation with the Parenteral Dual Modality Vaccine

To assess the protective efficacy of the parenteral dual modality vaccine, immunised mice were challenged with a pre-determined SLID of Tohama I two weeks after the second booster. The bacterial load in the lungs of the test and control groups of mice was then determined at three time points post-challenge (day 4, 7 and 14) to compare the rate of clearance. Mice immunised with the parenteral dual modality vaccine and DTaP completely cleared the experimental infection by day 14 post-challenge, and had a similar rate of clearance at each of the selected time points (Figure 8.14). Clearance curves indicated that both the parenteral dual modality vaccine and DTaP were more efficacious than the vector dual modality vaccine (Figure 8.14). When the clearance data from each group of mice was expressed as a percentage of the unimmunised mice, the bacterial loads in mice immunised with parenteral dual modality vaccine were effectively reduced to 0.63% of the untreated controls by day 4 and 0.42% by day 7 (Table 8.15).

The clearance data showed that mice immunised with the vector dual modality vaccine did not clear *B. pertussis* as effectively with a reduction to 1.23% of the untreated controls at day 4, 0.67% at day 7 and 2% at day 14 (Table 8.15). A comparison of clearance index (CI) values showed that the parenteral dual modality vaccine, vector dual modality vaccine and DTaP had a significantly enhanced rate of clearance compared to the placebo group (Table 8.15). There was found to be no significant difference in the rate of clearance between the two dual modality vaccines and DTaP (Table 8.15).

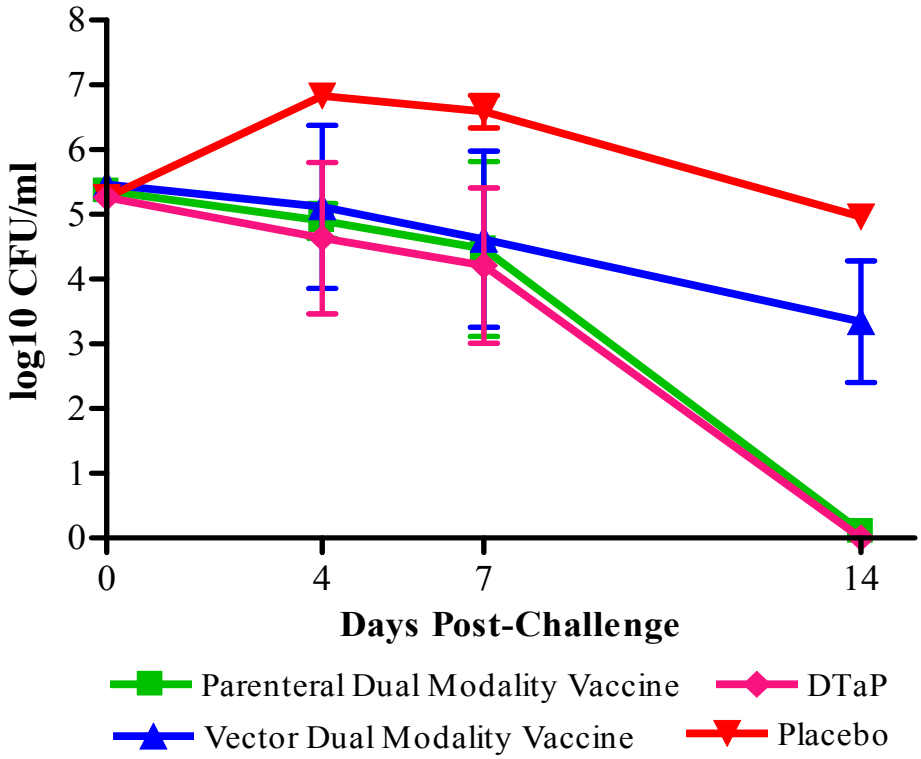

Figure 8.14: Clearance of *B. pertussis* challenge from the lungs of mice immunised with the parenteral dual modality vaccine. Data points represent mean count of CFU/lung from 4 mice +/- SE.

Table 8.15: Post-challenge clearance data from mice immunised with the parenteral dual modality vaccine.

Vaccine Group	% of Untreated Control[a] Day			CI[b]
	4	7	14	
Placebo	na	na	na	141.55 ± 1.92
Vector Dual Modality	1.23	0.67	2	44.68 ± 8.16 *
Parental Dual Modality Vaccine	0.63	0.42	0	26.93 ± 11.49 **
DTaP	0.64	0.08	0	22.15 ± 10.14 ***

[a] Percentage of bacteria cleared from the lungs relative to the untreated group of mice.

[b] Clearance Index ± SE = mean log10 number of *B. pertussis*/ml × days (van den Berg et al. 2001).

* Significantly different from placebo ($P < 0.005$).

** Significantly different from placebo ($P < 0.005$).

*** Significantly different from placebo ($P < 0.005$).

8.3.4 Immunogenicity and Protective Efficacy of the Oral Dual Modality Vaccine in Mice

8.3.4.1 Cytokine Production in Response to the Oral Dual Modality Vaccine

The levels of IFN-γ, IL-2 and IL-4 produced by the splenocytes of mice immunised with the oral dual modality vaccine were determined by ELISA as described in Section 2.16.1. The IFN-γ assays showed that the oral dual modality vaccine induced a strong Th1 response with high levels of IFN-γ detected in the culture supernatants of splenocytes that were stimulated with rFHAB1, rPRN, rPTS1.13L.129G or rCYAA (Figure 8.15). The IFN-γ response to stimulation with rFHAB1 was more than 4-fold greater than that of mice immunised with the oral combination DNA vaccine and 20-fold larger than the oral vector and DTaP groups. Similarly, the IFN-γ response to rPRN, rPTS1.13L.129G and rCYAAL58 was generally 5 to 6-fold larger than the oral combination DNA vaccine and 20 to 40-fold larger than in mice given the oral vector or DTaP.

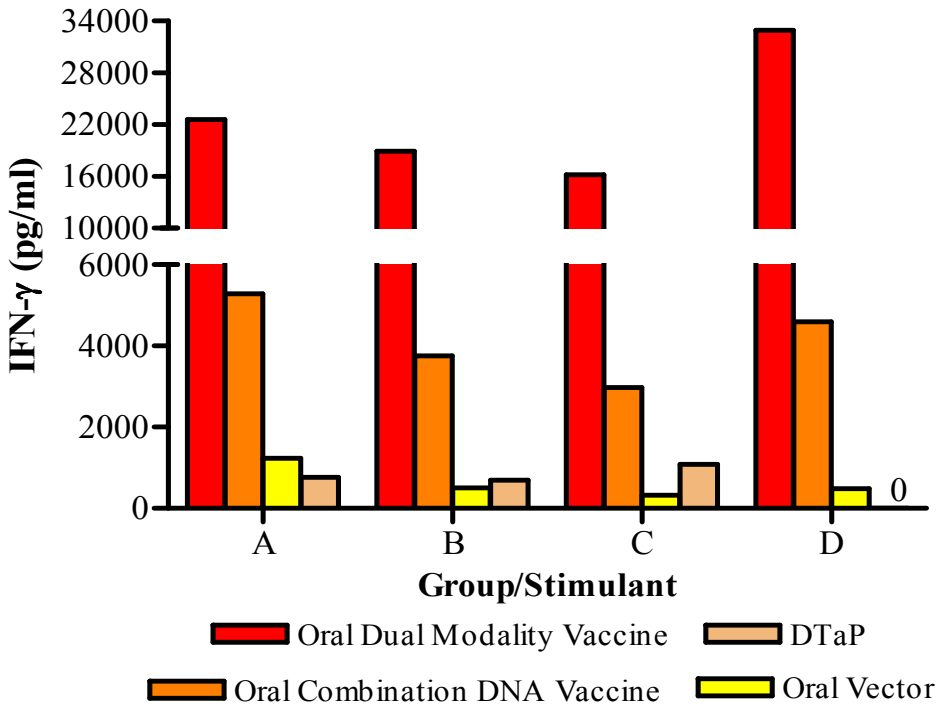

Figure 8.15: IFN-γ production from *in-vitro* re-stimulated splenocytes following immunisation with the oral dual modality vaccine. IFN-γ secreted from 5×10^6 splenocytes cultured for 72 hours in the presence of: A - 5μg/ml rFHAB1, B - 5μg/ml rPRN, C - 5μg/ml rPTS1.13L.129G, D - 5μg/ml rCYAAL58.

Analysis of IL-2 production from pooled splenocytes showed that the mice immunised with the oral dual modality vaccine produced far greater levels of the cytokine than DTaP-primed splenocytes upon stimulation with rFHAB1, rPRN, rPTS1.13L.129G and rCYAAL58 (Table 8.16). In fact, no IL-2 was detected in the supernatants of DTaP-primed splenocytes, except when stimulated with the acellular vaccine formulation (Appendix D.6). When compared to the oral combination DNA vaccine and oral vector, the oral dual modality vaccine induced a larger IL-2 response to stimulation with the aforementioned recombinant *B. pertussis* antigens. Stimulation with rFHAB1 resulted in a more than 60-fold increase in IL-2 levels compared to the oral combination DNA vaccine. The enhanced IL-2 response in mice immunised with oral dual modality vaccine was also observed following stimulation with rPRN, rPTS1.13L.129 and rCYAAL58. The levels of IL-2 induced following stimulation with a formalin-fixed *S. typhimurium* lysate were equivalent in all three oral vaccine groups (Table 8.16).

In addition to high levels of IFN-γ and IL-2, the oral dual modality vaccine appeared to induce a larger IL-4 response than the oral combination DNA vaccine, oral vector and even DTaP, which has been reported to generate a strong Th2 cytokine response in mice (Ryan et al. 1997) (Table 8.17). However, the levels of IL-4 produced in response to the *B. pertussis* antigen were typically 10 to 20-fold lower in comparison to stimulation with the natural T cell mitogen ConA (Table 8.17). Splenocytes from mice that received the oral combination DNA vaccine, oral vector and DTaP produced either low or undetectable levels of IL-4 in response to the recombinant *B. pertussis* antigens.

Table 8.16: IL-2 production from *in-vitro* re-stimulated splenocytes following immunisation with the oral dual modality vaccine.

Stimulant	µg/ml	IL-2 (pg/ml)			
		Oral Dual Modality Vaccine	Oral Combination DNA Vaccine	Oral Vector	DTaP
rFHAB1	5	767.7	11.4	0	0
rPRN2	5	440.1	0	0	0
rPTS1.13L.129G	5	172.1	54.8	0	0
rCYAAL58	5	237.4	172.3	0	0
FFST	5	228.8	138.8	185.6	0
ConA	2	4000	3760	881	973

Table 8.17: IL-4 production from *in-vitro* re-stimulated splenocytes following immunisation with the oral dual modality vaccine.

Stimulant	µg/ml	IL-4 (pg/ml)			
		Oral Dual Modality Vaccine	Oral Combination DNA Vaccine	Oral Vector	DTaP
rFHAB1	5	62.5	0	0	0
rPRN2	5	28.8	0	0	0.8
rPTS1.13L.129G	5	15.8	0	0	0
rCYAAL58	5	19.2	0	0	0
DTaP	10	19	0	0	35
DTaP	5	43.7	0	0	73.9
FFST	5	34	6.3	11	0.7
ConA	2	1000	540.8	932.5	837

8.3.4.2 Serum Antibody Response to the Oral Dual Modality Vaccine

Mice immunised with the oral dual modality vaccine generated significantly higher titers of serum IgG to rFHAB1 than the oral combination DNA vaccine ($P < 0.001$), oral vector ($P < 0.001$) and DTaP ($P < 0.01$) (Table 8.18). Interestingly, despite generating a high anti-rFHAB1 titer, no IgG specific for native FHA could be detected in the serum of mice immunised with the oral dual modality vaccine (Table 8.18).

Mice immunised with the oral dual modality vaccine and DTaP generated a significantly higher anti-rPRN IgG response than mice immunised with the oral combination DNA vaccine ($P < 0.05$) and the oral vector ($P < 0.005$) (Figure 8.16).

With respect to the serum IgG response to pertussis toxin, the oral dual modality vaccine generated significantly higher titers of anti-PT IgG than the oral combination DNA vaccine ($P < 0.05$), oral vector ($P < 0.005$) and DTaP ($P < 0.05$) (Figure 8.17). The oral dual modality vaccine also generated significantly higher titers of IgG to rPTS1.13L.129G compared to the oral combination DNA vaccine ($P < 0.005$), oral vector ($P < 0.005$) and DTaP ($P < 0.05$) (Figure 8.17).

No IgG specific for the rCYAAL58 fusion protein or native adenylate cyclase toxin was detected in the oral dual modality vaccine group (Table 8.19).

As expected, a significantly higher ($P < 0.05$) titer of anti-*S. typhimurium* IgG was detected in the serum of mice immunised with the oral dual modality vaccine compared to DTaP-immunised mice (Table 8.19). Overall, the oral dual modality vaccine generated a lower *S. typhimurium* IgG response than the oral combination DNa vaccine and oral vector but the differences were not statistically significant (Table 8.19).

Table 8.18: Endpoint titers of anti-rFHAB1 and anti-FHA IgG in sera of mice immunised with the oral dual modality vaccine. * Significantly different from DTaP ($P < 0.01$), oral combination DNA vaccine ($P < 0.001$) and oral vector ($P < 0.001$).

Group	Anti-rFHAB1		Anti-FHA	
	Titer	SE	Titer	SE
Oral Dual Modality Vaccine	17920*	3135	0	0
Oral Combination DNA Vaccine	0	0	0	0
Oral Vector	0	0	0	0
DTaP	2560	392	960	640

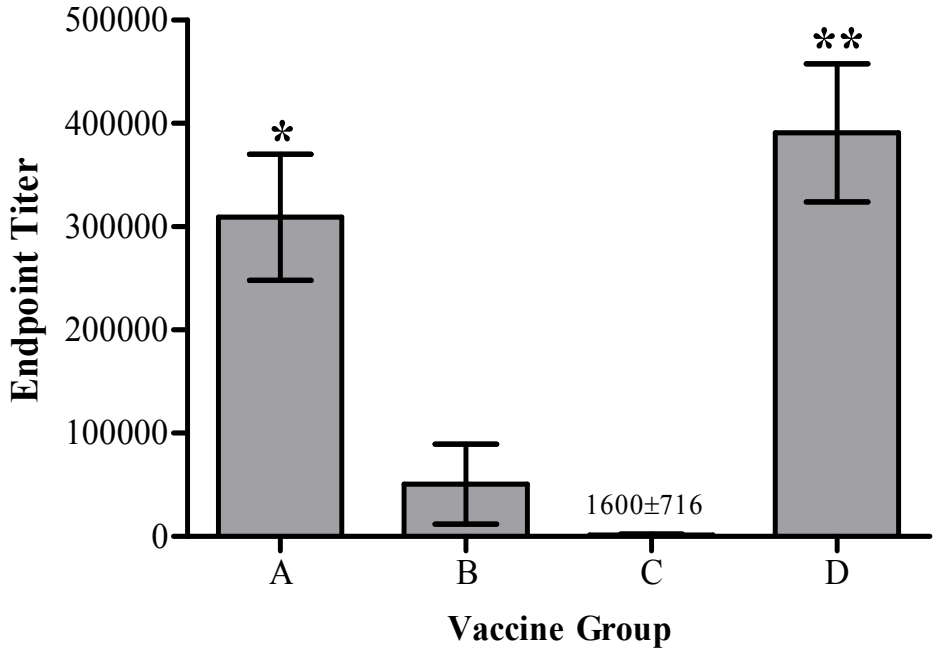

Figure 8.16: Endpoint titers of anti-rPRN IgG in sera of mice immunised with the oral dual modality vaccine. Columns represent mean scores from five mice +/- SE immunised with: A - oral dual modality vaccine, B – oral combination DNA vaccine, C – oral Vector and D - DTaP. * Significantly different from oral combination DNA vaccine ($P < 0.05$) and oral vector ($P < 0.005$), ** Significantly different from oral combination DNA vaccine ($P < 0.05$) and oral vector ($P < 0.005$).

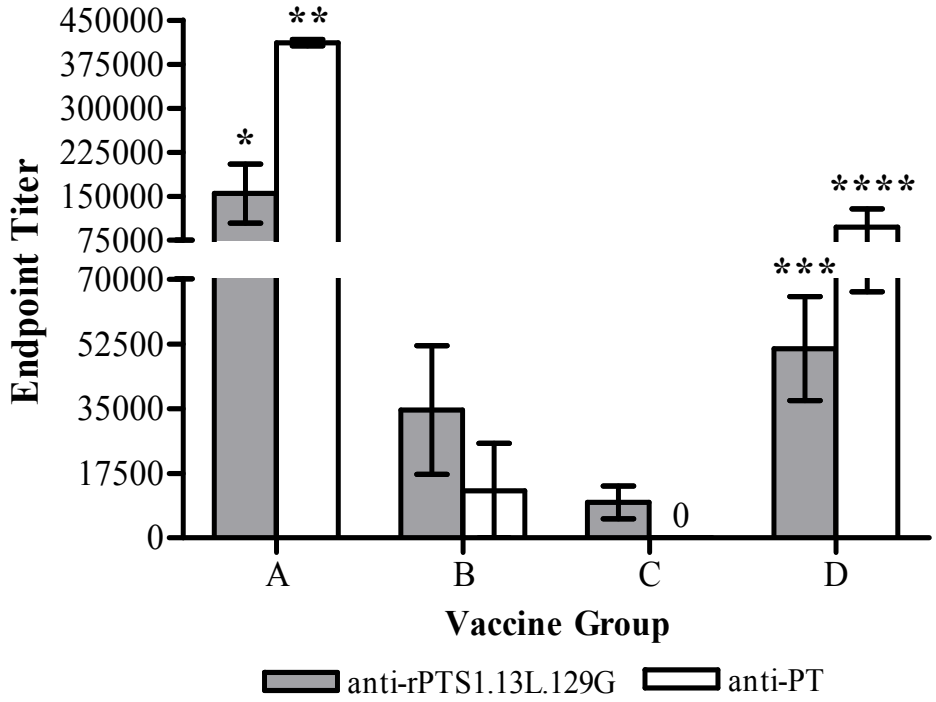

Figure 8.17: Endpoint titers of anti-pertussis toxin IgG in the sera of mice immunised with the oral dual modality vaccine. Columns represent mean scores from five mice +/- SE immunised with: A - oral dual modality vaccine, B – oral combination DNA vaccine, C – oral vector and D – DTaP. * Significantly different from oral combination DNA vaccine ($P < 0.05$), oral vector ($P < 0.005$) and DTaP ($P < 0.05$). ** Significantly different from oral combination DNA vaccine ($P < 0.005$), oral vector ($P < 0.005$) and DTaP ($P < 0.05$). *** Significantly different from oral vector ($P < 0.05$). **** Significantly different from oral combination DNA vaccine ($P < 0.05$) and oral vector ($P < 0.005$).

Table 8.19: Endpoint titers of anti-adenylate cyclase toxin and anti-*S. typhimurium* SL3261 IgG in sera of mice immunised with the oral dual modality vaccine. * Significantly different from DTaP ($P < 0.05$).

Group	anti-rCYAAL58		anti-AC-Hly		anti-SL3261	
	Titer	SE	Titer	SE	Titer	SE
Oral Dual Modality Vaccine	0	0	0	0	63047*	32810
Oral Combination DNA Vaccine	0	0	1127	373	211912*	68234
Oral Vector	405	230	599	367	88948*	33987
DTaP	0	0	0	0	0	0

Analysis of the serum IgG response to the oral dual modality vaccine showed a strong bias towards the generation of IgG1, with very high titers of IgG1 raised against all *B. pertussis* antigens, except rCYAAL58 and AC-Hly (Table 8.20). Titers of IgG1 were generally much higher in mice given the oral dual modality vaccine than in mice immunised with the oral combination DNA vaccine and oral vector. The majority of IgG generated against *S. typhimurium* by the oral vaccines was found to be IgG1 (Table 8.20). There also appeared to be some cross-reactivity, with IgG1-specific for *S. typhimurium* detected in the serum of mice immunised with DTaP (Table 8.20).

Table 8.20: Serum IgG1 and IgG2a titers in mice vaccinated with the oral dual modality vaccine. Figures represent pooled serum from five mice. FFST – formalin-fixed *S. typhimurium* lysate.

Antigen	Oral Dual Modality Vaccine		Oral Combination DNA Vaccine		Oral Vector		DTaP	
	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a
rFHAB1	102400	800	3200	800	0	100	12800	0
rPRN	505468	3200	1600	1600	1600	400	516608	3200
rPTS1.13L.129G	220063	800	102400	6400	25600	3200	203481	100
rCYAAL58	0	100	0	200	0	100	0	0
FHA	12800	800	0	50	0	0	3200	400
PTX	970597	800	0	0	0	0	502903	800
AC-Hly	400	0	0	50	0	100	0	0
FFST	102400	6400	460256	41168	204800	12800	6400	0

8.3.4.3 Mucosal Antibody Response to the Oral Dual Modality Vaccine

No antigen-specific IgA was detected in the BAL fluid of mice immunised with the oral dual modality vaccine (Appendix D.6). Nevertheless, low titers of IgG specific for rFHAB1, FHA, rPRN, PT, rCYAAL58, AC-Hly and *S. typhimurium* were detected in the lung washes of mice immunised with the oral dual modality vaccine (Tables 8.21 and 8.22). A low level of IgG specific for rFHAB1, FHA, rPRN and PT was also detected in the lung washes of DTaP-immunised mice (Table 8.21).

Table 8.21: Antigen-specific IgG in BAL fluid of mice immunised with the oral dual modality vaccine.

Vaccine Group	Anti-rFHAB1		Anti-FHA		Anti-rPRN		Anti-PT	
	Titer	SE	Titer	SE	Titer	SE	Titer	SE
Oral Dual Modality Vaccine	760	240	190	87	480	150	2400	1103
Oral Combination DNA Vaccine	0	0	0	0	0	0	0	0
Oral Vector	0	0	0	0	0	0	0	0
DTaP	180	66	360	183	1040*	240	1280	480

Table 8.22: Anti-rCYAAL58 and anti-*S. typhimurium* SL3261 IgG in the BAL fluid of mice immunised with the oral dual modality vaccine.

Vaccine Group	Anti-rCYAAL58		Anti-SL3261	
	Titer	SE	Titer	SE
Oral Dual Modality Vaccine	80	34	420	169
Oral Combination DNA Vaccine	10	10	340	125
Oral Vector	0	0	340	189
DTaP	0	0	0	0

8.3.4.4 Clearance of SLID following Immunisation with the Oral Dual Modality Vaccine

Mice immunised with the oral dual modality vaccine completely cleared the experimental *B. pertussis* challenge at a rate that was equivalent to DTaP-immunised mice. Clearance curves indicated that the oral dual modality vaccine and DTaP were more effective at eliminating *B. pertussis* from the lungs than the oral combination DNA vaccine and the oral vector (Figure 8.18). Interestingly, the number of days required to clear B. pertussis post-challenge was 14 days for both oral dual modality vaccine and the DTaP.

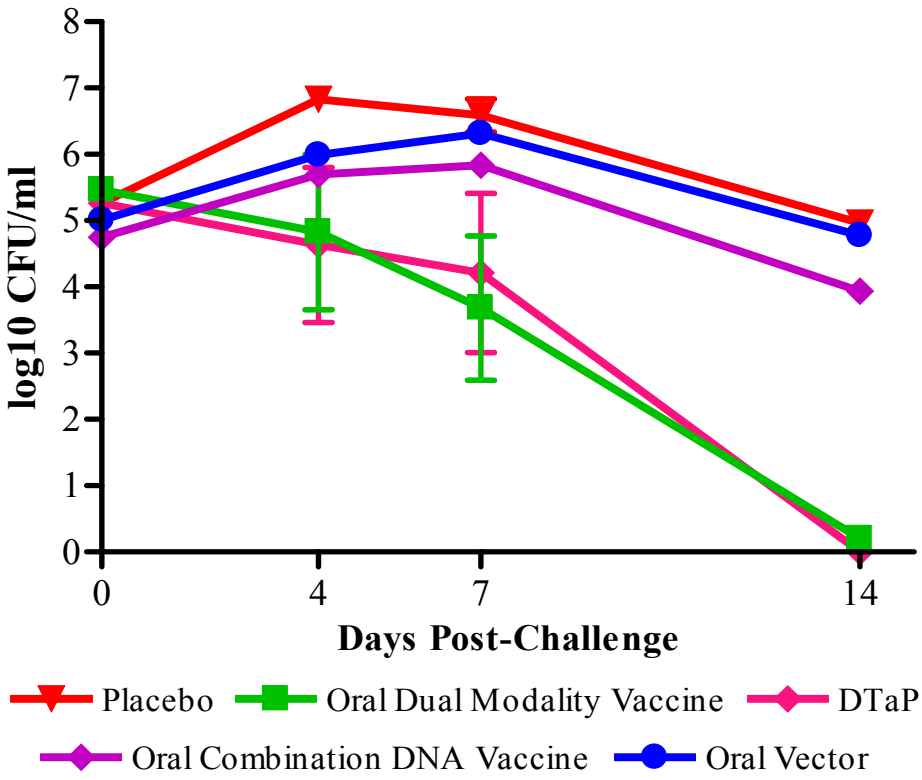

Figure 8.18: Clearance of *B. pertussis* challenge from the lungs of mice vaccinated with the oral dual modality vaccine. Data points represent mean count of CFU/lung from 4 mice +/- SE.

The clearance data showed that mice immunised with the oral dual modality vaccine reduced the lung bacterial counts to 0.94% of the untreated controls by day 4 and 0.6% by day 7 (Table 8.23). In contrast, mice immunised with the oral combination DNA vaccine failed to effectively clear *B. pertussis* from the lungs with a reduction of only 71% of the untreated controls at day 7, and 37% at day 14 post-challenge (Table 8.11).

A comparison of clearance index (CI) values showed that the oral dual modality vaccine had a significantly improved rate of clearance compared to the oral combination DNA vaccine ($P < 0.005$), oral vector ($P < 0.005$) and placebo ($P < 0.005$) groups (Table 8.23). DTaP was significantly more efficacious at clearing the experimental infection than the oral combination DNA vaccine ($P < 0.005$), oral vector ($P < 0.005$) and placebo ($P < 0.005$), but when compared to oral dual modality vaccine, there was no significant difference (Table 8.23).

Table 8.23: Post-challenge clearance data from mice immunised with the oral dual modality vaccine.

Vaccine Group	% of Untreated Control[a] Day			CI[b]
	4	7	14	
Placebo	na	na	na	141.55 ± 1.92
Oral Dual Modality Vaccine	0.94	0.6	0	34.83 ± 9.88 *
Oral Combination DNA Vaccine	2.9	71.1	37.8	132.4 ± 1.43 **
Oral Vector	25.9	95.6	117.2	141 ± 0.75
DTaP	0.64	0.42	0	22.15 ± 10.14 ***

[a] Percentage of bacteria cleared from the lungs relative to the untreated group of mice.

[b] Clearance Index ± SE = mean log10 number of *B. pertussis*/ml × days (van den Berg et al. 2001).

* Significantly different from placebo ($P < 0.005$), oral combination DNA vaccine ($P < 0.005$) and oral vector ($P < 0.005$).

** Significantly different from placebo ($P < 0.005$) and oral vector ($P < 0.005$).

*** Significantly different from placebo ($P < 0.005$), oral combination DNA vaccine ($P < 0.005$) and oral vector ($P < 0.005$).

8.4 Discussion

The majority of research into the prevention of pertussis has determined that multi-component formulations typically provide a more efficacious response than single antigen vaccines (Olin 1997; Plotkin & Cadoz 1997; Halperin 1999; Decker & Edwards 2000; Mills 2001). Whilst the goal of demonstrating proof of principle was achieved with the single antigen DNA vaccines, it was clear that they provided less than optimal protection and a combination approach was needed to establish whether DNA vaccination was a genuine alternative to DTaP. Hence, the immunogenicity and protective efficacy of two five-gene combination DNA vaccines were evaluated in mice. The IM combination DNA vaccine consisted of a mixture of five single antigen DNA vaccines (pcDNA3.1D/*fhaB1*; pcDNA3.1D/*prn*; pcDNA3.1D/*pts1.13L.129G*; pcDNA3.1D/*cyaAL58*; and pcDNA3.1D/*cyaC*), delivered by direct injection into the quadriceps. A second combination DNA vaccine was designed for mucosal delivery via the oral route and consisted of a suspension of five recombinant *S. typhimurium* SL3261 each harbouring one of the aforementioned plasmids.

It was observed that there was a difference in total IgG and IgG1/IgG2a titers to rFHAB1 in the oral combination DNA vaccine group. Whilst no anti-rFHAB1 IgG titers were observed, surpisingly both IgG1 and IgG2a were detectable. A possible explanation is that although the ELISA protocol used to determine total IgG and IgG1/IgG2a titers was the same, the secondary antibodies used to detect the respective IgG, IgG1 or IgG2a in the serum of mice vaccinated with the oral combination DNA vaccine were different. The anti-mouse IgG antibodies were supplied by Pierce, whereas the anti-mouse IgG1-HRP and anti-mouse IgG2a-HRP antibodies were supplied by Zymed Laboratories. Therefore, a possible explanation for the detection of low IgG1 and IgG2a titers but no total IgG in these samples could be that the anti-IgG1 and anti-IgG2a antibodies from Zymed were of a much higher affinity than the anti-IgG antibody from Pierce. An increased affinity of the secondary antibody would result in an increased sensitivity and therefore the observed variation between the two assays.

However, overall the efficacy of the combination DNA vaccines was variable and appeared to be dependent on the route of delivery. The IM five-gene combination DNA vaccine generated a strong Th1 response against FHA, pertussis toxin S1 and AC-Hly with high titers of serum IgG to pertactin. This collective response to each of the four pertussis antigens resulted in an improved level of protection compared to the respective single-antigen DNA vaccines. Moreover, the rate of clearance of the aerosol-induced infection approached that of DTaP with complete elimination of *B. pertussis* from the lungs within 14 days. However, the improved level of protection observed with the IM combination DNA vaccine could not be reproduced with the oral combination DNA vaccine i.e. the experimental infection with *B. pertussis* Tohama I could not be cleared within 14 days of challenge and the bacterial counts were only marginally lower than the vector and placebo-immunised mice. This poor protective efficacy was surprising considering the oral combination DNA vaccine typically induced a larger systemic response than the IM combination DNA vaccine.

With the exception of pertactin, immunisation of mice with the IM combination DNA vaccine induced relatively low titers of serum IgG but a potent CMI response, as seen by high levels of IFN-γ production from *in vitro*-stimulated splenocytes. In line with the response to immunisation with pcDNA3.1D/*prn* alone, the pertactin component of the combination DNA vaccine stimulated much larger titers of serum IgG (mostly

IgG1) and lower Th1 cytokine levels, indicative of a Th2-driven response. As previously discussed, a likely explanation for the poor humoral response to FHA, PT-S1 and AC-Hly was the natural bias of IM DNA vaccination towards activation of Th1 cells and $CD8^+$ Tc cells (Cornell et al. 1999; Rodriguez et al. 2001; Scheiblhofer et al. 2003). It is widely recognised that an IgG anti-toxin response, whether primed by vaccination or induced by natural infection is essential to confer protective immunity against pertussis (Taranger et al. 2001). However, the results of this investigation have clearly demonstrated that a *B. pertussis* infection, albeit a sub-lethal challenge, can be effectively cleared in the absence of a primary anti-PT IgG response.

Vaccines delivered by parenteral routes typically induce a strong systemic response with little or no mucosal immunity (Thomas et al. 1989; Dougan, Huett & Clare 2002; Rigano & Walmsley 2005). It is also accepted that the generation of an effective secretory response requires the presentation of antigen (or DNA) to a mucosal surface (Dougan 1994; Holmgren & Czerkinsky 2005). As *B. pertussis* enters and colonises the respiratory tract and does not naturally disseminate beyond the lungs, it is likely that the generation of an IgA response would limit both colonisation and disease. The benefits of mucosal priming have been confirmed with detection of specific IgA in the tracheal secretions of animals and humans following natural infection and the recognition of its important role in defence (Hellwig et al. 2001). Oral immunisation has proven to be an effective means of stimulating antibody production at both local and distant mucosal surfaces (Challacombe et al. 1997; Fagan et al. 2001). More specifically, mucosal and systemic antibody responses have been generated by *aro*A *S. typhimurium* expressing three of the four pertussis antigens evaluated in this study, namely FHA, pertussis toxin and pertactin (Guzman et al. 1991; Walker et al. 1992, Anderson, Dougan & Roberts 1996). Some other attributes of attenuated bacteria that make them attractive candidates for vaccine development include the: ease of delivery (patient compliance), low cost of production, elimination of local side reactions, and/or the potential for genetic manipulation for future improvements to antigen presentation or DNA delivery.

The main goal of oral immunisation was to induce a secretory IgA response in the lungs via stimulation of the common mucosal pathway. Although the oral combination DNA vaccine generated a CMI response to all four pertussis antigens equivalent to that observed with the IM combination DNA vaccine group and high serum IgG titers to

recombinant S1 and native PT, no secretory IgA or IgG could be detected in the BAL fluid of immunised mice. However, surprisingly this did not translate to the level of protection conferred by a similar systemic response to the IM combination DNA vaccine or DTaP. Our failure to detect secretory IgA response in the lungs of orally-immunised mice confirms similar observations reported by other investigators (Forrest et al. 1991; Mukkur & Walker 1992; Shimoji et al. 2003).

There are numerous factors that may have contributed to the poor mucosal antibody response to the oral combination DNA vaccine. The strain of mouse used has been reported to affect the nature of an immune response by directing towards a specific compartment. For instance, Soo et al. (1998) found that mice carrying the wild-type Nramp1 allele (*Ity*^{r}) mounted a predominantly Th1 response to vaccination with *S. typhimurium* expressing the gp63 antigen of *Leishmania major*, whereas mice possessing the mutant Nramp1 allele (*Ity*^{s}) induced a Th2 response to the same vaccine delivered via the same route under identical conditions. Dunstan, Simmons & Strugnell (1998) reported that the nature of an immune response to a heterologous antigen could also be influenced by the strain of *S. typhimurium* used. Of particular relevance was the observation that an *aroA* strain of *S. typhimurium* (SL3261) induced up to a five times higher IFN-γ levels to a heterologous antigen than *purA*, *ompR*, *htrA* or *cya crp* mutants. Similarly, Valentine et al. (1998) evaluated the response to four distinct *S. typhimurium* mutants from a transposon-generated library and found that three of the four mutant strains induced higher serum IgG and secretory IgA titers than an *aro*A mutant. The CL288, CL401 and CL558 mutants, which have insertions in the genes encoding an osmoregulated transferase, a putative methionyl-tRNA formyltransferase and malate oxidoreductase respectively, were also shown to confer a greater level of protection than the *aro*A *S. typhimurium* strain (Valentine et al. 1998).

Given the favourable IgA induction in previous studies that have used *aro*A *S. typhimurium* as the vehicle for delivery of pertussis antigens in Balb/C mice (Guzman et al. 1991; Walker et al. 1992), a more likely explanation for the poor secretory IgA response to oral DNA immunisation was the tendency for this type of vaccine to induce a Th1 response. Heterologous antigens expressed by the *S. typhimurium* are processed with MHC class II receptors for presentation to Th2 effector cells, whereas endogenous expression through DNA vaccination typically leads to MHC class I presentation and

induction of a Th1-driven cellular response (Constant & Bottomly 1997). It has been reported that activated Th2 and dendritic cells in the Peyer's patches release IL-5 which promotes IgA class switching in plasma cells (Hobson et al. 2003). Alternatively, Th1 cells stimulated by DNA vaccination produce cytokines such as IL-2 and IFN-γ and as a consequence there are low levels of IL-4 and IL-5, which are necessary for serum and mucosal antibody production.

Although the common mucosal pathway has been proven to generate a secretory response at distant mucosal surfaces, it has been observed that the largest mucosal response occurs at the site of initial antigen presentation (Hobson et al. 2003; Zuercher 2003). Considering that the portal of entry for *B. pertussis* is the respiratory tract and a poor protective response to oral DNA vaccination with attenuated *S. typhimurium* was observed in this investigation, it may be worthwhile exploring intranasal delivery as the route of delivery for future mucosal DNA vaccines. Vecino et al. (2002) found that intranasal immunisation with attenuated *Shigella flexneri* was superior to oral immunisation with an attenuated (*aro*D) strain of *S. typhimiurium* for induction of a protective mucosal response to a HIV antigen. Attenuated *Shigella flexneri* has also been used as a carrier of antigen or DNA for intranasal immunisation against measles, HIV and enterotoxigenic *E. coli* (Fennelly et al. 1999; Shata et al. 2001; Barry et al. 2003). Molecular supports such as liposomes are yet another option for intranasal delivery and pose a much lower risk than live attenuated pathogens. Klavinskis et al. (1997) reported that expression of a reporter enzyme (firefly luciferase) was enhanced in nasal tissue by coating DNA with cationic lipids, compared to intranasal administration of naked DNA. Moreover, Guzman et al. (1993) immunised mice with FHA and PT coated liposomes and found serum and mucosal antibody responses were significantly higher than in mice immunized with free antigen. Conway et al. (2001) found that, when trapped in biodegradable particles, orally-delivered *B. pertussis* antigens protected mice from an aerosol challenge. It was also reported that antigens delivered via larger microparticles elicited a largely Th1 response, whereas antigens encased in smaller nanoparticles induced a Th2 antibody response (Conway et al. 2001).

A concern with the combination DNA vaccines was a potential for antigen-induced suppression, also known as antigenic competition, in which the simultaneous presentation of two or more antigens results in depression of the immune response

towards the non-dominant antigen(s) (Brody & Siskind 1969; Eidinger et al. 1971). It was clear from the data presented in this study that antigenic competition had not occurred following IM or oral administration of the combination DNA vaccines. Antibody titers to all antigens except FHA were equivalent or greater than the responses to each individual DNA vaccine as were the levels of IFN-γ and IL-2 produced by *in vitro*-cultured splenocytes. Han et al. (1999) found that a combination DNA vaccine of four Papillomavirus (PV) early antigens (E1, E2, E6 and E7) completely protected rabbits from a lethal challenge with Cottontail Rabbit Papillomavirus (CRPV) more effectively than either gene alone. When co-administered as an equimolar mixture of plasmids, there was no apparent antigenic competition (Han et al. 1999). There have also been no reports of antigenic competition with the multi-component Pa or Pw vaccines.

In the final phase of the DNA vaccine experiments, two different DNA vaccine prime/acellular vaccine boost regimens were tested in an attempt to stimulate both arms of the immune system. Priming with a vaccine DNA followed by boosting with a subunit or whole-cell vaccine, or vice-versa, has been effective against certain complex pathogens. For instance, *Helicobacter pylori* is a prevalent gastric pathogen of humans for which development of an efficacious vaccine has so far been elusive. Londono-Arcila et al. (2002) achieved a protective Th1/Th2 response against a mouse-adapted strain of *H. pylori* by mucosal priming with *Salmonella enterica* serovar Typhi expressing the urease antigen followed by parenteral boosting with urease plus alum, after immunisation with each modality alone could not confer protection. Other studies have demonstrated that boosting with protein or attenuated pathogen supports the Th1 response of a DNA vaccine and enhances the overall efficacy against *Mycobacterium tuberculosis*, simian HIV and Hepatitis C Virus (Tanghe et al. 2001; Doria-Rose et al. 2003; Pancholi et al. 2003). The idea of a dual modality approach to pertussis immunisation arose from evidence of complementary roles for both humoral and cellular responses in immunity to pertussis (Mills et al. 1998). A strong CMI response elicited following DNA vaccination and the potent antibody response to the acellular vaccine provided the means to explore this option.

It is accepted that the Th1 cytokine IFN-γ and the Th2 cytokine IL-4 are antagonistic towards each other and arises from activation of opposing sets of transcription factors and signalling cascades in T cells (Neurath, Finotto & Glimcher 2002; Holmgren & Czerkinsky 2005). Thus it was considered that protein boosting may diminish the strong CMI response elicited by DNA vaccination. The data collected on the parenteral and oral dual modality vaccines showed that the Th1 responses to DNA priming were not adversely affected by boosting with the in-house subunit vaccine (with alum). In fact, the Th1 response to DNA was found to be somewhat augmented as a single priming dose of DNA induced a cytokine profile that was roughly equivalent or greater than three doses of the combination DNA vaccine. Tanghe et al. (2001) compared the immunogenicity and protective efficacy of three different tuberculosis vaccine formulations: DNA, protein with alum and a DNA prime-protein boost. They found a two to four-fold increase in proliferation of IL-2 and IFN-γ-producing $CD4^+$ cells in the spleen after injection of 30-100μg of purified Ag85 protein into DNA vaccine primed mice compared to immunisation with DNA vaccine alone.

Londono-Arcila et al. (2002) evaluated attenuated *Salmonella enterica* serovar *typhi* as part of a mucosal priming-parenteral boosting regimen against *H. pylori*. As with our oral dual modality results, they found that intranasal (IN) priming with the recombinant strains initially skewed the systemic response towards Th1 and the response was only shifted to a mixed Th1/Th2 phenotype after parenteral boosting with purified antigen in alum. They also found that despite an early Th1-driven systemic response, an antigen-specific IgA response was detected in the BAL fluid. Expression of the urease antigen under the control of a prokaryotic rather than a eukaryotic promoter may explain why their vaccine stimulated a positive IgA response whereas our oral DNA prime-protein boost approach could not.

It was beyond the scope of this project to evaluate all the possible prime-boost regimens, so in line with early dual modality studies priming with the five-gene combination DNA vaccine was followed with two protein boosters. Eo et al. (2001) compared DNA and attenuated virus as a dual modality vaccine against herpes simplex virus (HSV) but focussed on mucosal rather than parenteral delivery of both the priming and booster doses. They found that optimal immunity to a systemic dual modality vaccine occurred when mice were primed with DNA and boosted with antigen. In

contrast, the mucosal response peaked after intranasal priming with purified protein followed up by intranasal boosting with DNA. Doria-Rose et al. (2003) tested a DNA prime-protein boost immunisation for simian HIV and also the reverse approach of protein prime-DNA boost. Whilst both regimens protected over 90% of macaques from disease, virus primed-DNA boosted animals had stronger cellular and humoral responses which correlated with significantly lower viral loads. Therefore, it would be interesting to analyse the immune response generated by intranasal administration of the in-house acellular pertussis vaccine followed by two mucosal doses of the combination DNA vaccine in a future experiment.

Mucosal priming with the oral combination DNA vaccine and parenteral boosting with the subunit vaccine (oral dual modality vaccine) generated a strong systemic response but no anti-pertussis IgA. This observation was not surprising considering that three oral doses of the combination DNA vaccine failed to stimulate an IgA response to the pertussis antigens (rFHA, rPRN, rPTS1.13L.129G and rCYAAL58). Eo et al. (2001) found that IN priming with a DNA vaccine followed by IN rather than IM boosting with antigen induced a local IgA response. It was also reported that significantly higher secretory IgA and serum IgG titers were produced with the opposite approach, intranasal priming with antigen followed by intranasal boosting with naked DNA (Eo et al. 2001). Direct IN immunisation with FHA and pertactin can stimulate a mucosal IgA and IgG response (Roberts et al. 1993), which suggests it would be worthwhile to investigate mucosal priming with the combination DNA vaccine followed by mucosal boosting with a subunit vaccine and vice-versa. Although no IgA was detected in the BAL fluid of mice that received the oral dual modality vaccine, a specific mucosal IgG response was elicited. Given there were nearly identical titers in mice given the parenteral DTaP and the IM dual modality vaccine it also pointed towards transudation from a high serum concentration rather than local production from IgG-producing cells in the respiratory mucosa. Whilst there would be an obvious benefit from a mucosal IgG response for clearance of an experimental challenge in mice considering the infection is localised in the lungs, where IgG-mediated opsonisation is the primary method of acquired defence, there are several factors that must be considered when interpreting this data. Unlike the lower respiratory tract infection seen in mice after aerosol or intranasal challenge, humans typically develop a URTI rather than pneumonia following exposure to *B. pertussis* (Sato et al. 1980). IgA and innate

mechanisms are the main defences against colonisation in the upper respiratory tract, whereas IgG in association with alveolar macrophages protect the deeper mucosal surfaces (Kaltreider & Chan 1976). Hence, the IgG in the lungs would be far less beneficial in humans, which rely heavily on the induction of IgA (Hellwig et al. 2001).

As with the single antigen DNA vaccines, DTaP was used as a positive control and performance indicator for the test vaccines. DTaP induced a systemic Th2 response with high IgG titers to PT and rPRN, but a lower than expected level of anti-FHA IgG. The levels of IgA and IgG in the lung secretions were determined for the acellular vaccine with some surprising results. No IgA was detected but there was pertussis-specific IgG in the BAL fluid of mice immunised with DTaP and the dual modality vaccines (IM and oral). There appear to be two possible explanations for this response. As the levels of antibody to specific antigens was associated with high titers of serum IgG, it is likely that all or a large proportion of the mucosal antibody was derived by transudation from circulation (Russell et al. 1996). Alternatively, it is possible that after subcutaneous injection of the vaccines, antigen could have entered or been transported to the draining cervical lymph nodes that are in close proximity to the site of injection. Upon priming, committed B cells may have homed to mucosal effector sites in addition to entering circulation (Brokstad et al. 1995; el-Madhun et al. 1998; Coffin et al. 1999).

One of the considerations when comparing the efficacy of the dual modality vaccines to DTaP, particularly with respect to the humoral immune response, was the differences in their composition. Although the same antigen concentrations were used in the DTaP and aP acellular vaccines, the detoxification method for PT were different, which could have resulted in differences in immune response. PT in Infanrix™ is inactivated by treatment with formalin and glutaraldehyde, whereas EDAC was used for inactivation of the PT included in the in-house aP version. The glaring difference between to two acellular vaccines was in relation to the humoral response. Mice immunised with the dual modality vaccines (aP booster) generated higher titers of serum IgG to FHA and PT but a lower response to pertactin than DTaP. Interestingly, these higher titers to FHA and PT combined with a strong Th1 response to all antigens did not translate to an improved level of protection for the dual modality vaccines over DTaP. Despite the varying immune responses, both the aP-boosted dual modality vaccines and DTaP conferred an equivalent degree of protection with clearance within 14 days.

Another interesting observation with the commercial DTaP was the disparity in its protective efficacy across the two DNA vaccine trials. An identical vaccine batch, dosage, route of delivery and method of challenge was employed yet there was variation with the immunogenicity and bacterial clearance data. Overall the cytokine levels were similar but there was a clear difference in serum IgG titers with a much lower anti-FHA response which was counteracted by a 2-3 fold increase in anti-pertactin and anti-pertussis toxin titers. A slower rate of clearance was also seen in second experiment with clearance between 7 and 14 days compared with 4-7 days in the first experiment. The most likely explanation for this inconsistency is individual to individual variation in the induction of immune response in mice.

When the humoral response generated by the IM five gene combination DNA vaccine were compared to the parenteral dual modality vaccine, the latter induced substantially higher titers to FHA, PT and PRN. This outcome was expected considering that dual modality vaccination involved boosting with an in-house acellular vaccine (aP), which is known to generate a strong Th2 response. However, it was somewhat surpising that both the parenteral dual modality vaccine and vector dual modality vaccine generated significantly higher titers of serum IgG to recombinant FHA and native PT than three doses of the Infanrix™ DTaP. As previously indicated the method of detoxification of PT that was different for DNA vaccination with S1 (genetic), in-house aP (EDAC) and DTaP (formalin/glutaraldehyde) may have contributed to the observed differences in antibody levels (Figure 8.11). It is possible that chemical inactivation of PT with formalin and glutaraldehyde reduces the immunogenicity to a greater extent than EDAC, which would account for the higher anti-PT titers in the modality vaccine groups. This difference in the mode of inactivation of PT may also explain the enhanced anti-FHAB1 response to the dual modality vaccines. PT has been found to exhibit immunomodulatory effects on co-administered antigens (Mills 2001). For instance, it has been demonstrated that PT can enhance IgG1 and Th2 responses to tetantus toxoid and ovalbumin (Samore & Siber 1996). Hence, the genetically inactivated S1 presented by the DNA vaccine and/or the EDAC-inactivated PT of the in-house aP booster may have retained greater immunomodulatory properties than the formalin/glutaraldehyde-inactivated PT of DTaP and enhanced the response to FHA.

The purpose of the dual modality vaccines was to generate both a Th1 and Th2 response and thereby improve on the clinically proven efficacy of DTaP. This goal was realised with the generation of a strong cell-mediated response, as indicated by the levels of IFN-γ and IL-2, as well as equivalent or higher serum IgG and mucosal IgG response compared to DTaP. WCV is a prime example of how effective a dichotomous Th1/Th2 response is against pertussis. Mills et al. (1998) have shown that mice given the WCV have a faster rate of clearance of *B. pertussis* than cohorts given DTaP. Interestingly, a mixed Th1/Th2 response induced by the parenteral and oral dual modality vaccines did not show this dominant trend over DTaP. Perhaps the benefit of the DNA-induced cellular response would be the longer-term protection against pertussis. The mechanism by which parenteral administration of the WCV vaccine can induce a Th1 response is not clear. By convention, parenterally delivered an antigen or in this case the WCV, resides in the extracellular milieu and is phagocytosed by local APC's for presentation to $CD4^+$ Th2 cells. As the target antigens are not produced within host cells the induction of Th1 or Tc cell response is limited. There have been instances in which $CD8^+$ Tc cells have been primed by MHC class I molecules that presented exogenous antigens in soluble or particulate forms but the mechanisms by which internalised antigens can gain access to the cytosol and ER are not understood (Wraith et al. 1987; Staerz et al. 1987; Harding & Song 1994; Falo et al. 1995; Pasetti et al. 1999).

In summary, the immunogenicity and protective efficacy of five different types of multi-component vaccines were evaluated in mice: (i) 0.2 SHD of a commercial DTaP, (ii) IM five-gene combination DNA vaccine, (iii) oral five-gene combination DNA vaccine, (iv) IM DNA prime-SC protein boost, and (v) oral DNA prime-SC protein boost. The response to DTaP and the combination DNA vaccines was polarised, with the generation of a purely Th2 humoral response in DTaP-immunised mice compared with a predominantly Th1 response in the DNA vaccinated animals, with the exception of pertactin. The protective efficacy of the IM combination DNA vaccine was encouraging with a significantly improved rate of clearance compared to the placebo and vector only controls and a rate of clearance that approached that of DTaP. However, DTaP still provided a significantly better degree of protection than the five-gene combination DNA vaccine, delivered via the IM route. Unfortunately, the oral combination DNA vaccine failed to induce an S-IgA response in the lungs and in terms of protection from an aerosol challenge offered little improvement compared to the

negative controls. In contrast to the single modality vaccines, the two DNA prime-protein boost regimens induced a dichotomous Th1/Th2 response or stimulation of both cellular and humoral effectors to each of the four *B. pertussis* antigens. This dual activation provided an excellent degree of protection that was equivalent to that provided by the DTaP. Whether the additional Th1 response elicited by the dual modality vaccines confers longer-term protection than the DTaP remains to be investigated.

Chapter 9 General Discussion

9.1 Summary

Whooping cough is a respiratory infection caused by the gram-negative bacterium *Bordetella pertussis*, a strictly human pathogen. Infection occurs in all age groups but due to the severe systemic complications and the paroxysmal cough is only a cause for concern in infants, young children and the elderly. Over time the epidemiology of pertussis has been influenced by several notable factors. Firstly, the advent of immunisation with a highly efficacious whole killed vaccine (WCV) caused a dramatic decrease in the incidence the advent of the whole-cell vaccine. After a sustained period of control over the pathogen, reports of rare but potentially fatal side reactions to the WCV emerged which dramatically reduced compliance rates in developed countries and resulted in a renaissance for pertussis. The most recent impact has been the introduction of the acellular vaccine, proven to be less reactogenic than the WCV whilst conferring an equivalent degree of protection. Of concern has been the rising endemic circulation of pertussis with regular epidemics even in industrialised countries despite a high coverage of the acellular vaccine. This persistence has been attributed to issues such as antigenic variation among key virulence determinants, sophisticated immune subversion by the pathogen, greater public and clinical awareness and increased surveillance (Mooi, van Loo & King 2001). A factor often overlooked among these epidemiological studies is that the efficacy current pertussis vaccines may be less than optimal.

There is evidence that both whole cell and acellular vaccines provide variable and sometimes less than optimal immunity, with estimates in double blind trials ranging from 36% to 85% efficacy (reviewed in Mills et al. 1998). It is interesting to note that the re-emergence of whooping cough has coincided with the steady replacement of the whole cell vaccine with the more marketable acellular vaccines, or in other terms substitution of a dichotomous Th1/Th2 response with a purely Th2 inducer (Sutter & Cochi 1992). Recent studies have revealed complementary roles for cellular and humoral responses in immunity to pertussis, and have found that CMI is necessary for rapid clearance and long-term protection (Mills et al. 1993; Redhead et al. 1993; Ausiello et al. 1998). Moreover, immunity to pertussis is not life-long and irrespective of natural exposure or vaccination begins to wane after 10 years (Oliver & Fernandez

2001). In adults, pertussis often presents as a cold-like illness with no identifiable symptoms and although unchecked infections pose no immediate health concerns, except in the elderly and severely immunocompromised, there are some serious implications. First and foremost it can lead to inadvertent carriage and a source of: (i) transmission to highly susceptible infants or children; and (ii) to a lesser extent to under-reporting, which has prevented an accurate measure of disease incidence and distribution. In spite of this awareness no extended vaccination or boosting schemes have been implemented, especially for adults in close contact with children. Excessive cost, a lack of clinical trial data and the potential for adverse side-reactions are some of the factors that have prevented the notion of vaccination beyond childhood (Cherry et al. 1998). The high cost of production has also limited availability of these safer vaccines to developing countries. DNA vaccines have proven to be effective against a wide range of human and veterinary pathogens and could emerge as a more stable and low cost alternative to the acellular vaccines. If sufficiently efficacious and safe, it would enable comprehensive vaccination in the truer sense, in developing countries and across all age groups.

A criticism of acellular vaccines is that they require multiple doses to achieve maximum efficiency and pose a significant risk of local side reactions after the fourth or fifth booster (Rennels et al. 2000; Gold et al. 2003). These two recent studies have revealed that local but not systemic side reactions are common in infants following multiple booster doses of the Pa vaccine. These local reactions include redness, extensive swelling of the injected limb, skin rash around the site of injection. Although much less severe than the systemic effects of encephalopathy or febrile seizures attributed to the WCV the local side reactions are a concern for future vaccine compliance (Robbins et al. 2005). The exact cause of these post-immunisation sequelae are not known but it believed to be an IgE-mediated hypersensitivity response to the large dosage of antigen given in modern acellular vaccine regimens (Robbins et al. 2005).

The early targets of DNA vaccine development have been viruses, intracellular parasites or intracellular bacteria purely because of their endogenous mode of action. DNA vaccines had not yet been assessed as a method for immunisation against pertussis but it can offer an immunological advantage over the acellular vaccines viz., a Th1-mediated and potentially Tc-mediated cellular response. Furthermore, a combination of these two

modalities could provide a much sought after dichotomous Th1/Th2 response. Hence, the goals of this study were to firstly demonstrate that DNA vaccination could induce an efficacious immune response and then whether this response could be enhanced using a mix of antigens delivered by parenteral and mucosal routes or in combination with an established acellular pertussis vaccine formulation.

To provide a meaningful outcome from this project it was imperative to use an effective challenge model. Indicators of an ideal model are reproducibility, similarity to natural infection and correlation with clinical data. As the murine aerosol challenge model satisfies these three criteria, it was considered superior to the other available methods, namely the Kendrick test and intranasal challenge (Sato et al. 1980; Guiso et al. 1999; Xing et al. 1999). Aerosol challenge has also been effectively used for delivery of both lethal and sub-lethal infectious doses, the latter of which was required to satisfy the ethical constraints of this project. For the purpose of this study a challenge chamber was custom-built from several reference designs (Sato et al. 1980; Xing *et al*. 1999; Canthaboo et al. 2000). As the in-house chamber had subtle modifications, it was necessary to validate its performance and determine the optimum SLID prior to use in the vaccination experiments. It was discovered that an optimal SLID was delivered in a short period of time and a reproducible infection was induced that would allow the protective efficacy of the DNA vaccines to be distinguished from positive and negative controls.

DNA vaccines have been reported to confer protective immunity against a variety of viral and protozoal pathogens in small animal models (Ward, Rieder & Mason 1997; Nascimento et al. 2002). Early clinical trials of DNA vaccines have been positive. For example, all 12 healthy volunteers seroconverted following a single intradermal dose of an influenza DNA vaccine (PowderMed Ltd. press release 2004). Moreover, a phase II trial of a malarial DNA vaccine (*Plasmodium falciparum*) showed that a specific T cell response was generated against the encoded antigens but importantly the dosage was safe and well tolerated (Vical Inc. press release 2001). In contrast to the many viral and parasitic targets, there has been far less effort directed at the development of DNA vaccines against bacterial infections. This project investigated some alternatives for immunisation against *B. pertussis* which included DNA vaccines delivered via parenteral or mucosal routes and dual modality vaccination. To allow for the best

possible outcome, the four most potent immunogens of *B. pertussis*, namely filamentous hemagglutinin, pertactin, pertussis toxin and adenylate cyclase hemolysin were selected. As the development of DNA vaccines against this pathogen had not been reported, with the recent exception of the pertussis toxin S1 subunit, the preliminary aspect of this work involved assessing each antigen as a separate entity prior to the development of a well-defined multi-component DNA vaccine.

Plasmid vaccines encompassing a variety of pertussis antigens or fragments thereof were constructed using established molecular biology techniques. Gene sequences encoding the target antigens or fragments were amplified from genomic DNA by PCR and ligated into a commercial eukaryotic expression vector. These identical gene inserts were simultaneously cloned in a prokaryotic expression vector for purification of recombinant proteins needed for use in immunological tests. Two of the four target antigens selected were toxins and as such site-directed mutations were required as a means of genetic inactivation (Pizza et al. 1989; Gross et al. 1992). Although both methods have been proven to abolish catalytic activity and retain immunogenicity, it was necessary to verify through *in vitro* cytotoxicity assays that the endogenous S1 and CYAA were inactive and safe for use as DNA vaccine antigens in animal experiments. As a final stage of DNA vaccine development, it was necessary to test that the selected antigens could be expressed within a mammalian system. Western blotting confirmed that each antigen was endogenously expressed.

Mice immunised with DNA vaccines encoding either the entire immunodominant region (pcDNA3.1D/*fhaB1*) or dominant B cell epitopes (pcDNA3.1D/*fhaB2*) of FHA generated a specific Th1-type response. In accordance with this response, both DNA vaccines had a significantly improved rate of clearance over the negative control mice. Vaccination with pcDNA3.1D/*fhaB1* showed superior protective efficacy compared with pcDNA3.1D/*fhaB2*, which indicated that DNA vaccine efficacy was adversely affected by focussing purely on B-cell determinants of FHA. In contrast to the DNA vaccines, DTaP-immunised mice generated very high titers of anti-FHA IgG in the serum but low IFN-γ production. More importantly, *B. pertussis* was completely cleared by day 14 post-challenge. It was not surprising that the challenge data indicated DTaP was superior to DNA vaccination considering the proprietary vaccine was a multi-component formulation compared with the mono-component DNA vaccines.

Overall the performance of the FHA DNA vaccines was encouraging considering that only naked DNA was administered without any added adjuvants. Therefore, it is quite clear that formulation and use of a systemic combination DNA vaccine with DTaP is highly likely to provide both early and long-term protection because of the contribution of both Th1 and Th2-mediated immune responses.

This study has provided the first data relating to the efficacy of a DNA vaccine against the pertactin antigen of *B. pertussis*. When administered via the IM route, mice generated a serum IgG response rather than the expected or prototypical cellular response. It was confirmed that P.69 was a potentially protective DNA vaccine antigen as seen with a significantly enhanced rate of clearance over negative controls. Western blot detection of rPRN with sera from DNA vaccinated mice and a lack of signal with non-immunised mouse sera confirmed that the antibody-mediated control of infection was a function of immunogenicity and not cross-reactivity.

Whilst a proof of principle for an S1-based DNA vaccine has been published (Kamachi et al. 2003), the data from this study has provided additional insight relating to the: (i) IM route of administration for an S1-based DNA vaccine; (ii) efficacy of DNA vaccination with a genetically inactivated S1 subunit; and (iii) protective efficacy following an aerosol challenge of immunised mice. Mice immunised with three doses of pcDNA3.1/*pts1.13L.129G* induced a Th1 response with strong CMI indicators but no antibody. This result was consistent with other studies in which naked DNA has generated a primarily Th1 response when delivered by injection into the quadriceps or tibialis anterior muscle (Johnson et al. 2000; Velikovsky et al. 2002). No cross-reactive antibodies to S1 were detected in the serum of vector-immunised mice. Again, three 0.2 standard human doses of DTaP elicited a strong Th2 response with high titers of serum IgG1 to native PT and rS1 plus elevated IL-4 production from *in vitro*-stimulated splenocytes. Challenge of both pcDNA3.1/*pts1.13L.129G* and DTaP-immunised mice showed a significantly improved rate of clearance compared to the placebo and vector only controls. However, only in the mice immunised with the Infanrix™ DTaP were the lungs completely cleared within 14 days. The results indicated that the S1 DNA vaccine strategy would be beneficial in a multi-component DNA vaccine.

Despite being a proven immunogen and having a crucial role in lung colonisation by *B. pertussis*, AC-Hly has not yet been included in any acellular vaccine formulation (Hormozi, Parton & Coote 1999). The reasons for its omission are not clear but perhaps AC-Hly has not been as immunologically well defined as the other protective antigens or there were concerns over potential cross-reactivity with the human adenyl cyclases. The results of immunisation of mice with the AC-Hly DNA vaccines were encouraging. An *in situ* cytotoxicity assay confirmed that site-directed mutagenesis of lysine 58 effectively neutralised AC activity of the endogenous antigen and that the DNA vaccine was safe to use *in vivo*. After a lengthy optimisation, rCYAAL58 was purified in sufficient quantity for use as an ELISA and splenocyte antigen. Three IM doses of pcDNA3.1D/*cyaA* alone or in combination with pcDNA3.1D/*cyaC* (accessory gene) generated a largely Th1-type response which significantly reduced the post-challenge lung counts compared to the negative controls. This has been the first study to demonstrate that a specific Th1 response against AC-Hly is potentially protective, albeit against challenge with a homogeneous strain. Our findings also supported previous studies which found CyaC-mediated palmitolyation enhanced the subsequent immune response with a clear improvement in all aspects of immunity including the humoral, cellular and protective responses after co-administration of the *cyaC* gene. In contrast to the response to DTaP, the single immunogen approach again failed to completely clear the challenge infection within 14 days. The basis for the improved response to co-administration of *cyaA* and *cyaC* versus immunisation with *cyaA* alone was not clear, especially considering that the action of CyaC, which modifies and activates the invasive properties of the toxin, would not be necessary following endogenous expression. Nevertheless, these preliminary results indicated that rCYAAL58 was a valuable candidate for further vaccine trials such as inclusion in a combination DNA vaccine, particularly when co-administered with CyaC.

Considering that all four candidate antigens performed well as single antigen DNA vaccines, it was anticipated that a multi-component vaccine may generate a cumulative response that could match the degree of protection conferred by DTaP. It was decided to first evaluate a five-gene combination DNA vaccine via IM injection to determine whether the response to each encoded antigen was enhanced, equivalent or diminished with respect to the homogenous DNA vaccines. For this purpose the five plasmid constructs were delivered as an equimolar mixture and the results exceeded

expectations. There was no significant IgG response towards FHA, PT or AC-Hly and a moderate anti-PRN titer, which mirrored the response to the single antigen DNA vaccines. Again there was a potent CMI response with high levels of IFN-γ and IL-2 detected in the cultre supernatants of re-stimulated splenocytes. More specifically, the IFN-γ response to rFHAB1 in the IM combination DNA vaccine group was equivalent to pcDNA3.1D/*fhaB1* but around two-fold lower in the oral combination DNA vaccine group. However, in response to stimulation with rPTS1.13L.129G and rCYAAL58 splenocytes of mice immunised with the five-gene combination DNA vaccines produced between two and four-fold less IFN-γ than pcDNA3.1D/*ptS1.13L.129G* and pcDNA3.1D/*cyaAL58*+pcDNA3.1D/*cyaC*, respectively. Interestingly, the response to rPRN increased by more than two-fold in mice that received the combination DNA vaccines compared to pcDNA3.1D/*prn*. In spite of an 80% reduction in the amount of each plasmid DNA delivered as a combination dose, the response to each antigen was equivalent or better than the respective individual DNA vaccines, suggesting the absence of potential antigenic competition.

Traditional pertussis vaccines have been delivered via the IM route. Parenteral administered vaccines are an effective means of generating a systemic response but are often poor inducers of mucosal immune responses. In most cases a secretory response can only be elicited by mucosal application of an antigen or pathogen. Various methods of oral and intranasal delivery of pertussis antigens have been evaluated with variable results. In a study by Molina and Parker (1990), the immunisation of mice with attenuated Salmonella expressing truncated FHA failed to generate a mucosal antibody response in respiratory secretions. On the other hand, Guzman et al. (1991) found that it was possible to generate a secretory IgA response at the lung surface with full-length FHA, which was attributed to an increase in the level of expression and immunogenicity of the native versus truncated antigen. Intranasal delivery of purified FHA and pertactin has also been shown to induce a dual mucosal and systemic antibody response with enhanced clearance of an aerosol challenge (Roberts et al. 1993). Walker et al. (1992) found that *aro*A *S. typhmurium* expressing the S1 subunit effectively primed both a specific lung and systemic response. However, no specific IgA or IgG could be detected in the lung washes of mice immunised with the same *aro*A strain that expressed the entire pertussis toxin operon and the purely systemic response could not protect against an intracerebral challenge (Dalla Pozza et al. 1998).

To assess whether a secretory response could be generated by oral DNA vaccination, a cohort of mice were orally gavaged with a combination of *aro*A *S. typhimurium* strains each harbouring one of the five DNA vaccine constructs. In response to oral vaccination, there was little or no anti-FHA or anti-AC-Hly IgG in the serum, but a low to moderate antibody response to pertactin and pertussis toxin. A strong IgG response to *S. typhimurium* was detected in the serum of orally-immunised mice using a formalin-fixed lysate of *S. typhimurium* as the ELISA antigen. The serum IgG to pertactin, PT and *S. typhimurium* were found to be predominantly IgG1. IFN-γ and IL-2 levels were much greater in mice immunised with the oral combination DNA vaccine compared to mice that received the oral vector (*S. typhimurium* harbouring pcDNA3.1D/V5-His-TOPO vector with no insert) or DTaP, and were equivalent to the levels seen with individual DNA vaccines delivered by the IM route. Overall, the main difference in immunogenicity between the IM and oral combination DNA vaccines was the high anti-PT titers generated by oral delivery, the reason for which was not clear. Despite a similar level of immunogenicity, IM delivery of the combination DNA vaccine conferred a much faster rate of clearance of *B. pertussis* than oral delivery via *aro*A *S. typhimurium*. In fact, the degree of protection conferred by the IM combination DNA vaccine approached that of DTaP, with complete clearance within 14 days and without an added adjuvant. When the clearance data from mice immunised with the oral combination DNA vaccine was compared to the rate of clearance in the vector only and placebo negative groups there was only a slight improvement, but this was found to be significant.

When mice were primed with an IM or oral combination DNA vaccine and boosted with an in-house acellular vaccine (aP), the level of protection obtained was similar to that of the DTaP. Unlike DTaP however, which elicited a Th2 response, the dual modality vaccines induced both cellular and humoral responses to each pertussis antigen. The in-house acellular vaccine was prepared based on the composition of the Infanrix™ DTaP using out-sourced FHA and PT combined with rPRN absorbed onto alum. Although the dual modality vaccines generated a broader immune response than DTaP, this did not correspond to an improved rate of clearance. In the first of the two vaccine trials in mice, DTaP-immunised mice were very effective in clearing aerosol-induced infection within 7 days post-challenge. The benefits in an improvement in this

already rapid rate of clearance would be negligible, but considering CMI responses are known to be more persistent than antibody, it is conceivable that the additional CMI component of the response to dual modality vaccination could provide longer-term protection than the response to DTaP.

The positive control for both mouse experiments was a 0.2 SHD of Infanrix™ DTaP, kindly provided by GlaxoSmithKline. Infanrix™ is a five-component formulation of diphtheria and tetanus toxoids with the FHA, pertussis toxoid and pertactin antigens of *B. pertussis*. Three sub-cutaneous injections were carried out at three-week intervals in-line with the test vaccines. DTaP-immunised mice induced high serum IgG titers against the available recombinant and native pertussis antigens, with the obvious exception of AC-Hly. Generally these titers were significantly greater than in DNA or sham-vaccinated mice. Further analysis of showed that this serum IgG consisted entirely of the IgG1 and coincided with a weak cellular response as seen by low levels of IFN-γ and IL-2 from cultured splenocytes. In a surprising result, specific IgG was also detected at the lung mucosal surface and a similar mucosal response was present following dual modality vaccination with the custom-made subunit vaccine. Considering the subcutaneous route of delivery, it was presumed that the response was derived from transudation from circulation (Russell et al. 1996). In the first and second mouse experiments, DTaP conferred a high degree protection with complete clearance of bacteria from the lungs within 7 and 14 days respectively. Overall, the acellular vaccine performed predicably well and was a good positive control and performance indicator for the DNA vaccination experiments.

This study has been the first to demonstrate that immunisation with DNA vaccines or a DNA/protein cocktail, against infection with *B. pertussis*, can match or even exceed the immunogenicity and protective efficacy of acellular vaccines. DNA vaccines offer several key advantages over acellular vaccines in that they are: easy and inexpensive to produce, homogeneous and heat-stable facilitating room temperature storage and transport. This was a preliminary study of DNA vaccine efficacy against *B. pertussis* infection and due to time constraints only two *in vivo* experiments were carried out. Clearly, it was beyond the scope of this study to cover other aspects of DNA vaccination against *B. pertussis* such as cross-protection, anamnestic responses including characterisation of memory B and T cell responses, safety, adjuvants and the

use of different animal models. Nevertheless, the results of this study have been promising and provide the basis for a more expansive research effort.

9.2 Conclusions

- Aerosol challenge with a sub-lethal infectious dose provided an effective and reproducible means of determining the protective efficacy of DNA vaccines, dual modality vaccines and their respective positive and negative controls.

- Inactivation of S1 and CYAA antigens by site-directed mutagenesis effectively neutralised the cytotoxic effects of both toxins following endogenous expression.

- Each of the *B. pertussis* antigens was constitutively expressed within cultured mammalian cells under the control of a CMV promoter.

- An FHA DNA vaccine induced a potent cellular response that provided a significantly improved level of protection over mice immunised with plasmid vector only or a placebo.

- Immunisation of mice with a DNA vaccine encompassing the immunodominant region of FHA was more immunogenic and conferred a superior level of protection than a smaller fragment that encompassed dominant B cell epitopes.

- Cross-reactivity to the filamentous hemagglutinin antigen was detected in the serum of vector-immunised but appeared to have no impact on protection against a virulent challenge.

- IM immunisation of mice with a DNA vaccine encompassing the full-length pertactin antigen elicited an unexpected serum antibody response albeit with a diminished CMI that nonetheless conferred a significantly improved level of protection over the sham-immunised mice. The results supported the inclusion of pertactin in future DNA vaccination strategies.

- An S1 DNA vaccine generated a purely Th1 response that provided a significantly improved level of clearance compared with the placebo and vector-immunised controls.

- Immunisation of mice with an AC-Hly DNA vaccine stimulated a high level of CMI that provided a significantly improved level of protection over the placebo and vector-immunised groups and the protective efficacy of CyaAL58 DNA vaccine was enhanced by co-administration of a eukaryotic expression construct that encoded CyaC, an accessory protein that post-translationally modifies and activates the protoxin.

- The immunogenicity of the IM five-gene combination DNA vaccine was collectively equivalent to each individual DNA vaccine with a very high Th1 response to FHA, S1 and AC-Hly and a moderate serum IgG response to pertactin.

- An oral five-gene combination DNA vaccine delivered by *aro*A *S. typhimurium* generated a strong systemic response but a poor mucosal antibody response to recombinant and native *B. pertussis* antigens.

- Immunisation of mice with a five gene combination DNA vaccine administered via the IM route completely cleared the challenge infection within 14 days. The IM combination DNA vaccine provided an improved level of protection compared to the oral combination DNA vaccine, which failed to clear the aerosol-induced infection within the allotted 14 day post-challenge period.

- Dual modality vaccination successfully induced a dichotomous Th1/Th2 response that conferred protection equivalent to that provided by the DTaP, irrespective of whether the DNA vaccine priming dose was delivered via direct IM injection of naked DNA or orally by *aro*A *S.typhimurium*.

- The Infanrix™ DTaP induced a potent Th2-mediated antibody response that conferred a high degree of protection.

- An IgG response was detected in the BAL fluid (lung washes) of mice following parenteral immunisation with the commercial DTaP or laboratory constituted (in-house) acellular vaccine.

9.3 Future Directions

Based on the positive outcomes thus far, the immediate future of this work would involve further testing of these vaccines in animal models with a view to the eventual evaluation in humans. There is currently very little information regarding the efficacy of DNA vaccines in humans, with the increased dosage and choice of an appropriate adjuvant being just two examples of adjustments that would need to be made. Species differences are another factor that may influence DNA vaccine efficacy in humans as it is well established that there are numerous variations between the immune systems of mice and humans (MacDonald 2003). For instance, with respect to oral delivery of a vaccine there are several important physical differences to consider such as unlike humans, laboratory mice lack a vomit reflex, are coprophagous and have a consistent and basic diet. Physiologically, the Peyer's patches (PP) of mice are smaller with 6-10 follicles whereas humans have an expansive PP with hundreds of follicles. There are also an abundance of cryptopatches or small lymphoid aggregates between the PP of mice but none in humans (MacDonald 2003). Some other differences include strong delayed type hypersensitivity reactions (DTH) and the activation of STAT4 (main transcription factor involved in Th1 polarisation) by type I interferons, both of which are common to man but are at low levels or non-existent in mice (MacDonald 2003). Therefore, whilst a DNA vaccine may elicit a strong and protective immune response in mice or other lower order animals, this immunogenicity and protective efficacy may not necessarily be reciprocated in humans or larger animals.

First and foremost, the two *in vivo* experiments of individual DNA vaccines need to be repeated to validate the efficacy of this mode of immunisation against *B. pertussis*. Moreover, the inclusion of additional controls consisting of mice immunized with the respective purified protein (i.e. mice immunised with purified FHA only) would provide a more complete evaluation of the efficacy of DNA vaccination than using DTaP, in which factors such as adjuvanticity and immune modulation by co-administered antigens are influential. For example, both FHA and PT have been reported to exhibit immunomodulatory effects (Mills et al. 2001). In this pilot study, these additional control groups were not included purely from the animal ethics standpoint and financial constraints of the research project. However, considering the promising results of pertussis DNA vaccination, expanded mouse trials can now be justified.

With exception of the pertactin antigen, the DNA vaccines evaluated in this study generated a strong cellular response that was associated with bacterial clearance. Rather than rely on indicators of CMI such as IFN-γ production in re-stimulated splenocytes, it would have been desirable to perform a more detailed analysis of the T cell responses to DNA and dual modality vaccination i.e. which specific T cell populations that were stimulated. A lack of access to flow cytometry and other specialised equipment prevented this from being accomplished but future studies may seek to obtain this data.

Considering that acquired immunity to pertussis wanes over time, it would be valuable to determine the duration of immune response and protection afforded by our novel DNA vaccines, as well as the generation of an anamnestic response. It has been reported that specific T cell responses are far more persistent than humoral responses (Mills 2001). Furthermore, it has also been observed that DNA vaccination with the pcDNA3 vector (cytomegalovirus promoter) was stable in transfected muscle cells for more than a year over which time it continued to induce a specific immune response (Wolff et al. 1992). Clearly it would be beneficial for future marketability and public acceptance to establish that the strong Th1 responses elicited by DNA vaccines could offer longer-term protection than acellular vaccines.

Prior to testing in higher animals and humans, the IgG cross-reactivity to FHA and AC-Hly detected in the serum of vector-immunised mice would need to be investigated. It was beyond the scope of this project to identify the nature of this cross-reactivity but it does not seem to be a co-incidence that the two largest and most complex of the *B. pertussis* antigens were the sources of the cross-reactivity. However, the implications from this response to FHA and AC-Hly cross-reactivity appear to be minimal. FHA has been a key component in licensed Pa preparations and no complications associated with antibody cross-reactivity have been reported. Similarly, no specific cross-reactivity related concerns have been observed following natural infection or Pw vaccination, in which the recipient is exposed to both FHA and AC-Hly. The short-term and safe option regarding the issue of cross-reactivity to AC-Hly would be to exclude this antigen from the combination DNA vaccines or dual modality formulations as with all modern DTaP formulations. It would be interesting to determine whether the combination DNA and dual modality vaccines would be less or equally efficacious in the absence of AC-Hly.

Another consideration if this work is to be taken further, will be the use minimalistic vectors as outlined by Moreno et al. (2004). The antibiotic resistance markers for neomycin and ampicillin and other immunologically redundant sequences of the pcDNA3.1D/V5-His-TOPO are not suitable for use in humans, presumably due to fears regarding the transfer of resistance genes to both pathogenic and non-pathogenic bacteria in the normal microbial flora or the environment and other unknown effects (McNeill et al. 2000; Ertl & Thomsen 2003). Cross-reactivity with FHA and to a lesser extent AC-Hly in the serum of negative control mice was attributed to neomycin phosphotranferase (Neo^R gene) of the DNA vaccine vector. Testing with a minimalistic vector would confirm or dismiss this as the source of cross-reactivity.

As the interest surrounding DNA vaccines grows, existing vectors are likely to be improved and new strategies developed. For instance, it has been shown that inclusion of one or more introns can enhance the expression levels of prokaryotic genes in a mammalian system and therefore can potentially enhance the subsequent immune response (Chapman et al. 1991). For our study, an equal mixture of the individual plasmids was chosen as the method of delivery for the multiple antigenic genes because of its simplicity and theoretical predictability of response. However, there are numerous other methods of delivering more than one antigen gene including the use of bicistronic vectors, fusion proteins or polyepitope sequences. Polyepitope or polytope vaccines, which involve the synthetic combination of numerous epitopes as a single polypeptide chain, have shown promise and allow for coverage across a broad range of antigens or serotypes with a single plasmid construct. Woodberry et al. (1999) showed that mice immunised with a HIV type 1 polytope that encompassed seven CD8+ T cell epitopes and successfully generated a specific CTL response to the respective antigens. It is obvious that a polytope antigen can only be developed after the protective response to a pathogen has been well characterised and the dominant epitopes from all protective antigens are identified, with the latter being the limitation for the development of such vaccines against pertussis. Although B cell determinants have been identified within each of the four target antigens, FHA is currently the only pertussis antigen in which the dominant B cell epitopes have been mapped (Charles et al. 1991; Chong et al. 1992; Wilson, Siebers & Finlay 1998; Lee et al. 1999; Piatti 1999). There is even less information published that relates to the location of T cell determinants, with only a hand-full of studies that have identified epitopes in the S1 and S4 subunits of pertussis

toxin (De Magistris et al. 1989; Petersen et al. 1992). A further limitation with this polytope strategy surrounds the inclusion of the AC-Hly antigen, in which the entire polypeptide sequence is required to induce an optimum immune response (Betsou, Sebo & Guiso 1995)

Over the last decade DNA vaccination has become a proven method of inducing a humoral or cellular response or both depending on the antigen and the route of delivery. In this study, it was discovered that the humoral response to DNA vaccines alone was typically several orders of magnitude lower than the commercial acellular pertussis vaccine. Although low immunogenicity particularly in form of an antibody response is not necessarily indicative of protective immunity, any increase in DNA vaccine potency with adjuvant could be desirable (Scheerlinck 2001).

Adjuvants can be divided into two broad classes: conventional or genetic. Traditional adjuvants such as alum have successfully enhanced the serum IgG response to naked DNA vaccines (Donnelly et al. 1997). Other agents such as cationic lipids and ubiquitin that improve transfection efficiency and intracellular processing rather than immune modulation have also shown real promise by enhancing the levels of antigen-specific IgG titers and production of Th1 cytokines by up to 10-fold compared with the injection of naked DNA (Delogu et al. 2000; D'Souza et al. 2002).

With recent advances in molecular biology and recombinant protein expression, there has been an increasing interest directed towards the use of genetic adjuvants such as cytokines, complement and transcriptional factors (Scheerlinck 2001). The response to co-administration of cytokines has not been surprising. For example, CMI including CTL proliferation has been found to be enhanced by co-administration of plasmids encoding IL-2, IL-12 and IFN-γ, whereas the co-delivery of Th2 cytokines such as IL-4, and IL-10 stimulated larger IgG1 titers while down-regulating the CMI responses (Scheerlinck 2001). Fusion of C3b to the hemagglutinin antigen of the influenza virus accelerated the antibody response to DNA vaccination as seen by higher avidity and neutralising activities compared with the response to antigen only (Ross et al. 2000).

DNA vaccines also possess an innate adjuvant activity due to the presence of unmethylated CpG motifs (McCluskie et al. 2001). These motifs are up to 20 times more common in bacterial DNA than in vertebrates. There is evidence that DNA containing CpG motifs stimulates immune cells to produce proinflammatory cytokines such as IL-1, IL-6, IL-12, IL-18, TNF-α and IFN-γ (Sasaki et al. 2003). It has been confirmed that CpG dinucleotides also increase the expression of MHC class II and co-stimulatory molecules (CD40, B7.2 and ICAM-1), the cumulative effect of which is activation of innate responses, promotion of APC functions and an acquired immune response dominated by Th1 cells (Sasaki et al. 2003). Herein lies a possible explanation for the polarised Th2 response to DNA vaccines delivered via biolisitc means versus the largely Th1 response to IM injection. Particle bombardment (gene gun) delivers DNA directly into the host cell cytoplasm whereas myocyte transfection after injection requires uptake of the DNA and thereby a possible interaction between CpG motifs and cell surface receptors that activate transcription of IL-2 and other cytokine genes. Also, by adding or removing these reactive motifs the immunogenicity of a DNA vaccine can be significantly improved or reduced respectively compared to an unmodified template (Klinman et al. 1997).

An interesting observation with the dual modality vaccines tested in this study was that one priming dose of DNA induced a Th1 response that was similar in magnitude to that that of three doses of DNA. Hence, the use of aluminium hydroxide in the subunit boosters of the dual modality vaccine did not appear to inhibit the induction of CMI and despite being well regarded as a Th2 adjuvant it may have had an influence on the induction of the early cellular response. Future research into a range of adjuvants could be pivotal in augmenting the anti-pertussis immune response to DNA vaccines alone, particularly when the end milestone is testing in humans and the inclusion of immunomodulators is the rule rather than the exception.

For the proof of concept, the protective antigen genes used for construction of the DNA vaccines were derived from the same strain that was used for challenge. In the future, the evaluation of cross-protection against alternative wild-type strains of *B. pertussis*, preferably currently circulating strains and those strains with polymorphisms recognised within PT and pertactin, would be essential.

Evaluation of other *B. pertussis* immunogens such as FIM and BrkA could be explored in the future. Serological data has shown that antibodies directed against FIM are second only to pertactin in terms of a correlation with protection (Cherry et al. 1998). Similarly, antibodies to BrkA have enhanced the bactericidal activity of anti-*B. pertussis* serum against the pathogen (Oliver & Fernandez (2001). In an extension of this theme, the immunodominant regions of the recognised virulence factors could be fused into several chimeric antigens and delivered as combination DNA vaccines. This would conceivably maximise the efficacy of both modes by concentrating delivery of only the reactive regions. This could also allow for the inclusion of fragments from other virulence determinants such as FIM, BrkA, and DNT, in addition to the FHA, pertactin, and PT. This form of molecular engineering would also allow the removal of superfluous or non-reactogenic regions of the antigen that show a certain degree of homology to host proteins and hence elimination of cross-reactivity.

Finally, due to the relatively recent advent of DNA vaccine technology, the safety issues surrounding this approach have not yet been fully investigated. Aside from the safety issues with all vaccines, such as local reactogenicity and systemic toxicity, the use of DNA raises concerns such as the induction of tolerance through long-term antigen presentation and autoimmunity from a potential anti-DNA response (Donnelly et al. 1997). Although Wolff et al. (1992) demonstrated a lack of chromosomal integration following IM injection of a DNA vaccine, there are still concerns about plasmid integration into host chromosomes and the activation of oncogenes or inactivation of tumour suppressor genes. Further insight into the mechanisms of action of DNA vaccines and future clinical trials in humans should provide the answers to these safety concerns.

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Appendix A

A.1 Materials and Suppliers

Agarose (Progen), Ammonium persulfate (Sigma), Ampicillin (Progen and ICN), Acetic acid – glacial (BDH), Agar (Acumedia), p-aminobenzoic acid (Sigma), 30% Acrylamide/bisacrylamide (BioRad), Ascorbic acid (Sigma), Aluminium hydroxide gel (Sigma)

Bordet Gengou agar base (Difco), Boric acid (Ajax), β-mercaptoethanol (Sigma), Bromophenol blue (Sigma)

Chloramphenicol (Progen), Casamino acids – technical (Difco), Casamino acids – low NaCl and iron (Difco), Coomassie brilliant blue R250 (Sigma), Calcium chloride (Sigma), Cysteine (Sigma)

DMEM base (Invitrogen), DMSO (Sigma), 2,3-Dihydroxybenzoic acid (Aldrich), Dimethyl formamide (Sigma)

Ethanol (Sigma), EDTA - disodium (Sigma)

Foetal bovine serum (Invitrogen), F12 base media (Invitrogen), Formaldehyde (Sigma)

Glycerol (ICN), Guanidine hydrochloride (Lancaster), Glycine (Lancaster), D-Glucose (Sigma), L-Glutamic acid (Sigma), Glutathione (ICN)

Horse blood – defibrinated (Oxoid), 1 M HEPES buffer solution (Invitrogen), Hydrochloric acid (Sigma), p-Hydroxybenzoic acid (Sigma), Heptakis-2-cyclodextrin (Sigma)

Isopropanol (ICN), IPTG (ICN), Iron sulfate heptahydrate (Sigma)

LB broth base (Difco), LB agar base (Difco)

Methanol (BDH), Magnesium sulfate (Sigma), Magnesium chloride hexahydrate (ICN)

Nicotinic acid/Niacin (Sigma)

OPTI-4CN substrate kit (BioRad)

PMSF (Invitrogen), Protease inhibitor cocktail (Roche), PennicillinG/Streptomycin solution (Invitrogen), Polymyxin B (Sigma), Phenylalanine (ICN), 10 x PBS (Invitrogen), Potassium phosphate – monobasic (Sigma), L-Proline (Sigma), Potassium chloride (Sigma), 2-Phenoxyethanol (Lancaster)

RbCl/$CaCl_2$ transformation salts (Qbiogene)

Sodium hydrogen carbonate (Ajax), Skim milk (generic), Sheep blood – defibrinated (Oxoid), Sodium phosphate – monobasic (Sigma), Sodium hydroxide (ICN), Sodium Chloride (ICN), Sodium phosphate – dibasic (Sigma), Sodium dodecyl sulfate – SDS (ICN), Starch (Difco), Sodium carbonate (Sigma), 0.2µM Steritop 500ml filtration units (Millipore), Super-Comp media (Qbiogene), 0.2µM syringe filters (Millipore).

Tris.HCl (Invitrogen), Tryptone (Acumedia), Thiamin (Sigma), Tryptophan (Sigma), Tyrosine (Sigma), TEMED (Sigma)

Urea (ICN)

Water ultra-pure (Millipore), Water Dnase/Rnase free (Invitrogen)

X-gal/5-bromo-4-chloro-3-indolyl-β-D-galactosidase (Sigma)

Yeast extract (Acumedia)

A.2 Buffers, Solutions, Media and Markers

Agarose gel

0.8-2.0% (w/v) Agarose

1.0X TAE Buffer

0.25µg/ml Ethidium bromide

10% Ammonium Persulfate (w/v)

50mg of Ammonium Persulfate was dissolved in 500µl of ultra-pure water.

Antibiotic Concentrations in Media

For antibiotic selection, LB broth or agar was supplemented with 100µg/ml of ampicillin and 170µg/ml of chloramphenicol. A 50mg/ml (w/v) stock solution of ampicillin was prepared in ultra-pure water, whereas a 35mg/ml (w/v) chloramphenicol stock solution was prepared in 70% Ethanol. The ampicillin stock was sterilised by 0.2µM syringe filtration. Stock solutions were stored at –20°C.

BAL Wash Buffer

2mM of PMSF and 15 protease inhibitor cocktail tablets (Pierce) were dissolved in 150ml of 1X PBS.

Bicarbonate Buffer

30mM Sodium carbonate

20mM Sodium hydrogen carbonate

Adjusted to pH 9.6 with 1M NaOH.

Bicarbonate-buffered Saline

3g of sodium hydrogen carbonate was dissolved in 100ml of saline and sterilised by 0.2µM filteration.

Blocking and Antibody Diluent Buffer (ELISA and Western Blot)

5g of skim milk powder was dissolved in 100ml 1X PBST for 5% (w/v).

Bordet Gengou Agar

30g of Bordet Gengou agar base was resuspended in 950ml of 1% glycerol (in ultra-pure water). The suspension was heated until agar dissolved. The base was sterilised by autoclave at 121°C for 15 minutes and cooled to 55°C in a water bath. 50ml of defibrinated sheep or horse blood was added for 5% (v/v), mixed well and poured.

0.5M Boric acid

3.092g of H_3BO_3 was dissolved in 100ml of ultra-pure water and sterilised by 0.2μM filtration.

Buffer A (Affinity Purification)

100mM NaH_2PO_4	13.8g $NaH_2PO_4.H_2O$ (MW 137.99g/mol)
10mM Tris	1.2g Tris base (MW 121.1g/mol)
6M GuHCl	573g guanidine hydrochloride

Dissolved in 900ml ultra-pure water and pH adjusted to 8.0 with 1M NaOH.

Added ultra-pure water up to 1L and filtered through 0.2μM membrane under vacuum (Millipore).

Buffer C (Affinity Purification)

100mM NaH_2PO_4	13.8g $NaH_2PO_4.H_2O$ (MW 137.99g/mol)
10mM Tris	1.2g Tris base (MW 121.1g/mol)
8M Urea	480.5g Urea (MW 60.06g/mol)

Dissolved in 900 ml ultra-pure water and pH adjusted to 6.3 with 1M HCl.

Added ultra-pure water up to 1 L and filtered through 0.2μM membrane under vacuum (Millipore).

Buffer D (Affinity Purification)

100mM NaH_2PO_4	13.8g $NaH_2PO_4.H_2O$ (MW 137.99g/mol)
10mM Tris	1.2g Tris base (MW 121.1g/mol)
8M Urea	480.5g Urea (MW 60.06g/mol)

Dissolved in 900ml ultra-pure water and pH adjusted to 5.9 with 1M HCl.

Added ultra-pure water up to 1L and filtered through 0.2μM membrane under vacuum (Millipore).

Buffer E (Affinity Purification)

100mM NaH_2PO_4	13.8g $NaH_2PO_4.H_2O$ (MW 137.99g/mol)
10mM Tris	1.2g Tris base (MW 121.1g/mol)
8M Urea	480.5g Urea (MW 60.06g/mol)

Dissolved in 900ml ultra-pure water and pH adjusted to 4.5 with 1M HCl

Added ultra-pure water up to 1L and filtered through 0.2μM membrane under vacuum (Millipore).

1% Casien (Technical Grade)

1g of technical grade casamino acids (acid-hydrolysed casein) was dissolved in 100ml saline. Sterilised at 121ºC for 15 minutes and stored at 4ºC.

1% Casein (*in vivo*)

1g of casamino acids (low NaCl and iron) was dissolved in 100ml saline. Sterilised at 121ºC for 15 minutes and stored at 4ºC.

Coating Buffer (ELISA)

40ml of 1.5M NaCl, 50ml of 0.5M Boric acid (H_3BO_3) and 12ml of 1M NaOH was added to 348ml of ultra-pure water. The pH was adjusted to 9.0 and the volume made up to 500ml with ultra-pure water.

Coomassie Brilliant Blue Stain

0.25g of Coomassie Brilliant Blue R250 was dissolved in 100ml of destaining solution.

Destaining Solution

Ultra-pure water	450ml
Methanol	450ml
Glacial Acetic Acid	100ml

DP (Denaturing protein) Buffer

100mM Sodium phosphate (monobasic)

10mM Tris

8M Urea

Dissolved in 900ml ultra-pure water and pH adjusted to 5.2 with 1M HCl

Added ultra-pure water up to 1L and filtered through 0.2µM membrane under vacuum (Millipore).

Dulbecco's Modified Eagle Media (DMEM)

For routine culture (complete DMEM): 10% foetal bovine serum, 25mM HEPES, 200 units/ml Penicillin G and 200µg/ml Streptomycin were added through a 0.2µM filter to 500ml DMEM base with high glucose, L-glutamine, sodium pyruvate and pyroxidine hydrochloride, and stored as 50ml aliquots at 4ºC.

For transfection complete DMEM was prepared without antibiotics. For splenocyte culture (sDMEM) complete DMEM was supplemented with 50µM of β-mercaptoethanol (50µl of a 50mM stock) and 10µg/ml polymyxin B (10µl of a 50mg/ml stock). For liquid N_2 storage complete DMEM was supplemented with 10% DMSO (v/v).

0.5M EDTA

186.1g of disodium ethylenediaminetetra-acetate.$2H_2O$ was added to 800ml of ultra-pure water and dissolved by adjustment of the pH to 8.0 with 1 M NaOH and vigorous stirring.

F12 (Ham's) Media

For routine culture (complete F12): 10% foetal bovine serum, 25mM HEPES, 200 units/ml Penicillin G and 200µg/ml Streptomycin were added through a 0.2µM filter to 500ml F12 base with L-glutamine and stored as 50ml aliquots at 4ºC.

For toxicity testing complete F12 was prepared without antibiotics. For liquid N_2 storage complete F12 was supplemented with 10% DMSO (v/v).

2M Glucose

18.02g of D-glucose was dissolved in 50ml of ultra-pure water and sterilised by 0.2µM filtration (Millipore).

1M HCl

37% (10M) hydrochloric acid was diluted to 1M with ultra-pure water.

1M IPTG

2.38g of isopropylthio-β-D-galactoside was dissolved in ultra-pure water, sterilised by 0.2µM filtration and dispensed into 1ml aliquots and stored at –20ºC.

LB Broth or Agar

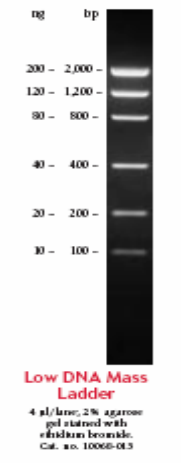
Yeast Extract	1g
Tryptone	2g
NaCl	1g
+/- Agar	3g
Ultra-pure Water	up to 200ml

LBA refers to LB with 100µg/ml ampicillin.

LBAG refers to LB with 100µg/ml ampicillin and 0.05% glucose.

LBACG refers to LB with 100µg/ml ampicillin, 170µg/ml chloramphenicol and 0.05% glucose.

Low DNA Mass Ladder (Source: http://www.invitrogen.com/downloads/713011008FAcidMarker.pdf)



M9 Minimal Salts Agar Base

2.0g of M9 salts and 3.0g of Agar were dissolved in 200ml ultra-pure water, then autoclaved at 121°C for 15 minutes. Following sterilisation, the temperature of the solution was maintained at 55°C in a water bath and the following 0.2µM-filtered solutions were added:

NaCl (0.1g/ml)	1ml
$CaCl_2$ (0.0222g/ml)	0.1ml
$MgSO_4$ (0.2465g/ml)	0.4ml
Glucose (0.3g/ml)	2.86ml
Thiamin (0.02g/ml)	0.1ml

M9 Minimal Salts + Aromatic Mix Agar Base

The following filter sterilised aromatic amino acids were added to M9 minimal salts agar base whilst the temperature of the agar base was maintained at 55°C:

2,3-DHBA (10mg/ml, dissolved in 40% v/v Ethanol)	200µl for 10µg/ml
pHBA (10mg/ml, dissolved in 40% v/v Ethanol)	200µl for 10µg/ml
pABA (10mg/ml, dissolved in 40% v/v Ethanol)	200µl for 10µg/ml
Tryptophan (50mg/ml, dissolved in 1M NaOH)	80µl for 20µg/ml
Phenylalanine (10mg/ml, dissolved in ultra-pure water)	800µl for 40µg/ml
Tyrosine (4mg/ml, dissolved in 1M NaOH)	2ml for 40µg/ml

0.9% NaCl (Saline)

9g NaCl was dissolved in 1L ultra-pure water and sterilised at 121°C for 15 minutes.

10mM NaCl

0.06g of NaCl was dissolved in 100ml of ultra-pure water and sterilised by 0.2µm filtration.

1.5M NaCl

2.92g of NaCl was dissolved in 100ml of ultra-pure water and sterilised by 0.2µm filtration.

1M NaOH

20g of sodium hydroxide was dissolved in 500ml of ultra-pure water.

Opti-4CN HRP Visualisation Solution

For each 10cm x 10cm membrane:

Opti-4CN Diluent	1ml
Ultra-pure water	9ml
Opti-4CN Substrate	0.2ml

PAGE Loading Buffer

Milli Q Plus water	6.089ml
90 % Glycerol (v/v)	1.111ml
1 M Tris-HCl, pH 6.8	600µl
β-mercaptoethanol	100µl
100 mM PMSF	100µl
0.1 % Bromophenol Blue (w/v)	0.5ml

1X PBS (Commercial)

100ml of 10X PBS (Invitrogen) was added to 900ml ultra-pure water. A 1X solution was sterilised at 121°C for 15 minutes for use in ELISA and western blot buffers.

1X PBS (In-house)

16g of NaCl, 0.4g of KCl, 2.3g of Na_2HPO_4 and 0.4g of KH_2PO_4 were dissolved in 1.8L of ultra-pure water. The pH was adjusted to 7.2 and made up to 2L with ultra-pure water. In house 1X PBS was used in all applications other than the preparation of ELISA and western blot buffers.

1X PBST

0.01ml (Tween20) was added to 1L of 1X PBS (commercial) for a 0.1% (v/v) final concentration of Tween20.

100mM PMSF

87mg of phenylmethylsulonyl fluoride was dissolved in 5 ml of 100% isopropanol.

PS Buffer

25mM Tris

0.5M NaCl

50% Glycerol

0.303g of Tris base and 2.92g of NaCl was dissolved in 100ml of 50% glycerol and sterilised by 0.2µm filtration.

10% SDS (w/v)

10g of SDS was dissolved in 100ml of ultra-pure water.

PAGE Gel Preparation

	Resolving Gel (12%)	Stacking Gel (5%)
Ultra-pure water	3.3ml	3.4ml
1.5M Tris-HCl, pH 8.8	2.5ml	-
0.5M Tris-HCl, pH 6.8	-	630µl
10% SDS (w/v)	100µl	50µl
30% Acrylamide/bisacrylamide	4.0ml	830µl
10% Ammonium Persulfate (w/v)	100µl	50µl
TEMED	8µl	5µl

Components were added in order shown above

SOB

Tryptone	20g
Yeast extract	5g
NaCl	0.5g
Ultra-pure water	up to 980ml

Sterilised at 121˚C for 15 minutes

10ml of filter-sterilised 1M $MgCl_2$ and 10ml of 1M $MgSO_4$ was added prior to use.

SOC

1ml of filter-sterilised 2M glucose was added to 99ml of SOB base.

Stainer-Scholte Broth (Modified)

Part A

L-Proline	240mg
L-Glutamic acid	670mg
NaCl	2500mg
KH_2PO_4	500mg
KCl	200mg
$MgCl_2.6H_2O$	100mg
$CaCl_2$	20mg
Tris	6075mg
Heptakis-2-cyclodextrin (ICN)	1000mg

Added 900ml Ultra-pure dH_2O.

Adjusted to pH 7.6 with 1M HCl.

Added Ultra-pure dH_2O up to 960 ml and sterilised at 121°C for 15 minutes.

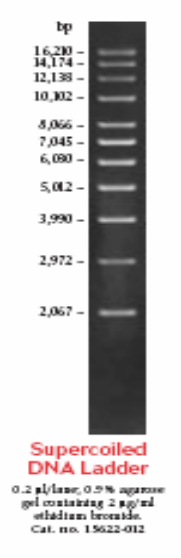
Part B

Cysteine	40mg
Glutathione	100mg
Ascorbic acid	20mg
Niacin	4mg
$FeSO_4.7H_2O$	10mg

Dissolved in 40ml ultra-pure dH_2O.

Sterilised by 0.2μM filtration, and added to autoclaved part A component after it was cooled to RT.

Supercoiled DNA Ladder (Source: http://www.invitrogen.com/downloads/713011008FAcidMarker.pdf)



2X SUV Buffer

NaCl	0.18g
2-Phenoxyethanol (100mg)	100µl
$AlOH_3$ (20mg)	1.5ml of aluminium hydroxide gel (13mg/ml)
Ultra-pure water	up to 10ml

50X TAE Buffer

242g of Tris base was dissolved in 842.9ml of ultra-pure water, to which 57.1ml of glacial acetic acid and 100ml of 0.5 M EDTA (pH 8.0) was added. For a 1X working solution 500ml of 50X TAE was diluted up to 25L with ultra-pure water.

25mM Tris/50mM $CaCl_2$

0.61g of Tris and 1.11g of $CaCl_2$ were dissolved in 200ml of ultra-pure water.

5X Tris – Glycine Electrophoresis Running Buffer

15.1g of Tris and 94g of Glycine were dissolved in 900ml of ultra-pure water. 50ml of 10% SDS (w/v) was added, with the final volume made up to 1L with ultra-pure water. For a 1X working solution, 300ml of 5X stock was diluted up to 1.5L with ultra-pure water.

25mM Tris-HCl, pH 7.5

0.61g of Tris base was dissolved in 150ml of ultra-pure water. Adjusted pH to 7.5 with 1M HCl and made up to 200ml with ultra-pure water.

1.5M Tris-HCl, pH 8.8

90.82g of Tris base was dissolved in 375ml of ultra-pure water. Adjusted pH to 8.8 with 1M HCl and made up to 500ml with ultra-pure water.

0.5M Tris-HCl, pH 6.8

12g of Tris base was dissolved in 150ml of ultra-pure water. Adjusted pH to 6.8 with 1M HCl and made up to 200ml with ultra-pure water.

Transfer Buffer (Western Blotting)

9.1g of Tris base and 43.25g of Glycine were dissolved in 2L of ultra-pure water and 450ml of reagent grade Methanol. Adjusted pH to 8.3 and made up to 2L with ultra-pure water.

Verwey *et al.* Broth (Modified by D.J. Farrell unpublished)

Casamino acids, technical grade	3.5 g
KCl	50 mg
KH_2PO_4	125 mg
$MgCl_2.6H_2O$	25 mg
Nicotinic acid	5 mg
Glutathione	2.5 mg
Starch	250 mg
Heptakis-2-cyclodextrin	250 mg
Ultra-pure dH_2O	200 ml

Adjusted pH to 6.8 using 1M KOH and made up to 250ml with Ultra-pure water.

X-gal Stock

200mg of 5-Bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) was dissolved in 10ml of dimethylformamide for 20mg/ml. One ml aliquots were stored at –20˚C.

Appendix B

B.1 Extended Methodology

B.1.1 Counting of Mammalian Cells

Viable cell counts were performed using a haemocytometer (Neubauer). A 10 µl aliquot of cell suspension was diluted 1:10 with culture media. An equal volume of 0.14% trypan blue (Invitrogen) was added for a dilution factor of 1:20. Both chambers of the haemocytometer were filled by capillary action and an average count of the four large outer squares was taken. The number of viable cells per ml was calculated by multiplying the average large square count by the dilution and conversion factors. The depth of the haemocytometer used in this study was 0.1 mm (conversion factor 10,000).

B.1.2 Staining of PAGE Gels

PAGE gels were stained in a Coomassie brilliant blue solution (Appendix A) for upwards of 8 hours at room temperature with gentle shaking. Following removal of the staining solution, the gel was gently shaken at room temperature in the de-staining solution (Appendix A) until the background of the gel was clear.

B.1.3 Alternative Western Blot Protocol

Following transfer to PVDF, the membrane was removed from the transfer cassette and incubated in 10 mls of 2.5% skim milk in PBS for 2 hours at RT (with gentle agitation). After the blocking the membrane was washed twice in 0.1% Tween 20 in PBS for 10 minutes per wash, followed by a third 10 minute rinse in PBS. The membrane was then incubated for 3 to 4 hours with either: (i) a 1:10,000 dilution of anti-histidine HRP conjugated antibody (Invitrogen) or a 1:5000 dilution anti-histidine AP conjugated antibody (Invitrogen) in 2.5% skim milk in PBS. Recombinant proteins expressed from mammalian cells were detected using a 1:2000 dilution of anti-V5 Alkaline Phosphatase conjugated antibody (Invitrogen). Alternatively, for detection of IgG in serum samples or monoclonal antibody, incubation with a secondary anti-mouse IgG HRP conjugate (Pierce) was included. The washing step was repeated to remove unbound primary and secondary antibody. Detected proteins were visualised using Opti-4CN (BioRad) for HRP conjugates or NBT/BCIP (Pierce) for AP conjugated secondary antibodies in accordance with the manufacturer's instructions.

B.1.4 Optimal Lysis Conditions for CHO-K1 Cells

Two 24-well plates were seeded with CHO-K1 cells at a density 1.2 x 10^5 cells/well in 500 µl complete F12 without antibiotics. Plates were incubated at 37°C with 5% CO_2 until wells were 100% confluent. Culture media was removed and the various lytic test solutions were added to their respective wells in duplicate plates. The layout of the plate has been summarised in Table B.1. One plate was incubated for 30 minutes on ice, whereas the duplicate plate for 30 minutes at RT. Each well was visually inspected for lysis at 10 minute intervals and documented.

Table B.1: Outline of CHO-K1 Cell Lysis Experiment

Well	Vol. (ml)	Lysis Solution	Well	Vol. (ml)	Lysis Solution
1	0.5	0.1 M HCl	13	0.5	0.1M HCL/0.1% Triton
2	0.25	0.1 M HCl	14	0.25	0.1M HCL/0.1% Triton
3	0.5	0.5 M HCl	15	0.5	0.1M HCL/1.0% Triton
4	0.25	0.5 M HCl	16	0.25	0.1M HCL/1.0% Triton
5	0.5	1.0 M HCl	17	0.5	1.0M HCL/0.1% Triton
6	0.25	1.0 M HCl	18	0.25	1.0M HCL/0.1% Triton
7	0.5	0.1% Triton X100	19	0.5	1.0M HCL/1.0% Triton
8	0.25	0.1% Triton X100	20	0.25	1.0M HCL/1.0% Triton
9	0.5	0.5% Triton X100	21	0.5	Tris/SDS/DTT buffer
10	0.25	0.5% Triton X100	22	0.25	Tris/SDS/DTT buffer
11	0.5	1.0% Triton X100	23	0.5	1 x PBS
12	0.25	1.0% Triton X100	24	0.0	No lysis buffer

B.1.5 Preparation of RbCl/$CaCl_2$ Competent Cells

One ml from an overnight LB culture of P9121 was diluted into 25 ml Super-Comp broth (Qbiogene) and incubated at 37°C and 200 rpm until OD_{600} reached 0.8. Cells were centrifuged for 10 minutes at 3000 x g and 4°C. Supernatant was removed and the pellet resuspended in 15 ml of ice-cold 1 x RbCl/$CaCl_2$ salts (Qbiogene). After 15 minute incubation on ice, the cells were pelleted and gently resuspended in 2 ml of ice-cold 1 x RbCl/$CaCl_2$ salts. To increase transformation efficiency by 4-6 fold, the appropriate volume of suspension was stored at 4°C for 24 hours prior to heat shock. For the residual cell suspension, glycerol was added to a final concentration of 20% (v/v) with aliquots of 50 µl stored at -80°C.

B.1.6 Preparation of Electrocompetent *S. typhimurium* SL3261 Cells

The protocol used for the preparation of electrocompetent *S. typhimurium* strain SL3261 was adapted from the instruction manual provided with the BTX Electro Cell Manipulator 600 (BTX). An overnight culture of SL3261 was diluted 1:100 in 50 ml of LB media and incubated at 37°C and 200 rpm until the OD_{600} reached 1.0. Cells were pelleted at 6000 x g and 4°C for 12 minutes and then washed twice with 10ml of ultra-pure water. The washed pellet was resuspended in 1.25 ml of Ultra-pure water and transferred to a 1.7 ml microfuge tube. After a final centrifuge step of 6000 x g for 3 minutes the pellet was resuspended in 1 ml of 10% glycerol and stored at -80°C in 40 µl aliquots.

B.1.7 Concentration and Dialysis of Target Proteins

The vivaspin20 column (Vivaflow) was used for concentration and buffer exchange of protein samples according to the manufacturers' recommendations. Protein samples were loaded into the upper chamber and reduced through a 5000 MWCO filter at 6000 x g at 4°C. For dialysis/buffer exchange four volumes of the end buffer was passed through the column. Concentrated or dialysed samples were analysed by PAGE and modified Bradford assay to confirm yield.

B.2 Codon Usage Data

Bordetella pertussis Tohama I [gbbct]: 3498 CDS's (1145165 codons)

fields: [triplet] [frequency: per thousand] ([number])

```
UUU  4.0(  4577)  UCU  0.7(   824)  UAU  6.7(  7715)  UGU  1.0(  1136)
UUC 28.6( 32787)  UCC  9.8( 11279)  UAC 17.8( 20431)  UGC  7.9(  9093)
UUA  0.2(   251)  UCA  0.8(   934)  UAA  0.5(   526)  UGA  1.9(  2137)
UUG  8.6(  9871)  UCG 19.0( 21776)  UAG  0.7(   835)  UGG 14.1( 16164)

CUU  2.0(  2324)  CCU  2.6(  2934)  CAU  8.0(  9169)  CGU  5.0(  5680)
CUC 12.3( 14101)  CCC 20.2( 23147)  CAC 15.8( 18106)  CGC 54.7( 62630)
CUA  1.2(  1365)  CCA  1.5(  1750)  CAA  6.6(  7612)  CGA  2.4(  2781)
CUG 82.7( 94723)  CCG 29.0( 33231)  CAG 34.6( 39602)  CGG 12.2( 13979)

AUU  4.1(  4683)  ACU  1.3(  1466)  AAU  5.5(  6242)  AGU  1.1(  1240)
AUC 38.1( 43665)  ACC 28.5( 32661)  AAC 19.1( 21868)  AGC 17.3( 19827)
AUA  1.5(  1690)  ACA  1.2(  1326)  AAA  4.0(  4594)  AGA  0.5(   595)
AUG 23.2( 26591)  ACG 17.4( 19909)  AAG 23.1( 26404)  AGG  1.5(  1756)

GUU  2.9(  3326)  GCU  4.2(  4834)  GAU 13.0( 14858)  GGU  4.2(  4799)
GUC 27.7( 31721)  GCC 75.8( 86823)  GAC 40.1( 45903)  GGC 66.8( 76531)
GUA  2.9(  3334)  GCA  4.3(  4963)  GAA 20.1( 22988)  GGA  3.6(  4115)
GUG 42.8( 49025)  GCG 51.4( 58892)  GAG 29.3( 33499)  GGG 10.1( 11567)
```

Coding GC 68.10% 1st letter GC 69.01% 2nd letter GC 47.22% 3rd letter GC 88.06%

Figure B.2: Codon Usage Table of *E. coli* K12

Source: Codon Usage Database (www.kazusa.or.jp/codon/)

Escherichia coli K12 [gbbct]: 5095 CDS's (1609357 codons)

fields: [triplet] [frequency: per thousand] ([number])

```
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UUC 16.6( 26700)  UCC  8.6( 13858)  UAC 12.3( 19740)  UGC  6.4( 10360)
UUA 13.9( 22389)  UCA  7.2( 11519)  UAA  2.0(  3250)  UGA  0.9(  1469)
UUG 13.7( 22092)  UCG  8.9( 14383)  UAG  0.2(   379)  UGG 15.3( 24639)

CUU 11.0( 17771)  CCU  7.1( 11346)  CAU 12.9( 20748)  CGU 21.0( 33729)
CUC 11.0( 17739)  CCC  5.5(  8921)  CAC  9.7( 15610)  CGC 22.0( 35337)
CUA  3.9(  6215)  CCA  8.5( 13724)  CAA 15.4( 24848)  CGA  3.6(  5722)
CUG 52.6( 84728)  CCG 23.2( 37353)  CAG 28.8( 46359)  CGG  5.4(  8692)

AUU 30.4( 48851)  ACU  9.0( 14408)  AAU 17.7( 28485)  AGU  8.8( 14100)
AUC 25.0( 40207)  ACC 23.4( 37646)  AAC 21.7( 34941)  AGC 16.1( 25859)
AUA  4.3(  6971)  ACA  7.1( 11381)  AAA 33.6( 54137)  AGA  2.1(  3343)
AUG 27.7( 44659)  ACG 14.4( 23144)  AAG 10.2( 16414)  AGG  1.2(  1990)

GUU 18.4( 29602)  GCU 15.4( 24740)  GAU 32.2( 51885)  GGU 24.9( 40045)
GUC 15.2( 24493)  GCC 25.5( 41016)  GAC 19.0( 30649)  GGC 29.4( 47336)
GUA 10.9( 17523)  GCA 20.3( 32686)  GAA 39.5( 63575)  GGA  7.9( 12783)
GUG 26.2( 42233)  GCG 33.6( 54023)  GAG 17.7( 28551)  GGG 11.0( 17714)
```

Coding GC 51.80% 1st letter GC 58.89% 2nd letter GC 40.72% 3rd letter GC 55.78%

Figure B.3: Codon Usage Tables of *E. coli* K12

Source: Codon Usage Database (www.kazusa.or.jp/codon/)

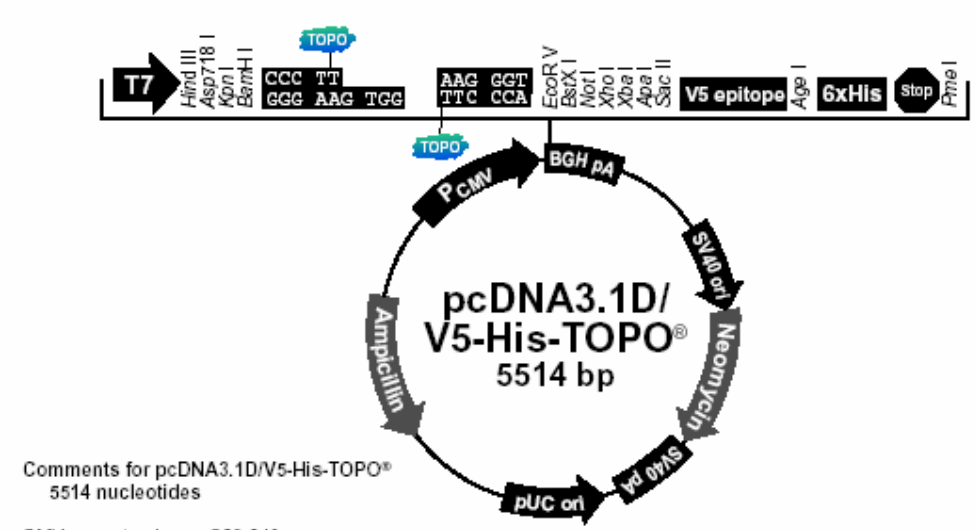
Appendix C

Plasmid Vectors

C.1 pcDNA3.1D/V5-His-TOPO

- DNA vaccine vector
- Eukaryotic expression vector

Map



Comments for pcDNA3.1D/V5-His-TOPO®
5514 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
TOPO® recognition site 1: bases 930-934
Overhang sequence (complementary strand): bases 935-938
TOPO® recognition site 2: bases 939-943
V5 epitope: bases 1011-1052
Polyhistidine (6xHis) tag: bases 1062-1079
BGH reverse priming site: bases 1102-1119
BGH polyadenylation signal: bases 1108-1332
SV40 early promoter and origin: bases 1833-2142
Neomycin resistance gene: bases 2217-3011
SV40 early polyadenylation signal: bases 3189-3319
pUC origin: bases 3700-4373 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4518-5378 (complementary strand)
bla promoter: bases 5379-5477 (complementary strand)

Figure C.1: Plasmid Map of pcDNA3.1D/V5-His-TOPO

Source: Invitrogen Corporation (Carlsbad, California)

http://www.invitrogen.com/content/sfs/vectors/pcdna3_1dv5histopo_map.pdf

Sequence

GACGGATCGGGAGATCTCCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGT
ATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTG
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SFPCSEQF→

ACTGCTTACTGGCTTATCGAAAT**TAATACGACTCACTATAGGG**AGACCCAAGCTGGCTAGTTAAGCTTGGTACCGA
GCTCGGATCCAGTACCCTTCACCAAGGGTCAAGACAATTCTGCAGATATCCAGCACAGTGGCGGCCGCTCGAGTCTA
GAGGGCCCGCGGTTCGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGCGTACCGGTCATCATCAC
CATCACCA

←SFPCSEQR

TTGAGTTTAAACCCGCTGATCAG**CCTCGACTGTGCCTTCTA**GTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCC
TTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAG
GTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCT
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CGTC

Figure C.2: Sequence of pcDNA3.1D/V5-His-TOPO. Forward and Reverse primer sites for sequencing of cloned inserts are indicated as magenta.

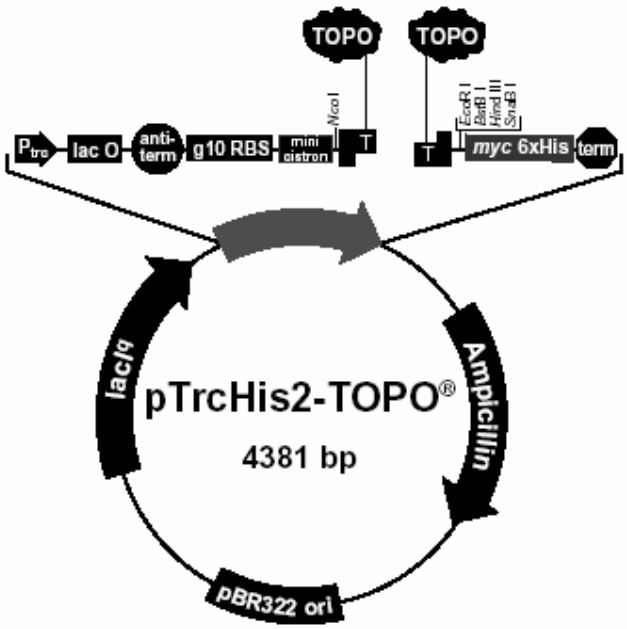
Source: Invitrogen (Carlsbad, California)

http://www.invitrogen.com/content/sfs/vectors/pcdna3_1dv5histopo_seq.txt

C.2 pTrcHis2-TOPO

- Prokaryotic expression vector
- Expression of recombinant proteins in *E. coli*

Map



Comments for pTrcHis2-TOPO®
4381 nucleotides

trc promoter and 5': bases 190-382
-35 region: bases 193-198
-10 region: bases 216-221
lac operator (*lacO*): bases 228-248
*rrn*B antitermination signal: bases 264-333
gene 10 region: bases 346-354
Ribosome binding site: bases 369-373
pTrcHis Forward priming site: bases 370-390
Minicistron ORF: bases 383-409
Reinitiation RBS: bases 398-403
Initiation ATG: bases 413-415
TOPO® Cloning site: bases 421-422
myc epitope: bases 446-475
Polyhistidine region: bases 491-508
pTrcHis2 Reverse priming site: bases 564-581
*rrn*B T1 and T2 transcriptional terminators: bases 614-771
bla promoter: bases 993-1050
Ampicillin resistance gene (*bla*): bases 1051-1911
pBR322-derived origin: bases 2056-2729
Lac Repressor (*lacI*q) ORF: bases 3258-4342

Figure C.3: Plasmid Map of pTrcHis2-TOPO

Source: Invitrogen Corporation (Carlsbad, CA)

http://www.invitrogen.com/content/sfs/vectors/ptrchis2topo_map.pdf

Sequence

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←SFTRCSEQR

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TGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAG
ATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATC
AAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGC
CGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTG
CTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGC
TGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCT
ATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGA
GCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCG
TCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGC
CTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGT
GAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCTGAT
GCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCC
GCATAGTTAAGCCAGTATACACTCCGCTATCGCTACGTGACTGGGTCATGGCTGCGCCCCGACACCCGCCAACACCC
GCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATG
TGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGGCAGCAGATCAATTCGCGCGCGAAGGCGAAGCGGCATGC
ATTTACGTTGACACCATCGAATGGTGCAAAACCTTTCGCGGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAG
GGTGGTGAATGTGAAACCAGTAACGTTATACGATGTCGCAGAGTATGCCGGTGTCTCTTATCAGACCGTTTCCCGCGT
GGTGAACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAGTGGAAGCGGCGATGGCGGAGCTGAATTACATT
CCCAACCGCGTGGCACAACAACTGGCGGGCAAACAGTCGTTGCTGATTGGCGTTGCCACCTCCAGTCTGGCCCTGCA
CGCGCCGTCGCAAATTGTCGCGGCGATTAAATCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTCGATGGTAG

AACGAAGCGGCGTCGAAGCCTGTAAAGCGGCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGATCATTAAC
TATCCGCTGGATGACCAGGATGCCATTGCTGTGGAAGCTGCCTGCACTAATGTTCCGGCGTTATTTCTTGATGTCTCTG
ACCAGACACCCATCAACAGTATTATTTTCTCCCATGAAGACGGTACGCGACTGGGCGTGGAGCATCTGGTCGCATTG
GGTCACCAGCAAATCGCGCTGTTAGCGGGCCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGGCTGGCTGGCATAAA
TATCTCACTCGCAATCAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGGAGTGCCATGTCCGGTTTTCAACAAAC
CATGCAAATGCTGAATGAGGGCATCGTTCCCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCTGGGCGCAATGC
GCGCCATTACCGAGTCCGGGCTGCGCGTTGGTGCGGATATCTCGGTAGTGGGATACGACGATACCGAAGACAGCTCA
TGTTATATCCCGCCGTCAACCACCATCAAACAGGATTTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAA
CTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCTGGCGCC
CAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAA
GCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCGCGAATTGATCTG

Figure C.4: Sequence of pTrcHis2-TOPO. Forward and Reverse primer sites for sequencing of cloned inserts are indicated as magenta.

Source: Invitrogen (Carlsbad, CA)
http://www.invitrogen.com/content/sfs/vectors/ptrchis2topo_seq.txt

Appendix D

Complementary Data for Chapters 3 to 8

D.1 Chapter 3

Table D.1: Determination of SLID for aerosol challenge with Tohama I.

Group	Mouse	CFU/lung	log10 CFU/lung
1	1	3.35E+05	5.525044807
	2	2.15E+05	5.33243846
	3	2.45E+05	5.389166084
	4	3.30E+05	5.51851394
	5	1.25E+05	5.096910013
	6	1.65E+05	5.217483944
2	1	5.25E+05	5.720159303
	2	1.15E+06	6.058805487
	3	6.60E+05	5.819543936
	4	4.95E+05	5.694605199
	5	3.00E+05	5.477121255
	6	3.00E+05	5.477121255
3	1	1.50E+06	6.176091259
	2	8.25E+05	5.916453949
	3	7.95E+05	5.900367129
	4	9.40E+05	5.973127854
	5	1.35E+06	6.130333768
	6	1.08E+06	6.031408464
	7	6.95E+05	5.841984805
4	1	1.16E+06	6.064457989
	2	5.00E+05	5.698970004
	3	1.34E+06	6.125481266
	4	9.55E+05	5.980003372
	5	1.13E+06	6.053078443
	6	9.05E+05	5.956648579
	7	1.73E+06	6.238046103
	8	1.36E+06	6.131939295
5	1	0	0
	2	0	0
	3	0	0
	4	0	0

D.2 Chapter 4

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   1 GAATTCCTGC GCTGGCACCC GCGGCGGGCC GGGGAGCGGG TTGTCGGCGC
  51 ACGCCTATAC GTGCCGGACA GGGTTTGATG GTTTGACTAA GAAATTTCCT
 101 ACAAGTCTTG TATAAATATC CATTGATGGA CGGGATCATT ACTGACTGAC
 151 GAAGTGCTGA GGTTTATCCA GACTATGGCA CTGGATTTCA AAACCTAAAA
 201 CGAGCAGGCC GATAACGGAT TCTGCCGATT ACTTCACTTC GCTGGTCGGA
 251 ATATGAACAC GAACCTGTAC AGGCTGGTCT TCAGCCATGT TCGCGGCATG
 301 CTTGTTCCCG TGAGCGAGCA TTGCACCGTC GGAAACACCT TCTGTGGGCG
 351 CACGCGTGGT CAAGCGCGAA GTGGGGCCCG CGCCACGAGC CTGTCCGTAG
 401 CGCCCAATGC GCTGGCCTGG GCCCTGATGT TGGCGTGTAC GGGTCTTCCG
 451 TTAGTAACGC ACGCCCAGGG CTTGGTTCCT CAGGGGCAGA CACAGGTGCT
 501 GCAGGGCGGG AACAAGGTTC CCGTTGTCAA TATCGCCGAC CCAAATTCCG
 551 GCGGCGTCTC GCACAACAAG TTCCAGCAGT TCAACGTCGC CAACCCTGGC
 601 GTGGTCTTCA ACAACGGCCT GACCGACGGC GTGTCCAGGA TCGGCGGGGC
 651 GCTGACCAAG AACCCCAACC TGACTCGCCA GGCCTCGGCC ATTCTTGCCG
 701 AAGTCACGGA CACTTCGCCC AGTCGCCTGG CCGGTACGCT CGAAGTCTAT
 751 GGCAAGGGCG CCGACCTCAT CATCGCCAAC CCCAACGGCA TCAGCGTCAA
 801 CGGCCTGAGC ACGCTCAACG CCAGCAACCT GACGCTCACG ACGGGGCGTC
 851 CCAGCGTCAA CGGCGGCCGC ATCGGCCTTG ATGTCCAACA GGGCACCGTC
 901 ACGATCGAAC GAGGCGGCGT CAATGCCACC GGCCTGGGCT ATTTCGACGT
 951 GGTGGCGCGC CTGGTCAAGC TGCAGGGTGC CGTGTCGAGC AAGCAGGGCA
1001 AGCCCCTGGC CGACATCGCG GTGGTCGCCG GCGCCAACCG GTACGACCAC
1051 GCAACCCGCC GCGCCACGCC GATCGCCGCA GGCGCGCGCG GCGCCGCCGC
1101 GGGCGCCTAC GCGATTGACG GCACGGCGGC GGGCGCCATG TACGGCAAGC
1151 ACATCACGCT GGTGTCCAGC GATTCAGGCC TGGGCGTGCG CCAGCTCGGC
1201 AGCCTGTCCT CGCCATCGGC CATCACCGTG TCGTCGCAGG GCGAAATCGC
1251 GCTGGGCGAC GCCACGGTCC AGCGCGGCCC GCTCAGCCTC AAGGGCGCGG
1301 GGGTCGTGTC GGCCGGCAAA CTGGCCTCCG GGGGGGGGGC GGTGAACGTC
1351 GCGGGCGGCG GGGCGGTGAA GATCGCGTCG GCCAGCAGCG TTGGAAACCT
1401 CGCGGTGCAA GGCGGCGGCA AGGTACAGGC CACGCTGTTG AATGCCGGGG
1451 GGACGTTGCT GGTGTCGGGC CGCCAGGCCG TCCAGCTTGG CGCGGCGAGC
1501 AGCCGTCAGG CGCTGTCCGT GAACGCGGGC GGCGCCCTCA AGGCGGACAA
1551 GCTGTCGGCG ACGCGACGGG TCGACGTGGA TGGCAAGCAG GCCGTCGCGC
1601 TGGGGTCGGC CAGCAGCAAT GCGCTGTCGG TGCGTGCCGG CGGCGCCCTC
1651 AAGGCGGGCA AGCTGTCGGC GACGGGGCGA CTGGACGTGG ACGGCAAGCA
1701 GGCCGTCACG CTGGGTTCGG TTGCGAGCGA CGGTGCGCTG TCGGTAAGCG
1751 CTGGCGGAAA CCTGCGGGCG AACGAATTGG TCTCCAGTGC CCAACTTGAG
1801 GTGCGTGGGC AGCGGGAGGT CGCGCTGGAT GACGCTTCGA GCGCACGCGG
1851 CATGACCGTG GTTGCCGCAG GAGCGCTGGC GGCCCGCAAC CTGCAGTCCA
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1901 AGGGCGCCAT CGGCGTACAG GGTGGAGAGG CGGTCAGCGT GGCCAACGCG
1951 AACAGCGACG CGGAATTGCG CGTGCGCGGG CGCGGCCAGG TGGATCTGCA
2001 CGACCTGAGC GCAGCGCGCG GCGCGGATAT CTCCGGCGAG GGGCGCGTCA
2051 ATATCGGCCG TGCGCGCAGC GATAGCGATG TGAAGGTCTC CGCGCACGGC
2101 GCCTTGTCGA TCGATAGCAT GACGGCCCTC GGTGCGATCG GCGTCCAGGC
2151 AGGCGGCAGC GTGTCGGCCA AGGATATGCG CAGCCGTGGC GCCGTCACCG
2201 TCAGCGGCGG CGGCGCCGTC AACCTGGGCG ATGTCCAGTC GGATGGGCAG
2251 GTCCGCGCCA CCAGCGCGGG CGCCATGACG GTGCGAGACG TCGCGGCTGC
2301 CGCCGACCTT GCGCTGCAGG CGGGCGACGC GTTGCAGGCC GGGTTCCTGA
2351 AATCGGCCGG TGCCATGACC GTGAACGGCC GCGATGCCGT GCGACTGGAT
2401 GGCGCGCACG CGGGCGGGCA ATTGCGGGTT TCCAGCGACG GGCAGGCTGC
2451 GTTGGGCAGT CTCGCGGCCA AGGGCGAGCT GACGGTATCG GCCGCGCGCG
2501 CGGCGACCGT GGCCGAGTTG AAGTCGCTGG ACAACATCTC CGTGACGGGC
2551 GGCGAACGCG TGTCGGTTCA GAGCGTCAAC AGCGCGTCCA GGGTCGCCAT
2601 TTCGGCGCAC GGCGCGCTGG ATGTAGGCAA GGTTTCCGCC AAGAGCGGTA
2651 TCGGGCTCGA AGGCTGGGGC GCGGTCGGAG CGGACTCCCT CGGTTCCGAC
2701 GGCGCGATCA GCGTGTCCGG GCGCGATGCG GTCAGGGTCG ATCAAGCCCG
2751 CAGTCTTGCC GACATTTCGC TGGGGGCGGA AGGCGGCGCC ACGCTGGGCG
2801 CGGTGGAGGC CGCCGGTTCG ATCGACGTGC GCGGCGGATC CACGGTGGCG
2851 GCGAACTCGC TGCACGCCAA TCGCGACGTT CGGGTCAGCG GCAAGGATGC
2901 GGTGCGCGTA ACGGCCGCCA CCAGCGGGGG CGGTCTGCAT GTGTCGAGCG
2951 GCCGCCAGCT CGATCTGGGC GCCGTGCAGG CGCGCGGCGC GCTGGCCCTG
3001 GACGGAGGCG CCGGCGTGGC GCTGCAATCG GCCAAGGCTA GCGGCACGCT
3051 GCATGTGCAG GGCGGCGAGC ACCTGGACCT GGGCACGTTG GCCGCCGTAG
3101 GGGCGGTGGA CGTCAATGGC ACGGGAGACG TGCGCGTTGC GAAGCTGGTG
3151 AGCGATGCAG GCGCCGATCT GCAAGCGGGG CGCTCCATGA CGCTGGGTAT
3201 CGTCGACACG ACCGGCGATC TGCAGGCGCG CGCGCAGCAG AAGCTGGAGC

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3251 TCGGGTCG**GT TAAGAGCGAT GGCGGCCTTC AG**GCGGCCGC CGGCGGGGCC
3301 CTCAGCCTGG CGGCGGCGGA AGTCGCAGGG GCGCTGGAGC TCTCGGGCCA
3351 GGGCGTCACC GTGGACAGAG CCAGCGCTAG CCGGGCACGC ATCGACAGCA
3401 CCGGTTCGGT CGGCATCGGC GCGCTGAAGG CAGGCGCTGT CGAGGCCGCC
3451 TCGCCACGGC GGGCGCGCCG CGCGCTGCGG CAGGATTTCT TCACGCCCGG
3501 CAGCGTGGTG GTCCGCGCCC AGGGCAATGT CACGGTCGGG CGCGGCGATC

FHAB3577DNAF→

3551 CGCATCAGGG CGTGCTGGCC CAGGGC**GACA TCATCATGGA TGCGAAG**GGC
3601 GGCACCTTGC TGTTGCGCAA CGATGCCTTG ACCGAGAACG GGACGGTCAC
3651 CATATCGGCC GATTCGGCCG TGCTCGAGCA TTCCACCATC GAGAGCAAGA
3701 TCAGCCAGAG CGTGCTGGCT GCCAAAGGGG ACAAGGGCAA GCCGGCGGTG
3751 TCGGTGAAG**G TCGCGAAGAA GCTGTTTCTC** AATGGTACGT TGCGGGCCGT

3801 CAACGACAAC AACGAAACCA TGTCCGGGCG CCAGATCGAC GTCGTGGACG
3851 GACGTCCGCA GATCACCGAC GCGGTCACGG GCGAAGCGCG TAAGGACGAA
3901 TCGGTTGTGT CCGACGCCGC GCTCGTGGCC GATGGCGGTC CGATCGTGGT
3951 CGAGGCCGGC GAGCTGGTCA GCCATGCCGG CGGTATCGGC AACGGCCGCA
4001 ACAAGGAGAA TGGCGCCAGC GTCACCGTGC GCACGACTGG CAACCTGGTC
4051 AACAAGGGCT ACATCTCGGC CGGCAAGCAG GGCGTGCTCG AGGTGGGCGG
4101 CGCCTTGACG AACGAGTTCC TGGTCGGCTC GGACGGCACC CAGCGCATCG
4151 AGGCGCAGCG CATCGAGAAC AGGGGCACCT TCCAGAGCCA GGCTCCGGCG
4201 GGCACGGCCG GCGCCCTGGT GGTCAAGGCT GCCGAGGCCA TCGTGCACGA
4251 CGGCGTCATG GCC**ACCAAAG GCGAGATGCA GATC**GCCGGC AAGGGCGGCG
4301 GGTCTCCGAC CGTCACCGCC GGCGCAAAGG CGACGACCAG CGCGAACAAG
4351 CTGAGCGTCG ACGTGGCAAG CTGGGACAAC GCGGGAAGCC TGGATATCAA
4401 GAAGGGCGGC GCGCAGGTCA CGGTGGCCGG GCGCTATGCC GAGCACGGCG
4451 AGGTTTCGAT ACAGGGCGAT TACACCGTCT CGGCCGACGC CATCGCGCTG
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4601 ACGCCGGCGG GCCGGTGTCC AATACCGGCA ATCTGAAAGT GCGCGAGGGC
4651 GTGACCGTAA CGGCGGCGTC GTTCGACAAC GAGACCGGGG CCGAGGTCAT
4701 GGCCAAGAGC GCCACGCTGA CGACTTCCGG GGCCGCGCGC **AACGCGGGCA**
4751 **AGATGCAGGT C**AAGGAGGCC GCCACGATCG TTGCCGCCAG CGTTTCCAAT
4801 CCCGGCACGT TCACGGCCGG CAAGGATATC ACTGTTACCT CGCGCGGAGG
4851 ATTCGATAAC GAAGGCAAGA TGGAGTCCAA CAAGGACATC GTCATCAAGA
4901 CGGAACAGTT CAGCAATGGC AGGGTTCTCG ACGCCAAGCA TGATCTGACG
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5051 CCGGCCACGA CGCCACGCTG AAGGCGCCGC ACCTGCGCAA TACGGGCCAG
5101 GTCGTAGCCG GGCACGACAT CCATATCATC AACAGCGCCA AGCTGGAGAA
5151 CACCGGGCGC GTGGATGCGC GCAACGACAT CGCTCTGGAT GTGGCG**GATT**
5201 **TCACCAACAC GGGATCC**CTC TACGCCGAGC ATGACGCGAC GCTGACGCTT
5251 GCGCAAGGCA CGCAGCGCGA TCTGGTGGTG GACCAGGATC ATATCCTGCC
5301 GGTGGCGGAG GGGACGTTAC GCGTCAAGGC CAAGTCGCTG ACCACCGAAA
5351 TCGAGACCGG CAATCCCGGC AGCCTGATCG CCGAGGTGCA GGAAAATATC
5401 GACAACAAGC AGGCCATCGT CGTCGGCAAG GACCTGACGC TGAGTTCGGC
5451 GCACGGCAAC GTGGCCAACG AAGCGAACGC GCTGCTGTGG GCCGCCGGGG
5501 AGCTGACCGT CAAGGCGCAG AACATCACCA ATAAACGGGC CGCGCTGATC
5551 GAGGCGGGCG GCAACGCCCG GCTGACGGCG GCCGTTGCCT TGCTCAACAA
5601 GCTGGGCCGC ATTCGCGCGG GCGAGGACAT GCACCTGGAT GCGCCGCGCA
5651 TCGAGAACAC CGCGAAACTG AGCGGCGAGG TGCAACGCAA AGGCGTGCAG

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5701 GACGTCGGGG GAGGCGAGCA CGGCCGCTGG AGCGGT**ATCG GCTATGTCAA**

5751 **CTACTGGTTG** CGCGCCGGCA ATGGGAAGAA GGCGGGAACC ATCGCCGCGC
5801 CGTGGTATGG CGGTGATCTG ACGGCGGAGC AGTCGCTCAT CGAGGTCGGC
5851 AAGGATCTCT ATCTGAATGC CGGAGCGCGC AAGGACGAAC ATCGCCATCT
5901 GCTCAATGAA GGCGTGATCC AGGCGGGCGG CCATGGCCAC ATCGGCGGCG
5951 ACGTGGACAA CCGGTCGGTG GTGCGCACCG TGTCCGCCAT GGAGTATTTC
6001 AAGACGCCTC TTCCGGTGAG CCTGACTGCC CTGGACAATC GTGCCGGCTT
6051 GTCTCCGGCG ACCTGGAACT TCCAGTCCAC GTATGAACTC CTGGATTATC
6101 TGCTGGACCA GAATCGCTAC GAGTACATTT GGGGGCTGTA TCCGACCTAC
6151 ACCGAATGGT CGGTGAATAC GCTGAAGAAC CTCGACCTGG GCTACCAGGC
6201 CAAGCCGGCT CCCACTGCGC CGCCGATGCC CAAGGCTCCC GAACTCGACC
6251 TGCGTGGCCA TACGCTGGAG TCGGCCGAAG GCCGG**AAGAT CTTTGGCGAG**
6301 **TACAAGAAGC TG**CAAGGCGA GTACGAGAAG GCGAAGATGG CCGTCCAGGC
6351 CGTGGAGGCT TACGGCGAGG CTACTCGGCG CGTCCATGAT CAGCTGGGCC
6401 AACGTTATGG TAAGGCCCTG GGCGGCATGG ATGCCGAGAC CAAGGAGGTC
6451 GACGGCATCA TCCAGGAGTT CGCCGCGGAT CTGCGAACGG TCTATGCGAA
6501 GCAGGCCGAC CAGGCGACCA TCGACGCAGA GACGGACAAG GTCGCCCAGC
6551 GCTACAAGTC GCAGATCGAC GCGGTGCGGC TGCAGGCGAT CCAGCCTGGC
6601 CGGGTCACGC TGGCCAAGGC GCTGTCGGCG GCGCTGGGCG CCGACTGGCG
6651 CGCGCTGGGT CACTCCCAAT TGATGCAGCG CTGGAAGGAT TTCAAGGCGG

←FHAB6750R

6701 GCAAGCGCGG CGCGGAAATC GCG**TTCTATC CCAAGGAACA AACCGTGCTG**
6751 GCCGCCGGCG CCGGTTTGAC CCTGTCCAAC GGGGCGATCC ACAACGGCGA
6801 GAACGCCGCG CAGAATCGCG GCCGGCCGGA AGGCCTGAAA ATCGGCGCAC
6851 ATTCGGCGAC TTCGGTGAGC GGCTCGTTCG ACGCCTTGCG CGACGTGGGG
6901 CTGGAAAAGC GGCTGGATAT CGACGATGCG CTGGCTGCC**G TGCTCGTGAA**

←FHAB6966R

6951 **TCCGCATATT TTCACG**CGGA TCGGGGCGGC TCAGACATCC CTTGCCGACG
7001 GCGCCGCCGG GCCGGCGCTG GCGCGCCAGG CCAGGCAAGC GCCGGAGACC
7051 GACGGCATGG TGGATGCGCG AGGGCTGGGC AGCGCCGATG CGCTCGCTTC
7101 CCTGGCCAGC TTGGACGCGG CGCAAGGGCT GGAGGTATCC GGCAGGCGCA
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7251 GGTCGACCAG CCTGTCGTGG CGGTCGGGCT CGAGCAGCCT GTCGCGACGG
7301 TCCGGGTCGC GCCGCCAGCC GTCGCGTTGC CGCGGCCGCT GTTCGAAACC
7351 CGCATCAAGT TTATCGACCA GAGCAAATTC TACGGCTCGC GTTATTTCTT
7401 CGAGCAGATC GGCTACAAGC CCGATCGCGC CGCGCGGGTG GCGGGCGACA
7451 ACTATTTCGA TACCACGCTG GTGCGCGAGC AGGTGCGGCG CGCCCTGGGC
7501 GGCTATGAAA GCCGCCTGCC CGTGCGCGGT GTCGCGTTGG TGGCCAAGCT
7551 GATGGATTCG GCCGGGACGG TCGGCAAGGC GCTGGGCCTG AAGGTGGGTG
7601 TCGCGCCGAC CGCGCAGCAG CTCAAGCAGG CCGACCGCGA TTTCGTCTGG

7651 TACGTGGATA CCGTGATCGA CGGCCAGAAG GTTCTCGCTC CCCGGCTGTA
7701 CCTGACCGAG GCGACGCGCC AGGGCATCAC GGATCAGTAC GCCGGCGGCG
7751 GGGCGCTGAT TGCCTCCGGC GGCGACGTAA CTGTCAATAC GGACGGCCAT
7801 GACGTCAGTT CGGTCAACGG GCTGATCCAG GGCAGGAGCG TCAAGGTGGA
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7901 GCATCGAGGC CGATGACGAG GTCGACGTCT CAGGCCGGGA TATCGGCATC
7951 GAGGGCGGCA AGCTGCGCGG CAAGGATGTC AGGCTCAAGG CCGACACGGT
8001 CAAGGTCGCG ACCTCGATGC GTTACGACGA CAAGGGCAGG CTGGCGGCGC
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8101 AAGCGCCTGG AGACGGCCGG CGCGACGCTC AAGGGCGGCA AGGTGAAGCT
8151 GGATGTCGAT GACGTCAAGT TGGGCGGCGT GTACGAGGCG GGGTCCAGCT
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8301 CATCGAAGCC GGTACGCTGG AAGGAAAGAT GCAGAACCTG GAGATCGAAG
8351 GCGGTTCGGT CGATGCCGCG CATACGGACC TGTCCGTGGC CCGCGACGCG
8401 AGGTTCAAGG CCGCCGCGGA TTTCGCGCAC GCCGAGCATG AGAAGGATGT
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8501 GCTTCAGCCT GGGCAGCGAA AGCGGTCTGG AAGCGCACGC CGGCCGCGGT
8551 ATGACCGCGG GCGCTGAAGT CAAGGTAGGT TATCGGGCAT CGCACGAACA
8601 GTCCTCGGAA ACCGAAAAGT CCTATCGCAA CGCGAACCTC AATTTCGGTG
8651 GAGGCTCCGT CGAGGCTGGC AATGTCCTGG ATATCGGCGG CGCCGACATC
8701 AACCGGAACC GGTACGGCGG CGCCGCGAAG GGGAACGCCG GGACCGAGGA
8751 GGCCTTGCGC ATGCGCGCCA AGAAGGTCGA GTCCACCAAG TACGTCAGCG
8801 AGCAGACGAG CCAGAGCTCC GGCTGGAGCG TGGAGGTGGC ATCGACGGCC
8851 AGTGCCCGTT CCAGCCTGCT GACGGCCGCC ACGCGCCTGG GCGACAGCGT
8901 GGCGCAGAAT GTCGAGGACG GCCGCGAGAT CCGCGGCGAG CTGATGGCTG
8951 CGCAAGTCGC CGCGGAGGCC ACGCAACTGG TAACCGCCGA CACGGCGGCG
9001 GTGGCACTGA GTGCCGGAAT CAGCGCCGAC TTCGACAGCA GCCACAGCCG
9051 CTCCACCTCG CAGAATACCC AATATCTGGG CGGAAACTTG TCCATCGAGG
9101 CCACCGAGGG CGATGCGACG CTGGTGGGCG CGAAGTTCGG CGGTGGCGAC
9151 CAGGTCAGCT TGAAGGCAGC GAAGAGCGTG AACCTCATGG CGGCCGAATC
9201 GACCTTCGAA TCGTACTCGG AGAGCCACAA CTTCCACGCC TCCGCCGACG
9251 CGAACCTTGG CGCCAACGCC GTGCAGGGCG CCGTTGGCCT GGGGTTGACT
9301 GCGGGTATGG GGACGTCGCA TCAGATTACC AACGAAACCG GCAAGACCTA
9351 TGCCGGAACC TCGGTGGATG CGGCGAACGT GTCGATCGAT GCAGGCAAGG
9401 ATCTGAACCT TTCCGGGTCC CGCGTGCGGG GTAAGCATGT TGTCCTGGAT
9451 GTCGAGGGCG ATATCAATGC GACCAGCAAG CAGGATGAAC GCAACTACAA
9501 CTCCAGCGGT GGCGGTTGGG ACGCCTCGGC AGGGGTGGCG ATTCAGAACC
9551 GCACGTTGGT TGCGCCCGTG GGGTCTGCCG GCTTCAATTT CAATACGGAA
9601 CACGACAATT CGCGCCTGAC CAATGACGGG GCGGCGGGTG TCGTTGCCAG

9651 CGACGGGTTG ACGGGCCATG TGAAAGGCGA CGCCAACCTG ACCGGCGCGA
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9751 AACGCGCAGA ACCTGAAAGA CTACCGCGAC AAGGACGGCG GCAGCGGCGG
9801 CCTGAACGTG GGCATCTCGT CGACCACGCT GGCGCCCACC GTGGGCGTGG
9851 CGTTCGGCAG GGTGGCCGGA GAGGATTATC AGGCCGAGCA GCGCGCCACG
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10051 TCACTGAAGA AGAAGAACCA GGTCCGCCCG GTGGAGACGC CGACGCCGGA
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10401 GGTCCAGCCC CAGCTTGCCA AGGTGGAGAC GGTGCAGCCG GTGAAGCCCG
10451 AAACCACCAA GCCGTTGCCC AAGCCGCTGC CGGTGGCGAA GGTGACGAAA
10501 GCGCCGCCGC CGGTTGTGGA GACCGCCCAG CCGCTGCCGC CGGTCAAGCC
10551 ACAGAAGGCG ACCCCCGGCC CCGTGGCTGA GGTGGGCAAG GCTACGGTCA
10601 CGACGGTGCA GGTGCAGAGT GCGCCGCCCA AGCCGGCCCC GGTGGCCAAG
10651 CAGCCCGCGC CTGCACCGAA GCCCAAGCCC AAGCCCAAGC CCAAGGCCGA
10701 GCGTCCGAAG CCGGGCAAAA CGACGCCCTT GAGCGGGCGC CACGTGGTGC
10751 AACAGCAGGT GCAGGTCTTG CAGCGGCAAG CGAGTGACAT CAACAACACC
10801 AAGAGCCTGC CTGGCGGGAA GCTGCCCAAG CCGGTCACCG TGAAGCTGAC
10851 CGACGAGAAC GGCAAGCCGC AGACGTATAC GATCAACCGG CGCGAGGATC
10901 TGATGAAGCT CAACGGCAAG GTGCTGTCCA CCAAGACGAC ACTGGGCCTG
10951 GAGCAGACCT TCCGCCTGCG GTCGAGGATA TCGGCGGCAA GAACTACCGG
11001 GTCTTCTATG AAACCAACAA ATAGGTAGTC GCGGCCTGCC GCGGCTCGGC
11051 GCATGGGGAT TCGCAGGGTT CTCATGCGCC GGCCAATGCC GGATAGCGGT
11101 GCAATTGCCG ACCATTTCGC GCACCGCGCT CAAGGACGTA GGGTCGACGG
11151 CAGGCGGGAC AGTTTTTGAC GTGAAACTGA CCGAGTGTCC GCAGGCATTG
11201 AATGGTCAGC AAGTGGGATT GTTCTTCGAA TCTGGTGGCA CGGTTGACTA
11251 TACGTCGGGA AACCTGTTTG CGTATCGGGC CGATAGTCAG GGCGTCGAAC
11301 AGGCTACCGC AGAGCGAAAG CCGACAACGT GCAAGCCAAT CTGGATGGTT
11351 CCGCTATTCA TTTGGGCCGC AACAAGGGTG CGCAGGCTGC TCAGACGTTT
11401 CTGGTATCGC AGACGGCTGG GTCGTCGACG TACGGGGCGA CCCTGCGCTA
11451 TCTGGCATGC TACATCCGTT CGGGCGCTGG TTCCATTGTT GCGGGGAATC
11501 TCCGCAGTCA GGTGGGGTTC TCCGTGATGT ATCCGTAGCC CGTGAAAGAG
11551 GGGTCACCCA CTGCGGGGGG CCCCGGTACG GGATGGTCGG CTTGTCACGA
11601 GATTCTTGTT TTCCATTTCT TTCTTTTCAC TCGGTCGCAG CGCCGGCTTG

11651 ATGCATGCAA AGCATCGATA GCTACGAACG GCCGCGATTC TTGAATCATG

11701 AATACATACG CTTGTGACGG GGCGCTCGCG AGAGCCGGCC CCAGGGATGG

11751 TTTACGCCTG CATTTACGGT AAAGCGGCAA GGCGGCATGG CGCGCTGGCG

11801 GCGGCTGGGC GTCGCGGCGC TGGGCCATGC

Figure D.1: Genbank sequence of *fhaB* (accession # M60351.1). PCR primer sites are highlighted in blue and sequencing primer sites are highlighted or underlined in magenta.

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              3481      3491      3501      3511      3521      3531
BPEFHAB1      CAGGATTTCTTCACGCCCGGCAGCGTGGTGGTCCGCGCCCAGGGCAATGTCACGGTCGGG
fhaB1_pcDN    ------------------------------------------------------------
fhaB2_pcDN    ------------------------------------------------------------

              3541      3551      3561      3571      3581      3591
BPEFHAB1      CGCGGCGATCCGCATCAGGGCGTGCTGGCCCAGGGCGACATCATCATGGATGCGAAGGGC
fhaB1_pcDN    ---------------------------CACCATGG--ACATCATCATGGATGCGAAGGGC
fhaB2_pcDN    ------------------------------------------------------------

              3601      3611      3621      3631      3641      3651
BPEFHAB1      GGCACCTTGCTGTTGCGCAACGATGCCTTGACCGAGAACGGGACGGTCACCATATCGGCC
fhaB1_pcDN    GGCACCTTGCTGTTGCGCAACGATGCCTTGACCGAGAACGGGACGGTCACCATATCGGCC
fhaB2_pcDN    ------------------------------------------------------------

              3661      3671      3681      3691      3701      3711
BPEFHAB1      GATTCGGCCGTGCTCGAGCATTCCACCATCGAGAGCAAGATCAGCCAGAGCGTGCTGGCT
fhaB1_pcDN    GATTCGGCCGTGCTCGAGCATTCCACCATCGAGAGCAAGATCAGCCAGAGCGTGCTGGCT
fhaB2_pcDN    ------------------------------------------------------------

              3721      3731      3741      3751      3761      3771
BPEFHAB1      GCCAAAGGGGACAAGGGCAAGCCGGCGGTGTCGGTGAAGGTCGCGAAGAAGCTGTTTCTC
fhaB1_pcDN    GCCAAAGGGGACAAGGGCAAGCCGGCGGTGTCGGTGAAGGTCGCGAAGAAGCTGTTTCTC
fhaB2_pcDN    ------------------------------------------------------------

              3781      3791      3801      3811      3821      3831
BPEFHAB1      AATGGTACGTTGCGGGCCGTCAACGACAACAACGAAACCATGTCCGGGCGCCAGATCGAC
fhaB1_pcDN    AATGGTACGTTGCGGGCCGTCAACGACAACAACGAAACCATGTCCGGGCGCCAGATCGAC
fhaB2_pcDN    ------------------------------------------------------------

              3841      3851      3861      3871      3881      3891
BPEFHAB1      GTCGTGGACGGACGTCCGCAGATCACCGACGCGGTCACGGGCGAAGCGCGTAAGGACGAA
fhaB1_pcDN    GTCGTGGACGGACGTCCGCAGATCACCGACGCGGTCACGGGCGAAGCGCGTAAGGACGAA
fhaB2_pcDN    ------------------------------------------------------------

              3901      3911      3921      3931      3941      3951
BPEFHAB1      TCGGTTGTGTCCGACGCCGCGCTCGTGGCCGATGGCGGTCCGATCGTGGTCGAGGCCGGC
fhaB1_pcDN    TCGGTTGTGTCCGACGCCGCGCTCGTGGCCGATGGCGGTCCGATCGTGGTCGAGGCCGGC
fhaB2_pcDN    ------------------------------------------------------------

              3961      3971      3981      3991      4001      4011
BPEFHAB1      GAGCTGGTCAGCCATGCCGGCGGTATCGGCAACGGCCGCAACAAGGAGAATGGCGCCAGC
fhaB1_pcDN    GAGCTGGTCAGCCATGCCGGCGGTATCGGCAACGGCCGCAACAAGGAGAATGGCGCCAGC
fhaB2_pcDN    ------------------------------------------------------------

              4021      4031      4041      4051      4061      4071
BPEFHAB1      GTCACCGTGCGCACGACTGGCAACCTGGTCAACAAGGGCTACATCTCGGCCGGCAAGCAG
fhaB1_pcDN    GTCACCGTGCGCACGACTGGCAACCTGGTCAACAAGGGCTACATCTCGGCCGGCAAGCAG
fhaB2_pcDN    ------------------------------------------------------------

              4081      4091      4101      4111      4121      4131
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BPEFHAB1      GGCGTGCTCGAGGTGGGCGGCGCCTTGACGAACGAGTTCCTGGTCGGCTCGGACGGCACC
fhaB1_pcDN    GGCGTGCTCGAGGTGGGCGGCGCCTTGACGAACGAGTTCCTGGTCGGCTCGGACGGCACC
fhaB2_pcDN    ------------------------------------------------------------

              4141      4151      4161      4171      4181      4191
BPEFHAB1      CAGCGCATCGAGGCGCAGCGCATCGAGAACAGGGGCACCTTCCAGAGCCAGGCTCCGGCG
fhaB1_pcDN    CAGCGCATCGAGGCGCAGCGCATCGAGAACAGGGGCACCTTCCAGAGCCAGGCTCCGGCG
fhaB2_pcDN    ------------------------------------------------------------

              4201      4211      4221      4231      4241      4251
BPEFHAB1      GGCACGGCCGGCGCCCTGGTGGTCAAGGCTGCCGAGGCCATCGTGCACGACGGCGTCATG
fhaB1_pcDN    GGCACGGCCGGCGCCCTGGTGGTCAAGGCTGCCGAGGCCATCGTGCACGACGGCGTCATG
fhaB2_pcDN    ------------------------------------------------------------

              4261      4271      4281      4291      4301      4311
BPEFHAB1      GCCACCAAAGGCGAGATGCAGATCGCCGGCAAGGGCGGCGGGTCTCCGACCGTCACCGCC
fhaB1_pcDN    GCCACCAAAGGCGAGATGCAGATCGCCGGCAAGGGCGGCGGGTCTCCGACCGTCACCGCC
fhaB2_pcDN    ------------------------------------------------------------

              4321      4331      4341      4351      4361      4371
BPEFHAB1      GGCGCAAAGGCGACGACCAGCGCGAACAAGCTGAGCGTCGACGTGGCAAGCTGGGACAAC
fhaB1_pcDN    GGCGCAAAGGCGACGACCAGCGCGAACAAGCTGAGCGTCGACGTGGCAAGCTGGGACAAC
fhaB2_pcDN    ------------------------------------------------------------

              4381      4391      4401      4411      4421      4431
BPEFHAB1      GCGGGAAGCCTGGATATCAAGAAGGGCGGCGCGCAGGTCACGGTGGCCGGGCGCTATGCC
fhaB1_pcDN    GCGGGAAGCCTGGATATCAAGAAGGGCGGCGCGCAGGTCACGGTGGCCGGGCGCTATGCC
fhaB2_pcDN    ------------------------------------------------------------

              4441      4451      4461      4471      4481      4491
BPEFHAB1      GAGCACGGCGAGGTTTCGATACAGGGCGATTACACCGTCTCGGCCGACGCCATCGCGCTG
fhaB1_pcDN    GAGCACGGCGAGGTTTCGATACAGGGCGATTACACCGTCTCGGCCGACGCCATCGCGCTG
fhaB2_pcDN    ------------------------------------------------------------

              4501      4511      4521      4531      4541      4551
BPEFHAB1      GCGGCGCAGGTCACCCAGCGCGGAGGCGCCGCGAACCTGACCTCGCGGCACGACACCCGT
fhaB1_pcDN    GCGGCGCAGGTCACCCAGCGCGGAGGCGCCGCGAACCTGACCTCGCGGCACGACACCCGT
fhaB2_pcDN    ------------------------------------------------------------

              4561      4571      4581      4591      4601      4611
BPEFHAB1      TTCTCCAACAAGATTCGCCTGATGGGGCCGTTGCAGGTCAACGCCGGCGGGCCGGTGTCC
fhaB1_pcDN    TTCTCCAACAAGATTCGCCTGATGGGGCCGTTGCAGGTCAACGCCGGCGGGCCGGTGTCC
fhaB2_pcDN    ------------------------------------------------------------

              4621      4631      4641      4651      4661      4671
BPEFHAB1      AATACCGGCAATCTGAAAGTGCGCGAGGGCGTGACCGTAACGGCGGCGTCGTTCGACAAC
fhaB1_pcDN    AATACCGGCAATCTGAAAGTGCGCGAGGGCGTGACCGTAACGGCGGCGTCGTTCGACAAC
fhaB2_pcDN    ------------------------------------------------------------

              4681      4691      4701      4711      4721      4731
BPEFHAB1      GAGACCGGGGCCGAGGTCATGGCCAAGAGCGCCACGCTGACGACTTCCGGGGCCGCGCGC
fhaB1_pcDN    GAGACCGGGGCCGAGGTCATGGCCAAGAGCGCCACGCTGACGACTTCCGGGGCCGCGCGC
fhaB2_pcDN    ------------------------------------------------------------

              4741      4751      4761      4771      4781      4791
BPEFHAB1      AACGCGGGCAAGATGCAGGTCAAGGAGGCCGCCACGATCGTTGCCGCCAGCGTTTCCAAT
fhaB1_pcDN    AACGCGGGCAAGATGCAGGTCAAGGAGGCCGCCACGATCGTTGCCGCCAGCGTTTCCAAT
fhaB2_pcDN    ------------------------------------------------------------

              4801      4811      4821      4831      4841      4851
BPEFHAB1      CCCGGCACGTTCACGGCCGGCAAGGATATCACTGTTACCTCGCGCGGAGGATTCGATAAC
fhaB1_pcDN    CCCGGCACGTTCACGGCCGGCAAGGATATCACTGTTACCTCGCGCGGAGGATTCGATAAC
fhaB2_pcDN    ------------------------------------------------------------

              4861      4871      4881      4891      4901      4911
BPEFHAB1      GAAGGCAAGATGGAGTCCAACAAGGACATCGTCATCAAGACGGAACAGTTCAGCAATGGC
fhaB1_pcDN    GAAGGCAAGATGGAGTCCAACAAGGACATCGTCATCAAGACGGAACAGTTCAGCAATGGC
fhaB2_pcDN    ------------------------------------------------------------
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              4921      4931      4941      4951      4961      4971
BPEFHAB1      AGGGTTCTCGACGCCAAGCATGATCTGACGGTCACGGCGAGCGGGCAGGCGGACAACCGG
fhaB1_pcDN    AGGGTTCTCGACGCCAAGCATGATCTGACGGTCACGGCGAGCGGGCAGGCGGACAACCGG
fhaB2_pcDN    ------------------------------------------------------------

              4981      4991      5001      5011      5021      5031
BPEFHAB1      GGCAGCCTGAAGGCAGGCCACGATTTCACGGTGCAGGCCCAGCGTATCGACAATAGCGGA
fhaB1_pcDN    GGCAGCCTGAAGGCAGGCCACGATTTCACGGTGCAGGCCCAGCGTATCGACAATAGCGGA
fhaB2_pcDN    ------------------------------------------------------------

              5041      5051      5061      5071      5081      5091
BPEFHAB1      ACCATGGCCGCCGGCCACGACGCCACGCTGAAGGCGCCGCACCTGCGCAATACGGGCCAG
fhaB1_pcDN    ACCATGGCCGCCGGCCACGACGCCACGCTGAAGGCGCCGCACCTGCGCAATACGGGCCAG
fhaB2_pcDN    ------------------------------------------------------------

              5101      5111      5121      5131      5141      5151
BPEFHAB1      GTCGTAGCCGGGCACGACATCCATATCATCAACAGCGCCAAGCTGGAGAACACCGGGCGC
fhaB1_pcDN    GTCGTAGCCGGGCACGACATCCATATCATCAACAGCGCCAAGCTGGAGAACACCGGGCGC
fhaB2_pcDN    ------------------------------------------------------------

              5161      5171      5181      5191      5201      5211
BPEFHAB1      GTGGATGCGCGCAACGACATCGCTCTGGATGTGGCGGATTTCACCAACACGGGATCCCTC
fhaB1_pcDN    GTGGATGCGCGCAACGACATCGCTCTGGATGTGGCGGATTTCACCAACACGGGATCCCTC
fhaB2_pcDN    ------------------------------------------------------------

              5221      5231      5241      5251      5261      5271
BPEFHAB1      TACGCCGAGCATGACGCGACGCTGACGCTTGCGCAAGGCACGCAGCGCGATCTGGTGGTG
fhaB1_pcDN    TACGCCGAGCATGACGCGACGCTGACGCTTGCGCAAGGCACGCAGCGCGATCTGGTGGTG
fhaB2_pcDN    ------------------------------------------------------------

              5281      5291      5301      5311      5321      5331
BPEFHAB1      GACCAGGATCATATCCTGCCGGTGGCGGAGGGGACGTTACGCGTCAAGGCCAAGTCGCTG
fhaB1_pcDN    GACCAGGATCATATCCTGCCGGTGGCGGAGGGGACGTTACGCGTCAAGGCCAAGTCGCTG
fhaB2_pcDN    ------------------------------------------------------------

              5341      5351      5361      5371      5381      5391
BPEFHAB1      ACCACCGAAATCGAGACCGGCAATCCCGGCAGCCTGATCGCCGAGGTGCAGGAAAATATC
fhaB1_pcDN    ACCACCGAAATCGAGACCGGCAATCCCGGCAGCCTGATCGCCGAGGTGCAGGAAAATATC
fhaB2_pcDN    ------------------------------------------------------------

              5401      5411      5421      5431      5441      5451
BPEFHAB1      GACAACAAGCAGGCCATCGTCGTCGGCAAGGACCTGACGCTGAGTTCGGCGCACGGCAAC
fhaB1_pcDN    GACAACAAGCAGGCCATCGTCGTCGGCAAGGACCTGACGCTGAGTTCGGCGCACGGCAAC
fhaB2_pcDN    ------------------------------------------------------------

              5461      5471      5481      5491      5501      5511
BPEFHAB1      GTGGCCAACGAAGCGAACGCGCTGCTGTGGGCCGCCGGGGAGCTGACCGTCAAGGCGCAG
fhaB1_pcDN    GTGGCCAACGAAGCGAACGCGCTGCTGTGGGCCGCCGGGGAGCTGACCGTCAAGGCGCAG
fhaB2_pcDN    ------------------------------------------------------------

              5521      5531      5541      5551      5561      5571
BPEFHAB1      AACATCACCAATAAACGGGCCGCGCTGATCGAGGCGGGCGGCAACGCCCGGCTGACGGCG
fhaB1_pcDN    AACATCACCAATAAACGGGCCGCGCTGATCGAGGCGGGCGGCAACGCCCGGCTGACGGCG
fhaB2_pcDN    ------------------------------------------------------------

              5581      5591      5601      5611      5621      5631
BPEFHAB1      GCCGTTGCCTTGCTCAACAAGCTGGGCCGCATTCGCGCGGGCGAGGACATGCACCTGGAT
fhaB1_pcDN    GCCGTTGCCTTGCTCAACAAGCTGGGCCGCATTCGCGCGGGCGAGGACATGCACCTGGAT
fhaB2_pcDN    ------------------------------------------------------------

              5641      5651      5661      5671      5681      5691
BPEFHAB1      GCGCCGCGCATCGAGAACACCGCGAAACTGAGCGGCGAGGTGCAACGCAAAGGCGTGCAG
fhaB1_pcDN    GCGCCGCGCATCGAGAACACCGCGAAACTGAGCGGCGAGGTGCAACGCAAAGGCGTGCAG
fhaB2_pcDN    ------------------------------------------------------------
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              5701      5711      5721      5731      5741      5751
BPEFHAB1      GACGTCGGGGGAGGCGAGCACGGCCGCTGGAGCGGTATCGGCTATGTCAACTACTGGTTG
fhaB1_pcDN    GACGTCGGGGGAGGCGAGCACGGCCGCTGGAGCGGTATCGGCTATGTCAACTACTGGTTG
fhaB2_pcDN    --------------------------------CACCATGGGCTATGTCAACTACTGGTTG

              5761      5771      5781      5791      5801      5811
BPEFHAB1      CGCGCCGGCAATGGGAAGAAGGCGGGAACCATCGCCGCGCCGTGGTATGGCGGTGATCTG
fhaB1_pcDN    CGCGCCGGCAATGGGAAGAAGGCGGGAACCATCGCCGCGCCGTGGTATGGCGGTGATCTG
fhaB2_pcDN    CGCGCCGGCAATGGGAAGAAGGCGGGAACCATCGCCGCGCCGTGGTATGGCGGTGATCTG

              5821      5831      5841      5851      5861      5871
BPEFHAB1      ACGGCGGAGCAGTCGCTCATCGAGGTCGGCAAGGATCTCTATCTGAATGCCGGAGCGCGC
fhaB1_pcDN    ACGGCGGAGCAGTCGCTCATCGAGGTCGGCAAGGATCTCTATCTGAATGCCGGAGCGCGC
fhaB2_pcDN    ACGGCGGAGCAGTCGCTCATCGAGGTCGGCAAGGATCTCTATCTGAATGCCGGAGCGCGC

              5881      5891      5901      5911      5921      5931
BPEFHAB1      AAGGACGAACATCGCCATCTGCTCAATGAAGGCGTGATCCAGGCGGGCGGCCATGGCCAC
fhaB1_pcDN    AAGGACGAACATCGCCATCTGCTCAATGAAGGCGTGATCCAGGCGGGCGGCCATGGCCAC
fhaB2_pcDN    AAGGACGAACATCGCCATCTGCTCAATGAAGGCGTGATCCAGGCGGGCGGCCATGGCCAC

              5941      5951      5961      5971      5981      5991
BPEFHAB1      ATCGGCGGCGACGTGGACAACCGGTCGGTGGTGCGCACCGTGTCCGCCATGGAGTATTTC
fhaB1_pcDN    ATCGGCGGCGACGTGGACAACCGGTCGGTGGTGCGCACCGTGTCCGCCATGGAGTATTTC
fhaB2_pcDN    ATCGGCGGCGACGTGGACAACCGGTCGGTGGTGCGCACCGTGTCCGCCATGGAGTATTTC

              6001      6011      6021      6031      6041      6051
BPEFHAB1      AAGACGCCTCTTCCGGTGAGCCTGACTGCCCTGGACAATCGTGCCGGCTTGTCTCCGGCG
fhaB1_pcDN    AAGACGCCTCTTCCGGTGAGCCTGACTGCCCTGGACAATCGTGCCGGCTTGTCTCCGGCG
fhaB2_pcDN    AAGACGCCTCTTCCGGTGAGCCTGACTGCCCTGGACAATCGTGCCGGCTTGTCTCCGGCG

              6061      6071      6081      6091      6101      6111
BPEFHAB1      ACCTGGAACTTCCAGTCCACGTATGAACTCCTGGATTATCTGCTGGACCAGAATCGCTAC
fhaB1_pcDN    ACCTGGAACTTCCAGTCCACGTATGAACTCCTGGATTATCTGCTGGACCAGAATCGCTAC
fhaB2_pcDN    ACCTGGAACTTCCAGTCCACGTATGAACTCCTGGATTATCTGCTGGACCAGAATCGCTAC

              6121      6131      6141      6151      6161      6171
BPEFHAB1      GAGTACATTTGGGGGCTGTATCCGACCTACACCGAATGGTCGGTGAATACGCTGAAGAAC
fhaB1_pcDN    GAGTACATTTGGGGGCTGTATCCGACCTACACCGAATGGTCGGTGAATACGCTGAAGAAC
fhaB2_pcDN    GAGTACATTTGGGGGCTGTATCCGACCTACACCGAATGGTCGGTGAATACGCTGAAGAAC

              6181      6191      6201      6211      6221      6231
BPEFHAB1      CTCGACCTGGGCTACCAGGCCAAGCCGGCTCCCACTGCGCCGCCGATGCCCAAGGCTCCC
fhaB1_pcDN    CTCGACCTGGGCTACCAGGCCAAGCCGGCTCCCACTGCGCCGCCGATGCCCAAGGCTCCC
fhaB2_pcDN    CTCGACCTGGGCTACCAGGCCAAGCCGGCTCCCACTGCGCCGCCGATGCCCAAGGCTCCC

              6241      6251      6261      6271      6281      6291
BPEFHAB1      GAACTCGACCTGCGTGGCCATACGCTGGAGTCGGCCGAAGGCCGGAAGATCTTTGGCGAG
fhaB1_pcDN    GAACTCGACCTGCGTGGCCATACGCTGGAGTCGGCCAAAGGCCGGAAGATCTTTGGCGAG
fhaB2_pcDN    GAACTCGACCTGCGTGGCCATACGCTGGAGTCGGCCAAAGGCCGGAAGATCTTTGGCGAG

              6301      6311      6321      6331      6341      6351
BPEFHAB1      TACAAGAAGCTGCAAGGCGAGTACGAGAAGGCGAAGATGGCCGTCCAGGCCGTGGAGGCT
fhaB1_pcDN    TACAAGAAGCTGCAAGGCGAGTACGAGAAGGCGAAGATGGCCGTCCAGGCCGTGGAGGCT
fhaB2_pcDN    TACAAGAAGCTGCAAGGCGAGTACGAGAAGGCGAAGATGGCCGTCCAGGCCGTGGAGGCT

              6361      6371      6381      6391      6401      6411
BPEFHAB1      TACGGCGAGGCTACTCGGCGCGTCCATGATCAGCTGGGCCAACGTTATGGTAAGGCCCTG
fhaB1_pcDN    TACGGCGAGGCTACTCGGCGCGTCCATGATCAGCTGGGCCAACGTTATGGTAAGGCCCTG
fhaB2_pcDN    TACGGCGAGGCTACTCGGCGCGTCCATGATCAGCTGGGCCAACGTTATGGTAAGGCCCTG

              6421      6431      6441      6451      6461      6471
BPEFHAB1      GGCGGCATGGATGCCGAGACCAAGGAGGTCGACGGCATCATCCAGGAGTTCGCCGCGGAT
fhaB1_pcDN    GGCGGCATGGATGCCGAGACCAAGGAGGTCGACGGCATCATCCAGGAGTTCGCCGCGGAT
fhaB2_pcDN    GGCGGCATGGATGCCGAGACCAAGGAGGTCGACGGCATCATCCAGGAGTTCGCCGCGGAT
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               6481      6491      6501      6511      6521      6531
BPEFHAB1       CTGCGAACGGTCTATGCGAAGCAGGCCGACCAGGCGACCATCGACGCAGAGACGGACAAG
fhaB1_pcDN     CTGCGAACGGTCTATGCGAAGCAGGCCGACCAGGCGACCATCGACGCAGAGACGGACAAG
fhaB2_pcDN     CTGCGAACGGTCTATGCGAAGCAGGCCGACCAGGCGACCATCGACGCAGAGACGGACAAG

               6541      6551      6561      6571      6581      6591
BPEFHAB1       GTCGCCCAGCGCTACAAGTCGCAGATCGACGCGGTGCGGCTGCAGGCGATCCAGCCTGGC
fhaB1_pcDN     GTCGCCCAGCGCTACAAGTCGCAGATCGACGCGGTGCGGCTGCAGGCGATCCAGCCTGGC
fhaB2_pcDN     GTCGCCCAGCGCTACAAGTCGCAGATCGACGCGGTGCGGCTGCAGGCGATCCAGCCTGGC

               6601      6611      6621      6631      6641      6651
BPEFHAB1       CGGGTCACGCTGGCCAAGGCGCTGTCGGCGGCGCTGGGCGCCGACTGGCGCGCGCTGGGT
fhaB1_pcDN     CGGGTCACGCTGGCCAAGGCGCTGTCGGCGGCGCTGGGCGCCGACTGGCGCGCGCTGGGT
fhaB2_pcDN     CGGGTCACGCTGGCCAAGGCGCTGTCGGCGGCGCTGGGCGCCGACTGGCGCGCGCTGGGT

               6661      6671      6681      6691      6701      6711
BPEFHAB1       CACTCCCAATTGATGCAGCGCTGGAAGGATTTCAAGGCGGGCAAGCGCGGCGCGGAAATC
fhaB1_pcDN     CACTCCCAATTGATGCAGCGCTGGAAGGATTTCAAGGCGGGCAAGCGCGGCGCGGAAATC
fhaB2_pcDN     CACTCCCAATTGATGCAGCGCTGGAAGGATTTCAAGGCGGGCAAGCGCGGCGCGGAAATC

               6721      6731      6741      6751      6761      6771
BPEFHAB1       GCGTTCTATCCCAAGGAACAAACCGTGCTGGCCGCCGGCGCCGGTTTGACCCTGTCCAAC
fhaB1_pcDN     GCGTTCTATCCCAAGGAACAAACCGTGCTGGCCGCCGGCGCCGGTTTGACCCTGTCCAAC
fhaB2_pcDN     GCGTTCTATCCCAAGGAACAAACCGTGCTG------------------------------

               6781      6791      6801      6811      6821      6831
BPEFHAB1       GGGGCGATCCACAACGGCGAGAACGCCGCGCAGAATCGCGGCCGGCCGGAAGGCCTGAAA
fhaB1_pcDN     GGGGCGATCCACAACGGCGAGAACGCCGCGCAGAATCGCGGCCGGCCGGAAGGCCTGAAA
fhaB2_pcDN     ------------------------------------------------------------

               6841      6851      6861      6871      6881      6891
BPEFHAB1       ATCGGCGCACATTCGGCGACTTCGGTGAGCGGCTCGTTCGACGCCTTGCGCGACGTGGGG
fhaB1_pcDN     ATCGGCGCACATTCGGCGACTTCGGTGAGCGGCTCGTTCGACGCCTTGCGCGACGTGGGG
fhaB2_pcDN     ------------------------------------------------------------

               6901      6911      6921      6931      6941      6951
BPEFHAB1       CTGGAAAAGCGGCTGGATATCGACGATGCGCTGGCTGCCGTGCTCGTGAATCCGCATATT
fhaB1_pcDN     CTGGAAAAGCGGCTGGATATCGACGATGCGCTGGCTGCCGTGCTCGTGAATCCGCATATT
fhaB2_pcDN     ------------------------------------------------------------

               6961      6971      6981      6991      7001      7011
BPEFHAB1       TTCACGCGGATCGGGGCGGCTCAGACATCCCTTGCCGACGGCGCCGCCGGGCCGGCGCTG
fhaB1_pcDN     TTCACG------------------------------------------------------
fhaB2_pcDN     ------------------------------------------------------------

               7021      7031      7041      7051      7061      7071
BPEFHAB1       GCGCGCCAGGCCAGGCAAGCGCCGGAGACCGACGGCATGGTGGATGCGCGAGGGCTGGGC
fhaB1_pcDN     ------------------------------------------------------------
fhaB2_pcDN     ------------------------------------------------------------
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Figure D.2: Nucleotide sequence alignment of published *fhaB* Genbank sequence and pcDNA3.1D/*fhaB1* and pcDNA3.1D/*fhaB2* insert sequences. ClustalW program (BioManager) was used to compare the two sequences. BPEFHAB1 refers to Genbank sequence of *fhaB* (accession # M60351.1), fhaB1_pcDN refers to the sequenced insert of pcDNA3.1D/*fhaB1*., and fhaB2_pcDN refers to the sequenced insert of pcDNA3.1D/*fhaB2*.

Table D.2: Cytokine production from *in-vitro* re-stimulated splenocytes from mice immunised with pcDNA3.1D/*fhaB1*. Values represent mean (pg/ml) from duplicate wells. Nd – Not Determined.

Vaccine Group	Stimulant	IFN-γ	IL-2	IL-4
pcDNA3.1D/*fhaB1*	rFHAB1 (5μg/ml)	8490	166.8	39.7
pcDNA3.1D/*fhaB1*	DTaP (10μg/ml)	227	11.6	8.7
pcDNA3.1D/*fhaB1*	HKBP (2 x 10^6)	428	Nd	Nd
pcDNA3.1 vector	rFHAB1 (5μg/ml)	492	47.9	1
DTaP (Infanrix™)	rFHAB1 (5μg/ml)	343	46.2	75.9
pcDNA3.1D/*fhaB1*	ConA (2μg/ml)	6000	10779.7	474

Table D.3: Cytokine production from *in-vitro* re-stimulated splenocytes from mice immunised with pcDNA3.1D/*fhaB2*. Nd – Not determined.

Vaccine Group	Stimulant	IFN-γ	IL-2	IL-4
pcDNA3.1D/*fhaB2*	rFHAB2 (5μg/ml)	2051	77.2	0
pcDNA3.1D/*fhaB2*	DTaP (10μg/ml)	199	1	0
pcDNA3.1D/*fhaB2*	HKBP (2 x 10^6)	176	Nd	Nd
pcDNA3.1 vector	rFHAB2 (5μg/ml)	773	39.4	0
DTaP (Infanrix™)	rFHAB2 (5μg/ml)	406	30.8	72.9
pcDNA3.1D/*fhaB2*	ConA (2μg/ml)	6367	11344.5	689.2

Table D.4: Summary of serum anti-rFHAB1 IgG. Nd – Not determined for individual mice (tested as pooled serum sample).

Vaccine Group	Individual Mice					Mean	SE
	M1	M2	M3	M4	M5		
pcDNA3.1D/*fhaB1*	3122	6423	5055	6627	5781	5402	633
pcDNA3.1D vector	Nd	Nd	Nd	Nd	Nd	17046	0
DTaP	113499	51200	3200	800	51200	43980	20573

Table D.5: Summary of serum anti-rFHAB2 IgG. Nd – Not determined for individual mice (tested as pooled serum sample).

Vaccine Group	Individual Mice					Mean	SE
	M1	M2	M3	M4	M5		
pcDNA3.1D/*fhaB2*	200	3200	0	800	1600	1160	581
pcDNA3.1D vector	Nd	Nd	Nd	Nd	Nd	24470	0
DTaP	113499	51200	3200	800	51200	43980	20573

Table D.6: Summary of serum anti-FHA IgG. Nd – Not determined for individual mice (tested as pooled serum sample).

Vaccine Group	Individual Mice					Mean	SE	Signif. (P)
	M1	M2	M3	M4	M5			
pcDNA3.1D/*fhaB1*	50	0	0	0	50	20	12	
pcDNA3.1D/*fhaB2*	25	0	25	100	25	35	17	
pcDNA3.1D vector	nd	nd	nd	nd	nd	800	0	
DTaP	51200	51200	12800	51200	25600	38400	8095	*0.009

* = significantly > pcDNA3.1D/*fhaB1* and pcDNA3.1D/*fhaB2*

Table D.7: Clearance of *B. pertussis* from lungs following aerosol challenge (SLID). Bacterial load expressed as log10 CFU/lung

Group	Day	Mouse1	Mouse2	Mouse3	Mouse4
pcDNA3.1D/*fhaB1*	0	5.699	5.602	5.778	5.699
	4	5.057	6.352	6.632	6.104
	7	0	5.228	2.301	5.037
	14	0	0	0	3.415
pcDNA3.1D/*fhaB2*	0	5.699	5.602	5.778	5.699
	4	6.386	4.833	5.845	6.176
	7	3	4.968	5.238	2.954
	14	0	4.491	0	0
pcDNA3.1 Vector	0	5.699	5.602	5.778	5.699
	4	6.439	6.778	6.303	6.41
	7	5.663	6.799	6.991	6.212
	14	5.255	6.204	5.716	5.959
Placebo	0	5.699	5.602	5.778	5.699
	4	5.217	6.318	6.442	5.217
	7	6.756	5.764	6.672	6.176
	14	6.27	6.079	5.7	5.996
DTaP	0	5.699	5.602	5.778	5.699
	4	5.58	5.613	5.613	5.602
	7	0	0	0	0
	14	0	0	0	0

Table D.8: Raw ELISA data of serum anti-rFHAB1 IgG from mice vaccinated with pcDNA3.1D/*fhaB1*.

Dilution	25	50	100	200	400	800	1600	3200	6400	12800
P 9a	0.056	0.04	0.022	0.016	0.015	0.014	0.007	0.004	0.004	0
P 9b	0.055	0.037	0.025	0.016	0.013	0.01	0.004	0.002	0.002	0.001
P 8a	1.526	1.443	1.263	1.048	0.772	0.585	0.353	0.189	0.101	0.05
P 8b	1.468	1.452	1.205	1.119	0.765	0.554	0.358	0.2	0.106	0.057
P 1a	1.068	0.725	0.338	0.192	0.092	0.048	0.045	0.019	0.012	0.012
P 1b	0.906	0.736	0.373	0.189	0.112	0.067	0.05	0.035	0.027	0.021
M 1a	0.097	0.049	0.031	0.025	0.018	0.013	0.018	0.004	0.001	0
M 1b	0.081	0.041	0.029	0.02	0.016	0.015	0	0	0	0
M 2a	1.187	1.07	0.715	0.44	0.234	0.1	0.055	0.026	0.018	0.007
M 2b	1.325	0.992	0.742	0.431	0.215	0.104	0.061	0.028	0.014	0.001
M 3a	0.096	0.067	0.06	0.056	0.047	0.041	0.037	0.031	0.012	0.008
M 3b	0.076	0.053	0.041	0.032	0.025	0.021	0.016	0.015	0.015	0.007
M 4a	1.21	0.938	0.6	0.373	0.199	0.108	0.055	0.032	0.015	0.011
M 4b	1.176	0.919	0.583	0.346	0.179	0.094	0.048	0.015	0.002	0
M 5a	0.042	0.032	0.019	0.013	0.008	0.004	0.001	0	0	0
M 5b	0.028	0.026	0.016	0.008	0.06	0.014	0.007	0	0	0

Table D.9: Raw ELISA data of serum anti-FHA IgG from mice vaccinated with pcDNA3.1D/*fhaB1*.

Dilution	25	50	100	200	400	800	1600	3200	6400	12800
P 9a	0.034	0.018	0.014	0.009	0.009	0.007	0.004	0.003	0.001	0
P 9b	0.037	0.014	0.014	0.013	0.012	0.009	0.006	0.003	0	0
P 8a	0.282	0.18	0.133	0.104	0.07	0.044	0.023	0.015	0.004	0
P 8b	0.289	0.192	0.14	0.103	0.081	0.051	0.026	0.017	0.005	0.004
M 1a	0.067	0.034	0.016	0.013	0.011	0.007	0.006	0.006	0.006	0.004
M 1b	0.065	0.035	0.032	0.021	0.02	0.019	0.019	0.018	0.016	0.016
M 2a	0.029	0.024	0.018	0.015	0.01	0.007	0.005	0.005	0.003	0.003
M 2b	0.052	0	0	0	0	0	0	0	0	0
M 3a	0.052	0.027	0.017	0.012	0.006	0.004	0.004	0.001	0	0
M 3b	0.055	0.027	0.017	0.009	0.007	0	0	0	0	0
M 4a	0.048	0.036	0.025	0.023	0.021	0.02	0.018	0.015	0.015	0.012
M 4b	0.048	0.029	0.024	0.02	0.015	0.014	0.01	0.006	0.005	0.002
M 5a	0.099	0.055	0.03	0.02	0.016	0.015	0.009	0.008	0.005	0.004
M 5b	0.076	0.035	0.019	0.004	0.001	0	0	0	0	0

Table D.10: Raw ELISA data of serum anti-rFHAB2 IgG from mice vaccinated with pcDNA3.1D/*fhaB2*.

Dilution	25	50	100	200	400	800	1600	3200	6400	12800
P 9a	0.056	0.04	0.022	0.016	0.015	0.014	0.007	0.004	0.004	0
P 9b	0.055	0.037	0.025	0.016	0.013	0.01	0.004	0.002	0.002	0.001
P 8a	1.507	1.426	1.354	1.258	1.149	0.953	0.813	0.423	0.218	0.124
P 8b	1.526	1.336	1.329	1.305	1.05	0.89	0.759	0.389	0.233	0.133
P 2a	0.55	0.463	0.286	0.222	0.146	0.124	0.085	0.038	0.025	0.016
P 2b	0.452	0.374	0.255	0.228	0.163	0.117	0.109	0.06	0.041	0.027
M 1a	0.121	0.083	0.053	0.047	0.035	0.03	0.025	0.018	0.017	0.012
M 1b	0.104	0.073	0.032	0.016	0.005	0.003	0	0	0	0
M 2a	0.053	0.033	0.028	0.019	0.018	0.018	0.013	0	0	0
M 2b	0.062	0.039	0.025	0.024	0.02	0.018	0.017	0.013	0.003	0.001
M 3a	0.049	0.02	0.016	0.015	0.009	0.006	0.004	0.003	0	0
M 3b	0.062	0.042	0.028	0.023	0.017	0.013	0.012	0.012	0.009	0.007
M 4a	0.231	0.122	0.08	0.044	0.034	0.023	0.017	0.011	0.01	0.009
M 4b	0.218	0.133	0.087	0.054	0.041	0.031	0.029	0.026	0.017	0.016
M 5a	0.333	0.23	0.125	0.073	0.042	0.031	0.024	0.023	0.011	0.009
M 5b	0.291	0.209	0.094	0.057	0.028	0.016	0.008	0.001	0	0

Table D.11: Raw ELISA data of serum anti-FHA IgG from mice vaccinated with pcDNA3.1D/*fhaB2*.

Dilution	25	50	100	200	400	800	1600	3200	6400	12800
P 9a	0.034	0.018	0.014	0.009	0.009	0.007	0.004	0.003	0.001	0
P 9b	0.037	0.014	0.014	0.013	0.012	0.009	0.006	0.003	0	0
P 8a	0.563	0.382	0.307	0.224	0.176	0.094	0.075	0.046	0.021	0.01
P 8b	0.644	0.531	0.345	0.272	0.208	0.118	0.095	0.058	0.034	0.019
P 2a	0.259	0.214	0.148	0.096	0.07	0.035	0.024	0.017	0.012	0.012
P 2b	0.255	0.214	0.126	0.096	0.069	0.048	0.04	0.034	0.027	0.021
M 1a	0.059	0.034	0.018	0.016	0.013	0.012	0.012	0.008	0.006	0.005
M 1b	0.067	0.045	0.025	0.01	0.004	0	0	0	0	0
M 2a	0.04	0.029	0.007	0.004	0.001	0	0	0	0	0
M 2b	0.044	0.02	0.012	0.007	0.005	0	0	0	0	0
M 3a	0.049	0.031	0.016	0.013	0.012	0.006	0.003	0.003	0.001	0
M 3b	0.059	0.038	0.031	0.022	0.017	0.017	0.016	0.01	0.01	0.004
M 4a	0.075	0.066	0.051	0.019	0.013	0.012	0.011	0.01	0.009	0.008
M 4b	0.121	0.054	0.045	0.03	0.022	0.02	0.02	0.019	0.017	0.017
M 5a	0.187	0.063	0.034	0.03	0.016	0.013	0.011	0.007	0.014	0.007
M 5b	0.054	0.018	0.012	0.008	0	0	0	0	0	0

Table D.12: Raw ELISA data of serum anti-rFHAB1 IgG from mice vaccinated with DTaP.

Dilution	400	800	1600	3200	6400	12800	25600	51200	102400	204800
P 9a	0.021	0.014	0.014	0.01	0.01	0.01	0.008	0.008	0.006	0.005
P 9b	0.064	0.025	0.025	0.021	0.021	0.02	0.018	0.015	0.013	0.013
P 10a	1.187	0.931	0.637	0.413	0.248	0.145	0.075	0.041	.	.
P 10b	1.234	0.978	0.622	0.385	0.237	0.138	0.078	0.042	.	.
M 1a	0.697	0.38	0.215	0.126	0.095	0.044	0.031	0.035	0.031	0.028
M 1b	0.716	0.377	0.186	0.146	0.114	0.06	0.049	0.044	0.042	0.041
M 2a	0.343	0.181	0.14	0.097	0.042	0.033	0.028	0.027	0.024	0.024
M 2b	0.371	0.181	0.156	0.082	0.056	0.049	0.015	0.014	0.008	0.007
M 3a	0.427	0.255	0.13	0.084	0.052	0.033	0.027	0.017	0.015	0.01
M 3b	0.475	0.243	0.107	0.061	0.032	0.021	0.02	0.017	0.009	0.009
M 4a	0.027	0.011	0.004	0	0	0	0	0	0	0
M 4b	0.04	0.024	0.013	0.012	0.011	0.009	0.008	0.007	0.005	0.002
M 5a	0.293	0.176	0.085	0.045	0.03	0.027	0.018	0.015	0.006	0.002
M 5b	0.345	0.185	0.089	0.056	0.039	0.03	0.027	0.024	0.021	0.019

Table D.13: Raw ELISA data of serum anti-FHA IgG from mice vaccinated with DTaP.

Dilution	400	800	1600	3200	6400	12800	25600	51200	102400	204800
M 1a	0.303	0.214	0.171	0.11	0.074	0.047	0.026	0.025	0.021	0.019
M 1b	0.33	0.23	0.184	0.138	0.083	0.071	0.061	0.046	0.037	0.034
M 2a	0.278	0.238	0.18	0.151	0.135	0.087	0.053	0.041	0.039	0.012
M 2b	0.252	0.226	0.139	0.109	0.097	0.061	0.05	0.016	0	0
M 3a	0.209	0.174	0.088	0.058	0.034	0.021	0.01	0.007	0.007	0
M 3b	0.223	0.164	0.096	0.058	0.034	0.032	0.019	0.015	0	0
M 4a	0.248	0.212	0.115	0.087	0.046	0.032	0.024	0.021	0.015	0.012
M 4b	0.327	0.275	0.15	0.106	0.061	0.044	0.031	0.028	0.028	0.019
M 5a	0.436	0.344	0.207	0.154	0.093	0.063	0.041	0.033	0.027	0.023
M 5b	0.459	0.343	0.207	0.154	0.1	0.075	0.054	0.046	0.037	0.035
M 1c	0.269	0.254	0.224	0.182	0.13	0.074	0.032	0.031	0.016	0.015
M 1d	0.292	0.228	0.198	0.136	0.122	0.058	0.047	0.042	0.026	0.024
M 2c	0.252	0.216	0.193	0.145	0.086	0.065	0.059	0.045	0.015	0.01
M 2d	0.266	0.208	0.178	0.133	0.087	0.05	0.031	0.006	0.001	0
M 3c	0.277	0.211	0.159	0.113	0.057	0.03	0.014	0.014	0.003	0.001
M 3d	0.268	0.176	0.137	0.094	0.054	0.023	0.017	0.012	0.002	0
M 4c	0.227	0.168	0.155	0.097	0.066	0.047	0.034	0.021	0.013	0.01
M 4d	0.247	0.19	0.177	0.151	0.112	0.091	0.065	0.056	0.049	0.023
M 5c	0.287	0.218	0.192	0.161	0.093	0.049	0.042	0.041	0.034	0.033
M 5d	0.247	0.207	0.184	0.132	0.094	0.07	0.059	0.044	0.041	0.029

Table D.14: Raw ELISA data of serum anti-rFHAB1/anti-FHA IgG1.

Dilution	100	200	400	800	1600	3200	6400	12800	25600	51200
P 9a	1.435	1.328	1.045	0.73	0.52	0.339	0.216	0.15	0.107	0.084
P 9b	1.422	1.359	1.154	0.812	0.526	0.363	0.235	0.167	0.129	0.085
P 8a	1.657	1.591	1.587	1.58	1.424	1.143	0.828	0.57	0.347	0.26
P 8b	1.672	1.628	1.555	1.514	1.386	1.061	0.757	0.526	0.355	0.225
P 1a	1.439	1.165	0.696	0.429	0.237	0.132	0.074	0.049	0.022	0.015
P 1b	1.502	1.053	0.703	0.44	0.232	0.115	0.106	0.05	0.011	0.012
P 7a	1.385	0.967	0.65	0.398	0.254	0.23	0.154	0.146	0.139	0.104
P 7b	1.541	0.992	0.509	0.311	0.193	0.131	0.085	0.068	0.05	0.046
P 10a	1.966	1.899	1.844	1.694	1.476	0.938	0.674	0.403	0.251	0.193
P 10b	1.896	1.767	1.747	1.587	1.316	0.879	0.55	0.373	0.184	0.092
P 10c	2.073	1.964	1.961	1.836	1.761	1.767	1.564	1.366	1.067	0.721
P 10d	2.112	2.068	2.062	1.953	1.944	1.885	1.698	1.58	1.143	0.759

Table D.15: Raw ELISA data of serum anti-rFHAB1/anti-FHA IgG2a.

Dilution	100	200	400	800	1600	3200	6400	12800	25600	51200
P 9a	0.338	0.167	0.1	0.073	0.038	0.024	0.022	0.019	0.018	0.019
P 9b	0.276	0.178	0.103	0.096	0.047	0.031	0.024	0.037	0.022	0.007
P 8a	0.112	0.063	0.035	0.019	0.019	0.015	0.009	0.005	0.012	0.015
P 8b	0.096	0.067	0.056	0.022	0.023	0.017	0.006	0.021	0.023	0.01
P 1a	0.094	0.063	0.031	0.008	0.006	0	0	0	0	0
P 1b	0.078	0.035	0.004	0	0	0	0	0	0	0
P 7a	0.055	0.042	0.034	0.036	0.02	0.024	0.033	0.02	0.02	0.044
P 7b	0.043	0.027	0.022	0.016	0.012	0.013	0.026	0.023	0.02	0.005
P 10a	0.097	0.057	0.042	0.027	0.021	0.021	0.019	0.015	0.017	0.018
P 10b	0.089	0.049	0.02	0.016	0.006	0.004	0.003	0.007	0.006	0.004
P 10c	0.268	0.167	0.086	0.047	0.027	0.017	0.015	0.015	0.006	0.009
P 10d	0.263	0.173	0.096	0.063	0.038	0.028	0.025	0.024	0.022	0.021

Table D.16: Growth curve data of SL3261-pcDNA3.1D/*fhaB1* for the preparation of the oral combination DNA vaccine.

Transformant	OD_{600}	Mean Count (CFU)	Dil. Factor	Adj.Count (CFU/ml)	Adj. Count (log10CFU/ml)
SL3261-pcDNA3.1D/*fhaB1*	0.325	50	2.00E+06	1.00E+08	8
	0.55	22	2.00E+07	4.40E+08	8.643452676
	1.12	42	2.00E+07	8.40E+08	8.924279286
	1.38	72	2.00E+07	1.44E+09	9.158362492
SL3261-pcDNA3.1 Vector	0.182	50	2.00E+06	1.00E+08	8
	0.67	24	2.00E+07	4.80E+08	8.681241237
	1.12	46	2.00E+07	9.20E+08	8.963787827
	1.39	64	2.00E+07	1.28E+09	9.10720997
SL3261	0.218	46	2.00E+06	9.20E+07	7.963787827
	0.515	20	2.00E+07	4.00E+08	8.602059991
	0.998	41	2.00E+07	8.20E+08	8.913813852
	1.22	92	2.00E+07	1.84E+09	9.264817823

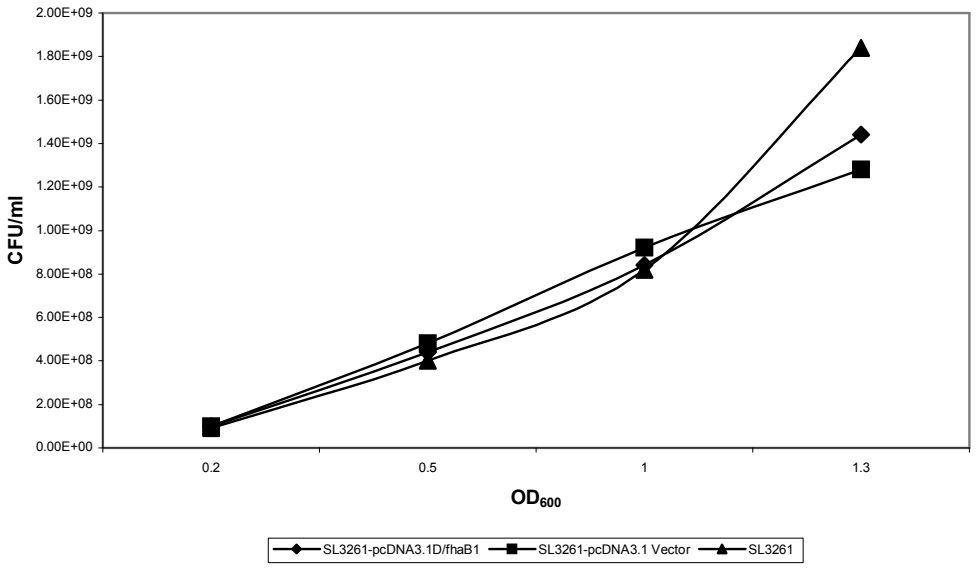

Figure D.3: Growth curve of SL3261-pcDNA3.1D/*fhaB1* for the preparation of the oral combination DNA vaccine.

D.3 Chapter 5

1 TGCAGCGGTT CTGGATGGCG TTATTCGTAC TTTTGCTGCG CCCATTCTTC
51 CCTGTTCCAT CGCGGTGCGG GCATGGCGGG CGTCTGCTCT CCACCTGGCA
101 TCCAATGAAC ATGTCTCTGT CACGCATTGT CAAGGCGGCG CCCCTGCGCC
151 GCACCACGCT GGCCATGGCG CTGGGCGCGC TGGGCGCCGC CCCGGCG**GCG**

PRN198DNAF/PRN198TRCF→

201 **CATGCCGACT GGAACAACCA GTCCATC**GTC AAGACCGGTG AGCGCCAGCA
251 TGGCATCCAT ATCCAGGGCT CCGACCCGGG CGGCGTACGG ACCGCCAGCG
301 GAACCACCAT CAAGGTAAGC GGCCGTCAGG CCCAGGGCAT CCTGCTAGAA
351 AATCCCGCGG CCGAGCTGCA GTTCCGGAAC GGCAGTGTCA CGTCGTCGGG
401 ACAGTTGTCC GACGATGGCA TCCGGCGCTT TCTGGGCACC GTCACCGTCA
451 AGGCCGGCAA GCTGGTCGCC GATCACGCCA CGCTGGCCAA CGTTGGC**GAC**
501 **ACCTGGGACG ACGAC**GGCAT CGCGCTCTAT GTGGCCGGCG AACAGGCCCA
551 GGCCAGCATC GCCGACAGCA CCCTGCAGGG CGCTGGCGGC GTGCAGATCG
601 AGCGCGGCGC CAATGTCACG GTCCAACGCA GCGCCATCGT CGACGGGGGC
651 TTGCATATCG GCGCCCTGCA GTCATTGCAG CCGGAAGACC TTCCGCCCAG
701 CCGGGTGGTG CTGCGCGACA CCAACGTGAC CGCCGTGCCC GCCAGCGGCG
751 CGCCCGCGGC GGTGTCTGTG TTGGGGGCCA GTGAGCTTAC GCTCGACGGC
801 GGGCACATCA CCGGCGGGCG GGCAGCGGGG GTGGCGGCCA TGCAAGGGGC
851 GGTCGTGCAT CTGCAGCGCG CGACGATACG GCGCGGGGAC GCGCCTGCCG
901 GCGGTGCGGT TCCCGGCGGT GCG**GTTCCCG GTGGTGCGGT T**CCCGGCGGC
951 TTCGGTCCCG GCGGCTTCGG TCCCGTCCTC GACGGCTGGT ATGGCGTGGA
1001 CGTATCGGGC TCCAGCGTGG AGCTCGCCCA GTCGATCGTC GAGGCGCCGG
1051 AGCTGGGCGC CGCAATCCGG GTGGGCCGCG GCGCCAGGGT GACGGTGTCG
1101 GGCGGCAGCT TGTCCGCACC GCACGGCAAT GTCATCGAGA CCGGCGGCGC
1151 GCGTCGCTTT GCGCCTCAAG CCGCGCCCCT GTCGATCACC TTGCAGGCCG
1201 GCGCGCATGC CCAGGGGAAA GCGCTGCTGT ACCGGGTCCT GCCGGAGCCC
1251 GTGAAGCTGA CGCTGACCGG GGGCGCCGAT GCGCAGGGCG ACATCGTCGC
1301 GACGGAGCTG CCCTCCATTC CCGGCACGTC GATCGGGCCG CTCGACGTGG
1351 CGCTGGCCAG CCAGGCCCGA TGGACGGGCG CTACCCGCGC GGTCGAC**TCG**
1401 **CTGTCCATCG ACAAC**GCCAC CTGGGTCATG ACGGACAACT CGAACGTCGG
1451 TGCGCTACGG CTGGCCAGCG ACGGCAGCGT CGATTTCCAG CAGCCGGCCG
1501 AAGCTGGGCG GTTCAAGGTC CTGACGGTCA ATACGCTGGC GGGTTCGGGG
1551 CTGTTCCGCA TGAATGTCTT CGCGGACCTG GGGCTGAGCG ACAAGCTGGT
1601 CGTCATGCAG GACGCCAGCG GCCAGCACAG GCTGTGGGTC CGCAACAGCG
1651 GCAGCGAGCC GGCCAGCGCC AACACCCTGC TGCTGGTGCA GACGCCACTA
1701 GGCAGCGCGG CGACCTTTAC CCTTGCCAAC AAGGACGGCA AGGTCGATAT
1751 CGGTACCTAT CGCTATCGAT TGGCCGCCAA CGGCAATGGG CAGTGGAGCC
1801 TGGTGGGCGC GAAGGCGCCG CCGGCGCCCA AGCCCGCGCC GCAGCCGGGT

1851 CCCCAGCCGC CGCAGCCGCC GCAGCCGCAG CCGGAAGCGC CGGCGCCGCA
1901 ACCGCCGGCG GGCAGGGAGT TGTCCGCCGC CGCCAACGCG GCGGTCAACA
1951 CGGGTGGGGT GGGCCTGGCC AGCACGCTCT GGTACGCCGA AAGCAATGCG
2001 TTGTCCAAGC GCCTGGGCGA GTTGCGCCTG AATCCGGACG CCGGCGGCGC
2051 CTGGGGCCGC GGCTTCGCGC AACGCCAGCA GCTGGACAAC CGCGCCGGGC
2101 GGCGCTTCGA CCAGAAGGTG GCCGGCTTCG AGCTGGGCGC CGACCACGCG
2151 GTGGCGGTGG CCGGCGGACG CTGGCACCTG GGCGGGCTGG CCGGCTATAC
2201 GCGCGGCGAC CGCGGCTTCA CCGGCGACGG CGGCGGCCAC ACC**GACAGCG**

←**PRN2273R**

2251 **TGCATGTCGG GGGCTATGCC ACA**TATATCG CCGACAGCGG TTTCTACCTG
2301 GACGCGACGC TGCGCGCCAG CCGCCTGGAG AATGACTTCA AGGTGGCGGG
2351 CAGCGACGGG TACGCGGTCA AGGGCAAGTA CCGCACCCAT GGGGTGGGCG
2401 CCTCGCTCGA GGCGGGCCGG CGCTTTACCC ATGCCGACGG CTGGTTCCTC
2451 GAGCCGCAGG CCGAGCTGGC GGTATTCCGG GCCGGCGGCG GTGCGTACCG
2501 CGCGGCCAAC GGCCTGCGGG TGCGCGACGA AGGCGGCAGC TCGGTGCTGG
2551 GTCGCCTGGG CCTGGAGGTC GGCAAGCGCA TCGAACTGGC AGGCGGCAGG
2601 CAGGTGCAGC CATACATCAA GGCCAGCGTG CTGCAGGAGT TCGACGGCGC
2651 GGGTACGGTA CACACCAACG GCATCGCGCA CCGCACCGAA CTGCGCGGCA
2701 CGCGCGCCGA ACTGGGCCTG GGCATGGCCG CCGCGCTGGG CCGCGGCCAC
2751 AGCCTGTATG CCTCGTACGA GTACTCCAAG GGCCCGAAGC TGGCCATGCC
2801 GTGGACCTTC CACGCGGGCT ACCGGTACAG CTGGTAAAGC GAGGAGGGTC
2851 TATCCCCCGC GGAGGAGGTT TTCCTGGAGC TTGGCCGGTG CCAGTCTCCA
2901 GGCTCAGGCG GCCAGGGCCT GCGGGCCGGG CAGGCCGCGC TGGTGCTGG

Figure D.4: Genbank sequence of *prn* (accession # AJ006158). PCR primer sites are highlighted in blue and sequencing primer sites in magenta.

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               1         11        21        31        41        51
BPA006158_     TGCAGCGGTTCTGGATGGCGTTATTCGTACTTTTGCTGCGCCCATTCTTCCCTGTTCCAT
prn2_pcDNA     ------------------------------------------------------------

               61        71        81        91        101       111
BPA006158_     CGCGGTGCGGGCATGGCGGGCGTCTGCTCTCCACCTGGCATCCAATGAACATGTCTCTGT
prn2_pcDNA     ------------------------------------------------------------

               121       131       141       151       161       171
BPA006158_     CACGCATTGTCAAGGCGGCGCCCCTGCGCCGCACCACGCTGGCCATGGCGCTGGGCGCGC
prn2_pcDNA     ------------------------------------------------------------

               181       191       201       211       221       231
BPA006158_     TGGGCGCCGCCCCGGCGGCGCATGCCGACTGGAACAACCAGTCCATCGTCAAGACCGGTG
prn2_pcDNA     ----------------GCGCATGCCGACTGGAACAACCAGTCCATCGTCAAGACCGGTG

               241       251       261       271       281       291
BPA006158_     AGCGCCAGCATGGCATCCATATCCAGGGCTCCGACCCGGGCGGCGTACGGACCGCCAGCG
prn2_pcDNA     AGCGCCAGCATGGCATCCATATCCAGGGCTCCGACCCGGGCGGCGTACGGACCGCCAGCG

               301       311       321       331       341       351
BPA006158_     GAACCACCATCAAGGTAAGCGGCCGTCAGGCCCAGGGCATCCTGCTAGAAAATCCCGCGG
prn2_pcDNA     GAACCACCATCAAGGTAAGCGGCCGTCAGGCCCAGGGCATCCTGCTAGAAAATCCCGCGG

               361       371       381       391       401       411
BPA006158_     CCGAGCTGCAGTTCCGGAACGGCAGTGTCACGTCGTCGGGACAGTTGTCCGACGATGGCA
prn2_pcDNA     CCGAGCTGCAGTTCCGGAACGGCAGTGTCACGTCGTCGGGACAGTTGTCCGACGATGGCA

               421       431       441       451       461       471
BPA006158_     TCCGGCGCTTTCTGGGCACCGTCACCGTCAAGGCCGGCAAGCTGGTCGCCGATCACGCCA
prn2_pcDNA     TCCGGCGCTTTCTGGGCACCGTCACCGTCAAGGCCGGCAAGCTGGTCGCCGATCACGCCA

               481       491       501       511       521       531
BPA006158_     CGCTGGCCAACGTTGGCGACACCTGGGACGACGACGGCATCGCGCTCTATGTGGCCGGCG
prn2_pcDNA     CGCTGGCCAACGTTGGCGACACCTGGGACGACGACGGCATCGCGCTCTATGTGGCCGGCG

               541       551       561       571       581       591
BPA006158_     AACAGGCCCAGGCCAGCATCGCCGACAGCACCCTGCAGGGCGCTGGCGGCGTGCAGATCG
prn2_pcDNA     AACAGGCCCAGGCCAGCATCGCCGACAGCACCCTGCAGGGCGCTGGCGGCGTGCAGATCG

               601       611       621       631       641       651
BPA006158_     AGCGCGGCGCCAATGTCACGGTCCAACGCAGCGCCATCGTCGACGGGGGCTTGCATATCG
prn2_pcDNA     AGCGCGGCGCCAATGTCACGGTCCAACGCAGCGCCATCGTCGACGGGGGCTTGCATATCG

               661       671       681       691       701       711
BPA006158_     GCGCCCTGCAGTCATTGCAGCCGGAAGACCTTCCGCCCAGCCGGGTGGTGCTGCGCGACA
prn2_pcDNA     GCGCCCTGCAGTCATTGCAGCCGGAAGACCTTCCGCCCAGCCGGGTGGTGCTGCGCGACA

               721       731       741       751       761       771
BPA006158_     CCAACGTGACCGCCGTGCCCGCCAGCGGCGCGCCCGCGGCGGTGTCTGTGTTGGGGGCCA
prn2_pcDNA     CCAACGTGACCGCCGTGCCCGCCAGCGGCGCGCCCGCGGCGGTGTCTGTGTTGGGGGCCA

               781       791       801       811       821       831
BPA006158_     GTGAGCTTACGCTCGACGGCGGGCACATCACCGGCGGGCGGGCAGCGGGGGTGGCGGCCA
prn2_pcDNA     GTGAGCTTACGCTCGACGGCGGGCACATCACCGGCGGGCGGGCAGCGGGGGTGGCGGCCA

               841       851       861       871       881       891
BPA006158_     TGCAAGGGGCGGTCGTGCATCTGCAGCGCGCGACGATACGGCGCGGGGACGCGCCTGCCG
prn2_pcDNA     TGCAAGGGGCGGTCGTGCATCTGCAGCGCGCGACGATACGGCGCGGGGACGCGCCTGCCG

               901       911       921       931       941       951
BPA006158_     GCGGTGCGGTTCCCGGCGGTGCGGTTCCCGGTGGTGCGGTTCCCGGCGGCTTCGGTCCCG
prn2_pcDNA     GCGGTGCGGTTCCCGGCGGTGCGGTTCCCGGTGGTGCGGTTCCCGGCGGCTTCGGTCCCG

               961       971       981       991       1001      1011
BPA006158_     GCGGCTTCGGTCCCGTCCTCGACGGCTGGTATGGCGTGGACGTATCGGGCTCCAGCGTGG
prn2_pcDNA     GCGGCTTCGGTCCCGTCCTCGACGGCTGGTATGGCGTGGACGTATCGGGCTCCAGCGTGG
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               1021      1031      1041      1051      1061      1071
BPA006158_     AGCTCGCCCAGTCGATCGTCGAGGCGCCGGAGCTGGGCGCCGCAATCCGGGTGGGCCGCG
prn2_pcDNA     AGCTCGCCCAGTCGATCGTCGAGGCGCCGGAGCTGGGCGCCGCAATCCGGGTGGGCCGCG

               1081      1091      1101      1111      1121      1131
BPA006158_     GCGCCAGGGTGACGGTGTCGGGCGGCAGCTTGTCCGCACCGCACGGCAATGTCATCGAGA
prn2_pcDNA     GCGCCAGGGTGACGGTGTCGGGCGGCAGCTTGTCCGCACCGCACGGCAATGTCATCGAGA

               1141      1151      1161      1171      1181      1191
BPA006158_     CCGGCGGCGCGCGTCGCTTTGCGCCTCAAGCCGCGCCCCTGTCGATCACCTTGCAGGCCG
prn2_pcDNA     CCGGCGGCGCGCGTCGCTTTGCGCCTCAAGCCGCGCCCCTGTCGATCACCTTGCAGGCCG

               1201      1211      1221      1231      1241      1251
BPA006158_     GCGCGCATGCCCAGGGGAAAGCGCTGCTGTACCGGGTCCTGCCGGAGCCCGTGAAGCTGA
prn2_pcDNA     GCGCGCATGCCCAGGGGAAAGCGCTGCTGTACCGGGTCCTACCGGAGCCCGTGAAGCTGA

               1261      1271      1281      1291      1301      1311
BPA006158_     CGCTGACCGGGGGCGCCGATGCGCAGGGCGACATCGTCGCGACGGAGCTGCCCTCCATTC
prn2_pcDNA     CGCTGACCGGGGGCGCCGATGCGCAGGGCGACATCGTCGCGACGGAGCTGCCCTCCATTC

               1321      1331      1341      1351      1361      1371
BPA006158_     CCGGCACGTCGATCGGGCCGCTCGACGTGGCGCTGGCCAGCCAGGCCCGATGGACGGGCG
prn2_pcDNA     CCGGCACGTCGATCGGGCCGCTCGACGTGGCGCTGGCCAGCCAGGCCCGATGGACGGGCG

               1381      1391      1401      1411      1421      1431
BPA006158_     CTACCCGCGCGGTCGACTCGCTGTCCATCGACAACGCCACCTGGGTCATGACGGACAACT
prn2_pcDNA     CTACCCGCGCGGTCGACTCGCTGTCCATCGACAACGCCACCTGGGTCATAACGGACAACT

               1441      1451      1461      1471      1481      1491
BPA006158_     CGAACGTCGGTGCGCTACGGCTGGCCAGCGACGGCAGCGTCGATTTCCAGCAGCCGGCCG
prn2_pcDNA     CGAACGTCGGTGCGCTACGGCTGGCCAGCGACGGCAGCGTCGATTTCCAGCAGCCGGCCG

               1501      1511      1521      1531      1541      1551
BPA006158_     AAGCTGGGCGGTTCAAGGTCCTGACGGTCAATACGCTGGCGGGTTCGGGGCTGTTCCGCA
prn2_pcDNA     AAGCTGGGCGGTTCAAGGTCCTGACGGTCAATACGCTGGCGGGTTCGGGGCTGTTCCGCA

               1561      1571      1581      1591      1601      1611
BPA006158_     TGAATGTCTTCGCGGACCTGGGGCTGAGCGACAAGCTGGTCGTCATGCAGGACGCCAGCG
prn2_pcDNA     TGAATGTCTTCGCGGACCTGGGGCTGAGCGACAAGCTGGTCGTCATGCAGGACGCCAGCG

               1621      1631      1641      1651      1661      1671
BPA006158_     GCCAGCACAGGCTGTGGGTCCGCAACAGCGGCAGCGAGCCGGCCAGCGCCAACACCCTGC
prn2_pcDNA     GCCAGCACAGGCTGTGGGTCCGCAACAGCGGCAGCGAGCCGGCCAGCGCCAACACCCTGC

               1681      1691      1701      1711      1721      1731
BPA006158_     TGCTGGTGCAGACGCCACTAGGCAGCGCGGCGACCTTTACCCTTGCCAACAAGGACGGCA
prn2_pcDNA     TGCTGGTGCAGACGCCACTAGGCAGCGCGGCGACCTTTACCCTTGCCAACAAGGACGGCA

               1741      1751      1761      1771      1781      1791
BPA006158_     AGGTCGATATCGGTACCTATCGCTATCGATTGGCCGCCAACGGCAATGGGCAGTGGAGCC
prn2_pcDNA     AGGTCGATATCGGTACCTATCGCTATCGATTGGCCGCCAACGGCAATGGGCAGTGGAGCC

               1801      1811      1821      1831      1841      1851
BPA006158_     TGGTGGGCGCGAAGGCGCCGCCGGCGCCCAAGCCCGCGCCGCAGCCGGGTCCCCAGCCGC
prn2_pcDNA     TGGTGGGCGCGAAGGCGCCGCCGGCGCCCAAGCCCGCGCCGCAGCCGGGTCCCCAGCCGC

               1861      1871      1881      1891      1901      1911
BPA006158_     CGCAGCCGCCGCAGCCGCAGCCGGAAGCGCCGGCGCCGCAACCGCCGGCGGGCAGGGAGT
prn2_pcDNA     CGCAGCCGCCGCAGCCGCAGCCGGAAGCGCCGGCGCCGCAACCGCCGGCGGGCAGGGAGT

               1921      1931      1941      1951      1961      1971
BPA006158_     TGTCCGCCGCCGCCAACGCGGCGGTCAACACGGGTGGGGTGGGCCTGGCCAGCACGCTCT
prn2_pcDNA     TGTCCGCCGCCGCCAACGCGGCGGTCAACACGGGTGGGGTGGGCCTGGCCAGCACGCTCT

               1981      1991      2001      2011      2021      2031
BPA006158_     GGTACGCCGAAAGCAATGCGTTGTCCAAGCGCCTGGGCGAGTTGCGCCTGAATCCGGACG
prn2_pcDNA     GGTACGCCGAAAGCAATGCGTTGTCCAAGCGCCTGGGCGAGTTGCGCCTGAATCCGGACG
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               2041      2051      2061      2071      2081      2091
BPA006158_     CCGGCGGCGCCTGGGGCCGCGGCTTCGCGCAACGCCAGCAGCTGGACAACCGCGCCGGGC
prn2_pcDNA     CCGGCGGCGCCTGGGGCCGCGGCTTCGCGCAACGCCAGCAGCTGGACAACCGCGCCGGGC

               2101      2111      2121      2131      2141      2151
BPA006158_     GGCGCTTCGACCAGAAGGTGGCCGGCTTCGAGCTGGGCGCCGACCACGCGGTGGCGGTGG
prn2_pcDNA     GGCGCTTCGACCAGAAGGTGGCCGGCTTCGAGCTGGGCGCCGACCACGCGGTGGCGGTGG

               2161      2171      2181      2191      2201      2211
BPA006158_     CCGGCGGACGCTGGCACCTGGGCGGGCTGGCCGGCTATACGCGCGGCGACCGCGGCTTCA
prn2_pcDNA     CCGGCGGACGCTGGCACCTGGGCGGGCTGGCCGGCTATACGCGCGGCGACCGCGGCTTCA

               2221      2231      2241      2251      2261      2271
BPA006158_     CCGGCGACGGCGGCGGCCACACCGACAGCGTGCATGTCGGGGGCTATGCCACATATATCG
prn2_pcDNA     CCGGCGACGGCGGCGGCCACACCGACAGCGTGCATGTCGGGGGCTATGCCACA-------

               2281      2291      2301      2311      2321      2331
BPA006158_     CCGACAGCGGTTTCTACCTGGACGCGACGCTGCGCGCCAGCCGCCTGGAGAATGACTTCA
prn2_pcDNA     ------------------------------------------------------------

               2341      2351      2361      2371      2381      2391
BPA006158_     AGGTGGCGGGCAGCGACGGGTACGCGGTCAAGGGCAAGTACCGCACCCATGGGGTGGGCG
prn2_pcDNA     ------------------------------------------------------------
```

Figure D.5: Nucleotide sequence alignment of published *prn* Genbank Sequence and pcDNA3.1D/*prn* insert sequence. ClustalW program (BioManager) was used to compare the two sequences. BPA0061458_ refers to Genbank sequence of *prn* (accession # AJ006158) and prn2_pcDN refers to the sequenced insert of pcDNA3.1D/*prn*.

Table D.17: Cytokine production from *in-vitro* re-stimulated splenocytes from mice immunised with pcDNA3.1D/*prn*. Values represent mean (pg/ml) from duplicate wells. Nd – Not determined.

Vaccine Group	Stimulant	IFN-γ	IL-2	IL-4
pcDNA3.1D/*prn*	rPRN	1486	54.8	1
pcDNA3.1D/*prn*	DTaP	596	18.8	8.7
pcDNA3.1D/*prn*	HKBP	521	Nd	Nd
pcDNA3.1 vector	rPRN	359	12	16.8
DTaP (Infanrix™)	rPRN	1039	111.2	58.9
pcDNA3.1D/*prn*	ConA	6000	14670.8	455.1

Table D.18: Summary of serum anti-rPRN IgG. Nd – Not determined for individual mice (tested as pooled serum sample).

Vaccine Group	Individual Mice					Mean	SE	Signif. (P)
	M1	M2	M3	M4	M5			
pcDNA3.1D/*prn*	18909	42361	10472	21014	84337	35419	13311	
pcDNA3.1D vector	Nd	Nd	Nd	Nd	Nd	400	0	
DTaP	185897	142629	116388	135104	166787	149361	12195	*

* = significantly different from pcDNA3.1D/*prn* ($P < 0.005$)

Table D.19: Clearance of *B. pertussis* from lungs following aerosol challenge (SLID). Bacterial load expressed as log10 CFU/lung

Group	Day	Mouse1	Mouse2	Mouse3	Mouse4
pcDNA3.1D/*prn*	0	5.699	5.602	5.778	5.699
	4	5.505	4.929	5.111	5.699
	7	4.42	4.117	4.778	4.42
	14	4.38	0	4	0
pcDNA3.1 Vector	0	5.699	5.602	5.778	5.699
	4	6.439	6.778	6.303	6.41
	7	5.663	6.799	6.991	6.212
	14	5.255	6.204	5.716	5.959
Placebo	0	5.699	5.602	5.778	5.699
	4	5.217	6.318	6.442	5.217
	7	6.756	5.764	6.672	6.176
	14	6.27	6.079	5.7	5.996
DTaP	0	5.699	5.602	5.778	5.699
	4	5.58	5.613	5.613	5.602
	7	0	0	0	0
	14	0	0	0	0

Table D.20: Growth curve of SL3261-pcDNA3.1D/*prn* for the preparation of the oral combination DNA vaccine.

Transformant	OD_{600}	Mean Count (CFU)	Dil. Factor	Adj.Count (CFU/ml)	Adj. Count (log10CFU/ml)
SL3261-pcDNA3.1D/*prn*	0.265	44	2.00E+05	8.80E+06	6.944482672
	0.609	90	2.00E+05	1.80E+07	7.255272505
	1	24	2.00E+06	4.80E+07	7.681241237
	1.45	63	2.00E+06	1.26E+08	8.100370545
SL3261-pcDNA3.1 Vector	0.182	50	2.00E+06	1.00E+08	8
	0.67	24	2.00E+07	4.80E+08	8.681241237
	1.12	46	2.00E+07	9.20E+08	8.963787827
	1.39	64	2.00E+07	1.28E+09	9.10720997
SL3261	0.218	46	2.00E+06	9.20E+07	7.963787827
	0.515	20	2.00E+07	4.00E+08	8.602059991
	0.998	41	2.00E+07	8.20E+08	8.913813852
	1.22	92	2.00E+07	1.84E+09	9.264817823

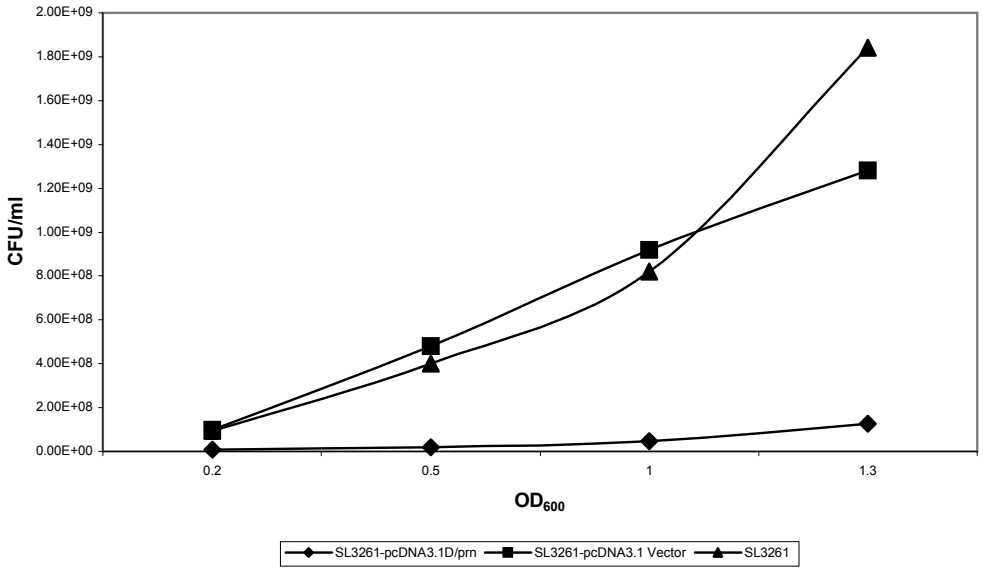

Figure D.6: Growth curve of SL3261-pcDNA3.1D/*prn* for the preparation of the oral combination DNA vaccine.

D.4 Chapter 6

1 GAATTCGTCG CCTCGCCCTG GTTCGCCGTC ATGGCCCCCA AGGGAACCGA
51 CCCCAAGATA ATCGTCCTGC TCAACCGCCA CATCAACGAG GCGCTGCAGT
101 CCAAGGCGGT CGTCGAGGCC TTTGCCGCCC AAGGCGCCAC GCCGGTCATC
151 GCCACGCCGG ATCAGACCCG CGGCTTCATC GCAGACGAGA TCCAGCGCTG
201 GGCCGGCGTC GTGCGCGAAA CCGGCGCCAA GCTGAAGTAG CAGCGCAGCC
251 CTCCAACGCG CCATCCCCGT CCGGCCGGCA CCATCCCGCA TACGTGTTGG
301 CAACCGCCAA CGCGCATGCG TGCAGATTCG TCGTACAAAA CCCTCGATTC
351 TTCCGTACAT CCCGCTACTG CAATCCAACA CGGCATGAAC GCTCCTTCGG
401 CGCAAAGTCG CGCGATGGTA CCGGTCACCG TCCGGACCGT GCTGACCCCC
451 CTGCCATGGT GTGATCCGTA AAATAGGCAC CATCAAAACG CAGAGGGGAA

PTX507DNAF/PTX507TRCF→

501 GACGGG**ATGC GTTGCACTCG GGCAATTCGC** CAAACCGCAA GAACAGGCTG
551 GCTGACGTGG CTGGCGATTC TTGCCGTCAC GGCGCCCGTG ACTTCGCCGG

PTXS1-13LF/PTXS1-13LR

601 CATGGGCCGA CGATCCTCCC GCCACCGTA**T ACCGCTATGA CTCCCGCCCG**
651 **CCGGAGGAC**G TTTTCCAGAA CGGATTCACG GCGTGGGGAA ACAACGACAA
701 TGTGCTCGAC CATCTGACCG GACGTTCCTG CCAGGTCGGC AGCAGCAACA
751 GCGCTTTCGT CTCCACCAGC AGCAGCCGGC GCTATACCGA GGTCTATCTC
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851 CGGCCACTTC ATCGGCTACA TCTACGAAGT CCGCGCCGAC AACAATTTCT
901 ACGGCGCCGC CAGCTCGTAC TTCGAATACG TCGACACTTA TGGCGACAAT

PTXS1-129GF/PTXS1-129GR

951 GCCGGCCGTA TCCTCGCCGG CGCGCTG**GCC ACCTACCAGA GCGAATATCT**
1001 **GGCACACCGG** CGCATTCCGC CCGAAAACAT CCGCAGGGTA ACGCGGGTCT
1051 ATCACAACGG CATCACCGGC GAGACCACGA CCACGGAGTA TTCCAACGCT
1101 CGCTACGTCA GCCAGCAGAC TCGCGCCAAT CCCAACCCCT ACACATCGCG
1151 AAGGTCCGTA GCGTCG**ATCG TCGGCACATT GGTG**CGCATG GCGCCGGTGA
1201 TAGGCGCTTG CATGGCGCGG CAGGCCGAAA GCTCCGAGGC CATGGCAGCC

←PTX1313DNAR/PTX1313TRCR

1251 TGGTCCGAAC GCGCCGGCGA GGCGATGGTT CTCGTG**TACT ACGAAAGCAT**
1301 **CGCGTATTCG TTC**TAGACCT GGCCCAGCCC CGCCCAACTC CGGTAATTGA
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1701 ATGACCACGC GCAATACGGG TCAACCCGCA ACGGATCACT ACTACAGCAA
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1801 TCTTCGTCAG AAGCGGGCAA CCGGTCATTG GCGCCTGCAC CAGCCCGTAT
1851 GACGGCAAGT ACTGGAGCAT GTACAGCCGG CTGCGGAAAA TGCTTTACCT
1901 GATCTACGTG GCCGGCATCT CCGTACGCGT CCATGTCAGC AAGGAAGAAC
1951 AGTATTACGA CTATGAGGAC GCAACGTTCG AGACTTACGC CCTTACCGGC
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2201 GTAGCCATGA AGCCGTATGA AGTCACCCCG ACGCGCATGC TGGTCTGCGG
2251 CATCGCCGCC AAACTGGGCG CCGCGGCCAG CAGCCCGGAC GCGCACGTGC
2301 CGTTCTGCTT CGGCAAGGAT CTCAAGCGTC CCGGCAGCAG TCCCATGGAA
2351 GTCATGTTGC GCGCCGTCTT CATGCAACAA CGGCCGCTGC GCATGTTTCT
2401 GGGTCCCAAG CAACTCACTT TCGAAGGCAA GCCCGCGCTC GAACTGATCC
2451 GGATGGTCGA ATGCAGCGGC AAGCAGGATT GCCCCTGAAG GCGAACCCCA
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2551 GCTGACGTCG CCGGCTTGCC GACCCATCTG TACAAGAACT TCACTGTCCA
2601 GGAGCTGGCC TTGAAACTGA AGGGCAAGAA TCAGGAGTTC TGCCTGACCG
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2751 GTATGCGCTC AAGAGCCGGA TCGCGCTGAC GGTGGAAGAC TCGCCGTATC
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3351 CCACGCGCCT GCTCGCCAGC ACCAACAGCA GGCTGTGCGC GGTATTCGTC
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3451 GTACAGAGAC ATGTACGACG CGCTGCGGCG CCTGCTGTAC ATGATCTATA
3501 TGTCCGGCCT TGCCGTACGC GTCCACGTCA GCAAGGAAGA GCAGTATTAC
3551 GACTACGAGG ACGCCACATT CCAGACCTAT GCCCTCACCG GCATTTCCCT
3601 CTGCAACCCG GCAGCGTCGA TATGCTGAGC CGCCGGCTCG GATCTGTTCG
3651 CCTGTCCATG TTTTTCCTTG ACGGATACCG CGAATGAATC CCTTGAAAGA

3701 CTTGAGAGCA TCGCTACCGC GCCTGGCCTT CATGGCAGCC TGCACCCTGT
3751 TGTCCGCCAC GCTGCCCGAC CTCGCCCAGG CCGGCGGCGG GCTGCAGCGC
3801 TGTCAACCAC TTCATGGCGA GCATCGTGGT CGTACTGCCG CGGCGGTCAG
3851 TGGCCACGGT GACCATCGCC ATAATCTGGG CGGGCTACAA GCTGCTGTTC
3901 CGGCACGCCG ATGTGCTGGA CGTGGTGCGT GTGGTGCTGG CGGGAGCTGC
3951 TGATCGGCGC ATCGGCCGAA ATCGCTCGTT ATCTGCTGAC CTGAATCCTG
4001 GACGTATCGA ACATGCGTGA TCCGCTTTTC AAGGGCTGCA CCCGGCGCCG
4051 CGATGCTGAT GGCGTACCCG CCACGGCAGG CCGTGTGCAG CCGGCACCAT
4101 TCCCTGCTGG GCCATCTCGG TTCAGCATCC GCTTTCTGGC CTTGTTTCCC
4151 GTGGCATTGC TGGCGATGCG GATCATGATC CGGCGCGATG ACCAGCAGTT
4201 CCGCCTGATC

Figure D.7: Genbank sequence of *ptx* gene (accession # M13223). PCR primer sites are highlighted in blue, SDM primer site in red and sequencing primer sites in magenta.

```
               421       431       441       451       461       471
BPETOX         CCGGTCACCGTCCGGACCGTGCTGACCCCCCTGCCATGGTGTGATCCGTAAAATAGGCAC
PTS1.13L.1     ------------------------------------------------------------

               481       491       501       511       521       531
BPETOX         CATCAAAACGCAGAGGGGAAGACGGGATGCGTTGCACTCGGGCAATTCGCCAAACCGCAA
PTS1.13L.1     ----------------------CACCATGCGTTGCACTCGGGCAATTCGCCAAACCGCAA

               541       551       561       571       581       591
BPETOX         GAACAGGCTGGCTGACGTGGCTGGCGATTCTTGCCGTCACGGCGCCCGTGACTTCGCCGG
PTS1.13L.1     GAACAGGCTGGCTGACGTGGCTGGCGATTCTTGCCGTCACGGCGCCCGTGACTTCGCCGG

               601       611       621       631       641       651
BPETOX         CATGGGCCGACGATCCTCCCGCCACCGTATACCGCTATGACTCCCGCCCGCCGGAGGACG
PTS1.13L.1     CATGGGCCGACGATCCTCCCGCCACCGTATACCGCTATGACTCCCTGCCGCCGGAGGACG

               661       671       681       691       701       711
BPETOX         TTTTCCAGAACGGATTCACGGCGTGGGGAAACAACGACAATGTGCTCGACCATCTGACCG
PTS1.13L.1     TTTTCCAGAACGGATTCACGGCGTGGGGAAACAACGACAATGTGCTCGACCATCTGACCG

               721       731       741       751       761       771
BPETOX         GACGTTCCTGCCAGGTCGGCAGCAGCAACAGCGCTTTCGTCTCCACCAGCAGCAGCCGGC
PTS1.13L.1     GACGTTCCTGCCAGGTCGGCAGCAGCAACAGCGCTTTCGTCTCCACCAGCAGCAGCCGGC

               781       791       801       811       821       831
BPETOX         GCTATACCGAGGTCTATCTCGAACATCGCATGCAGGAAGCGGTCGAGGCCGAACGCGCCG
PTS1.13L.1     GCTATACCGAGGTCTATCTCGAACATCGCATGCAGGAAGCGGTCGAGGCCGAACGCGCCG

               841       851       861       871       881       891
BPETOX         GCAGGGGCACCGGCCACTTCATCGGCTACATCTACGAAGTCCGCGCCGACAACAATTTCT
PTS1.13L.1     GCAGGGGCACCGGCCACTTCATCGGCTACATCTACGAAGTCCGCGCCGACAACAATTTCT

               901       911       921       931       941       951
BPETOX         ACGGCGCCGCCAGCTCGTACTTCGAATACGTCGACACTTATGGCGACAATGCCGGCCGTA
PTS1.13L.1     ACGGCGCCGCCAGCTCGTACTTCGAATACGTCGACACTTATGGCGACAATGCCGGCCGTA

               961       971       981       991       1001      1011
BPETOX         TCCTCGCCGGCGCGCTGGCCACCTACCAGAGCGAATATCTGGCACACCGGCGCATTCCGC
PTS1.13L.1     TCCTCGCCGGCGCGCTGGCCACCTACCAGAGCGGCTATCTGGCACACCGGCGCATTCCGC

               1021      1031      1041      1051      1061      1071
```

```
BPETOX          CCGAAAACATCCGCAGGGTAACGCGGGTCTATCACAACGGCATCACCGGCGAGACCACGA
PTS1.13L.1      CCGAAAACATCCGCAGGGTAACGCGGGTCTATCACAACGGCATCACCGGCGAGACCACGA

                1081      1091      1101      1111      1121      1131
BPETOX          CCACGGAGTATTCCAACGCTCGCTACGTCAGCCAGCAGACTCGCGCCAATCCCAACCCCT
PTS1.13L.1      CCACGGAGTATTCCAACGCTCGCTACGTCAGCCAGCAGACTCGCGCCAATCCCAACCCCT

                1141      1151      1161      1171      1181      1191
BPETOX          ACACATCGCGAAGGTCCGTAGCGTCGATCGTCGGCACATTGGTGCGCATGGCGCCGGTGA
PTS1.13L.1      ACACATCGCGAAGGTCCGTAGCGTCGATCGTCGGCACATTGGTGCGCATGGCGCCGGTGA

                1201      1211      1221      1231      1241      1251
BPETOX          TAGGCGCTTGCATGGCGCGGCAGGCCGAAAGCTCCGAGGCCATGGCAGCCTGGTCCGAAC
PTS1.13L.1      TAGGCGCTTGCATGGCGCGGCAGGCCGAAAGCTCCGAGGCCATGGCAGCCTGGTCCGAAC

                1261      1271      1281      1291      1301      1311
BPETOX          GCGCCGGCGAGGCGATGGTTCTCGTGTACTACGAAAGCATCGCGTATTCGTTCTAGACCT
PTS1.13L.1      GCGCCGGCGAGGCGATGGTTCTCGTGTACTACGAAAGCATCGCGTATTCGTTC-------

                1321      1331      1341      1351      1361      1371
BPETOX          GGCCCAGCCCCGCCCAACTCCGGTAATTGAACAGCATGCCGATCGACCGCAAGACGCTCT
PTS1.13L.1      ------------------------------------------------------------
```

Figure D.8: Nucleotide sequence alignment of published *ptx* Genbank sequence and pcDNA3.1D/*pts1.13L.129G* insert sequence. ClustalW program (BioManager) was used to compare the two sequences. BPETOX refers to Genbank sequence of *ptx* (accession # M13223) and PTS1.13L.1 refers to the sequenced insert of pcDNA3.1D/*pts1.13L.129G*.

Table D.21: Cytokine production from *in-vitro* re-stimulated splenocytes from mice immunised with pcDNA3.1D/*pts1.13L.129G*. Values represent mean (pg/ml) from duplicate wells. Nd – Not determined.

Vaccine Group	Stimulant	IFN-γ	IL-2	IL-4
pcDNA3.1D/ *pts1.13L.129G*	rPTS1.13L.129G	5253	35.6	15.3
pcDNA3.1D/ *pts1.13L.129G*	DTaP	450	5	9.5
pcDNA3.1D/ *pts1.13L.129G*	HKBP	1629	Nd	Nd
pcDNA3.1 vector	rPTS1.13L.129G	647	9.4	12.8
DTaP (Infanrix™)	rPTS1.13L.129G	1755	38.5	5.3
pcDNA3.1D/ *pts1.13L.129G*	ConA	6000	12388.3	476.7

Table D.22: Summary of serum anti-rPTS1.13L.129G IgG. Nd – Not determined.

Vaccine Group	Individual Mice					Mean	SE
	M1	M2	M3	M4	M5		
pcDNA3.1D/*pts1.13L.129G*	0	0	0	0	0	0	0
pcDNA3.1D vector	Nd	Nd	Nd	Nd	Nd	0	0
DTaP	6400	3200	6400	3200	51200	14080	9308

Table D.23: Summary of serum anti-PTX IgG. Nd – Not determined.

Vaccine Group	Individual Mice					Mean	SE	Signif. (P)
	M1	M2	M3	M4	M5			
pcDNA3.1D/*pts1.13L.129G*	0	0	0	0	0	0	0	
pcDNA3.1D vector	Nd	Nd	Nd	Nd	Nd	0	0	
DTaP	138487	51200	102400	51200	118559	92369	17753	*

* = significantly different from pcDNA3.1D/*pts1.13L.129G* ($P < 0.01$)

Table D.24: Clearance of *B. pertussis* from lungs following aerosol challenge (SLID). Bacterial load expressed as log10 CFU/lung

Group	Day	Mouse1	Mouse2	Mouse3	Mouse4
pcDNA3.1D/*pts1.13L.129G*	0	5.699	5.602	5.778	5.699
	4	4.623	5.294	6.892	5.158
	7	4.057	4.987	4.82	3.944
	14	0	2.505	0	0
pcDNA3.1 Vector	0	5.699	5.602	5.778	5.699
	4	6.439	6.778	6.303	6.41
	7	5.663	6.799	6.991	6.212
	14	5.255	6.204	5.716	5.959
Placebo	0	5.699	5.602	5.778	5.699
	4	5.217	6.318	6.442	5.217
	7	6.756	5.764	6.672	6.176
	14	6.27	6.079	5.7	5.996
DTaP	0	5.699	5.602	5.778	5.699
	4	5.58	5.613	5.613	5.602
	7	0	0	0	0
	14	0	0	0	0

Table D.25 Growth curve of SL3261-pcDNA3.1D/*pts1.13L.129G* for the preparation of the oral combination DNA vaccine.

Transformant	OD_{600}	Mean Count (CFU)	Dil. Factor	Adj.Count (CFU/ml)	Adj. Count (log10CFU/ml)
SL3261-pcDNA3.1D/*pts1.13L.129G*	0.236	52	2.00E+06	1.04E+08	8.017033339
	0.5	117	2.00E+06	2.34E+08	8.369215857
	1.08	37	2.00E+07	7.40E+08	8.86923172
	1.38	64	2.00E+07	1.28E+09	9.10720997
SL3261-pcDNA3.1 Vector	0.182	50	2.00E+06	1.00E+08	8
	0.67	24	2.00E+07	4.80E+08	8.681241237
	1.12	46	2.00E+07	9.20E+08	8.963787827
	1.39	64	2.00E+07	1.28E+09	9.10720997
SL3261	0.218	46	2.00E+06	9.20E+07	7.963787827
	0.515	20	2.00E+07	4.00E+08	8.602059991
	0.998	41	2.00E+07	8.20E+08	8.913813852
	1.22	92	2.00E+07	1.84E+09	9.264817823

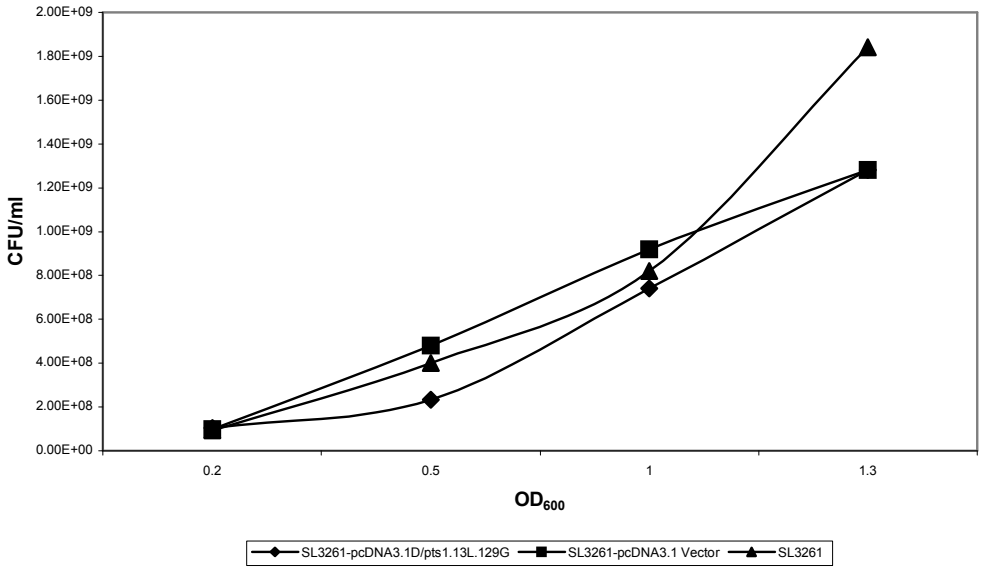

Figure D.9: Growth curve of SL3261-pcDNA3.1D/*pts1.13L.129G* for the preparation of the oral combination DNA vaccine.

D.5 Chapter 7

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   1  CGATCATTCG GCATGTACGG TCCAGCTGCG CGCGAGCGGC GGCCGCGTCC
  51  AGCGCGCGGC CTCGGTACTC CTTGACGCGC GCGGTGTCGC CGCCGCGCCG
 101  AACGCGCAGC GAACGGCCCA CGCTGTCGGG GTGCCGTTCG GCCAGCGCGC
 151  GGCGCAGCGC ACGATTGTCG TCGCGCGAGA ATGGCGCGAT CCAGTCGATG
 201  ATCCACAGTC GGTCGCCGCA GTTCCAGGCA TTCCCGCCCA GCGACGAGGG
 251  CGCCATGACA TAGGAGAGTT CGGTGTCGGC GTCCATTAGG GCCCAGCTGC
 301  AGTATGCAAC CGGCACGTCA TTGCATCGCA GCAGAATGTA TTGGCCCAGT
 351  TGAATCGGCG CGAGCGCTGT TGCGTGCGAG CAGATGCACC GGCCAGTCGC
 401  GGTGCATGGG AGAGTTCATC CACAGCCAGG CAATATTGCC CAGTGCCGCG
 451  AAGTCGTCGG TGGGATTGAG GAGGGAGGGC GCTTGGGCGG ACGGAAGCAT
 501  GACATCGGTG CATGGTGGAG CGGGGGGCAT ATTCCGTGTT GGGTGCGCGC
 551  ATGGCAAGCC GCCGGCGCAT CATGGTTGCG CCGGAATGGC TTTTCTTACA
 601  TGTTTCCAGG ATATGTCCGT ATTTCGGGCG ATGCCTCGGT CGCGGCGCCT
 651  GCTTTTGTCG AACATGTGCA ATGTTGTTGT CGCGATCGCG TTGGCGCTTG
 701  CTCGCTTATT TATCTCCCTT GAAGCCTTGT TCTTCTTTTC ATTAGAAAGA
 751  AATATGCGCT TTGTGTTTAG GATGATTTTC CTGTCCGAGT AGGGTGGATC
 801  CAAATTTTCC GGATTGGTGG GAATTTGTGC ATTTTCACTG CGAATGTTGG
                                  CYAA883TRCF/CYAA883DNAF→
 851  AATAATTTCG CCCATCGTCA TACGACATGC TGGATGTTTG GTTCTTGCAG
 901  AAGGATGAGG TTCTGAGCGC TACACACCGG TTGCGTCGGT GCGAATCCGT
 951  TCAATCGACT ACTTATCGAC AGATCCACAT GCAGCAATCG CATCAGGCTG
1001  GTTACGCAAA CGCCGCCGAC CGGGAGTCTG GCATCCCCGC AGCCGTACTC
1051  GATGGCATCA AGGCCGTGGC GAAGGAAAAA AACGCCACAT TGATGTTCCG
                                  CYAA-58LF/CYAA-58LR
1101  CCTGGTCAAC CCCCATTCCA CCAGCCTGAT TGCCGAAGGG GTGGCCACCA
1151  AAGGATTGGG CGTGCACGCC AAGTCGTCCG ATTGGGGGTT GCAGGCGGGC
1201  TACATTCCCG TCAACCCGAA TCTTTCCAAA CTGTTCGGCC GTGCGCCCGA
1251  GGTGATCGCG CGGGCCGACA ACGACGTCAA CAGCAGCCTG GCGCATGGCC
1301  ATACCGCGGT CGACCTGACG CTGTCGAAAG AGCGGCTTGA CTATCTGCGG
1351  CAAGCGGGCC TGGTCACCGG CATGGCCGAT GGCGTGGTCG CGAGCAACCA
1401  CGCAGGCTAC GAGCAGTTCG AGTTTCGCGT GAAGGAAACC TCGGACGGGC
1451  GCTATGCCGT GCAGTATCGC CGCAAGGGCG GCGACGATTT CGAGGCGGTC
1501  AAGGTGATCG GCAATGCCGC CGGTATTCCA CTGACGGCGG ATATCGACAT
1551  GTTCGCCATT ATGCCGCATC TGTCCAACTT CCGCGACTCG GCGCGCAGTT
1601  CGGTGACCAG CGGCGATTCG GTGACCGATT ACCTGGCGCG CACGCGGCGG
1651  GCCGCCAGCG AGGCCACGGG CGGCCTGGAT CGCGAACGCA TCGACTTGTT
1701  GTGGAAAATC GCTCGCGCCG GCGCCCGTTC CGCAGTGGGC ACCGAGGCGC
1751  GTCGCCAGTT CCGCTACGAC GGCGACATGA ATATCGGCGT GATCACCGAT
```

1801 TTCGAGCTGG AAGTGCGCAA TGCGCTGAAC AGGCGGGCGC ACGCCGTCGG
1851 CGCGCAGGAC GTGGTCCAGC ATGGCACTGA GCAGAACAAT CCTTTCCCGG
1901 AGGCAGATGA GAAGATTTTC GTCGTATCGG CCACCGGTGA AAGCCAGATG
1951 CTCACGCGCG GGCAACTGAA GGAATACATT GGCCAGCAGC GCGGCGAGGG
2001 CTATGTCTTC TACGAGAACC GTGCATACGG CGTGGCGGGG **AAAAGCCTGT**
2051 **TCGACGAT**GG GCTGGGAGCC GCGCCCGGCG TGCCGAGCGG ACGTTCGAAG
2101 TTCTCGCCGG ATGTACTGGA AACGGTGCCG GCGTCACCCG GATTGCGGCG
2151 GCCGTCGCTG GGCGCAGTGG AACGCCAGGA TTCCGGCTAT GACAGCCTTG
2201 ATGGGGTGGG ATCGCGATCG TTCTCGTTGG GCGAGGTGTC CGACATGGCC
2251 GCCGTGGAAG CGGCGGAACT GGAAATGACC CGGCAAGTCT TGCACGCCGG
2301 GGCGCGGCAG GACGATGCCG AGCCGGGCGT GAGCGGTGCG TCGGCGCACT
2351 GGGGGCAGCG GGCGCTGCAG GGCGCCCAGG CGGTGGCGGC GGCGCAGCGG
2401 CTGGTTCATG CCATTGCCCT GATGACGCAA TTCGGCCGGG CCGGTTCCAC
2451 CAACACGCCG CAGGAAGCGG CCTCGTTGTC GGCGGCCGTG TTCGGCTTGG
2501 GCGAGGCCAG CAGCGCCGTG GCCGAA**ACCG TGAGCGGTTT TTTC**CGCGGG
2551 TCTTCGCGCT GGGCCGGCGG TTTCGGCGTG GCTGGCGGCG CGATGGCGCT
2601 GGGAGGCGGC ATCGCCGCGG CCGTTGGCGC CGGGATGTCG TTGACCGATG
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3201 AAACCTGCAG GCGCGTCAC**G AACAACTGGC CAATTCG**GAC GGCCTACGGA
3251 AAATGCTGGC CGACCTGCAG GCCGGTTGGA ACGCCAGCAG CGTGATCGGG
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3451 GGCGGCATCG ATATCGCCAG CCGCAAGGGC GAGCGGCCGG CGCTGACGTT
3501 CATCACGCCG CTGGCCGCGC CAGGAGAAGA GCAGCGCCGG CGCACGAAAA
3551 CGGGCAAGAG CGAATTCACC ACATTCGTCG AGATCGTGGG CAAGCAGGAC
3601 CGCTGGCGCA TCCGGGACGG CGCGGCCGAC ACCACCATCG ATCTGGCCAA
3651 GGTGGTGTCG CAACTGGTCG ACGCCAATGG CGTGCTC**AAG CACAGCATCA**
3701 **AACTGGAT**GT GATCGGCGGA GATGGCGATG ACGTCGTGCT TGCCAATGCT
3751 TCGCGCATCC ATTATGACGG CGGCGCGGGC ACCAACACGG TCAGCTATGC

3801 CGCCCTGGGT CGACAGGATT CCATTACCGT GTCCGCCGAC GGGGAACGTT
3851 TCAACGTGCG CAAGCAGTTG AACAACGCCA ACGTGTATCG CGAAGGCGTG
3901 GCTACCCAGA CAACCGCCTA CGGCAAGCGC ACGGAGAATG TCCAATACCG
3951 CCATGTCGAG CTGGCCCGTG TCGGGCAAGT GGTGGAGGTC GACACGCTCG
4001 AGCATGTGCA GCACATCATC GGCGGGGCCG GCAACGATTC GATCACCGGC
4051 AATGCGCACG ACAACTTCCT AGCCGGCGGG TCGGGCGACG ACAGGCTGGA
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4151 TCATCGGCGG CGCCGGCGAC GACGTATTCC TGCAGGACCT GGGGGTATGG
4201 AGCAACCAGC TCGATGGCGG CGCGGGCGTC GATACCGTGA AGTACAACGT
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4301 ATGCCGATCT TCAAAAGGGC ACGGTCGAGA AGTGGCCGGC CCTGAACCTG
4351 TTCAGCGTCG ACCATGTCAA GAATATCGAG AATCTGCACG GCTCCCGCCT
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4701 CAAGGCGTCC GCGCTGGGCG TGGACTATTA CGATAATGTC CGCAATGTCG
4751 AAAACGTCAT CGGTACGAGC ATGAAGGATG TGCTCATCGG CGACGCGCAA
4801 GCCAATACCC TGATGGGCCA GGGCGGCGAC GATACCGTGC GCGGCGGCGA
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4901 ACGCCGGCAA CGACACCCTC TACGGGGGGC TGGGCGACGA TACCCTTGAA
4951 GGCGGCGCGG GCAACGATTG GTTCGGCCAG ACGCAGGCGC GCGAGCATGA
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5101 GACCTGGGCG CCGGCCGCGT CGACAAGCTG GGCGAGGCCG GCAGCAGCGC
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5201 ACCGCATCAC GGGCGATGCG CAGGCCAACG TGCTGCGCGG CGCGGGTGGC
5251 GCCGACGTGC TTGCGGGCGG CGAGGGCGAC GATGTGCTGC TGGGCGGCGA
5301 CGGCGACGAC CAGCTGTCGG GCGACGCCGG ACGCGATCGC TTGTACGGCG
5351 AAGCCGGTGA CGACTGGTTC TTCCAGGATG CCGCCAATGC CGGCAATCTG
5401 CTCGACGGCG GCGACGGCCG CGATACCGTG GATTTCAGCG GCCCGGGCCG
5451 GGGCCTCGAC GCCGGCGCAA AGGGCGTATT CCTGAGCTTG GGCAAGGGGT
5501 TCGCCAGCCT GATGGACGAA CCCGAAACCA GCAACGTGTT GCGCAATATC
5551 GAGAACGCCG TGGGCAGCGC GCGTGATGAC GTGCTGATCG GCGACGCAGG
5601 CGCCAACGTC CTCAATGGCC TGGCGGGCAA CGACGTGCTG TCCGGCGGCG
5651 CTGGCGACGA TGTGCTGCTG GGCGACGAGG GCTCGGACCT GCTCAGCGGC
5701 GATGCGGGCA ACGACGATCT GTTCGGCGGG CAGGGCGATG ATACTTATCT
5751 GTTCGGGGTC GGGTACGGGC ACGACACGAT CTACGAATCG GGCGGCGGCC

5801 ATGACACCAT CCGCATCAAC GCGGGGGCGG ACCAGCTGTG GTTCGCGCGC
5851 CAGGGCAACG ACCTGGAGAT CCGCATTCTC GGCACCGACG ATGCACTTAC
5901 CGTGCACGAC TGGTATCGCG ACGCCGATCA CCGGGTGGAA ATCATCCATG
5951 CCGCCAACCA GGCGGTAGAC CAGGCAGGCA TCGAAAAGCT GGTCGAGGCA
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←**CYAA6096R**

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6151 CGTTCTTCTC CGAGGTATTT CCCATCATGA CGTCGCCCGG GGCGCAATGC
6201 GCCAGCGTGC CCGATTCCGG GTTGCTCTGC CTGGTCATGC TGGCTCGCTA
6251 TCACGGATTG GCAGCCGATC CCGAGCAGTT GCGGCATGAG TTCGCCGAGC
6301 AGGCATTCTG TAGCGAAACG ATACAGCCTG GCGGCGCGCC GGGTCGGCCT
6351 GAAAGTGCGG CGGCACCGAC CCGCGCCGGC GCGGCTGCCA CGCGCGCCGC
6401 TGCCGGCCAT CGCGCTGGAC CGGCAGGGCG GCTACTTTGT T

Figure D.4: Genbank sequence of *cyaA* (accession # A14850). PCR primer sites are highlighted in blue, SDM primer site in red and sequencing primer sites in magenta.

1 ACAAAAGCAG GCGCCGCGAC CGAGGCATCG CCCGAAATAC GGACATATCC
51 TGGAAACATG TAAGAAAAGC CATTCCGGCG CAACCATGAT GCGCCGGCGG

CYAC111DNAF→ **CYAC135TRCF→**

101 CTTGCCATGC **GCGCACCCAA CACGG**AATAT GCCC**CCCGCT CCACCATGCA**
151 **CCGATGTC**AT GCTTCCGTCC GCCCAAGCGC CCTCCCTCCT CAATCCCACC
201 GACGACTTCG CGGCACTGGG CAATATTGCC TGGCTGTGGA TGAACTCTCC
251 CATGCACCGC GACTGGCCGG TGCATCTGCT CGCACGCAAC ACGCTCGCGC
301 CGATTCAACT GGGCCAATAC ATTCTGCTGC GATGCAATGA CGTGCCGGTT
351 GCATACTGCA GCTGGGCCCT AATGGACGCC GACACCGAAC TCTCCTATGT
401 CATGGCGCCC TCGTCGCTGG GCGGGAATGC CTGGAACTGC GGCGACCGAC
451 TGTGGATCAT CGACTGGATC GCGCCATTCT CGCGCGACGA CAATCGTGCG
501 CTGCGCCGCG CGCTGGCCGA ACGGCACCCC GACAGCGTGG GCCGTTCGCT
551 GCGCGTTCGG CGCGGCGGCG ACACCGCGCG CGTCAAGGAG TACCGAGGCC
601 GCGCGCTGGA CGCGGCCGCC ACTCGCGCGC AGCTGGACCG CTACCATGCC
651 GAACTGATCG CAGGACTGCG CGCGAGCAAC GGCGGATACG CGCCG**CGAGG**

←**CYAC713R**

701 **CCGGGGCACC GCC**TGA

Figure D.10: Genbank sequence of *cyaC* (accession # M57286). PCR primer sites are highlighted in blue. Insert sequencing primer sites were not required.

```
                781       791       801       811       821       831
BPCYA           TCCTGTCCGAGTAGGGTGGATCCAAATTTTCCGGATTGGTGGGAATTTGTGCATTTTCAC
cyaAL58_pc      ------------------------------------------------------------

                841       851       861       871       881       891
BPCYA           TGCGAATGTTGGAATAATTTCGCCCATCGTCATACGACATGCTGGATGTTTGGTTCTTGC
cyaAL58_pc      ------------------------------------CACCATGGATGTTTGGTTCTTGC

                901       911       921       931       941       951
BPCYA           AGAAGGATGAGGTTCTGAGCGCTACACACCGGTTGCGTCGGTGCGAATCCGTTCAATCGA
cyaAL58_pc      AGAAGGATGAGGTTCTGAGCGCTACACACCGGTTGCGTCGGTGCGAATCCGTTCAATCGA

                961       971       981       991       1001      1011
BPCYA           CTACTTATCGACAGATCCACATGCAGCAATCGCATCAGGCTGGTTACGCAAACGCCGCCG
cyaAL58_pc      CTACTTATCGACAGATCCACATGCAGCAATCGCATCAGGCTGGTTACGCAAACGCCGCCG

                1021      1031      1041      1051      1061      1071
BPCYA           ACCGGGAGTCTGGCATCCCCGCAGCCGTACTCGATGGCATCAAGGCCGTGGCGAAGGAAA
cyaAL58_pc      ACCGGGAGTCTGGCATCCCCGCAGCCGTACTCGATGGCATCAAGGCCGTGGCGAAGGAAA

                1081      1091      1101      1111      1121      1131
BPCYA           AAAACGCCACATTGATGTTCCGCCTGGTCAACCCCCATTCCACCAGCCTGATTGCCGAAG
cyaAL58_pc      AAAACGCCACATTGATGTTCCGCCTGGTCAACCCCCATTCCACCAGCCTGATTGCCGAAG

                1141      1151      1161      1171      1181      1191
BPCYA           GGGTGGCCACCAAAGGATTGGGCGTGCACGCCAAGTCGTCCGATTGGGGGTTGCAGGCGG
cyaAL58_pc      GGGTGGCCACCATGGGATTGGGCGTGCACGCCAAGTCGTCCGATTGGGGGTTGCAGGCGG

                1201      1211      1221      1231      1241      1251
BPCYA           GCTACATTCCCGTCAACCCGAATCTTTCCAAACTGTTCGGCCGTGCGCCCGAGGTGATCG
cyaAL58_pc      GCTACATTCCCGTCAACCCGAATCTTTCCAAACTGTTCGGCCGTGCGCCCGAGGTGATCG

                1261      1271      1281      1291      1301      1311
BPCYA           CGCGGGCCGACAACGACGTCAACAGCAGCCTGGCGCATGGCCATACCGCGGTCGACCTGA
cyaAL58_pc      CGCGGGCCGACAACGACGTCAACAGCAGCCTGGCGCATGGCCATACCGCGGTCGACCTGA

                1321      1331      1341      1351      1361      1371
BPCYA           CGCTGTCGAAAGAGCGGCTTGACTATCTGCGGCAAGCGGGCCTGGTCACCGGCATGGCCG
cyaAL58_pc      CGCTGTCGAAAGAGCGGCTTGACTATCTGCGGCAAGCGGGCCTGGTCACCGGCATGGCCG

                1381      1391      1401      1411      1421      1431
BPCYA           ATGGCGTGGTCGCGAGCAACCACGCAGGCTACGAGCAGTTCGAGTTTCGCGTGAAGGAAA
cyaAL58_pc      ATGGCGTGGTCGCGAGCAACCACGCAGGCTACGAGCAGTTCGAGTTTCGCGTGAAGGAAA

                1441      1451      1461      1471      1481      1491
BPCYA           CCTCGGACGGGCGCTATGCCGTGCAGTATCGCCGCAAGGGCGGCGACGATTTCGAGGCGG
cyaAL58_pc      CCTCGGACGGGCGCTATGCCGTGCAGTATCGCCGCAAGGGCGGCGACGATTTCGAGGCGG

                1501      1511      1521      1531      1541      1551
BPCYA           TCAAGGTGATCGGCAATGCCGCCGGTATTCCACTGACGGCGGATATCGACATGTTCGCCA
cyaAL58_pc      TCAAGGTGATCGGCAATGCCGCCGGTATTCCACTGACGGCGGATATCGACATGTTCGCCA

                1561      1571      1581      1591      1601      1611
BPCYA           TTATGCCGCATCTGTCCAACTTCCGCGACTCGGCGCGCAGTTCGGTGACCAGCGGCGATT
cyaAL58_pc      TTATGCCGCATCTGTCCAACTTCCGCGACTCGGCGCGCAGTTCGGTGACCAGCGGCGATT

                1621      1631      1641      1651      1661      1671
BPCYA           CGGTGACCGATTACCTGGCGCGCACGCGGCGGGCCGCCAGCGAGGCCACGGGCGGCCTGG
cyaAL58_pc      CGGTGACCGATTACCTGGCGCGCACGCGGCGGGCCGCCAGCGAGGCCACGGGCGGCCTGG

                1681      1691      1701      1711      1721      1731
BPCYA           ATCGCGAACGCATCGACTTGTTGTGGAAAATCGCTCGCGCCGGCGCCCGTTCCGCAGTGG
cyaAL58_pc      ATCGCGAACGCATCGACTTGTTGTGGAAAATCGCTCGCGCCGGCGCCCGTTCCGCAGTGG

                1741      1751      1761      1771      1781      1791
BPCYA           GCACCGAGGCGCGTCGCCAGTTCCGCTACGACGGCGACATGAATATCGGCGTGATCACCG
cyaAL58_pc      GCACCGAGGCGCGTCGCCAGTTCCGCTACGACGGCGACATGAATATCGGCGTGATCACCG
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             1801      1811      1821      1831      1841      1851
BPCYA        ATTTCGAGCTGGAAGTGCGCAATGCGCTGAACAGGCGGGCGCACGCCGTCGGCGCGCAGG
cyaAL58_pc   ATTTCGAGCTGGAAGTGCGCAATGCGCTGAACAGGCGGGCGCACGCCGTCGGCGCGCAGG

             1861      1871      1881      1891      1901      1911
BPCYA        ACGTGGTCCAGCATGGCACTGAGCAGAACAATCCTTTCCCGGAGGCAGATGAGAAGATTT
cyaAL58_pc   ACGTGGTCCAGCATGGCACTGAGCAGAACAATCCTTTCCCGGAGGCAGATGAGAAGATTT

             1921      1931      1941      1951      1961      1971
BPCYA        TCGTCGTATCGGCCACCGGTGAAAGCCAGATGCTCACGCGCGGGCAACTGAAGGAATACA
cyaAL58_pc   TCGTCGTATCGGCCACCGGTGAAAGCCAGATGCTCACGCGCGGGCAACTGAAGGAATACA

             1981      1991      2001      2011      2021      2031
BPCYA        TTGGCCAGCAGCGCGGCGAGGGCTATGTCTTCTACGAGAACCGTGCATACGGCGTGGCGG
cyaAL58_pc   TTGGCCAGCAGCGCGGCGAGGGCTATGTCTTCTACGAGAACCGTGCATACGGCGTGGCGG

             2041      2051      2061      2071      2081      2091
BPCYA        GGAAAAGCCTGTTCGACGATGGGCTGGGAGCCGCGCCCGGCGTGCCGAGCGGACGTTCGA
cyaAL58_pc   GGAAAAGCCTGTTCGACGATGGGCTGGGAGCCGCGCCCGGCGTGCCGAGCGGACGTTCGA

             2101      2111      2121      2131      2141      2151
BPCYA        AGTTCTCGCCGGATGTACTGGAAACGGTGCCGGCGTCACCCGGATTGCGGCGGCCGTCGC
cyaAL58_pc   AGTTCTCGCCGGATGTACTGGAAACGGTGCCGGCGTCACCCGGATTGCGGCGGCCGTCGC

             2161      2171      2181      2191      2201      2211
BPCYA        TGGGCGCAGTGGAACGCCAGGATTCCGGCTATGACAGCCTTGATGGGGTGGGATCGCGAT
cyaAL58_pc   TGGGCGCAGTGGAACGCCAGGATTCCGGCTATGACAGCCTTGATGGGGTGGGATCGCGAT

             2221      2231      2241      2251      2261      2271
BPCYA        CGTTCTCGTTGGGCGAGGTGTCCGACATGGCCGCCGTGGAAGCGGCGGAACTGGAAATGA
cyaAL58_pc   CGTTCTCGTTGGGCGAGGTGTCCGACATGGCCGCCGTGGAAGCGGCGGAACTGGAAATGA

             2281      2291      2301      2311      2321      2331
BPCYA        CCCGGCAAGTCTTGCACGCCGGGGCGCGGCAGGACGATGCCGAGCCGGGCGTGAGCGGTG
cyaAL58_pc   CCCGGCAAGTCTTGCACGCCGGGGCGCGGCAGGACGATGCCGAGCCGGGCGTGAGCGGTG

             2341      2351      2361      2371      2381      2391
BPCYA        CGTCGGCGCACTGGGGGCAGCGGGCGCTGCAGGGCGCCCAGGCGGTGGCGGCGGCGCAGC
cyaAL58_pc   CGTCGGCGCACTGGGGGCAGCGGGCGCTGCAGGGCGCCCAGGCGGTGGCGGCGGCGCAGC

             2401      2411      2421      2431      2441      2451
BPCYA        GGCTGGTTCATGCCATTGCCCTGATGACGCAATTCGGCCGGGCCGGTTCCACCAACACGC
cyaAL58_pc   GGCTGGTTCATGCCATTGCCCTGATGACGCAATTCGGCCGGGCCGGTTCCACCAACACGC

             2461      2471      2481      2491      2501      2511
BPCYA        CGCAGGAAGCGGCCTCGTTGTCGGCGGCCGTGTTCGGCTTGGGCGAGGCCAGCAGCGCCG
cyaAL58_pc   CGCAGGAAGCGGCCTCGTTGTCGGCGGCCGTGTTCGGCTTGGGCGAGGCCAGCAGCGCCG

             2521      2531      2541      2551      2561      2571
BPCYA        TGGCCGAAACCGTGAGCGGTTTTTTCCGCGGGTCTTCGCGCTGGGCCGGCGGTTTCGGCG
cyaAL58_pc   TGGCCGAAACCGTGAGCGGTTTTTTCCGCGGGTCTTCGCGCTGGGCCGGCGGTTTCGGCG

             2581      2591      2601      2611      2621      2631
BPCYA        TGGCTGGCGGCGCGATGGCGCTGGGAGGCGGCATCGCCGCGGCCGTTGGCGCCGGGATGT
cyaAL58_pc   TGGCTGGCGGCGCGATGGCGCTGGGAGGCGGCATCGCCGCGGCCGTTGGCGCCGGGATGT

             2641      2651      2661      2671      2681      2691
BPCYA        CGTTGACCGATGACGCGCCGGCCGGACAGAAGGCCGCCGCCGGCGCCGAGATCGCGCTGC
cyaAL58_pc   CGTTGACCGATGACGCGCCGGCCGGACAGAAGGCCGCCGCCGGCGCCGAGATCGCGCTGC

             2701      2711      2721      2731      2741      2751
BPCYA        AGTTGACAGGTGGAACGGTCGAGCTGGCTTCTTCCATCGCGTTGGCGCTGGCCGCGGCGC
cyaAL58_pc   AGTTGACAGGTGGAACGGTCGAGCTGGCTTCTTCCATCGCGTTGGCGCTGGCCGCGGCGC

             2761      2771      2781      2791      2801      2811
BPCYA        GCGGCGTGACCAGCGGCTTGCAGGTGGCCGGGGCGTCGGCCGGGGCGGCTGCCGGCGCAT
cyaAL58_pc   GCGGCGTGACCAGCGGCTTGCAGGTGGCCGGGGCGTCGGCCGGGGCGGCTGCCGGCGCAT
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             2821      2831      2841      2851      2861      2871
BPCYA        TGGCCGCGGCGCTCAGTCCCATGGAGATCTACGGCCTGGTGCAGCAATCGCACTATGCGG
cyaAL58_pc   TGGCCGCGGCGCTCAGTCCCATGGAGATCTACGGCCTGGTGCAGCAATCGCACTATGCGG

             2881      2891      2901      2911      2921      2931
BPCYA        ATCAGCTGGACAAGCTGGCGCAGGAATCGAGCGCATACGGTTACGAGGGCGACGCCTTGC
cyaAL58_pc   ATCAGCTGGACAAGCTGGCGCAGGAATCGAGCGCATACGGTTACGAGGGCGACGCCTTGC

             2941      2951      2961      2971      2981      2991
BPCYA        TGGCCCAGCTGTATCGCGACAAGACGGCCGCCGAGGGCGCCGTCGCCGGCGTCTCCGCCG
cyaAL58_pc   TGGCCCAGCTGTATCGCGACAAGACGGCCGCCGAGGGCGCCGTCGCCGGCGTCTCCGCCG

             3001      3011      3021      3031      3041      3051
BPCYA        TCCTGAGCACGGTGGGGGCGGCGGTGTCGATCGCCGCGGCGGCCAGCGTGGTAGGGGCCC
cyaAL58_pc   TCCTGAGCACGGTGGGGGCGGCGGTGTCGATCGCCGCGGCGGCCAGCGTGGTAGGGGCCC

             3061      3071      3081      3091      3101      3111
BPCYA        CGGTGGCGGTGGTCACTTCCTTGCTGACCGGGGCTCTCAACGGCATCCTGCGCGGCGTGC
cyaAL58_pc   CGGTGGCGGTGGTCACTTCCTTGCTGACCGGGGCTCTCAACGGCATCCTGCGCGGCGTGC

             3121      3131      3141      3151      3161      3171
BPCYA        AGCAGCCCATCATCGAAAAGCTGGCCAACGATTACGCTCGCAAGATCGACGAGCTGGGCG
cyaAL58_pc   AGCAGCCCATCATCGAAAAGCTGGCCAACGATTACGCTCGCAAGATCGACGAGCTGGGCG

             3181      3191      3201      3211      3221      3231
BPCYA        GGCCGCAAGCGTACTTCGAGAAAAACCTGCAGGCGCGTCACGAACAACTGGCCAATTCGG
cyaAL58_pc   GGCCGCAAGCGTACTTCGAGAAAAACCTGCAGGCGCGTCACGAACAACTGGCCAATTCGG

             3241      3251      3261      3271      3281      3291
BPCYA        ACGGCCTACGGAAAATGCTGGCCGACCTGCAGGCCGGTTGGAACGCCAGCAGCGTGATCG
cyaAL58_pc   ACGGCCTACGGAAAATGCTGGCCGACCTGCAGGCCGGTTGGAACGCCAGCAGCGTGATCG

             3301      3311      3321      3331      3341      3351
BPCYA        GGGTGCAGACGACAGAGATCTCCAAGTCGGCGCTCGAACTGGCCGCCATTACCGGCAACG
cyaAL58_pc   GGGTGCAGACGACAGAGATCTCCAAGTCGGCGCTCGAACTGGCCGCCATTACCGGCAACG

             3361      3371      3381      3391      3401      3411
BPCYA        CGGACAACCTGAAATCCGTCGACGTGTTCGTGGACCGCTTCGTCCAGGGCGAGCGGGTGG
cyaAL58_pc   CGGACAACCTGAAATCCGTCGACGTGTTCGTGGACCGCTTCGTCCAGGGCGAGCGGGTGG

             3421      3431      3441      3451      3461      3471
BPCYA        CCGGCCAGCCGGTGGTCCTCGACGTCGCCGCCGGCGGCATCGATATCGCCAGCCGCAAGG
cyaAL58_pc   CCGGCCAGCCGGTGGTCCTCGACGTCGCCGCCGGCGGCATCGATATCGCCAGCCGCAAGG

             3481      3491      3501      3511      3521      3531
BPCYA        GCGAGCGGCCGGCGCTGACGTTCATCACGCCGCTGGCCGCGCCAGGAGAAGAGCAGCGCC
cyaAL58_pc   GCGAGCGGCCGGCGCTGACGTTCATCACGCCGCTGGCCGCGCCAGGAGAAGAGCAGCGCC

             3541      3551      3561      3571      3581      3591
BPCYA        GGCGCACGAAAACGGGCAAGAGCGAATTCACCACATTCGTCGAGATCGTGGGCAAGCAGG
cyaAL58_pc   GGCGCACGAAAACGGGCAAGAGCGAATTCACCACATTCGTCGAGATCGTGGGCAAGCAGG

             3601      3611      3621      3631      3641      3651
BPCYA        ACCGCTGGCGCATCCGGGACGGCGCGGCCGACACCACCATCGATCTGGCCAAGGTGGTGT
cyaAL58_pc   ACCGCTGGCGCATCCGGGACGGCGCGGCCGACACCACCATCGATCTGGCCAAGGTGGTGT

             3661      3671      3681      3691      3701      3711
BPCYA        CGCAACTGGTCGACGCCAATGGCGTGCTCAAGCACAGCATCAAACTGGATGTGATCGGCG
cyaAL58_pc   CGCAACTGGTCGACGCCAATGGCGTGCTCAAGCACAGCATCAAACTGGATGTGATCGGCG

             3721      3731      3741      3751      3761      3771
BPCYA        GAGATGGCGATGACGTCGTGCTTGCCAATGCTTCGCGCATCCATTATGACGGCGGCGCGG
cyaAL58_pc   GAGATGGCGATGACGTCGTGCTTGCCAATGCTTCGCGCATCCATTATGACGGCGGCGCGG

             3781      3791      3801      3811      3821      3831
BPCYA        GCACCAACACGGTCAGCTATGCCGCCCTGGGTCGACAGGATTCCATTACCGTGTCCGCCG
cyaAL58_pc   GCACCAACACGGTCAGCTATGCCGCCCTGGGTCGACAGGATTCCATTACCGTGTCCGCCG
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             3841      3851      3861      3871      3881      3891
BPCYA        ACGGGGAACGTTTCAACGTGCGCAAGCAGTTGAACAACGCCAACGTGTATCGCGAAGGCG
cyaAL58_pc   ACGGGGAACGTTTCAACGTGCGCAAGCAGTTGAACAACGCCAACGTGTATCGCGAAGGCG

             3901      3911      3921      3931      3941      3951
BPCYA        TGGCTACCCAGACAACCGCCTACGGCAAGCGCACGGAGAATGTCCAATACCGCCATGTCG
cyaAL58_pc   TGGCTACCCAGACAACCGCCTACGGCAAGCGCACGGAGAATGTCCAATACCGCCATGTCG

             3961      3971      3981      3991      4001      4011
BPCYA        AGCTGGCCCGTGTCGGGCAAGTGGTGGAGGTCGACACGCTCGAGCATGTGCAGCACATCA
cyaAL58_pc   AGCTGGCCCGTGTCGGGCAACTGGTGGAGGTCGACACGCTCGAGCATGTGCAGCACATCA

             4021      4031      4041      4051      4061      4071
BPCYA        TCGGCGGGGCCGGCAACGATTCGATCACCGGCAATGCGCACGACAACTTCCTAGCCGGCG
cyaAL58_pc   TCGGCGGGGCCGGCAACGATTCGATCACCGGCAATGCGCACGACAACTTCCTAGCCGGCG

             4081      4091      4101      4111      4121      4131
BPCYA        GGTCGGGCGACGACAGGCTGGATGGCGGCGCCGGCAACGACACCCTGGTTGGCGGCGAGG
cyaAL58_pc   GGTCGGGCGACGACAGGCTGGATGGCGGCGCCGGCAACGACACCCTGGTTGGCGGCGAGG

             4141      4151      4161      4171      4181      4191
BPCYA        GCCAAAACACGGTCATCGGCGGCGCCGGCGACGACGTATTCCTGCAGGACCTGGGGGTAT
cyaAL58_pc   GCCAAAACACGGTCATCGGCGGCGCCGGCGACGACGTATTCCTGCAGGACCTGGGGGTAT

             4201      4211      4221      4231      4241      4251
BPCYA        GGAGCAACCAGCTCGATGGCGGCGCGGGCGTCGATACCGTGAAGTACAACGTGCACCAGC
cyaAL58_pc   GGAGCAACCAGCTCGATGGCGGCGCGGGCGTCGATACCGTGAAGTACAACGTGCACCAGC

             4261      4271      4281      4291      4301      4311
BPCYA        CTTCCGAGGAGCGCCTCGAACGCATGGGCGACACGGGCATCCATGCCGATCTTCAAAAGG
cyaAL58_pc   CTTCCGAGGAGCGCCTCGAACGCATGGGCGACACGGGCATCCATGCCGATCTTCAAAAGG

             4321      4331      4341      4351      4361      4371
BPCYA        GCACGGTCGAGAAGTGGCCGGCCCTGAACCTGTTCAGCGTCGACCATGTCAAGAATATCG
cyaAL58_pc   GCACGGTCGAGAAGTGGCCGGCCCTGAACCTGTTCAGCGTCGACCATGTCAAGAATATCG

             4381      4391      4401      4411      4421      4431
BPCYA        AGAATCTGCACGGCTCCCGCCTAAACGACCGCATCGCCGGCGACGACCAGGACAACGAGC
cyaAL58_pc   AGAATCTGCACGGCTCCCGCCTGAACGACCGCATCGCCGGCGACGACCAGGACAACGAGC

             4441      4451      4461      4471      4481      4491
BPCYA        TCTGGGGCCACGATGGCAACGACACGATACGCGGCCGGGGCGGCGACGACATCCTGCGCG
cyaAL58_pc   TCTGGGGCCACGATGGCAACGACACGATACGCGGCCGGGGCGGCGACGACATCCTGCGCG

             4501      4511      4521      4531      4541      4551
BPCYA        GCGGCCTGGGCCTGGACACGCTGTATGGCGAGGACGGCAACGACATCTTCCTGCAGGACG
cyaAL58_pc   GCGGCCTGGGCCTGGACACGCTGTATGGCGAGGACGGCAACGACATCTTCCTGCAGGACG

             4561      4571      4581      4591      4601      4611
BPCYA        ACGAGACCGTCAGCGATGACATCGACGGCGGCGCGGGGCTGGACACCGTCGACTACTCCG
cyaAL58_pc   ACGAGACCGTCAGCGATGACATCGACGGCGGCGCGGGGCTGGACACCGTCGACTACTCCG

             4621      4631      4641      4651      4661      4671
BPCYA        CCATGATCCATCCAGGCAGGATCGTTGCGCCGCATGAATACGGCTTCGGGATCGAGGCGG
cyaAL58_pc   CCATGATCCATCCAGGCAGGATCGTTGCGCCGCATGAATACGGCTTCGGGATCGAGGCGG

             4681      4691      4701      4711      4721      4731
BPCYA        ACCTGTCCAGGGAATGGGTGCGCAAGGCGTCCGCGCTGGGCGTGGACTATTACGATAATG
cyaAL58_pc   ACCTGTCCAGGGAATGGGTGCGCAAGGCGTCCGCGCTGGGCGTGGACTATTACGATAATG

             4741      4751      4761      4771      4781      4791
BPCYA        TCCGCAATGTCGAAAACGTCATCGGTACGAGCATGAAGGATGTGCTCATCGGCGACGCGC
cyaAL58_pc   TCCGCAATGTCGAAAACGTCATCGGTACGAGCATGAAGGATGTGCTCATCGGCGACGCGC

             4801      4811      4821      4831      4841      4851
BPCYA        AAGCCAATACCCTGATGGGCCAGGGCGGCGACGATACCGTGCGCGGCGGCGACGGCGATG
cyaAL58_pc   AAGCCAATACCCTGATGGGCCAGGGCGGCGACGATACCGTGCGCGGCGGCGACGGCGATG
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             4861      4871      4881      4891      4901      4911
BPCYA        ATCTGCTGTTCGGCGGCGACGGCAACGACATGCTGTATGGCGACGCCGGCAACGACACCC
cyaAL58_pc   ATCTGCTGTTCGGCGGCGACGGCAACGACATGCTGTATGGCGACGCCGGCAACGACACCC

             4921      4931      4941      4951      4961      4971
BPCYA        TCTACGGGGGGCTGGGCGACGATACCCTTGAAGGCGGCGCGGGCAACGATTGGTTCGGCC
cyaAL58_pc   TCTACGGGGGGCTGGGCGACGATACCCTTGAAGGCGGCGCGGGCAACGATTGGTTCGGCC

             4981      4991      5001      5011      5021      5031
BPCYA        AGACGCAGGCGCGCGAGCATGACGTGCTGCGCGGCGGAGATGGGGTGGATACCGTCGATT
cyaAL58_pc   AGACGCAGGCGCGCGAGCATGACGTGCTGCGCGGCGGAGATGGGGTGGATACCGTCGATT

             5041      5051      5061      5071      5081      5091
BPCYA        ACAGCCAGACCGGCGCGCATGCCGGCATTGCCGCGGGTCGCATCGGGCTGGGCATCCTGG
cyaAL58_pc   ACAGCCAGACCGGCGCGCATGCCGGCATTGCCGCGGGTCGCATCGGGCTGGGCATCCTGG

             5101      5111      5121      5131      5141      5151
BPCYA        CTGACCTGGGCGCCGGCCGCGTCGACAAGCTGGGCGAGGCCGGCAGCAGCGCCTACGATA
cyaAL58_pc   CTGACCTGGGCGCCGGCCGCGTCGACAAGCTGGGCGAGGCCGGCAGCAGCGCCTACGATA

             5161      5171      5181      5191      5201      5211
BPCYA        CGGTTTCCGGTATCGAGAACGTGGTGGGCACGGAACTGGCCGACCGCATCACGGGCGATG
cyaAL58_pc   CGGTTTCCGGTATCGAGAACGTGGTGGGCACGGAACTGGCCGACCGCATCACGGGCGATG

             5221      5231      5241      5251      5261      5271
BPCYA        CGCAGGCCAACGTGCTGCGCGGCGCGGGTGGCGCCGACGTGCTTGCGGGCGGCGAGGGCG
cyaAL58_pc   CGCAGGCCAACGTGCTGCGCGGCGCGGGTGGCGCCGACGTGCTTGCGGGCGGCGAGGGCG

             5281      5291      5301      5311      5321      5331
BPCYA        ACGATGTGCTGCTGGGCGGCGACGGCGACGACCAGCTGTCGGGCGACGCCGGACGCGATC
cyaAL58_pc   ACGATGTGCTGCTGGGCGGCGACGGCGACGACCAGCTGTCGGGCGACGCCGGACGCGATC

             5341      5351      5361      5371      5381      5391
BPCYA        GCTTGTACGGCGAAGCCGGTGACGACTGGTTCTTCCAGGATGCCGCCAATGCCGGCAATC
cyaAL58_pc   GCTTGTACGGCGAAGCCGGTGACGACTGGTTCTTCCAGGATGCCGCCAATGCCGGCAATC

             5401      5411      5421      5431      5441      5451
BPCYA        TGCTCGACGGCGGCGACGGCCGCGATACCGTGGATTTCAGCGGCCCGGGCCGGGGCCTCG
cyaAL58_pc   TGCTCGACGGCGGCGACGGCCGCGATACCGTGGATTTCAGCGGCCCGGGCCGGGGCCTCG

             5461      5471      5481      5491      5501      5511
BPCYA        ACGCCGGCGCAAAGGGCGTATTCCTGAGCTTGGGCAAGGGGTTCGCCAGCCTGATGGACG
cyaAL58_pc   ACGCCGGCGCAAAGGGCGTATTCCTGAGCTTGGGCAAGGGGTTCGCCAGCCTGATGGACG

             5521      5531      5541      5551      5561      5571
BPCYA        AACCCGAAACCAGCAACGTGTTGCGCAATATCGAGAACGCCGTGGGCAGCGCGCGTGATG
cyaAL58_pc   AACCCGAAACCAGCAACGTGTTGCGCAATATCGAGAACGCCGTGGGCAGCGCGCGTGATG

             5581      5591      5601      5611      5621      5631
BPCYA        ACGTGCTGATCGGCGACGCAGGCGCCAACGTCCTCAATGGCCTGGCGGGCAACGACGTGC
cyaAL58_pc   ACGTGCTGATCGGCGACGCAGGCGCCAACGTCCTCAATGGCCTGGCGGGCAACGACGTGC

             5641      5651      5661      5671      5681      5691
BPCYA        TGTCCGGCGGCGCTGGCGACGATGTGCTGCTGGGCGACGAGGGCTCGGACCTGCTCAGCG
cyaAL58_pc   TGTCCGGCGGCGCTGGCGACGATGTGCTGCTGGGCGACGAGGGCTCGGACCTGCTCAGCG

             5701      5711      5721      5731      5741      5751
BPCYA        GCGATGCGGGCAACGACGATCTGTTCGGCGGGCAGGGCGATGATACTTATCTGTTCGGGG
cyaAL58_pc   GCGATGCGGGCAACGACGATCTGTTCGGCGGGCAGGGCGATGATACTTATCTGTTCGGGG

             5761      5771      5781      5791      5801      5811
BPCYA        TCGGGTACGGGCACGACACGATCTACGAATCGGGCGGCGGCCATGACACCATCCGCATCA
cyaAL58_pc   TCGGGTACGGGCACGACACGATCTACGAATCGGGCGGCGGCCATGACACCATCCGCATCA

             5821      5831      5841      5851      5861      5871
BPCYA        ACGCGGGGGCGGACCAGCTGTGGTTCGCGCGCCAGGGCAACGACCTGGAGATCCGCATTC
cyaAL58_pc   ACGCGGGGGCGGACCAGCTGTGGTTCGCGCGCCAGGGCAACGACCTGGAGATCCGCATTC
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               5881      5891      5901      5911      5921      5931
BPCYA          TCGGCACCGACGATGCACTTACCGTGCACGACTGGTATCGCGACGCCGATCACCGGGTGG
cyaAL58_pc     TCGGCACCGACGATGCACTTACCGTGCACGACTGGTATCGCGACGCCGATCACCGGGTGG

               5941      5951      5961      5971      5981      5991
BPCYA          AAATCATCCATGCCGCCAACCAGGCGGTAGACCAGGCAGGCATCGAAAAGCTGGTCGAGG
cyaAL58_pc     AAATCATCCATGCCGCCAACCAGGCGGTAGACCAGGCAGGCATCGAAAAGCTGGTCGAGG

               6001      6011      6021      6031      6041      6051
BPCYA          CAATGGCGCAGTATCCGGACCCCGGCGCGGCGGCGGCTGCCCCGCCGGCGGCGCGCGTGC
cyaAL58_pc     CAATGGCGCAGTATCCGGACCCCGGCGCGGCGGCGGCTGCCCCGCCGGCGGCGCGCGTGC

               6061      6071      6081      6091      6101      6111
BPCYA          CGGACACGCTGATGCAGTCCCTGGCTGTCAACTGGCGCTGAAGCGCCGTGAATCACGGCC
cyaAL58_pc     CGGACACGCTGATGCAGTCCCTGGCTGTCAACTGGCGC----------------------

               6121      6131      6141      6151      6161      6171
BPCYA          CGCCTGCCTCGCGCGGCGGCGCCGTCTCTTTGCGTTCTTCTCCGAGGTATTTCCCATCAT
cyaAL58_pc     ------------------------------------------------------------
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Figure D.11: Nucleotide sequence alignment of published *cyaA* Genbank sequence and pcDNA3.1D/*cyaAL58* insert sequence. ClustalW program (BioManager) was used to compare the two sequences. BPCYA refers to Genbank sequence of *cyaA* (accession # A14850) and cyaAL58_pc refers to the sequenced insert of pcDNA3.1D/*cyaAL58*.

```
               1         11        21        31        41        51
BPECYACA       ACAAAAGCAGGCGCCGCGACCGAGGCATCGCCCGAAATACGGACATATCCTGGAAACATG
cyaC_pcDNA     ------------------------------------------------------------

               61        71        81        91        101       111
BPECYACA       TAAGAAAAGCCATTCCGGCGCAACCATGATGCGCCGGCGGCTTGCCATGCGCGCACCCAA
cyaC_pcDNA     -------------------------------------------CACCATG-GCGCACCCAA

               121       131       141       151       161       171
BPECYACA       CACGGAATATGCCCCCCGCTCCACCATGCACCGATGTCATGCTTCCGTCCGCCCAAGCGC
cyaC_pcDNA     CACGGAATATGCCCCCCGCTCCACCATGCACCGATGTCATGCTTCCGTCCGCCCAAGCGC

               181       191       201       211       221       231
BPECYACA       CCTCCCTCCTCAATCCCACCGACGACTTCGCGGCACTGGGCAATATTGCCTGGCTGTGGA
cyaC_pcDNA     CCTCCCTCCTCAATCCCACCGACGACTTCGCGGCACTGGGCAATATTGCCTGGCTGTGGA

               241       251       261       271       281       291
BPECYACA       TGAACTCTCCCATGCACCGCGACTGGCCGGTGCATCTGCTCGCACGCAACACGCTCGCGC
cyaC_pcDNA     TGAACTCTCCCATGCACCGCGACTGGCCGGTGCATCTGCTCGCACGCAACACGCTCGCGC

               301       311       321       331       341       351
BPECYACA       CGATTCAACTGGGCCAATACATTCTGCTGCGATGCAATGACGTGCCGGTTGCATACTGCA
cyaC_pcDNA     CGATTCAACTGGGCCAATACATTCTGCTGCGATGCAATGACGTGCCGGTTGCATACTGCA

               361       371       381       391       401       411
BPECYACA       GCTGGGCCCTAATGGACGCCGACACCGAACTCTCCTATGTCATGGCGCCCTCGTCGCTGG
cyaC_pcDNA     GCTGGGCCCTAATGGACGCCGACACCGAACTCTCCTATGTCATGGCGCCCTCGTCGCTGG

               421       431       441       451       461       471
BPECYACA       GCGGGAATGCCTGGAACTGCGGCGACCGACTGTGGATCATCGACTGGATCGCGCCATTCT
cyaC_pcDNA     GCGGGAATGCCTGGAACTGCGGCGACCGACTGTGGATCATCGACTGGATCGCGCCATTCT

               481       491       501       511       521       531
BPECYACA       CGCGCGACGACAATCGTGCGCTGCGCCGCGCGCTGGCCGAACGGCACCCCGACAGCGTGG
cyaC_pcDNA     CGCGCGACGACAATCGTGCGCTGCGCCGCGCGCTGGCCGAACGGCACCCCGACAGCGTGG

               541       551       561       571       581       591
BPECYACA       GCCGTTCGCTGCGCGTTCGGCGCGGCGGCGACACCGCGCGCGTCAAGGAGTACCGAGGCC
cyaC_pcDNA     GCCGTTCGCTGCGCGTTCGGCGCGGCGGCGACACCGCGCGCGTCAAGGAGTACCGAGGCC
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               601        611        621        631        641        651
BPECYACA       GCGCGCTGGACGCGGCCGCCACTCGCGCGCAGCTGGACCGCTACCATGCCGAACTGATCG
cyaC_pcDNA     GCGCGCTGGACGCGGCCGCCGCTCGCGCGCAGCTGGACCGCTACCATGCCGAACTGATCG

               661        671        681        691        701        711
BPECYACA       CAGGACTGCGCGCGAGCAACGGCGGATACGCGCCGCGAGGCCGGGGCACCGCCTGA
cyaC_pcDNA     CAGGACTGCGCGCGAGCAACGGCGGATACGCGCCGCGAGGCCGGGGCACCG-----
```

Figure D.12: Nucleotide sequence alignment of published *cyaC* genbank sequence and pcDNA3.1D/*cyaC* insert sequence. ClustalW program (BioManager) was used to compare the two sequences. BPCYACA refers to Genbank sequence of *cyaC* (accession # M57286) and cyaC_pcDNA refers to the sequenced insert of pcDNA3.1D/*cyaC*.

Table D.26: Cytokine production from *in-vitro* re-stimulated splenocytes from mice immunised with pcDNA3.1D/*cyaAL58*. Values represent mean (pg/ml) from duplicate wells.

Vaccine Group	Stimulant	IFN-γ	IL-2	IL-4
pcDNA3.1D/*cyaAL58*	rCYAAL58	4504	258.6	0
pcDNA3.1D/*cyaAL58*	HKBP	370	0	7.5
pcDNA3.1 vector	rCYAAL58	1107	135.2	19.4
pcDNA3.1D/*cyaAL58*	ConA	7044	15452.8	406.4

Table D.27: Cytokine production from *in-vitro* re-stimulated splenocytes from mice immunised with pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC*. Values represent mean (pg/ml) from duplicate wells.

Vaccine Group	Stimulant	IFN-γ	IL-2	IL-4
pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC*	rCYAAL58	7890	515.4	5.7
pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC*	HKBP	456	8.3	4
pcDNA3.1 vector	rCYAAL58	1546	40.2	10.5
pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC*	ConA	6000	16968.6	462.8

Table D.28: Summary of serum anti-rCYAAL58 IgG.

Vaccine Group	Individual Mice					Mean	SE
	M1	M2	M3	M4	M5		
pcDNA3.1D/*cyaAL58*	0	0	0	0	0	0	0
pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC*	6400	3200	6400	3200	51200	14080	9308
pcDNA3.1D vector	0	0	4734	9507	9507	4750	2126

Table D.29: Summary of serum anti-AC-Hly IgG.

Vaccine Group	Individual Mice					Mean	SE
	M1	M2	M3	M4	M5		
pcDNA3.1D/*cyaAL58*	0	0	0	0	0	0	0
pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC*	12800	12800	0	0	0	5120	3135
pcDNA3.1D vector	0	0	12800	25600	25600	12800	5724

Table D.30: Clearance of *B. pertussis* from lungs following aerosol challenge (SLID). Bacterial loads expressed as log10 CFU/lung

Group	Day	Mouse1	Mouse2	Mouse3	Mouse4
pcDNA3.1D/*cyaA*	0	5.699	5.602	5.778	5.699
	4	4.8	6.643	6.561	6.382
	7	5.791	4.845	4.17	3.505
	14	3.531	4.699	4.201	0
pcDNA3.1D/*cyaA* + pcDNA3.1/*cyaC*	0	5.699	5.602	5.778	5.699
	4	6.441	5.575	6.167	6.449
	7	4.292	3.38	5.412	3.748
	14	3.699	0	0	0
pcDNA3.1 Vector	0	5.699	5.602	5.778	5.699
	4	6.439	6.778	6.303	6.41
	7	5.663	6.799	6.991	6.212
	14	5.255	6.204	5.716	5.959
Placebo	0	5.699	5.602	5.778	5.699
	4	5.217	6.318	6.442	5.217
	7	6.756	5.764	6.672	6.176
	14	6.27	6.079	5.7	5.996
DTaP	0	5.699	5.602	5.778	5.699
	4	5.58	5.613	5.613	5.602
	7	0	0	0	0
	14	0	0	0	0

Table D.31: Growth curve data of SL3261-pcDNA3.1D/*cyaAL58* and SL3261-pcDNA3.1D/*cyaC* for the preparation of the oral combination DNA vaccine.

Transformant	OD_{600}	Mean Count (CFU)	Dil. Factor	Adj.Count (CFU/ml)	Adj. Count (log10CFU/ml)
SL3261-pcDNA3.1D/*cyaA*	0.137	131	2.00E+05	2.62E+07	7.418301291
	0.67	100	2.00E+06	2.00E+08	8.301029996
	0.94	36	2.00E+07	7.20E+08	8.857332496
	1.33	48	2.00E+07	9.60E+08	8.982271233
SL3261-pcDNA3.1D/*cyaC*	0.15	20	2.00E+06	4.00E+07	7.602059991
	0.57	87	2.00E+06	1.74E+08	8.240549248
	1	162	2.00E+06	3.24E+08	8.51054501
	1.26	34	2.00E+07	6.80E+08	8.832508913
SL3261-pcDNA3.1 Vector	0.182	50	2.00E+06	1.00E+08	8
	0.67	24	2.00E+07	4.80E+08	8.681241237
	1.12	46	2.00E+07	9.20E+08	8.963787827
	1.39	64	2.00E+07	1.28E+09	9.10720997
SL3261	0.218	46	2.00E+06	9.20E+07	7.963787827
	0.515	20	2.00E+07	4.00E+08	8.602059991
	0.998	41	2.00E+07	8.20E+08	8.913813852
	1.22	92	2.00E+07	1.84E+09	9.264817823

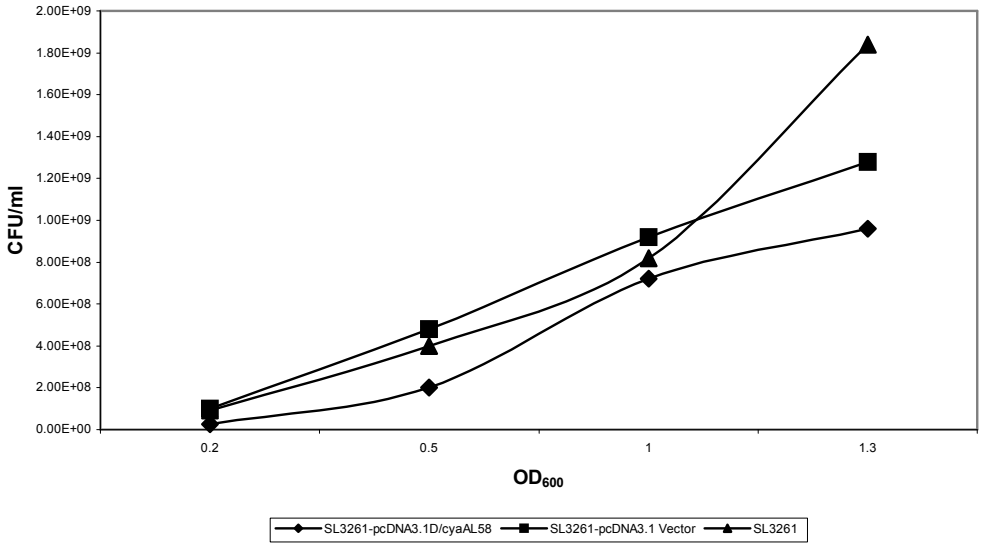

Figure D.13: Growth curve of SL3261-pcDNA3.1D/*cyaAL58* for the preparation of the oral combination DNA vaccine.

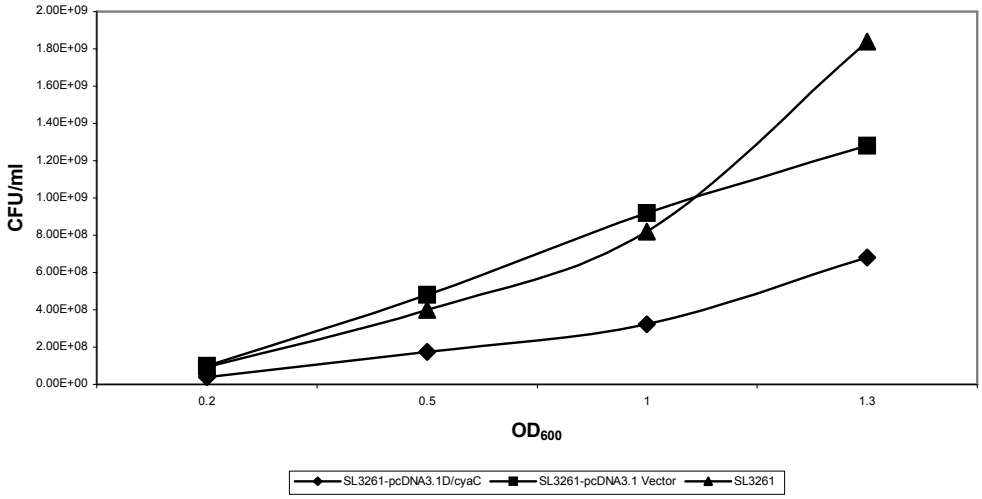

Figure D.14: Growth curve of SL3261-pcDNA3.1D/*cyaC* for the preparation of the oral combination DNA vaccine.

Table D.32: Raw ELISA data of serum anti-rCYAAL58 IgG from mice vaccinated with pcDNA3.1D/*cyaAL58* via the IM route.

Dilution	25	50	100	200	400	800	1600	3200	6400	12800
P 9a	1.939	1.818	1.637	1.43	1.091	0.732	0.448	0.22	0.12	0.063
P 9b	2.016	1.949	1.8	1.532	1.039	0.686	0.496	0.232	0.123	0.068
P 8a	1.882	1.836	1.696	1.387	1.078	0.697	0.484	0.236	0.118	0.057
P 8b	1.811	1.773	1.726	1.415	1.082	0.734	0.488	0.243	0.118	0.072
P 5a	2.035	1.928	1.77	1.415	1.137	0.816	0.62	0.272	0.15	0.1
P 5b	2.002	1.965	1.844	1.496	1.129	0.846	0.607	0.285	0.139	0.074
M 1a	2.004	1.946	1.858	1.737	1.491	1.144	0.896	0.483	0.275	0.163
M 1b	2.055	2.045	1.856	1.753	1.501	1.159	0.654	0.506	0.282	0.173
M 2a	1.743	1.736	1.685	1.586	1.554	1.077	0.875	0.551	0.309	0.182
M 2b	2.041	1.669	1.611	1.452	1.299	1.136	0.766	0.599	0.325	0.183
M 3a	1.5	1.25	1.104	0.958	0.65	0.449	0.282	0.18	0.092	0.046
M 3b	1.5	1.378	1.375	0.998	0.65	0.397	0.277	0.145	0.097	0.052
M 4a	1.133	1.131	0.804	0.51	0.3	0.166	0.094	0.042	0.022	0.01
M 4b	1.118	0.978	0.746	0.512	0.267	0.161	0.066	0.03	0.012	0.001
M 5a	1.083	0.893	0.586	0.359	0.205	0.121	0.061	0.042	0.033	0.017
M 5b	1.193	0.988	0.587	0.404	0.211	0.125	0.063	0.035	0.019	0.011

Table D.33: Raw ELISA data of serum anti-AC-Hly IgG from mice vaccinated with pcDNA3.1D/*cyaAL58* via the IM route.

Dilution	100	200	400	800	1600	3200	6400	12800	25600	51200
P 9a	1.591	1.44	0.807	0.512	0.298	0.185	0.141	0.114	0.09	0.065
P 9b	2.082	1.434	0.845	0.541	0.309	0.207	0.15	0.116	0.106	0.076
P 8a	1.824	1.737	1.788	1.475	1.13	0.748	0.476	0.272	0.179	0.113
P 8b	1.878	1.825	1.852	1.537	1.357	0.817	0.521	0.275	0.187	0.126
M 1a	1.893	1.377	0.913	0.541	0.344	0.205	0.153	0.104	0.084	0.067
M 1b	1.764	1.351	0.837	0.521	0.305	0.178	0.112	0.088	0.071	0.038
M 2a	1.765	1.416	0.871	0.56	0.326	0.194	0.128	0.086	0.061	0.048
M 2b	1.841	1.327	0.915	0.529	0.333	0.203	0.12	0.081	0.057	0.051
M 3a	1.272	0.787	0.481	0.284	0.163	0.117	0.089	0.069	0.064	0.055
M 3b	1.362	0.844	0.512	0.297	0.194	0.126	0.1	0.079	0.069	0.062
M 4a	0.861	0.494	0.302	0.176	0.123	0.104	0.088	0.077	0.068	0.067
M 4b	0.926	0.55	0.332	0.209	0.153	0.111	0.098	0.093	0.089	0.087
M 5a	0.844	0.474	0.264	0.162	0.122	0.091	0.079	0.082	0.066	0.061
M 5b	0.793	0.447	0.246	0.135	0.101	0.068	0.062	0.054	0.043	0.041

Table D.34: Raw ELISA data of serum anti-rCYAAL58 IgG from mice vaccinated with pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC* via the IM route.

Dilution	25	50	100	200	400	800	1600	3200	6400	12800
P 9a	0.597	0.327	0.158	0.07	0.04	0.02	0.013	0.01	0.005	0.001
P 9b	0.421	0.303	0.13	0.071	0.047	0.047	0.022	0.023	0.015	0.014
P 8a	1.508	1.436	1.3	1.318	1.293	1.146	0.979	0.757	0.56	0.315
P 8b	1.724	1.337	1.09	1.294	1.242	1.054	0.877	0.725	0.543	0.298
P 6a	1.605	1.552	1.332	1.23	1.211	1.049	0.704	0.434	0.26	0.144
P 6b	1.289	1.275	1.249	0.994	0.875	0.678	0.66	0.442	0.274	0.163
M 1a	1.113	1.097	0.897	0.813	0.559	0.285	0.183	0.101	0.045	0.015
M 1b	1.426	1.139	1.128	0.898	0.604	0.369	0.21	0.111	0.065	0.047
M 2a	1.572	1.557	1.287	1.059	0.862	0.562	0.364	0.221	0.113	0.057
M 2b	1.43	1.285	1.266	1.077	0.804	0.588	0.366	0.233	0.149	0.078
M 3a	1.464	1.393	1.352	1.152	1.01	0.671	0.435	0.314	0.165	0.096
M 3b	1.483	1.456	1.337	1.3	1.053	0.689	0.435	0.271	0.168	0.095
M 4a	1.632	1.493	1.486	1.453	1.396	1.274	1.121	0.851	0.563	0.367
M 4b	1.546	1.535	1.529	1.52	1.466	1.263	1.029	0.906	0.593	0.385
M 5a	1.592	1.55	1.547	1.472	1.46	1.364	1.327	1.315	1.142	0.85
M 5b	1.644	1.558	1.379	1.362	1.351	1.335	1.281	1.234	1.081	0.82

Table D.35: Raw ELISA data of serum anti-AC-Hly IgG from mice vaccinated with pcDNA3.1D/*cyaAL58* + pcDNA3.1D/*cyaC* via the IM route.

Dilution	100	200	400	800	1600	3200	6400	12800	25600	51200
P 9a	1.591	1.44	0.807	0.512	0.298	0.185	0.141	0.114	0.09	0.065
P 9b	2.082	1.434	0.845	0.541	0.309	0.207	0.15	0.116	0.106	0.076
P 8a	1.824	1.737	1.788	1.475	1.13	0.748	0.476	0.272	0.179	0.113
P 8b	1.878	1.825	1.852	1.537	1.357	0.817	0.521	0.275	0.187	0.126
M 1a	2.062	1.943	1.874	1.503	1.119	0.726	0.458	0.275	0.168	0.108
M 1b	1.938	1.853	1.803	1.54	1.082	0.712	0.477	0.275	0.168	0.111
M 2a	2.058	1.901	1.707	1.255	0.85	0.51	0.316	0.199	0.133	0.091
M 2b	2.068	1.916	1.644	1.216	0.809	0.523	0.319	0.206	0.137	0.101
M 3a	0.316	0.197	0.124	0.099	0.089	0.084	0.083	0.08	0.07	0.07
M 3b	0.347	0.203	0.13	0.116	0.097	0.091	0.085	0.085	0.081	0.079
M 4a	1.781	1.253	0.773	0.457	0.284	0.186	0.137	0.101	0.096	0.067
M 4b	1.86	1.248	0.759	0.449	0.276	0.164	0.109	0.074	0.063	0.05
M 5a	1.716	1.091	0.651	0.387	0.243	0.155	0.094	0.083	0.06	0.055
M 5b	1.63	1.033	0.656	0.379	0.256	0.155	0.104	0.074	0.06	0.052

Table D.36: Raw ELISA data of serum anti-rCYAAL58 IgG1

Dilution	100	200	400	800	1600	3200	6400	12800	25600	51200
P 9a	1.936	1.858	1.785	1.843	1.744	1.746	1.589	1.503	0.921	0.701
P 9b	1.961	1.889	1.832	1.801	1.686	1.625	1.592	1.348	1.076	0.661
P 8a	1.762	1.729	1.661	1.645	1.594	1.567	1.471	1.411	1.362	1.117
P 8b	1.702	1.673	1.638	1.624	1.561	1.555	1.481	1.435	1.287	1.036
P 5a	2.087	1.827	1.334	0.858	0.44	0.254	0.191	0.148	0.104	0.124
P 5b	2.163	1.899	1.304	0.809	0.434	0.304	0.148	0.099	0.08	0.066
P 6a	2.192	2.031	1.981	1.855	1.391	0.975	0.856	0.486	0.316	0.198
P 6b	2.108	1.972	1.887	1.825	1.752	0.967	0.767	0.468	0.362	0.256
P 7a	2.152	2.091	2.014	1.712	1.531	0.947	0.646	0.402	0.249	0.154
P 7b	2.022	2.019	1.961	1.904	1.801	1.226	0.693	0.508	0.306	0.272

Table D.37: Raw ELISA data of serum anti-rCYAAL58 IgG2a

Dilution	100	200	400	800	1600	3200	6400	12800	25600	51200
P 9a	0.535	0.238	0.122	0.079	0.055	0.019	0.008	0.003	0	0
P 9b	0.524	0.307	0.166	0.079	0.049	0.038	0.027	0.021	0.011	0.009
P 8a	0.771	0.609	0.442	0.268	0.174	0.101	0.064	0.038	0.02	0.02
P 8b	0.766	0.579	0.421	0.254	0.146	0.093	0.046	0.026	0.018	0.013
P 5a	0.546	0.305	0.184	0.093	0.047	0.038	0.029	0.027	0.021	0.011
P 5b	0.591	0.308	0.176	0.082	0.039	0.014	0.007	0.004	0.002	0
P 6a	0.38	0.203	0.091	0.048	0.021	0.008	0.006	0.003	0	0
P 6b	0.397	0.193	0.108	0.054	0.033	0.027	0.022	0.015	0.014	0.007
P 7a	1.342	0.793	0.583	0.295	0.181	0.093	0.058	0.024	0.013	0.007
P 7b	1.279	0.991	0.537	0.389	0.211	0.103	0.055	0.032	0.013	0.01

D.6 Chapter 8

Table D.38: IFN-γ production from *in-vitro* re-stimulated splenocytes from mice immunised with combination DNA vaccines and dual modality vaccines. Values represent mean (pg/ml) from duplicate wells. rCYAAL58 + C – 5µg/ml of rCYAAL58 + 5µg/ml of rCYAC; FFST – formalin-fixed *S. typhimurium* lysate; HKBP – heat-killed *B. pertussis* lysate; ConA – concanavalin A.

Stimulant	µg/ml	Group			
		Oral Dual Modality	**IM Dual Modality**	**Vector Dual Modality**	**DTaP**
rFHAB1	5	22572	9916	1860	763
rPRN2	5	18900	5254	1669	697
rPTS1.13L.129G	5	16192	4856	1297	1082
rCYAAL58	5	21235	1489	222	0
rCYAAL58+C	5	32956	4900	410	0
DTaP h	10	4632	1828	125	1744
DTaP l	5	4334	2241	183	2111
FFST	5	33135	1155	701	80
HKBP	5	8791	660	720	522
ConA	2	30000	30000	30000	30000

Stimulant	µg/ml	Group			
		Oral vector	**Oral Combo DNA vaccine**	**IM vector**	**IM Combo DNA vaccine**
rFHAB1	5	1238	5280	7790	492
rPRN2	5	501	3758	3175	359
rPTS1.13L.129G	5	324	2970	2892	647
rCYAAL58	5	482	4593	2090	1107
rCYAAL58+C	5	0	5784	823	1546
DTaP	10	0	0	3189	76
DTaP	5	0	0	3067	221
FFST	5	6205	6205	nd	nd
HKBP	5	0	410	1615	1026
ConA	2	30000	30000	6000	7033

Table D.39: IL-2 production from *in-vitro* re-stimulated splenocytes from mice immunised with combination DNA vaccines and dual modality vaccines. Values represent mean (pg/ml) from duplicate wells.

Stimulant	µg/ml	Group			
		Oral Dual Modality	IM Dual Modality	Vector Dual Modality	DTaP
rFHAB1	5	767.7	881	333.6	0
rPRN2	5	440.1	247.4	190.5	0
rPTS1.13L.129G	5	172.1	180.4	113	0
rCYAAL58	5	311.6	423.9	266.7	0
rCYAAL58+C	5	237.4	246.7	181.2	0
DTaP h	10	189.1	126.2	15.4	281.8
DTaP l	5	227.1	204.2	62.6	456.8
FFST	5	228.8	104.7	65.4	0
HKBP	5	155.2	81.7	39.4	5.6
ConA	2	1096	1336	1376	973

Stimulant	µg/ml	Group			
		Oral vector	Oral Combo DNA Vaccine	IM vector	IM Combo DNA Vaccine
rFHAB1	5	0	11.4	47.9	249.5
rPRN2	5	0	0	12	90.7
rPTS1.13L.129G	5	0	54.8	9.4	42.8
rCYAAL58	5	0	172.3	135.2	134.3
rCYAAL58+C	5	0	71.1	40.2	6.8
DTaP	10	0	0	16.3	106.9
DTaP	5	0	0	16.3	83.8
FFST	5	185.6	138.8	nd	nd
HKBP	5	0	74.9	16.3	19.7
ConA	2	881	3760	25360.2	16134.2

Table D.40: IL-4 production from *in-vitro* re-stimulated splenocytes from mice immunised with combination DNA vaccines and dual modality vaccines. Values represent mean (pg/ml) from duplicate wells.

Stimulant	µg/ml	Group			
		Oral dual modality vaccine	Parenteral dual modality vaccine	Vector dual modality	DTaP
rFHAB1		62.5	53.6	21.1	0
rPRN2	5	28.8	11.6	6	0.8
rPTS1.13L.129G	5	15.8	0	0	0
rCYAAL58	5	19.2	0	0	0
rCYAAL58+C	5	1.4	0	0	0
DTaP h	5	19	6.3	7.2	35
DTaP l	10	43.7	22.2	11.8	73.9
FFST	5	34	3	2.7	0.7
ConA	2	1000	1000	824.3	837

Stimulant	µg/ml	Group			
		Oral vector	Oral combination DNA vaccine	IM vector	IM combination DNA vaccine
rFHAB1		0	0	0	22.5
rPRN2	5	0	0	16.8	19.2
rPTS1.13L.129G	5	0	0	12.8	16.7
rCYAAL58	5	0	0	19.4	7.8
rCYAAL58+C	5	0	0	10.5	2.5
DTaP h	10	0	0	6.4	14.3
DTaP l	5	0	0	6	23
FFST	5	11	6.3	nd	nd
ConA	2	932.5	540.8	509.4	481.3

Table D.41: IgG titers in serum of mice vaccinated with five-gene combination DNA vaccines and controls

Vaccine group	Description	Antigen	Titer				
			M1	M2	M3	M4	M5
1a	Oral vector	rFHAB1	0	0	0	0	0
		rPRN	1600	3200	0	3200	0
		rPTS1.13L.129G	3200	3200	3200	25600	12800
		rCYAAL58	845	1075	0	103	0
		AC-Hly	1525	1472	0	0	0
		SL3261 lysate	25600	179275	163064	51200	25600
2a	Oral combination DNA vaccine	rFHAB1	0	0	0	0	0
		rPRN	6400	3200	204800	25600	12800
		rPTS1.13L.129G	6400	25600	102400	12800	25600
		rCYAAL58	0	0	0	0	0
		FHA	0	0	0	0	0
		PTX	0	0	6400	0	0
		AC-Hly	1744	1600	475	0	1817
		SL3261 lysate	199792	102400	472865	102400	182104
Exp2 G7a	IM combination DNA vaccine	rFHAB1	3200	12800	0	0	6400
		rPRN	44705	44932	9073	51056	44595
		rPTS1.13L.129G	0	100	150	400	1600
		rCYAAL58	119258	91250	44242	56356	127524
Exp2 G8a	M vector	rFHAB1	12800	12800	12800	12800	12800
		rPRN	0	0	0	0	0
		rPTS1.13L.129G	800	800	800	800	800
		rCYAAL58	83246	83246	83246	83246	83246
7a	DTaP (Infanrix™)	rFHAB1	1600	1600	3200	3200	3200
		rPRN	502383	219599	235422	514120	482991
		rPTS1.13L.129G	51200	25600	51200	102400	25600
		rCYAAL58	0	0	0	0	0
		FHA	0	0	0	3200	1600
		PTX	102400	102400	51200	204800	25600
		AC-Hly	0	0	0	0	0

Table D.42: IgG titers in serum of mice immunised with dual modality vaccines and controls.

Vaccine group	Description	Antigen	Titer				
			M1	M2	M3	M4	M5
3a	Oral dual modality vaccine	rFHAB1	12800	12800	12800	25600	25600
		rPRN	204800	468398	447552	220397	204800
		rPTS1.13L.129G	12800	246146	230944	231068	51200
		rCYAAL58	0	0	0	0	0
		FHA	0	0	0	0	0
		PTX	414261	417028	390012	414538	421865
		AC-Hly	0	0	0	0	0
		SL3261 lysate	12800	102400	174434	12800	12800
4a	Parenteral dual modality vaccine	rFHAB1	12800	12800	1600	51200	25600
		rPRN	25600	204800	25600	204800	3200
		rPTS1.13L.129G	12800	204800	6400	51200	1600
		rCYAAL58	0	0	0	0	0
		FHA	1600	1600	0	3200	0
		PTX	480265	565625	414022	497420	51200
		AC-Hly	0	0	0	0	0
5a	Vector dual modality vaccine	rFHAB1	0	12800	25600	0	0
		rPRN	6400	25600	123029	3200	3200
		rPTS1.13L.129G	12800	12800	213555	12800	3200
		rCYAAL58	0	0	0	0	0
		FHA	3200	3200	1600	3200	1600
		PTX	196714	413942	402118	407187	409906
		AC-Hly	0	0	0	0	0
7a	DTaP	rFHAB1	1600	1600	3200	3200	3200
		rPRN	502383	219599	235422	514120	482991
		rPTS1.13L.129G	51200	25600	51200	102400	25600
		rCYAAL58	0	0	0	0	0
		FHA	0	0	0	3200	1600
		PTX	102400	102400	51200	204800	25600
		AC-Hly	0	0	0	0	0

Table D.43: Serum IgG1 and IgG2a titers for oral combination DNA vaccine and dual modality vaccines

Antigen	Oral five-gene combination DNA vaccine		Oral vector		DTaP	
	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a
rFHAB1	3200	800	0	100	12800	0
rPRN	1600	1600	1600	400	516608	3200
rPTS1.13L.129G	102400	6400	25600	3200	203481	100
rCYAAL58	0	200	0	100	0	0
FHA	0	50	0	0	3200	400
PTX	0	0	0	0	502903	800
AC-Hly	0	50	0	100	0	0
SL3261 lysate	460256	41168	204800	12800	6400	0

Antigen	Oral dual modality vaccine		IM dual modality vaccine		Vector dual modality vaccine	
	IgG1	IgG2a	IgG1	IgG2a	IgG1	IgG2a
rFHAB1	102400	800	51200	400	51200	200
rPRN	505468	3200	228571	6400	204800	800
rPTS1.13L.129G	220063	800	207352	100	51200	100
rCYAAL58	0	100	0	0	0	0
FHA	12800	800	12800	100	12800	400
PTX	970597	800	831524	400	520556	800
AC-Hly	400	0	400	0	0	0
SL3261 lysate	102400	6400	800	0	800	0

Table D.44: IgG Titers in BAL fluid of mice vaccinated with oral combination DNA vaccine and dual modality vaccines

Vaccine group	Description	Antigen	Titer				
			M1	M2	M3	M4	M5
1a	Oral vector	rPTS1.13L.129G	3200	800	100	0	0
		rCYAAL58	0	50	0	0	0
		AC-Hly	0	100	0	0	0
		SL3261 lysate	100	200	800	200	400
2a	Oral combination DNA vaccine	rPTS1.13L.129G	400	0	200	800	0
		rCYAAL58	0	0	0	0	0
		SL3261 lysate	800	0	800	100	0
3a	Oral dual modality vaccine	rFHAB1	400	200	1600	800	800
		rPRN	400	400	800	0	800
		rPTS1.13L.129G	800	800	3200	400	800
		rCYAAL58	50	50	200	0	100
		FHA	100	50	400	0	400
		PTX	800	800	3200	800	6400
		SL3261 lysate	0	400	100	800	800
4a	Parenteral dual modality vaccine	rFHAB1	800	1600	400	200	200
		rPRN	100	0	50	100	0
		rPTS1.13L.129G	100	0	50	100	0
		FHA	50	200	50	50	0
		PTX	800	800	800	800	400
5a	Vector dual modality vaccine	rFHAB1	1600	0	800	0	800
		FHA	100	200	400	200	400
		PTX	800	1600	3200	3200	3200
7a	DTaP	rFHAB1	200	200	100	400	0
		rPRN	1600	1600	800	800	400
		rPTS1.13L.129G	0	200	200	800	100
		FHA	800	800	0	200	0
		PTX	800	3200	800	800	800

Table D.45: IgA titers in pooled BAL fluid for oral five-gene combination DNA vaccine and dual modality vaccines

Vaccine group	Description	Antigen	Titer
1a	Oral vector	rFHAB1	0
		rPRN	0
		rPTS1.13L.129G	0
		rCYAAL58	0
		FHA	0
		PTX	0
		AC-Hly	0
		SL3261 lysate	200
2a	Oral combination DNA vaccine	rFHAB1	0
		rPRN	0
		rPTS1.13L.129G	0
		rCYAAL58	0
		FHA	0
		PTX	0
		AC-Hly	0
		SL3261 lysate	50
3a	Oral dual modality vaccine	rFHAB1	25
		rPRN	25
		rPTS1.13L.129G	0
		rCYAAL58	0
		FHA	0
		PTX	25
		AC-Hly	0
		SL3261 lysate	25
4a	Parenteral dual modality vaccine	rFHAB1	0
		rPRN	25
		rPTS1.13L.129G	0
		rCYAAL58	0
		FHA	0
		PTX	0
		AC-Hly	0
		SL3261 lysate	0
5a	Vector dual modality	rFHAB1	0
		rPRN	0
		rPTS1.13L.129G	0
		rCYAAL58	0
		FHA	0
		PTX	0
		AC-Hly	0
		SL3261 lysate	0
7a	DTaP	rFHAB1	0
		rPRN	0
		rPTS1.13L.129G	0
		rCYAAL58	0
		FHA	25
		PTX	25
		AC-Hly	0
		SL3261 lysate	0

Table D.46: Clearance of *B. pertussis* from lungs following aerosol challenge of mice immunised with IM combination DNA vaccine. Bacterial load expressed as log10 CFU/lung

Group	Day	Mouse1	Mouse2	Mouse3	Mouse4
IM Combination DNA Vaccine	0	5.699	5.602	5.778	5.699
	4	5.908	5.748	5.792	5.954
	7	3.278	3.204	2.845	3.301
	14	0	0	0	0
pcDNA3.1 Vector	0	5.699	5.602	5.778	5.699
	4	6.439	6.778	6.303	6.41
	7	5.663	6.799	6.991	6.212
	14	5.255	6.204	5.716	5.959
Placebo	0	5.699	5.602	5.778	5.699
	4	5.217	6.318	6.442	5.217
	7	6.756	5.764	6.672	6.176
	14	6.27	6.079	5.7	5.996
DTaP	0	5.699	5.602	5.778	5.699
	4	5.58	5.613	5.613	5.602
	7	0	0	0	0
	14	0	0	0	0

Table D.47: Clearance of *B. pertussis* from lungs following aerosol challenge of mice immunised with oral combination DNA vaccine and dual modality vaccines

Group	Day	Mouse1	Mouse2	Mouse3	Mouse4
1	0	2.77E+05	1.51E+05	1.61E+05	1.44E+05
	4	2.98E+06	1.88E+06	7.50E+05	1.36E+06
	7	3.68E+06	4.26E+06	4.17E+06	2.70E+06
	14	8.40E+04	1.33E+05	1.06E+05	1.06E+05
2	0	2.30E+05	2.04E+05	1.52E+05	3.15E+05
	4	1.88E+06	2.80E+06	1.93E+06	1.28E+06
	7	2.50E+06	4.00E+06	9.50E+05	3.54E+06
	14	2.11E+04	3.10E+04	6.90E+04	1.70E+04
3	0	2.77E+05	1.51E+05	1.61E+05	1.44E+05
	4	1.09E+05	1.02E+05	3.40E+04	8.10E+03
	7	4.70E+04	0.00E+00	4.60E+04	0.00E+00
	14	0.00E+00	0.00E+00	0.00E+00	0.00E+00
4	0	2.77E+05	1.51E+05	1.61E+05	1.44E+05
	4	7.50E+04	7.90E+03	0.00E+00	8.80E+04
	7	6.80E+03	0.00E+00	0.00E+00	5.30E+03
	14	0.00E+00	0.00E+00	0.00E+00	0.00E+00
5	0	2.77E+05	1.51E+05	1.61E+05	1.44E+05
	4	7.70E+04	1.14E+05	1.41E+05	0.00E+00
	7	5.50E+04	0.00E+00	4.90E+04	0.00E+00
	14	0.00E+00	0.00E+00	0.00E+00	5.50E+03
6	0	2.77E+05	1.51E+05	1.61E+05	1.44E+05
	4	1.12E+07	4.60E+06	4.80E+06	6.30E+06
	7	1.39E+06	4.28E+06	9.10E+06	7.00E+05
	14	5.80E+04	1.24E+05	5.80E+04	1.25E+05
7	0	2.77E+05	1.51E+05	1.61E+05	1.44E+05
	4	7.30E+04	7.50E+03	0.00E+00	9.20E+04
	7	0.00E+00	6.50E+04	0.00E+00	0.00E+00
	14	0.00E+00	0.00E+00	0.00E+00	0.00E+00

Key:

Group 1: Oral Vector (SL3261-pcDNA3.1D vector)

Group 2: Oral Combination DNA vaccine

Group 3: IM Dual Modality (IM combination DNA vaccine prime/subunit boosters

Group 4: Oral Dual Modality (Oral combination DNA vaccine prime/subunit boosters

Group 5: Vector Dual Modality (IM vector prime/subunit boosters

Group 6: Placebo (Saline)

Group 7: DTaP