Strategies for delivery of alpha toxin variants designed to inhibit *Clostridium perfringens* infections

Fiona Baird
Doctor of Philosophy

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RMIT
Strategies for delivery of alpha toxin variants designed to inhibit *Clostridium perfringens* infections

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

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Declaration

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

Fiona Baird
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<tr>
<td>Δ</td>
<td>Deletion</td>
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<tr>
<td>λ</td>
<td>Lambda phage DNA</td>
</tr>
<tr>
<td>λ-PstI</td>
<td>Lambda DNA digested with the restriction enzyme PstI</td>
</tr>
<tr>
<td>μg</td>
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<td>Ampicillin</td>
</tr>
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<td>ANGIS</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>°C</td>
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<td>C-terminus</td>
<td>Carboxyl terminus domain of an amino acid sequence/protein</td>
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<td>Hour</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<td>H₂O</td>
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<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>IFN-γ</td>
<td>Interferon gamma</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IMAC</td>
<td>Immobilised metal affinity chromatography</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kbp</td>
<td>Kilobase pairs</td>
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<tr>
<td>kDa</td>
<td>One thousand Daltons</td>
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<tr>
<td>L</td>
<td>Litre</td>
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<tr>
<td>LD₅₀</td>
<td>The 50% lethal dose, the concentration required to kill 50% of a population</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>M</td>
<td>Molarity</td>
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Abstract

Alpha toxin is expressed by *Clostridium perfringens* during necrotic enteritis infection of chickens and causes great cellular damage in its active conformation. This project focussed on designing constructs with modifications to the gene sequence that resulted in inactivation of the toxin whilst retaining the immunogenicity of the native toxin. Three constructs were designed: plcInv, deletion of a section of the catalytic domain that inactivates the active site; plc104, the highly immunogenic carboxyl domain of the alpha toxin; and plc2, a multiple point mutagenesis designed to inactivate two key amino acids in the active site. Purification of the proteins demonstrated that plc2 had superior expression and was more stable than plcInv deletion protein. These constructs were tested as both purified proteins and cloned to be expressed constitutively from an attenuated vaccine strain STM1, a *Salmonella enterica* serovar Typhimurium vaccine approved for poultry usage, using a hemolysin secretion system vector.

As purified protein vaccines, the plc2 construct provided partial protection from gas gangrene infection in mice; while the other constructs elicited good humoral responses they did not induce a significant delay of disease onset. In mice, the delivery of these constructs via STM1 did not result in protection from gas gangrene challenge. In chicken immunogenicity trials the responses elicited by the STM1 delivered constructs were determined to be non-specific and cross-reactive with the STM1 vector. In the presence of pre-existing immunity to the most commonly isolated commensal, *Salmonella enterica* serovar Sofia, the vaccines and constructs did result in lower responses for the extracellularly delivered construct.

In conclusion, these constructs based on the alpha toxin when delivered by STM1 were not effective immunogens in eliciting specific protective responses to gas gangrene challenge in mice and did not elicit strong responses in the immunogenicity trials in chickens. This failure to induce high antigen-specific responses does not preclude the use of the hemolysin system in STM1 as a...
vaccine delivery system with a more immunogenic antigen. Ideally the most effective way to investigate these responses would be in a necrotic enteritis chicken model as this would determine if the responses are indeed ultimately protective.
1.1 Introduction

Globally, the burden of disease and infection is diverse and inescapable. It is a shared affliction for all humanity and one that is constantly moderated by better hygiene, enhanced education, and improved vaccines and drug treatments. In terms of disease management, it is always more beneficial to prevent a disease or infection from occurring than to treat and cure it. The development of vaccines is dependent on the knowledge of: what pathogen causes the disease; how it establishes itself in the host; how the host’s innate and cell-mediated immunity responds to pathogens; and how it maintains ongoing protection after the disease using antibodies. Whilst there are many successful vaccines currently available, there are still no registered vaccines for the two most prevalent global diseases – malaria and human immunodeficiency virus (HIV).

Although we have made enormous progress in medicine over the last 300 years since the practice of vaccination first began, there are still diseases that are killing millions of people globally which desperately require a vaccine. Furthermore, there is a multitude of diseases that affect the veterinary sector- these are costly and cause animal suffering. This review will explore the progression from empirical vaccines to the more recent novel vaccines and how recent advancements can be used to combat two poultry diseases: necrotic enteritis and salmonellosis caused by Clostridium perfringens and Salmonella species, respectively.

1.2 A brief history of vaccination

The first crude attempt at disease control was the procedure of variolation where the inoculated person stood a good chance at surviving both the procedure and later exposure to the pathogen.
Variolation consisted of directly transferring the infection from a sick person to a healthy person, through direct contact or by infectious matter such as pus, saliva or blood (Dinc and Ulman, 2007). This form of vaccination is believed to have begun in either ancient China or India, but was only brought to the United Kingdom by the wife of a British diplomat, Lady Wortley-Montagu in 1721 (Figure 1.1 A) (Dinc and Ulman, 2007).

Lady Wortley-Montagu had observed that harem girls in Constantinople had pox-free faces which was attributed to them being variolated, hence she had her son variolated in Istanbul in 1718 (Figure 1.1B) to save him from experiencing what she had as a young adult – smallpox. Later she also variolated her daughter in London; however this was only after she had confirmed that it did not result in death or disease in eleven orphans and six convicted murderers from Newgate Prison (Dinc and Ulman, 2007). Lady Wortley-Montagu was so impressed that she implored her surgeon, Dr Charles Maitland, to learn the technique and demonstrate it to the Royal British Court (Stewart and Devlin, 2006). After this demonstration, 200 upper-class members of British society - including members of the royal family – were subjected to the procedure, and in 1729 a further 897 more inoculations were performed with only 17 deaths post-procedure which is an insignificant number compared to the smallpox mortality at the time (Dinc and Ulman, 2007).

Even though Lady Wortley-Montagu was severely criticised for bringing the procedure to Britain, it was slowly implemented throughout the United Kingdom over the following years, and in 1757 a young boy named Edward Jenner would be variolated against smallpox (Dinc and Ulman, 2007). This ultimately saved countless lives from smallpox; the most devastating disease of the time. However there were two issues with variolation: 1) it could impair the patient or even kill them if the dosage was incorrect or if they were not physically fit enough to withstand the infection, and 2) whilst the patient would be protected from further infections they would become contagious as they were having a true progression of the disease (Bazin, 2003).
A) Lady Mary Wortley-Montagu was responsible for variolation being introduced from Turkey to Britain. B) She had her son variolated in Istanbul by an old wise Turkish woman and her Embassy surgeon, Dr Charles Maitland in 1718. Figure obtained from (Dinc and Ulman, 2007).

Figure 1.1: Historical depictions of Lady Mary Wortley-Montagu.
Although variolation was popular, in the English countryside there were many rumours that if you had a case of cowpox you were protected from the deadly smallpox, subsequently a farmer named Benjamin Jesty inoculated his wife and two sons with cowpox. Even though his wife became very ill, she and the whole family survived and went on to survive many exposures to smallpox. This transpired a full 20 years before scientist Jenner began his experiment with a boy called James Phipps (Pead, 2003). It is believed that Jenner was also aware of the rumours of cowpox protecting against smallpox, and that was the inspiration for his experiment, resulting in him being the first to document that a person infected with cowpox would survive subsequent exposure to smallpox (Stewart and Devlin, 2006). This technique evolved into using cow inoculums as the vaccine, which did provide immunity to smallpox although not to the same degree as natural disease or variolation. This discovery was heralded as the new age of vaccines and instigated new research into other common diseases.

A couple of centuries later, medicine would again make another huge step forward with the separate works of scientists Louis Pasteur and Robert Koch and the publication of germ theory. The most famous of these works would be Pasteur’s and his attenuation of the bacteria Pasteurella multocida, which causes fowl cholera by exposing the cultures to air and room temperature for extended periods of time (Bordenave, 2003). He proved that whilst the bacteria were avirulent, they provided full protection from the virulent strain of the bacteria, which was a revolutionary idea at the time. Pasteur also went on to attenuate the rabies virus by passage through rabbits (Bazin, 2003). Koch, on the other hand, would discover the bacterial agents of anthrax, tuberculosis and cholera whilst also compiling postulates with fellow scientist Jacob Henle that would transform the world of microbiology (Kaufmann and Schaible, 2005). All of these discoveries led to the development of immunology and non-empirical vaccines.
The first whole cell vaccine was produced by Salmon and Smith in 1886 and was based on a *Salmonella* strain that was killed by heat and injected into pigeons to provide immunity (Bazin, 2003). Around the same time, others were looking at bacterial components and methods to purify them. This was the beginning of traditional vaccine methodology. During this era there were many great innovations in the field of immunology and vaccinology, such as the discovery of toxins and the consequent inactivation of toxins by heat and formalin, killed vaccines, adjuvants, sub-unit or acellular vaccines, tissue culture and live attenuated vaccines. With the establishment of molecular biology and genetic engineering in the late 1950s, a new era began where vaccine development no longer needed to be empirical and bacterial components could be produced artificially or even *in vivo* by unrelated vectors.

### 1.3 Vaccines in the modern era

#### 1.3.1 What makes a good vaccine?

The traditional definition of a vaccine is one that protects against a particular (or group of) infectious agent(s), however these days there are many vaccines that could be designated as therapeutic agents against diseases such as cancer (Bergman *et al.*, 2006), although the goal is still to prevent illness. The global market for vaccines is estimated to be around US$8 billion per year whilst the cost to develop each vaccine from concept to commercialism is around US$300—800 million (Plotkin, 2005). The reason for the high expenditure is that each vaccine has to be rigorously tested before commercial release and the average time it takes to fully develop a vaccine is between 15 and 20 years (Arntzen *et al.*, 2005). A successful vaccine is measured by its effectiveness, its spectrum of protection, the duration of immunity and the strength of immunological memory that it induces. Secondary considerations of a good vaccine are its stability, ease of administration and storage, achievable mass production and its toxicity. The
most recent and highly publicised vaccine is the quadvalent human papillomavirus vaccine against cervical cancer, marketed as Gardasil®, which prevents the premalignant disease that leads to cervical cancers and fulfils all the above criteria of being a successful vaccine (Govan, 2008).

Biotechnology is a rapidly developing area which allows continued improvement into the exploration of antigens suitability as vaccine candidates. Choosing the right antigen is a core decision in the development of a vaccine candidate as some antigens that are immunogenic in vivo may not elicit long term protection. The same antigen may also vary in structure and sequence between strains, limiting its usefulness. Some antigens are also hard to express and purify on a large scale which is required for mass production (Mora et al., 2003). This is where novel vaccine methodology hopes to improve how vaccines are made and administered; this will be examined subsequently. The most common areas of vaccine design are shown in Figure 1.2.
Figure 1.2: Traditional and novel vaccinology.

Traditional vaccinology is represented by killed vaccines, live attenuated vaccines and subunit vaccines. Novel vaccinology is represented by live carriers which deliver heterologous antigens and reverse vaccinology which screens genomes for potential antigens. Figure from (Scarselli et al., 2005)
1.3.2 Routes of administration

1.3.2.1 Subcutaneous vaccination and intramuscular routes

The oldest technique for vaccination is that of subcutaneous delivery via scarification and one of the newest techniques is intramuscular injection (Bazin, 2003). Whilst the intramuscular route of vaccination is quite standard today in developed countries, it is an inconvenient method of application as it requires sterile needles and syringes, and usually a medical physician to administer it. This is the major drawback of vaccines that rely on intramuscular injections to be effective. In one study, a viral vectored vaccine was found to elicit stronger systemic and detectable mucosal responses via a single intramuscular injection than if it was applied via the oral route. The oral route proved to stimulate suboptimal T-cell responses and did induce a higher level of mucosal antibody than the intramuscular route (Lin et al., 2007).

1.3.2.2 Nasal and oral routes

Nasal and oral administration routes of a vaccine are desirable as they are non-invasive, painless, not required to be sterile and do not require a physician for administration. This final point is most important as it is one of the reasons that Third World countries have the lowest level of immunisations in the world (Costantino et al., 2007). Nasal immunisation would place the vaccine in contact with the large surface of the nasal mucosa which consists of the nasal-associated lymphoid tissue (NALT), which can lead to both humoral and cellular immune responses (Zuercher et al., 2002; Costantino et al., 2007). The most well-known nasal vaccine is FluMist®; a live cold-adapted influenza virus. It can be given as one or two doses from a syringe sprayer, is licensed for use in the U.S.A. for persons aged 5–50 and has shown high efficacy from its inception (Plotkin, 2005; Costantino et al., 2007). However one of the detrims of a nasal vaccine is that an unpleasant taste and nasal discomfort can occur often discouraging repeated use (Atmar et al., 2007).
Oral administration is a practical method of application if it can be achieved without diminishing the effectiveness of the vaccine and immunity can be achieved with a single dose. The objective of oral vaccines is to mimic a natural infection and provide mucosal immunity. Orally delivered vaccines can induce suboptimal T-cell responses with high levels of mucosal antibody than the intramuscular route; however the vaccine must be very stable as it will have to survive the acidic environment of the stomach before it reaches the M cells of the intestinal wall where it can be processed by antigen presenting cells (APCs) (Lin et al., 2007).

1.3.3 Adjuvants

Adjuvants are defined as compounds that influence the immune system into mounting a Th1 or Th2 response and whilst doing so, greatly enhances the magnitude of immune response against the antigen (Marciani, 2003). They are an important aspect of vaccines due to their tendency to make an ineffective antigen become effective. It is vital that adjuvants have the following properties as a components of vaccines – a non-toxic nature or have minimal toxicity at the dosage to elicit effective adjuvanticity; able to stimulate a strong humoral and/or T-cell immune response; provide good immunological memory or long-term protection; not induce autoimmunity; are non-mutagenic, non-carcinogenic, non-teratogenic and non-pyrogenic; and be stable under broad ranges of storage time, temperature and pH levels (Marciani, 2003). The most popular adjuvants are aluminium-based and were first described and published in 1926 (Glenny et al., 1926). The only adjuvant that is currently licensed for human use is aluminium hydroxide, also known as alum (Davies and Flower, 2007). It is well established that aluminium adjuvants stimulate the production of IgE and a Th2 immune profile, yet for some diseases this would not be adequate protection against pathogens as a Th1 response would be required (Lindblad, 2004).
Adjuvants are used to lengthen the dissemination time of the antigen from the site of injection which allows the antigen to be released over a prolonged period improving the effectiveness of the vaccine. This feature is called the depot effect and is observed with most aluminium-based adjuvants (Lindblad, 2004). Other methods in which adjuvants improve the immune response are to form complexes with the antigen and to target the vaccine towards specific receptors. For example, the use of mannose in the adjuvant is recognised by pattern recognition receptors (PRRs) that initiate endocytosis and antigen processing (Stahl and Ezekowitz, 1998). The use of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and CpG-DNA, and of synthetic low-MW imidazoquinolines in adjuvants, all trigger innate immune responses that lead to a Th1 or Th2 response in the vaccinated person (Marciani, 2003). Other adjuvants consist of cytokines (Cheng et al., 2007) and glycolipids (Singh et al., 1999; Ko et al., 2005) and other immunomodulators (Morrow et al., 2004) that bind to highly specific receptors on T cells which activate them.

1.3.4 Political and global aspects of vaccine usage

When a vaccine is designed, it is assumed that if it proves effective it will be used in countries around the world to vaccinate the population; however this is not always the case. Within each country there are government agencies, industry and community health advocates, and outside agencies such as the World Health Organization (WHO) that will make recommendations for vaccination strategies. Often this process will result in a successful vaccination strategy such as the global eradication of smallpox (Stewart and Devlin, 2006), but it can also lead to confusion and scepticism in the chosen strategy. One such example was the choice of pertussis vaccine for a national vaccination campaign in the Netherlands.
Originally, the Dutch government chose to use a whole cell vaccine based on the *Bordetella pertussis* bacterium, however after speculation that the vaccine could cause brain damage, alternative vaccines were sought. At this time, acellular vaccines comprising three to five bacterial components were being used by many countries in Europe as they were comparable in protection to the whole cell vaccines and appeared to have minimal side effects (Blume and Zanders, 2006). Over the course of seven years, the debate over the new vaccine became very convoluted as many government agencies, drug companies and consumer groups presented opposing studies and evidence. There was also external pressure from neighbouring countries and global non-profit groups including WHO and UNICEF for the Dutch government to make a decision. Concurrently, many parents had lost faith in the old vaccine strategy; hence an epidemic of pertussis ensued. To combat the growing epidemic the Dutch government chose an acellular vaccine which was used in primary vaccinations in 2005, however the Health Minister advised that this decision was not based on recommendations and evidence provided by the Dutch Health Council, but on the need to appease parents and re-establish their confidence in the vaccine strategy (Blume and Zanders, 2006). By contrast in areas where any disease is endemic and the health system is overwhelmed, often the vaccination strategy proposed by governing bodies will be accepted by the population and acquiesced as mandatory (Chalmers, 2006).

### 1.4 Traditional vaccine methodology

The early development of vaccines focused on using killed organisms, inactivated toxins or modified organisms, but currently there are many different approaches to vaccine development, which will be examined subsequently. As these approaches were empirical in design, these types of vaccines — whilst being successful — are now viewed as being traditional vaccines. These can be divided into three different types: a) killed vaccines; b) attenuated vaccines; and c) sub-unit vaccines (Table 1.1).
1.4.1 Killed vaccines

A killed or ‘inactivated’ vaccine is developed by the pathogen being grown and then being made inactive by means of heat, chemical or radiation treatment and was the basis of most vaccines until the 1980s. This results in the pathogen being unable to cause disease whilst providing the immune system with stimulation via its normal antigenic epitopes on its cell surface. One major disadvantage of this approach is that, whilst these vaccines are immunogenic, they do not replicate \textit{in vivo} limiting the spectrum of the immunity acquired as the agent is incapable of going through its normal antigenic variation over the course of the infection. This results in decreased immunity and a requirement of booster shots to maintain immunity (Moylett and Hanson, 2003). Another disadvantage of this vaccine type is that during the inactivation process the antigenic epitopes can be modified resulting in a less efficacious vaccine (Tano \textit{et al.}, 2007). Despite these limitations, killed vaccines are commonly used today with the typhoid, Salk poliomyelitis and seasonal influenza vaccines still being administered (Bazin, 2003; Palese, 2006).

1.4.2 Attenuated vaccines

Among the more efficacious of the traditional vaccines are the attenuated ones. In this case, a pathogen is subjected to altered growth conditions, is passaged through a host or is genetically modified to eliminate its virulence, yet retaining its ability to replicate albeit at a greatly reduced rate. These vaccines are more successful at eliciting a robust lifelong immunity than other traditional vaccines. This can be attributed to their ability to cause an asymptomatic infection which stimulates both the humoral and cellular branches of the immune system.
Table 1.1: Types of traditional vaccines (adapted from (Griffin, 2002)).

<table>
<thead>
<tr>
<th>Types of vaccine</th>
<th>Target micro-organism</th>
<th>Type of Immunity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Killed</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial</td>
<td><em>Salmonella, Yersinia, Leptospires</em></td>
<td>IgG</td>
</tr>
<tr>
<td>Virus</td>
<td>Polio, Influenza</td>
<td>IgG</td>
</tr>
<tr>
<td><strong>Attenuated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial</td>
<td><em>Salmonella, Tuberculosis (BCG)</em></td>
<td>IgA, CMI</td>
</tr>
<tr>
<td>Virus</td>
<td>Mumps, Measles, Polio, Rubella</td>
<td>IgA, CMI</td>
</tr>
<tr>
<td><strong>Sub-unit</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial</td>
<td>Clostridial, Fusiformis — toxoids, <em>Haemophilus influenzae type B</em></td>
<td>IgG</td>
</tr>
<tr>
<td>Virus</td>
<td>Foot and Mouth Disease Virus — capsid</td>
<td>IgG</td>
</tr>
</tbody>
</table>
However this ability to replicate carries the greatest risk as the vaccine can persist in immune-compromised persons or the elderly due to limited immune responses. A benefit to these vaccines is they express their own immunogenic antigens which stimulate the immune system strongly thereby negating the need for an adjuvant to be used (Loessner et al., 2008). The most commonly used attenuated vaccine is the MMR vaccine which protects children worldwide against measles, mumps and rubella and with subsequent boosters provides lifelong immunity (Vandermeulen et al., 2007). Attenuated vaccines have further progressed into carrier vaccines where they can deliver heterologous antigens (Bachtiar et al., 2003; Lotter et al., 2008; Schoen et al., 2008). For live carrier vaccines that deliver multiple heterologous antigens, there is a risk that the host immune system will dampen the immune response to the heterologous antigens by misdirecting the immune response against the carrier (Berzofsky et al., 2004).

### 1.4.3 Subunit vaccines

Traditionally, it was thought that the only way to protect against a disease was to use the whole organism to vaccinate the host. However it was elucidated that specific parts of the organisms, when purified or isolated, proved to be immunogenic. These components could be the capsule, the flagella or even an outer membrane protein of the cell wall. These types of vaccines are known as subunit vaccines or acellular vaccines. These vaccines are not able to cause the disease and in comparison to whole cell killed vaccines they are not as efficacious. This is both an advantage as they are safe for immune-compromised patients and a disadvantage as they do not elicit long-term immunity and will often require multiple vaccinations to maintain immunity (Schmitt et al., 2008). An advantage of this type of vaccine is that it can be engineered to protect against various strains of the organism. An example of a successful subunit vaccine is the *Haemophilus influenzae* type b (HiB) conjugate vaccine which consists of a polysaccharide-protein
conjugate. This vaccine has eliminated or significantly reduced this disease in children in regions of South America (Ribeiro et al., 2007; Franco-Paredes et al., 2008) and Africa (Adegbola et al., 2005; Muganga et al., 2007) where it was once endemic. In the UK, the success of this vaccination program was compromised by a highly publicised paper (which has now been retracted) that linked autism to early childhood vaccination which lead to a rise in HiB infections as parents chose not to vaccinate; however subsequent booster campaigns by the NHS has seen a reduction in infection rates again (Ladhani et al., 2008).

1.5 Novel vaccine methodology

1.5.1 In silico screening

In silico screening is a recent technique that has only become accessible to scientists in the last 25 years due to the increasing power of computers and the sequencing of complete genomes. In silico screening is based on the use of algorithms to search genomes. A potential use is to search for putative antigens termed reverse vaccinology (Mora et al., 2003). There are four main processes to screening genomes for putative antigens: 1) a primary screen for coding regions; 2) all open reading frames (ORFs) are detected and used for homology searches using such programs as BLASTx and BLASTn; 3) examine if the potential antigen has not been characterised; and 4) identify the cellular location of the potential antigen. A major advantage of this technique is the efficient exploration of all ORFs in a genome. The primary disadvantage of in silico screening for reverse vaccinology is that the algorithms are sometimes unable to predict whether the protein is intracellular or extracellular, as extracellular proteins tend to be more immunogenic.

The first published use of in silico screening, in terms of reverse vaccinology, was the identification of vaccine candidates for Neisseria meningitidis serogroup B meningococcus (Pizza et al., 2000). Pizza and colleagues identified 570 genes for possible surface-exposed antigens of
which 350 were successfully cloned and expressed. Of these characterised proteins, 85 were found to be surface-exposed and 22 were able to induce an antibody response (Pizza et al., 2000). This technique has also been used for other organisms such as *Streptococcus pneumoniae* (Wizemann et al., 2001), *Porphyromonas gingivalis* (Ross et al., 2001) and *Chlamydia pneumoniae* (Montigiani et al., 2002).

Other uses of in silico screening are: B cell and T cell epitope prediction based on forecasting peptide fragments that can be presented by T cells using BIMAS or EpiDirect programs; predicting the surface region of antigens that will bind to antibodies using ePitope or Discotope programs; screening proteins that have IgE epitopes that lead to allergies and sensitivities using AlgPred or AllerPredict programs; in cancer vaccinology, algorithms can show proteins that might have an effect on tumour growth; and adjuvant hunting using Immunogrid where small proteins are screened against cell receptors and ligands to see if they can elicit an immune response (Mora et al., 2003).

1.5.2 Plant-based oral vaccines

One of the most novel and innovative fields of current vaccine experimentation is the exploration of plant-derived vaccines and antibodies. This revolves around the production of antigens or antibodies within the stems or leaves of a plant. The main advantage of this technique is that plants can perform post-translational modifications, opposed to other systems such as bacteria that are unable to. Other advantages include minimised risk of contamination from human pathogens, the elimination of purification steps for food based vaccines, convenient storage, the elimination of health professionals if the vaccine can be given orally, and a renewable source of the vaccine (Arntzen et al., 2005; Shams, 2005; van der Laan et al., 2006). The first scientific report describing the potential of plant-derived vaccines was the expression of a Hepatitis B
surface antigen in a transgenic tobacco plant (Mason et al., 1992). There are three major strategies to induce plants to express foreign antigens – stable genetic transformation of the nuclear genome, stable transformation of the chloroplast genome, or transient expression using viral vectors. However these three strategies differ in how much protein is expressed and to which plant species the strategy can be applied (Arntzen et al., 2005). The majority of these studies have been carried out in model tobacco plants but the longer term usefulness of these transgenics is limited due to the high alkaloid content which would not pass vaccine regulations for orally delivered vaccines (Arntzen et al., 2005; van der Laan et al., 2006). One persistent concern regarding plant-derived vaccines is that the antigen is ingested like food proteins, which could lead to tolerance of the antigen and thus would not provide protection against infection (van der Laan et al., 2006). However there has been recent articles reporting that when given orally, plant derived vaccines induce both mucosal and humoral immunity (Hale et al., 2006; Youm et al., 2007). Another significant aspect of plant-derived vaccines is the production of antibodies in transgenic plants, first reported in 1989, where plants expressing either single gamma or kappa immunoglobulin chains were crossed to yield tobacco plant progeny in which both chains were expressed simultaneously (Hiatt et al., 1989). This production of antibody could be used as an edible prophylaxis vaccine after exposure to infectious gastrointestinal pathogens to which standard vaccines do not exist.

1.5.3 Synthetic peptides

Synthetic peptides are another enhancement of traditional sub-unit vaccines. Whilst sub-unit vaccines focussed on reproducing whole proteins as the antigen, synthetic peptides focussed in on small regions of the antigens that were presented by T-cells. This epitope-based strategy relies on confirmation that the epitope is immunogenic and the peptide being able to elicit a strong cell-
mediated immune response (van der Burg et al., 2006). Delivery of a large dose of peptides leads to a larger magnitude immune response as they will activate a greater number of lymphocytes.

Synthetic peptides are easily produced, stable, deficient of oncogenic potential and are free of bacterial or viral contaminating substances (Jiang et al., 2006; van der Burg et al., 2006). The first reports of this type of vaccine occurred in 1991 where a peptide provided protection from a viral challenge (Kast et al., 1991; Schulz et al., 1991). Since then there have been many improvements which have increased the efficacy of these peptides such as the modification of peptides to improve its binding efficiency to major histocompatibility complex (MHC). Another is to include multiple peptide epitopes that induce both CD8+ and CD4+ T-cell activity which results in a stronger immune response. Promiscuous overlapping synthetic peptides are one application that uses multiple peptides. The peptides are designed to represent sequential portions of a known antigen which subsequently elicits both CD4+ and CD8+ immune responses as both types of epitopes are represented (Jiang et al., 2006). One difficulty associated with synthetic peptides is their short nature, in vivo they are automatically exposed to the indigenous proteases which may render the peptide inert if cleavage occurs. One way to avoid this scenario is to make the peptide longer but consequently it will reduce its ability to bind to MHC. However it has been found that although the peptide loses its binding ability, it will still be taken up and processed by dendritic cells which will result in an immune response (van der Burg et al., 2006). An advantage of synthetic peptides is that multiple peptides can be administered to protect against a multitude of antigens; but they must be coupled with the right adjuvant that will attract APCs to the site of injection. Currently, two synthetic peptide vaccines are being tested in Phase I trials against malaria (Hermsen et al., 2007; Peduzzi et al., 2008).
1.5.4 DNA vaccines

DNA vaccines differ from traditional vaccines as they do not consist of a protein or a cell component but only the DNA that encodes an immunogenic antigen within a plasmid vector. The plasmid can be administered by injection, gene gun, electroporation or aerosol delivery, upon which the host’s immune cells, usually dendritic cells, will sample the plasmid and express the encoded antigens. These antigens are then degraded by the cell into peptides and presented via MHC class I and class II molecules depending on the mode of administration and the cell type. From this, both antibody and cellular responses can be induced (Forde, 2005).

The first reported use of a plasmid DNA vaccine outside of trial or experimental conditions was in 2003 and was a desperate attempt to save an endangered species from extinction (Bouchie, 2003). The vaccine was for the highly endangered California condors against the lethal West Nile virus. West Nile virus had emerged in New York in 1999 and spread to 41 out of the 50 US states killing birds from 138 species in a matter of years. It was believed that if the virus spread to California, the remaining 200 or so condors would face extinction. The US Centers for Disease Control and Prevention expedited the manufacture of an experimental vaccine and permitted the condors to be vaccinated with it (Bouchie, 2003). The DNA vaccine expressed West Nile virus premembrane/membrane and envelope proteins. The vaccinated condors were monitored and it was observed that the DNA vaccination stimulated protective immunity in adults, nestlings and newly hatched chicks. Following two intramuscular vaccinations, the condors demonstrated excellent neutralising antibodies 60 days post-vaccination with a continued increase until approximately one year post-vaccination. It was also noted that the birds did not show any unusual behaviours, health changes or side effects post-vaccination (Chang et al., 2007). The first two DNA vaccines for veterinary use were granted US approval in 2005 for West Nile virus vaccine for horses and haematopoietic necrosis vaccine for farm-reared Atlantic salmon (Chalmers, 2006).
1.6 Veterinary vaccines

Veterinary vaccines have slightly different objectives to those of human vaccines. They share the same aim of prevention and control of infectious agents with one major difference. In veterinary vaccinology, reducing the cost of food production whilst maintaining animal welfare is paramount as disease results in great economic losses (Shams, 2005). These vaccines have different but more stringent requirements such as they do not induce local reactions diminishing the value of the food product, induce long term and stable herd immunity, and vaccine-initiated infections can be differentiated from naturally acquired infections, known as marker vaccines. Most current licensed veterinary vaccines are killed or inactivated types such as porcine reproductive and respiratory syndrome virus (Zuckermann et al., 2007) or live modified vaccines such as foot and mouth virus (Mason et al., 1997). Another avenue of veterinary vaccines being developed is that of fertility controls, where the vaccines can either increase or inhibit ovulation in females (Rangel et al., 2005; Medan et al., 2006), which could be useful in times of drought or food shortages. In most cases, commercial veterinary vaccination is purely available to sustain the health of the animal so that premium price is ensured after slaughter.

1.7 Diseases in the poultry industry

1.7.1 Salmonella infections

Chickens are extremely susceptible to Salmonella infections in the first days of life and prevention of infection post-hatch is the primary and most important objective of poultry vaccine researchers worldwide. It is very important to control the amount of Salmonella harboured in the gut of chickens for several reasons; firstly chicken is an important source of affordable protein around the world, (Zhang-Barber et al., 1999) as on average, Americans alone consume 234 eggs/person/year (Babu et al., 2004). Secondly, the global poultry meat and egg industry is worth
more than US$2 billion annually (Collier et al., 2008) and responsible for many jobs internationally. In Australia, the gross value of egg production in 2007-08 was A$463 million (ABS, 2009) with egg consumption fluctuating between 129 – 140 eggs/person/year between 1998 and 2000 highlighting the significance of the local poultry industry (ABS, 2000; Bell, 2001). Vaccination against *Salmonella* in poultry is important both for the poultry’s health and also for reducing contamination of the food chain later on.

There are over 2400 different *Salmonella* serovars but only a few will infect poultry, causing septicaemia and rapid mortality in flocks (Zhang-Barber et al., 1999). The risk of this occurring can be severely reduced by stringent cleaning measures and by identifying risk factors that increase the horizontal and vertical spread of *Salmonella*. There are three stages in avian systemic salmonellosis. The first is the invasion of the gastrointestinal tract with the second being the establishment of a systemic infection resulting in *Salmonella* invading the circulating macrophages and disseminating to the internal organs for further replication. The third stage of infection is dependent on the level of *Salmonella* replication that occurs in the organs. There are three possible outcomes: 1) uncontrolled replication in the organs that leads to a systemic infection ending in mortality; 2) levels of replication are moderate and a persistent infection will result with the chicken becoming a carrier; and 3) the replication does not outpace the immune system’s ability to clear the infection and the chicken recovers (Barrow et al., 1990; Zhang-Barber et al., 1999; Chappell et al., 2009).

In poultry, *Salmonella* Gallinarum and *Salmonella* Pullorum produce severe systemic disease with limited faecal excretion (Barrow, 2007), so the risk of carcass contamination at slaughter is minimal and does not cause food poisoning in humans (Zhang-Barber et al., 1999). These host specific serotypes can be vaccinated against in poultry. However *Salmonella* Enteritidis and *Salmonella* Typhimurium, both host non-specific serotypes, can cause transient infections in
poultry which colonise the gastrointestinal tract of the chicken and enter the human food chain potentially causing food poisoning (Zhang-Barber et al., 1999). The main routes of human infection are from the ingestion of meat from colonised or contaminated carcasses or eggs infected from colonised ovaries or by Salmonella-contaminated faeces during laying (Barrow et al., 1990).

Salmonella Enteritidis is the most commonly found serovar in poultry and can cause systemic infections in young chicks and laying hens accompanied by prolonged faecal shedding (Suzuki, 1994; Zhang-Barber et al., 1999; Barrow, 2000). The typical route of poultry infection is when Salmonella is ingested by the chicken. It then passes through the acidic environment of the crop, where the acid adaptation mechanisms are initiated that help the Salmonella survive the subsequent passage through the proventriculus, the gizzard and eventually the gastrointestinal tract (Chappell et al., 2009). During the initial infection symptoms can include depression, anorexia, diarrhoea, and reduced egg production in layers and can lead to high mortality in young chicks of up to 21% (Gorham et al., 1991; Suzuki, 1994). If the chicken is unable to clear the infection it will become a persistent carrier of the organism and expose the rest of the flock to the infection.

After the initial infection, S. Enteritidis will colonise the large blind ceca, which are the largest secondary lymphoid organs in chickens, which branch off the distal ileum just before the colon (Chappell et al., 2009). Once the caeca are colonised in the chicks that survive, they will constantly shed S. Enteritidis in the faecal matter (Suzuki, 1994). Upon dissection these persistent carriers of S. Enteritidis will have the organism recovered from the heart, liver, spleen, caeca, yolk sac, ovary, oviduct, peritoneum, eggs and intestines (Suzuki, 1994). In older layers that have had a sub-clinical infection there are often deformed, shrunken, discoloured and/or congested ovaries and ovules with malformed follicles (Suzuki, 1994). These dissections occurred at typical market
age at 42 days showing that the risk to humans of ingesting contaminated meats is quite high if the carcasses are not examined carefully during slaughter (Gorham et al., 1991; Suzuki, 1994). Through the colonisation of the oviduct, which is dictated by the induction of multiple genes on the virulence plasmid, *S. enterica* can directly contaminate eggs through the shell and can be found in both the yolk and the albumen (Gantois et al., 2008).

Ingestion of *Salmonella*-contaminated materials is the key cause of these infections and often it is the ingestions of their own faecal matter by chickens that leads to infection. A study of 74 poultry farms over the course of a year found that *S. enterica* serovar Enteritidis was the only persistent serotype found in flocks and that environmental contamination with this serovar increased significantly over time (Wales et al., 2007). Interestingly, all of the flocks were vaccinated against *Salmonella* with the majority being vaccinated using either a live *S. Enteritidis* vaccine with or without a live *Salmonella Typhimurium* component, an inactivated *S. Enteritidis* vaccine with *Salmonella Typhimurium* or a live *Salmonella Gallinarum* that provided cross-protection against *S. Enteritidis*. They had also been administered with an oral competitive exclusion treatment, however these flocks still showed signs of being persistent carriers (Wales et al., 2007). Wildlife vectors such as rodents and flies (Mian et al., 2002; Garber et al., 2003) had also introduced *Salmonella* into poultry farms and were found to be an indicator of the *Salmonella* status of the chickens. During sampling, when the rodent faecal matter and the flies tested negative for the presence of *Salmonella*, the flock tested negative as well and the reverse was found that if the result was positive, the flock would also be positive (Wales et al., 2007).

The reduction of *Salmonella*-colonisation in the poultry industry is a major objective although the costly and time-consuming measures are not often embraced by the industry. Control and reduction of *Salmonella* in flocks can be achieved through sophisticated housing measures, constant surveillance on feed quality, strict and regular hygiene management and administration
of competitive exclusion and vaccine measures in chicks (Zhang-Barber et al., 1999). As the poultry industry often uses high density housing and have a high output, it has been found that continuously used housing which have multi-aged flocks living in them were heavily contaminated with *Salmonella* (Wales et al., 2007). A modification that has the potential to limit the accumulation of *Salmonella* in this scenario is to organise a break in the rearing of the chicks in the henhouse to allow proper decontamination of the cages and the associated fixtures. Regular and thorough cleaning is of the utmost importance to reduce horizontal transmission within flocks. The physical removal of the organic matter, the thorough and regular application of disinfectants, strict rodent control and constant surveillance of risk factors such as continuous use of henhouses and older flocks all contribute to reduction of carriage rates (Wales et al., 2007).

The second modification that would reduce the proliferation of *Salmonella* is changing the density of which the chickens are housed and the housing itself. Chickens are sensitive to change and stresses can often make the infection worse such stresses include vaccinations, transfer of birds, cold wet weather and rearing at a high density (Suzuki, 1994; Zhang-Barber et al., 1999). If the poultry farms made their henhouses more insulated and reared at a lower density, there could be a reduction in prevalence of *Salmonella*. A distinct seasonal pattern for environmental contamination is seen quite clearly among the free range flocks than the caged flocks, supporting the fact that stress of cold weather and wildlife vectors can cause an increase in *Salmonella* excretion (Wales et al., 2007). However these modifications are costly and rearing at a lower density directly affects the poultry farm’s ability to earn income.

As poultry is consumed on such a regular basis, most countries have taken steps to regulate the poultry industry to minimise health impacts on the public. In Australia, this began in the 1990s when changes were made to the regulation of poultry hygiene, with Australian Standards and meat processors who were required to operate a Hazard Analysis and Critical Control Points
(HACCP) program introduced (Sumner et al., 2004). Since the implementation of these standards the microbiological status of poultry has improved with human pathogens such as *S. Typhimurium*, along with non-pathogenic strains such as *S. Sofia*, being isolated less often in meat samples (Sumner et al., 2004). The only controversy with these changes is that they were made to reduce the incidence of salmonellosis in the human population; however statistics in the decade after the introduction indicated the numbers of infections have remained stable over the last decade rarely changing from 5000—6000 cases per annum (Baker et al., 1998; Sumner et al., 2004). This can be attributed to three possible causes – 1) the failure of retail food suppliers to follow health regulations whilst serving patrons, 2) inadequate handling and storage of food products in the home and 3) non-food sources of contamination such as contaminated water and contact with pets/farm animals faecal matter (Sumner et al., 2004). Improvement in clinical diagnosis and reporting may also contribute to the consistent annual trend.

In the European Union (EU), in order to regulate their poultry industry, legislation was introduced at the beginning of 2009 with the aim to reduce the prevalence of *S. Enteritidis* and *S. Typhimurium* to 1% or less in all broiler and layer flocks by the end of 2011 (Cooper, 2008). In the UK, the current overall rate is 8% whilst in other parts of the EU the overall *Salmonella* prevalence can range from 0% in Sweden to 68% in Hungary with an average of 24% with the EU (Cooper, 2008). In their most recent report, the European Food Safety Authority have advised that even with the preceding recommendations 15 member states have already been able to get their *Salmonella* prevalence to below the reduction target of 1% laid down by the new EU legislation (EFSA, 2009).
1.7.2 *Clostridium perfringens*, gas gangrene and necrotic enteritis

1.7.2.1 *Clostridium perfringens* and the alpha toxin

The genus *Clostridium* contains bacteria that are Gram-positive, obligately anaerobic, spore-forming bacilli (Hatheway, 1990) and the first clostridia species, *Clostridium butyricum*, was identified by Louis Pasteur in 1861 (Rekha and Gopalan, 2007). They can be differentiated from *Bacillus* species by their lack of catalase activity and are ubiquitously found in soil, sewage, decaying biological matter and the intestinal tracts of humans and other animals (Smith and Gardner, 1949; Hatheway, 1990). Of this genus the most omnipresent is *Clostridium perfringens* which has previously been known as *Bacillus aerogenes capsulatus* and *C. welchii* after one of its discoverers, William H. Welch (Hatheway, 1990). *Clostridium perfringens* produces five different types of major toxins (A-E) of which the alpha (α), beta (β), epsilon (ε) toxins are prevalent (McDonel, 1980). Each strain of *C. perfringens* causes different disease as they express differing toxins in differing quantities, with Type A strains producing large amounts of alpha toxin whilst lacking expression of the other major toxins (Petit et al., 1999). Type A strains of *C. perfringens* are often responsible for causing gas gangrene and food borne illnesses in humans and livestock (Hatheway, 1990).

Characterisation of the alpha toxin showed that it was a phospholipase C which was the first time that a toxin had been shown to be enzymatic (Macfarlane and Knight, 1941). Alpha toxin is a zinc-metallo-phospholipase which has two domains, the first amino-terminus domain (N-domain) composed of α-helices and the carboxyl-domain (C-domain) composed of β sheets (Naylor et al., 1998). Alpha toxin is a potent toxin with a MLD <0.1 µg/mouse (Naylor et al., 1998) which is attributed to the fact that alpha toxin can hydrolyse phospholipids and lyses erythrocytes from various species (Titball et al., 1999). Crystallographic analysis of the alpha toxin has elucidated that the putative active site is situated within a cleft that has three essential zinc ions held in
place by the N-terminus tryptophan residue, a glutamic acid residue, two aspartic acid residues and five histidine residues (Figure 1.3) (Naylor et al., 1998).

Gene analysis of the alpha toxin gene, known as plc or cpa, has shown that the gene is highly conserved between isolates from diseased chickens. Despite that conservation, there are 28 different published types of alpha toxin genes and studies have shown that there is no correlation between PFGE type, plc sequence type, health status of the host chickens and level of alpha toxin production (Abildgaard et al., 2009). The differences between these sequences were shown to be just single base substitutions resulting in synonymous changes that did not significantly alter the encoded proteins (Sheedy et al., 2004; Abildgaard et al., 2009).

Of the C. perfringens strains that can induce lesions, the production of alpha toxin is quite variable and similar levels can be produced by normal gut isolates that do not cause disease making it likely that certain C. perfringens strains engage in host-specific interactions that lead to disease (Timbermont et al., 2009). Effects on mammalian cells has shown that at high concentrations, alpha toxin leads to the disruption of the plasma membrane whilst at low concentrations it disrupts phospholipid metabolism and several signal transduction processes such as diacylglycerol generation, protein kinase C activation, calcium mobilisation and activation of arachidonic acid metabolism (Flores-Díaz et al., 2004). All of these effects go on to cause inhibition of neutrophils in infected tissues and can cause thrombotic events that enhance the anaerobic conditions that C. perfringens thrive in (Flores-Díaz et al., 2004).

1.7.2.2 Gas gangrene in humans to necrotic enteritis in poultry

Gas gangrene is an acute and critical infection caused by clostridial species, C. perfringens being the most frequently isolated from gangrenous wounds (Flores-Díaz and Alape-Giron, 2003). Observations of this disease can be found in the oldest editions of our modern journals with one
dating back as far as 1914 where it was noted that gas gangrene was caused by wound contamination with “street mud” (Dudgeon, 1914). Another early report of gas gangrene came from army doctors who had experience with shrapnel wounds in the First World War (Bowlby and Rowlands, 1914). Bowlby and Rowlands isolated a bacillus from such wounds and found that when a guinea pig was inoculated with it, gangrenous cellulitis occurred. They also noted that of
Figure 1.3: Tertiary structure and mode of action of alpha toxin.

A) Protein model of alpha toxin showing the two domains. B) Mode of membrane destruction by alpha toxin. Figure taken from Sakurai et al. (2004).
all the organisms isolated from these wounds were anaerobic spore-bearers. The development of the disease was observed to appear in a few days or even a few h depending on the severity of the injury sustained by the soldier. Symptoms included severe pain caused by swelling, discolouration of the skin to green then black (Figure 1.4), an overpowering smell coming from the offending limb and death due to cardiac failure (Bowlby and Rowlands, 1914; Stevens, 2000). Typical incubation times for gas gangrene is less than 24 h after trauma but extremes such as 1 hour to 6 weeks have been reported in civilian cases (Rekha and Gopalan, 2007). The description of this disease has altered very little over the course of the last 95 years, and unfortunately neither has the treatment.

Treatment of gas gangrene during the First World War included making incisions into the affected areas, flushing them with hydrogen peroxide and removing any contaminating materials in the wound, and as a last resort, amputation, which was the most successful treatment (Bowlby and Rowlands, 1914). Currently, treatment consists of aggressive use of antibiotics, most often penicillin and clindamycin in combination (Brook, 2008); debridement surgery where longitudinal incisions are made throughout the deep fascia to assist with drainage (Schwartz, 1978; Brook, 2008) leading to surgical amputation in 15% to 20% of cases (Rekha and Gopalan, 2007); and if diagnosed soon enough hyperbaric oxygen therapy (Mortensen, 2008). However as the necrosis can spread at a speed of up to 15 cm/h, hyperbaric oxygen therapy is a rather controversial treatment (Mortensen, 2008). It has been known that well perfused tissue is resistant to infection and can help inhibit the spread (Schwartz, 1978; Mortensen, 2008) and that the thousand-fold increase in oxygen saturation in the infected tissue leads to a bactericidal effect and improved wound healing (Brook, 2008). However to date there has been no controlled studies of the treatment leading to much speculation that it is not a suitable treatment for such a rapidly developing disease (Brook, 2008). One of the most promising aspects of this treatment is that it
Figure 1.4: Patient with gas gangrene affecting her left leg after a laceration to her foot.

Characteristic darkening of the skin during gas gangrene with swelling apparent around the ankle. Figure from Rekha and Goplan (2007).
can inhibit formation of toxins and at a pO$_2$ of 250 mmHg, there is no production of toxins by the
_C. perfringens_ (Mortensen, 2008) which may limit cellular damage and prevent shock.

The relationship between the toxins that _C. perfringens_ express and the progression of gas
gangrene has not been fully elucidated and early treatments found that antitoxin treatments
were ineffective as a prophylaxis (Schwartz, 1978). Toxin filtrates of _C. perfringens_ were tested to
see if signs of gangrene could be induced and it was observed that the filtrates could alter the
epinephrine-sensitivity in the blood vessels and inhibit phagocytosis at the site of infection, a
unique characteristic of gas gangrene (Ganley _et al._, 1955). As alpha toxin is the predominant
toxin produced by _C. perfringens_ Type A strains (Petit _et al._, 1999), it was a natural progression to
explore said toxin as a vaccine candidate against gas gangrene especially as it exhibits myotoxic
activity, induces platelet aggregation, affects leukocyte proliferation and leads to a rapid
reduction in arterial pressure (Stevens, 2000; Flores-Diaz and Alape-Giron, 2003). Later, alpha
toxin was purified in recombinant form and shown to induce myonecrosis and many
histopathological signs of gas gangrene when injected intramuscularly in mice (Bunting _et al._,
1997). The C-terminus domain of the alpha toxin was found to be an efficacious vaccine against
challenge with lethal doses of either alpha toxin or _C. perfringens_ in a gas gangrene mouse model
(Williamson and Titball, 1993). These studies indicated that the alpha toxin was a virulence factor
in the development of gas gangrene (Williamson and Titball, 1993; Bunting _et al._, 1997). Recently,
it was reported that the _C. perfringens_ alpha toxin was able to stimulate gas gangrene when
expressed in a _C. septicum_ background further confirming that it is an important virulence factor
in gas gangrene (Kennedy _et al._, 2009).

Another important disease caused by _C. perfringens_ Type A, and occasionally Type C, is necrotic
enteritis which occurs in poultry and other avian species worldwide (Parish, 1961; Songer, 1996).
The disease is characterised by a sudden increase in flock mortality without any clear indications
of illness, however development of wet litter can sometimes be an early warning sign. At necropsy, the small intestinal mucosa will have large necrotic lesions (Figure 1.5a) and in the severest cases the whole mucosa will show extensive necrosis of the lumen (Figure 1.5b) (Parish, 1961; Van Immerseel et al., 2009). There are two forms of the disease, clinical and sub-clinical, where in both there is chronic damage to the mucosa affecting weight gain and feed-conversion ratios (Kaldhusdal et al., 2001). Sub-clinical disease is very common and occurs in flocks with neither substantially increased mortality nor an increased number of ill birds. Sub-clinical disease is only detected upon discovery of lesions on either the intestines or liver which condemns the carcass at slaughter and can increase condemnations from 0.5% of the flock to 1.5% which translates to a large loss in production value (Kaldhusdal and Lovland, 2000). As the global poultry industry is estimated to be greater than US$2 billion annually (Collier et al., 2008) this production loss can translate into millions of dollars in loss of revenue every year. It has also been associated with increased feed-conversion ratio and retarded growth rate (Lovland and Kaldhusdal, 2001).

Necrotic enteritis reached epidemic proportions in Scandinavia in 1995 when the proportion of affected flocks rose to between 25–40% after the withdrawal of antimicrobial growth promoters from feed (Kaldhusdal and Lovland, 2000).

Although the pathogenesis of necrotic enteritis is not fully elucidated, it is known that toxigenic strains of *C. perfringens* displace the normal non-necrotic enteritis strains in the gut of broiler chickens and actively inhibit their re-establishment by producing substances such as bacteriocins (Barbara et al., 2008). It has been found that competitive exclusion against *C. perfringens* can reduce mortality. One study showed that giving affected birds doses of lactic-acid producing bacterial strains (*Lactobacillus acidophilus, Enterococcus faecium, Lactobacillus plantarum and Pediococcus acidilactici*) lowered mortality from 60% to 30% (Hofacre et al., 2003) and also reduced colonisation rates of *S. Typhimurium* (van der Sluis, 2003).
Figure 1.5: Necrotic lesions of the lumen in broilers suffering from severe necrotic enteritis.

A) Patches of lesions throughout the intestine; B) Severe extensive necrosis of the lumen surface (picture from Van Immerseel et al., 2009).
Experimentally, necrotic enteritis has been very difficult to reproduce as high pre-experiment \textit{C. perfringens} colonisation of commercial birds and spontaneous disease interferes with the validity of the results obtained (Kaldhusdal \textit{et al.}, 1999). Having high numbers of \textit{C. perfringens} in the broiler chickens gut is not sufficient to induce necrotic enteritis even when the broiler has predisposing factors such as mucosal damage caused by coccidial pathogens (Collier \textit{et al.}, 2008) and a diet high in protein (Timbermont \textit{et al.}, 2009). It has also been found that strains isolated from broilers suffering from necrotic enteritis have different virulence factor that may be required to induce the disease whereas strains that come from healthy broilers lack them although they have not all been characterised (Engstrom \textit{et al.}, 2003; Timbermont \textit{et al.}, 2009). Contradicting these reports, a study by Gholamiandekhordi and colleagues (2006) discovered that production of alpha toxin \textit{in vitro} does not show a positive correlation with the disease status of the flock (Gholamiandekhordi \textit{et al.}, 2006).

\subsection*{1.7.2.3 Virulence factors in necrotic enteritis}

Whilst the role of alpha toxin has been accepted as an important virulence factor in gas gangrene, its role in necrotic enteritis is still being debated. Throughout the literature, there are many contrasting studies that have reached differing views on how to approach necrotic enteritis. The three main hypotheses will be examined here. The first hypothesis is that alpha toxin is involved in pathogenesis of necrotic enteritis and should not be discounted as a vaccine candidate. The second is that alpha toxin is not essential to the development of necrotic enteritis and that there are other more efficacious immunogens that should be investigated. Finally the third is just emerging and is based on the idea that there may not be just one main factor in necrotic enteritis but multiple ones acting synergistically. As a result of this continuing debate all groups that are looking into a successful vaccine agree that necrotic enteritis is a multifactorial disease which has
increased in areas where antimicrobial growth promoters have been banned from use (Van Immerseel et al., 2009).

One of the first studies that linked alpha toxin to necrotic enteritis showed that intestinal lesions could be induced by administrating *C. perfringens* culture supernatant (Al-Sheikhly and Truscott, 1977), however the supernatant was not pure alpha toxin hence the relationship could not be definitively established. In a later report, germ-free chickens inoculated with a dose of purified alpha toxin died and upon necropsy, intestinal damage was observed that was consistent with necrotic enteritis (Fukata et al., 1988). These two articles appear to have laid the foundation of the hypothesis that alpha toxin was a virulence factor involved in intestinal damage of necrotic enteritis.

Consequently, researchers focused on collecting isolates from chickens suffering from necrotic enteritis to show that alpha toxin was essential to the disease. Upon characterisation of isolates taken from healthy flocks and those affected by necrotic enteritis, one study reported that “alpha toxin was produced in significantly larger amounts by isolates from birds with necrotising enteritis than by isolates from birds without the disease, regardless of bird species” (Hofshagen and Stenwig, 1992). This claim supported the theory that alpha toxin was involved in the pathogenesis and many subsequent reports demonstrated success in using the toxin as a vaccine. Immunisation with alpha toxoid followed with boosting with active toxin offered the best protection and induced epitope-specific neutralising antibodies (Kulkarni et al., 2007). Recombinant alpha toxin has also been reported as generating a strong IgG response when administrated subcutaneously which gave partial protection from whole organism challenge with *C. perfringens* (Cooper et al., 2009).
One of the most recent articles to support the argument that alpha toxin should not be dismissed as a virulence factor reported that protective responses against necrotic enteritis was induced using a *Salmonella* vaccine expressing the carboxyl-terminus domain of the alpha toxin (Zekarias *et al.*, 2008). Suppression of active growth of *C. perfringens* in broth culture by the serum acquired from these vaccinated chickens indicated that alpha toxin may be retained on the cell wall which reiterates that it is an important immunogen in this disease. This group found that their vaccine when administered orally induced toxin-neutralising antibodies, reduced lesion development and increased weight gain in the broiler chickens challenged with *C. perfringens* (Zekarias *et al.*, 2008). Delivery of the alpha toxin carboxyl-terminus domain using *Bacillus subtilis* endospores administered orally or nasally has also induced protective immune responses against alpha toxin challenge (Hoang *et al.*, 2008).

So why is there still debate over the role of alpha toxin? Firstly, in immunisation studies, a naturally occurring mutant strain of *C. perfringens* which expressed a non-toxic alpha toxin variant, lacking enzymatic, haemolytic and lethal activity, induced protective immune responses against wild-type alpha toxin challenge in a murine model (Schoepe *et al.*, 2006). Another study reported that two out of four avirulent alpha toxin deficient mutants were able to protect birds against experimental necrotic enteritis which showed that there are other immunogens important to pathogenesis (Thompson *et al.*, 2006). Finally a virulent alpha toxin mutant was shown to produce necrotic enteritis in an experimental chicken model (Keyburn *et al.*, 2006). All these studies further supported the idea that alpha toxin may not be the sole virulence factor because if it was crucial to pathogenesis, these particular mutant strains would not have been able to confer immunity. Furthermore, as stated previously, in contradiction to Hofshagen and Shenwig’s study from 1992, a group reported that they demonstrated no apparent correlation between the disease status of the flock and the amount of alpha toxin produced by the isolates as
in vitro expression of alpha toxin did not vary between normal flora isolates and disease isolates (Gholamiandekhordi et al., 2006).

Keyburn and colleagues focused on other immunogens when they could not reproduce necrotic enteritis using other Type A strains of C. perfringens even though they also expressed alpha toxin as well (Keyburn et al., 2006). The most recently reported toxin that has been identified by this group is NetB which is a pore-forming toxin that displayed cytotoxic activity in tissue culture assays and has been found in C. perfringens type A strains isolated from chickens suffering from necrotic enteritis (Keyburn et al., 2008). When a NetB mutant was tested for virulence it was unable to produce necrotic enteritis in an experimental model, however, during classification of the isolates from necrotic enteritis infected chickens four were found to be NetB negative yet were still able to cause necrotic enteritis in the experimental model (Keyburn et al., 2008) showing that like alpha toxin, NetB may not be an essential nor critical virulence factor. Furthermore, when non-Australian C. perfringens isolates were tested for the NetB gene, it was present in both strains isolated from healthy chickens and those suffering from necrotic enteritis (Martin and Smyth, 2009). There were also strains recovered directly from lesions in affected birds that were NetB negative (Martin and Smyth, 2009) adding to the evidence against NetB as an essential virulence factor.

For more than 20 years, the majority of necrotic enteritis research has focused on the alpha toxin. Recently, there have been reports of other immunogens that have shown potential as vaccine candidates. Intramuscular immunisation with other C. perfringens secreted proteins such as pyruvate:ferredoxin oxidoreductase (PFOR), glyceraldehyde-3-phosphate dehydrogenase, fructose 1,6-biphosphate aldolase (FBA) and hypothetical protein (HP) all conferred protection to mild challenge whilst FBA and HP protected against a moderate challenge (Kulkarni et al., 2007) These results suggest that there may be a synergistic relationship between multiple secreted
proteins in the pathogenesis of necrotic enteritis. Subsequently, these secreted proteins were also tested in *Salmonella* vectored vaccines and shown to induce significant protective immune responses (Kulkarni *et al.*, 2008). Metalloproteinases and collagenolytic enzymes are soon to be investigated as vaccine candidates as it is proposed that they may have a major role in early lesion development (Olkowski *et al.*, 2008).

1.8 *Salmonella* vaccination in livestock

1.8.1 Current strategies to protect against *Salmonella*

Vaccination is the simplest way to reduce bacterial carriage rates in animals. In livestock this is of particular importance as many of them become food products and have the potential to elicit food poisoning in humans. In other cases, infections by *Salmonella* can provoke unnecessary complications in rearing and farming. In swine, *Salmonella enterica* serovar Choleraesuis is a highly invasive, host-adapted pathogen that lead to sepsis, pneumonia, enterocolitis and hepatitis resulting in significant economic losses in the swine industry (Ku *et al.*, 2005). In rare cases, it can also result in intestinal infections and infective aneurysms in humans after ingestion of contaminated products (Chiu *et al.*, 2005). This strain has become a focus of researchers due to its increasing antibiotic resistance to conventional livestock prophylaxis attributed to its many pseudogenes which complement lateral gene acquisition for improved virulence and broader antibiotic resistance (Chiu *et al.*, 2005). Homologous protection against *Salmonella enterica* serovar Choleraesuis in swine has been tested using *S. Choleraesuis* *phoP* and *rpoS* mutants. This study showed that the *S. Choleraesuis* mutants were successful at proliferating within the primary intestinal macrophages, were able to competitively exclude the wild type strain of *S. Choleraesuis* from the pigs and induced good humoral immune responses (Domínguez-Bernal *et al.*, 2008). Recently the same group has shown that the *S. Choleraesuis* *phoP* and *rpoS* mutants are
successful at delivering DNA vaccines using a model epitope to the intestinal immune system in mice (Bartolomé et al., 2010).

Although the primary product derived from sheep is wool, not food products, vaccination against specific strains of *Salmonella* serovars are currently being investigated. One such strain is *Salmonella enterica* subspecies *enterica* serovar Abortusovis, an ovine-restricted serovar which causes contagious late-term abortions in ewes mainly in Europe and Western Asia (Cagiola et al., 2007). Killed vaccines have been effective against this disease (Pardon et al., 1990) and most affected ewes usually abort once then mount a successful immune response against the disease, which may be linked to a IFN-γ mediated immune response (Cagiola et al., 2007). Use of a live attenuated *S. Typhimurium* as a single dose vaccination reduced the abortion rate in ewes from 33.4% in the unvaccinated to ≤ 0.1% in over 100,000 deliveries when compared to inactivated vaccines (Linde et al., 1992).

Cattle are another livestock that require vaccination against *Salmonella* species. Persistent colonisation with *Salmonella* species in calves decreases their appetite resulting in lower feed: weight ratios and higher costs for the farmers (Dueger et al., 2003). *Salmonella enterica* serovar Typhimurium lacking DNA adenine methylase has been used to induce both early and late protective responses in calves to protect them from becoming carriers (Dueger et al., 2003). Cross-protection against *S. enterica* Dublin has also been shown (Mohler et al., 2006) as well as *S. enterica* serovar Newport which is important as it has become multi-drug resistant and is an emerging relevant pathogen in cattle (Mohler et al., 2008).

Poultry is one of the biggest reservoirs of *Salmonella* (Zhang-Barber et al., 1999) and reducing carriage rates has been an ongoing objective. Over the past decade the UK has seen a large decline in salmonellosis outbreaks since the implementation of the Lion Code Egg scheme which
requires compulsory vaccination against S. Enteritis, strict quality control and independent auditing of its eggs (reviewed by (Gormley et al., 2011)). Successful vaccination has been achieved using both killed and live *Salmonella* vaccines. The existing commercial live *Salmonella* vaccines have exhibited an ability to induce significant CD8+ T cell and IgA responses that resulted in almost no *Salmonella* being detected in the caecum where they normally colonise (Carvajal et al., 2008). In one study the use of a live attenuated *Salmonella* vaccine resulted in more effective clearance of S. Enteritidis in chickens than the killed vaccine. This was attributed to an increased cell-mediated response which lead to reduced shedding of the organism and stronger responses upon re-exposure (Babu et al., 2004). A recent study used a bivalent killed vaccine in laying hens which significantly reduced vertical egg transmission of *S. enterica* serovars Enteritidis, Typhimurium and Gallinarum biovar Pullorum (Okamura et al., 2007). The evolution of live vaccines continues and is now focusing on making them multivalent by delivering heterologous antigens.

### 1.8.2 Heterologous antigen presentation via *Salmonella* vectors

Pioneer studies into heterologous antigen presentation using *Salmonella* species as carriers were conducted in the early 1980s by Formal and colleagues. The first used the existing attenuated typhoid vaccine, *Salmonella typhi* Ty21a to present the form I antigen from *Shigella sonnei* which conferred protection against both infections when tested in mice (Formal et al., 1981). This system where the heterologous antigen is actually expressed by the vector demonstrated to be an effective system. Upon further investigation *Salmonella* was validated as an obvious choice in regards to delivering heterologous antigens ensuing from its ability to transfer expression plasmids to host cells even though the bacteria were unable to escape the phagocytic vacuole (Darji et al., 1997). This is the basis of most modern day DNA vaccines that are not expressed but just carried by the *Salmonella* vector. *Salmonella* can also persist in dendritic cells and are usually...
sampled directly from the intestinal lumen by dendritic cells after ingestion (Rescigno et al., 2001) indicating heterologous antigens delivered orally would be presented to the immune system earlier.

Most of the attenuated strains used in early vaccine research were auxotrophic strains in which essential genes for synthesis of purine (pur), aromatic acids (aro) or guanidine (gua) had been deleted or mutated. These mutations gave the desired attenuation of the strain whilst maintaining its immunogenicity. Early research was most promising, for example, the administration of an attenuated aroA mutant Salmonella Typhimurium carrying an eukaryotic expression plasmid encoding the listerial protein, listeriolysin, was given orally and strong cytotoxic and helper T cell production were elicited after the first dose (Darji et al., 2000). Further investigation demonstrated that the heterologous antigen delivered could be detected in Peyer’s patches and the spleen several days after the vaccination with the attenuated Salmonella even though the expression plasmid was lost soon after administration. It was also evident that although this loss occurred, mice that were immunised four times orally were protected against a lethal challenge of Listeria monocytogenes (Darji et al., 2000). Although the transference of the plasmid to the host nucleus occurs very early on, stabilisation of the plasmid in vitro and in vivo is still one critical criteria of a successful live carrier and this can be achieved by using low copy number plasmids in the Salmonella vector (Loessner et al., 2008). Another application that has been applied to Salmonella vaccines is to have the expression vector as a single copy in vitro but upon administration becomes a multicopy plasmid. This has been achieved by having two promoters in the plasmid, trfA and sifA, but only sifA is activated upon invasion of the cell and is able to dictate both expression and plasmid amplification (Loessner et al., 2008).

The strength of heterologous antigen expression determines the efficacy of a successful live carrier vaccine. However as protein expression places a burden on the metabolic status of the
carrier, this can lead to a loss of virulence and reduced viability, which decreases the immune response (Loessner et al., 2008). Early investigations into using the Salmonella Type I (ATP-binding cassette) secretion system to express heterologous antigens demonstrated that it had a low secretion capability, complicated genetic regulation and structural limitations of the secreted antigen, which all hinder in vivo delivery and uptake affecting immunogenicity of the antigen hence it is unsuitable for heterologous antigen expression (Hahn and von Specht, 2003). Salmonella pathogenicity islands are of importance in terms of carrier vaccines as their Type III secretion system can be exploited to reduce metabolic burden of the heterologous antigen and deliver it to the host cells (Cheminay and Hensel, 2008). The Salmonella Type III system has been demonstrated to deliver heterologous antigens to the host-cell cytosol which results in MHC class I-restricted immune responses which is useful against viral infections (Rüssmann et al., 1998).

Another aspect of using a carrier to deliver an antigen is how antigen accumulates in the cell as the magnitude of the immune response is affected by inefficient presentation to the immune system. Hence, many studies have shown that when an antigen is successfully secreted extracellularly the immune response to the antigen can be as much as $10^4$ times higher than when the antigen is accumulated in the cytoplasm of the carrier bacterial cell (Boyle et al., 1997; Kang and Curtiss, 2003). In one study, the expressed amount of recombinant Streptococcus pneumoniae PspA was similar in both Salmonella vaccines despite one being localised in the cytoplasm and the other being secreted (Kang and Curtiss, 2003). Another study which used an aro-attenuated Salmonella vaccines as a vector successfully protected against plague by expressing the V antigen from Yersinia pestis (Garmory et al., 2003). It has also been found that specific immune responses triggered against the heterologous antigen are often masked by the dominant immune response to the Salmonella vector antigens (Hahn and von Specht, 2003). Another limitation of experimental infection trials is the use of a large challenge dose, often $10^9$ CFU, which may not reflect the normal conditions under which an animal would be exposed to Salmonella over the
course of its lifetime (Zhang-Barber et al., 1999). This means that some poultry vaccines are
deemed failures even though they may protect against low infectious doses which is typical in
horizontal spread of \textit{Salmonella} in flocks.

There have been studies published that have utilised heterologous antigen presentation from a
live \textit{Salmonella} vector with the final application to be applied to livestock. Anthrax in cattle is one
such target. One study showed that using a vector expressing \textit{Bacillus anthracis} protective
antigen protected mice against aerosolised \textit{B. anthracis} spores when they were vaccinated orally
(Stokes et al., 2007) and has previously protected against subcutaneously delivered spore
challenge (Coulson et al., 1994). Using \textit{Salmonella} as a delivery system for DNA vaccines has also
proven successful against viral infections in chickens. Delivery of a hemagglutinin DNA vaccine
against H9 avian influenza virus was administered to specific pathogen free (SPF) chickens
followed by a killed avian influenza vaccine, this combination provided complete protection from
intranasal H9N2 challenge (Pan et al., 2009). It is thought that the stabilised mucosal DNA vaccine
coupled with a boost by a conventional killed vaccine provided enough stimulation for both a
strong and persistent humoral and cellular immune response (Pan et al., 2009).

As examined earlier, selected \textit{C. perfringens} secreted proteins as well as alpha toxin have been
delivered in \textit{Salmonella} vectored vaccines (Kulkarni et al., 2008; Zekarias et al., 2008). Both
studies used an attenuated Δ\textit{pabA} and Δ\textit{pabB} \textit{Salmonella Typhimurium} with a chromosomal
deletion of the aspartate B-semi-aldehyde dehydrogenase \textit{asd} which is complemented with a
\textit{asd}+ plasmid which ensures that the plasmid is steadily maintained and expression upheld when
induced (Kulkarni et al., 2008). The first assessed the immunogenicity of three immunogens
(PFOR, HP and FBA) each secreted under a \textit{lacI} promoter (Kulkarni et al., 2008) whilst the second
used the carboxyl-terminus domain of the alpha toxin under various promoters (Zekarias et al.,
2008). Both studies achieved partial protection against challenge with \textit{C. perfringens} and good
levels of specific antibodies against *Salmonella* antigens (Kulkarni *et al.*, 2008; Zekarias *et al.*, 2008). Both of these groups suggest that better expression of foreign antigens from the selected *Salmonella* vectors will lead to better induction of immune responses.

1.9 Concluding remarks

Vaccine development has progressed dramatically over the past couple of hundred years, from initial variolation techniques (Dinc and Ulman, 2007) to the latest DNA vaccination methods (Chang *et al.*, 2007). There have been many successes such as the smallpox vaccines (Stewart and Devlin, 2006) and the recent HPV vaccines (Govan, 2008). The pursuit of an efficacious vaccine to induce protection against multiple diseases would be of enormous value to the poultry industry. Many studies have shown that using attenuated vectors to deliver heterologous antigens will induce significant immune responses to both the vector and to the heterologous antigen (Darji *et al.*, 2000; Bachtiar *et al.*, 2003; Garmory *et al.*, 2003; Cheminay and Hensel, 2008; Kulkarni *et al.*, 2008; Zekarias *et al.*, 2008). Over the past decade, even with many conflicting reports about alpha toxin as an essential virulence factor, there has been progress in the elucidation of the pathogenesis and aetiology of necrotic enteritis. By utilising the best vector technologies and by selecting immunogenic antigens, necrotic enteritis could be eliminated from the poultry industry globally saving millions of dollars in product and improving the lives of poultry.

1.10 Rationale of the project

Live attenuated *Salmonella* vaccines are already established in the field of modern medicine for both human and veterinary use. As they are usually delivered in the same manner in which a natural infection would occur, the subsequent immune responses are often protective and targeted at specific points of the natural infection. In order to take advantage of this immune
targeting, an established *Salmonella* Typhimurium vaccine (STM1) which has been used in the livestock industry to prevent salmonellosis in poultry (Coloe *et al.*, 1995) was chosen as the carrier vector. Previously, STM1 was demonstrated to have the ability to express a model heterologous antigen, ovalbumin. Upon vaccination in mice, the dendritic cells not only up took the STM1 but was able to stimulate CD8+ cells specific to the dominant ovalbumin epitope, SIINFEKL and mucosal IgA responses (Bachtar *et al.*, 2003). It is these characteristics that have lead to STM1 being used in the current study. For the heterologous antigen, the alpha toxin from *C. perfringens* was chosen as it had been shown to be a stable immunogenic antigen when purified fragments had been used to vaccinate mice against gas gangrene (Williamson and Titball, 1993). Pre-existing immunity to heterologous and homologous *Salmonella* strains has been reported to not impede the efficiency of this vaccine in mice when delivering a model heterologous antigen, ovalbumin (Saxena *et al.*, 2009).

The objectives of this project focused on creating alpha toxin variants which retain their immunogenicity but lose their toxicity. Several forms of variants were made and tested in various animal trials. The effectiveness of STM1 expressing these variants was evaluated in both mice and chickens. The efficacy of the STM1 expressing variants was also determined when the chickens had prior colonisation with *Salmonella enterica* serovar Sofia, which is Australia’s most commonly isolated commensal *Salmonella* serovar.
2.1 General procedures

All chemicals used were of analytical laboratory reagent grade. All glassware, media and solutions were sterilised by autoclaving at 121°C (15 lbs/in²) for 20 min unless otherwise stated. All solutions were prepared with deionised water obtained from a Millipore Milli-Q® water system (mH₂O) (Liquipure, Melbourne, Australia) excluding media, which was prepared with distilled water (dH₂O). Glassware was washed with Pyroneg detergent (Diversey-Lever, Pty. Ltd., Australia), rinsed twice with tap water, and then twice with deionised water.

Solutions were dispensed using the Finnpipette® digital pipette range (Thermo Labsystems) which included a 0.5-10 μL, 5.0-40 μL, 20-200 μL, 200-1000 μL, 1-5 mL, 5-10 mL digital pipette and a 50-300 μL multi-channel pipette. Volumes of 1.5 mL or less were centrifuged with the Eppendorf microcentrifuge 5415C or 5415D. Greater volumes of up to 50 mL were separated by centrifugation in a Beckman Allegra 21R centrifuge. Volumes of up to 200 mL were separated by centrifugation in a Beckman J2-21 M/E centrifuge. All products of less than 2 g were weighed on a Sartorius analytical top loading balance. Reagents of greater than 2 g were weighed on an ISSCO model 300 top loading balance. Media containing supplements were autoclaved and cooled to 55°C before the addition of the supplement(s). All media was prepared using aseptic technique or in a lamina flow cabinet and stored at 4°C until required.
2.2 General chemicals and equipment

2.2.1 Equipment and reagents

ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit: Perkin-Elmer Corp., USA.
Balances:
   Analytical balance: Sartorius Gottingen, Germany.
   Balance (0.1-500 g): U-Lab, Australia.
BALB/c mice (female): Animal Resource Centre, Canningvale, Western Australia.
Centrifuge tubes:
   1.5 mL Eppendorf centrifuge tubes. Treff AG, Switzerland.
   10 mL centrifuge tubes Sarstedt, Germany.
   50 mL centrifuge tubes Greiner Labortechnik, Germany.
   200 mL autoclavable centrifuge bottles Nalgene Nunc International, USA.
Chromatography paper (0.18 mm thick): Whatman, England.
Cover slips: Mediglass, Australia.
Cryovials (1.8 mL): Nalgene Company, USA.
Dry block heater: Ratek, Australia.

Electrophoresis power supply:
   PowerPac 300 Biorad Laboratories, USA.
   EPS 3000xi BioRad Laboratories, USA.
   EPS600 Amersham Pharmacia LKB, Sweden.
Electrophoresis units - DNA:
   (a) Mini gel (GNA-100) Pharmacia LKB, Sweden.
   Protein:
      (a) Mini Protean III gel system BioRad Laboratories, USA.
Electroporation cuvettes: 0.2 cm cuvettes chilled at -20°C before use, BioRad Laboratories, USA.
Filters: Acrodisc 0.2 μm and 0.45μm, Pall, USA.
Gel Doc image system: BioRad Laboratories, USA.
Geneclean® kit: QBIOgene, USA.
Microplate reader (96-well) MR7000: Dynatech (now Dynex), USA.
Microscopes:
   Light microscope (CH2) Olympus Optical Co., Japan.
   Inverted microscope Olympus, Japan.
CHAPTER 2

Microscope slides: LOMB Scientific Co., Australia.

Petri dishes: BioLab, Australia.

Microtitre plate (96-well, flat bottom): Nunc, Denmark.

Platform shaker: Ratek, Australia.

Needles (19, 21, and 26 gauge): Terumo Pty, Ltd., Australia.

PCR machines:

GeneAmp2400 PCR system PerkinElmer, USA.

PCR Express gradient thermal cycler: Thermohybaid, UK.

PCR Express gradient thermal cycler: Thermohybaid, UK.

Nitrocellulose membrane (Trans-Blot®) 0.2 μm: BioRad Laboratories, USA.

Pulse controller & Gene pulser apparatus: BioRad Laboratories, USA.

Trans-blot electrophoretic transfer cell (mini): BioRad Laboratories, USA.

Syringe (1, 5, 10, 20, and 60 mL): Terumo Pty, Ltd, Australia.

QIAprep® spin miniprep kit: QIAGEN, Australia.

Transilluminator (UV): Novex, Australia.

Waterbath: Ratek, Australia.

2.2.2 Materials

2.2.2.1 General media

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

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

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

Cooked meat medium (CMM): 1.0 g granules of dehydrated CMM (Oxoid, England) were added to bottles and filled to 10 mL with dH₂O.

Sheep blood agar (SBA): CA cooled to 55°C and supplemented with 5% (v/v) defibrinated sheep blood (Oxoid, England).
**Nagler plate**: CA supplemented with 10% egg yolk solution (v/v). The egg yolk was first mixed with saline in a 1:1 ratio and centrifuged at 5,500 x g for 10 min. The supernatant was used for preparation of the plates.

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

### 2.2.2.2 General solutions

**β-mercaptoethanol**: electrophoresis purity (Biorad Laboratories, USA).

**λ-DNA marker**: A solution of 0.1 μg/μL digested λ-DNA was prepared by incubating 20 μg λ-DNA (Promega, Australia), 18 μL Buffer H (Promega, Australia), 10U Pst1 (Promega, Australia) and mH2O up to 180 μL, at 37°C overnight. The digestion was ceased by heating at 65°C for 15 min and twenty microlitres of 11x DNA loading buffer was then added. The marker was stored at -20°C and 10 μL aliquots used on a DNA agarose gel.

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

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

**Ammonium persulphate (APS)**: 10% (w/v) ammonium persulphate (Sigma-Aldrich, Germany) stored at 4°C.

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

**BCIP/NBT solution for detection of alkaline phosphatase antibodies**: 0.48 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 0.51 mM nitro blue tetrazolium (NBT) (Amresco, USA).

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

**Blocking solution (ELISA)**: 5% skim milk in PBST.
Bovine serum albumin fraction V (BSA): 1 mg/mL (Sigma-Aldrich, Germany), stored at -20°C.

Bradford reagent: 100 mg Coomassie Brilliant G-250 (Sigma-Aldrich, Germany) dye was dissolved in 50 mL 95% (v/v) ethanol, then mixed with 100 mL 85% (v/v) phosphoric acid, and brought up to 1 L with dH₂O. The solution was stored at 4°C, and filtered through a 0.45 μm filter before use.

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

Calcium chloride (CaCl₂): 1 M CaCl₂ (BDH, USA) filter sterilised.

Cell lysis buffer: 25 mM Tris, pH 8.0, 0.5 M NaCl, 50 mM imidazole. Lysozyme (4mg/mL) was added to the buffer immediately prior to use.

Chloroform: 100% (v/v) chloroform (BDH, USA).

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

CIAP Stop buffer: 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (Sigma-Aldrich) (pH 7.5), 200 mM NaCl (BDH, USA), 0.5% SDS (BDH, USA).

Coating buffer: 0.016 M Na₂CO₃, 0.034 M NaHCO₃, pH 9.6.

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

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

Detection buffer: 100 mM Tris, 100 mM NaCl, pH9.5

Dextran: 10 mg/mL stored at -20°C (Sigma-Aldrich, Germany)

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

dNTPs (deoxynucleotide triphosphates): 25 mM each of dATP, dGTP, dTTP and dCTP (Bioline, Denmark)

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

Ethanol: 70% (v/v), 95% (v/v), 100% (v/v) analytic ethanol (BDH, USA).
Ethidium bromide (EtBr): A stock solution of 10 mg/mL EtBr (BM). This solution was not autoclaved.

Ethylene-diaminetetra-acetate (EDTA) buffer: 0.25 M EDTA (BDH, USA), pH 8.0.

Folin’s reagent: Folin & Ciocalteaus reagent (BDH, USA) diluted 1:1 in mH₂O, stored at 4°C away from light.

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

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

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

Hydrogen peroxide (H₂O₂): 30% (w/v) H₂O₂ (BDH, USA).

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

Isopropanol: Propan-2-ol (BDH, USA).

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

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

Lowry reagent B: 4% (w/v) copper sulphate (Ajax Chemicals Ltd., Australia)

Magnesium chloride: BDH, USA

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

Nickel Sulphate (0.2 M): Nickel sulphate (2.6 g) (BDH, USA) was dissolved in 50 mL of mH₂O.

Phenol (saturated): Supplied by Astral Scientific, Australia.

Phosphate-buffered saline (PBS): 1 tablet Dulbecco’s A PBS (Oxoid, England) in 100 mL mH2O (sodium chloride 0.8%, potassium chloride 0.02%, disodium hydrogen phosphate 0.115% potassium dihydrogen phosphate 0.02%).

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

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

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

Plasmid isolation solution III: 60 mL of 5 M potassium acetate (BDH, USA) (final concentration 3 M), 11.5 mL glacial acetic acid (BDH, USA) (final concentration 5 M) and 28.5 mL mH2O.

Protein lysis buffer 1: 25 mM Tris, pH 8.0, 0.5 M NaCl, 50 mM imidazole. Lysozyme (1 mg/mL) was added to the solution just before use.

Saline: 0.85% sodium chloride (BDH, USA).

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

SDS-PAGE Separating gel (12.5%): 375 mM Tris. HCl (pH 8.8), 0.1% w/v SDS , 12.5% v/v bis acrylamide with setting agents added prior to pouring: 0.06% w/v ammonium persulfate, 0.03% v/v TEMED.

SDS-PAGE Stacking gel (4.5%): 125 mM Tris. HCl (pH 6.8), 0.1% (w/v) SDS and 4.5% bis acrylamide with setting agents added prior to overlaying of separating gel: 0.06% (w/v) ammonium persulfate and 0.03% (v/v) TEMED.

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

Skim milk: Bonlac Foods Limited, Australia.
Sodium acetate: 3 M (BDH, USA), pH 4.6.

Sodium chloride: 5 M stock solution of sodium chloride.

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

Sodium hydroxide (NaOH): 10 M stock solution (BDH, USA).

Sulphuric acid (H₂SO₄) (TMB stop solution): 1.0 M sulphuric acid (BDH, USA).

TCA: 100% (w/v) Trichloroacetic acid

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

TEMED (N,N,N.,N.-tetramethylethylenediamine): Electrophoresis purity (BioRad Laboratories, Australia).

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

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

Tris (hydroxymethyl) aminomethane (Tris): 2 M stock solution (BDH, USA)

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

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

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


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

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

Western blot substrate solution (for HRP antibodies): Chloro-1-naphthol (30 mg) (Sigma-Aldrich, Germany) was dissolved in 10 mL methanol (BDH, USA), brought up to 50 mL with TBS, mixed with 30 μL hydrogen peroxide (H₂O₂) and used immediately for the development of Western blots probed with horseradish peroxidase (HRP) conjugates.

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

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

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

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

**DNA polymerase, Platinum Taq**: 5U/μL (Invitrogen, USA)

**Restriction enzymes**: See Table 2.4

**Calf intestinal phosphatase (CIAP)**: 10U/μL (Promega, USA), stored at 4°C.

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

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

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

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

2.2.2.4 Antibodies

**Horse anti-α-toxin polyclonal antisera (gas-gangrene antisera)**: CSL, Australia

**Rabbit anti-chicken IgA-HRP**: Sapphire Bioscience, Australia

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

**Goat anti-chicken IgG-HRP**: Sapphire Bioscience, Australia

**Goat anti-mouse IgG-HRP**: ICN, USA

**Rabbit anti-horse IgG (H+L)-alkaline-phosphatase**: Sigma-Aldrich, Germany.
2.3 Microbiological methods

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

The description of all the bacterial strains used throughout this study is listed in Table 2.1. A description of all plasmid vectors developed throughout the study is listed in Table 2.2.

2.3.2 Bacterial storage

_E. coli_ and _Salmonella_ strains were stored at -70°C in 50% glycerol. _C. perfringens_ strains were stored at -70°C in a solution containing 10% skim milk, 1% tryptone, and 10 mM Tris, pH 7.5.

2.3.3 Culture conditions

All _E. coli_ and _Salmonella_ strains were grown on solid microbiological media under aerobic conditions at 37°C for 16 h. In instances where broth cultures were used, the strains were grown aerobically at 37°C for 16-20 h on a Ratek orbital shaker set at 120 rotations per min. All clostridial species grown on solid agar were placed in airtight jars and an anaerobic environment was maintained with an Anaerogen gas pack (Oxoid, England) containing ascorbic acid as the active component. _Clostridia_ species were grown at 37°C for 16-20 h.
Table 2.1: Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Genotype/Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clostridium perfringens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>Type A</td>
<td>NE isolate from a chicken</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOP10F’</td>
<td>F’ (lacI, Tn10(Tet)) mcrA mrr (mrr-hsdRMS-mcrBC) Δ80lac2 Δ M15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG</td>
<td>Invitrogen, Australia</td>
</tr>
<tr>
<td>BL21</td>
<td>F’ ompT hsdSa(r5m8) gal dcm (DE3)</td>
<td>Invitrogen, Australia</td>
</tr>
<tr>
<td>DH5α</td>
<td>F’/endA1, hsdR17(r5m8), subE44, thi-1, recA1, gyrA (NalR), relA1, Δ(lacZYA-argF)Δ1569 (m80lacZΔM15)</td>
<td>(Hanahan, 1983)</td>
</tr>
<tr>
<td><strong>Salmonella spp</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmonella Typhimurium LT2-9121</td>
<td>leu hsdL trpD2 rpsL120 ilvS2 metE551 metA22 hsdA hsdB</td>
<td>Prof. P. Reeves, Department of Microbiology, The University of Sydney</td>
</tr>
<tr>
<td>Salmonella Typhimurium STM1</td>
<td>ΔaroA ΔserC</td>
<td>RMIT University</td>
</tr>
<tr>
<td>Salmonella Typhimurium 82/6915</td>
<td>Wild type Inv’</td>
<td>(Alderton et al., 1991)</td>
</tr>
<tr>
<td>Salmonella enterica serovar Sofia</td>
<td>Wild type Bt8</td>
<td>Dr. Margaret McKenzie, Inghams Enterprises Pty Ltd., Springwood, Queensland</td>
</tr>
</tbody>
</table>
Table 2.2: Plasmid vectors used in this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description/Genotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR2.1</td>
<td>3.9 kb PCR cloning plasmid, Amp⁶, Km⁶, lacZ</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pCRplc</td>
<td>1.24 kb amplified fragment from <em>C. perfringens</em> strain 61 containing the complete <em>plc</em> with a 6xCAT (coding for 6xHis) at the 5’ end, cloned into pCR2.1 using TA cloning</td>
<td>Dr Xenia Gatsos</td>
</tr>
<tr>
<td>pCRplc2</td>
<td>Two point mutation with two base changes at each of sites 84 (D→A) and 96 (H→S).</td>
<td>This study</td>
</tr>
<tr>
<td>pCRplcInv</td>
<td>Inverse amplified and self-ligated pCRplc (with nucleotides 250-309 of <em>plc</em> deleted)</td>
<td>Dr Xenia Gatsos</td>
</tr>
<tr>
<td>pRSETA</td>
<td>2.9 kb protein expression plasmid: Amp⁶, N-terminus 6xHis, T7 promoter</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pRplc104</td>
<td>DNA sequence coding for the C-terminus of <em>plc</em> (nucleotides 882-1243) cloned into the <em>PstI</em> and <em>EcoRI</em> sites of pRSETA</td>
<td>Dr Xenia Gatsos</td>
</tr>
<tr>
<td>pMOhly1</td>
<td>11.9 kb protein expression plasmid containing the <em>E. coli</em> hemolysin secretion system. Genes of interest are ligated into a truncated hlyA’, immediately upstream of the C-terminus secretion signal and downstream of the initiation codon.</td>
<td>Dr I. Gentschev</td>
</tr>
<tr>
<td>pMO104</td>
<td>DNA sequence coding for the C-terminus of <em>plc</em> (nucleotides 882-1243) in the hlyA’ expression gene in pMOhly1</td>
<td>This study</td>
</tr>
<tr>
<td>pMOInv</td>
<td>Full <em>plc</em> sequence with deletion of nucleotides 250-309 inserted into hlyA’ expression gene in pMOhly1</td>
<td>This study</td>
</tr>
<tr>
<td>pMNIInv</td>
<td><em>plc</em> (deletion of nucleotides 205-309) with additional deletion of internal signal peptide (nucleotides 1-84) inserted into hlyA’ expression gene in pMOhly1</td>
<td>This study</td>
</tr>
<tr>
<td>pMOplc2</td>
<td>Full <em>plc2</em> inserted into pMOhly1 vector</td>
<td>This study</td>
</tr>
<tr>
<td>pMNPlc2</td>
<td><em>plc2</em> with additional deletion of internal signal peptide (nucleotides 1-84)</td>
<td>This study</td>
</tr>
</tbody>
</table>
Broths for clostridial cultivation were used immediately, once cooled from sterilisation. If not used immediately, day old broths were boiled for ten min prior to *C. perfringens* inoculation to remove any dissolved oxygen.

### 2.4 Methods of DNA analysis

#### 2.4.1 Alkaline lysis plasmid extraction (miniprep)

Plasmids used in restriction digests were purified by using the alkaline lysis method described in Current Protocols in Molecular Biology (Ausubel, et al., 1994). One and a half millilitres of an overnight culture was placed in a microfuge tube and the cell pellet collected by centrifugation at 16,000 x *g* for 2 min. The pellet was resuspended in 100 μL of plasmid isolation solution I and left at room temperature for 5 min followed by cell lysis using 200 μL of plasmid isolation solution II. The solution was placed on ice for 5 min followed by the addition of 150 μL ice cold plasmid isolation solution III to precipitate chromosomal DNA and protein. The precipitate was separated by centrifugation at 16,000 x *g* for 10 min at 4°C and the supernatant transferred to a new microfuge tube. Fifty microlitres of PCI was added to the tube and mixed by vortex. The upper aqueous phase was collected by centrifugation at 16,000 x *g* for 2 min and mixed with 50 μL of CI. The solution was mixed by vortex and pelleted by centrifugation as above. The aqueous phase was collected and 2 volumes of 96% ethanol added to precipitate the plasmid DNA. Plasmid DNA was pelleted by centrifugation at 16,000 x *g* for 5 min, the supernatant decanted and the pellet air-dried before the addition of 40 μL 10 mM Tris, pH 8.0 and stored at -20°C.

#### 2.4.2 Plasmid extraction for DNA sequencing

Plasmid DNA required for sequence determination was purified using the QIAprep® spin miniprep kit as per the manufacturer’s instructions.
2.4.3 Extraction of DNA from agarose

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

2.4.4 Agarose gel electrophoresis

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

The DNA products were visualised with an UV illuminator and photographed using the Geldoc imaging system (BioRad Laboratories). Sizes of DNA fragments were estimated by the addition of lambda (λ) DNA previously digested with PstI (λ-PstI) to the gel prior to electrophoresis. A diagram of the λ-DNA sizes is located in Appendix 1.

2.4.5 Spectrophotometric quantification of DNA

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

Lambda-DNA previously digested with PstI was separated on an agarose gel along with sample DNA fragments. The quantity of DNA within each digested fragment of λ-DNA is listed in Appendix 1 and was compared with intensities of unknown DNA samples.

2.5 DNA manipulation

2.5.1 Restriction enzymes

The restriction enzymes (RE) used throughout the study are listed in Table 2.3. Approximately 1 μg of plasmid DNA was digested with 1U of enzyme containing 1x buffer in a total volume of 10-20 μL at 37°C for 2 h. Enzymes were heat inactivated at 65°C for 15 min when DNA was to be used in cloning experiments. All restriction enzymes were purchased from Promega, USA.

2.5.2 Amplification of DNA

2.5.2.1 Primers

Primers were designed with the aid of the software program Sci Ed Central for Windows 95. Primers were designed to have a guanine & cytosine (GC) content between 40-60% with a melting temperature (Tm°C) in the range of 55-75°C. Primers were obtained as lyophilised samples from a number of companies (Table 2.4).
Table 2.3: List of restriction enzymes (RE) used in this study.

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Recognition sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alu</em>I</td>
<td>AG\textsuperscript{CT}</td>
</tr>
<tr>
<td><em>BamHI</em></td>
<td>G\textsuperscript{GATCC}</td>
</tr>
<tr>
<td><em>NsiI</em></td>
<td>ATG\textsuperscript{CAT}</td>
</tr>
<tr>
<td><em>PstI</em></td>
<td>CTG\textsuperscript{AG}</td>
</tr>
<tr>
<td><em>SalI</em></td>
<td>GTC\textsuperscript{GAC}</td>
</tr>
</tbody>
</table>
Table 2.4: List of primers used throughout this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’ → 3’)</th>
<th>Description</th>
<th>Tm°C*</th>
</tr>
</thead>
<tbody>
<tr>
<td>plcInv-PstIRev</td>
<td>CCGCCGCTGCAGCTTTTATATT</td>
<td>Reverse primer for amplification of plcInv</td>
<td>69.1</td>
</tr>
<tr>
<td>plcInv-PstIForw</td>
<td>CGCCTGCAGCGAAAAAGAAAGA</td>
<td>Forward primer for amplification of plcInv</td>
<td>70.5</td>
</tr>
<tr>
<td>NSPInvPstl Forw</td>
<td>ATACTGCAGCGTGGGATGGAAGA</td>
<td>Forward primer to remove internal signal peptide for amplification of plcInv</td>
<td>60.4</td>
</tr>
<tr>
<td>plc104-NsilForw</td>
<td>CGCATGCATCCGAAAAAGATG</td>
<td>Forward primer for amplification of plc104</td>
<td>69.4</td>
</tr>
<tr>
<td>NP104-Nsil Rev</td>
<td>GCGGCCCAGTGCATCTTTTATATT</td>
<td>Reverse primer for amplification of plc104</td>
<td>67.9</td>
</tr>
<tr>
<td>SEQFpMOhly1</td>
<td>ATTGCACCTCAGCAGGAC</td>
<td>Forward sequencing primer of pMOhly1</td>
<td>57.1</td>
</tr>
<tr>
<td>SEQRpMOhly1</td>
<td>GAAGCTACCTGCAGCTGA</td>
<td>Reverse sequencing primer of pMOhly1</td>
<td>59.4</td>
</tr>
</tbody>
</table>

*Calculated by company of synthesis

Bases highlighted are enzyme recognition sites with:

**Pink sequence indicating a PstI recognition site**

**Green sequence indicating an NsiI recognition site**
2.5.2.2  **Polymerase chain reaction (PCR)**

General PCR was performed according to the polymerase manufacturer’s instructions. The general reaction mix and programs used are listed in Table 2.5, 2.6 and 2.7. The annealing temperature is specified in the relevant chapters. PCR products for cloning purposes were amplified using *Pfu* polymerase (Promega, USA), Platinum *Taq* polymerase, or Expand Long template PCR system (Roche, Germany) to reduce errors. PCR for general detection of fragments was prepared with Platinum *Taq* polymerase.

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

2.5.3  **Phosphatase treatment of vector DNA**

Digested vector, up to 1 μg, was treated with 0.04U of CIAP and incubated for 30 min at 37°C. A further 0.04U was added and incubated for another 30 min. A 200 μL volume of CIAP Stop buffer was added and the DNA solution cleaned with PCI. The DNA was precipitated with 95% w/v ethanol and collected by centrifugation at 16,100 x *g* for 15 min and resuspended in 10 μL of molecular grade water.
Table 2.5: Standard reaction conditions for amplification of DNA by PCR.

<table>
<thead>
<tr>
<th>PCR reagent</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template</td>
<td>10-100 ng</td>
</tr>
<tr>
<td>dNTP</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>Primer A</td>
<td>0.2 μM</td>
</tr>
<tr>
<td>Primer B</td>
<td>0.2 μM</td>
</tr>
<tr>
<td>10 x PCR buffer (supplied with polymerase)</td>
<td>1 x</td>
</tr>
<tr>
<td>MgCl$_2$ (Platinum Taq only)</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>Polymerase:</td>
<td></td>
</tr>
<tr>
<td><em>Pfu</em></td>
<td>1.5U/50 μL</td>
</tr>
<tr>
<td>Platinum <em>Taq</em></td>
<td>1.0U/50 μL</td>
</tr>
<tr>
<td>Expand Long Template range</td>
<td>2.5U/50 μL</td>
</tr>
</tbody>
</table>
Table 2.6: Standard amplification conditions of PCR with Platinum Taq and Expand Long range polymerase.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (s)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>120</td>
<td>1</td>
</tr>
<tr>
<td>94</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Specific Annealing °C</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>68</td>
<td>Taq 60/kbp</td>
<td>35</td>
</tr>
<tr>
<td>68</td>
<td>Expand 60/kbp</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 2.7: Standard amplification conditions with *Pfu* polymerase.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (s)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td>120</td>
<td>1</td>
</tr>
<tr>
<td>94</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Specific Annealing °C</td>
<td>45</td>
<td>35</td>
</tr>
<tr>
<td>72</td>
<td>120/kB</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>10 min</td>
<td>1</td>
</tr>
</tbody>
</table>
2.5.4 Ligation

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

2.5.4.1 Purification of DNA from ligation for electroporation

DNA ligations were precipitated from their buffers in order to prevent arcing during electrottransformation. This was done by the addition of 0.1 vol of 3 M sodium acetate, 2.5 vol of 100% ethanol, and 1 μL dextran (of a 10 μg/μL stock) directly into the ligation. The reaction was mixed by vortex and left on ice for 10 min. The DNA was then precipitated by centrifugation at 16,000 x g for 5 min. The sample was washed in 70% ethanol and the centrifugation process repeated. The DNA was resuspended in 10 μL molecular grade water and transformed via electrottransformation as described below.

2.5.5 Electrocompetent cell preparation

Bacterial cells were prepared for electrottransformation by the method supplied with the Gene Pulser apparatus user’s manual (BioRad, Laboratories). Briefly, 200 mL of Luria-Bertani broth (LB) was inoculated with 1/100 volume of an overnight bacterial culture. The cells were grown with vigorous shaking at 37°C to an OD₆₀₀ of 0.5-0.7 (early to mid-log phase). The culture was chilled on ice for 15 to 30 min and the culture pelleted by centrifugation in 50 mL tubes at 5,445 x g for 15 min at 4°C. The supernatant was drained and the pellet resuspended in an equal volume of ice-
cold mH$_2$O. The centrifuge process was repeated and the cells resuspended in a total volume of 100 mL of ice-cold mH$_2$O. The cells were pelleted as above and resuspended in 4 mL ice-cold 10% glycerol. The cells were pelleted once again and resuspended with 10% glycerol in a final volume of 400 μL. Aliquots of 40 μL were distributed to 1.5 mL tubes and stored at -70°C until required.

2.5.6 Electrotransformation

The method supplied with the Gene Pulser apparatus user’s manual (BioRad Laboratories) was employed for the high efficiency electrotransformation of *E. coli* and *S. Typhimurium*. Frozen electrocompetent cells were thawed on ice for 15 min and mixed with up to 2 μL of unpurified DNA or 10 μL of purified DNA and transferred to an ice-cold electrouvette with a 0.2 cm gap. The pulse settings used to deliver DNA into the cells were 2.5 kV, 25 μF and 200 Ω.

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

2.5.7 Sequence analysis

DNA was amplified using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (PerkinElmer). DNA from pRSETA was amplified using primers pRSETRev and T7promoter, and DNA from the pMOhly1 plasmid was amplified using primers SEQFpMOhly1 and SEQRpMOhly1. The amplified DNA was then collected by precipitation with 2.5 vol of absolute ethanol and 0.1 vol of 3 M sodium acetate as described in the manufacturer’s protocol and was sequenced using
the ABI Prism 377 DNA Sequencer with XL Upgrade (PerkinElmer) at Monash University (Clayton Campus), Victoria, Australia.

2.6 Protein methods

2.6.1 Preparation of whole cell lysates

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

2.6.2 Protein determination

2.6.2.1 Bradford assay

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

One millilitre of Bradford reagent was added to 100 μL of sample, or standard, mixed by inversion and left to stand for 2 min. When required, samples and standards were diluted in 0.15 M NaCl. Bovine serum albumin fraction V (BSA) was used as the standard protein solution. Two hundred microlitres of processed sample was added to the wells of a 96-well microtitre plate. The OD of samples was determined with a Dynatech ELISA plate reader at 600 nm (OD_{600}), and a standard curve constructed by plotting the concentrations of the bovine serum albumin standards (μg) versus absorbance was used to determine the protein content of unknowns.
2.6.2.2 Lowry assay

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

2.6.3 SDS-PAGE

SDS-PAGE was performed using a discontinuous buffer system (Bollag and Edelstein, 1991). The gels were prepared using SDS stacking gel buffer and SDS separating gel buffer. Proteins were mixed with SDS loading buffer and heated to 100°C in a dry heating block for 5 min. The proteins were separated by electrophoresis at 80 V for 30 min and then at 200 V for 35 min in an electrophoresis unit (BioRad Laboratories) containing SDS running buffer. Two gels were run at the same time in this system. Gels that were not transferred onto nitrocellulose membranes for immunoblotting were stained with Coomassie blue. The SeeBlue Plus2 protein marker was separated alongside protein samples and used for the determination of relative protein mass (Appendix 2).
2.6.4 Coomassie staining

SDS PAGE gels were stained in Coomassie blue staining solution for 30 min. Gels were rinsed under dH₂O and destained in the Coomassie destain solution for up to 2 h. A tissue was folded and placed into the destaining solution to assist with the removal of the stain.

2.6.5 Immunoblotting

2.6.5.1 Protein electrophoretic transfer and immunoblotting

Proteins resolved by SDS-PAGE gels were transferred by electrophoresis onto a nitrocellulose membrane padded with 4 sheets of Whatman chromatography paper and a scotch-brite pad on both sides of the cassette. Transfer buffer was added to the tank and the electrophoretic transfer was carried out at 70 V for 1.5 h. A container of ice was placed in the buffer to maintain a cool temperature to prevent overheating of sample transfer. After electrophoretic transfer, the nitrocellulose membrane was blocked by incubation with 5% (w/v) skim milk in TBS for 1 h on a rotary shaker. The skim milk solution was removed and the membrane washed twice by gentle agitation in TBS for 2 min. The membrane was then incubated overnight at 4°C on a rotary shaker with 25 mL of TST containing adsorbed antiserum. The membrane was then washed 2 times in TBS buffer for 5 min. Horseradish peroxidase-conjugated (HRP) antibodies diluted 1:5000 or alkaline phosphatase-conjugated (AP) antibodies diluted 1:15000 in TBS containing 1% skim milk were used as the secondary antibodies and were incubated with the membrane for 1 h shaking at 22°C. The membranes were washed 3 times in TBS for a total of 5 min and for HRP antibodies, the bound peroxidase was visualised by incubating in the Western blot substrate solution for approximately 5-15 min. For blots with AP as the secondary antibody, the blot was equilibrated in detection buffer for 10 min and then the AP labelled bands developed in BCIP/NBT solution. The reaction was stopped by washing the blot in dH₂O.
2.6.6 Enzyme-linked immunosorbent assay

Ninety-six well flat bottomed sterile plates (Greiner, Germany) were coated with 100 μL of 5 μg/mL purified protein or 10 μg/mL of a mixed protein sample diluted in PBS and incubated for 1 h at 37°C or 4°C overnight. Unbound protein was removed with 3 washes in PBST and the plate coated with 200 μL of blocking buffer. After a further hour incubation at 37°C, plates were washed as above, patted dry on absorbent paper towelling, and 100 μL of primary antibody two-fold serially diluted in diluent (1% skim milk, PBST) was added. Plates were incubated at 37°C with vigorous shaking on an ELISA shaker platform for 2 h. The solution was discarded, wells washed 4 times in PBST and patted dry. Secondary antibody conjugated to HRP was diluted 1:5000 in PBST (1% skim milk) and 100 μL added to the wells of the ELISA plate. Following a 1 h incubation at 37°C on a shaking platform, the plates were washed 4 times in PBST followed by a wash in dH₂O and then patted dry on absorbent paper. Tetramethylbenzidine (TMB) substrate was prepared and 100 μL added to all wells. The development of the reaction was allowed to proceed for up 30 min and the reaction was stopped with 50 μL of 1 M sulphuric acid. The OD₄₅₀ of the wells was determined in a Dynatech ELISA microplate reader. The endpoint was determined as the dilution at which the OD₄₅₀ was 3 times the background level (OD≥0.2). All samples were tested in duplicate.

2.7 Animal studies

2.7.1 Mouse studies

Specific pathogen free (SPF) six to eight week old female BALB/c mice were purchased from the Animal Resource Centre, Canningvale, Western Australia. Mice were acclimatised for a minimum of 1 week prior to the start of any experimentation.
Mice were bled from the retro-orbital vein using a capillary tube coated with heparin. Blood samples were separated by centrifugation at 6,000 x g for 10 min and the sera was collected and stored at -20°C until required for assays. These mouse studies were approved by the RMIT Animal Ethics Committee under AEC# 602 and 729.

### 2.7.2 Chicken studies

Newly hatched Cobb 500 broiler chicks were obtained from Inghams Farms, Pakenham, Victoria. The chicks were allowed to acclimatise for 2 days prior to the start of any experimentation. Chicks were bled from the wing vein using a 25 gauge needle and a 1 mL syringe. Blood samples were separated by centrifugation at 6,000 x g for 10 min and the sera was collected and stored at -20°C until required for assays. These chicken studies were approved by the RMIT Animal Ethics Committee under AEC# 729.

### 2.8 Bioinformatics

All the Bioinformatics programs used in this investigation were located at the Australian National Genomic Information Service (ANGIS) within WebANGIS and BioManager (www.angis.org.au). The program Sci Ed Central was used for the design of primers and vectors.
CHAPTER 3

CLONING, CHARACTERISATION & OPTIMISATION OF EXPRESSION OF ALPHA TOXIN CONSTRUCTS

3.1 Introduction

The alpha toxin gene is highly conserved among C. perfringens isolates and is known as plc or cpa in published literature, and hereafter will be referred to as plc. Despite that conservation, there are 28 different published variants of plc genes (Sheedy et al., 2004; Abildgaard et al., 2009) which all retain their toxicity. Purified alpha toxin can be neutralised by formalin treatment and used as a vaccine (Amimoto et al., 2002), however care must be taken to remove all traces of the formalin as it is also displays toxicity. The plc gene translates into a 370 amino acid protein with an α-helical N-terminus domain and a β-sandwich C-terminus domain as shown by x-ray crystallisation studies (Naylor et al., 1998). The key features of this protein are: the presence of the N-terminus domain providing phospholipase catalytic activity; the C-terminus domain where calcium ions bind to activate haemolytic and sphingomyelinase activities; and the three catalytically essential zinc ions in the active site (Naylor et al., 1998; Naylor et al., 1999; Titball et al., 1999). The alpha toxin protein also has a signal peptide which are the first 28 residues of the N-terminus region that are cleaved upon release into the supernatant (a 3 kDa signal sequence), or in the case of E. coli into the periplasmic space (Leslie et al., 1989).

In order to eliminate the toxicity so that the toxin could be used as a vaccine candidate without harming the vaccination target, areas of the toxin were removed or altered as described in the following studies (Figure 3.1). The main alteration target is the active site and the carboxyl end which has been demonstrated to be immunogenic in previous studies (Williamson and Titball,
1993; Hoang et al., 2008; Zekarias et al., 2008). The first construct, plcInv, is based on the deletion of nucleotides 250 to 309 which removes a section encoding part of the catalytic domain and inactivates the active site of the alpha toxin (Figure 3.2). The second construct, plc104, is the carboxyl domain of the alpha toxin and the third, plc2, is based on a two point mutagenesis at two strategic points of the active site (Figure 3.3 and 3.4). These clones were constructed prior to the current study.

The constructed inserts were amplified and cloned into another vector, pMOhly1 (Gentschev et al., 1996). This vector has the hly operon which consists of four genes, hlyA’, hlyB, hlyC, hlyD, responsible for the hemolysin system used by E. coli to synthesise and transport proteins directly out of the cell (Koronakis et al., 1988). The pMOhly1 vector has the full sequence of the structural genes hlyC, hlyB and hlyD and the modified version of the hlyA gene, hlyA’ (Figure 3.5). The full hlyA gene is not required as the N-terminus 34 amino acids and the C-terminus 61 amino acids are the regions that direct translocation of the protein through the cytoplasm to the extracellular space (Gentschev et al., 1996). This vector expresses protein constitutively without the need for induction (Gentschev et al., 1996). As the alpha toxin has its own signal peptide, two constructs were assembled: one with the alpha toxin signal peptide and the hlyA’ signal peptide (clones names pMOxxx – for details refer to Table 2.2) and one with only the hlyA’ signal peptide (named pMNxxx - for details refer to Table 2.2). This was performed for both the plcInv and plc2 constructs as they both possess the internal signal peptide, however was not necessary for the plc104 clone due to its removal during the original cloning process. The clones that were designed were pMOInv, pMNInv, pMO104, pMOplc2 and pMNplc2 and their stability in the cloning E. coli strain DH5α and the vaccine strain STM1 were ascertained.
Figure 3.1: Representation of the three alpha toxin constructs used in this study.

White filled areas indicate a deletion of nucleotide sequence; yellow filled areas indicate substitution mutagenesis.
Figure 3.2: Graphical representation of plcInv deletion as indicated by red section on a tertiary structure representation of alpha toxin.
Figure 3.3: Graphical representation of plc104 protein on a tertiary structure representation of alpha toxin.

Red highlighted section indicates that the N-terminus has been deleted.
Figure 3.4: Graphical representation of plc2 substitution modification.

Amino acid changes indicated by the yellow circles on a tertiary structure representation of alpha toxin: pink – aspartic acid to alanine; green – histidine to serine.
Figure 3.5: Map of pMOhly1 vector.

Inserts are cloned in-frame into the Nsil site.
Vaccination by administration of bacterial antigens and toxoids is a successful strategy in preventing disease. The construction of these alpha toxin constructs was performed to determine which one will provide protective responses against *C. perfringens* infection. In order to do this, the proteins must be purified so that they can be tested in an immunogenicity trial. The alpha toxin constructs that were cloned using an inducible vector will be purified using the 6xHis-tag that was added to make IMAC possible. This would be the basis of a subunit vaccine used in Chapter 4. Other existing toxoid based vaccines are the diphtheria toxoid vaccine and tetanus toxoid vaccine which are both continually being investigated to reduce adverse reactions after use (Relyveld *et al.*, 1998; Lee *et al.*, 2009).

The other method of vaccination being investigated is the use of live attenuated vectors expressing heterologous antigens. Over 13 different heterologous antigens have been expressed using different hemolysin expression vectors all with different expression levels in different bacterial vectors (Hahn and von Specht, 2003; Gentschev *et al.*, 2004; Zhu *et al.*, 2006; Hotz *et al.*, 2009). The pMOhly1 plasmid was chosen for this reason as it is a suitable vector that can be used in both *E. coli* and *Salmonella* strains as the hemolysin expression system is active in both. The Hly operon (Figure 3.6) consists of the following genes: *hlyA’, hlyB, hlyC* and *hlyD* (Koronakis *et al.*, 1988) and the pMOhly1 plasmid contains the entire operon with an restriction site *NsiI* in the middle of the *hlyA’. The *hlyA’* was derived from the cytotoxic hemolysin gene but has been modified to act as a transporter. The *hlyA’* gene consists of two sections of the original gene.
Figure 3.6: The hly operon and the fusion construct.
The first is 34 amino acids at the N-terminus region which assists with the activation of the hemolysin system. The second section is the $hlyA'$, secretion signal which is the 61 amino acids located at the end of the $hlyA$ gene; this is then added to the C-terminus of the inserted coding region which will then export the protein across the cellular membrane.

HlyC assists in the synthesis of the fusion constructs and is an acetylase required for post-translational activation of the hemolysin system. The other proteins, hlyB and hlyD, encoded in the operon are responsible for the transport of the fusion constructs through the bacterial membranes. The $hlyB$ gene is a translocase protein that will be located at the C-terminus of the fusion construct and works in conjunction with the hlyD protein. HlyD is a membrane-fusion protein which interacts directly with outer membrane proteins and Tol-C receptors as well as hlyB translocase to form the conduit through the cytosol to the extracellular space (Koronakis et al., 1988; Balakrishnan et al., 2001; Hahn and von Specht, 2003). The inclusion of these proteins can add up to 11 kDa in size to the fusion constructs if they remain after the translocation of the constructs across the bacterial membranes (Li et al., 2002; Hahn and von Specht, 2003). This may occur when the cleavage site becomes inaccessible to proteases during exportation due to changes in the proteins conformation.

Having successfully cloned the alpha toxin constructs into pMOhly1, the clones were further investigated for protein expression. The objectives of this part of the study is to a) purify sufficient quantities of the plcInv, plc2 and plc proteins using IMAC with minimal contamination whilst investigating possible sugar supplements to increase yield, and b) demonstrate that the hemolysin vector, pMOhly1, is capable of extracellularly expressing fusion products that react with anti-alpha toxin serum.
3.2 Materials and methods

3.2.1 Background development of constructs

Early studies on the design and development of the alpha toxin constructs were carried out by Dr Xenia Gatsos and Abdulkareem Elbaz. The plc gene, a 1.24 kb amplified fragment, from the chromosomal DNA of C. perfringens 61 was amplified using Pfu polymerase (Promega, USA) with a 6xCAT (coding for 6xHis) at the 5’ end, cloned into pCR2.1 using TA cloning kit (Invitrogen). This plasmid pCRplc (Figure 3.7: A)) was the template for all subsequent clones described in this study. The plasmid pCRplcInv (Figure 3.7: B)) was constructed using inverse PCR with Pfu polymerase (Promega, USA) with blunt ended self ligation and pCRplc2 (Figure 3.7: D)) by the same method using primers with the double base pair substitution site designed to change the aspartate amino acid to an alanine at position 56 and histidine to serine at position 68. The insert plc104 (Figure 3.7: C)) was developed with Pfu polymerase (Promega, USA) amplification using specific primers and then ligated into the PstI and EcoRI sites of pRSETA (Invitrogen). All of these plasmids incorporated a 6x Histidine tag onto the insert and as these studies focus on expression from a vector strain, not purification using IMAC, the primers were designed to omit the 6x Histidine tag. The presence of an NsiI site at base position 263 of the alpha toxin sequence resulted in the use of another restriction enzyme site for the plcInv and plc2 primers. This NsiI site was not present in the plc104 sequence as bases 1-881 were already deleted. In the plcInv and plc2 primers, the NsiI restriction site of ATGCAT was substituted for CTGCAT which is recognised and cut by PstI. This resulted in complementary overhangs of ACGT for the ligation.
Figure 3.7: Maps of alpha toxin construct plasmids- A) pCRplc, B) pCRplcInv.
Figure 3.7 continued: Maps of alpha toxin construct plasmids- C) pRplc104, D) pCRplc2.
3.2.2 Cloning of plcInv and NSPplcInv into pMOhly1

The primers plcInv-PstIRev and plcInv-PstIForw were used to amplify full length plcInv. The primers plcInv-PstIRev and NSPInv-PstIForw were used to amplify the plcInv insert without the internal signal peptide (nucleotides 1-84), NSPplcInv. The amplifications were carried out as described in Section 2.4.2.2 using Platinum Taq polymerase with both carried out at an annealing temperature of 40.5˚C and elongation for 2 min, with a final elongation time of 5 min. Ten microlitres of each amplified product was analysed on a 1% agarose gel, and the remaining used for digestion and ligation into pMOhly1 (Section 2.4.4). The PCR product was then digested with PstI for 2 h whilst pMOhly1 was digested with NsiI. After the ligation, the DNA was purified (Section 2.4.4.1) and 2 µL was used for electroporation (Section 2.4.6) into electrocompetent E. coli DH5α cells. Colonies containing putative clones that grew on the LB agar with 100 µg/mL ampicillin were chosen for further analysis.

3.2.3 Cloning of plc104 into pMOhly1

The primers NPplc104-NsiIRev and plc104-NsiIForw were used to amplify the plc104 insert. The amplifications were carried out as described in Section 2.4.2.2 using Pfu polymerase with an annealing temperature of 42.7˚C and elongation for 1 min, with a final elongation time of 5 min. Ten microlitres of each amplified product was analysed on a 1% agarose gel, and the remaining used for digestion and ligation into pMOhly1 (Section 2.4.4). The PCR product was then digested with NsiI for 2 h whilst pMOhly1 was digested with NsiI. After the ligation, the DNA was purified (Section 2.4.4.1) and 2 µL was used for electroporation (Section 2.4.6) into electrocompetent E. coli DH5α cells. Colonies containing putative clones that grew on the LB agar with 100 µg/mL ampicillin were chosen for further analysis.
3.2.4 Cloning of plc2 and NSPplc2 into pMOhly1

3.2.4.1 Direct cloning strategy

The primers plcInv-PstIRev and plcInv-PstIForw were used to amplify full length plc2 insert. The primers plcInv-PstIRev and NSPInv-PstIForw were used to amplify the plc2 fragment without the internal signal peptide (nucleotides 1-84), NSPplc2. The amplifications were carried out as described in Section 2.4.2.2 using Expand polymerase with both at an annealing temperature of 54.5°C and elongation for 2 min, with a final elongation time of 7 min. Ten microlitres of each amplified product was analysed on a 1% agarose gel, and the remaining used for digestion and ligation into pMOhly1 (Section 2.4.4). The digested vector was used both with and without phosphatase treatment (Section 2.4.3) during the cloning studies. The PCR product was then digested with PstI for 2 h whilst pMOhly1 was digested with NsI. After the ligation, the DNA was purified (Section 2.4.4.1) and 2 µL was used for electroporation (Section 2.4.6) into electrocompetent E. coli DH5α cells. Clones that grew on the LB agar with 100 µg/mL ampicillin were chosen for further analysis.

3.2.4.2 TA cloning strategy

The Expand PCR product was also cloned directly into the TA cloning vector pCR2.1 according to the manufacturer’s instructions (Invitrogen). Briefly, 30 ng of PCR product was mixed with 50 ng of pCR2.1 vector along with 4U of T4 DNA ligase and ligase buffer. This was incubated overnight at 14°C in waterbath. Two microlitres of the ligation product was then transformed into chemically competent E. coli TOP10F’ cells, supplied with the TA cloning kit.

These clones were then grown and the plasmid isolated via alkaline lysis plasmid extraction (Section 2.3.1). The PCR fragment was then digested out of pCR2.1 using PstI or EcoRI, and run on a 0.5 % agarose gel and the appropriate fragment excised from the gel and purified using the
Geneclean kit as per the manufacturer’s instructions. The fragment was then directly ligated into the previously *Nsii* digested pMOhly1 (Section 2.4.4). The digested pMOhly1 vector was used both with and without phosphatase treatment (Section 2.4.3) during the cloning studies. After the ligation, the DNA was purified (Section 2.4.4.1) and 2 µL was used for electroporation (Section 2.4.6) into electrocompetent *E. coli* DH5α cells. Colonies containing putative clones that grew on the LB agar with 100 µg/mL ampicillin were chosen for further analysis.

### 3.2.5 Screening of clones

Plasmids were isolated from the clones using the alkaline lysis plasmid extraction (Section 2.3.1) after being grown overnight shaking at 200 rotations per min at 37°C. The plasmids were then linearised via digestion with *SalI* and analysed on a 1% agarose gel. Plasmids showing an increase in size were then subjected to a screening PCR. When the difference in size was difficult to determine visually, the plasmids were subjected to a multiple site digestion using either *AluI* or *BamHI*. Plasmids showing a different restriction profile from the original empty plasmid were subjected to further screening. To determine the presence of the inserts and to confirm directionality, PCR was carried out using the following combinations of primers (Table 3.1). Once orientation was confirmed, the clone was then subjected to sequence determination (Section 2.4.8) to confirm the insert had no PCR introduced errors.

### 3.2.6 Passage of plasmids

The construct plasmid was isolated from the cloning strain of *E. coli* (either TOP10F’ or DH5α) using alkaline lysis extraction method (Section 2.3.1) and electrotransformation (Section 2.4.6) was used to transform the plasmid into the intermediate vector strain, *S. Typhimurium* LT2-9121.
Table 3.1: Screening PCR primer combinations for confirmation of orientation.

<table>
<thead>
<tr>
<th>Primer combination</th>
<th>Inferred Insert Orientation if PCR product is detected</th>
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<tbody>
<tr>
<td><strong>plcInv</strong></td>
<td></td>
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<tr>
<td>plcInv</td>
<td></td>
</tr>
<tr>
<td>SEQFpMOhly1 + plcInv-PstIRev</td>
<td>Correct</td>
</tr>
<tr>
<td>SEQFpMOhly1 + plcInv-PstIForw</td>
<td>Incorrect</td>
</tr>
<tr>
<td>SEQRpMOhly1 + plcInv-PstIForw</td>
<td>Correct</td>
</tr>
<tr>
<td>SEQRpMOhly1 + plcInv-PstIRev</td>
<td>Incorrect</td>
</tr>
<tr>
<td><strong>NSPplcInv</strong></td>
<td></td>
</tr>
<tr>
<td>NSPplcInv</td>
<td></td>
</tr>
<tr>
<td>SEQFpMOhly1 + plcInv-PstIRev</td>
<td>Correct</td>
</tr>
<tr>
<td>SEQFpMOhly1 + NSPInvPstI Forw</td>
<td>Incorrect</td>
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<tr>
<td>SEQRpMOhly1 + NSPInvPstI Forw</td>
<td>Correct</td>
</tr>
<tr>
<td>SEQRpMOhly1 + plcInv-PstIRev</td>
<td>Incorrect</td>
</tr>
<tr>
<td><strong>plc104</strong></td>
<td></td>
</tr>
<tr>
<td>plc104</td>
<td></td>
</tr>
<tr>
<td>SEQFpMOhly1 + NP104-Nsil Rev</td>
<td>Correct</td>
</tr>
<tr>
<td>SEQFpMOhly1 + plc104-NsilForw</td>
<td>Incorrect</td>
</tr>
<tr>
<td>SEQRpMOhly1 + plc104-NsilForw</td>
<td>Correct</td>
</tr>
<tr>
<td>SEQRpMOhly1 + NP104-Nsil Rev</td>
<td>Incorrect</td>
</tr>
<tr>
<td><strong>plc2</strong></td>
<td></td>
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<tr>
<td>plc2</td>
<td></td>
</tr>
<tr>
<td>SEQFpMOhly1 + plcInv-PstIRev</td>
<td>Correct</td>
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<tr>
<td>SEQFpMOhly1 + plcInv-PstIForw</td>
<td>Incorrect</td>
</tr>
<tr>
<td>SEQRpMOhly1 + plcInv-PstIForw</td>
<td>Correct</td>
</tr>
<tr>
<td>SEQRpMOhly1 + plcInv-PstIRev</td>
<td>Incorrect</td>
</tr>
<tr>
<td><strong>NSPplc2</strong></td>
<td></td>
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<tr>
<td>NSPplc2</td>
<td></td>
</tr>
<tr>
<td>SEQFpMOhly1 + plcInv-PstIRev</td>
<td>Correct</td>
</tr>
<tr>
<td>SEQFpMOhly1 + NSPInvPstI Forw</td>
<td>Incorrect</td>
</tr>
<tr>
<td>SEQRpMOhly1 + NSPInvPstI Forw</td>
<td>Correct</td>
</tr>
<tr>
<td>SEQRpMOhly1 + plcInv-PstIRev</td>
<td>Incorrect</td>
</tr>
</tbody>
</table>
The plasmids were then isolated a second time using the alkaline lysis extraction method (Section 2.4.1) from the *S. Typhimurium* LT2-9121 and electrotransformation (Section 2.5.6) was used again to incorporate these passaged plasmids into the STM1 vaccine strain.

### 3.2.7 Plasmid stability assays

Stocks of STM1 harbouring the pMOhly1 based vectors were streaked onto LB agar with 100 µg/mL ampicillin to obtain single colonies. Three to four well isolated colonies were selected for plasmid stability assays and incubated in 10 mL of LB broth with 100 µg/mL ampicillin at 37°C on an orbital shaker for 18 h. The culture was 10-fold diluted from $10^{-1}$ to $10^{-6}$ in LB broth and 100 µL of each dilution spread plate onto LB agar with 100 µg ampicillin in duplicate, to determine the initial plate count. The $10^{-6}$ dilution (10 mL) was incubated for 24 h at 37°C in the absence of ampicillin. This culture was again serially 10-fold diluted to $10^{-6}$ and 100 µL of each dilution spread onto LB agar with 100 µg/mL ampicillin and without ampicillin, in duplicate. The $10^{-6}$ dilution (10 mL) was incubated at 37°C for 24 h and the process of dilution and spread plating repeated. The process was repeated for a minimum time of 5 days (approximately 100 generations) or until less than 0.05-0.002% of the culture retained the plasmid.

### 3.2.8 Alpha toxin assays

#### 3.2.8.1 Lecithinase reaction

Nagler plates were inoculated with a single streak of *E. coli* colonies transformed with vector containing *plc* constructs. The plates were incubated at 37°C overnight and the diameter of the zone of turbidity measured. Samples with no observable signs of hydrolysis were left at 37°C for up to 24 h.
3.2.8.2 Haemolysis assay

Sheep blood agar was prepared as described in Section 2.1.2.1 with the addition of 100 μg/mL ampicillin to the molten agar. Fifty microlitres from a stock solution of 100 mM IPTG was spread onto the SBA plates and *E. coli* colonies transformed with vector containing *plc* constructs were streaked onto the agar and incubated for 18 h at 37°C. Plates were then incubated at 4°C for 2 h prior to determining the presence of incomplete haemolysis surrounding the colonies.

3.2.9 Protein purification

3.2.9.1 Media supplement evaluation

One percent (v/v) of an overnight culture of *E. coli* harbouring pCRplc or pCRplcInv was used to inoculate 10 mL LB broth containing 100 μg/mL ampicillin supplemented with 0.5% (w/v) glucose, 0.5% (w/v) fructose, 0.5% (w/v) sucrose or 0.5% (w/v) maltose. The broth was incubated at 37°C with vigorous shaking until mid-log phase of growth was reached (an OD600 of between 0.4-0.6). IPTG, to a final concentration of 1 mM, was added to the culture and incubated for a further 16 h. One millilitre hourly samples of culture were taken pre and post induction, cells collected by centrifugation and lysed using the whole cell lysis method (Section 2.6.1). Protein concentration of the supernatant was measured and 15-30 micrograms of lysed cell supernatant was analysed using SDS-PAGE and immunoblotting (Section 2.6.3 – 2.6.5). Cell supernatant was also incubated for 18 – 48 h at 4°C to check the stability of the proteins.

3.2.9.2 Pilot intracellular expression of α-toxin and α-toxin constructs

One percent (v/v) of an overnight culture of *E. coli* harbouring *plc* constructs was used to inoculate 10 mL LB broth containing 100 μg/mL ampicillin. The broth was incubated at 37°C with vigorous shaking until mid-log phase of growth was reached (an OD600 of between 0.4-0.6). IPTG
to a final concentration of 1 mM was added to the culture and incubated for a further 16 h. One millilitre hourly samples of culture were taken pre- and post-induction, pelleted by centrifugation and lysed using the whole cell lysis method (Section 2.6.1). Fifteen to thirty micrograms of lysed cell supernatant was analysed using SDS-PAGE and immunoblotting (Section 2.6.3 – 2.6.5).

3.2.9.3 Isolation of proteins

One percent of an overnight culture (v/v) was used to inoculate 100-500 mL fresh LB broth containing 100 μg/mL ampicillin. The culture was grown until an OD600 of 0.4-0.6 was reached and protein expression induced with 1 mM IPTG. The culture was incubated for a further 3 h and then pelleted by centrifugation. The bacteria were resuspended in 1/25th the volume of protein lysis buffer I and incubated at 4°C for 30 min on a rocking platform. The bacteria were then treated with a repeated freeze/thaw cycle up to 4 times, with the addition of 5U of DNase following the first thaw cycle. Debris was separated by centrifugation at 5,445 x g for 40 min at 4°C. The supernatant was filter sterilised through a 0.2 μm membrane and loaded onto an IMAC gravity flow column.

3.2.9.4 Immobilised Metal Affinity Chromatography (IMAC)

Chelating fast flow sepharose was prepared according to the manufacturer’s instructions (Amersham Biosciences) using 1 and 5 mL gravity flow columns (QIAGEN). Once packed and washed with H₂O, the column was charged using half a column volume (CV) of a 0.2 M solution of the transitional metal nickel sulphate (NiSO₄) (BDH, USA). The column was washed with at least 5 CV of 0.45 μm filtered mH₂O and equilibrated with 5 CV of binding buffer. Up to 60 mL of supernatant containing his-tagged protein was applied to the column. The column was washed with 10 CV of wash buffer containing between 60 - 80 mM imidazole depending on the optimisation, following protein binding and eluted with 10 CV of elution buffer. One millilitre
fractions were collected during the elution step, and analysed using SDS-PAGE. The flow through from the column was then placed into a second 1 mL IMAC column and the process repeated to purify any recombinant protein that flowed through the first column.

3.2.9.5 Cleaning in place and regeneration of column

Gravity flow columns were re-used up to 5 times and cleaned between each use to remove hydrophobic and ionic bound contaminants. Cleaning in place involved the application of 10 CV of 2 M NaCl, followed by 10 CV of 1 M NaOH, and finished with 10 CV of 70% ethanol (v/v). Columns were washed with dH₂O between each solution and Ni²⁺ was stripped with 2 CV 0.05 M EDTA, 0.5 M NaCl, pH 7.0. A 20% ethanol solution was added to the sepharose when stored for later use.

3.2.9.6 Buffer exchange of proteins

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

3.2.9.7 Quantification of protein fractions

Quantification of purified proteins was done using both Bradford (Section 2.6.2.1) and Lowry assay methods (Section 2.6.2.2) and using a spectrophotometric measurement at 280nm on an Eppendorf Biophotometer. After quantification, the proteins were run on a SDS-PAGE gel to confirm integrity of the protein.
3.2.10 Trichloroacetic acid (TCA) precipitation of extracellularly expressed proteins

A volume of 1 mL from a 5 mL overnight culture was pelleted at 16,000 x g for 5 min. The cell pellet was discarded and the supernatant transferred to a new microcentrifuge tube. A volume of 200μL of 100% w/v TCA was added to the supernatant and the tube inverted. The tube was incubated on ice for a period of 30 min and the precipitated proteins collected by centrifugation at 16,000 x g for 15 min. The precipitated pellet was washed with 100% w/v acetone, recovered by centrifugation at 16,000 x g for 15 min and dried at RT for 30 min. The pellet was then dissolved in protein sample buffer, boiled at 100°C for 10 min and subjected to SDS-PAGE and immunoblotting (Section 2.6.3 – 2.6.5).
3.3 Results

3.3.1 Cloning of plcInv and plcNSPInv and construction of pMOInv and pMNInv plasmids

The Platinum Taq PCR amplification of plcInv using an annealing temperature of 40.5°C resulted in a single strong band at the expected size of 1.144 kbp (Figure 3.8 A). This was subsequently digested with PstI and ligated into the NsiI digested pMOhly1 vector using T4 DNA ligase. The resulting plasmid was named pMOInv (Figure 3.8 B). The Platinum Taq polymerase PCR amplification of plcNSPInv using the same annealing temperature of 40.5°C resulted in a single band at the expected size of 1.060 kbp (Figure 3.9 A). This PCR product was then digested with PstI and ligated into the NsiI digested pMOhly1 vector using T4 DNA ligase. The resulting plasmid was named pMNInv (Figure 3.9 B). Quite a number of colonies obtained from both ligations showed the presence of the inserts. The orientation of the inserts was determined using a restriction digest profile using the four base cutter AluI (Figure 3.10). The clones that showed a different profile from the pMOhly1 digest were sent for sequencing. The sequencing results showed that both clones were: missense-error free, in the correct orientation, and in-frame with the hlyA’ gene and the rest of the hly operon.
A) PCR amplification of the plcInv using Taq. Lane 1: PstI/Lambda DNA marker, Lane 2: Negative PCR control, Lanes 3 & 4: plcInv at an annealing temperature of 40.5˚C.

B) Map of the completed plasmid.

Figure 3.8: Development of pMOInv plasmid.

A) PCR amplification of the plcInv using Taq. Lane 1: PstI/Lambda DNA marker, Lane 2: Negative PCR control, Lanes 3 & 4: plcInv at an annealing temperature of 40.5˚C. B) Map of the completed plasmid.
Figure 3.9: Development of pMNInv plasmid.

A) PCR amplification of the plcNSPInv using Taq. Lane 1: NSPplcInv at annealing temperature of 40.5°C, Lane 2: PstI/Lambda DNA marker. B) Map of the completed plasmid.
Figure 3.10: Screening for insert orientation using AluI restriction profiles.

Lane 1: PstI/Lambda DNA marker, Lane 2: pMOhly1, Lane 3-12: Unsuccessful clones, Lane 13: pMNInv, Lane 14: pMOInv.
3.3.2 Cloning of plc104 and construction of pMO104 plasmid

The *Pfu* polymerase PCR amplification of plc104 using an annealing temperature of 42.7°C resulted in a strong band at the expected size of 0.322 kbp (Figure 3.11 A)). This was subsequently digested with *Nsi*I and ligated into the *Nsi*I digested pMOhly1 vector using T4 DNA ligase. The resulting plasmid was named pMO104 (Figure 3.11 B)). Resulting clones were screened for the direction of the insert using PCR using the primer combinations outlined in Table 3.1. As shown in Figure 3.12, Clone 1 had bands for both the primer combinations for the correct orientation whilst also having one band from an incorrect combination of primers. Clone 3 showed two bands for the incorrect orientation primers combinations however also had a band of same intensity for a correct orientation combination. Both of these clones were sequenced and it was shown that Clone 1 was in the correct orientation without errors and Clone 2 was in the reverse orientation.
Figure 3.11: Development of pMO104.

A) PCR amplification of the plcNSPInv using *Pfu*. Lane 1: plc104 at annealing temperature of 42.7°C, Lane 2: PstI/Lambda DNA marker. B) Map of the completed plasmid.
Four PCR reactions were set up for each clone according to the primer combinations listed in Table 3.1. Ticks indicate a PCR product is expected if the insert is in the correct orientation, crosses indicate a PCR product is expected if the insert is in the incorrect orientation.
3.3.3 Cloning of plc2 and NSPplc2 and construction of pMOplc2 and pMNplc2

3.3.3.1 PCR amplification of plc2 and NSPplc2 inserts

PCR amplification of the plc2 insert and NSPplc2 insert was accomplished using Expand Long Template system at an annealing temperature of 54.5°C as shown in Figure 3.13 A). The PCR products generated during these reactions were ample and specific without any other contaminating bands. Once the products were digested using the enzyme PstI, they retained their size compared to the original products and did not appear to degrade (Figure 3.13(B)).

3.3.3.2 Direct method: Ligation of products into pMOhly1

The first cloning strategy used was a direct method where the PCR products were digested, cleaned (Section 2.5.4.1) then immediately used in the ligation. Ligations using a digested phosphatase-treated vector and a digested only vector were tested with varying results. The ligation frequency was quite low and many of the colonies screened contained the empty vector when the vector was not phosphatase treated. The ligation frequency increased slightly with the use of the phosphatase-treated pMOhly1. Increasing the vector to insert ratios from 1:3 to 1:15 and 1:50 for both reactions did not increase the frequency of positive colonies obtained from the ligation, nor did altering the ligation timeframe or incubation temperature.

All colonies found to have an insert in the pMOhly1 were screened using PCR with various primer combinations to show the orientation of the insert (Table 3.1). The PCR results showed a product at the expected size of approximately 1.2 kbp for the primer combination for the incorrect
orientation, yet there were also multiple bands in the correct orientation primer combination (Figure 3.14 A)). This pattern occurred for every clone tested from the transformations and upon sequencing, the orientation of the inserts were shown to be in the incorrect orientation. Over five hundred clones were screened and each one of them was in the incorrect orientation, hence the production of pMOplc2 and pMNplc2 was unsuccessful using this method.

3.3.3 Indirect method: Ligation of products into pMOhly1 via pCR2.1

The alternative indirect strategy was used to increase the probability of the insert going into pMOhly1 in the correct orientation; by ensuring all ends of the insert would have the correct overhang (a certain proportion of PCR products that were directly digested may not have been digested). Both inserts were ligated into the pCR2.1 vector, transformed into TOP10F’ cells via electroporation according to the manufacturer’s instructions and then isolated using the alkaline lysis method. The insert was then digested out of the pCR2.1 using EcoRI, the fragments excised from an agarose gel and further digested using PstI and used for the ligation. This resulted in more colonies on the transformation plates than the direct method.

The PCR screening for the directionality using the primer combinations in Table 3.1 showed the same distinct pattern that occurred with the direct method of cloning. The PCR results showed a product at the expected size of approximately 1.2 kbp for the primer combination for the incorrect orientation, and again there were also multiple bands in the correct orientation primer combination (Figure 3.14 (B)).
Figure 3.13: Development of pMOplc2 and pMNplc2.

A) PCR amplification of the plc2 and NSPplc2 using Expand template system. Lane 1: plc2 at annealing temperature of 54.5°C, Lane 2: NSPplc2 at an annealing temperature of 54.5°C, Lane 3: PstI/Lambda DNA marker. B) Digestions of products and plasmid, Lane 1: PstI/Lambda DNA marker, Lane 2: Empty, Lane 3: NsiI digested pMOhly1, Lane 4: plc2 at annealing temperature of 54.5°C, Lane 5: NSPplc2 at an annealing temperature of 54.5°C. C) Map of the proposed pMOplc2 plasmid. D) Map of the proposed pMNplc2 plasmid.
Four PCR reactions were set up for each clone according to the primer combinations listed in Table 3.1. Ticks indicate a PCR product is expected if the insert is in the correct orientation, crosses indicate a PCR product is expected if the insert is in the incorrect orientation. A) plc2 screening with all clones showing the insert in the incorrect orientation. B) NSPplc2 screening with all clones showing the insert in the incorrect orientation.

Figure 3.14: Directional screening of potential clones.
Sequencing of these clones (data not shown) revealed that the majority of them that showed the inclusion of the insert had the correct insert of either plc2 or NSPplc2 in the reverse direction. This left a small portion of the clones that had another unknown insert of similar size that did not match with either of the plc2 insert or the NSPplc2 insert. When these unknown fragments were sequenced they were found to be contaminating pCR2.1 fragments. Over three hundred colonies were screened from this method and not one successful clone of pMOplc2 or pMNplc2 was ever found.

3.3.4 Stability of the plasmids in the vaccine strain STM1

The successful clones of pMOInv, pMNInv and pMO104 were passaged through the intermediate strain of *S. Typhimurium* LT2-9121 into STM1 so that transformation would occur at a higher frequency between two related bacterial strains. The stability of each of these was measured over a period of 6 days which is over 100 generations of the host strain. The stability of the plasmids were tested in both *E. coli* and STM1 strains as the hemolysin secretion system originated from the *E. coli* and the STM1 strain is the vaccine strain that will be used in later studies. Overall, the plasmids were retained by at least 50% of the STM1 over the six day timeframe in the absence of any selective pressure normally supplied by the presence of 100 µg/mL ampicillin.

The plasmid pMOInv was most stable in the *E. coli* with an average retention of 103% (i.e. no loss) while in STM1 the average was 84% (Figure 3.15). In *E. coli*, the pMOInv plasmid had the highest retention at Day 4 whilst the other two plasmids, pMNInv and pMO104, experienced theirs on Day 1 in both strains. Carriage of the plasmid steadily declined over the six day period with only
58% of STM1 retaining it on Day 6, whilst in *E. coli* it rose between Day 5 and 6 from 88% to more than 100%.

The plasmid pMNInv was more stable in STM1 with an average retention of 86% than in the *E. coli* at 60%. In STM1, the plasmid was retained at 80% on Day 6 whilst in the *E. coli* it had rapidly declined to only 16% (Figure 3.16). Similar to the pMOInv plasmid, there was a rise in retention by *E. coli* on Day 4 from 61% to 69% before declining sharply to 32%. The plasmid pMNInv was maintained by STM1 very well and only had a very slow decline over the six days. The final plasmid pMO104 was quite stable over the six days in both *E. coli* and STM1 with averages of 80% and 81%, respectively. In both strains there was an increase in retention on Day 3 with STM1 going from 91% to 98% and *E. coli* improved from 85% to 89% (Figure 3.17). On the final day, STM1 retained 66% of the plasmid whilst *E. coli* retained only 42%.
Figure 3.15: Stability assays for the clone pMOInv in *E. coli* and STM1.
Figure 3.16: Stability assays for the clone pMNInv in *E. coli* and STM1.
Figure 3.17: Stability assays for the clone pMO104 in *E. coli* and STM1.
3.3.5 Translational changes in the alpha toxin constructs

Mature alpha toxin is destructive due to its haemolytic and enzymatic activities which ruptures cell membranes (Sakurai et al., 2004). The molecular cloning changes to prevent this toxicity were confirmed by sequence analysis and the following proteins were purified. The first protein, plcInv, has a 20 amino acid deletion from position 56 – 75 removing the amino acids, aspartic acid (56) and histidine (68) which are important to zinc binding, and tyrosine (57, 64) and threonine (73) which assist in binding of the active site to host membranes (Nagahama et al., 1995; Flores-Díaz et al., 2004). The second protein is plc2 which has 2 point mutations with two amino acid changes at position 56, aspartic acid to alanine, and position 68, histidine to serine (Figure 3.18). The aspartic acid at position 56 was chosen as it is an essential amino acid which interacts with an essential zinc cation in the catalytic site whilst the conformation of the binding site of the alpha toxin is dependent on the histidine at position 68. Each of these changes was done with two nucleotide substitutions to minimise the possibility of spontaneous reversion to wildtype. These two proteins along with mature alpha toxin were used for immunogenicity trials.

The clones pMOInv, pMNInv and pMO104 were not designed for high level protein purification but as low expression vaccine plasmids that could be delivered via STM1. The plasmid pMO104 contains the coding region for protein plc104 which is 104 amino acids of the C domain of the mature alpha toxin. pMOInv has the plcInv coding region in its entirety whilst pMNInv has the 28 amino acid signal peptide removed. As these clones were not designed for purification, this 6 histidine tag was removed for constructs ligated into pHlyh. The presence of the hlyA’ signal peptide along with the inserts altered the expected size of the expressed constructs. Table 3.2 shows the expected sizes and the features of the proteins explored in this chapter.
Figure 3.18: Amino acid sequence alignment for alpha toxin with alpha toxin constructs.

Grey shaded area represents the N-terminus α-helical domain and the open-boxed area the C-terminus β-sheet domain. The first 28 amino acids are a signal peptide. The yellow highlighted amino acids are the two site mutagenesis changes in the pCRplc2 plasmid.
Table 3.2: Description of alpha toxin variants.

<table>
<thead>
<tr>
<th>Expression vector</th>
<th>Protein</th>
<th>No. of aa in protein</th>
<th>Deletion (Δ) or Change Positions</th>
<th>Estimated MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCRplc</td>
<td>plc</td>
<td>370</td>
<td>—</td>
<td>46.90</td>
</tr>
<tr>
<td>pCRplc2</td>
<td>plc2</td>
<td>370</td>
<td>Change Positions 56 (D→A) &amp; 68 (H→S.</td>
<td>46.80</td>
</tr>
<tr>
<td>pCRplcInv</td>
<td>plcInv</td>
<td>350</td>
<td>Δ Position 56 – 75</td>
<td>44.00</td>
</tr>
</tbody>
</table>

Without hlyA  | With hlyA
--- | ---

| pMO104         | plc104  | 104                  | Δ Position 1 – 266               | 12.21 | 23.22 |
| pMOInv         | plcInv  | 350                  | Δ Position 56 – 75               | 43.00 | 53.01 |
| pMNInv         | plcNSPInv | 322                  | Δ Position 1 – 28                | 40.00 | 50.01 |
|                |         |                      | Δ Position 56 – 75               |       |      |
3.3.6 Enzymatic properties of the alpha toxin constructs

Mature alpha toxin shows hemolytic activity when exposed to red blood cells and this was assessed using sheep blood agar plates and induction of protein expression using IPTG. This assessed the ability of the transformed E. coli strain to produce hemolytic protein. The alpha toxin clone showed typical β–hemolytic activity with complete clearing of the media around the transformed colonies (Figure 3.19 A)). The construct plcInv displayed no hemolytic activity (Figure 3.19 B)) indicating that the 20 amino acid deletion severely compromises the protein function, as expected. The protein plc2 also showed no hemolytic activity on the sheep blood agar (Figure 3.19 C)) which demonstrated that the 2 site mutagenesis was adequate in deactivating the properties of the toxin. Lecithinase activity is also a well documented feature of alpha toxin and was measured using Nagler plates. This activity is evident for alpha toxin (Figure 3.20 A)) but absent for both the plcInv (Figure 3.20 B)) and plc2 (Figure 3.20 C)) proteins. Therefore, the molecular changes made either via deletion or substitution renders the toxin inactive.

3.3.7 Purification studies using sugar supplements

Purification of the mature alpha toxin and the plcInv construct from E. coli was assessed using four different sugar supplements at a consistent concentration of 0.5% (w/v) in LB broth. Alpha toxin and plcInv were examined as higher quantities from the purification would be needed for vaccine trials. Purification of alpha toxin on average yielded 3.5 mg/L of E. coli grown in LB broth without any supplement over a 5 hour induction. The purification of plcInv was always lower than alpha toxin with an average yield of 1.5 mg/L of culture grown to the same conditions. Four sugars were chosen based on their ability to be digested without placing a high metabolic load on the E. coli. The first two are glucose and fructose which are monosaccharides followed by maltose and sucrose which are disaccharides. Each supplement was tested in duplicate.
Figure 3.19: Haemolysis reaction on SBA for alpha toxin constructs.

A) β-haemolysis from *E. coli* harbouring pCRplc. B) Lack β-haemolysis of *E. coli* harbouring pCRplc2 and C) Lack β-haemolysis of *E. coli* harbouring pCRplcInv.
Figure 3.20: Lecithinase activity on Nagler plates.

A) Characteristic opaque halo from *E. coli* harbouring pCRplc showing Lecithinase activity. B) Lack of lecithinase activity of *E. coli* harbouring pCRplc2 and C) Lack of lecithinase activity of *E. coli* harbouring pCRplcInv.
3.3.7.1 Alpha toxin purification with sugar supplements

Over a 5 h induction the amount of protein typically increases over time, with purification done shortly after. Each culture was sampled after each hour until 5 samples had been taken and the semi-quantitative western blot reflects changes to the expression level at each time point by differing reactivity to the anti-serum. Alpha toxin in the whole cell lysate acquired from *E. coli* grown in LB broth after 5 h yielded a single band as visualised by SDS-PAGE (Figure 3.21 A)) with minimal breakdown of the protein. In the presence of glucose there is a slight increase in expression at 5 h to however there are small amounts of breakdown (Figure 3.21 B)).

With the inclusion of fructose in the media, the lowest expression is observed with the band showing less reactivity with breakdown at each induction time point (Figure 3.21 C)). As maltose breaks down to two glucose molecules it was expected that there would be an increase in expression as was observed for the glucose supplement. However the western blot shows a marked decrease in expression across all time points (Figure 3.21 D)). Supplemented sucrose increased the expression of alpha toxin after 5 h induction, compared to the whole cell lysate grown in LB broth, but there is still a slight amount of breakdown evident at 5 h (Figure 3.21 E)).

3.3.7.2 plcInv purification with sugar supplements

The purification of plcInv from *E. coli* consistently gave lower yields than the alpha toxin purification and typically over a 5 h induction would give an increasing expression. In this experiment, the LB broth whole cell lysate showed decreased expression at 5 h induction (Figure 3.22 A)) but retained its stability over 18 h at 4˚C. The highest expression was at 4 h induction.
Figure 3.21: Western blots of alpha toxin obtained from whole cell lysate grown in LB media supplemented with four different sugars.

A) No supplement, B) 0.5% (w/v) Glucose, C) 0.5% (w/v) Fructose, D) 0.5% (w/v) Maltose and E) 0.5% (w/v) Sucrose. Numbers (0-5) above blot are h post-induction, M: SeeBlue Plus2 protein standard marker.
Figure 3.22: plcInv purified from media supplemented with different sugars.

A) No supplement, B) 0.5% (w/v) Glucose, C) 0.5% (w/v) Fructose, D) 0.5% (w/v) Maltose and E) 0.5% (w/v) Sucrose; Numbers (0-5) above blot are h post-induction, M: SeeBlue Plus2 protein standard marker.
When glucose was added to the media there was an increase in expression over all time points with the highest being at 5 h (Figure 3.22 B)). However there was increased degradation after the 2 h induction sample. The same pattern was observed for the media supplemented with fructose (Figure 3.22 C)). *E. coli* grown in maltose supplemented media expressed plcInv at a higher rate at 5 h induction (Figure 3.22 D)). There was no breakdown apparent at any of the time points. Higher expression rates were observed in LB media supplemented with sucrose with breakdown observed after 3 h induction (Figure 3.22 E)). Overall in the presence of any of these sugars, *E. coli* expressed the plcInv at a higher rate over the entire 5 h induction. Glucose was used as a supplement for subsequent purification experiments.

### 3.3.8 IMAC purification for vaccine studies

Large scale purification was done using IMAC with a Ni\(^{2+}\) loaded column using *E. coli* cultures ranging in size from 500 mL to 1.2 L. Soluble protein were recovered from the cell lysate in substantial quantities and the cell pellet revealed no inclusion bodies detected by western blot (not shown). Optimisation of the wash buffer was carried out using imidazole concentrations ranging from 60 mM to 100 mM and the buffer that removed the most contaminating bands with the smallest loss of the target protein was used. Large contaminating bands varying in size from 80 – 85 kDa that were not reactive to the anti-alpha toxin sera were binding to the column. Optimisation of the wash buffer showed that concentrations higher than 70 mM imidazole would remove more alpha toxin than the contaminating bands (Figure 3.23 A)). Both plc2 and plcInv bound to the column more readily, Figures 3.23 B) & C) respectively, hence tolerated a higher imidazole concentration at 80 mM with minimal loss of the target protein.
After optimisation of the wash buffers, the contaminating bands were no longer appearing in the fractions eluted from the columns. The alpha toxin was purified from the column in multiple fractions with minimal contaminating bands. Approximately 3.6 mg of plc was obtained per litre of *E. coli* following IMAC purification. Protein plcInv bound well to the column and no contaminating bands were found in the fractions eluted using 200 mM imidazole. An average yield of approximately 1.6 mg of plcInv was obtained per litre of *E. coli* following IMAC purification. Protein plc2 bound to the column exceedingly well and was eluted from the column over multiple fractions using 200 mM imidazole. The highest average yield of plc2 from IMAC purification was approximately 4.4 mg per litre of *E. coli* culture. Fractions were combined and subjected to buffer exchange to remove the imidazole prior to use in a vaccine trial.

Although the large contaminating protein bands varying in size from 80 – 85 kDa were removed from the individual fractions taken from the column upon combination and concentration they were apparent (Figure 3.24 A)) but remained non-reactive to the anti-alpha toxin sera (Figure 3.24 B)). Attempts were made to separate these proteins from the target proteins using Centricon centrifugal filter devices with a cut off of 50 kDa with the flow-through containing the target proteins. This was unsuccessful as the target proteins were too close to the cut off resulting in a large loss of the target protein. It was also observed that plc2 ran slower on the SDS-PAGE gel than expected from its predicted mass of 46.9 kDa (Figure 3.24). Buffer exchange was carried out in the Centricon devices of 5 and 10 kDa cut-off and less than 10% of the protein was lost. Although not a high percentage in the final protein mixture, the minor contaminating proteins were taken into consideration when the alpha toxin proteins were used for the vaccine trials.
Figure 3.23: Optimisation of IMAC wash buffers.

A) Alpha toxin - Imidazole concentrations, Lane 1: 70mM, Lane 2: 80mM, Lane 3: 90mM, Lane 4: 100mM and Lane 5 & 6: 200mM; B) plc2 - Imidazole concentrations, Lane 1: 60mM, Lane 2: 70mM, Lane 3: 80mM, Lane 4: 90mM, Lane 5: 100mM and Lane 6 & 7: 200mM; and C) plcinv - Imidazole concentrations, Lane 1: 60mM, Lane 2: 70mM, Lane 3: 80mM, Lane 4: 90mM, Lane 5: 100mM and Lane 6 & 7: 200mM. M: SeeBlue Plus2 protein standard marker.
Figure 3.24: Concentrated fractions of plcInv, plc2 and alpha toxin.

A) Coomassie stained gel - M= SeeBlue Plus2 protein standard marker, Lane 1: plcInv, Lane 2: plc2, Lane 3: plc. B) Western blot - M= SeeBlue Plus2 protein standard marker, Lane 1: plcInv, Lane 2: plc2, Lane 3: plc.
3.3.9 TCA precipitation studies of hemolysin secreted alpha constructs

The detection of the plcInv, plcNSPInv and plc104 fusion proteins from the pMOHly1 vector was difficult for two reasons. The first was the deliberate removal of the 6xHis-tag at the genetic level as this was for purification means via IMAC and was not essential for the protein expressed in the pMOhly1. Hence with the removal of the His-tag the easiest method to concentrate the expressed proteins was eliminated so other methods had to be investigated. The second difficulty was the level of expression from the hemolysin vector as it has been reported to be as little as 25 to 100 µg/L (Hahn and von Specht, 2003). Three bacterial vectors, E. coli, S. Typhimurium LT2-9121 and STM1 harbouring the pMOHly1 constructs were grown in broth culture and the supernatant was tested for the expressed constructs.

TCA precipitation studies were carried out on 10 mL cultures. These samples were taken from both mid-log phase and overnight cultures. This method of concentration only yielded a minute amount of precipitated protein for both cultures. This was then completely dissolved and used for the SDS-PAGE and the western blot. For the mid-log phase cultures no reaction was observed from any of the bacterial vectors.

For the overnight cultures, there was a reaction for each of the bacterial hosts with the reactions shown for the vaccine vector STM1 (Figure 3.25). The pMNInv fusion construct expressed the highest level and showed a band at 50 kDa. There was a second band below the expected one at approximately 48 kDa which would indicate that the N-terminus part of the hlyA' has been removed. It would appear that this cleavage is not 100% efficient. The pMOInv fusion construct also expressed well and had a single band lower than the expected 53 kDa. It appears to be approximately 46 kDa which would indicate that the C-terminus portion of the hlyA' has been
removed. The pMO104 construct was also expressed but not as strongly by the other constructs. There was a weak band expressed at the predicted size of 23.22 kDa with a stronger one at approximately 12 kDa which is the predicted size of the pMO104 protein without either the N-terminus or C-terminus hlyA secretion signals. Expression of these fusion constructs was confirmed in all bacterial vectors and at two different temperatures. Normal growth and expression was observed at 37°C (Figure 3.25) as well as at 42°C (not shown) which is a significant temperature as it is the core body temperature of chickens. For the mid-log phase no bands were detected (not shown).
Figure 3.25: TCA precipitation of extracellular expressed proteins from STM1.

M: SeeBlue Plus2 protein standard marker, N: Empty STM1 vector.
3.4 Discussion

3.4.1 Cloning difficulties with pMOhly1

Difficulties were encountered in the construction of some of the recombinant clones. The major difficulty encountered was with the vector pMOhly1. In order to utilise the hly operon in its most efficient form, the NsI site is the ideal place to insert a coding region to be expressed. Restriction profiling proved useful in determining orientation of the insert in this case where the restriction enzyme, Alul, was used for its four-base recognition site. This resulted in clear profiles for the empty pMOhly1 and for the clones that had the insert (Figure 3.10). A difficulty encountered for pMO104 screening is that as the NsI sites remained, when the plc104 was digested out using NsI, it confirms the presence of the insert but not the directionality of it.

PCR determination of the orientation (Figure 3.12 and Figure 3.14) was achieved using different primer combinations by mixing pMOhly1 sequencing primers and the insert primers (Table 3.1). In theory, if the insert was present in either the correct or incorrect orientation there should only be two bands present from the four PCR reactions. In the pMO104 screening, each clone produced three bands and the one that proved to be the correct clone, after sequencing, amplified two bands for the correct orientation and one for the incorrect (Figure 3.12). In the screening of pMOplc2 and pMNplc2, there was a single robust band for the incorrect orientation and then multiple bands for the correct orientation but none at the expected size of 1.2-1.3 kbp (Figure 3.14). These other bands can be attributed to non-specific binding of the primers on the pMOhly1 plasmid. Though the PCR screening was at times unpredictable and the restriction profiling time consuming, when combined they proved successful in identifying the correct pMOInv, pMNInv and pMO104 constructs.
Ligation of the plc2 and NSPplc2 inserts into the pMOhly1 vector was not successful. The size of the insert should not have impeded the insertion of plc2 and NSPplc2 into pMOhly1 as larger inserts have been successfully expressed and reported in the literature (Gentschev et al., 1996; Spreng et al., 2000; Gentschev et al., 2004; Zhu et al., 2006; Hotz et al., 2009). The most probable reason that the inserts were not compatible with the pMOhly1 plasmid is the fact that expression is constitutively controlled. The original plasmid pCRplc2 has an inducible T7 promoter which is not activated until the presence of IPTG, whereas the hly operon is activated upon successful transformation (Gentschev et al., 1996). The production of the toxoids is unlikely to have proved toxic to the host cell as expression in the previous vector accumulated in the cytoplasm of the host cell without being toxic. So as the toxoids were being exported directly to the outer membrane with hemolysin expression, it should not have killed the host cell. The expression of the toxoid-hemolysin complex may have been excessive and caused the host cell to die when the interaction of the HlyD and Tol-C receptors formed too many trans-periplasmic export channels causing leakage of the cytoplasm (Thanabalu et al., 1998; Balakrishnan et al., 2001). If this was the case, the metabolic load of this expression system was the most probable cause of the host cells demise when the insert was in the correct orientation.

3.4.2 Stability assays

Due to the metabolic load that the hemolysin secretion system can impose on a host cell, it was important to verify that the host cell can retain the plasmid without the assistance of selective pressure. The more stable the plasmid is in the vector strain the more likely it is to persist in a vaccinated animal increasing the host immune response. Retention rates of more than 100% can be attributed to sampling during log-phase growth and standard pipetting error. As the plasmids range in size from 11.966 kbp to 12.783 kbp (i.e. relatively large), the retention of the plasmids could be decreased in the STM1 vaccination strain. Overall the pMNIInv plasmid was retained by
STM1 the longest with an average of 86% over the six day period. The pMOInv construct was comparable with an average of 84% but had a lower STM1 retention rate of 58% on Day 6 (Figure 3.15) where pMNInv was still high at 80% (Figure 3.16). These two constructs are very similar and the presence of two signal peptides in pMOInv may be responsible for the slight decrease in stability in STM1. The pMO104 was not as stable as the other constructs with an average of 81% which is surprising as it is the smallest expressed protein yet had the steepest decline in retention (Figure 3.17). However, these levels of retention of plasmid are relatively high, and as the expression is constitutive should enable good amounts of recombinant protein to be secreted and available to the host immune system.

3.4.3 Expression and purification of the alpha toxin constructs

The purpose of this study was to construct non-toxic stable proteins based on the alpha toxin which while inactive will still retain immunogenic epitopes. This has been achieved by a) partial deletion of the active site or b) site mutagenesis of two amino acids at the DNA level. These molecular changes resulted in inactivation of the toxin’s two main activities – haemolysis (Figure 3.19) and lecithinase (Figure 3.20). Variants of alpha toxins have been designed in previous studies with site mutagenesis changing one amino acid to an alternative one (for a complete summary of refer to (Flores-Díaz et al., 2004)). Individual changes to the histidine at position 68 and aspartic acid at position 56 reduced lecithinase activities to 0.2% of that of the original toxin, and no activity, respectively (Nagahama et al., 1995; Guillouard et al., 1996; Nagahama et al., 1997). By having these two amino acids changed to serine and alanine, respectively (changing two nucleotides in each codon), this ensured that the protein plc2 would be highly unlikely to revert to full toxicity as four mutation events would be required in the plasmid pCRplc2. The protein plc2 displayed full inactivation of haemolysis and lecithinase activities yet was fully reactive to the anti-alpha toxin serum. This demonstrated that whilst the protein was inactivated
the key immunogenic epitopes were retained. The immunogenicity of plc2, along with plcInv and inactivated alpha toxin will be further explored in Chapter 4.

The use of supplements in media to increase growth of bacterial vectors is well known for large scale batch cultures (Yee and Blanch, 1992). The same idea was applied to these small scale cultures to increase expression of the recombinant protein. The sugars were chosen as either monosaccharides (glucose and fructose) or disaccharides (maltose and sucrose) due to the ease of digestion. As a simple carbon source all of these sugars had an effect on the expression of alpha toxin and plcInv. Glucose was used as a supplement in subsequent purification experiments.

For the expression of alpha toxin, supplemented sucrose had the most positive effect by increasing the expression over the 5 h induction period (Figure 3.21 (E)). Degradation of the alpha toxin was unexpected as when purified from culture grown in LB broth breakdown was rarely observed. For the protein plcInv, degradation was often observed after purification hence why supplements were investigated to see if a more stable product could be purified. The glucose supplemented culture produced more protein overall however a small proportion of this was degraded plcInv (Figure 3.22 (E)).

The higher migration of the plc2 protein than the predicted size of 46.8 kDa was unexpected. This had not been experienced with the other constructs as the deletion of sections of the proteins resulted in the clear reduction of the predicted size of protein. In other published studies, substitutions of one amino acid at similar positions in the alpha toxin have also shown a similar occurrence (depicted in Figure 3.24) using western blot analysis with anti-alpha toxin serum (Nagahama et al., 1995; Guillouard et al., 1996; Nagahama et al., 1997). In those studies, as here, this minor size difference did not adversely affect the purification or the stability of the variants.
3.4.4 Expression of alpha toxin constructs using a hemolysin expression system

The expression of the plcInv, plcNSPInv and plc104 was successful from the pMOhly1 plasmid and was observed from all of the bacterial vectors. The expression from STM1 vector is the most important as this is the vector which will be used for vaccination trials. Although it is an efficient recombinant expression system, it has been observed that expression levels can vary from 25 to 100 µg/L of culture supernatant (Hahn and von Specht, 2003).

Hahn and colleagues (2003) found that culture size greatly affected the protein expression rate in the hemolysin expression system due to the reduction in oxygen saturation levels in small culture volumes. This activation of the hemolysin system under reduced molecular oxygen might be beneficial under in vivo conditions as the gastrointestinal tract is a low oxygen environment. It could result in a higher level of expression of the fusion products which potentially could increase the potency of the immune responses.

All of the fusion-constructs were expressed successfully from the STM1 vector and were reactive with the anti-alpha toxin sera (Figure 3.25). The maintenance of the immunogenic epitopes on the fusion constructs is essential if these plasmids were to be used to stimulate protective immune responses in mice and chickens against C. perfringens. This will be explored in the subsequent experiments using a vaccine trial using a mouse model and immunological studies in chickens. The protective responses invoked by the IMAC purified proteins will also be tested in a gas gangrene mouse model.
4.1 Introduction

The bacterium \emph{Clostridium perfringens} can cause gas gangrene in humans (Flores-Diaz and Alape-Giron, 2003) and necrotic enteritis in poultry (Al-Sheikhly and Truscott, 1977). Both of these diseases are difficult to treat due to the location of the infection, hence why an efficacious vaccine is required. The mouse lethal dose of alpha toxin has been previously reported as <0.1 µg/mouse which makes it a highly potent toxin (Titball \emph{et al.}, 1989). As discussed in Chapter 1, alpha toxoid has been demonstrated to be immunogenic in previous studies (Amimoto \emph{et al.}, 2002; Zekarias \emph{et al.}, 2008; Cooper \emph{et al.}, 2009), with varying levels of protection achieved.

Although the ultimate objective is to find a vaccine for use in poultry, the vaccine candidates were first tested in a commonly used gas gangrene model carried out in mice. Gas gangrene is characterised by the production of gas within the tissues as a result of metabolic activities of the multiplying \emph{C. perfringens} and the production of numerous toxins (Shimizu \emph{et al.}, 2002). The clostridial toxins reduce the oxygen saturation in the infected area which increases proliferation of \emph{C. perfringens} resulting in further spreading of the infection and more extensive tissue damage (Shimizu \emph{et al.}, 2002). Alpha toxin assists by causing intravascular coagulation, reduced perfusion of neutrophils and the development of thrombosis (Bunting \emph{et al.}, 1997; Flores-Diaz \emph{et al.}, 2004; Sakurai \emph{et al.}, 2004), which maintains the anaerobic conditions required for the infection to continue. Cyanosis ensues followed by necrosis of the deoxygenated tissues, leaving the area swollen, necrotic and black and without treatment the infection will be fatal (Stevens, 2000).
Two different types of vaccines were evaluated in these mouse model studies. The first was the protein constructs in purified sub-unit form described in Chapter 3. The second vaccine type is an attenuated STM1 harbouring the hemolysin expression vector encoding three different toxin-constructs. The hemolysin expression operon and its genes have been described in Section 4. The main function of this system is to transport the fusion proteins outside of the vaccine strain so that host humoral immune responses can be efficiently induced even if only during the extracellular phase \textit{in vivo} (Gentschev \textit{et al.}, 1996). Expression levels of hemolysin fusion proteins from the carrier strains has varied from as low as $<0.01 \, \mu g/mL$ to $3.5 \, \mu g/mL$ (as reviewed in Hahn and Specht, 2003). The hemolysin system will express the majority of hemolysin fusion protein into the supernatant however small amounts can be found in the periplasm and the cytoplasm (Zhu \textit{et al.}, 2006). The hemolysin secretion system in a heterologous delivery vector, such as STM1, is shown in Figure 4.1.

To confirm that the alpha toxin variants are immunogenic when delivered alone or as a fusion construct, the two different vaccines were tested in the gas gangrene mouse model. The objectives for these animal trials are: a) to determine the effect of the protein vaccine on the development of gas gangrene; b) to determine the level of protective immune response induced by the protein vaccines; c) to ascertain if the level of expression of the fusion proteins is sufficient to provide a level of protection; and d) to establish if the use of a carrier strain influences the immunogenicity of the fusion proteins.
In STM1, the fusion proteins are expressed as secreted protein (Figure 3.25). Surface-bound fusion protein (which assists in pathogen detection by APC) and cytoplasmic fusion proteins were not detected by Western blot on whole cell lysates (not shown). Figure obtained from (Gentschev et al., 2002).
4.2 Materials and methods

4.2.1 Protein preparation

Proteins plc, plcInv and plc2, described in Chapter 3 (Table 4.1) were purified to homogeneity as described in Section 3.2.9. Tris was removed from the protein fractions by buffer exchange with PBS using Centricon centrifugal devices (Section 3.2.9.6) and the protein fractions concentrated using the same devices.

4.2.2 Toxoid preparation

Formaldehyde (Sigma-Aldrich, Germany) was added directly to a solution of plc (prepared in PBS at a concentration of 1 mg/mL) to give a final concentration of 0.4% (v/v) formaldehyde. The mixture was incubated at 37°C overnight on a rotating platform. Toxin inactivation was confirmed by the addition of 40 μL of formaldehyde treated sample to wells bored into a Nagler and SBA plate in the presence of 10 mM CaCl₂. Residual formaldehyde was removed by buffer exchange with PBS in Centricon centrifugal filters and the protein content of the solution was reconfirmed by the Bradford method and spectrometry, as protein losses during buffer exchange could be quite high.

4.2.3 Adjuvant preparation

Prior to vaccinations, inactivated plc (toxoid), site deletion protein plcInv, and site mutagenesis protein plc2 were prepared in an equal volume of Freund’s incomplete adjuvant (FIA) (Sigma) following the protocol outlined in Ausubel (1994). Briefly, an emulsion was prepared by drawing up and expelling equivalent volumes of protein and FIA through a 21 gauge needle in a 3 mL glass syringe until the fluidic suspension increased markedly in viscosity and was no longer able to
Table 4.1: Purified proteins used to vaccinate mice via the intraperitoneal route.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
<th>Location of His tag</th>
</tr>
</thead>
<tbody>
<tr>
<td>plc</td>
<td>The complete α-toxin of <em>C. perfringens</em> (inactivated prior to vaccination)</td>
<td>C-terminus</td>
</tr>
<tr>
<td>plcInv</td>
<td>Plc with an internal deletion of aa residues 56-75 which encompass two zinc binding residues essential for phospholipase C activity.</td>
<td>C-terminus</td>
</tr>
<tr>
<td>plc2</td>
<td>Plc with a two site mutagenesis of amino acid position 56 (aspartic acid to alanine) and position 68 (histidine to serine).</td>
<td>C-terminus</td>
</tr>
</tbody>
</table>
disperse when a drop was placed in a glass beaker of cold water. Vaccines prepared in adjuvant were used within 6 h of preparation.

4.2.4 Mouse vaccine trial using protein vaccine candidates

Four groups, each containing 6 female BALB/c mice (6-8 weeks old), were vaccinated via the intraperitoneal route using a 1 mL syringe and 21 gauge needle with 100 μL of protein: adjuvant emulsion. The groups consisted of a toxoid plc:FIA, plcInv:FIA, plc2:FIA and PBS:FIA control. Mice were vaccinated on days 1, 15 and 30 with 25 μg of protein at each vaccination. Mice were bled prior to each vaccination.

4.2.5 Mouse vaccine trial using STM1 expressing the engineered toxin variants

Four groups, each containing 6 female BALB/c mice (6-8 weeks old), were vaccinated via the subcutaneous route using a 1 mL syringe and 21 gauge needle with 100 μL of STM1 carrying the constructs pMO104, pMNInv, pMOInv along with the empty vector pMOhly1. Another four groups, each containing 6 female BALB/c mice (6-8 weeks old), were vaccinated via the oral route using a 1 mL syringe and a oral gavage needle with 100 μL of STM1 carrying the constructs pMO104, pMNInv, pMOInv along with the empty vector pMOhly1. All oral groups were vaccinated on days 1, 15, and 30 with $10^8$ CFU of STM1 harbouring each of the vaccine constructs at each vaccination. Subcutaneous groups were vaccinated on the same days but with $10^6$ CFU of STM1 harbouring each of the vaccine constructs at each vaccination. Mice were bled prior to each vaccination.
4.2.6 *C. perfringens* challenge

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

The culture was separated by centrifugation in sterilised 250 mL autoclave bottles at 10,000 x g for 10 min, the supernatant discarded and the pellet washed in sterile saline, pelleted as above and resuspended in a final volume of 3 mL saline (including bacterial volume). Mice received 50 µL containing approximately $10^9$ cells by an intramuscular injection to the left thigh. The right thigh was left unchallenged as an internal control. Mice were monitored closely for the first 4 h, and every 6-8 h for the following 72 h. Severity of gangrene in the mice was determined using an index calculating the severity of infection of challenged mice (Table 4.2). Mice were examined and scored according to Table 4.2 every 8 h for the first 48 h post-challenge, or more frequently if disease progression was acute.
Table 4.2: The index used to score mice infected with *C. perfringens*.

The reduced perfusion of vessels and migration of *C. perfringens* is indirectly observed via the swelling (oedema of infected thigh and gas production by *C. perfringens*) and blackening (cyanosis) of the injection site, and eventual gas-gangrene development towards the foot pad.

<table>
<thead>
<tr>
<th>Score</th>
<th>Clinical signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Swelling/limping of infected thigh</td>
</tr>
<tr>
<td>1</td>
<td>Cyanosis (blackening) at the injection site / Swelling of the foot pad with pink colouring</td>
</tr>
<tr>
<td>2</td>
<td>Spread of cyanosis to the thigh surrounding injection site</td>
</tr>
<tr>
<td>3</td>
<td>Spreading of cyanosis to ankle / Swelling of foot pad with dark red colouring</td>
</tr>
<tr>
<td>4</td>
<td>Blackening of complete foot pad (sacrifice) / Mouse found dead</td>
</tr>
</tbody>
</table>
4.2.7 ELISA protocols

Humoral antibody responses of vaccinated mice were measured using the ELISA as outlined in Section 2.6.6 with some modifications. Wells were coated with 3 μg/mL of purified alpha toxin (plc). Doubling dilutions of sera obtained from vaccinated mice were used as the primary antibody, and goat anti-mouse IgG-HRP (diluted 1:5000 in PBST/1% skim milk) was used as the secondary conjugated antibody. Following the addition of the substrate TMB, plates were incubated for 15 min before the addition of 50 μL of a 1 M solution of sulphuric acid. The endpoint was determined as the dilution at which the optical density (OD) at a wavelength of 450 nm (OD$_{450}$), was 3 times the background level.

4.2.8 Adsorption of sera to STM1

Four millilitres of TE buffer were used to flood an agar plate confluent with STM1 grown for 18 h at 37°C. The cells were harvested and separated by centrifugation for 10 min at 5,445 x g. The cells were washed in TE buffer and were ruptured on ice using 6 x 20 s bursts of sonication with a 30 s cooling off period between each cycle. Insoluble matter was separated by centrifugation at 14,000 x g for 5 min and the supernatant filtered through a 0.45 μm filter. Two millilitres of the supernatant was used to adsorb 15 μL of anti-sera previously diluted in 1 mL TST. This was left shaking at room temperature for 4 h or at 4°C for 24 h. The adsorbed anti-serum was then stored at -20°C until required.
4.2.9 Tissue sampling

At necropsy, spleens were removed and stored in sterile tubes on ice. Spleens were then homogenised with 1mL sterile PBS and spread onto LB plates and LB plates with 100 μg/mL ampicillin with serial dilutions of each homogenate. Plates were then grown overnight at 37°C and the colonies enumerated. Colonies grown on LB plates with 100 μg/mL ampicillin were then subjected to alkaline lysis plasmid extraction (Section 2.3.1) and the plasmids screened for constructs (Section 3.1.5). Homogenates were stored at 4°C for no longer than 48 h whilst the screening took place.

4.2.10 Statistical analysis

All statistical analysis was calculated using the program Microsoft Excel 2007 using the Data Analysis add-in. The results of all ELISA assays for the detection of anti-alpha toxin antibodies were analysed for equal variance within the data set. If unequal variances were encountered this was taken into account and a Welch’s T-test was used. Results displaying a normal distribution and equal variances were analysed using Student’s T-test and ANOVA methods.
4.3 Results

4.3.1 *Clostridium perfringens* challenge against protein vaccinated mice

Prior to challenge, sera was taken from all mice and analysed for the presence of anti-alpha toxin antibodies. All mice vaccinated with the purified protein vaccines of alpha toxoid, plc2 and plcInv developed an anti-alpha toxin antibody response, whilst the mice vaccinated with PBS:FIA did not develop a detectable response above the background (Figure 4.2). The group with the highest IgG titres was the plcInv vaccinated group; with the plc2 inducing a lower level and alpha toxoid was the least immunogenic of the three proteins. Each of the protein vaccinated groups induced significantly higher titres than negative control of PBS, the plcInv and the plc2 groups also induced significantly higher titres (p<0.05) than the toxoid.

The index used to score the development of gas gangrene was based on previous challenge trials (Awad et al., 2000; Awad et al., 2001) and is outlined in Table 4.2. All mice including the toxoid vaccinated mice displayed localised swelling at the site of challenge injection and limping in the injected thigh so these clinical symptoms were excluded from the index. Mice that developed cyanosis from the thigh to the footpad (Figure 4.3) were euthanized due to animal welfare considerations.
Figure 4.2: Elicited IgG response after three vaccinations with purified protein vaccines.

Bars with * indicate significant difference at p < 0.05 in comparison to the PBS control group.
Figure 4.3: Clinical signs of *C. perfringens* gas gangrene in mice.

A) Swelling of the thigh, ankle and foot caused by gross vasodilation of blood vessels, cyanosis is visible on the upper thigh and abdomen indicative of a score of 3 according to the index (Table 4.3). B) Cyanosis of the footpad indicative of a score of 4 according to the index (Table 4.3). C) Comparison of left and right thighs of a mouse infected with *C. perfringens*. Inflammation and swelling is prominent in the infected thigh on the left.
Development of gas gangrene was observed in all vaccinated groups. In the current study, two mice displayed cyanosis on the affected thigh and an open wound from the injection site at 77 h post-challenge. A third mouse developed an open wound at the conclusion of the observation period and was euthanized. In the plcInv group, four mice developed a black foot pad indicating an index score of 4 after 40 h post-challenge. Mice that were not vaccinated with protein but PBS:FIA developed signs of gangrene similar to that of plcInv vaccinated mice (Figure 4.4). The plcInv group did not have a delayed onset of gas gangrene despite having high anti-alpha toxin antibodies prior to challenge (Figure 4.5).

The plc2 vaccinated group did display a delay in onset of gas gangrene with no mice displaying higher than a CI score of 2 for the first 32 h post-challenge (Figure 4.4). This was despite having a lower detected level of anti-alpha toxin antibodies than the plcInv group which had a higher antibody titre but a higher cumulative CI score over the 88 hour observation period. In the plc2 vaccinated groups there were 4 mice out of 6 that displayed no signs of gas gangrene (CI score 0) until the conclusion of the trial.
Figure 4.4: Progression of gas gangrene over 88 h in mice vaccinated with purified toxin proteins.

Group name with * indicates significant reduction (p<0.01) from the PBS control group. % CI Score is the sum of CI Index scores for all mice in the group over the maximum CI Score possible (i.e. 5 x n) expressed as a percentage.
Figure 4.5: IgG response in BALB/c mice (grouped data, n=6) to purified alpha toxin vaccines.

All groups displayed a significantly higher titre (p<0.05) that the PBS control group (not shown) which displayed no reactivity to the alpha toxin specific ELISA.
4.3.2 *Clostridium perfringens* challenge against STM1 carrying pMOhly1 constructs vaccinated mice

Prior to challenge, sera was collected from all groups and tested for reactivity against both STM1 and alpha toxin. It was found that after adsorption of the sera to STM1 (Section 4.2.8) no reactivity was observed against the alpha toxin. The same was observed when the sera were adsorbed with alpha toxin instead. As will be seen, this compounds the analysis; therefore it was proposed that STM1 contains epitopes cross-reactive with alpha toxin. As this cross reactivity masks the specific antibodies against alpha toxin, all the ELISAs were carried out on normal sera with no adsorption.

The humoral response induced by oral vaccinations with pMO104, pMOInv and STM1 carrying pMOhly1 were relatively higher than with their subcutaneous vaccinations (Figure 4.6). The pMO104 construct elicited the highest IgG antibody response with pMNInv eliciting the lowest response. However, none of the vaccinated groups, neither oral nor subcutaneously delivered, displayed a significantly increased response when compared to the STM1 carrying pMOhly1 (p>0.05). All groups had induced IgG responses against the vector strain, STM1 (Figure 4.7). The IgA response was higher in the orally vaccinated mice than the subcutaneous vaccinated group but they did not differ significantly (p>0.05) (Figure 4.8).

The challenge culture was grown to an estimated $5 \times 10^8$ CFU/mL to give challenge inoculums of $10^9$ CFU. Retrospective plate counting of the challenge inocula on SBA revealed that mice were given $4.6 \times 10^8$ CFU/mL instead of the calculated dose of $10^9$ CFU per mouse. The reduced inoculum was adequate in producing severe signs of gas gangrene. Mice that developed cyanosis from the thigh to the footpad (Figure 4.3) were euthanized due to animal welfare considerations.
Figure 4.6: IgG responses (obtained from pooled sera, 1:25 dilution) against alpha toxin from oral and subcutaneous STM1 vaccination carrying the following constructs.
Figure 4.7: IgG responses against STM1 from oral and subcutaneous STM1 vaccinated mice.
Figure 4.8: Alpha toxin specific IgA after vaccinations Days 1, 14 and 28.

No significant differences were observed between any of the groups.
After the challenge, progression of gas gangrene was rapid (Figure 4.4). Both the oral and subcutaneous groups that were vaccinated with STM1 carrying the pMOhly1 vector developed a CI score of 4 within 14 h of challenge (Figure 4.8). The group with the lowest CI score at the conclusion of the observation period was the orally vaccinated pMOInv group at 83.33%. There was no significant difference in overall progression of the disease at 31 h post-challenge as all groups followed the same trend. Upon necropsy, spleens were recovered and tested for the presence of STM1. STM1 was recovered from one third of the mice and of these, 31.25% harboured the construct plasmids. The most recovered plasmid from the spleen was pMNInv from two mice in the oral group and one in the subcutaneous group, with only one mouse positive for the pMO104 construct and one for the pMOhly1 construct (Figure 4.9). The best group for recovery of STM1 alone was the oral pMNInv where all 6 of the mice in the group tested positive for the STM1 in their spleens (Figure 4.10).
Figure 4.9: Progression of CI scores over 31 h post-challenge after oral or subcutaneous vaccinations.

No significant differences were observed between any of the groups. SC – subcutaneous route, Oral – oral route. % CI Score is the sum of CI Index scores for all mice in the group over the maximum CI Score possible (i.e. 5 x n) expressed as a percentage.
Mice from the oral pMNInv vaccinated group were positive for STM1 in significantly higher numbers (p<0.05) than the other groups (denoted by *).

Figure 4.10: Recovery of plasmids and STM1 from spleens.
4.4 Discussion

Two alpha toxin variants, one a deletion mutant and the other a substitution mutant were developed, expressed and purified as described in Chapter 3, were examined for their potential as vaccines against *C. perfringens*. Immunogenicity was determined using BALB/c mice when administered intraperitoneally with FIA. Both of the vaccines, plcInv and plc2, as well as the positive control alpha toxoid produced a significant titre of anti-alpha toxin IgG (Figure 4.2). The plcInv vaccine did induce a significantly higher IgG titre than the alpha toxoid which was unexpected as the alpha toxoid may have been expected to induce the highest titre as it is the same sequence as the native alpha toxin. It also had a disease progression similar to the unvaccinated PBS control group (Figure 4.4). This reduction in titre is probably caused by the formaldehyde in the inactivation incubation. The alpha toxins physical state is affected by the formaldehyde resulting in denatured protein with a subsequent loss of conformational epitopes. Therefore, some antibodies induced by the inactivated toxoid may not recognise epitopes on the native toxin in the ELISAs.

The challenge inoculum was lower than the anticipated $10^5$ CFU/dose; however it was adequate in reproducing the signs of gas gangrene. Mice that survived longer than 48 h either displayed a CI score of 3 over many h or showed no symptoms at all. Two thirds of the plc2 vaccinated group (4/6) demonstrated full protective responses as they did not develop any symptoms of gangrene. This indicates that the substitution mutant retains the same immunogenic level of alpha toxoid, even though the IgG levels were lower than the plcInv vaccine. This indicates the presence of alpha toxin neutralising antibodies which can result in the significant delay in developing a high CI score. The plcInv protein, which has part of the $\alpha$-helical region of the active site removed, is not as effective at eliciting neutralising protective responses, although the IgG titre was relatively high.
In the second challenge trial, three constructs pMO104, pMNInv and pMOInv, that were developed and confirmed for expression as described in Chapter 4, were tested in BALB/c mice for the induction of protective immune responses against alpha toxin from *C. perfringens*. These constructs were stable in the vaccine strain STM1 and were able to express detectable levels of the alpha toxin variants (Chapter 3). The main difficulty encountered was the lack of specific reactivity of the induced antibodies to the native alpha toxin as shown by the lack of reactivity when the serum was adsorbed with STM1 lysate. This lack of reactivity was observed in both the IgG (Figure 4.6) and IgA (Figure 4.7) ELISAs.

This lack of antigen specificity indicates one of three problems: non-specific binding, cross reactive binding or undetectable levels of responses. The relatively high levels of humoral responses to the control pMOhly1/STM1 compared to the toxin constructs indicate that there is non-specific induction of humoral responses. To determine if there is cross-reactive binding the alpha toxin protein sequence was compared against the *Salmonella* genome. A hypothetical protein (GenBank: BAE20151.1) demonstrated some sequence identity (44%) with alpha toxin. So there is a possibility that between these similar proteins the specific antibodies against alpha toxin are lost in the adsorption due to cross-specific binding. In other recent studies cross-reactivity has not been reported however these studies do not adsorb their sera against the carrier strain before testing for specific antibody responses (Gentschev *et al*., 2004; Zhu *et al*., 2006; Hotz *et al*., 2009). Finally, it may be that the expressed amount of fusion constructs is not substantial enough to induce a detectable level of humoral responses.

Despite there being difficulties identifying specific immune responses the ELISAs did show that the route of administration is important. For the IgG responses, the mice that were orally vaccinated showed a relatively higher level of alpha toxin specific and STM1 specific IgG than when the vaccine was delivered in a subcutaneous manner. This can be attributed to the STM1
being delivered in a manner akin to its natural infection route and the higher dose delivered to the mucosa-associated lymphoid tissue (MALT). For orally vaccinated mice it was expected that alpha toxin-specific IgA levels would be higher than in the subcutaneously vaccinated mice as the STM1 colonise the mucosal surfaces of the intestinal tract when delivered orally. However there was no significant difference in the alpha toxin-specific IgA levels measured from either the oral or subcutaneous vaccinated groups.

The progression of gas gangrene in the second challenge trial was more rapid than that of the first trial showing that the two different vaccine types induce different immune responses. As the ELISAs showed specific anti-alpha toxin antibodies could not be detected, it was unlikely that there would be a significant effect on the onset of symptoms. Both control groups that were only vaccinated with STM1 carrying pMOhly1 developed gas gangrene in all of the mice by 11.5 h post-challenge, which was the same for the subcutaneous vaccinations of pMO104 and pMOInv. The other test groups were somewhat more delayed with the majority of the mice having a CI score of 3 at 14 h for the pMNInv and pMOInv orally vaccinated and the subcutaneous vaccination with pMNInv. The last group to develop a mean CI score of 3 was the orally pMO104 vaccinated showing that the highest titre measured for the IgG (Figure 4.6) and IgA (Figure 4.7) may be true anti-alpha toxin antibodies that partially neutralised the active toxin.

The spleen is an important lymphoid organ in terms of vaccination as both innate and adaptive immune responses can be mounted in this one organ. The white pulp of the spleen houses APCs and undifferentiated T cells whereas the marginal zone has a large population of macrophages and B-cells (Mebius and Kraal, 2005). Consequently, when the STM1 is recovered from the spleen it is probable that both innate and adaptive immune responses are being elicited. The recovery of STM1 was significantly higher in pMNInv vaccinated mice than any of the other groups (p<0.05) as STM1 was recovered from each mouse. Overall, STM1 (with or without plasmid) were found in
one third of the mice in the trial. In terms of plasmid, pMNInv was recovered from 3 mice, two from the oral group and one from the subcutaneous group. This was expected as the stability of this construct was the highest at 86% after 6 days of non-selective culture (Figure 3.13). The pMO104 and pMOhly1 constructs were recovered just once each and pMOInv was not recovered at all. Both pMO104 and pMOInv were stable in the STM1 with 81% (Figure 3.17) and 84% (Figure 3.15) retention, respectively. This suggests that stability in vivo does not correlate with how the constructs will be recovered and disseminated throughout the body after vaccination.

The first mouse trial showed that the induction of partial protection by antigen-specific humoral responses could be achieved using the purified recombinant proteins. Partial protection was observed in the plc2 vaccinated group (Figure 4.4) and significantly high antibody levels were observed for the other variant, plcInv (Figure 4.2). However in the second trial, where the constructs were delivered via STM1, the lower expression levels by the hemolysin system negate this prophylactic potential against gas gangrene (Figure 4.8). In terms of protection against gas gangrene these STM1 vectored vaccines are not successful. However, the use of the gas gangrene mouse model to test for protective responses is not entirely appropriate as it is believed that necrotic enteritis is caused by prolonged exposure to small doses of C. perfringens (Kaldhusdal et al., 1999; Lovland and Kaldhusdal, 2001; Hofacre et al., 2003). So, what may be an inadequate neutralising immune response against gas gangrene in mice may be a sufficient neutralising immune response against alpha toxin and necrotic enteritis in chickens. Both diseases also affect completely different regions of the hosts anatomy – gas gangrene is a deep fascia infection whereas necrotic enteritis occurs in the intestinal tract. Hence these vectored vaccines will be tested in immunogenicity trials in the target animal, chickens.
5.1 Introduction

Poultry is one of the largest naturally occurring animal reservoirs for *Salmonella* (Zhang-Barber *et al*., 1999). The presence of *Salmonella* species in broiler and layer flocks has a devastating effect on the quality and price of the product. In 2010, the USA experienced its largest consumer egg recall when there was a four-fold increase in reported *Salmonella* Enteritidis infections nationally over the period of May to July (CDC, 2010). The recall was for any eggs produced by two Iowa farms and accounted for approximately 500 million eggs that had been sold nationally under multiple brand names (BBC, 2010). The use of attenuated *Salmonella* vaccines to eliminate *Salmonella* infections and reduce colonisation rates has been quite successful in other countries and eliminates the risk of such recalls occurring.

Several live attenuated commercial vaccines exist, including STM1, which when used in chickens cause no signs of illness and can provide heterologous protection (Barrow *et al*., 1990; Copper *et al*., 1992; Coloe *et al*., 1995; Mohler *et al*., 2006; Bohez *et al*., 2008). Modification of these attenuated vaccines into delivery vectors has been widely investigated utilising a diverse assortment of heterologous pathogen antigens (Coulson *et al*., 1994; Bachtiar *et al*., 2003; Garmory *et al*., 2003; Stokes *et al*., 2007; Pan *et al*., 2009; Bartolomé *et al*., 2010). There have been various degrees of success using *Salmonella* as a delivery vector depending on the host animal and the disease, including targeting necrotic enteritis in chickens using alpha toxin as a potentially protective antigen (Kulkarni *et al*., 2008; Zekarias *et al*., 2008). The method of heterologous delivery or DNA vaccines has also been investigated leading to the theory that
prokaryotic expression in the *Salmonella* vector (from cryptic bacterial promoters in the CMV promoter) is equally important as eukaryotic expression after transfection for inducing immune responses (Gahan et al., 2009).

Although the complete mechanism of pathogenesis of necrotic enteritis has yet to be fully elucidated, targeting of potential vaccines to the MALT and the gut associated lymphatic tissue (GALT) is the most logical choice as this is where lesions occur during the infection. This was taken into consideration when selecting the hemolysin expression system to used in STM1 so that induction of humoral responses in the MALT and GALT could be achieved. The first trial aimed to establish immunogenicity levels in chickens against both the STM1 vector and the *C. perfringens* alpha toxin via two vaccination routes, oral and subcutaneous injection. A necrotic enteritis challenge model was not conducted as repeated efforts to have a reproducible model at the university proved unsuccessful (personal communication, Jason Chang).

One potential impediment for the use of live attenuated *Salmonella* vaccines is pre-existing immunity as a result of pre-exposure to either a heterologous or homologous strain. For chickens *Salmonella* exposure can occur via both horizontal and vertical transmission and many different strategies have been utilised to reduce such transmission including feed and drinking water acidification, immune strategies based on passive and active immunity, nutrient composition to reduce susceptibility to *Salmonella* and improved hygiene practices (Vandeplas et al., 2010). It was reported that a bivalent killed vaccine in laying hens significantly reduced vertical *in ovo* transmission of *S. enterica* serovars Enteritidis, Typhimurium and Gallinarum biovar Pullorum (Okamura et al., 2007). However, the consequence of *in ovo* transmission, where the chicks will already be colonised prior to *Salmonella* vaccination is not yet clear.
In Australia, *Salmonella enterica* serovar Sofia is the most prevalent strain found in poultry and one study recently demonstrated that S. Sofia was the dominant isolate found in carcasses before processing with 45/89 (51%) isolations and 51/69 (74%) isolations after processing (Mellor *et al.*, 2010). This serovar is also found dominantly in the litter of poultry farms which would assist transmission between chickens (Chinivasagam *et al.*, 2010). This prevalence of S. Sofia could influence the efficacy of live *Salmonella* vectored vaccines, especially if vertical transmission occurs by *in ovo* transmission as well as horizontal transmission from other birds and environmental exposure.

Currently there is much speculation over the effect of pre-existing immunity on heterologous delivered antigens. There has been various reports that draw the conclusion that pre-existing immunity leads to an up-regulation of the immune response magnitude to the delivered antigen (Bao and Clements, 1991; Whittle and Verma, 1997; Kohler *et al.*, 2000; Kohler *et al.*, 2000; Jespersgaard *et al.*, 2001; Metzger *et al.*, 2004; Sevil Domenech *et al.*, 2008; Saxena *et al.*, 2009). However, there has been convincing reports that pre-existing immunity diminishes heterologous antigen-specific immune responses and that this effect is a restrictive limitation of this delivery system as exposure to *Salmonella* is common (Attridge *et al.*, 1997; Roberts *et al.*, 1999; Vindurampulle and Attridge, 2003; Vindurampulle and Attridge, 2003; Sevil Domenech *et al.*, 2007; Gahan *et al.*, 2008). These reports draw their conclusions from mouse models which is why this study was designed to test the effect of S. Sofia colonisation in a chicken immunogenicity trial to determine if either the humoral or cell-mediated immune responses to subsequent vaccines are affected.
5.2 Materials and methods

Throughout this chapter, STM1 is left out of the vaccine nomenclature for brevity.

5.2.1 Chickens and bacterial strains

Newly hatched Cobb 500 broiler chicks were obtained from Inghams Farms, Pakenham, Victoria. The strain of *S. Sofia* used in the colonisation study was kindly provided by Dr Margaret McKenzie, Inghams Enterprises Pty Ltd., Springwood, Queensland.

5.2.2 Immunogenicity trial via subcutaneous and oral vaccination routes

The immunogenicity of the pMO104/STM1, pMNInv/STM1, pMOInv/STM1 and pCRplc2/STM1 vaccines was determined in a chicken model. Four groups of six chickens were vaccinated via the oral route and four groups of six chickens were vaccinated via the subcutaneous route. Each vaccination consisted of $10^8$ CFU in a total volume of 1 mL for oral vaccinations and 0.5 mL for subcutaneous vaccinations. Chickens were vaccinated three times in a period of 28 days. Blood samples were taken from the wing vein prior to the first vaccination and at necropsy. Faecal samples were taken prior to the first vaccination and one day prior to necropsy for IgA analysis. Necropsy occurred two weeks after the final vaccination. Samples from the caecal contents, spleen, liver and cloaca were collected at necropsy (Section 6.1.4). Plates were incubated at 37°C for 18-24 h.

5.2.3 Immunogenicity trial using chicken colonised with *S. Sofia* prior to vaccination

Two vaccines were selected to determine if immune responses could still be induced in the presence of a common commensal, *S. Sofia*. The vaccines were pMO104/STM1 and
pCRplc2/STM1 with the empty vector pMOhly1 as a negative control. Three groups of five chickens (5 days old) were colonised with S. Sofia five days prior to the first oral vaccination and three groups of five chickens were left un-colonised. Faecal samples were collected for five consecutive days from all chickens and enumerated, and chickens observed twice daily for signs of abnormal behaviour and/or malaise. Briefly, the faecal matter was suspended in sterile LB broth and dilutions were made of the faecal suspension and plated out onto Hektoen and XLD media in duplicate. Plates were incubated at 37°C for 18-24 h. Subsequently, two vaccinations consisting of $10^8$ CFU in a total volume of 1 mL were administered over a period of 14 days. One week post-vaccination two chickens were euthanized and their organs analysed for the presence of S. Sofia and STM1 (Section 6.1.4) and the final three chickens two weeks post vaccination. Blood samples were collected at necropsy. Faecal samples were collected prior to the first vaccination and one day prior to necropsy for IgA analysis.

### 5.2.4 Recovery of *Salmonella* from the organs.

Standard microbiology methods were used to recover bacteria from the chicken organs (Copper *et al.*, 1992; Cox *et al.*, 2007). Each organ was removed aseptically at necropsy and stored at 4°C until processed. Samples were streaked onto selective media Hektoen Enteric agar and XLD agar (Oxoid). STM1 could be distinguished from S. Sofia from the lack of hydrogen sulphide production on both the Hektoen agar and XLD agar. Cloacal swabs were obtained and streaked onto selective media Hektoen Enteric agar and XLD agar. Cecal contents were diluted in LB broth and spread onto Hektoen Enteric agar and XLD agar plates. Vaccine constructs were isolated on ampicillin supplemented media (100 μg/mL) and confirmed by PCR amplification once isolated by Qiagen Miniprep (Qiagen, Australia).
5.2.6 ELISA protocols

Antibody responses of vaccinated chickens were measured using the ELISA as outlined in Section 2.5.7 with a few modifications. Wells were coated with 3 μg/mL of purified alpha toxin (plc). Doubling dilutions of sera obtained from vaccinated chickens were used as the primary antibody, and goat anti-chicken IgG-HRP (Sapphire Bioscience, Australia) diluted 1:5000 in PBST/1% skim milk, was used as the secondary conjugated antibody. Following the addition of the substrate TMB, plates were incubated for 15 min before the addition of 50 μL of a 1 M solution of sulphuric acid. The endpoint was determined as the highest dilution at which the OD at a wavelength of 450 nm (OD_{450}) was 3 times higher than the background level.

5.2.7 Preparation of faecal samples for IgA analysis

Faecal samples were collected within minutes of defecation over a period of an hour. Samples were weighed and then frozen at -20°C until processed. Standard faecal preparations of 1:10 dilutions (w/v) were made in PBS, and the samples were vortexed vigorously for 5 min and allowed to stand at room temperature for 1 h (Holt et al., 1999). After centrifugation at 15,000 x g for 15 min, the supernatant was decanted and added to the ELISA plate in triplicate. The ELISA protocol was carried out as per Section 6.1.6 with the secondary conjugated antibody of rabbit anti-chicken IgA – HRP diluted 1:5000 in PBST/1% skim milk (Sapphire Bioscience, Australia).

5.2.8 Chicken splenocyte preparation

The chicken splenocytes were homogenised by squashing the spleen using the back of a 3 mL syringe. The homogenised cells were resuspended in 5 mL of RPMI and filtered through a cell strainer (BD Falcon). The splenocytes were pelleted by centrifugation at 1,600 x g for 5 min at 4°C, the RPMI solution decanted and cells resuspended in 5 mL of ACK lysing buffer to lyse any
erythrocytes present. Following 5 min incubation on ice, 5 mL of RPMI was added to the lysing solution and the cells pelleted by centrifugation (1600 x g, 5 min, 4°C). The cells were washed 3 times in RPMI and resuspended in 1 mL of RPMI. Cell viability was calculated using Trypan Blue exclusion. Ten microlitres of cell suspension, 10 μL of Trypan blue (Sigma) and 80 μL of PBS were mixed and viable cells counted in a haemocytometer chamber using an inverted microscope. Cell concentration was adjusted to 10⁶ cells/ 90μL in RPMI. The splenocyte suspension was used to determine chicken IFN-γ levels (Section 6.1.7)

5.2.9 Determination of IFN-γ levels

Chicken IFN-γ levels were determined using the Chicken IFN-γ Cytoset kit (CAC1233, Biosource-Invitrogen, USA). A 96 well plate was coated overnight at 4°C with 100 μl anti-chicken IFN-γ antibody (1.0 μg/mL) in Coating Buffer (137 mM NaCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, pH 7.4); the wells washed twice with 400 μL Wash Buffer (1.5 mM KH₂PO₄, 8.3 mM K₂HPO₄, 1.4 mM EDTA, 0.05% v/v Tween 20, pH 7.4) and patted dry with autoclaved paper towelling. Unbound capture antibody was discarded and the plates rinsed once with 400 μL Wash Buffer. Excess liquid was removed from the plate by patting the bottom of the plate onto sterilised dry absorbent paper, and wells were blocked with 300 μL Assay Buffer (137 mM NaCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, 0.5 w/v BSA – fraction V, pH 7.4) for 1 h at room temperature. A volume of 100 μL of RPMI was added to each well whilst the chicken IFN-γ standards using RPMI and splenocyte samples were prepared (Section 6.1.6). The RPMI was discarded, plates were patted dry and 90 μL of splenocytes added to each well (containing 10⁶ cells/ 90 μL). All standard and sample wells were seeded in triplicate, as well as three wells per control. The addition of 10 μl of a stock (10 μg/mL) of concanavalin A (Sigma-Aldrich, Germany), a mitogen would serve as a positive control, 100 μg per well BSA would serve as a negative unrelated protein control and 10 μL of RPMI to confirm that there was no contamination
of the tissue culture media. Alpha-toxin was prepared in RPMI at a concentration of 250 μg/mL and 10 μL added to each sample well.

Subsequently, 50 μL of Detection Biotin antibody at a concentration of 0.5 μg/mL was added to each well and the plate incubated at room temperature shaking at 700 rpm. The plates were washed 5 times with 400 μL Wash Buffer and patted dry with autoclaved paper towelling. To each well 100 μL of working Streptavidin-HRP was added and the plate incubated at room temperature for 30 min shaking at 700 rpm. The plates were washed 5 times again with 400 μL Wash Buffer and patted dry with autoclaved paper towelling. To develop the assay, 100 μL of TMB (BD) was added to each well and incubated at room temperature with continual shaking at 700 rpm for 30 min. The assay was stopped with the addition of 100 μL 1.8 N sulphuric acid to each well and the absorbance at 450 nm measured. The amount of chicken IFN-γ was derived from a log-log standard curve. The independent Student’s t-test was used to determine any statistical differences between vaccine groups.

5.2.10 Statistical analysis

All statistical analysis was calculated using the program Microsoft Excel 2007 using the Data Analysis add-in. The results of all ELISA assays for the detection of anti-alpha toxin antibodies were analysed for equal variance within the data set. If unequal variances were encountered this was taken into account and a Welch’s T-test was used. Results displaying a normal distribution and equal variances were analysed using Student’s T-test and ANOVA methods.
5.3 Results

5.3.1 Immunogenicity trial via subcutaneous and oral vaccination routes

Samples of blood were taken prior to vaccination with STM1 to ensure that the chickens were immunologically naive to Salmonella at hatching and were not exposed by in ovo transmission. All pre-bleed sera and pre-vaccination faecal samples were negative (below background level) for the presence of Salmonella specific humoral responses (data not shown). Vaccination doses were confirmed by retrospective plate count and none were found to be over the $10^8$ CFU in the volume of 1 mL given for oral vaccinations and 0.5 mL dose given for subcutaneous vaccinations. They were not significantly less than the required dosage.

5.3.1.1 Humoral and cell mediated responses

The sera collected at necropsy was analysed for the presence of IgY humoral responses specific to both the alpha toxin and the STM1 vector in all chickens. All orally and subcutaneously vaccinated groups showed humoral IgY responses above the background levels of the prevaccination sera including the pMOhly1/STM1 vaccinated control group (Figure 5.1). The highest IgY alpha-toxin-specific immune response was obtained from the subcutaneously vaccinated pMOInv and this was a significant increase (p<0.05) when compared to the pMOhly1/STM1 vaccinated control group. The lowest IgY response from the subcutaneously vaccinated chickens was the pMOhly1/STM1 vaccinated control group. For the orally vaccinated groups there were no significant differences between any of the groups or with the pMOhly1/STM1 control group.

The presence of IgY humoral responses specific to STM1 was also determined (Figure 5.2). The highest IgY STM1-specific immune response was obtained from the subcutaneously vaccinated...
Figure 5.1: Anti-alpha toxin IgY humoral responses detected after three vaccinations via two different routes.

The pMOInv/STM1 induced humoral responses at a significantly higher level via the subcutaneous route (denoted by *) compared to the control construct of pMOhy1/STM1.
Figure 5.2: Anti-STM1 IgY humoral responses detected after 3 vaccinations via two different routes.

All constructs administered via the subcutaneous route induced a significantly higher humoral response than their oral counterpart (denoted by *).
chickens in the pMOhly1/STM1 vaccinated control group. All of the subcutaneously vaccinated groups demonstrated significantly higher (p<0.05) IgY STM1-specific immune responses than their orally vaccinated equivalent group. However, the IgY STM1-specific immune responses from the pMOInv/STM1 and pCRplc2/STM1 vaccinated groups were not above control levels. None of the orally vaccinated chickens showed IgY STM1-specific immune responses above the level observed in pMOhly1 vaccinated chickens.

Faecal IgA responses were determined against both the alpha toxin (Figure 5.3) and the STM1 vector (Figure 5.4). The alpha-toxin-specific IgA responses were below control level for the subcutaneously vaccinated groups with the exception of the pCRplc2/STM1 vaccinated group. This group had a significant increase (p<0.05) compared to the other test groups and the pMOhly1/STM1 vaccinated control group. The orally vaccinated test groups all had significant increased responses (p<0.05) when compared to the pMOhly1/STM1 vaccinated control group. The highest alpha-toxin-specific IgA responses occurred in the pMO104/STM1 groups and this was a significant increase when compared to the other test groups and the pMOhly1/STM1 vaccinated control group.

STM1-specific IgA responses were determined to be significantly higher in the orally vaccinated groups than the subcutaneously vaccinated equivalent which were below background level and showed no significant differences between the groups (p>0.05) (Figure 5.4). The highest STM1 specific IgA response was from the pMO104/STM1 orally vaccinated group and this was significantly higher (p<0.05) than in the pMNInv/STM1, pMOInv/STM1 and pCRplc2/STM1 vaccinated groups. When compared to the pMOhly1/STM1 vaccinated control group, all orally vaccinated test groups IgA responses were significantly higher (p<0.05).
Figure 5.3: Anti-alpha toxin IgA humoral responses detected after 3 vaccinations via two different routes.

A significant increase in IgA responses compared to the control was observed by all constructs delivered by oral vaccinations and pCRplc2 via the subcutaneous route (p<0.05, denoted by *).
Figure 5.4: Anti-STM1 IgA humoral responses detected after 3 vaccinations via two different routes.

All constructs delivered orally demonstrated significant increase in the IgA response when compared to the control (p<0.05, denoted by *).
Cell mediated responses were measured by the production of IFN\(_{\gamma}\) by splenocytes in response to stimulation by alpha toxin and BSA as a negative control (Figure 5.5). Orally and subcutaneously delivered pMO104/STM1 induced a significantly higher IFN\(_{\gamma}\) level than the counterpart pMOhly1 controls (p<0.05). Of the subcutaneously delivered groups the highest IFN\(_{\gamma}\) response was induced by from the pCRplc2/STM1 vaccine. There was no difference in the induced IFN\(_{\gamma}\) responses between the pMNInv/STM1 and pMOInv/STM1 vaccines and the pMOhly1 control delivered by either the oral or subcutaneous routes.

5.3.1.2 Presence of constructs and vector strain from tissue samples

The presence of the vaccine constructs and strains was determined at necropsy from the ceca, cloaca, liver and spleen (Table 5.1). This part of the study reports on the number of chickens whose particular organs contain STM1 with construct and also the number without the construct. This indicates how successfully the construct is retained and delivered by STM1 in organs where virulent wild-type Salmonella upon infection would invade. Recovery of STM1 could not be determined for some samples due to the presence of other bacterial and fungal species in the samples (particularly the cloacal swabs) which outgrew the STM1 on the plates. For the ceca, all vaccine constructs from both vaccination routes were able to be detected, the lowest being 7/8 in the subcutaneous pMO104/STM1 group. The vaccine strain alone without construct was also isolated although not to the same degree with the lowest being 2/8 chickens that were orally vaccinated with pMOhly1/STM1, and the highest being 6/7 orally vaccinated chicken with pMO104/STM1. This suggests that there is good persistence of the constructs when the STM1 disseminates to the organs.
Figure 5.5: Induced IFN-γ from vaccinations with STM1 carrying alpha toxin construct.

For oral delivered vaccines, pMO104 demonstrated a significant increase compared to the control group (p<0.05, denoted by *). In terms of subcutaneous delivery, the pCRplc2 construct significantly induced a higher IFN-γ response than the control group (p<0.05, denoted by *). Negative control splenocytes were stimulated with BSA as an unrelated protein control with minimal IFN-γ induced.
Table 5.1: Recovery of vaccine constructs and vaccine strain alone from individual chickens in each vaccination group.

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<th>CECA</th>
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<th>CLOACA</th>
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<th>LIVER</th>
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<td>Construct with STM1</td>
<td>STM1 alone</td>
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<tr>
<td>pMO104/STM1a</td>
<td>7/7</td>
<td>6/7</td>
<td>6/7</td>
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<td>2/7</td>
<td>3/7</td>
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<tr>
<td><strong>Subcutaneous Vaccination</strong></td>
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<tr>
<td>pMOhly1/STM1</td>
<td>8/8</td>
<td>3/8</td>
<td>7/7b</td>
<td>3/7b</td>
<td>0/8</td>
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a One chicken died during acclimatisation of a congenital defect prior to the commencement of the vaccinations.

b Recovery of STM1 could not be determined in some chickens (see Section 5.2.1.2)
From the cloacal swabs, the majority of the orally vaccinated chickens showed the presence of both the construct and the vector strain with the exception of the pMO104/STM1 group (6/7). The vector strain without the construct was recovered in lower numbers with the lowest being the pMOhly1 group (4/8). For the subcutaneously vaccinated groups, the recovery was still high with the construct and vector strain not dropping below half of the chickens in the group (lowest – 6/7, pMNInv/STM1 and pMOInv/STM1 groups). For the vector strain alone, the lowest recovery was 3/7 for the pMOhly1/STM1 group with the other groups remaining above three quarters of the individuals were positive for the vector strain. Again this indicated that the vector strain with plasmid is more prudent that the vector which has lost the plasmid.

The liver samples demonstrated some variation in the recovery rates. For the oral groups, the highest recovery of the construct was 4/8 in the pMOhly1 group and the lowest in the pMO104 group (2/7). For the vector strain alone, most groups were at 5/8 with the lowest being pMO104 group (3/7). In the subcutaneous groups, neither the pMOhly1 control construct nor the vector strain was isolated from any chicken livers. The highest recovery was of the vector strain in the pMO104 group (5/8) then the recovery of pMO104 and pMNInv constructs at 4/8 for both groups.

Recovery of the constructs from the spleen was still relatively high with the highest at 6/8 for pCRplc2 in both the orally and subcutaneous vaccinated groups, and the lowest was 4/8 for the pMNInv and pMOhly1 groups. Even the recovery of the vector alone was 7/8 and 6/8 for the pCRplc2 for the oral and subcutaneously groups, respectively and the lowest at 3/8 for pMOInv group for the oral group. Once again the pMOhly1 construct with vector strain and the vector strain alone could not be isolated from any of the spleen samples in the subcutaneous vaccinated group.
5.3.2 Immunogenicity trial using chickens colonised with S. Sofia prior to vaccination

Samples of blood were collected prior to colonisation with S. Sofia to ensure that the chickens were immunologically naive to *Salmonella* at hatching and were not exposed by *in ovo* transmission. All prevaccination sera and faecal samples were negative (below background level) for the presence of *Salmonella* specific humoral responses. Vaccination doses were confirmed by retrospective plate count and none were found to be over the $10^8$ CFU in the volume of 1 mL given for oral vaccinations and were not significantly less than the required dose.

5.3.2.1 Recovery of S. Sofia from faeces after colonisation

S. Sofia colonies were not recovered over the five day period from the faecal samples of the chickens that were not inoculated. All of the chickens that were colonised showed slight signs of salmonellosis in the first two days with their faecal matter having a more watery appearance and consistency than prior to colonisation. However, normal feeding behaviour and activity was observed from all subjects after inoculation. The highest level of faecal S. Sofia recovered was at 24 h post-inoculation at $1.32 \times 10^8$ CFU/g with the lowest being on the fifth day (120 h) post-inoculation at $1.24 \times 10^8$ CFU/g.

5.3.2.2 Humoral and cell mediated responses to vaccination

The sera collected at necropsy was analysed for the presence of IgY humoral responses specific to both the alpha toxin and the STM1 vector in all chickens. The IgY responses specific against alpha toxin did not register higher than the pMOhly1 control level for either the colonised or non-colonised groups (Figure 5.6). Each of the vaccine groups elicited an IgY response against STM1 higher than the prevaccination control level in chickens that were not colonised with
Figure 5.6: IgY responses induced in chickens specific to alpha toxin detected after two vaccinations at Days 12 and 26.

Responses measured were not significantly above control pMOhly1 levels.
S. Sofia prior to vaccination (Figure 5.7). The groups that were colonised prior to vaccination had significantly increased IgY responses to STM1 (p<0.05). The responses elicited from the two vaccines pMO104/STM1 and pCRplc2/STM1 were not significantly different (p>0.05) when compared to each other, as expected.

Faecal IgA responses were also determined against alpha toxin and STM1. Specific IgA responses to alpha toxin were elicited by both vaccines (Figure 5.8). The pMO104 group did not significantly vary between the colonised and the non-colonised groups (p>0.05). For the pCRplc2 vaccine there was a significant decrease (p<0.05) in the colonised group compared to the non-colonised group. The STM1 specific responses from the pMO104 construct was significantly increased (p<0.05) in the colonised group compared to the non-colonised group (Figure 5.9). The pCRplc2 construct elicited a significantly lower IgA response in the colonised group (p<0.05) compared to the non-colonised group.

Cell mediated responses were measured by the production of IFN-γ by splenocytes in response to stimulation by alpha toxin and BSA as a negative control (Figure 5.10). The levels of chicken IFN-γ were significantly different for each construct used between the colonisation and non-colonisation groups (p<0.05). The construct pMO104 induced a significantly lower level of IFN-γ when the chickens were colonised with S. Sofia (297 pg/mL; non-colonised at 421 pg/mL) whilst the pCRplc2 construct induced a significantly higher level of IFN-γ in the colonised group (417 pg/mL; non-colonised 269 pg/mL). The pMOhly1 control group demonstrated a significantly lower level of IFN-γ when the chickens were colonised with S. Sofia however this is not antigen-specific (207 pg/mL; non-colonised 660 pg/mL).
Figure 5.7: IgY responses induced in chickens specific to STM1 detected after two vaccinations at Days 12 and 26.

Significant differences were observed for the groups colonised with S. Sofia (p<0.05, denoted by *).
Figure 5.8: Faecal IgA responses specific to alpha toxin after two vaccinations at Days 12 and 26.

The pCRplc2 group showed a significant decrease in the colonised chickens (p<0.05, denoted by *).
Figure 5.9: Faecal IgA responses to STM1 after two vaccinations at Days 12 and 26.

The pMO104 group both showed a significant increase in responses when colonised with *S*. Sofia (*p*<0.05, denoted by *). The pCRplc2 group showed a significant decrease in IgA responses when colonised (*p*<0.05, denoted by *).
Figure 5.10: Cell-mediated responses as deduced by chicken IFN-γ production.

Each group is significantly ($p<0.05$) different from its colonisation counterpart (denoted by *), with reduction for the pMO104 construct and an increase for the pCRplc2 group. Negative control splenocytes were stimulated with BSA as an unrelated protein control with only 15 pg/mL IFN-γ induced which is below the recommended limits of the assay.
Recovery of constructs, vector strain and S. Sofia from tissue samples

Recovery of the vaccine constructs with and without the vector strain and S. Sofia was determined at necropsy from the ceca, cloaca, liver and spleen (Table 5.2). Chickens that were not inoculated with S. Sofia before vaccination were negative for S. Sofia strain in organs or tissues samples at necropsy. This was despite all groups being housed in the same room (but in separate pens). The colonisation strain S. Sofia was isolated overall from all colonised chickens most frequently from the spleens (73.33%, 11/15), then the livers (66.67%, 10/15), followed by the cloacal swabs (46.67%, 7/15), and the least in the ceca (27%, 4/15).

The ceca had relatively high recovery rates for the constructs for all three vaccines in the non-colonised groups: pMO104 – 3/5, pCRplc2 – 3/5 and pMOhly1 – 4/5. This was decreased significantly (p<0.05) in the S. Sofia colonised groups which had pMO104 vaccination (0/5) but not for the pCRplc2 (1/5) and pMOhly1 (2/5) groups. The same trend was observed for the isolation of the vector strain alone in the chickens that were colonised: significant decrease (p<0.05) for pMO104 group – 1/5, non-colonised 4/5; no significant difference for pCRplc2 – 1/5, non-colonised 3/5; and pMOhly1 – 2/5, non-colonised 4/5.

The constructs were isolated from the cloacal swabs from all non-colonised groups with the pMOhly1 group being identical (2/5, colonised: 2/5) and no significant difference for the pCRplc2 group (2/5, colonised: 4/5). There was a significant reduction for the pMO104 vaccinated group (p<0.05) in isolation from 3/5 of the non-colonised group versus 0/5 of the colonised group. For the isolation of the STM1 vector strain alone, there was a significant decline in the isolation from the colonised chickens (p<0.05). The biggest significant difference in the cloacal swabs was in the pMO104 group which was 3/5 for the non-colonised group compared to the colonised group at no isolations (0/5). Similarly, for both the pCRplc2 and pMOhly1 groups 2 out of 5 chickens were...
Table 5.2: Recovery of vaccine strains and vaccine constructs when the chickens are colonised with S. Sofia.

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Table 5.2 continued: Recovery of vaccine strains and vaccine constructs when the chickens are colonised with *S. Sofia*.

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<td>pMOhly1/STM1</td>
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positive for the STM1 strain alone when not colonised, but in the colonised groups no chickens tested positive (0/5).

In the liver, the constructs were isolated from all chickens with the exception of the pCRplc2 groups where 3/5 had the construct recovered. In the pCRplc2 group this recovery rate did not vary in the colonised group which remained at 3/5. The two other constructs had a significant reduction in the recovery in the colonised group (pMO104 – 5/5 versus 1/5; pMOhly1 – 5/5 versus 2/5). Similarly, this was also observed for the STM1 alone. All the groups had a significant decrease (p<0.05): pMO104 – 5/5 versus 1/5; pCRplc2 – 3/5 versus 0/5; and pMOhly1 – 5/5 versus 2/5.

For the spleen, significant reductions in the isolation of the vaccine constructs in the colonised chickens were observed for the pMO104 group (0/5; non-colonised 3/5) and the pCRplc2 group (3/5; non-colonised 5/5). The pMOhly1 group had no significant difference in recovery (3/5; non-colonised 3/5). Isolation of the STM1 vaccine strain was significantly reduced in the pMO104 group (0/5; non-colonised 3/5) and the pMOhly1 group (1/5; non-colonised 4/5). The pCRplc2 group had 4/5 isolations from the non-colonised chickens and 3/5 from the colonised chickens.
5.4 Discussion

5.4.1 Immunogenicity trial via subcutaneous and oral vaccination routes

Four vaccine constructs, pMO104, pMNInv, pMOInv and pCRplc2 were tested for immunogenicity in chickens using STM1 as a vector delivery strain via two different vaccination routes, oral and subcutaneous. In the following discussion, STM1 is left out of the vaccine nomenclature for brevity. The delivery of the hemolysin system was also being compared to a cytoplasm accumulation vector, pCRplc2 vaccine construct, where the plc2 was expressed into the cytoplasm unlike the hemolysin constructs which expressed extracellularly. Prior to any vaccination, the chickens were confirmed to be immunologically naive to Salmonella which confirmed that the source of our chickens had effective measures in place to prevent Salmonella colonisation and in ovo transmission. After three vaccinations via the two routes of delivery each of the vaccine constructs induced differing levels of responses for both humoral and cell mediated.

For IgY humoral responses specific to the alpha toxin all groups showed responses. As previously discussed in Section 4.3, humoral immune responses were detected in mice vaccinated with the control pMOhly1 that reacted with alpha toxin in the immunoglobulin ELISAs. These responses were considered to be either cross-reactive or non-specific by standard ELISA method. The same trend was observed in both chicken immunogenicity trials for the humoral responses; however the use of BSA in the chicken IFN-γ assays (Figure 5.5 and 5.10) as a negative control demonstrated that these responses in the pMOhly1 control chickens are most likely cross-reactive with alpha toxin, as splenocytes were not induced to produce IFN-γ in the presence of BSA.
The vaccine construct pMOInv delivered by the subcutaneous route elicited significantly higher IgY responses (p<0.05) in an alpha toxin ELISA than the other groups and was the highest elicited response of either vaccination routes (Figure 5.1). The pMOInv vaccine construct did not have the highest expression in the STM1 vector (Figure 4.9) so it was not expected to produce the highest elicited IgY response. However, it did appear to have complete cleavage of the hemolysin signal peptides which may have contributed to its immunogenicity by revealing more epitopes. The other construct of pMO104 which also had complete cleavage but was not expressed to the same degree, but it also induced moderate responses in both the oral and subcutaneous groups. This indicates that although the expression was not as high as the other constructs the plc104 construct is a strong immunogen even though it was only the C-terminus of the full length toxin.

The IgY responses against alpha toxin (Figure 5.1) were similar for both vaccination routes which indicate that the use of oral vaccination in the poultry industry is effective at eliciting the same magnitude of humoral responses as a subcutaneous vaccination. However, for STM1 specific IgY responses (Figure 5.2) the subcutaneous route was significantly higher (p<0.05) than the oral route for all vaccinated groups. This is to be expected as the subcutaneous route is more effective at eliciting systemic humoral and cell mediated responses (Muir et al., 2000). The oral route is not as effective at eliciting IgY responses but can elicit strong IgA mucosal responses which may be sufficient to protect against pathogens at the site of entry, in this case the intestinal tract.

The IgA responses specific to alpha toxin (Figure 5.3) were significantly higher for each of the orally delivered vaccine constructs compared to the subcutaneous groups. The highest response was from pMO104 demonstrating that the carboxyl terminus of the alpha toxin was being successfully delivered to the MALT and eliciting a strong IgA response despite the expression not being lower than the other two vaccine constructs (Figure 4.9). The IgA response elicited from the subcutaneously delivered pCRplc2 was inconsistent with the expectations of this vaccination.
route as subcutaneous delivery has no direct contact with the mucosal surfaces; however, this effect has been reported previously in chickens (Widders et al., 1996; Muir et al., 2000). The STM1-specific IgA elicited by the oral vaccinations were substantially higher ($p<0.05$) than that of the subcutaneously vaccinated groups which can be attributed to the higher magnitude of exposure of the GALT.

Cell mediated responses were measured by the induction of IFN-γ by splenocytes in the presence of alpha toxin (Figure 5.5). The most consistent result for both the oral and the subcutaneous routes was obtained from the pMO104 vaccinated group which were 217.24 pg/mL and 223 pg/mL respectively. Both of these were significantly higher ($p<0.05$) than their respective control groups. The pCRplc2 orally vaccinated group induced the highest response; however the oral group was the lowest of the vaccinated groups. The recovery of both of these constructs from the spleens of the chickens also showed that they were processed by the chickens’ lymphoid tissue and were colonising the chickens at a substantial level in the cloaca and ceca to induce both humoral and cell mediated immune responses.

Whilst the humoral responses in the mouse trials (Chapter 4) were clearly cross-reactive between a native STM1 protein and the alpha toxin variants, in the chicken trials there are instances where the level of non-specificity has subsided. For example, there are non-specific responses in the case of IgY (Figure 5.1 and 5.2) but in the case of IgA (Figure 5.3 and 5.4) there appears to be more specific responses to both alpha toxin and STM1. Due to this inconsistency, the responses measured in this chicken trial are not considered to be definitive indications of specific responses.
The two vaccine constructs of pMO104 and pCRplc2 were chosen to be tested in a chicken model where the chickens were colonised with S. Sofia prior to vaccination. The two constructs of pMOLinv and pMNInv were not examined in this trial. The vaccine construct pMO104 was chosen as it gave consistent humoral responses and a good IFN-γ response compared to the control groups (p<0.05). The pCRplc2 vaccine was chosen as it could induce significantly high levels of IFN-γ in the chickens demonstrating strong immunogenicity and a potential for stronger cell mediated responses if up-regulation occurred. An effect of either up-regulation or down-regulation of the specific immune responses, either humoral or cell mediated, should be determined with these two vaccine constructs.

5.4.2 Immunogenicity trial using chickens colonised with S. Sofia prior to vaccination

Colonisation with S. Sofia was easily achieved and demonstrated no long term effects besides minor short term diarrheal signs in the litter. The colonisation levels remained relatively high and stable over the period of five days post-inoculation with it not dropping below 10^8 CFU/g. This shows how successful S. Sofia is at colonising a naive chicken and contaminating the environment through the high levels of S. Sofia shed in the faecal matter.

Alpha toxin specific IgY in both the colonised and non-colonised groups (Figure 5.6) did not rise above background levels which was unlike the previous trial (Figure 5.1) which showed a higher magnitude humoral response. The cause of this is unknown but does show that oral vaccinations can be inconsistent for induction of humoral IgY responses. However this may be compensated by the higher level of IgA induced as IgA-mediated phagocytosis of Salmonella would occur much faster and prevent adhesion and invasion of the tissues.
The elicited IgY responses against STM1 (Figure 5.7) significantly increased in the presence of colonisation for both of the vaccine constructs, pMO104 and pCpRplc2 and the pMOhly1 control (p<0.05). The responses measured for the constructs and the control were almost the same magnitude despite there being two different vaccine constructs being delivered by STM1. This indicates that the delivery method of the heterologous antigen is not contributing to the overall immunogenicity of the STM1 although it may diminish slightly compared to the pMOhly1. Once STM1 is sampled by APC in the GALT and T helper cells are activated, IgY will be generated with a higher magnitude than in chickens that are not colonised by S. Sofia. This up-regulation of the serum IgY is the prime-boost response that some vaccines rely on to generate lifelong immunity. The colonisation of chickens by S. Sofia at a young age is priming the immune system for a higher magnitude and avidity response which is shown by the increased response of IgY when the chickens are subsequently exposed to STM1. These induced IgY responses are cross-reactive against both strains of Salmonella.

For an intestinal pathogen like Salmonella, the production of IgA can greatly assist in preventing colonisation and infection as shown in studies with heterologous-delivered Campylobacter jejuni antigens by Salmonella (Wyszynska et al., 2004; Buckley et al., 2010), hence why heterologous antigen delivery to the GALT and MALT is of great importance. For this study it was shown that prior colonisation can directly modulate the induced faecal IgA response against a heterologous antigen from a similar vector strain as the colonising serovar. The faecal IgA response specific to alpha toxin in the colonised group was significantly decreased (p<0.05) for the vaccine construct pCpRplc2 (Figure 5.8). As this vaccine construct accumulates in the cytoplasm and is released upon lysis, a more rapid and effective clearance of the vaccine would account for the reduction in faecal IgA response to alpha toxin, as there would be less accumulation at the time of phagocytosis. No significant difference in the specific-alpha toxin IgA responses was observed in the pMO104 colonised group compared to the non-colonised group. This indicates that the
extracellular expression of this heterologous antigen and recognition by the chickens’ immune system is not affected by prior colonisation by a similar vector strain.

The IgA responses to the vector strain showed a similar outcome for the pCRplc2 group (Figure 5.9). Again the IgA responses specific to STM1 was significantly (p<0.05) down-regulated in the pCRplc2 vaccinated group that was colonised. The elicited IgA response specific to STM1 was up-regulated in pMO104 vaccinated group that was colonised, as well as the pMOhly1 control group. The increase in the response for both the pMO104 construct and pMOhly1 control constructs can be attributed again to the prime-boost effect that is an established outcome of exposing the immune system to the same immunogen multiple times and getting a higher magnitude response on the second exposure. However the pCRplc2 vaccine construct did not exhibit this effect in the colonised chickens.

Cell mediated responses as indicated by the expression of IFN-γ did change with the presence of colonisation. The IFN-γ induced from the splenocytes of colonised chickens was significantly decreased for the pMO104 and pMOhly1 vaccinated groups (p<0.05) but significantly increased in the pCRplc2 group (p<0.05). This suggests that the expression system for the heterologous expression does affect what immune responses are elicited. Extracellularly expressed plc104 from the pMO104 vaccine construct induced higher IgA but less IFN-γ whilst cytoplasm accumulated plc2 from the pCRplc2 vaccine construct had a decreased IgA response but an increased IFN-γ response. The effectiveness of the immune responses induced by both of these vaccine constructs would have to be tested in a challenge trial in order to demonstrate how protective they are; however as discussed previously necrotic enteritis models are extremely difficult to replicate in laboratory settings as the full disease pathology has not been elucidated.
Recovery of vaccine constructs (Table 6.2) was successful from all of the organs but the most dramatic reductions were observed in the ceca of the colonised chickens. Once the chickens were colonised with S. Sofia the presence of the vaccine, both construct and vector strain alone, were unrecoverable in the ceca. This could be accredited to an invasion-inhibition effect by S. Sofia against the STM1 vector strain. This effect has only recently been described where homologous and heterologous strains can result in a lower influx of phagocytes to the caecal tissue and completely prevent invasion during a secondary exposure of a related Salmonella strain (Methner et al., 2010). In the liver and spleen, there were also reductions but not to the same degree as in the ceca. The importance of the spleen has been discussed previously and the presence of S. Sofia in the spleens of the majority of the chickens shows that although the strain does not cause disease, the level at which it colonised the tissue is quite high.

This study has shown that whilst the presence of a related strain of Salmonella serovar can have an effect on the magnitude of humoral and cell mediated responses, delivery of the heterologous antigen to the host system is more important. As shown by the two contrasting vaccine constructs, when the antigen is delivered extracellularly there is no modulation of the IgA response but a down-regulation of cell mediated responses and the opposite is true if the antigen is accumulated in the cytoplasm of the vector strain. The effectiveness of these different response profiles would need to be tested in a necrotic enteritis model to assess if they are protective against the ongoing exposure to low levels of C. perfringens; however as those models are difficult to maintain and reproduce perhaps alternative animal models should be investigated.
Necrotic enteritis is a subtle but devastating disease in the global poultry industry (Parish, 1961; Al-Sheikhly and Truscott, 1977; Songer, 1996; Kaldhusdal et al., 1999). The success rate of antimicrobials in affected flocks has been debatable with antimicrobial resistance observed in some countries (Gharaibeh et al., 2010) but alternative treatments such as competitive exclusion and probiotics feed products to promote balanced intestinal health have been only partially successful in field trials (Hofacre et al., 2003; Thanissery et al., 2010). The development of an efficacious vaccine to protect against the onset of necrotic enteritis would improve the husbandry of poultry and increase the productivity of poultry farms (Kaldhusdal and Lovland, 2000; Lovland and Kaldhusdal, 2001).

This study focused on using variants of the alpha toxin delivered by attenuated Salmonella vectors to elicit immune responses that would be protective against a related clostridial disease in mice, gas gangrene and necrotic enteritis in broiler chickens which is the most commonly affected species in poultry (Lovland and Kaldhusdal, 2001). When used as a subunit vaccine against gas gangrene in mice all recombinant protein variants elicited immune responses; however the plc2 variant was the only protein vaccine to significantly delay the onset of gas gangrene. Both the plc104 and plcInv proteins elicited detectable humoral responses, however these were not protective.

Incorporation of the two deletion variants of plc104 and plcInv into a hemolysin expression system using the pMOhly1 vector was successful and expression was demonstrated. It was unfortunate that the plc2 variant could not be ligated into this system as this variant showed the
most potential from the gas gangrene challenge trial. This outcome is probably caused by over-expression of the hemolysin operon causing either a high metabolic load for the host organism to sustain or excessive pore formation by the interaction of HlyD with Tol-C receptors during expression leading to cell death (Thanabalu et al., 1998; Balakrishnan et al., 2001). This could be examined further by introducing mutations into the HlyD gene to reduce the number of pores formed or into the other genes to reduce the level of expression. This has been successfully done to the HlyB gene to increase expression levels (Low et al., 2010), so a reduction in expression could theoretically be achieved. It has also been reported that expression levels of heterologous antigen from *Salmonella* vectored vaccines do not affect the magnitude and diversity of immune response levels generated by the expressed heterologous antigen (Pathangey et al., 2009) so decreasing the expression efficiency of the hemolysin operon will not decrease its effectiveness as a vaccine. Alternatively, inducible promoters could be used, as done by Saxena and colleagues (2009).

The inability to generate protective responses in mice against gas gangrene does not necessarily mean there will not be efficacy against necrotic enteritis, as the diseases are quite different in both their pathology and progression. In particular, the conditions that lead to the establishment of necrotic enteritis are not fully elucidated, and therefore the required array of immune responses needed to target the disease may be more complex that for gas gangrene, where neutralising antibody is proposed to be the effector mechanism. Alpha toxin is a protective antigen for gas gangrene and it has not been elucidated yet if there is a sole protective antigen for necrotic enteritis. Although alpha toxin was a good candidate for over twenty years, the variability of the literature regarding its role in necrotic enteritis indicates that a multifaceted approach to vaccination is required, including targeting strong T cell responses to selected antigens.
The chicken immunogenicity trial demonstrates that delivery by the STM1 vector is targeted to the MALT and GALT as shown by the IgA responses achieved (Figures 5.3) which could be protective against intestinal invasion by *C. perfringens*. Cell mediated responses were also generated by the pMO104 and pCRplc2 that were significantly higher than the pMOhly1 control (Figure 5.5) suggesting that with the right variant both strong humoral and cell mediated responses can be generated. STM1 was successful at delivering the construct not only to the gastrointestinal tract but to the spleen, ceca, cloaca and liver in the chickens confirming that this attenuated bacterial vector is an excellent delivery option for heterologous antigens.

Pre-existing immunity is a burgeoning issue for veterinary and medical vaccine development and unfortunately it affects all bacterial and viral vectors. The prevalence of avirulent *S. Sofia* was observed rising from approximately 5% in the chicken population in 1980 (Harrington *et al.*, 1991) to currently 51% (Mellor *et al.*, 2010) which reveals that as the levels are increasing over time a larger number of chickens will be exposed. The recovery of *S. Sofia* from the organs was much higher than anticipated considering that it is deemed to be an avirulent commensal. However others have found that similarly avirulent *Salmonella* species can also be recovered from organs (Desin *et al.*, 2009; Wisner *et al.*, 2010). The down-regulation of the cell mediated responses for the extracellularly expressed construct and up-regulation for the cytoplasmic-accumulated construct without modulation of the IgA responses implies that early detection of *S. Sofia* in commercial flocks is critical. If the cell mediated responses are down-regulated resulting in failure of the vaccine this leads to higher production costs and waste of the flock as they will be condemned at slaughter if infected with a human pathogen. Early detection and education of commercial farmers to the implication of pre-existing immunity will lead to more efficacious vaccine usage in poultry.
In conclusion, the responses generated by the alpha toxin variants delivered by STM1 were not protective in mice against gas gangrene and did not elicit high specific responses in chickens. This failure does not preclude the use of the hemolysin system as a heterologous delivery system in *Salmonella* vectors. This system is particularly appropriate for any gastrointestinal pathogens as targeted delivery was achieved with the high levels of IgA detected in the trials. Even though the responses were cross-reactive, there were significant differences which suggest that further examination is required. For this to occur, a consistent and reproducible necrotic enteritis model would have to be used to establish if the responses are protective, and consideration should be made for the inclusion of antigens other than, or in addition to, alpha toxin.
Appendix 1

Lambda DNA digested with PstI run on a 1.5% agarose gel.
Appendix 2

SeeBlue Plus2 protein standard marker by Invitrogen, Australia

<table>
<thead>
<tr>
<th>Protein</th>
<th>Approximate Molecular Weights (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tri-Glycine</td>
</tr>
<tr>
<td>Myosin</td>
<td>250</td>
</tr>
<tr>
<td>Phosphorylase</td>
<td>148</td>
</tr>
<tr>
<td>BSA</td>
<td>98</td>
</tr>
<tr>
<td>Glutamic Dehydrogenase</td>
<td>64</td>
</tr>
<tr>
<td>Alcohol Dehydrogenase</td>
<td>50</td>
</tr>
<tr>
<td>Carbonic Anhydrase</td>
<td>36</td>
</tr>
<tr>
<td>Myoglobin Red</td>
<td>22</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>15</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>6</td>
</tr>
<tr>
<td>Insulin, B Chain</td>
<td>4</td>
</tr>
</tbody>
</table>

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