Novel Immunisation Strategies Against

Salmonella

by

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Abstract

There are currently two vaccines licensed for use against *Salmonella enterica* serovar Typhi (*S. typhi*) infection, namely the live attenuated vaccine and the Vi polysaccharide sub-unit vaccine. Both vaccines induce protective immune responses, however they do so with variable efficacy. Considerable progress has been made in the development of alternative live attenuated *S. typhi* vaccines, however little progress has been made in the development of alternative sub-unit vaccines. Considerable progress has also been made in the comparatively new field of DNA vaccines, however very little progress has been made in their application to *Salmonella*.

This thesis presents two novel immunisation strategies against *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) in the mouse model. Firstly, mice immunised with multiple antigens in the form of a DNA vaccine have been shown to develop specific humoral and cellular responses to proteins encoded within the vaccine. Secondly, it has been shown that immunisation with multiple cytosolic antigens (CA) of *S. typhimurium* SL1344, formulated with the adjuvant dimethyl dioctadecyl ammoniumbromide (DDA), induces strong humoral and cellular responses. These responses have been characterised, and their ability to confer protection on mice challenged with a lethal dose of *S. typhimurium* SL1344 has been investigated.

The aim of DNA vaccination is to induce immune responses to protein antigens expressed *in vivo* by injecting plasmid DNA encoding the antigen sequence. Expression library immunisation (ELI) is a new technique that draws on DNA immunisation and can be developed to identify the key antigens of a pathogen that confer protection. ELI has previously been used in a number of disease models in mice and has been used in this thesis as a means of inducing immune responses against an unspecified subset of bacterial antigens. An expression library (EL) of *S. typhimurium* SL1344 was constructed using a mammalian expression vector encoding EGFP. The expression of *S. typhimurium* SL1344 antigens forming fusion proteins with EGFP was visualised as
green fluorescence in mouse fibroblast cells \textit{in vitro}. The EL consisted of 140,000 clones of which 14,000 were used for immunisation. The library was administered to both BALB/c and CBA mice to examine the effect of mouse strain and was administered both intradermally (ID) and intramuscularly (IM) to examine the effect of immunisation route. Analysis by Western blot and ELISA showed that humoral responses were induced in both BALB/c and CBA mice. ID and IM inoculation produced similar results in BALB/c mice. Responses included a significant Th1 component as judged by the presence of IgG2a in the serum and the secretion of IFN-\(\gamma\) when T cells from peripheral lymph nodes were stimulated by CA in culture. T cell proliferative responses confirmed the presence of activated, antigen specific T cells. The induction of significant humoral and cellular responses following ELI demonstrates the first successful DNA immunisation strategy that uses multiple antigens of \textit{Salmonella} and provides a foundation for work towards the identification of protective antigens.

In an extension to the ELI study, immunological responses of BALB/c mice to multiple cytosolic antigens (CA) of \textit{S. typhimurium} SL1344 formulated with the adjuvant DDA were examined and their role in protection was determined. Mice immunised subcutaneously with CA and DDA induced strong humoral responses, as determined by Western blot and ELISA, with significant increases in CA-specific IgG, IgG1 and IgG2a antibodies. The induction of a significant Th1 component in the response to CA and DDA was shown by the high level of IFN-\(\gamma\) production by T cells upon \textit{in vitro} stimulation with CA. The protective role of CA and DDA was assessed by immunising, as described above, and then challenging with graded doses of virulent \textit{S. typhimurium} SL1344. Although protection was only marginal, in that immunisation was not sufficient to protect BALB/c mice from doses of \textit{S. typhimurium} SL1344 in excess of \(1 \times 10^2\) colony forming units, a 70-fold increase in LD\(_{50}\) was identified.

Together, the two strands of this investigation have shown that mice may be immunised against multiple \textit{S. typhimurium} SL1344 antigens either by ELI, or by direct inoculation with CA together with a suitable adjuvant. Analysis of these responses have indicated
that components which might be expected to be protective in mouse models have been stimulated, notably the activation of Th1 lymphocytes. The failure to demonstrate convincing protection may relate to the \textit{in vivo} expression of antigens used in the CA preparation. However, the level of responses achieved suggest that ELI may be developed as a means of identifying key antigens in \textit{S. typhimurium} SL1344 infection, and both procedures make a novel contribution to the search for safer and more effective vaccines against \textit{Salmonella} infections.
Declaration

I declare that this thesis was composed by myself. The research presented is my own and has not been submitted for any other degree or professional qualification.
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Finally, I would like to thank my family and friends for their encouragement and support throughout my research and for keeping me smiling.
Dedication

To those in my life I love

my parents

my sister

my Mark

Thank you
Thesis Overview

This thesis describes advances made by the author in the field of vaccines for Salmonella, in particular the use of DNA vaccines and cytosolic antigen vaccines. Chapter 1 provides a background to the thesis by reviewing the literature on Salmonella pathogenesis and current Salmonella vaccines. The principles of DNA vaccination and expression library immunisation are also presented.

Chapter 2 provides details of all the materials and methods required to perform the work described in this thesis. The construction of a representative DNA expression library is then discussed in greater depth in the Chapter 3.

Immune responses generated by DNA vaccination with multiple antigens from an expression library of S. typhimurium SL1344 are analysed in Chapter 4, and the effects of the administration route on the resulting immune response are discussed. The effect of the mouse strain on the immune response to DNA immunisation is also studied in Chapter 4. Chapter 5 assesses the use of a cytosolic antigen preparation of S. typhimurium as a potential vaccination strategy.

Discussions and conclusions are drawn in Chapter 6 and recommendations for further work are given. All the references used in this thesis are written in Chapter 7.
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Table 5.4 Proliferative responses of T cells incubated with medium, CA, or Con A, from mice immunised with CA + DDA, DDA alone and from mice receiving NT

Table 5.5 Summary of humoral and cellular immunity induced following immunizations with CA+DDA, DDA alone and mice receiving NT
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>ampicillin resistant</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BGH</td>
<td>bovine growth hormone</td>
</tr>
<tr>
<td>CLIP</td>
<td>class II-associated invariant peptide</td>
</tr>
<tr>
<td>c.f.u.</td>
<td>colony forming units</td>
</tr>
<tr>
<td>Con A</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>CpG</td>
<td>cytidine-phosphate-guanosine</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CA</td>
<td>cytosolic antigen</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DDA</td>
<td>dimethyl dioctadecylammonium bromide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>dioxyribonucleic acid</td>
</tr>
<tr>
<td>dH&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>distilled water</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescence protein</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EL</td>
<td>expression library</td>
</tr>
<tr>
<td>ELI</td>
<td>expression library immunisation</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FAE</td>
<td>follicle-associated epithelium</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>HIS</td>
<td>histidine tag</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>ID</td>
<td>intradermal</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N’-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TBST</td>
<td>tris buffered saline tween</td>
</tr>
<tr>
<td>U/ml</td>
<td>units/millilitre</td>
</tr>
<tr>
<td>V</td>
<td>volts</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-indoyl-β-D-galactoside</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
Diarrhoeal diseases are the second largest cause of death worldwide today, contributing to over 3 million deaths each year (Robinson et al., 1996). *Salmonella enterica* is an important agent of diarrhoeal disease in humans and livestock and is responsible for 1.3 billion reported cases each year (Pang et al., 1995; Ivanoff et al., 1994). Concerns about food and water-borne pathogens, such as *Salmonella*, have increased significantly due to the emergence of antibiotic resistant strains (Parry et al., 2002). As a result, new methods to combat these bacteria are being investigated. DNA vaccination represents a novel means of expressing antigens in vivo for the generation of both humoral and cellular immune responses. DNA vaccines have been demonstrated to elicit protective immunity in a number of disease models (Reviewed Donnelly et al., 1997; Hasan et al., 1999; Gurunathan et al., 2000).

This Chapter consists of two main sections. The first section provides an overview of *Salmonella enterica*, its pathogenesis and current *Salmonella* vaccine strategies. The following section provides details of DNA immunisation, its mechanism of action and how DNA immunisation technology can be used to identify new antigens for pathogens such as *Salmonella*. 
1.1 *Salmonella*

The genus *Salmonella* is greatly heterogeneous, comprising of over 2,200 serotypes that differ in their host range and ability to cause disease (Chan *et al.*, 2003). Although appearing complex, *Salmonella* species are very similar genetically, with serotype differences based on surface antigen differences such as flagella and lipopolysaccharide (LPS). Originally, each different serotype was given a distinct species classification. However, this was later contested and led to the designation of a single species, *S. enterica*, with separate serotype designations (LeMinor and Popoff, 1987). Thus the agents of murine typhoid fever, *Salmonella typhimurium*, and human typhoid fever, *Salmonella typhi* became *S. enterica*, serovar Typhimurium and *S. enterica*, serovar Typhi respectively. However, it has recently been proposed that the genus *Salmonella* comprises of two distinct species, *Salmonella enterica* and *Salmonella bongori* (Baumler *et al.*, 1998; Chan *et al.*, 2003). For the purpose of this thesis, the old nomenclature *S. typhimurium* or *S. typhi* will be used.

*S. enterica* is a species of Gram negative, facultative intracellular bacterium (Briles *et al.*, 1993) that can infect both humans and animals. Infection with *S. enterica* typically arises from the oral ingestion of contaminated food or water, and generally takes the form of a diarrhoeal disease (Finlay and Falkow, 1989; Finlay, 1994). There are three major diseases caused by *Salmonella* in humans namely typhoid fever (caused by *S. typhi*), gastroenteritis (caused by most *Salmonella* strains such as *S. typhimurium* and *S. enteritidis*) and an invasive disease (caused by *S. cholerasuis*). Whilst some *Salmonella* species such as *S. typhi* are very host specific, others are capable of infecting a range of different hosts, causing a range of different diseases. For example, *S. typhimurium* was named for its ability to cause typhoid-like disease in mice, but in humans it causes gastroenteritis.

Some of the most important health problems faced by the world today are diarrhoeal diseases, of which non-typhoidal *Salmonella* infections contribute a staggering 3 million
deaths each year (Ivanoff et al., 1994; Pang et al., 1995). S. typhi also remains an important health threat for humans, with more than 16.6 million cases of typhoid fever and 600,000 deaths annually worldwide (Ivanoff et al., 1995). The incidence of typhoid fever is declining due to the industrialisation of developing countries, however ironically industrialisation has also led to a rapid increase in non-typhoidal Salmonella infections. Typhoid fever is a disease linked to overcrowding and poor sanitation, so improvements in clean water provisions, modern sewerage systems and more recently vaccination have led to its decline in humans. Industrial manufacturing techniques such as intensive animal breeding and large scale food production however provide opportunities for the spread of non-typhoidal Salmonella infections amongst livestock and subsequently through the food chain (Maurice, 1994, Coynault et al., 1996).

Many of the potential problems of S. enterica infection in both animals and humans have been overcome with the use of antibiotics such as chloramphenicol (Parry et al., 2002). However, concerns are mounting over the increase in multi-drug resistant bacteria, in particular the strain of S. typhimurium known as definitive type 104 (DT104) which is resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracycline (Mirza et al., 1996; Glynn et al., 1998; Parry et al., 2002). Non-typhoidal species cause serious disease in immunocompromised individuals, the young and the elderly, and these species are becoming increasingly resistant to many antibiotics. S. enterica therefore remains an important health concern and the need to develop new methods of treatment in animals and humans has become increasingly more important (Wallis, 2001; Parry et al., 2002).

1.1.1 Invasion of Epithelial Cells

Salmonella infection is usually initiated by the ingestion of contaminated food or water (Finlay, 1994; Parry et al., 2002). Internalised Salmonella pass through the stomach into the small intestine where they come into contact with enterocytes and membraneous
epithelial (M) cells of the intestinal epithelium. At this stage *S. enterica* demonstrates its pathogenicity by entering cells of the intestinal epithelium which are normally non-phagocytic (Finlay, 1994; Jepson and Clark, 2001).

M cells reside in the follicle-associated epithelium (FAE) which overlays gut-associated lymphoid tissues. These tissues form domed structures that protrude into the gut lumen between villi and are commonly known as a Peyer's patches (PP). M cells are specialised antigen sampling cells and are characterised by sparse, irregular microvilli on their apical surface, and a cytoplasmic invagination containing lymphocytes and macrophages on their basal surface. M cells transport material across the FAE to the underlying lymphoid tissues where protective immune responses are initiated (Siebers and Finlay, 1996, Gerbert, 1997).

Despite the role of M cells in the detection of foreign antigens, many enteric pathogens like *S. enterica*, target these M cells for initial infection. This is largely due to their specialised function in rapidly transporting internalised material across an already shortened distance from the apical membrane to the lymphoid cells in the basolateral invagination (Gerbert, 1997, Clark *et al.*, 1998). Additionally, reduced quantities of mucus at the FAE surface allow *Salmonella* to access M cells more easily, and the sparse, irregular microvilli on the M cells facilitates cytoskeletal rearrangements required for efficient *Salmonella* invasion (Gerbert, 1997, Jepson and Clark, 2001). In addition to M cells, *S. enterica* is also capable of penetrating neighbouring enterocytes in the FAE, providing an additional pathway for tissue invasion (Hohmann *et al.*, 1978; Kohbata *et al.*, 1986).

Upon contact with intestinal epithelial cells, *S. enterica* inject a variety of bacterial proteins into host cells via the bacterial *Salmonella* pathogenicity Island 1 (SPI-1) type III secretion system. Type III secretion systems are complex organelles composed of over 20 proteins that are organised into a needle structure that spans the bacterial envelope (Galan, 1996; Kubori *et al.*, 1998). Once the needle structure is formed, the
type III secretion system exports other type III secretion proteins encoded on SPI-1 into the cytosol of the host cell. At least 13 proteins delivered by the SPI-1 type III secretion system have been identified of which the functions of SipA, SipB, SipC, SopB, SopE, SopE2 and SptP are known (Zhou and Galan, 2001). Whilst the mechanism by which each protein exerts its function is not fully understood, the resultant effect is bacterial internalisation.

Studies using cultured epithelial cells have shown extensive redistribution of polymerised actin occurs at the site of bacterial-host contact on the apical membrane forming membrane protrusions termed ruffles (Pegues et al., 1995; Galan, 1996; Zhou and Galan, 2001). Membrane ruffling results in the loss of cell integrity and causes the subsequent pinocytosis of Salmonella into the cell. In vivo studies in mice and calves have also identified the ruffling phenomenon, further demonstrating that the primary site of S. enterica entry seems to be M cells, although enterocytes are also invaded by this bacterium.

Following S. enterica invasion of M cells, pinocytotic vesicles coalesce to form larger intracellular compartments and extensive bacterial multiplication occurs (Finlay, Gumbiner and Falkow, 1988; Gahring et al., 1990). Bacterial multiplication results in M cell destruction and the sloughing of the FAE which may in turn provide easy access to subepithelial tissues within this region (Penheiter et al., 1997, Clark et al., 1998). Additionally, the resulting infection of intestinal epithelial cells increases the expression of many cytokine genes such as IL-8 (Eckmann and Kagnoff, 2001), which have chemoattractant properties for neutrophils, monocytes/macrophages, specialised T cells and immature dendritic cells. All these cell types play an important role in the innate and acquired host defence against S. enterica.
1.1.2 Interaction of *Salmonella* with Phagocytic Cells

After invading and passing through the intestinal epithelium, *S. enterica* are engulfed by professional phagocytic cells such as macrophages, polymorphonuclear leukocytes (PMN) and dendritic cells (DC) found within the lamina propria and PP (Jones and Falkow, 1996). In addition to the classical phagocytosis methods of these cells, macrophages, PMN and DC can also take up *S. enterica* via the specific invasion pathway already described for non-phagocytic cells. This section describes the interactions of *Salmonella* with the various types of phagocytic cells and demonstrates that both bacterial and host factors determine the outcome of infection.

1.1.2.1 Monocytes/Macrophages

Monocytes/macrophages play an important role in controlling and clearing *Salmonella* infection from the host. Large numbers of macrophages are located in PP and many more are recruited to the site of infection via cytokines produced by infected intestinal epithelial cells (Eckmann and Kagnoff, 2001). Whilst macrophages, like neutrophils, are capable of destroying *Salmonella*, the interactions between *Salmonella* and macrophages are sometimes more prolonged due to the counteracting defences of *Salmonella* used to resist the killing mechanisms of macrophages.

Production of Cytokines from Monocytes/Macrophages

After the exposure of monocytes/macrophages to *Salmonella* or its products such as LPS, flagellin or porins, an increased production of proinflammatory and chemotactic cytokines is observed (Eckmann and Kagnoff, 2001). Cytokine production serves to recruit additional phagocytic cells to the site of infection and induces the early inflammatory response. Of the cytokines produced, interleukin-1α (IL-1α) and tumour necrosis factor-α (TNF-α) are the classical proinflammatory cytokines that have been
found in all *Salmonella* infected organs studied to date (Eckmann *et al.*, 1996). Additionally, these cytokines have been shown to contribute protective roles in animal models, whereby neutralisation of TNF-α by pharmacological or genetic approaches increases the severity of *Salmonella* infection and decreases host survival. Furthermore, treatment with IL-1 or TNF-α increases host survival following *Salmonella* infection (Morrissey *et al.*, 1995). Another important protective cytokine found during the course of *Salmonella* infection is interferon-γ (IFN-γ), produced by T cells and natural killer (NK) cells. Whilst IFN-γ is not produced by macrophages, it is induced through communication with macrophages via the cytokines IL-12 and IL-18. Again, both IL-12 and IL-18 have shown to be vital in decreasing bacterial numbers and increasing host survival (Eckmann and Kagnoff, 2001).

**Macrophage Cytotoxicity**

Following uptake into macrophages, *Salmonella* can either induce almost immediate cell death via caspase-1 (Monack *et al.*, 2001), or reside within a unique organelle called the *Salmonella* containing vacuole (SCV) (Gorvel *et al.*, 2001). The occurrence of either event is thought to be determined by the activation state of the macrophage and the expression of proteins encoded on SPI-1 (Monack *et al.*, 2001).

The induction of rapid cell death by *Salmonella* is dependant on SPI-1 and the host cell protein caspase-1. Caspases are a family of cysteine proteases that are synthesised as precursors and which undergo proteolytic processing to generate the active enzyme. Through mechanisms not completely understood (although shown to be dependant on the bacterial invasion protein SipB), *Salmonella* induces the activation of caspase-1 which then promotes early cell death in macrophages (Hersh *et al.*, 1999). Additionally, caspase-1 activation occurs in monocytes following the binding of bacterial components such as LPS and bacterial lipoproteins. Caspase-1 not only induces macrophage cell death, it also activates the potent proinflammatory cytokines IL-1β and IL-18 found in the cytosol of macrophages. The resultant increase in inflammation and proapoptotic
responses regulated by caspase-1 is essential for the initial colonisation of the PP (Monack et al., 2000; Monack et al., 2001) and the subsequent dissemination of Salmonella to the liver and spleen (Baumler et al., 1997). Caspase-1 however is not necessary for Salmonella virulence once the bacteria have breached the gastrointestinal barrier (Monack et al., 2000).

Macrophage death occurs rapidly when Salmonella are maximally expressing SPI-1 encoded type III secretion proteins during the early stages of infection. However, Salmonella has the ability to down-regulate the expression of SPI-1 encoded proteins and caspase-1 dependant macrophage cell death to allow for continued intracellular proliferation and systemic spread (Monack et al., 2001). After dissemination and replication, macrophages are killed via an SPI-2 dependant mechanism at sites distant to infection (van Der Velden et al., 2000). Both rapid and delayed macrophage cell death occurs under discreet conditions in vivo at distinct times and locations.

Salmonella Containing Vesicle in Macrophages

During periods of low SPI-1 protein expression, Salmonella are capable of replicating within macrophages without being detected by the immune system. Since the intracellular environment of macrophages is particularly hostile for intracellular bacteria such as Salmonella, the bacteria have developed different mechanisms for survival, one of which involves the biogenesis of the SCV (Reviewed Gorvel and Meresse, 2001).

The SCV is a unique organelle in which Salmonella reside following uptake by macrophages. The biogenesis of SCV has largely been studied in vitro using S. typhimurium and cultured epithelial and macrophage cells. It involves a complex process of maturation, requiring interactions with pre-existing and unique intracellular compartments which can not be detected in non-infected cells (Gorvel and Meresse, 2001). The initial stage in maturation involves the interactions of nascent SCV with early endosomes. This is demonstrated by the acquisition of early endosomal markers
such as the transferrin receptor and is regulated by SopE, a protein delivered by the SPI-1 type III secretion system (Grovel and Meresse, 2001). The intermediate SCV recycles the early endosomal markers and acquires vesicles and lysosomal glycoproteins from late endosomes to form a mature SCV. The acquisition of lysosomal glycoproteins without the mannose 6-phosphate receptor or cathepsin D indicates direct interactions between SCVs and late endosomal compartments do not occur, and that vesicles from the late endosome are independently recruited. This verifies that Salmonella subverts the intracellular trafficking system, so that it never meets the anti-microbial and degradative enzymes of the late endosome. This alteration in intracellular trafficking has been shown to be dependent on the SPI-2 encoded SpiC protein (Uchiya et al., 1999).

After a lag phase of 2-3 hours, Salmonella starts to replicate within the mature SCV. In order for intracellular Salmonella to replicate within a membrane bound vacuole, there must be a progressive net increase in the surface area of the vacuolar membrane. Studies on the virulence factors involved in intracellular replication within macrophages have identified the SPI-2 type III secretion system effector, SifA (Stein et al., 1996). SifA controls the maintenance of the SCV in macrophages and is necessary for the formation of membrane tubules termed Salmonella-induced filaments (Sifs) in epithelial cells. With the maintenance of the SCV, the enclosed Salmonella are protected from the cytosol and can replicate undetected by the immune system and subsequently enter the lymphatics and blood stream to spread to the liver and spleen (Vasquez-Torres et al., 1999).

Evasion of NADPH Phagocyte Oxidase in Macrophages

As previously discussed, Salmonella has developed a number of strategies to enhance its survival within macrophages. In addition to the formation of SCVs, Salmonella is capable of reducing the efficacy of reactive oxygen species with detoxifying enzymes such as superoxide dismutase and oxidative repair systems (Vazquez-Torres and Fang, 2001). Moreover, Salmonella has acquired a type III secretory system encoded by SPI-2
that interferes with the trafficking of vesicles containing functional NADPH phagocyte oxidase to the phagosome (Vazquez-Torres and Fang, 2001).

Following the phagocytosis of *Salmonella* into membrane-bound vacuoles, macrophages assemble a highly effective antimicrobial complex called the NADPH phagocyte oxidase. This enzymatic complex catalyses the reduction of molecular oxygen to reactive oxygen species (ROS) in quantities that exert anti-microbial activity and is critical in the defence against *Salmonella*.

\[
\begin{align*}
\text{O}_2 + e^- & \rightarrow \text{O}_2^- \\
\text{O}_2^- + e^- & \rightarrow \text{H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + e^- & \rightarrow \text{OH}^+.
\end{align*}
\]

*Figure 1.1 Fenton reaction for the generation of ROS in macrophages and neutrophils.*

By limiting the availability of essential nutrients and producing antimicrobial peptides and ROS, macrophages can kill or limit the replication of *Salmonella* in phagosomes. However, experiments have shown effector proteins secreted by the SPI-2 type III secretion system antagonise the NADPH phagocyte oxidase and may prevent the trafficking or targeting of NADPH phagocyte oxidase containing vesicles to the vicinity of the *Salmonella* phagosome (Vazquez-Torres *et al.*, 2000). Although still unclear, it has recently been suggested that the inhibition of NADPH phagocyte oxidase trafficking and phagosome-lysosome fusion, may be related via the SPI-2 protein SpiC (Vazquez-Torres and Fang, 2001).

**Nitric Oxide Production in Macrophages**

Nitric oxide (NO) is a biologically active molecule produced by NO synthases (NOS) and has also been shown to play an important role in the oxidative killing mechanism.
produced by macrophages (Nathan and Hibbs, 1991; Vidal et al., 1993; Pacelli et al., 1995). NO is induced in activated macrophages as a result of infection with intracellular pathogens such as *Salmonella*, and from the dual stimulation of LPS and IFNγ. Whilst NO is itself toxic, it can also potentiate the activity of other ROS.

Studies using an *aroA* attenuated *Salmonella* strain SL3235 have recently shown that the NO produced in response to IFNγ from NK cells generates beneficial microbicidal effects, however, it is also capable of detrimental immunosuppressive effects in adjacent lymphocytes (Eisenstein, 2001). Since the *aroA* SL3235 strain was still capable of inducing protection against virulent *Salmonella*, it was suggested that the NO induced immunosuppression was tolerated temporarily by the host in favour of eliminating the rapidly multiplying bacteria, and that the acquired immune response arose once the NO levels decreased.

The importance of NO production by macrophages in clearing *Salmonella* infection has been further demonstrated by Eisenstein through inhibiting NO in mice immunised with the attenuated *aroA* strain SL3235. Mice receiving the NO inhibitor, although not immunosuppressed, succumbed to the infection and had higher levels of *Salmonella* in the liver and spleen compared to controls 21 days post infection (Eisenstein, 2001).

1.1.2.2 Polymorphonuclear Leukocytes

Whilst many of the initial mechanisms of the early immune response to *Salmonella* rely on macrophages as the major effector cell against this pathogen, it is now well known that polymorphonuclear leukocytes (PMNs) also contribute to resistance (Fierer, 2001). PMNs are part of the innate immune system and appear in the sub-mucosa within hours of *Salmonella* infection in response to IL-8, a specific PMN chemokine produced by infected intestinal epithelial cells. Whilst *Salmonella* have developed several methods to
survive within macrophages, they neither avoid phagocytosis, nor are capable of surviving within PMNs.

The anti-microbial activity of neutrophils occurs before and after phagocytosis of *Salmonella* and involves both oxygen-dependant and oxygen-independent mechanisms (Adams and Hamilton, 1984; Hasset and Cohen, 1989). Similar to macrophages, the oxygen-dependant bacterial killing of neutrophils involves the production of ROS by the NADPH phagocyte oxidase complex (see Figure 1.1). ROS are released via a respiratory burst upon contact or engulfment of the *Salmonella* bacterium and cause fatal damage to bacterial nucleic acids, lipids and proteins.

There are numerous mechanisms for oxygen-independent killing, although many are not fully understood. One important anti-bacterial mechanism is the acidification of the phagolysosome following bacterial phagocytosis. The low pH conditions are inhibitory for a range of bacteria, yet provide optimal conditions for degradative enzymes such as lysozyme to destroy the internalised bacteria. Additionally a number of antimicrobial peptides are found in the phagolysosome which have cytotoxic activity that also aids bacterial killing within the PMNs (Lehrer et al., 1990; Gulig et al., 1996). In addition to the direct microbicidal activity, PMNs can also produce the proinflammatory cytokines IL-1β and TNFα, which serve to recruit other phagocytic cells of the immune system to the vicinity.

The importance of PMNs in *Salmonella* infection has only become evident from recent investigations (Fierer et al., 2001). Experiments have shown that whilst wild-type macrophages have sufficient antimicrobial activity to contain *Salmonella* that lack virulence plasmids, macrophages with a mutant allele of Slc11A1 (and therefore more susceptible to *Salmonella*) require the antimicrobial actions of PMN to combat infection. Since murine Slc11A1 mutants are the result of mouse inbreeding and have no known counterpart in animals in nature, these results have been interpreted to suggest that PMN
are necessary in providing resistance to more virulent *Salmonella* strains that are capable of multiplying within human host macrophages.

1.1.2.3 Dendritic Cells

Dendritic cells (DC), together with macrophages are particularly important in the interface between the innate and acquired the immune responses to *Salmonella* infections. In addition to their defensive role engulfing microbes, DC also act as antigen presenting cells (APC) which are critical for initiating the primary immune response. Microbes engulfed by DC are quickly degraded by proteolysis into peptide fragments, which are loaded onto major histocompatibility complex (MHC) molecules and displayed to T cells. Typically exogenous antigens such as internalised bacteria are presented to CD4+ T cells via MHC II molecules, which in turn help activate B cells for antibody production (Germain, 1994; Ramachandra et al., 1999). However, it is also possible for exogenous antigenic peptides to be displayed on MHC I molecules to CD8+ cytotoxic T cells, which can then destroy other cells infected with the bacterium (Rock et al., 1990; Pifiefer et al., 1993; Wick and Ljunggren, 1999; Yrlid et al., 2001).

In order for DC to be effective activators of naïve T cells, DC must be in a mature state. Immature DC reside in the peripheral tissues and are very efficient at phagocytosing bacteria and processing antigens, but are not effective in stimulating T cells. The maturation process involves downregulating phagocytic activity, and upregulating MHC and co-stimulatory molecule expression. In addition, there is an increased ability of DC to migrate from peripheral tissues to lymphoid organs (where they encounter T cells) and changes in chemokine production and surface receptor expression also occur (Yrlid et al., 2001). DC maturation can be initiated by a variety of factors. Factors include exposure to microbial products such as LPS and nucleic acids in the periphery, and pro-inflammatory cytokines such as TNFα and IL-1β produced by macrophages and infected epithelial cells (Banchereau et al., 2000).
As previously described with macrophages, \textit{S. typhimurium} is capable of surviving and replicating within DC (Svensson \textit{et al.}, 2000). In addition \textit{S. typhimurium} has also been observed to induce apoptosis in DC and influence the ability of DC to present \textit{Salmonella} derived antigens to naïve T cells (Yrlid \textit{et al.}, 2001). By inducing apoptosis in both macrophages and DC, \textit{Salmonella} is able to prevent the direct presentation of \textit{Salmonella} derived antigens on both MHC I and MHC II molecules, and initiate its release from APC so it can infect and replicate in neighbouring cells. This phenomenon allows \textit{Salmonella} to evade immune recognition for longer (Yrlid \textit{et al.}, 2001). However, following \textit{Salmonella} induced apoptosis, neighbouring DC ingest the apoptotic material, which includes \textit{Salmonella} derived antigens, and presents them to T cells. Interestingly it has been shown that macrophages are unable to present the \textit{Salmonella} derived antigens and may in turn inhibit or compete with bystander DC, reducing antigen presentation to T cells (Yrlid \textit{et al.}, 2001). This may be another event that further enables \textit{Salmonella} to evade the immune system.

\subsection{1.1.3 Systemic Distribution of \textit{Salmonella}}

\textit{S. typhimurium} has evolved to become an intracellular parasite that is capable of sensing changing environmental conditions, and co-ordinating the expression of numerous virulence genes which provide protection against anti-microbial agents and down regulate the host cell defence systems (Zaiger \textit{et al.}, 1993; Rathman \textit{et al.}, 1997). With the ability to survive and multiply within macrophages, PMN and DC, and the ability to reduce immune recognition, \textit{S. typhimurium} can be distributed throughout the blood and lymph systems and remain protected from extracellular attack by antibodies and complement (Gulig, 1996). Ultimately, when the infecting serotype of \textit{Salmonella} is virulent, or the individual is immunocompromised, \textit{Salmonella} can be transported to the liver, spleen and mesenteric lymph nodes where it can multiply extensively and cause systemic disease (Gulig, 1996).
1.1.4 Host Resistance to Salmonella

Interactions between an infectious agent and its host can result in one of three possible outcomes. The microbe can be eliminated from the host immediately, it can colonise and persist without causing adverse effects, or it can give rise to infection. The outcome of host/microbe interactions is determined by a number of complex factors that include microbial virulence, and the immune capability of the host.

1.1.4.1 Natural Resistance in Inbred Mouse Strains

Natural resistance to Salmonella in mice is a classical example of how resistance is genetically determined (Plant and Glynn, 1974; Hormaeche, 1979a; Hormaeche, 1979b). Using LD_{50} values on well defined inbred strains of mice, it was demonstrated how natural resistance varies from low, through intermediate to high, with BALB/c and B10 strains being the most susceptible and CBA and A/J the most resistant (Hormaeche, 1979a). The range of degrees of resistance does not suggest resistance is under single gene control, but rather suggests a number of genes act in concert throughout the infection process.

However, results concerning the early net growth rate of S. typhimurium C5 in the liver and spleen show that all strains of mice fall into two clear categories. Susceptible BALB/c and B10 mice allow ‘fast net growth rate’, whilst resistant CBA and A/J mice show a ‘slow net growth rate’ (Hormaeche, 1979a; Hormaeche, 1979b). The absence of intermediates illustrates the initial growth rate of the organism up to day 4 of infection is determined by a mechanism under single gene control. This was further confirmed through hybrid crosses, which additionally demonstrated that the host mechanism determining slow net growth rate was inherited as a dominant trait. It is important to note that whilst all strains allowing ‘fast net growth rate’ were susceptible, not all strains showing a ‘slow net growth rate’ were resistant. This demonstrates that overall
resistance to \textit{S. typhimurium} is polygenic and involves cell-mediated immunity which occurs towards the end of the first week of infection, and that the mechanism responsible for the differences in early net growth rate is less complex and involves innate immunity (Plant and Glynn, 1974; Horrnaeche, 1979a; Horrnaeche, 1979b).

Similar mouse strain infection patterns have also been reported for susceptibility to infection with other pathogens including \textit{Leishmania donovani} (Bradley, 1974) and \textit{Mycobacterium bovis} (Forget \textit{et al.}, 1981). In each case, the control of the early stages of infection with \textit{S. typhimurium}, \textit{L. donovani} and \textit{M. bovis} was reported to behave as a dominant trait encoded by a single gene. This gene was independently given the names \textit{Ity} (Plant and Glynn, 1974) \textit{Lsh} (Bradley, 1974) and \textit{Bcg} (Forget \textit{et al.}, 1981). Subsequent studies have since shown the genetic advantage of the resistant strains of mice was phenotypically expressed as the ability of macrophages to restrict the intracellular replication of each of these pathogens. The protein responsible was referred to as the natural resistance associated macrophage protein 1 (Nramp1), but is now known as Slc11A1 (Blackwell and Searle, 1999; Blackwell \textit{et al.}, 2001).

\textit{1.1.4.2 The function of Slc11A1}

Slc11A1 is an integral membrane protein and is expressed in professional phagocytic cells (Reviewed Govoni and Gros, 1998). The relationship between Slc11A1 and resistance to infection in inbred strains has been demonstrated by both gain-of-function and loss-of-function experiments (Govoni \textit{et al.}, 1996). Inbred strains with the susceptibility to infection were found to be associated with a single glycine-to-aspartic acid substitution at position 169 in the fourth transmembrane spanning region of the protein (Govoni \textit{et al.}, 1996). Interestingly, no Slc11A1 protein was detected in macrophages from susceptible mice, implying the mutation prevented folding and/or targeting of the protein, which in turn resulted in its rapid degradation (Vidal \textit{et al.}, 1996).
Functional studies using Slc11A1 transfected macrophage cell lines have shown that the *Slc11A1* gene plays a significant role in the early macrophage activation pathway of the innate immune system. The effect of Slc11A1 expression includes the up-regulation of chemokines, cytokines (TNFα and IL-1β), MHC II expression, respiratory burst activity, nitric oxide release, antigen processing and apoptosis (Blackwell and Searle, 1999; Dunstan *et al*., 2001). The mechanisms by which Slc11A1 exerts these effects on macrophage function are not fully understood, although it has been suggested that Slc11A1 stabilises the mRNA of genes associated with macrophage activation, sustaining the production of anti-microbial effector molecules (Brown *et al*., 1997).

Recent studies have shown Slc11A1 is a divalent cation transporter that can flux iron in either direction against a proton gradient (Blackwell *et al*., 2001), and whose expression is induced by LPS and IFNγ in a time and dose dependant manner (Zhang *et al*., 2000; Blackwell *et al*., 2001). Slc11A1 has been shown to localise with membranes of the late endosomal and lysosomal compartment of the macrophage, (Dunstan *et al*., 2001) where it delivers divalent cations from the cytosol to the acidic late endosomes and lysosomes and where the Fenton reaction generates toxic antimicrobial radicals (Blackwell *et al*., 2001). This provides another method by which Slc11A1 can provide resistance to intracellular pathogens such as *S. typhimurium*. Additionally, it has been proposed that Slc11A1 counteracts the ability of *Salmonella* to become secluded in a SCV that limits access of bactericidal agents, allowing the normal degradative pathway of the macrophage to proceed (Cuellar-Mata *et al*., 2002).

### 1.1.5 Vaccine Development

Despite the improvements in public health and awareness, personal hygiene, and the use of antibiotics, diseases caused by *S. enterica* continue to be a serious global health problem. In one year alone, 16.6 million cases of typhoid fever and 1.3 billion
incidences of gastroenteritis were recorded worldwide (Ivanoff et al., 1994). Furthermore, these figures are increasing, with some European countries witnessing a 20-fold increase in the number of salmonellosis cases over the past 10-15 years (World Health Organisation, 1997). Although antibiotics have proved useful in combating S. enterica infection in both humans and animals, there is a growing concern over the emergence of strains resistant to previously useful antibiotics. As a result, the demands for prophylactic therapies to prevent infections are intensifying, and vaccination is a cost-effective means for prevention. There are three main categories of active vaccines, namely live vaccines, subunit or inactivated vaccines and the more recent nucleic acid based vaccines. For each category of vaccines, there are a number of different designs, formulations and administration procedures. These vaccine designs are summarised in Figure 1.2. This section provides a brief history of the current vaccines available for S. typhi and new candidate vaccines undergoing clinical trials.

1.1.5.1 Killed Whole Cell Vaccines

The first human vaccines developed against typhoid fever were based on killed whole S. typhi cells inactivated by heat, phenol or acetone (Hornick et al, 1970). Subsequent investigations however, showed that inactivation by heat and phenol resulted in less immunogenic and protective vaccines than acetone inactivation, since they lacked the Vi capsular antigen of S. typhi (Reviewed Tacket and Levine, 1995).
Figure 1.2 Types of active vaccines

Although moderately efficacious, acetone-inactivated vaccines still only offered limited protection largely due to the fact that killed vaccines induce good humoral immune responses but insufficient Th1 type-cell responses (Collins, 1974). In addition, the acetone inactivated vaccine had excessive reactogenicity (Levine et al., 1989), and the protective effect generated could be overwhelmed if a large enough \((10^7\) colony forming units) bacterial inoculum was ingested (Hornick et al, 1970). Whilst the protective ability of whole cell killed vaccines was improved with the use of strong adjuvants such as Freund’s incomplete adjuvant, the protection generated was still limited.
1.1.5.2 Subunit Vaccines

With advances in technology from the 1970s, the bacterial capsular polysaccharide Vi, known to be the contributing factor in improving immune responses from killed whole cell vaccines, was able to be purified in large quantities without denaturation for use as a sub-unit vaccine. Phase II studies conducted in many countries showed a subcutaneous dose of 25μg of Vi stimulated significant rises in serum IgG Vi antibody in the majority of recipients. However, these responses were markedly reduced in children less than 4 years of age and the responses were not increased with a booster inoculum (Tacket et al., 1986). By refining the Vi polysaccharide vaccine, scientists discovered that conjugating the Vi polysaccharide to exoprotein A from Pseudomonas aeruginosa gives rise to higher antibody levels which are further increased with a booster inoculum. Moreover, the conjugated vaccine is highly immunogenic for infants of 2-4 years of age (Kossaczka et al., 1999).

Alternative sub-unit vaccines have been investigated and animals have been immunised with bacterial fractions including porins, outer membrane proteins, and O-polysaccharides. Inbred mice immunised with porins have been shown to be protective when challenged with a moderately virulent organism, and outbred mice have been protected when challenged with a very low dose of virulent organisms (Mastroeni et al., 2001). The use of outer membrane proteins as sub-unit vaccines has also been demonstrated to elicit high antibody responses and was shown to be protective in chickens when challenged with virulent Salmonella (Mastroeni et al., 2001). However, investigations using Salmonella O-polysaccharides obtained by the acid hydrolysis of LPS, have been shown not to be immunogenic unless coupled to protein carriers such as tetanus toxoid (Watson et al., 1992).

Although some inactivated Salmonella vaccines and sub-unit vaccines can confer protection against infection in several animal species (Mastroeni et al., 2001), the superiority of live attenuated vaccines in inducing cell mediated and humoral immune
responses has steered future vaccine developments towards the use of *Salmonella* mutants for humans and animals.

1.1.5.3 *Attenuated Vaccines*

The original motive for investigating the use of live attenuated bacteria in vaccination was to increase the length of protection against *S. typhi* (Collins, 1974). Dead cell vaccines, although safer since they are unable to cause disease, tend to induce only short lived humoral mediated immunity since they are processed solely as exogenous or circulating antigen. Live attenuated bacteria carry the risk of reversion to a virulent state, but their proteins are processed as both endogenous and exogenous antigens and so are capable of inducing both humoral and cell mediated immunity. Because of this and their longer persistence in the host, live attenuated bacteria therefore provide longer protection.

**Galactose Epimerase-less Mutants**

The first live attenuated *S. typhi* vaccine was a Ty21a galactose epimerase (*galE*) mutant (Germanier and Furer, 1975) and is currently the only licensed live oral vaccine. It was generated by nitrosoguanidine mutagenesis, and was thought to be defective in UDP-glucose-4-epimerase, an essential enzyme for the production of a complete LPS molecule. However, due to the non-specific nature of chemical mutagenesis, it is likely that the Ty2 strain has many mutations that collectively result in its attenuation. This is supported by the fact that a precise *galE* mutation in the same Ty2 parent strain retains it virulence and induces typhoid fever in several subjects (Hone *et al.*, 1988). Additionally, studies performed have further demonstrated that the attenuating factor was not the *galE* mutation, but was possibly a *rpoS* mutation (Robbe-Saule *et al.*, 1995). Nonetheless the Ty21a live-attenuated oral vaccine is the most widely used typhoid vaccine at present. It is well tolerated, can be administered orally in enteric coated capsules, and offers
protection for up to 7 years in 60% of subjects (Ivanoff et al., 1994). However, it confers only partial protection in humans and therefore multiple doses are required to ensure protective immunity. Continuing studies aimed at improving both the level and the duration of immunity conferred by Ty21a have shown the formulation of the vaccine and the immunisation regime can greatly influence the outcome (Wahdan et al., 1982; Levine et al., 1999). As a result, the current formulation of lyophilised Ty21a vaccine in enteric coated acid resistant capsules has been replaced by a liquid suspension of Ty21a in a number of countries, with increased protection for an increased number of subjects.

Auxotrophic Mutants

Following the success of the Ty21a live attenuated vaccine, additional clinical trials have been performed to identify alternative genes that can be mutated for alternative live attenuated vaccines. Auxotrophic mutants require metabolites that are unavailable in mammalian tissues. As a result they have a reduced ability to grow and are therefore promising candidates as vaccines for salmonellosis. Certain auxotrophic mutations led to the discovery that genes encoding precursors of the aromatic amino acid (aro) pathway are essential for bacterial virulence (Hoiseth and Stocker, 1981). The vaccine strain CVD908 originally from Ty2, contains deletions in the aroC and aroD genes, thereby rendering it nutritionally dependant on para-amino benzoic acid (PABA) and 2,3-dihydroxybenzoate. Since these compounds cannot be obtained from the host and are essential for producing folates, aromatic amino acids and enterochelin, the CVD908 strain has reduced growth rates and is therefore successfully attenuated (Hone et al., 1991; Tacket et al., 1992a). When administered during phase I trials, CVD908 was well tolerated clinically and triggered strong cell-mediated and humoral immune responses with one single dose (Tacket et al., 1992a). However, during days 4-8 after vaccination, silent self-limited vaccinemas in 50% of the subjects were observed. This was considered a limitation for CVD908 despite the vaccinemas disappearing after day 8 without antibiotics (Tacket et al., 1992a).
In a similar study, the *aroC* and *aroD* deletion was performed in a more recent wildtype *S. typhi* strain (ISP 1820). In this instance, the mutations did not achieve adequate attenuation, causing typhoid fever symptoms in the test subjects and discontinuation of the clinical trials (Tacket *et al*, 1992b). In an attempt to further attenuate CVD908 strain to prevent silent vaccinemias forming after immunisation, a precise deletion of the *htrA* gene in addition to *aroC* and *aroD* deletions was investigated (Chatfield *et al*, 1992). The resultant vaccine strain CVD908-*htrA* was well tolerated in phase I and II clinical trials (Tacket *et al*, 1997; Tacket *et al*, 2000). Moreover, it generated strong cell mediated and humoral immune responses, without causing silent vaccinemias in any subject.

Another attenuated Ty2 derivative strain, Ty800, harbours a deletion mutation in the *phoP-phoQ* two component regulatory system. This system is critical for the survival of *Salmonella* within the phagosomes of macrophages, and without which, renders *S. typhimurium* attenuated in a mouse model (Miller *et al*, 1989). As with the CVD908 and CVD908-*htrA* strains, Ty800 was well tolerated and elicited strong humoral immune responses with a single dose of the vaccine (Miller *et al*, 1989).

Mutations within the global regulatory system *cya, crp* of Ty2 generated the strain X3927. During phase II trials it was observed to elicit unacceptable typhoid fever symptoms and its trial was discontinued (Levine *et al*, 2001). However, with an additional mutation within the *cdt* gene, which is involved in *Salmonella* dissemination from the mucosal associated lymphoid tissue, strain X4073 was well tolerated in all subjects, although it was less immunogenic than CVD908-*htrA* and Ty800 (Levine *et al*, 2001).

Another live attenuated *S. typhi* vaccine currently undergoing phase I clinical trials is CVD909. In addition to the attenuation, the strain also constitutively expresses the Vi polysaccharide antigen. The combination of successful live attenuated and subunit vaccines demonstrates that alternative vaccination technologies are being explored.
Another alternative is through DNA vaccination. DNA vaccination has a number of advantages over conventional vaccination strategies, and shows great potential for future vaccines against *S. enterica*. 
1.2 DNA Vaccines

Vaccination against pathogenic microorganisms was discovered over 200 years ago and represents one of the most important advances in the history of medicine (Robinson et al. 1996; Gurunathan et al., 2000). Over the last century, technological advances have aided the development of vaccines for use against a range of infectious agents. However, for many pathogens such as human immunodeficiency virus (HIV), Leishmania, Salmonella and the agents of malaria and tuberculosis, there are currently no vaccines uniformly effective or available. The lack of effective vaccines may be largely due to the requirement to induce both cell-mediated immunity and humoral immunity. Since cellular immunity can only be efficiently derived from vaccination with live attenuated organisms, vaccine development encounters several safety constraints for serious pathogens such as HIV.

However, as technology continues to advance, novel vaccine strategies continue to appear. One strategy that has recently emerged is DNA vaccination. DNA vaccines are capable of inducing both cellular and humoral responses without the risks associated with live vaccines and therefore have the potential to revolutionise the way vaccines are designed and administered today (Donnelly et al., 1997; Lai and Bennett, 1998; Watts et al., 1999; Hasan et al., 1999; Gurunathan et al., 2000).

DNA vaccination involves the transfer of a promoter and a gene encoding an antigenic protein into a host organism that does not express the protein under normal circumstances. The expression of the protein in vivo leads to the induction of antigen-specific immune responses that are directed against the encoded protein. DNA vaccination is applicable for a wide range of proteins and in many cases can provide an organism with immunity against bacteria, viruses, parasites and tumours (Lai and Bennett, 1998; Gregoriadis, 1998; Hasan et al., 1999).
The role of DNA delivery therapeutically was originally observed in the 1950s when the injection of crude preparations of DNA from tumours led to tumour formation in experimental animals (Stasney et al., 1950; Ito, 1961). These observations were further supported when plasmid DNA encoding hepatitis B proteins was transfected into cells and produced the appropriate gene products with their corresponding functions (Will et al., 1982). Similar observations were also made with DNA encoding insulin (Nicolau et al., 1983) and reporter molecules (Wolf et al., 1990). It was not until 1992 however that the significance of gene expression from direct injection with DNA was realised. Plasmid DNA was observed to induce antigen-specific antibody responses to the encoded human growth hormone (Tang et al., 1992).

Subsequent experiments illustrated vaccination with DNA encoding the influenza nucleoprotein was capable of protecting mice and chickens when challenged with a live form of the influenza virus (Ulmer et al., 1993; Robinson et al., 1993). Additionally, results indicated that protection was generated from both humoral and cell-mediated immune responses (Ulmer et al., 1993; Fynn et al., 1993). Further evidence for DNA vaccination eliciting both cell-mediated and humoral immune responses has been reported (Wang et al., 1993; Coavis et al., 1994). The application of DNA vaccination for cancer immunotherapy has also been recognised, with the demonstration that injections of plasmids encoding tumour antigens elicited protective immune responses in animal models (Conray et al., 1995; Bright et al., 1996; Hipp et al., 2000).

The rapid advance in DNA vaccine development has generated an increasing number of plasmids encoding immunogens from bacterial, viral, and parasitic pathogens and from a variety of tumours (see Table 1.1). In addition, DNA immunisation has offered new perspectives for the treatment of allergy (Raz et al., 1996; Hartl et al., 1999). More significantly, clinical trials for HIV, herpes, influenza, hepatitis B, and carcinomas are already in progress (Watts et al., 1999).
<table>
<thead>
<tr>
<th>Host</th>
<th>Route</th>
<th>Challenged</th>
<th>Immune response</th>
<th>Protection</th>
</tr>
</thead>
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</tr>
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<td>G.G</td>
<td>Yes</td>
<td>CTL</td>
</tr>
<tr>
<td>Nucleoprotein of influenza</td>
<td>Mice</td>
<td>I.M, I.V, I.N, S.C, I.P, G.G</td>
<td>Yes</td>
<td>Antibodies and CMI</td>
</tr>
<tr>
<td>Nucleoprotein of influenza</td>
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<td>I.M</td>
<td>Yes</td>
<td>Antibodies and CTL</td>
</tr>
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<td>No</td>
<td>Antibodies and CTLs</td>
</tr>
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<td>I.M</td>
<td>Yes</td>
<td>Antibodies and CTL</td>
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<tr>
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<td>I.D</td>
<td>Yes</td>
<td>Antibodies and CTL</td>
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<td>I.M, I.D</td>
<td>No</td>
<td>Antibody and CMI</td>
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<td>Yes</td>
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<tr>
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<td>Yes</td>
<td>Antibodies, Th and CTL</td>
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<td>Yes</td>
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<td>No</td>
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<td>Yes</td>
<td>Humoral and DTH</td>
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<td>I.M</td>
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<td>I.M</td>
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<td>Yes</td>
<td>Antibodies</td>
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<td>No</td>
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<td>gp 53-Bovine virus</td>
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<td>DNA Vaccine</td>
<td>Species</td>
<td>Route</td>
<td>Immunogenicity</td>
<td>Response</td>
</tr>
<tr>
<td>------------</td>
<td>---------</td>
<td>-------</td>
<td>----------------</td>
<td>----------</td>
</tr>
<tr>
<td>Influenza A HA</td>
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<td>Antibodies</td>
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<tr>
<td>HIV-1 nef, rev and tat genes</td>
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<td>HIV-1 vif, vpr, vpu and nef genes</td>
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<td>gP E Varicella-Zoster Virus</td>
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</table>

**Bacterial Antigen**

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<th>DNA Vaccine</th>
<th>Species</th>
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<tbody>
<tr>
<td>Mycobacterium TB (ELI)</td>
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**Parasitic Antigen**

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<th>Immunogenicity</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malaria circumsporozoite protein</td>
<td>Mice</td>
<td>I.M</td>
<td>Yes</td>
<td>CTL, cytokines and NO</td>
</tr>
<tr>
<td>Malaria circumsporozoite protein</td>
<td>Mice</td>
<td>I.M, G.G</td>
<td>Yes</td>
<td>Antibodies</td>
</tr>
<tr>
<td>Schistosoma japonium</td>
<td>Mice</td>
<td>I.M</td>
<td>No</td>
<td>Antibodies</td>
</tr>
<tr>
<td>Leishmania major gp63</td>
<td>Mice</td>
<td>I.M</td>
<td>Yes</td>
<td>IL-2 and IFN gamma</td>
</tr>
<tr>
<td>Leishmania gp63</td>
<td>Mice</td>
<td>I.D</td>
<td>Yes</td>
<td>Antibodies and CMI</td>
</tr>
<tr>
<td>Leishmania major LACK Ag</td>
<td>Mice</td>
<td>I.D</td>
<td>Yes</td>
<td>Antibodies and CMI</td>
</tr>
<tr>
<td>Taenia solium (KETc7)</td>
<td>Mice</td>
<td>I.M</td>
<td>Yes</td>
<td>Antibodies</td>
</tr>
<tr>
<td>Malaria, 10 peptide sequences</td>
<td>Human</td>
<td>I.M</td>
<td>No</td>
<td>CTL</td>
</tr>
<tr>
<td>T. cruzi, TSA-1</td>
<td>Mice</td>
<td>I.M</td>
<td>Yes</td>
<td>Antibodies and CTL</td>
</tr>
<tr>
<td>Taenia crassiceps, KETc-T</td>
<td>Mice</td>
<td>I.M</td>
<td>Yes</td>
<td>Antibodies</td>
</tr>
<tr>
<td>Taenia crassiceps, ELI</td>
<td>Mice</td>
<td>I.M and S.C</td>
<td>Yes</td>
<td>CMI</td>
</tr>
</tbody>
</table>

*Table 1.1 DNA vaccines for infectious diseases. Adapted from Hasan et al., 1999.*
This section summarises the nature of DNA vaccines and the mechanism by which DNA vaccines induce immune responses. In addition, advantages and safety concerns of DNA vaccines will be discussed.

1.2.1 DNA Vaccine Constructs

DNA vaccines typically consist of an *E. coli* derived plasmid into which a foreign gene encoding the antigen of interest is cloned. For the optimal expression of this gene, the plasmid requires the following components (Chapter 3; Dertzbaugh, 1998):

1. An eukaryotic promoter to facilitate the transcription of the foreign gene. The promoters are usually derived from viruses such as the cytomegalovirus (CMV), Rous sarcoma virus (RSV), or the simian virus 40, since they provide the greatest gene expression.
2. A polyadenylation such as the bovine growth hormone (BGH). This is necessary at the 3' end of the foreign gene for the stabilisation of mRNA transcripts.
3. An origin of replication for amplification of the plasmid in bacteria. The *E. coli* Col LEI origin of replication is most commonly used since it provides large copy numbers in bacteria and high yields during purification.
4. A prokaryotic selectable marker such as an antibiotic resistance gene. This is required for plasmid selection in bacterial cultures and is most commonly the ampicillin resistance gene. For research purposes, some DNA vaccine plasmids also contain an eukaryotic selectable marker such as the neomycin resistant gene for plasmid selection in mammalian cells.

It has recently been discovered that DNA vaccines also contain a specific nucleotide motif that elicits an innate immune response and thereby plays an important role in the
immunogenicity of these vaccines (Yamamoto et al., 1992; Klinman et al., 1996; Sato et al., 1996; Thalhammer et al., 2001). The motif is present in bacterial DNA and consists of unmethylated cytidine-phosphate-guanosine (CpG) dinucleotides flanked by two 5’ purines and three 3’ pyrimidines (Sato et al., 1996; Krieg et al., 1995; Thalhammer et al., 2001). The action of the CpG motif is extensive and includes the up-regulation of antigen presentation by macrophages and dendritic cells, the increased production of cytokines from these antigen presenting cells, the induction of B cell proliferation, an increase in antibody production and an indirect activation of natural killer (NK) cells (Yamamoto et al., 1992; Sato et al., 1996; Krieg et al., 1995; Thalhammer et al., 2001; Yamamoto et al., 2002).

Finally, a variety of supplementary sequences can be co-administered with the DNA vaccine construct, which contribute to the effectiveness of a DNA vaccine. These include sequences that encode cytokines to direct the nature of the immune response (Chow et al., 1998; Sakai et al., 1999; Palendira et al., 2002; Katae et al., 2002), and co-stimulatory molecules (Iwasaki et al., 1997; Tsuji et al., 1997; Flo et al., 2000), which lead to immune activation rather than induction of tolerance (Ridge, 1996).

1.2.2 Routes of Administration

DNA vaccines can be introduced to the host via injection, or through a number of carrier-mediated methods (Tang et al., 1992; Sizemore et al., 1995; Haynes et al., 1996; Spier, 1996; Darji et al., 1997; Dietrich et al., 1999; Cui and Mumper, 2001; Vajdy and O’Hagan, 2001; McKeever et al., 2002; Cui and Mumper, 2002). Several routes are available for delivery via injection, and include intramuscular, intradermal, subcutaneous, intravenous, and intraperitoneal (Wolff et al., 1990; Raz et al., 1994; Fynan et al., 1993; Spier, 1996). The most studied method of immunisation is intramuscular injection of pure plasmid DNA and has been demonstrated to generate good results in animal models ranging from fish (Anderson et al., 1996), to chimpanzees.
(Davis et al., 1996a; Boyer et al., 1997). In the case of larger animals however, this route of immunisation may be less effective due to the lower efficiency of muscle cell transfection. One method commonly used to overcome such difficulties involves pre-treating muscle tissue with cardiotoxin (Davis et al., 1994) or bupivacaine (Wang et al., 1993). The resulting effect enhances DNA uptake by myocytes and improves responses.

In addition to the route of administration, induced immune responses are effected by other variables, such as the type of diluent, for example, distilled water, saline or sucrose (Chattergoon et al., 1997). Additionally, immune responses are effected by the amount of plasmid per dose, the number of doses, the different sites of each dose and finally the time intervals between doses (Spier, 1996; Fuller et al., 1997; Leitner et al., 1997). The optimal dose depends largely on the antigen and the animal model used, although typically 25 – 100μg of plasmid DNA is required when administered intramuscularly in mouse models (Gurunathan et al., 2000), increasing to 500 – 2500μg in primates and humans (Wang et al., 1998). It is worthy to note that immunisation by gene gun (a carrier mediated approach) requires only 0.1 – 1μg of plasmid DNA in mouse models (Gurunathan et al., 2000) and highlights the variation between administration routes and methods of immunisation. In most early studies mice were injected three times into the tibialis muscle with 2-3 weeks between injections. More recently there have been an increasing number of successful DNA immunisations that have been administered intradermally (Hasan et al., 1999; Gurunathan et al., 2000).

The success of DNA vaccination is dependent upon the DNA reaching immunologically relevant cells before being degraded by extracellular deoxyribonucleases (Lew et al., 1995; Gregoriadis, 1999). Carrier mediated approaches are therefore being investigated to more efficiently target DNA to host cells whilst also protecting the DNA from the extracellular environment. One such technique in DNA immunisation involves the use of a biolistic device called a gene gun (Tang et al., 1992; Fynan et al., 1993; Haynes et al., 1996). Plasmid DNA is coated onto microscopic gold beads that are in turn propelled, using a particle accelerator or compressed helium, into the cytosol of
keratinocytes. Once inside the host cells, DNA dissociates from the gold beads and is transported to the nucleus. Since the DNA directly penetrates the target cells, transgene expression levels are similar to those generated when greater amounts of pure DNA are injected as previously discussed (Tang et al., 1992).

The use of attenuated intracellular microbes as carriers of DNA vaccines has also been reported (Sizemore et al., 1995; Darji et al., 1997; Sizemore et al., 1997). The invasive intracellular bacteria containing the plasmid DNA are phagocytosed by antigen presenting cells (APCs), thereby delivering the DNA into the host cell cytosol. One such bacterium, Shigella flexneri is attenuated by an aspartic/semialdehyde dehydrogenase (Δasd) mutation that does not interfere with the ability of the organism to invade cells, but which causes it to burst open inside the cell, releasing expression plasmids into the cytoplasm of mucosal cells. Indeed, immunisation with S. flexneri carrying plasmids encoding β-galatosidase generated strong antigen specific cellular and humoral immune responses (Sizemore et al., 1997). Vaccination strategies using Salmonella typhimurium transformed with DNA encoding lysteriolysin have similarly been shown to induce specific antibody and T-cell responses (Darji et al., 1997). This method of carrier mediated immunisation allows plasmid DNA to be directly targeted to relevant immune cells allowing the efficient induction of immune responses at the site of pathogen entry.

Another method of carrier mediated DNA delivery is via the use of cochleates. Cochleates are rigid calcium-induced structures consisting of spiral bilayers of anionic phospholipids (Papahadjopoulos et al., 1975). To generate the spiral bilayers, large continuous sheets of anionic phospholipids roll back on themselves, capturing the DNA between the sheets during formation. The structure is then stabilised by the interaction of the divalent calcium ions. Due to its stability, cochleates are able to survive the stomach environment and can therefore be administered orally (Gould-Fogerite and Mannino, 1996). After passage through the stomach, cochleates reach the PP in the small intestine. Upon contact with the membrane of M cells, the outer layer of the cochleate fuses and releases the DNA directly into the cytosol of the cell. Reports using DNA cochleates
with plasmid encoding HIV-1, influenza and parainfluenza antigens have shown strong, long lived immune responses (Gould-Fogerite and Mannino, 1996).

The use of liposomes presents another method of carrier-mediated DNA immunisation (Gregoriadis, 1999; Gurunathan et al., 2000). Liposomes are bilayered membranes consisting of amphipathic molecules such as phospholipids, which can form unilayered or multilayered (lamellar) vesicles surrounding an aqueous core. The nature of the phospholipid allows the vesicles to complex with DNA upon mixing, and provides protection from deoxyribonuclease attack (Felgner and Rhodes, 1991; Gregoriadis, 1999). Since the composition of the liposomes are similar to that of the cell membrane, vesicles can fuse with the cell membrane and deliver DNA into the cytosol of the cell (Szoka et al., 1996; Gregoriadis, 1999). Many studies have shown only limited success with immune responses being similar or modestly higher than those achieved with naked DNA. However, intramuscular injection and intranasal administration of liposomes containing DNA encoding the hepatitis B surface antigen have generated higher antibody levels and increased cytokine levels compared with naked DNA (Klavinski et al., 1997; Gregoriadis et al., 1997). Also with the structural versatility of liposomes, improvements in transfection efficiency and the induction of immune responses through alterations in liposome size, charge, lipid content and the co-entrapment of cytokine genes or other adjuvants can be achieved, and is currently an active area of investigation (Gregoriadis, 1999; Gurunathan et al., 2000).

More recently, the entrapment of DNA in biodegradable polymeric microparticles has been investigated (Vajdy and O'Hagan, 2001; Cui and Mumper, 2001; McKeever et al., 2002; Cui and Mumper, 2002). Plasmid DNA trapped in these polymers (such as polylactide-coglycolides or chitosan), can be given systemically or to mucosal surfaces and has been demonstrated to induce systemic and mucosal immune responses (Jones et al., 1997; Chen et al., 1998).
1.2.3 Immune Responses to Foreign Proteins

Following exposure to foreign antigen, either directly from the microorganism or indirectly via DNA vaccines, two types of acquired immunity may be induced as a response, namely humoral immunity and cell-mediated immunity. For many viral and bacterial infections, protection is mediated by a humoral immune response. Whilst humoral immunity plays an important role in the immune responses to *S. enterica* infection (Mastroeni *et al.*, 1993), protection against *Salmonella* and a number of other intracellular pathogens also requires cell-mediated immunity (Lo *et al.*, 1999).

The humoral branch of the immune system involves the interaction of B cells with antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. There are three main functions of antigen-specific antibodies produced from activated B cells, namely the neutralisation of circulating pathogens and their toxins, the opsonisation of pathogens to facilitate their uptake by phagocytic cells and the activation of complement (Joiner *et al.*, 1984; Janeway and Travers, 1997). The cell-mediated branch of the immune response involves the generation of effector T cells including cytotoxic T lymphocytes (CTLs) and T helper (Th) cells. The function of CTLs is to destroy cells infected with the pathogen, whilst the T helper cells secrete a variety of cytokines to activate phagocytic cells to kill microorganisms more effectively and stimulate B cells (Collins, 1974; Kaufmann, 1993).

Cell-mediated immunity involves the recognition of processed antigens presented on cell surfaces via molecules encoded by the major histocompatibility complex (MHC) of genes by CD4+ and CD8+ T cells (Whitton, 1998; Zamoyska, 1998). Class I MHC presentation occurs on any nucleated self cell in which there is endogenous synthesis of an antigen, for example from live vaccines or DNA vaccines. Antigens made in the cell cytoplasm are degraded here into intracellular peptides by proteolytic enzymes. These peptides are then transported into the endoplasmic reticulum (ER) by two ATP binding cassette-transporters, TAP1 (transporter associated with antigen processing) and TAP2.
(Nandi et al., 1998; Watts et al., 1999). In the ER, MHC class I heterodimers consisting of a transmembrane heavy chain and a non-covalently associated light chain (β2-microglobulin) are loaded with the antigenic peptides. This trimolecular complex is exported through the Golgi and the secretory pathway to the surface of the cell where it can be recognised by CD8\(^+\) T cells, as shown in Figure 1.3 (Abbas et al., 1991; Germain, 1994; Lai and Bennett, 1998).

Class II MHC antigen presentation however, takes place exclusively on professional antigen-presenting cells (APCs) such as macrophages and dendritic cells, and usually presents antigens that are obtained exogenously (Abbas et al., 1991; Chapman, 1998; Watts et al., 1999). Vaccines accessing this pathway include whole killed/inactivated pathogens, recombinant and protein vaccines and DNA vaccines. Like MHC class I
molecules, MHC class II molecules are also assembled in the lumen of the ER. MHC-II molecules are heterodimers consisting of two transmembrane proteins. In the ER, the partially folded MHC-II protein is bound by an invariant chain (Ii) to form a trimer. The terminal section of Ii contains the Class II-associated invariant peptide (CLIP), which overlies the peptide-binding groove of MHC II heterodimers and prevents premature peptide loading (Cresswell, 1994). The MHC-II: CLIP heterotrimer leaves the ER through the Golgi apparatus, where the invariant chain diverts the complex towards the endosomal pathway. The efficient loading of MHC-II molecules with antigenic peptides takes place in special endosome/lysosome compartments where concurrent proteolytic processing of both the invariant chain and endocytosed proteins occurs (Ghosh et al., 1995). Once the peptides are captured by MHC-II, the complex is rapidly transported to the cell surface where it stimulates CD4+ T cells to become T helper cells (see Figure 1.4; Chapman, 1998; Watts et al., 1999).

![Figure 1.4 MHC class II pathway of antigen presentation. Adapted from Watts et al., 1999.](image-url)
When applying these two processing pathways to infectious agents, it was assumed that CD4\(^+\) T cells were responsible for controlling endosomal pathogens, such as bacteria which reside in phagosomes, whereas CD8\(^+\) T cells controlled cytosolic pathogens, such as viruses (Kaufmann, 1996; Reimann and Kaufmann, 1997). However, with the discovery that both MHC-II-restricted CD4\(^+\) T cells and MHC-I-restricted CD8\(^+\) T cells become activated during bacterial infection and both contribute to antibacterial protection, it has become apparent that antigen processing is more complex (Kaufmann, 1993). It has since been shown that an alternative MHC-I pathway exists, whereby phagosomal antigens are displayed in association with MHC-I molecules to CD8\(^+\) T cells (Rock et al., 1990; Pfeifer et al., 1993; Reimann and Kaufmann, 1997). This pathway also plays an important role in the mechanism of DNA vaccination.

The CD4\(^+\) T cell population has been subdivided into two basic types; T-helper-1 (Th1) or T-helper-2 (Th2) based on the profile of cytokines secreted after activation (Seder and Paul, 1994; Abbas et al., 1996; O’Garra, 1998; Liew, 2002). Among the cytokines secreted by the Th1 CD4\(^+\) cells are interferon \(\gamma\) (IFN-\(\gamma\)) and interleukin 2 (IL-2). Secretion of these cytokines results in T cell proliferation and the upregulation of MHC class II expression. The Th1 CD4\(^+\) cells contribute limited help for the activation of B cells, but they provide potent cell-mediated immune responses by stimulating CTL and increasing the phagocytic activity of monocytes and macrophages (Abbas et al., 1996; Watts et al., 1999). Resultant antibodies from a Th1 response in mice are of the IgG\(_{2a}\) subclass. Other cytokines associated with the Th1 response include interleukin 12 (IL-12), which suppresses the Th2 pathways and further stimulates the Th1 response, and TNF-\(\alpha\) (O’Garra, 1998; Watts et al., 1999). Alternatively, the Th2 CD4\(^+\) T cell population secretes cytokines such as interleukins 4 (IL-4), 5 (IL-5), 6 (IL-6) and 10 (IL-10) which play a major role in supplying B cell help in the generation of the humoral immune response (Romagnani, 1996; Abbas et al., 1996). These cytokines activate B cells and induce antibody isotype switching and secretion of antibodies of the IgG1 subclass in mice (Nauciel et al., 1992; Kelso, 1995; Romagnani, 1996).
The type of T helper cell generated in response to DNA vaccines can be influenced by a number of factors (Pertmer et al., 1996; Feltquate et al., 1997), although the most critical factor is the cytokines to which the naïve cells are exposed during antigenic stimulation (Xiang and Ertl, 1995; Chow et al., 1998). It is therefore possible to alter the nature of the induced immune response to that which is desired for protection, by the coadministration of cytokines (Bueler et al., 1996; Chow et al., 1997; Geissier et al., 1997; Iwasaki et al., 1997; Kim et al., 1997; Tsuji et al., 1997; Chow et al., 1998; Sakai et al., 1999; Palendira et al., 2002; Katae et al., 2002).

1.2.4. Mechanism of DNA Immunisation

The precise mechanisms by which direct injection of DNA into tissues induces both humoral and cell-mediated immune responses to encoded antigens has been the subject of numerous investigations and still remains to be elucidated. The majority of studies have concentrated on investigating the mechanism of DNA immunisation following intramuscular (IM) and intradermal (ID) inoculation of plasmid DNA.

The initial stage in DNA vaccination requires plasmid DNA to enter cells and be transported to the nucleus where the antigenic section of DNA can be transcribed. There is evidence that bacterial DNA enters cells by receptor-mediated endocytosis (RME), but it is unclear how subsequent degradation is avoided in the secondary lysosomes (Hefeneider et al., 1992). It has been suggested that some of the DNA escapes the lysosomotropic pathway and enters the nucleus via internal receptors where the encoded gene can then be transcribed (Gregoriadis, 1998). However, the mechanism of such a pathway is unknown and has not been widely investigated. Once the gene has been transcribed and translated, the foreign antigen can access the MHC class I and MHC class II pathways, as previously described.
It has been observed that after both IM and ID injection of plasmid DNA, antibody is produced and both MHC class I restricted antigen specific CTLs and MHC class II restricted CD4+ T cells secreting Type-I cytokines are activated (Fynan et al., 1993; Raz et al., 1994; Ulmer et al., 1993; Manickan et al., 1995; Xiang and Ertl, 1995; Davis et al., 1995; Raz et al., 1996).

After IM injection of a DNA vaccine, myocytes appear to be the predominant cell type transfected, and studies have shown the expression of DNA encoded antigens in myoblasts and myotubules (Triyatni et al., 1998). Since a low number of professional APCs are located in the muscle tissue, this would imply that muscle cells process and present the antigen and stimulate a primary immune response. However, muscle cells lack MHC class II expression and the necessary co-stimulatory molecules such as B7. Additionally muscle cells express only low levels of MHC class I molecules (Whalen et al., 1995). It is known that interferon upregulates MHC-I expression and is present during the inflammatory response caused by intramuscular injection. However, experiments to determine if the interferon at the site of injection locally upregulates MHC-I expression on myocytes for CTL priming were inconclusive (Whalen et al., 1995). In a different set of investigations however, it was demonstrated that neither the co-expression of antigen with the co-stimulatory molecule B7 nor the expression of cytokines was sufficient to induce nonhemopoietic cells to become effective APCs (Iwasaki et al., 1997). This indicates that an alternative method of inducing CTL responses exists.

One method by which MHC class I restricted CTL might be induced after DNA immunisation is by the direct transfection of APCs. The transfected APC synthesises the antigen endogenously, which can then enter the MHC class I processing pathway for recognition by CD8+ T cells. However, one study has shown that myocytes stably transfected with the gene for influenza nucleoprotein induced a CTL response when transplanted into compatible mice. This would indicate that the transfection of professional APCs was not required for the induction of CTL (Ulmer et al., 1996).
Under certain circumstances it has been demonstrated that exogenous antigens can be presented in the context of MZHC class I (Rock et al., 1990; Schirmbeck et al., 1995; Harding et al., 1995; Martinez-Kinader et al., 1995; Kaufmann, 1997). Consequently, it was suggested that CTL responses following intramuscular DNA immunisation were the result of antigen transfer from transfected myocytes to professional APCs (Donnelly et al., 1997; Gregoriadis, 1998; Whitton et al., 1999; Hasan et al., 1999). Indeed, by performing bone marrow chimera experiments in mice, Corr and colleagues and Iwasaki and colleagues demonstrated that specific CTL responses were produced as a result of antigen presentation by professional APC of bone marrow origin and not somatic cells at the site of plasmid DNA injection (Corr et al., 1996; Iwasaki et al., 1997). The mechanism by which transfer of antigen between somatic cells and professional APCs occurs is not fully known, but evidence indicates it is through the process of cross-priming (Carbone and Bevan, 1990; Whitton et al., 1999; Gurunathan et al., 2000).

Cross-priming is the ability of antigens produced by non-APCs to prime CTL via MHC molecules present only on professional APCs. The cellular mechanism by which cross-priming occurs remains to be defined, although it is thought to be via the secretion of unprocessed protein antigens or alternatively from the phagocytosis of apoptotic bodies derived from transfected tissue cells (Albert et al., 1998). The unprocessed antigens are delivered to the cytosol of APCs where they can be presented on MHC class I or MHC class II molecules and induce humoral and cell-mediated immunity. It has more recently been demonstrated that the CTL response to both intramuscular and intradermal DNA vaccination is also highly dependant upon the generation of CD4+ T cell help via a class II MHC-dependant pathway (Maecker et al., 1998).

Due to the high numbers of professional APCs such as Langerhans cells, macrophages and dendritic cells within the dermis of the skin, intradermal injection of DNA vaccines appear to be the most efficient route of administration (Corr et al., 1996; Davis et al., 1997; Morita and Takashima, 1999). Intradermal DNA vaccination has been shown to
induce the expression of gene products in cells with macrophage-like morphology as well as keratinocytes and dermal fibroblasts (Raz et al., 1994). However, the principle APCs initiating the immune response following DNA vaccination are dendritic cells (Akbari, et al., 1999; Morita and Takashima, 1999) and studies have shown that only small numbers of directly transfected DCs are required to elicit an immune response (Casares, et al., 1997). In addition to the mechanism of cross priming for inducing CTL responses, APCs that are transfected directly from intradermal injection can also process the translated antigen and can present it via MHC class I molecules to activate CTLs (Corr et al., 1996; Iwasaki et al., 1997; Whitton et al. 1999). Additionally the circulating APCs can acquire locally released antigen from keratinocytes and fibroblasts and process them via the MHC class II pathway for presentation to both T and B cells in the draining lymph nodes. Thus, both cell-mediated and humoral immunity can be induced following DNA immunisation.

In most models of DNA vaccines, there is a mixed Th1 and Th2 response, although a predominant Th1 response is more often reported (Raz et al., 1996; Davis et al., 1996b). Several factors determine whether a Th1 or Th2 response predominates, including the route and method of DNA administration and whether or not an adjuvant is used (Xiang and Ertl, 1995; Pertmer et al., 1996; Felquate et al., 1997; Johnston and Barry, 1997; Chow et al., 1998; Gurunathan et al., 2000). Overall however, DNA vaccines are capable of inducing strong and long lived humoral and cellular immune responses in most disease models (Robinson and Torres, 1997; Watts et al., 1999; Hasan et al., 1999; Gurunathan et al., 2000).

1.2.5. Considerations of DNA Vaccines

DNA vaccination is the most recent approach to activating the immune system against pathogens and has been demonstrated in numerous studies of infectious diseases in animal models, as shown in Table 1.1. Whilst DNA vaccines offer considerable
advantages over current vaccination strategies, there are also several disadvantages and safety considerations.

<table>
<thead>
<tr>
<th>Type of Vaccine Strategy</th>
<th>Production</th>
<th>Booster</th>
<th>Stability</th>
<th>Type of Immune Response</th>
<th>Reversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attenuated (live)</td>
<td>Grown under abnormal culture conditions, or mutated to select for non virulent types</td>
<td>No</td>
<td>Not very</td>
<td>Humoral and cell-mediated immunity</td>
<td>May revert to virulent form</td>
</tr>
<tr>
<td>Inactivated</td>
<td>Virulent pathogen inactivated by chemicals or γ-irradiation</td>
<td>Yes</td>
<td>Yes</td>
<td>Mainly humoral immunity</td>
<td>No</td>
</tr>
<tr>
<td>Recombinant protein</td>
<td>Gene from pathogen expressed in bacteria, yeast or mammalian cells and administered with an adjuvant</td>
<td>Yes</td>
<td>Yes</td>
<td>Mainly humoral immunity</td>
<td>No</td>
</tr>
<tr>
<td>Recombinant infectious vector</td>
<td>Genes encoding major antigens are placed into attenuated viruses or bacteria</td>
<td>Yes</td>
<td>Not very</td>
<td>Humoral and cell-mediated immunity</td>
<td>Vector may revert to virulent form</td>
</tr>
<tr>
<td>Synthetic peptides</td>
<td>Peptide sequences encoding immunodominant T or B cell epitopes are coupled to an immunogenic carrier</td>
<td>Yes</td>
<td>Yes</td>
<td>Humoral and cell-mediated immunity</td>
<td>No</td>
</tr>
<tr>
<td>DNA vaccine</td>
<td>Genes encoding antigenic proteins from a pathogen are inserted into plasmid vector</td>
<td>Possibly</td>
<td>Yes</td>
<td>Humoral and cell-mediated immunity</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 1.2 Comparison of current vaccine strategies. Adapted from Hasan et al., 1999.
1.2.5.1 Advantages of DNA Vaccines

Many of the advantages that DNA vaccines offer over current vaccine strategies are shown in Table 1.2. The ability of DNA vaccines to induce both cell-mediated and humoral immunity is particularly important when immunising against intracellular pathogens such as *Salmonella* for which both types of immunity are required (Collins, 1974; Mastroeni *et al.*, 1993). Whilst attenuated vaccines and recombinant infectious vectors are also capable of activating both arms of the immune response, DNA vaccines have no risk of reverting to a virulent form and causing disease since the plasmids are typically constructed not to replicate in mammalian cells or produce infectious particles. In addition, live attenuated vaccines are difficult to produce and store (Rabinovich, *et al.*, 1994; Fynan *et al.*, 1993), whilst supercoiled DNA is highly stable at a range of temperatures and can be stored as a dry pellet (Watts *et al.*, 1999; Hasan *et al.*, 1999; Thalhamer *et al.*, 2001). This is particularly advantageous for mass vaccination in developing countries where a cold chain is difficult to maintain (Ertl and Xiang, 1996). The preparation and purification of supercoiled circular DNA is also less labour intensive than the production and purification of recombinant infectious vectors and recombinant proteins and promises to be less expensive to produce on a large scale. Additionally, the speed and economics associated with the production of DNA vaccines could prove very useful with rapidly mutating viruses where frequent reformulation is required (Hasan *et al.*, 1999).

Whilst it has been demonstrated that direct IM injection of recombinant plasmids generated higher levels of expression than viral vectors (Hasan *et al.*, 1999), it was also demonstrated that DNA vaccination enabled a long-lived gene expression, eliminating the need for booster immunisations (Ulmer *et al.*, 1994). DNA vaccines can also be tailored to bias the desired immune response towards one of the two different types of T-cell help. This can ultimately determine how effectively the body clears a particular type of infection. The tailoring of a desired immune response can be achieved through alterations in the formulations, the route of immunisation or the use of co-adjuvants
Felquate et al., 1996; Ulmer et al., 1996; Johnston and Barry, 1997; Gurunathan et al., 2000). In addition, DNA vaccines can incorporate one or more genes encoding antigens of interest. It is therefore feasible that several antigens from different pathogens can be incorporated into a single vaccine (Boyle et al., 1998).

Finally, DNA immunisation technology has been extended to enable its use as a screening tool for identifying protective antigens of a pathogen, a technique termed 'expression library immunisation' (ELI) (Barry et al., 1995; Johnston and Barry, 1997; Johnston et al., 2002). ELI is discussed further in section 1.2.6.

1.2.5.2 Concerns and Limitations of DNA Vaccines

The use of DNA vaccines raises several important safety concerns. There is a possibility that the DNA vaccine plasmid could integrate into the host genome either randomly or by homologous recombination. This could lead to insertional mutagenesis causing activation of proto-oncogenes or the inactivation of tumour suppressor genes (Robinson et al., 1996). However, ongoing animal studies have failed to detect insertions of the injected plasmid in a number of different tissues (Nichols et al., 1995) and have shown the risk of integration is extremely low.

Another concern of DNA vaccines is that they may alter the immune state of the individual, inducing tolerance. In some experimental systems, the continuous expression of small amounts of antigen produced by a DNA vaccine can lead to the development of immune unresponsiveness or tolerance following pathogenic challenge (Donnelly et al., 1997a). Whilst some experiments have demonstrated that tolerance was induced (Mor et al., 1996), other similar experiments have instead generated protective immunity (Schirmbeck et al., 1995; Davis et al., 1996a). Further investigation is therefore needed before this concern can be fully assessed.
Additional safety investigations have addressed the issue of autoimmunity, whereby host cells producing the foreign antigen become targets for immune attack. However, both the destruction of one’s own cells and the expression of foreign antigens occur in the normal course of viral and bacterial infections, so DNA vaccines pose no greater risk of inducing autoimmunity than during natural infections. Additionally, autoimmunity as a result of DNA vaccination has not been detected, even in genetically susceptible strains of mice (Mor et al., 1997). One final concern for DNA vaccines is the possible induction of anti-DNA antibodies. The immune system would see plasmid DNA injected into the host as foreign and may cross-react with mammalian DNA. However, it has been reported that the likelihood of inducing anti-DNA antibodies is small and investigations so far have shown few or no anti-DNA antibodies are present following the DNA vaccination of healthy mice (Mor et al., 1997).

1.2.6. Expression Library Immunisation

DNA immunisation has recently emerged as a promising new approach to vaccination and has been labelled the third revolution in vaccines (Dixon, 1995). Over the past 8 years, the efficacy of DNA vaccines has been demonstrated in several animal models of infectious, viral, parasitic and bacterial diseases (Donnelly et al., 1994, Lai and Bennett, 1998; Hasan et al., 1999). In addition to the success of DNA immunisation, the technology has been extended to enable its use as a possible screening tool for identifying protective antigens of a pathogen, a technique termed ‘expression library immunisation’ (ELI).

1.2.6.1 Principle of Expression Library Immunisation

To construct a DNA vaccine against a pathogen such as S. typhimurium, genes encoding proteins that elicit a strong immune response when expressed in the host need to be
identified and cloned into an expression vector. However, identifying which gene or genes to incorporate into the expression vector is a major challenge. One possible solution to this problem is the technique of ELI, and has been applied to a number of different pathogens (Barry et al., 1995; Johnston and Barry, 1997; Manoutcharian et al., 1998; Alberti et al., 1998; Brayton et al., 1998; Piedrafita, et al., 1999; Lazowska et al., 2000; Smooker et al., 2000).

ELI makes use of DNA immunisation and the fact that all the antigens of a pathogen are encoded in its DNA and can be cloned into individual expression vectors to make a representative expression library. The initial stage of ELI therefore involves the preparation of a representative DNA expression library. This can be achieved by partial digestion or fragmentation of the pathogen's genomic DNA, and the fusion of these library fragments into expression vectors. Whole or partial libraries can then be used for immunisation and their ability to protect mice against the pathogen in question can be assessed. Through the sequential partitioning of these libraries into smaller protective sub-libraries, the individual expression vectors (plasmids) encoding protective antigens can be identified (see Figure 1.5).

1.2.6.2 Sensitivity of Expression Library Immunisation

For the successful application of ELI to identify all protective antigens of a pathogen, it is critical that the expression library (EL) constructed is representative of the entire genome of the pathogen. Additionally, the number of clones in each immunising library must be sufficient to encode different protective antigens, but not so many that the concentration of each plasmid is too dilute to elicit a protective immune response (Johnston and Barry, 1997). The sensitivity of ELI is dependent upon two factors, namely the threshold for protection of a plasmid and the number of protective plasmids in a library.
To determine the minimum concentration required for a plasmid to elicit a protective immune response, a plasmid known to induce a given humoral or CTL response was diluted with a mixture of plasmids and injected into mice (Johnston and Barry, 1997). Results showed that after a single immunisation of 10µg DNA with a gene gun, as little as 40ng of specific plasmid was required to induce a humoral response (Johnston and Barry, 1997). When looking for cellular responses, the results showed that as little as 0.4ng of plasmid could elicit CTL activity after a single gene gun immunisation. Additionally, the results of the induced cellular response showed a peak CTL activity using 40ng of plasmid DNA, which declined as the amounts of DNA increased further.

**Figure 1.5 The basis of expression library immunisation. Adapted from Johnston and Barry, 1997.**
Interestingly, the results demonstrated that the cellular response might be 100 times more sensitive than the humoral response in ELI.

Overall, the data predicted that libraries or sub-libraries with a complexity of $2.5 \times 10^4$ plasmids could be used in a single inoculum to induce specific humoral and cellular responses. In addition, experiments confirmed that a complexity of up to $2.7 \times 10^4$ was acceptable for EL screening, however, the protection conferred by the total library was lower than that conferred by smaller sub-libraries. This is consistent with the observation that lower plasmid concentrations elicit weaker immune responses and further highlights the importance of immunising with sub-libraries that have a complexity lower than $2.7 \times 10^4$ for generating humoral and cellular responses specific to the antigens encoded in the EL (Barry et al., 1995; Johnston and Barry, 1997).

Whilst there are still safety concerns surrounding DNA immunisation, DNA vaccines have been effective in generating immune responses and protection in a wide variety of preclinical models of viral, bacterial, parasitic infections, and cancer (Hasan, et al., 1999). Additionally, DNA immunisation technology has provided a new method for studying the mechanisms of antigen presentation and has provided a new technology for the discovery of novel vaccine antigens.
1.3 Motivation for this Work

Diseases caused by *S. enterica* infection are a global health problem. The most serious *S. enterica* related illness is typhoid fever and is caused by the serovar *typhi*. On a worldwide scale, typhoid fever is responsible for over 600,000 deaths annually (Ivanoff *et al.*, 1994). In addition, non-typhoidal *S. enterica* related disease accounts for 1.3 billion incidences of diarrhoeal disease in the world each year of which nearly 3 million result in death (Ivanoff *et al.*, 1994). Whilst antibiotics have proved useful in combating *S. enterica* infection in both humans and animals, antibiotic resistant strains are now emerging, making antibiotic therapy impractical and expensive (Pang *et al.*, 1995).

Vaccination is a powerful tool for the control of *Salmonella* infections, although safer and more effective *Salmonella* vaccines are needed (Mastroeni *et al.*, 2000). Killed vaccines and subunit vaccines have been used in the past with variable success. Recent vaccine development has focused on attenuating live bacteria since they are potentially superior to inactivated preparations due to their ability to induce cell-mediated immunity in addition to antibody responses. DNA vaccines however, mimic the effects of live attenuated vaccines in their ability to induce MHC class I restricted CD8+ T cell responses, whilst alleviating some of the safety concerns associated with live attenuated vaccines. So far DNA vaccines have been effective in generating immune responses and protection in a wide variety of pre-clinical models of viral, bacterial, parasitic infections, and cancer as shown in Table 1.1. No studies however, have investigated the possibility of DNA vaccines against *S. typhi*.

Using a mouse model of typhoid fever, this thesis presents novel work in the field of DNA vaccines for *Salmonella*. For the first time, the technique of ELI is applied to analyse the immune responses induced by DNA vaccines encoding multiple antigens for *S. typhimurium* SL1344. In addition the influence of the route of administration and the strain of mice on the outcome of the immune response is investigated. In addition, the
induction of specific cellular and humoral responses following immunisation with a
cytosolic preparation of *S. typhimurium* SL1344 formulated with DDA are investigated
and their role in protection assessed.
Chapter 2

Materials and Methods
2.1 Materials

The following section is subdivided into six parts and provides details of all the materials used and their manufacturers. Materials that were commonly used for a wide range of techniques are described in this section, whilst details of the more infrequently used materials are described under their relevant methods in section 2.2. Unless stated otherwise, all materials were of analytical grade or higher.

2.1.1 Enzymes, Chemicals and Isotopes

All the enzymes and DNA molecular markers used in this project were obtained from Roche. Antibiotics, bovine serum albumin (BSA), 30% (w/w) solution of hydrogen peroxide, lysozyme, glutathione, and protease inhibitors were purchased from Sigma. Isopropyl-β-D-thiogalactoside (IPTG) was purchased from Melford Laboratories, and 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) was purchased from Roche. SeaKem® LE agarose was purchased from BMA Products. Plasmid Wizard® ‘mini prep’ kits were supplied by Qiagen. Big Dye™ DNA sequencing kit was purchased from Novatech. A stabilised solution of [Methyl ³H]-thymidine (5mCi, 185MBq, 5ml, 2.0 Ci/mmol) was purchased from Amersham International. Glutathione Sepharose 4B was obtained from Amersham Pharmacia Biotech. Printal Filtermats and Meltilex scintillent sheets were supplied by Wallac. Dulbecco’s Modified Eagle Medium (DMEM), lipofectAMINE™ and bovine calf serum of USA origin was purchased from Gibco Life Technologies. All standard laboratory chemicals were supplied from Sigma, BDH Chemicals and Fisons Scientific Equipment.
2.1.2 Antibodies, Western Blots and ELISAs

Rabbit anti-His IgG and rabbit anti-GFP IgG antibodies for Western blotting were purchased from Santa Cruz Biotechnologies, whilst horseradish peroxidase conjugated sheep anti mouse IgG was obtained from SAPU. Goat anti mouse IgG1 and bovine anti mouse IgG2a for serum subtype analyses were purchased from Serotec. O-phenylenediamine (OPD) was purchased from Sigma. For cytokine enzyme-linked immunosorbent assay (ELISA), rat anti mouse IFN-γ IgG1 monoclonal antibody, anti mouse IL-4 11B11 monoclonal antibody, biotinylated rat anti mouse IFN-γ IgG1 antibody and biotinylated rat anti mouse IL-4 IgG1 antibody was obtained from BD Pharmingen. Recombinant IFN-γ and IL-4 was purchased from Sigma. Extravidin alkaline phosphatase conjugate and p-Nitrophenyl phosphate (pNPP) tablets were purchased from Sigma. The ECL + plus Western blotting detection system was purchased from Amersham Biosciences. Nitrocellulose membrane was obtained from Schleicher and Schuell. Medical film was supplied by Konica. Flat bottom, 96 well Immunlon 2 HB microtiter plates were purchased from Dynex Technologies.

2.1.3 Oligonucleotides

Primers for polymerase chain reaction (PCR) and sequencing reactions were obtained from Genosys Biotechnologies. All oligonucleotide primers used are listed in Table 2.1. The numbers shown below primer reference refer to the position at which each of the primers lay with respect to the sequence from which they were derived; T7 and SP6 (Promega).
<table>
<thead>
<tr>
<th>Primer Reference (Position)</th>
<th>Primer Sequence</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7 PROMOTER (2984-3003)</td>
<td>5'-TAATACGACTCACTATAGGG</td>
<td>Sequencing EGFP-His from pGEM-T</td>
</tr>
<tr>
<td>SP6 PROMOTER (143-134)</td>
<td>5'-TATTTAGGTGACACTATAG</td>
<td>Sequencing EGFP-His from pGEM-T</td>
</tr>
<tr>
<td>SEQ PVCN2 FOR (803-823)</td>
<td>5'-CTATATAAGCAGAGCTTCTCTG</td>
<td>Sequencing EGFP-His from pVCN2</td>
</tr>
<tr>
<td>SEQ PVCN2 REV (1893-1873)</td>
<td>5'-GGGGCAAAACACAGATGGCTG</td>
<td>Sequencing EGFP-His from pVCN2</td>
</tr>
<tr>
<td>BglII.GFP (991-1013)</td>
<td>5'-TAGATCTCCGGGTACCGGTCGCC</td>
<td>PCR of EGFP-His from pVCN2</td>
</tr>
<tr>
<td>BamHI.HIS (1792-1770)</td>
<td>5'-ATCTCGAGGGATCCGCGCCTGTG</td>
<td>PCR of EGFP-His from pVCN2</td>
</tr>
<tr>
<td>ZKC3 INSERT F (1642-1661)</td>
<td>5'-GCAAAGACCCCAACGAGAAG</td>
<td>PCR of S. typhimurium DNA from pZKC3</td>
</tr>
<tr>
<td>ZKC3 INSERT R (1851-1870)</td>
<td>5'-GGCAACTAGAAGGCACAGTC</td>
<td>PCR of S. typhimurium DNA from pZKC3</td>
</tr>
</tbody>
</table>

Table 2.1 Primers used during the course of the work

2.1.4 Bacterial Strains and Media

_E. coli_ and _S. typhimurium_ strains and their derivatives used during the course of this study are described, (see Table 2.2). All bacterial cells were grown in Luria-Bertani (LB) medium excluding JM109 cells, which were grown in Spitzizen minimal medium.

**Luria-Bertani (LB) Medium and Agar:**

LB medium consisted of 10g Difco bacto-tryptone, 5g Difco yeast extract and 10g NaCl, dissolved in 1 litre of dH2O. The pH of the medium was adjusted to 7.2 with 5M NaOH and then sterilised by autoclaving. LB agar was formed by adding 15g Difco Bacto-agar to 1 litre of LB medium.
<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Genotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> DH5α</td>
<td><em>SupE44 ΔlacU169 (φ80 lacZΔM15) endA1 hsdR17 recA1 gyrA96 thi-1 relA1</em></td>
<td>Hanahan (1985)</td>
</tr>
<tr>
<td><em>E. coli</em> JM109</td>
<td><em>endA1 recA1 gyrA96 thi hsdR17 (rK-, mK⁺) relA1 supE44 Δ(lac-proAB) [F' traD36 proAB lacIqZΔM15]</em></td>
<td>Yanisch-Perron <em>et al.</em> (1985)</td>
</tr>
<tr>
<td><em>E. coli</em> BL21DE3</td>
<td><em>hsdS gal (λcIts857 ind1 sam7 nin5 lacUV5-T7 gene1)</em></td>
<td>Studier <em>et al.</em> (1990)</td>
</tr>
<tr>
<td><em>S. typhimurium</em> SL1344</td>
<td><em>his</em></td>
<td>Hoiseth and Stocker (1981)</td>
</tr>
</tbody>
</table>

**Table 2.2 Bacterial strains and their genotypes**

**Spitzizen Minimal Medium:**

Spitzizen minimal medium (Spitzizen, 1958) consisted of 300ml dH₂O, 80ml (4x) Spitzizen Salts (7g K₂HPO₄, 10g (NH₄)SO₄, 3g KH₂PO₄, 5g sodium citrate and 1g MgSO₄.7H₂O dissolved in 1 litre of water), 10ml 20% (w/v) glucose and 0.5ml thiamine B1 (1mg/ml).

**SOC Medium:**

SOC medium consisted of 20g Difco bacto-tryptone, 5g Difco yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgSO₄ and 20mM glucose dissolved in 1 litre of dH₂O. SOC medium was sterilised by autoclaving and was stored at 4°C.

**2.1.5 Plasmids**

The plasmids and their derivatives used in this thesis are described, (see Table 2.3).
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant Features</th>
<th>Source Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM-T</td>
<td>ColE1 replicon, Amp&lt;sup&gt;R&lt;/sup&gt;, LacZα</td>
<td>Promega</td>
</tr>
<tr>
<td>pcDNA3</td>
<td>ColE1 replicon, Amp&lt;sup&gt;R&lt;/sup&gt;, Neo&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pVCN2</td>
<td>pcDNA3 derivative containing the 0.8kb egfp gene subcloned from pEGFP (Clontech), and a synthetic 50bp fragment downstream of eGFP encoding a His tag; Amp&lt;sup&gt;R&lt;/sup&gt;, Neo&lt;sup&gt;R&lt;/sup&gt;</td>
<td>V. C. North</td>
</tr>
<tr>
<td>pZKC3</td>
<td>pVCN2 derivative but with the position of the BamHI site altered using primers BglII.GFP and BamHI.His</td>
<td>This project</td>
</tr>
<tr>
<td>pZKC5</td>
<td>pZKC3 derivative containing 545bp of the ybbA gene from &lt;i&gt;S. typhimurium&lt;/i&gt;</td>
<td>This project</td>
</tr>
<tr>
<td>pZKC6</td>
<td>pZKC3 derivative containing the complete traD gene (563bp) from &lt;i&gt;S. typhimurium&lt;/i&gt;</td>
<td>This project</td>
</tr>
<tr>
<td>pZKC7</td>
<td>pZKC3 derivative containing 811bp of a putative outer membrane protein of &lt;i&gt;S. typhimurium&lt;/i&gt;</td>
<td>This project</td>
</tr>
<tr>
<td>pTMB18</td>
<td>pUC19 plasmid encoding a glutathione-S-transferase EGFP fusion protein; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>T. Martin (2003)</td>
</tr>
</tbody>
</table>

*Table 2.3 Plasmids used during the course of the work.*

### 2.1.6 Solutions

Unless stated otherwise, all solutions were made up in dH<sub>2</sub>O and sterilised by autoclaving at a pressure of 15 lb/inch<sup>2</sup> and a temperature of 110°C for 20 minutes prior to use.

**Tris.Cl:**

Tris hydroxymethyl aminomethane (Tris base) was dissolved to the desired molarity in dH<sub>2</sub>O and was adjusted to the required pH using concentrated HCl.

**EDTA:**

Ethylenediaminetetraacetic acid di-sodium salt (EDTA) was dissolved in dH<sub>2</sub>O to a concentration of 0.5M and was adjusted to pH 8.0 using 1M NaOH.
**TE buffer:**
TE buffer consisted of 10mM Tris.Cl (pH 8.0) and 1mM EDTA in dH₂O.

**TBE buffer:**
TBE buffer contained 90mM Tris.Cl (pH 7.5), 90mM Boric acid and 2mM EDTA in dH₂O.

**Sodium Acetate:**
Sodium acetate was dissolved in dH₂O to a final concentration of 3M and the pH was adjusted to 5.0 using glacial acetic acid.

**Phosphate buffered saline:**
Phosphate buffered saline (PBS) pH 7.2 was prepared by dissolving 8g NaCl, 0.2g KCl, 1.44g Na₂HPO₄ and 0.24g NaH₂PO₄ in 1 litre dH₂O.

**5x Tris glycine SDS buffer:**
Tris glycine sodium dodecyl sulphate (SDS) buffer was prepared by dissolving 144g glycine, 30g Tris-base, and 5g SDS in 1 litre dH₂O. This buffer was not autoclaved prior to use, and was stored at 4°C.

**Carbenicillin antibiotic solution:**
A stock solution of 200mg/ml carbenicillin antibiotic was made in dH₂O and was diluted to a working concentration of 50μg/ml in LB medium. Carbenicillin solutions were stored at -20°C prior to use.

**5x DNA loading buffer:**
Loading buffer for DNA gel electrophoresis comprised of a 20% (w/v) sucrose solution in dH₂O, which was autoclaved prior to adding 0.25% (w/v) bromophenol blue.
2x Laemmli protein loading buffer:
Laemmli loading buffer for SDS-polyacrylamide gel electrophoresis comprised of 5mM Tris.Cl, pH 6.8, containing 0.4% (w/v) SDS, 2% (v/v) glycerol, 0.2M DTT and 0.002% (w/v) bromophenol blue. Laemmli protein loading buffer was not autoclaved prior to use and was stored at 4°C.

2.1.7 Molecular weight standard markers

Molecular weight markers for SDS polyacrylamide gel electrophoresis (SDS-PAGE) are shown in Table 2.4.

<table>
<thead>
<tr>
<th>Protein Standard</th>
<th>Approximate Molecular Weight (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Lactalbumin</td>
<td>14.2</td>
</tr>
<tr>
<td>Trypsin Inhibitor</td>
<td>20.1</td>
</tr>
<tr>
<td>Trypsinogen</td>
<td>24.2</td>
</tr>
<tr>
<td>Carbonic Anhydrase (bovine)</td>
<td>29</td>
</tr>
<tr>
<td>Glyceraldehyde-3-Phosphate Dehydrogenase</td>
<td>36</td>
</tr>
<tr>
<td>Albumin (egg)</td>
<td>45</td>
</tr>
<tr>
<td>Albumin (bovine)</td>
<td>66</td>
</tr>
</tbody>
</table>

Table 2.4. Molecular weight markers for SDS-PAGE
2.2 Methods

This section provides details of the methods used to pursue this work. It is divided into six sections with methods of a similar nature grouped together under the appropriate heading.

2.2.1 Manipulation of Bacteria

2.2.1.1 Growth of Bacterial Cultures

Cultures of *E. coli* and *S. typhimurium* were prepared by inoculating 5ml LB broth (including antibiotic where appropriate) with a single bacterial colony. Cultures were grown for 16 hours at 37°C with rotational shaking at 200rpm in a New Brunswick Scientific controlled environment incubator. Larger volumes of bacterial cultures were prepared by diluting an overnight bacterial culture 1:100 into the required volume of LB broth. Typically cultures were grown in flasks 5 fold larger than the final culture volume, with growth conditions as stated above.

2.2.1.2 Storage of Bacterial Cultures

For long term storage of bacteria, a 1ml volume of an overnight culture was mixed with 75μl of DMSO and stored in a sterile cryovial at −80°C. Stored cells were recovered by streaking a sample onto LB agar plates containing the appropriate antibiotic and incubating at 37°C for 16 hours. Following the 16 hours incubation, single colonies were picked from the plates and a fresh culture propagated. For short-term storage (3-4 weeks), bacteria were streaked onto LB agar plates with the necessary antibiotic and maintained at 4°C.
2.2.1.3 Transformation of Bacteria by Electroporation

A culture of the bacterial strain to be transformed was grown for 16 hours in 5ml LB broth then diluted 1:100 into 500ml LB and grown until an optical density of 0.5 at 600nm. The cells were chilled at 4°C for 1 hour before being pelleted at 4,000g for 10 minutes at 4°C. The cells were gently resuspended in 150ml of chilled sterile dH2O, before being pelleted again at 4,000g. A further three chilled water washes were performed before the cells were finally resuspended in 0.5ml of sterile dH2O and aliquoted into 100μl volumes on ice. To each 100μl aliquot of competent cells, between 0.5ng and 0.5μg of DNA was added and mixed thoroughly. The cells and DNA mix was then transferred to pre-chilled electroporation cuvettes ensuring no air bubbles were present. Cells underwent electroporation with a voltage pulse of 2.5 kV with a resistance of 2000 using a BIORAD ‘Gene-pulser’. Following electroporation, the cells were mixed with 800μl of SOC medium and shaken at 37°C for 1 hour. Cells were then plated onto LB plates containing 50μg/ml carbenicillin.

2.2.1.4 Transformation of Bacteria by Heat Shock

A small 5ml culture of the bacterial strain to be transformed was grown for 16 hours in LB broth then diluted 1:100 into 250ml LB containing 20mM MgSO4, and grown at 37°C until an optical density of 0.5 at 600nm. The cells were chilled at 4°C for 20 minutes before being centrifuged at 4,000g for 10 minutes at 4°C. The cells were then resuspended in 100ml of chilled TFB1 (30mM KOAc, 10mM CaCl2, 50mM MnCl2, 100mM RbCl2, 15% [v/v] glycerol in dH2O, pH 5.8) and incubated at 4°C for 5 minutes. Following incubation, the cells were pelleted again at 4,000g for 10 minutes before finally being resuspended in 10ml of chilled TFB2 (10mM PIPES, 75mM CaCl2, 2.5mM RbCl2, 15% [v/v] glycerol in dH2O, pH 6.5). Cells were incubated at 4°C for 45 minutes, following which aliquots of 200μl were snap-frozen on dry ice for storage.
Prior to transformation, aliquots of competent cells were thawed slowly at 4°C and incubated with between 0.5 and 5μg of DNA for 20 minutes. The cells were then transferred to a water bath at 42°C for 45 seconds, then immediately returned to 4°C for a further 2 minutes. Following the heat shock procedure, cells were mixed with 450μl of LB medium and incubated at 37°C with shaking at 200rpm for 1 hour. The transformed cells were then plated on LB agar containing 50μg/ml carbenicillin.

2.2.2 DNA Manipulation and Detection

2.2.2.1 Agarose Gel Electrophoresis

DNA was resolved by electrophoresis on a 1.0% agarose (w/v) gel in 1 x TBE buffer containing 0.5μg/ml ethidium bromide. DNA samples were prepared for loading by mixing with 5 x loading buffer at a ratio of 4:1 (DNA: loading buffer). The samples were electrophoresed at 100V in a 110cm x 150mm in a horizontal submarine electrophoresis tank for approximately 1 hour. Nucleic acid size was determined by comparison to the position of pre-digested HindIII phage lambda DNA fragments (molecular weight marker II, from Roche). DNA was visualised under an UV trans-illuminator at a wavelength around 313nm and was photographed.

2.2.2.2 Recovery of DNA from Agarose Gels

The DNA fragment of interest was resolved by agarose gel electrophoresis, and the appropriate band was cut from the gel, weighed, and stored in an eppendorf. The DNA was purified from the agarose using a Qiagen Gel Extraction Kit and followed the manufacturer’s guidelines.
2.2.2.3 Ethanol Precipitation of DNA

DNA was precipitated from solution by mixing the DNA solution with 3M sodium acetate buffer, pH 5.0 and 95% (v/v) ethanol in a ratio of 10:1:25 respectively. Following thorough mixing, the solution was incubated at —80°C for 1 hour and the DNA was then pelleted by centrifugation at 20,000g for 15 minutes. The DNA pellet was washed with 1 volume of 70% ethanol (v/v) in dH2O and then re-centrifuged for a further 10 minutes at 20,000g. Following a second washing step with 70% ethanol, the DNA pellet was dried under vacuum and resuspended to the required concentration in dH2O.

2.2.2.4 Phenol/Chloroform Extraction of DNA

DNA was purified from cell debris and proteins by vortexing one volume of DNA solution with an equal volume of phenol saturated with 10mM Tris.Cl, pH 8.0 and 1mM EDTA. The upper aqueous phase containing the DNA was separated from the phenolic phase by centrifugation at 20,000g for 10 minutes. The aqueous phase was then transferred to an equal volume of saturated phenol/chloroform (1:1), vortexed and centrifuged again for 10 minutes at 20,000g. Again the upper aqueous phase was removed and transferred to an equal volume of chloroform, where it was mixed and separated as previously stated. Finally, the upper aqueous layer was transferred to a new eppendorf and stored at —20°C.

2.2.2.5 Restriction Endonuclease Digestion of DNA

Restriction endonuclease digestion was performed in dH2O containing between 0.1μg and 10μg of DNA, 1/10th volume of a 10 x enzyme buffer (appropriate to the enzyme), and 1.0 unit of the appropriate enzyme. The digestion reaction was incubated at 37°C for
2 hours, then at 65°C for 20 minutes in order to thermally inactivate the enzyme. In the case of dual digests where the enzymes required different buffers, the DNA was first cleaved with the enzyme requiring the lowest salt, then the higher salt buffered enzyme.

Partial digests of *S. typhimurium* chromosomal DNA were performed in 1ml volumes containing between 200μg and 300μg chromosomal DNA, 1 x *Sau3AI* buffer and 0.6 units of *Sau3AI* enzyme. Digests were incubated at 37°C for 30 minutes then heat inactivated at 65°C for 20 minutes.

2.2.2.6 Dephosphorylation of Vector DNA

Plasmid vectors digested with restriction enzymes were treated with shrimp alkaline phosphatase (SAP) to prevent the direct religation of the vector ends during ligation reactions. Vector dephosphorylation was performed in a 20μl volume containing 0.2 - 10μg vector DNA, 1/10th volume of 10 x SAP buffer and 1.0 unit of SAP. The vector DNA and SAP was then incubated at 37°C for 1 hour before the SAP was inactivated by incubation at 65°C for 20 minutes.

2.2.2.7 Ligation of DNA

Ligations were performed in dH₂O containing the appropriate amounts of vector and insert, as calculated by equation 1, 1/10th volume of 10 x T4 DNA ligase buffer and 1.0 unit of T4 DNA ligase. Ligation reactions were incubated at 16°C for 16 hours.

The amount of insert in the ligation mix can be determined by:

\[ M_i = \frac{M_v \times S_i}{S_v} \times I : V \]  

(1)
where $Mi$ is the mass of insert in ng, $Mv$ is the mass of vector in ng, $Si$ is the size of insert in kb, $Sv$ is the size of vector in kb, and $I: V$ is the insert: vector molar ratio (Sambrook et al., 1989). Typically an insert: vector molar ratio of 5:1 is most efficient for ligation (Sambrook et al., 1989).

2.2.2.8 Polymerase Chain Reaction

The oligonucleotide primers used in this project are listed in table 2.1. The polymerase chain reaction (PCR) was performed in 100μl volumes containing, 10mM Tris-HCl, 50mM KCl, 1.1mM MgCl2, 0.01% gelatin, 1.0 unit of Taq polymerase, 10mM dNTPs, 0.3nM of each primer and 1μg pVCN2 plasmid DNA. The cycle for amplification was as follows: Initial denaturation: 10 min, 95°C; then 25 cycles of (1) denaturation 1 min, 95°C; (2) annealing 1 min, 65°C; (3) polymerisation 1min, 72°C. A final elongation at 72°C was carried out for a further 10 min. The PCR product was purified from excess dNTPs, primers and enzymes using a Qiagen PCR Purification Kit following manufacturer’s instructions.

2.2.2.9 Plasmid Preparation

For small scale preparations, plasmid DNA was purified from cultured cells by the alkaline lysis method (Birnboim and Doly, 1979). Bacterial cultures grown in 5ml LB broth for 16 hours at 37°C were centrifuged in 1.5ml eppendorf tubes at 14,000g for 10 minutes. Pellets were resuspended at room temperature for 5 minutes in 100μl of Solution I (25mM Tris.Cl pH 8.0, containing 50mM glucose and 10mM EDTA), then mixed carefully with 200μl of fresh Solution II (0.2M NaOH, containing 1% [w/v] SDS) and incubated at 4°C for 5 minutes. The suspensions were neutralised with 300μl Solution III (3M potassium, 5M acetate, pH 4.8), incubated at 4°C for a further 5 minutes, then centrifuged at 20,000g for 5 minutes. The supernatant was removed and
mixed with an equal volume of phenol: chloroform. Following centrifugation at 20,000g for 10 minutes, the top aqueous layer was removed and mixed with two volumes of 95% ethanol then re-centrifuged at 20,000g. The DNA pellet was then washed with 70% ethanol and dried under vacuum. The plasmid DNA was finally resuspended in 100μl of nuclease-free water and stored at -20°C. For the preparation of plasmid DNA for sequencing reactions, Promega Wizard DNA Preparation Kits were used according to the manufacturer’s guidelines.

To prepare large quantities of pure plasmid DNA, the protocols of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981) were employed. A bacterial colony containing the plasmid of interest was used to inoculate 500ml LB broth containing 50μg/ml carbenicillin antibiotic and incubated at 37°C for 16 hours. For purifying DNA from the expression library, a frozen 0.5ml aliquot was thawed and used to inoculate 500ml of LB broth. Cells were centrifuged at 4,000g for 10 minutes then resuspended in 18ml of Solution I (see above). Following incubation for 5 minutes at room temperature, the cell suspension was mixed thoroughly with 40ml of freshly prepared Solution II and incubated at 4°C for a further 5 minutes. A 20ml volume of chilled Solution III was then added to the sample and mixed thoroughly. The sample was then incubated for 15 minutes at 4°C, and centrifuged at 9,800g for 10 minutes to pellet the cell debris. The supernatant was filtered through glass wool to remove any large cell debris, then mixed with 48ml of isopropanol. The plasmid DNA was pelleted by centrifugation at 15,300g for 10 minutes, and was washed with 70% ethanol. The DNA pellet was then left to air dry for approximately 15 minutes before it was resuspended in exactly 9ml of 1 x TE buffer. Once the plasmid DNA was in solution, 9.2g of CsCl and 0.5ml of ethidium bromide solution was added. The sample was gently mixed by inversion until all the CsCl had dissolved and then centrifuged at 6,000g for 10 minutes to remove final traces of protein. For the successful separation of DNA following ultracentrifugation, it was important to ensure the supernatant weighed 1.55g/ml. For the supernatants weighing less than 1.55g/ml, additional CsCl was added until this weight was reached. The
supernatant was then transferred to 2 polypropylene ultracentrifuge tubes (Kendro) and centrifuged at 118,000g for 16 hours.

The lower band of plasmid DNA was easily visualised under natural light, and was extracted using a sterile needle and 1ml syringe. Ethidium bromide was quickly extracted from the plasmid DNA by mixing the plasmid DNA solution with an equal volume of TE saturated butan-1-ol and removing the uppermost ethidium bromide/butanol layer. This extraction process was repeated 6 times until all the ethidium bromide colouration had been removed. The purified plasmid DNA was then ethanol precipitated, as described above, before being pelleted by centrifugation at 18,000g for 15 minutes. The DNA pellet was then washed twice with 70% ethanol. The plasmid DNA was air dried before being resuspended in PBS. The DNA solution was then dialysed for 2 hours against 1 litre PBS at 4°C. This dialysis stage was repeated a further 4 times to remove all traces of CsCl salt before the purified DNA was stored at -70°C. Plasmid DNA was quantified using a spectrophotometer at wavelengths of 260nm and 280nm.

2.2.2.10 Preparation of Chromosomal DNA

To prepare chromosomal DNA, the protocol of Silhavy et al., 1984, was employed. A 100ml culture of the desired bacterium was grown overnight at 37°C with shaking at 300rpm. The cells were pelleted by centrifugation at 7,500g for 10 minutes then resuspended in 5ml of 50mM Tris.Cl solution containing 50mM EDTA, before being stored at -20°C. A sample of fresh lysozyme (10mg/ml in 0.25M Tris.HCl, pH 8.0) was prepared and added to the frozen cells. Once the cells had just thawed, the sample was incubated at 4°C for 45 minutes. Following incubation, 1ml of STEP solution (50mM Tris.Cl pH 8.0, 0.4M EDTA, 0.5% [w/v] SDS, and 1mg/ml (final concentration) proteinase K) was added and mixed thoroughly. The sample was then incubated at 50°C for 1 hour with occasional mixing. A 6ml volume of equilibrated phenol was added to
the sample and gently mixed for 5 minutes before being centrifuged at 4,500g for 15 minutes. The top aqueous layer containing the DNA was transferred to a clean tube and precipitated by ethanol precipitation, as previously described. The DNA was spooled using a glass pipette into a clean tube containing 5ml of 50mM Tris.Cl buffer, pH 7.5, containing 1mM EDTA, and 200μg/ml RNase A. The DNA pellet was dissolved following overnight incubation at 4°C with gentle rocking. Once completely dissolved, an equal volume of chloroform was mixed with the DNA and the sample was centrifuged at 4,500g for 15 minutes. The aqueous layer (top) containing the DNA was then re-precipitated to leave a pellet of chromosomal DNA. Finally the DNA was dissolved in 2ml of 50mM Tris.Cl buffer, pH 7.5, containing 1M EDTA and was stored at —20°C.

2.2.2.11 Sucrose Density Gradient Centrifugation

Sucrose solutions containing 40%, 35%, 30%, 25%, 20%, 15%, and 10% (w/v) sucrose were made up in gradient buffer (20mM Tris.Cl buffer, pH 7.1, containing 20mM EDTA, 1M NaCl, and 0.3% [w/v] Sarcosyl) and were autoclaved at 100°C for 10 minutes. Gradients were prepared by layering equal volumes of each sucrose solutions into SW28 Beckman tubes giving a total volume of 16ml/tube, and allowing them to diffuse for 12-16 hours at 4°C. Following overnight diffusion, 1ml of partially digested *S. typhimurium* chromosomal DNA was carefully layered onto each gradient, and balanced using sterile water. Each gradient was centrifuged at 80,000g for 16 hours at 10°C in a Beckman SW28 rotor.

Sucrose gradients were fractionated using a BIORAD Econo pump attached to tubing inserted at the bottom of the gradient. Fractions of approximately 500μl were collected into 1.5ml eppendorf tubes and stored at 4°C. From each fraction, a 15μl sample was taken and examined by electrophoresis on a 0.5% [w/v] agarose gel. Fractions containing fragments of approximately 1-2kb were identified by comparison to DNA
molecular weight markers. Since high levels of salt affect DNA mobility, the molecular weight marker was constituted with salt and sucrose levels equivalent to those of the 30% sucrose solution so comparisons between the size of the DNA fractions and the marker could be made accurately. The fractions containing DNA fragments between 1-2 kb in size were then dialysed against 500 ml of TE buffer for 2 hours at 4°C. The dialysis process was repeated a further 4 times before the DNA was precipitated and resuspended in sterile dH2O to a final concentration of 0.3 μg/μl and stored at —20°C.

2.2.2.12 Construction of a S. typhimurium SL1344 DNA Expression Library

Partially digested fragments of S. typhimurium DNA from strain SL1344 approximately 1-2 kb in size were ligated into the pZKC3 expression vector, previously digested with BamHI and dephosphorylated using SAP. Ligations were performed in 10 μl volumes each containing 400 ng of pZKC3 vector and 600 ng of S. typhimurium SL1344 insert DNA. Ligation reactions were incubated at 16°C for 16 hours, after which they were transferred to 65°C for 20 minutes to thermally inactivate the T4 DNA ligase. Following thermal inactivation, each ligation mix was chilled for 10 minutes at 4°C before being mixed with competent cells. Competent cells were then heat shocked as previously described, before being incubated in 450 μl of LB for 1 hour at 37°C with shaking at 200 rpm. The transformed cells were plated onto LB agar, containing 50 μg/ml carbenicillin, and were incubated at 37°C for 16 hours. Colonies appearing on each plate were then thoroughly mixed with 1.5 ml LB and stored at —70°C in three aliquots of 0.5 ml, each containing 35 μl of DMSO.
2.2.3 Protein Overexpression and Purification

2.2.3.1 Induction of Transformed Bacterial Cells

A single colony was inoculated into 5ml of LB broth containing the appropriate antibiotic and grown for 16 hours at 37°C with shaking. The culture was then diluted 1:100 into LB broth containing antibiotic, and grown at 37°C with shaking to an optical density of 0.5 at 600nm. Cells were then induced with 1mM isopropyl-β-D-thiogalactoside (IPTG) and grown for a further 3 hours with shaking at 37°C. A 2ml sample of bacteria was collected every 30 minutes starting from the time of induction, and was pelleted at 20,000g for 3 minutes and drained before being stored at —20°C for later analysis. Following the 3 hours induction period, the remaining culture was centrifuged at 4,000g for 10 minutes in a Beckman JA-14 rotor at 4°C. The pellet was then resuspended in 20ml of chilled Buffer A (see below) before being lysed using a French Press at 5,000 lb/inch². Cellular debris was then removed by centrifugation at 20,000g for 30 minutes at 4°C in a Beckman JA-20. The remaining supernatant was retained and stored at —70°C.

2.2.3.2 Purification of Polyhistidine Tagged Proteins

Proteins containing a polyhistidine (His) tag were purified from non-His-tagged proteins using nickel-nitrilotriacetic acid (Ni²⁺-NTA) resin (Qiagen). Columns were prepared at 4°C using 1.5ml of Ni²⁺ NTA resin which formed a 1ml column. Once the resin had settled, the column was washed 3 times with 10ml of dH₂O and equilibrated with 10ml of Buffer A (see below). A 10ml aliquot of supernatant from IPTG induced bacterial cells (2.2.3.1), was defrosted on ice and applied to the column. The resin and supernatant in the capped column was mixed on a blood wheel at 4°C for 1.5 hours to allow His-tagged proteins to bind to the Ni²⁺ groups of the resin. Following this incubation, the column was fixed on a clamp stand to allow the resin to settle. Once settled, the cap at
the bottom of the column was removed to allow the supernatant to flow through the column. The column was washed 5 times with 2ml of Buffer B to remove any non-His-tagged proteins. His-tagged proteins were then eluted 3 times with 0.5ml of Buffer C and stored at −70°C. Typically 20μl samples from each phase in the purification process were analysed by SDS polyacrylamide gel electrophoresis (2.2.4.1) to ensure the purification process was successful.

Following elution of the desired protein, the columns were regenerated to allow future purification processes. Columns were washed 3 times with 10ml of Buffer R before being stored at 4°C in dH2O. The manufacturers (Qiagen) recommended a maximum of 5 purification events before changing the resin.

**Buffer A:**
50mM Tris.Cl, pH 8.0, containing 50mM NaCl, 5mM EDTA, 5μg/ml leupeptin, 5μg/ml pepstatin A, 5μg/ml aproptinin, 1mM (AEBSF), 10mM β-Mecaptoethanol, 0.01% (v/v) Triton X-100, 5μg/ml RNase and 10μg/ml lysozyme.

**Buffer B:**
20mM Tris.Cl, pH 8.0, containing 20% (v/v) glycerol, 100mM KCl, 1mM AEBSF and 20mM Imidazole.

**Buffer C:**
20mM Tris.Cl, pH 8.0, containing 20% (v/v) glycerol, 100mM KCl, 1mM AEBSF and 80mM Imidazole.

**Buffer D:**
50mM Tris.Cl, pH 8.0, containing 20mM glutathione and 100mM NaCl.

**Buffer R:**
6M Guanidine HCl, containing 0.2M acetic acid.
2.2.3.4 Purification of Glutathione-S-Transferase Proteins

Proteins expressed as fusions to GST were purified from bacterial cell lysates by their affinity for glutathione sepharose. Columns were prepared at 4°C with 1.5ml of GST sepharose, which settled to form a 1ml column. The sepharose column was washed 3 times with 10ml of dH2O then equilibrated with 10ml Buffer A (see above). A 10ml aliquot of supernatant from induced bacterial cells, prepared by French pressing and centrifugation (2.2.3.1), was then defrosted on ice and applied to the column. The sepharose and supernatant were mixed within the sealed column on a blood wheel at 4°C for 1.5 hours to allow the binding of GST fusion proteins to the glutathione sepharose. Following this incubation, the column was fixed on a clamp stand to allow the sepharose to settle. Once settled, the cap at the end of the column was removed to allow the supernatant to flow through the column. The column was then washed 5 times with 2ml of Buffer A before being transferred to room temperature in preparation for the elution stage. Once the column had equilibrated to room temperature, 0.5ml Buffer D was added to the column. The column was then left to incubate for a further 15 minutes at room temperature before the GST fusion proteins were eluted. This final elution step was repeated a further two times to ensure all the protein had been eluted. Purified proteins were then stored at —70°C. Typically 20µl samples from each phase of the purification process were collected and analysed by SDS polyacrylamide gel electrophoresis, as described in section 2.2.4.1, to ensure the purification process was successful.

Following elution of the desired protein, the columns were regenerated at 4°C to allow future purification processes. Columns were washed 3 times with 2ml 3M NaCl in PBS, 3 times with 2ml PBS, 3 times with 3ml of Buffer R, then an additional 3 times with 2ml PBS. The column was stored under 20% ethanol at 4°C until required.
2.2.4 Protein Detection Procedures

2.2.4.1 SDS-Polyacrylamide Gel Electrophoresis of Proteins

SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). The process involved denaturing the proteins and separating them according to size. Proteins were resolved on a 6cm x 9cm, 0.75mm thick SDS- polyacrylamide gel using the mini-PROTEAN II slab gel apparatus (BIORAD). The gel was composed of a 12.5% (v/v) polyacrylamide resolving gel and a 5% (v/v) polyacrylamide stacking gel. The compositions for a 10ml resolving gel and a 10ml stacking gel are shown below:

**Resolving Gel (12.5% [v/v] polyacrylamide):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% (w/v) acrylamide/bisacrylamide (19:1)</td>
<td>4ml</td>
</tr>
<tr>
<td>1.5M Tris.Cl, pH 8.8</td>
<td>2.5ml</td>
</tr>
<tr>
<td>20% (w/v) SDS</td>
<td>50μl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>3.4ml</td>
</tr>
<tr>
<td>10% Ammonium persulphate (APS)</td>
<td>50μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5μl</td>
</tr>
</tbody>
</table>

**Stacking Gel (4% [v/v] polyacrylamide):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% (w/v) acrylamide/bisacrylamide (19:1)</td>
<td>1.4ml</td>
</tr>
<tr>
<td>0.5M Tris.Cl, pH 6.8</td>
<td>2.3ml</td>
</tr>
<tr>
<td>20% (w/v) SDS</td>
<td>50μl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>6.2ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>100μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10μl</td>
</tr>
</tbody>
</table>

The resolving gel was poured between the two glass plates, leaving a 3cm gap from the top of the largest glass plate for the stacking gel. The resolving gel was overlaid with TE
saturated butanol and left to polymerise for 30 minutes at room temperature. Following polymerisation, all traces of the butanol overlay were removed using dH₂O and blotting paper and the stacking gel was layered onto the resolving gel. A comb was quickly inserted into the stacking gel to generate wells. The gel was then left to polymerise for 30 minutes at room temperature.

Samples to be loaded were mixed with 2 x Laemmli protein loading buffer, heated to 99°C for 5 minutes, then spun in a microcentrifuge at 18,000g for 30 seconds. A 20μl volume of each sample was loaded into each well and molecular weight markers were loaded at the ends of the gel for molecular weight analysis. The proteins were electrophoresed at 12 mA (per gel) and 250V in 1 x Tris glycine buffer for 1.5 hours.

2.2.4.2 Staining of SDS-Polyacrylamide Gels with Coomassie Brilliant Blue

Following SDS-PAGE, polypeptides were visualised by staining with Coomassie Brilliant Blue. Gels were removed from the mini-PROTEAN II apparatus and immersed in staining solution (0.1% [w/v] Coomassie Brilliant Blue R250 (BIORAD), dissolved in 45% [v/v] methanol, 10% [v/v] glacial acetic acid, 45% dH₂O) for 60 minutes with mild shaking at room temperature. Excess stain was then removed by immersing the gel into destaining solution (45% [v/v] methanol, 10% [v/v] glacial acetic acid, 45% dH₂O) for 4-8 hours at room temperature until individual bands could be visualised clearly. Gels were then photographed using the UVP chemi-darkroom apparatus.

2.2.4.3 Immunodetection of Proteins

Immunological detection of proteins was performed using a Western blot procedure. The SDS-polyacrylamide gel was removed from the micro-PROTEAN II apparatus and soaked in 1 x transfer buffer (25mM Tris.Cl, pH 8.3, containing 150mM Glycine, and
20% [v/v] methanol) for 2 minutes at 4°C. Blotting paper previously cut to 6cm x 9cm and soaked in 1 x transfer buffer was then layered onto a semi-dry TRANS-BLOT CELL (BIORAD). A piece of 6cm x 9cm nitro-cellulose membrane was also soaked in 1 x transfer buffer then layered onto the blotting paper. The SDS-polyacrylamide gel was then carefully layered onto the nitro-cellulose membrane and covered with more pre-soaked blotting paper. The polypeptides were then electrophoresed at 15V, 500mA for 50 minutes at 4°C onto the solid nitro-cellulose support (Towbin et al., 1979).

Following electrophoresis, the nitro-cellulose membrane was soaked in 10ml of Ponceau S (2g Ponceau S, 30g trichloroacetic acid, 30g sulphosalicylic acid, dissolved in 100ml dH₂O) for 5 minutes to reversibly stain the polypeptides. This was to ensure the polypeptides had successfully been transferred to the nitro-cellulose membrane and to ascertain the positions of the molecular weight markers for later analysis. Subsequently, Ponceau S stain was removed from the nitro-cellulose membrane by rinsing four times over a period of 20 minutes with PBST (0.25% [v/v] polyoxyethylene sorbitan monolaurate [Tween 20], in PBS).

Following the washes, the nitro-cellulose membrane carrying the electrophoresed polypeptides was incubated at room temperature in 20ml blocking solution (5% [w/v] dried milk powder (Safeway), 0.25% [v/v] Tween 20, in 100ml PBS) for 1 hour with gentle agitation. After this time, the nitro-cellulose membrane was washed a further four times in wash solution, then incubated at room temperature with 10ml of primary antibody solution (1:5000 dilution of rabbit anti-GFP antibody or 1:2000 dilution of rabbit anti-His antibody or 1:200 dilution of mouse sera, 0.1% [w/v] dried milk powder, 0.05% [v/v] Tween 20 in 1 x PBS). Following a one hour incubation, the nitro-cellulose membrane was washed four times with PBST to remove any unbound antibody, then incubated for a further hour at room temperature in secondary antibody solution (1:1000 dilution of horse radish peroxidase conjugated anti-rabbit or sheep anti-mouse antibody, 0.1% dried milk powder, 0.25% [v/v] Tween 20 in PBS). During this period, chemiluminescence solution A (50mg sodium luminol, 62ml H₂O₂, in 200ml 0.1M
Tris.Cl, pH 8.6) was mixed in a 100:1 ratio with solution B (11mg p-HO-coumaric acid, 10ml dimethylsulphoxide [DMSO]) and incubated at room temperature in the dark for 30 minutes. Following incubation with secondary antibody, the nitro-cellulose membrane was washed four times with PBST and once in PBS, then incubated with 10 ml of chemiluminescence solution (Knecht and Diamond, 1984). The nitro-cellulose membrane was dabbed dry then wrapped in cling film before being exposed to medical photographic film and developed in a dark room. Molecular weight markers were labelled on the photographic film for subsequent analysis of any band detected. Alternatively, luminescent bands on the nitro-cellulose membrane could be detected using the UVP epichem darkroom apparatus. For this latter method of detection, the nitro-cellulose membrane was submerged in ECL-PLUS chemiluminescence solution (Amersham) for a minimum of five minutes before being detected.

2.2.5 Mammalian Cell Studies

2.2.5.1 Recovery of L-Cells from Liquid Nitrogen Storage

Cryovials containing mouse fibroblast L-cells obtained from Dr. J. Guy (Kundig et al., 1995), were carefully removed from liquid nitrogen storage and quickly defrosted in a water bath at 37°C. Cells stored in liquid nitrogen are mixed with 10% DMSO (v/v), to prevent crystal formation. The DMSO is toxic to the cells, and must be removed quickly once the cells have thawed.

To 1ml of thawed cells, 9ml of cell culture medium (Dulbecco’s modified Eagle’s medium (DMEM) containing 10% [v/v] bovine calf serum and 1% [v/v] Streptomycin/penicillin) was added, and the sample was centrifuged at 300g for 5 minutes to pellet the cells. The supernatant was removed by aspiration using a vacuum pump and the cells were resuspended in 10ml of fresh cell medium. The cell suspension was then transferred to a 75cm² tissue culture flask (Corning) and an additional 15ml of
cell medium was added. The flask was then incubated at 37°C in a humidified atmosphere containing 5% CO₂ and air.

2.2.5.3 Trypsinisation of Adherent Cells

To prevent contact inhibition, cells were split 1:10 every 3 days. The cell medium was removed by aspiration and the remaining adherent cells were rinsed with 10ml of PBS for 30 seconds to remove all traces of serum, which inhibits the action of trypsin. The PBS was removed by aspiration, after which the cells were incubated for 2-3 minutes at 37°C with 2ml of trypsin solution (PBS, pH 7.2, containing 0.05% [w/v] trypsin and 0.53mM EDTA), and then gently agitated to loosen the cells from the flask. Once all the cells were detached, 8ml of cell medium was added to the flask to reduce the deleterious effects of trypsin, and 1ml was transferred to a new 75cm² flask containing 24ml of fresh, warm cell medium. The cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ and air.

2.2.5.4 Cell Counting

Cells were detached from a 75cm² flask using trypsin and made up to 10ml with cell medium as described above. A 30µl sample of cell suspension was carefully placed onto a haemocytometer and viewed under the microscope. The cells were counted over 25 central squares and the value obtained was converted to cells/ml using the equation below:

\[
\text{Cells/ml} = \text{Cell number} \times 1 \times 10^4
\]  

(2)
2.2.5.5 Transfection Procedure

The day prior to transfection, cells were trypsinised and counted, then plated at a cell density of $5 \times 10^5$ cells/well into a 6 welled plate, each containing sterile coverslips. During this time, all antibiotics in the cell medium were avoided to limit stresses on the cells.

For each well of cells, 3µg of DNA was diluted into 100µl of serum free OPTI-MEM I medium (GibcoB) and incubated at room temperature for 15 minutes. Similarly, for each well of cells, 8µl of LipofectAMINE (GibcoB) was diluted into 100µl of serum free OPTI-MEM I medium and incubated for 15 minutes at room temperature. Following the incubation stages, the diluted DNA was mixed with the diluted LipofectAMINE and incubated at room temperature for a further 15 minutes before being diluted to a total volume of 1ml/well using OPTI-MEM I medium. Immediately before transfection, the cells were washed 3 times with 1ml of PBS, and then the DNA/LipofectAMINE complexes were added gently to each well. The cells were then incubated for 16 hours at 37°C in 5% CO₂. After incubation, 3ml of cell medium and 100µl of foetal calf serum was added to each well of cells. The cells were then incubated for a further 24 hours at 37°C in a humidified atmosphere with 5% CO₂.

Following the final incubation, the coverslips were transferred to a new 6 well plate and washed 3 times with 1ml of PBS. After the third wash, cells were fixed to the coverslips by incubation at 37°C for 30 minutes with 1ml of fixing medium (3.5% [w/v] para-formaldehyde [PFA] in PBS). Once the cells were fixed, each coverslip was washed a further 3 times with PBS, dried carefully and mounted onto slides with one drop of Vectorshield (Vector Laboratories). The cells were then observed under an UV microscope (Zeiss) at x100 magnification using a FITSC filter, and images were captured using a digital camera and computer imaging software.
2.2.6 Murine Model Studies

Innately susceptible (*ity*<sup>i</sup>) female BALB/c mice of 8-10 weeks of age were used for all immunisation experiments. Mice were obtained from Edinburgh University and were segregated into cages with a maximum of five mice per cage. Mice were supplied with a commercial pellet diet and water *ad libitum*.

2.2.6.1 Preparation of *S. typhimurium* SL1344 Proteins for Injection into Mice

*S. typhimurium* strain SL1344 was grown to stationary phase in 250ml of LB broth. Cells were pelleted by centrifugation at 4,000g for 10 minutes in a bench top centrifuge then resuspended in 25ml of Buffer A (see section 2.2.3.2). The cell suspension was then passed through a pre-chilled French Press twice at 5,000lbs/inch<sup>2</sup> to lyse the cells. The membraneous components containing LPS were then removed by two high-speed centrifugation steps at 100,000g, each for 1 hour. Following each spin, the pellet containing the membrane fraction was removed and the remaining soluble fraction, termed cytosolic antigen (CA), was dialysed against 5 litres of PBS. The protein concentration was then determined using the Bradford assay (Bradford, 1976), using a BIORAD standard protein assay kit as described by the manufacturer. Proteins were aliquoted and stored at −70°C until required.

Prior to immunisation, the CA was mixed thoroughly with the adjuvant dimethyl dioctadecylammonium bromide (DDA) (Lindblad et al., 1997) and the preservative thimerosal. A 25mg/ml solution of adjuvant and 0.1% solution of thimerosal were prepared in sterile dH<sub>2</sub>O immediately prior to each immunisation. DDA was heated at 80°C for 10-20 minutes until fully dissolved then left to cool to room temperature to form micelles. Once cooled, the DDA was mixed thoroughly with the *S. typhimurium* cytosolic antigen, thimerosal and filter sterilised PBS, so as to inject each animal with 250µg DDA, 100µg cytosolic antigen and 0.01% thimerosal in a total volume of 200µl.
Mice were then injected subcutaneously into two sites (left and right) in the lumbar region. Injections were repeated on days 14 and 28 so each mouse received a total of 3 immunisations with cytosolic antigen.

2.2.6.2 Preparation of S. typhimurium SL1344 EL DNA for Injection into Mice

Frozen 0.5ml aliquots for 7 sub-libraries were individually inoculated into 1.5 liters of LB medium and incubated at 37°C with shaking for 16 hours. Large scale plasmid preparations were performed for each of the 7 sub-libraries, as described in section 2.2.2.10. The DNA concentration and cleanliness was determined using a spectrophotometer at 260nm and 280nm and was diluted using sterile PBS to a final concentration of 1µg/µl. Equal volumes of each DNA library aliquot were then mixed at a ratio of 1:1 to ensure an equal representation of DNA from each fraction. Mice were immunised either intramuscularly into the tibialis muscles, or intradermally at the base of the tail, with a total of 100μl of the DNA solution divided between two (left and right) sites. To ensure the mice were immunised at the correct site and in the correct manner, the fur covering the tibialis muscles and lumber regions was shaved off using a BaByliss beard trimmer. Injections were then repeated on days 14, 28 and 42 with each mouse receiving a total of 4 DNA immunisations.

2.2.6.3 Preparation of Bacterial Cells for Injection into Mice for LD₅₀ Studies

S. typhimurium strain SL1344 was grown in 5ml LB medium for exactly 16 hours at 37°C with shaking at 200rpm. Bacteria were pelleted by centrifugation at 4,000g for 10 minutes in a bench top centrifuge, then resuspended in 5ml sterile PBS. The process of pelleting the bacteria and resuspending in 5ml PBS was repeated an additional two times to wash the cells thoroughly before serially diluting the cell suspension. Serial dilutions were then performed in PBS until the required bacterial concentration had been
obtained. The bacterial inoculum was determined by plating 100μl aliquots from the serial dilution series onto LB plates. Mice were injected via the intraperitoneal (IP) route with 100μl bacterial suspension ranging from 10^5-10^6 organisms to 10^0-10^1 organisms. The survival of mice in each group was then monitored for at least 28 days and the lethal dose (LD_{50}) subsequently established (Reed and Muench, 1938).

2.2.6.4 Collection of Serum

Mice receiving injections with either the DNA expression library from *S. typhimurium* SL1344 or CA were bled on days 7 and 12 following the final immunisation (see results). Mice from each group were anaesthetised and suitable volumes of blood were collected from each mouse by venipuncture. Blood samples were then left to clot at room temperature for 3-4 hours, following which the clotted components were removed by centrifugation at 1,800g for 15 minutes. Where appropriate, serum samples were combined to give a pooled sample for each group. Pooled and individual serum samples were then aliquoted and stored at —70°C.

2.2.6.5 Antibody Analysis

Flat bottomed 96-well plates (Immulon 2HB) were coated overnight at 4°C with 100μl of 15μg/ml CA or 100μl of 2μg/ml GST-EGFP in coating buffer (15 mM Na_{2}CO_{3}, 35 mM NaHCO_{3}, and 0.01% [v/v] sodium azide, pH 9.5). Following overnight incubation, plates were washed three times with 200μl of wash buffer (0.05% Tween 20, 0.01% sodium azide in PBS, pH 7.2) using the MW plate washer to remove excess antigen. Any unoccupied protein binding sites were then blocked for 1 hour at 37°C with 200 μl per well blocking buffer (1% (w/v) skimmed milk powder in coating buffer). During this incubation, pooled serum samples were prepared by doubling dilutions starting at 1 in 16 continuing through to 1 in 32,768. Plates were washed three times as previously
mentioned, before adding 100μl of the diluted serum samples in duplicate to the plate. The 96-well plates were then incubated at room temperature for 4 hours. Following a further 3 washes, sheep anti-mouse IgG, goat anti mouse IgG1 or bovine anti mouse IgG2a HRP conjugated antibody was added to the appropriate wells at dilutions of 1:500, 1:2000 and 1:500 respectively in diluting buffer (0.05% Tween 20, 0.01% sodium azide, 0.25% [w/v] milk powder in PBS). For controls, 6 wells received 1.5μg of antigen and buffer alone, and 6 wells received 1.5μg of antigen and HRP conjugated antibody only. Plates were then incubated at 37°C for 30 minutes before undergoing 3 final washes in wash buffer. All traces of wash solution were removed from the wells by forceful flicking before adding 100μl of freshly prepared substrate buffer (70mM Citric acid, 7.5mM Na₂HPO₄, 0.04% [v/v] H₂O₂, 0.4mg/ml O-phenylenediamine (OPD) in dH₂O). Following substrate addition, the plate was incubated in the dark at room temperature for 15 minutes, before the reaction was stopped with 25μl 2M H₂SO₄. The results were recorded as optical density at 490nm using a Dynatech plate reader. Results from the pooled serum samples were plotted on a graph to establish a dilution of serum at which to analyse the individual serum samples. To test for differences in the median OD values for each group of mice, the Mann Whitney statistical test was applied. A $P \leq 0.05$ value was considered statistically significant.

2.2.6.6 T Cell Proliferation Assay

The inguinal, brachial and axillary lymph nodes were collected from individual mice previously immunised with either plasmid DNA, or CA or from control mice (Boyle et al., 1996). The lymph nodes were temporarily stored in a petri-dish containing 5ml of cell culture medium (DMEM supplemented with 10% [v/v] heat-inactivated foetal calf serum (USA), 2mM glutamine, 25mM HEPES and 50μM 2-ME, penicillin and streptomycin) at 4°C. Once in a sterile flow cabinet, the lymph nodes were gently crushed between the rough edges of two microscope slides, and the emerging lymph node cells (LNC) were washed with cell culture medium into a sterile petri-dish.
containing 5ml of fresh cell culture medium. The LNC were then transferred to a 15ml universal tube and centrifuged at 300g for 10 minutes. The supernatant was removed and the pelleted LNC were resuspended in 15ml of cell culture medium and re-centrifuged for a further 10 minutes at 300g. Following the second wash, the cell pellet was resuspended in 2ml of medium and 10μl was taken for viable cell counting. LNC from each mouse were plated out in triplicate wells at 5x10^5 cells/well in a volume of 100μl and incubated with 10μg/ml CA, prepared as described in section 2.2.6.1. In addition, LNCs from each mouse were incubated with medium alone to assess unstimulated control responses or medium containing 1μg/ml concanavalin A (ConA, Sigma), as a positive control for T cell proliferation. Following 72 hours incubation at 37°C with 5% CO₂, 100μl of supernatant was removed from selected lymphoproliferative cultures. Supernatants were stored at −70°C and subsequently used for cytokine analysis. Following the removal of supernatants, 100μl of medium containing 1μCi of [Methyl ³H] thymidine was added to each well. After a further 24 hours incubation, LNC were harvested onto Wallac filter paper using a Tomtec Mach III M harvester. Radioactive [Methyl ³H] thymidine incorporation was counted using a Beta Trilux scintillation counter. Individual values for T cell proliferations were logged and the means were compared using the Students two-tailed t-test. A P ≤ 0.05 was considered to be statistically significant. Proliferation was also expressed as a stimulation index (SI). Values for SI were calculated as the mean [³H] thymidine incorporation of cells stimulated with antigen / the mean incorporation in the absence of antigenic stimulation. SI values were compared between groups using the Mann Whitney statistical test. A P ≤ 0.05 value was considered to be statistically significant.

2.2.6.7 IFN-γ Sandwich Enzyme-Linked Immunosorbent Assay

Supernatants taken from the T cell proliferation assays and stored at −70°C were analysed for the secretion of IFN-γ by sandwich Enzyme-Linked Immunosorbent Assay (ELISA). Using a 96 well plate, each well was coated with 50μl of 3μg/ml rat anti-
mouse IFN-γ monoclonal antibody in bicarbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, in dH₂O pH 9.2) and left for 20 hours at 4°C. Following incubation, all traces of fluid were removed from the wells by forceful flicking. Wells were then each blocked with 150μl of 5% (w/v) BSA in bicarbonate buffer and incubated at 37°C for 2 hours. During the incubation, a standard curve of recombinant IFN-γ was prepared by doubling dilution. A total of 12 dilutions were prepared with concentrations from 500Units (U)/ml through to 0.244 U/ml.

Following the blocking incubation, the plate was washed with TBST (Tris buffered saline, 0.05% [v/v] Tween 20) using a 96 well ELISA plate washer. All traces of wash solution were removed from the wells by forceful flicking, and 50μl of each dilution of recombinant IFN-γ was added to the appropriate well. A total of 18 negative control wells per plate were set up, each containing 50μl of DMEM supplemented with 5% [v/v] foetal calf serum. Supernatants taken from each culture during the T cell proliferation assay were thawed, and 50μl of each supernatant was added to the appropriate wells in duplicate for each stimulant (medium, antigen or Con A) being tested. The plate was then covered and left for 16 hours at 4°C.

Following the 16 hour incubation, the plate was washed with TBST and 50μl of 0.5μg/ml biotinylated rat anti mouse IFN-γ IgG1 antibody in TBST containing 5% (v/v) FCS was added to each well. The plate was then incubated at 37°C for 1 hour before being washed with TBST and incubated for a further 45 minutes at 37°C with 75μl of extravidin alkaline phosphatase conjugate (1:25,000 dilution in TBST and 5% (v/v) FCS). During the final incubation, pNPP substrate solution was prepared by dissolving a 5mg pNPP tablet in 5ml of distilled water. The plate was then washed with TBST, twice with dH₂O, then 95μl of pNPP substrate was added to each well. The plate was kept in the dark at room temperature for 1 hour before the optical densities were read at 405nm. The plate was then stored at 4°C and read again after 3 hours and again at 24 hours. The cytokine levels were calculated according to the standard curve obtained for each ELISA
Values for cytokine levels were compared using the Student’s two-tailed t-test and a $P \leq 0.05$ was considered to be statistically significant.

2.2.6.8 IL-4 Sandwich Enzyme-Linked Immunosorbent Assay

Supernatants taken from the T cell proliferation assays and stored at $-70^\circ$C were analysed for the secretion of IL-4 by ELISA. Using a 96 well plate arrangement, each well was coated with 50µl of 2µg/ml anti-mouse IL-4 11B11 monoclonal antibody in bicarbonate buffer (15 mM Na$_2$CO$_3$, 35 mM NaHCO$_3$, in dH$_2$O pH 9.2) and left for 20 hours at 4°C. Following incubation, all traces of fluid were removed from the wells by forceful flicking. Wells were then each blocked with 150µl of 5% (v/v) BSA in bicarbonate buffer and incubated at 37°C for 2 hours. During this incubation, a standard curve of recombinant IL-4 was prepared by doubling dilution. A total of 12 dilutions were prepared with concentrations from 8ng/ml, to 3.906pg/ml.

Following the blocking incubation, the plate was washed with TBST (Tris buffered saline, 0.05% [v/v] Tween 20) using a 96 well ELISA plate washer. All traces of wash solution were removed from the plate as previously mentioned, and 50µl of each dilution of recombinant IL-4 was added to the appropriate wells. A total of 18 negative wells were prepared, each containing 50µl of DMEM supplemented with 5% (v/v) foetal calf serum. Supernatants taken from each culture during the T cell proliferation assay were thawed, and 50µl of each supernatant was added to the appropriate wells in duplicate for each stimulant (medium, antigen or Con A) being tested. The plate was then covered and left for 16 hours at 4°C.

Following the 16 hour incubation, the plate was washed again with TBST and 50µl of 0.5µg/ml biotinylated rat anti mouse IL-4 antibody in TBST supplemented with 5% (v/v) FCS was added to each well. The plate was incubated at 37°C for 1 hour before being washed with TBST and incubated for a further 45 minutes at 37°C with 75µl of
extravidin alkaline phosphatase conjugate (1:25,000 dilution in TBST containing 5% (v/v) FCS). During the final incubation, pNPP substrate solution was prepared by dissolving a 5mg pNPP tablet in 5ml of distilled water. The plate was then washed with TBST, twice with dH$_2$O, then 95μl of pNPP substrate was added to each well. The plate was kept in the dark at room temperature for 1 hour before the optical densities were read at 405nm. The plate was then stored at 4°C and read again after 3 hours and again at 24 hours. The cytokine levels were calculated according to the standard curve obtained for each ELISA plate. Values for cytokine levels were compared using the Student’s two-tailed t-test and a $P \leq 0.05$ was considered to be statistically significant.
Chapter 3

Construction of a DNA Expression Library
DNA immunisation has recently emerged as a promising new approach to vaccination, and protective responses from DNA vaccines have been demonstrated against several pathogens (Donnelly, et al., 1997; Lai and Bennet, 1998; Hasan et al., 1999; Gurunathan et al., 2000). To construct a DNA vaccine against a particular pathogen, genes encoding proteins that elicit a strong immune response when expressed in the host need to be identified and cloned into an expression vector. However, as with many other vaccine strategies, discovering which gene or genes mediate immunity still remains a problem. One possible solution termed 'expression library immunisation' (ELI) has been proposed and involves the sequential portioning of DNA expression libraries into smaller and smaller protective sub-libraries until individual expression vectors encoding protective antigens are identified (Chapter1; Johnston and Barry, 1997). The initial stage in ELI requires the construction of a representative expression library (EL) for the pathogen of interest in a mammalian expression vector. This chapter describes the requirements for a representative EL, and outlines the construction process. In addition, details are given for the construction of the mammalian expression vector pZKC3 and the *S. typhimurium* SL1344 EL. A description is provided of how key variables in the construction process are optimised. Finally the library is analysed to ensure it is representative of the *S. typhimurium* SL1344 genome and is tested for antigen expression in mammalian cells.
3.1 Construction of the Expression Vector pZKC3

The construction of an EL ideally requires all the genes from a pathogen to be cloned into an expression vector. It is therefore crucial that these vectors are capable of high-level expression of recombinant proteins in mammalian cells. For this reason, the expression vector pcDNA3 from Invitrogen was chosen for the work performed in this thesis since it induces high-level constitutive transcription from mammalian enhancer-promoter sequences and has transcription termination and polyadenylation signals (see Figure 3.1). In addition, reports have demonstrated the successful use of pcDNA3 in EL construction (Alberti et al., 1998; Manoutcharian et al., 1998; Manoutcharian et al., 2000; Melby et al., 2000).

![Figure 3.1 Expression vector pcDNA3](image-url)
3.1.1 Construction of pVCN2

Whilst the expression of the ampicillin and neomycin resistance genes encoded in pcDNA3 enables selection and maintenance of the vector in bacterial and mammalian cells, it was beneficial to clone into the pcDNA3 vector a reporter gene for the easy determination of gene expression. The expression vector was constructed so that any pathogenic DNA of interest cloned into the vector formed a heterologous fusion protein with the reporter gene when expressed. Therefore, the presence of the reporter gene signaled the expression of the pathogenic DNA of interest.

The reporter gene cloned into pcDNA3 was the enhanced green fluorescent protein (EGFP) amplified by PCR from the pEGFP vector (Clontech Laboratories Inc., Palo Alto, CA). EGFP is a variant of the wild-type green fluorescent protein (GFP) which has been optimised for higher expression in mammalian cells and brighter fluorescence at a convenient excitation wavelength of 488nm (Cormack et al., 1996). The GFP of the jellyfish *Aequorea victoria* has been widely used as a reporter in the determination of gene expression and protein localisation in numerous cells and organisms. GFP has many characteristics that make it useful for expression studies, primarily its ability to fluoresce when fused to polypeptides without the addition of exogenously added substrates (Phillips, 2001). In addition GFP is a stable protein with a half-life greater than 24 hours and has low toxicity (Clontech, 1998). More recently it has also been reported that GFP can increase protein production of the heterologous antigen when fused to poorly expressed sequences (Wu and Barry, 2000). This is particularly advantageous for DNA vaccines where problems can arise with protein sequences that are toxic to host cells or are difficult to translate by mammalian cells or evade immune presentation.

A further addition to the pcDNA3 vector was the insertion of a polyhistidine tag (HIS-Tag) downstream of the EGFP gene. This was to enable foreign proteins fused to EGFP-
3.1.2 Construction of pZKC3

In order to clone DNA fragments of *S. typhimurium* created by partial digestion of chromosomal DNA with *Sau3A*, it was necessary to have a single complementary restriction site such as *BamHI* downstream of EGFP-HIS. It was therefore important to disrupt the *BamHI* in pVCN2 at position 993 (see Figure 3.2) upstream of EGFP-HIS and generate a new *BamHI* site downstream of EGFP-HIS. This was performed by PCR using the primers described in Chapter 2. The PCR product encoded EGFP-HIS, but now had a *BglII* restriction site at the 5' end and a *BamHI* towards the 3' end (see Figure 3.3). The PCR product was digested with *BglII* and *XhoI* before being cloned back into pVCN2 previously digested with *BamHI* and *XhoI*. The resulting vector, pZKC3 (see Figure 3.4), was used for construction of the *S. typhimurium* SL1344 EL.

![Figure 3.3 PCR product of pVCN2](image)

To ensure the expression vector pZKC3 was functional in both prokaryotic and eukaryotic cells, *E. coli* BL21 cells were transformed and induced with IPTG and EGFP-HIS expression was analysed by SDS PAGE and Western blot. In addition, mouse fibroblast cells were transfected *in vitro* with pZKC3 and EGFP-HIS expression was visualised as green fluorescence under a microscope.
Ampicillin (Carbenicillin) resistance
CoIE1 origin
CMV Promoter
T7 Promoter
eGFP+His
Sp6 Promoter
BamHI (1777)
XhoI (1783)
BGH poly A
f1 ori
SV40 poly A
Neomycin Resistance

Figure 3.4 Expression vector pZKC3
3.2 Requirements for a Representative DNA Expression Library

For the technique of ELI to identify protective antigens of a pathogen, it is a requirement that the EL is representative of the entire pathogen’s genome, that is, the EL encodes and expresses every gene within the pathogen’s genome. To be certain that a DNA EL is representative, the presence of every gene in the pathogen’s genome should be identified. Full identification of every gene is beyond the scope of this work, however, the presence of every gene can be assumed with a certain level of confidence, by making a library with an appropriately large number of clones (Clarke and Carbon, 1976). The following equation can be used to calculate the number of clones required for a given level of confidence that a DNA library is representative (Clarke and Carbon, 1976). The number of clones, \( N_c \), is given by

\[
N_c = \frac{\ln(1 - L_c)}{\ln \left(1 - \frac{S_f}{S_g} \right)}
\]

(2)

where \( L_c \) is the level of confidence required, \( S_f \) is the size of fragments in base pairs (bp) and \( S_g \) is the size of the pathogen’s genome in base pairs. A useful fragment size is around 2,000 bp since this is slightly larger than the average \( S. typhimurium \) SL1344 open reading frame (ORF). The equation assumes that all genes are represented equally and that all genes are equally likely to be cloned. The number of clones required to have 99% confidence that the entire \( S. typhimurium \) SL1344 genome of \( 4.857 \times 10^6 \) bp (based on the LT2 strain (McClelland et al., 2001)), has been covered in 2,000 bp fragments was calculated to be 11,200, as shown below:

\[
\frac{\ln(1 - 0.99)}{\ln \left(1 - \frac{2000}{4.857 \times 10^6} \right)} = 11,200
\]

(3)
To construct a functional EL, the *S. typhimurium* SL1344 gene fragments must ligate into the expression vector in the correct orientation and frame to be expressed. Assuming the orientation and frame for the coding regions of *S. typhimurium* SL1344 are selected at random, statistically, only one clone in every six will encode *S. typhimurium* SL1344 gene fragments in the correct orientation and frame to be expressed. The size of the EL must therefore be greater than six times the size of a DNA library to account for the frame, orientation and non-coding regions of *S. typhimurium* SL1344 chromosomal DNA. The number of recombinant clones required to give 99% confidence that the entire *S. typhimurium* SL1344 genome of $4.857 \times 10^6$ bp has been represented and expressed is therefore 67,200.

In summary, there are three requirements to be 99% confident that an EL is representative (see Figure 3.5). Firstly, chromosomal DNA must be digested evenly so that gene fragments are not ligated into a preferential frame. Secondly, gene fragments must ligate with the vector at random so that each gene fraction is equally represented. Finally, the library must contain over 67,200 clones to be confident that each gene from the *S. typhimurium* SL1344 genome is present within the library. The three requirements are investigated later in this Chapter to determine whether the EL constructed was representative.
Number of clones > 67,200

Representative
*S. typhimurium*
SL1344 EL

Random cleavage of *S. typhimurium* SL1344 DNA

Random ligation of *S. typhimurium* SL1344 DNA fragments

*Figure 3.5 Requirements for a representative DNA expression library for S. typhimurium SL1344*
3.3 Investigation of Variables Involved in Constructing a DNA Expression Library for *S. typhimurium* SL1344

Construction of an EL involves a number of different processes. These include vector preparation, vector digestion, vector dephosphorylation, insert preparation, insert and vector ligation, and transformation (see Figure 3.6). Since a large number of clones were required for the EL to be representative, it was important to optimise the efficiency of each reaction in the construction process. This section evaluates the effect of variables involved in constructing a *S. typhimurium* SL1344 EL and optimises those that effect the process significantly.

*Figure 3.6 Process for the construction of a *S. typhimurium* SL1344 DNA EL. The vector pZKC3 was prepared by CsCl density gradient centrifugation and purified by dialysis. The vector was then digested with BamHI and dephosphorylated with shrimp alkaline phosphatase (SAP). The insert DNA was prepared by partial digestion of genomic *S. typhimurium* SL1344 DNA with Sau3A and separated according to size by sucrose density gradient centrifugation. Fragments of approximately 2,000 bp were identified by agarose gel electrophoresis and were purified by dialysis. Vector DNA and insert DNA was then ligated with T4 DNA ligase and *E. coli* DH5α cells were transformed with the recombinant vector.*
3.3.1 Effect of DNA Purity on the Efficiency of Transformation

Construction of a representative EL for *S. typhimurium* SL1344 requires the random ligation of *S. typhimurium* SL1344 gene fragments into a vector, such as pZKC3, and efficient transformation of recipient cells. A guideline figure of 2,000 colonies per transformation was set to enable an entire library consisting of over 67,200 clones to be constructed efficiently. Moreover, 2,000 was approximately the same number of colonies in each sub-library of the *Leishmania* EL (Melby et al., 2000) and is an optimal number of transformants to incubate on a standard sized petri-dish. To obtain colony numbers within the region of this guideline figure, attempts were made to maximise the efficiency of both ligation and transformation by increasing the purity of DNA at each stage in the process. It is well known that the presence of contaminants such as salts, in solutions of DNA can reduce the efficiency of ligation, since enzymes involved in ligation require specific salt and pH levels (Maniatis et al., 1982; Sambrook et al., 1989). Additionally, the levels of salt also affect the efficiency of transformation, with a reduction in the number of transformants observed with increasing salt concentrations. It was therefore necessary to remove such contaminants from both solutions of vector DNA (pZKC3) and the insert DNA (partially digested *S. typhimurium* SL1344 chromosomal DNA).

3.3.1.1 Effect of Vector Purity on the Efficiency of Transformation

The vector pZKC3 was prepared by CsCl density gradient centrifugation and was repeatedly dialysed against dH₂O to remove all traces of CsCl salt that may have been present from the procedure. However, prior to the ligation reaction, the pZKC3 vector DNA had undergone restriction digestion with the enzyme *Bam*HI and dephosphorylation with the enzyme shrimp alkaline phosphatase (SAP). It was therefore of concern that the buffers used in these reactions and the enzymes themselves would interfere with the ligation reaction and reduce the number of transformants generated,
since linearised DNA does not efficiently transform bacterial cells (see Figure 3.7, Group B and C).

Initial experiments were therefore conducted to analyse the effects of purifying vector DNA from the restriction and dephosphorylation enzymes and their corresponding buffers by phenol/chloroform extraction immediately prior to ligation. The number of colonies obtained following transformation was used to assess the purity of vector DNA on the efficiency of ligation and transformation.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Nature of pZKC3 vector DNA prior to transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Uncut vector</td>
</tr>
<tr>
<td>B</td>
<td>BamHI digested vector</td>
</tr>
<tr>
<td>C</td>
<td>BamHI digested vector purified by phenol/chloroform extraction</td>
</tr>
<tr>
<td>D</td>
<td>BamHI digested vector, re-ligated</td>
</tr>
<tr>
<td>E</td>
<td>BamHI digested vector, re-ligated and purified by phenol/chloroform extraction</td>
</tr>
<tr>
<td>F</td>
<td>BamHI digested vector, dephosphorylated with SAP, and ligated directly with <em>S. typhimurium</em> SL1344 fragments</td>
</tr>
<tr>
<td>G</td>
<td>BamHI digested vector, dephosphorylated with SAP, and purified by phenol/chloroform extraction then ligated directly with <em>S. typhimurium</em> SL1344 fragments</td>
</tr>
<tr>
<td>H</td>
<td>BamHI digested vector, dephosphorylated with SAP, and purified by phenol/chloroform extraction then ligated directly with <em>S. typhimurium</em> SL1344 fragments and purified again by phenol/chloroform extraction</td>
</tr>
</tbody>
</table>

Table 3.1. Key to groups in Figures 3.7 and 3.8. Groups A-E function as controls for the experiments. The quantity of pZKC3 vector DNA used in each group for ligation was 0.5µg. In the groups where pZKC3 vector was ligated with *S. typhimurium* SL1344 insert DNA, the quantity of insert used in the ligation reaction was 0.3µg.

The number of transformants obtained for experimental Group A was not included in Figure 3.7, but the number was in excess of $10^5$ (as determined by the presence of a complete lawn of cells), indicating the competency of the freshly prepared *E. coli* DH5α cells was reasonably high for all the experimental groups. Examination of the results in Figure 3.7 shows that the number of transformants obtained for experimental Groups B
and C was very low. This would indicate that the pZKC3 vector was almost completely digested with *Bam*HI and the few transformants observed were the consequence of incomplete vector digestion. The number of false positives in the experimental Groups F, G and H contributing to transformant numbers were therefore low, with the majority arising from vector and insert ligation.

It was evident from experimental Groups D and E, that ligation was successful, since 1,500 and 1,000 transformants were counted following transformation with re-ligated pZKC3 vector respectively. However, these results also demonstrated that purification of the re-ligated vector (Group E) by phenol/chloroform extraction reduced the efficiency of transformation, since fewer transformants were observed for experimental Group E compared to Group D.

*Figure 3.7 Effect of vector DNA purity on the efficiency of ligation and transformation. The values indicate the colonies counted following transformation of 200μl of electrocompetent *E. coli* DH5α cells. Experimental groups are described in Table 3.1.*
However, analysis of samples of the ligation mixtures by DNA slab gel electrophoresis showed that the quantity of re-ligated vector DNA in Group E was reduced during purification by phenol/chloroform extraction. This could explain why a lower number of transformants were obtained. The loss of DNA following phenol/chloroform purification was also evident for Group H, as shown in Figure 3.8. In this case, the second purification event following the ligation reaction resulted in the loss of the majority of ligated pZKC3 and S. typhimurium SL1344 DNA compared to experimental Groups F and G which received only one phenol/chloroform purification event before the ligation reaction. Again, this in-turn reduced the number of colonies observed following transformation.

![Ethidium bromide stained agarose gel of DNA from experimental groups](image)

**Figure 3.8 Ethidium bromide stained agarose gel of DNA from experimental groups.** With the exception of the molecular weight marker, each lane was loaded with 10μl of DNA, whilst only 1μl was used in each transformation event. Lane MW: Molecular weight marker III, Lane S: Sau3A partially digested S. typhimurium SL1344 DNA. All other experimental groups are described in Table 3.1.

The comparison of transformant numbers between Groups F and G in Figure 3.7, implies that purification of vector DNA from BamHI and SAP by phenol/chloroform
extraction in Group G, increased the efficiency of ligation and transformation as shown by the increased number of transformants for similar levels of DNA. However, the number of transformants obtained from vector DNA ligated with \textit{S. typhimurium} SL1344 DNA (Group F) was considerably lower than for the re-ligated vector (Group D). This would imply that the ligation of \textit{S. typhimurium} SL1344 DNA was limiting the efficiency of ligation and transformation. Attempts to further increase the number of transformants therefore concentrated on increasing the purity of the \textit{S. typhimurium} SL1344 DNA.

3.3.1.2 Effect of \textit{S. typhimurium} SL1344 DNA Purity on the Efficiency of Transformation

Chromosomal DNA was purified from stationary phase \textit{S. typhimurium} SL1344 cells by the method described by Silhavy \textit{et al.}, (1984). The chromosomal DNA was then partially digested with \textit{Sau3A} (see Figure 3.9) and fragments of approximately 2,000 bp were isolated by sucrose density gradient centrifugation (Maniatis \textit{et al.}, 1982). In performing density gradient centrifugation, \textit{S. typhimurium} SL1344 DNA was mixed with high concentrations of sucrose and sarcosyl (see Chapter 2). It was therefore highly likely that the sucrose and sarcosyl solutions were interfering with the ligation and/or transformation events.

To analyse the effect of \textit{S. typhimurium} SL1344 DNA (insert) purity on the overall efficiency of transformation, partially digested \textit{S. typhimurium} SL1344 DNA was purified by repeated dialysis against dH2O prior to ligation. Dialysis was chosen in preference to phenol/chloroform extraction so as to minimise the loss of \textit{S. typhimurium} SL1344 DNA. The pZKC3 vector DNA used for this experiment was however purified by phenol/chloroform extraction where stated.
The number of transformants obtained from Group A was in excess of $10^5$ (as determined by the observation of a complete lawn of cells), indicating that the cells were reasonably competent for this investigation. Looking at the results in Figure 3.10, it was very clear that the purification of insert *S. typhimurium* SL1344 DNA by dialysis greatly increased the overall efficiency of transformation, since the number of transformants obtained for Group B was substantially higher than the number of transformants obtained from Group C.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Nature of insert <em>S. typhimurium</em> SL1344 DNA</th>
<th>Nature of pZKC3 vector DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>Uncut vector</td>
</tr>
<tr>
<td>B</td>
<td>Purified</td>
<td>Purified</td>
</tr>
<tr>
<td>C</td>
<td>Not purified</td>
<td>Purified</td>
</tr>
<tr>
<td>D</td>
<td>Purified</td>
<td>Not purified</td>
</tr>
<tr>
<td>E</td>
<td>Not purified</td>
<td>Not purified</td>
</tr>
</tbody>
</table>

Table 3.2. Key for experimental groups in Figure 3.10. *S. typhimurium* SL1344 DNA was purified by dialysis prior to ligation with pZKC3. In each case the pZKC3 vector was digested with BamHI and dephosphorylated prior to ligation, except for Group A, which functioned as an internal control to ensure the competency of the E. coli DH5α cells. Where stated the pZKC3 vector was purified immediately preceding ligation by phenol/chloroform extraction.
Figure 3.10 Effect of insert and vector purity on the efficiency of ligation and transformation. The values indicate the number of colonies counted following transformation of 200μl of electrocompetent E. coli DH5α cells. Experimental groups are described in Table 3.2.

In addition, the results showed that the removal of BamHI and SAP from pZKC3 DNA by phenol/chloroform extraction also increased the number of transformants, since Group B produced more transformants than Group D. Despite the number of transformants being increased to over 800, this figure was still below the desirable figure of 2,000 transformants that was required for the efficient construction of a representative EL. Therefore alternative factors involved in the ligation and transformation process were investigated.
3.3.2 The Influence of Insert: Vector Mass Ratios on Transformation Efficiency

To maximise the number of clones obtained following transformation it was important to optimise the ligation of pZKC3 vector DNA and *S. typhimurium* SL1344 insert DNA further, since only circularised DNA could be used to successfully transform bacterial cells. Another variable known to affect the efficiency of ligation and therefore the efficiency of transformation is the relative quantity of vector and insert DNA in the ligation reaction (Maniatis *et al.*, 1982). The following section identifies the optimal quantities of vector and insert DNA for ligation by analysing the number of recombinant clones obtained following transformation. In this section, the vector DNA refers to the expression vector pZKC3 and the insert DNA refers to the partially digested chromosomal *S. typhimurium* SL1344 DNA.

Chromosomal *S. typhimurium* SL1344 DNA prepared from stationary phase cell cultures was partially digested with *Sau3A*. The restriction enzyme *Sau3A* was chosen to partially digest the chromosomal DNA since *Sau3A* recognises the commonly arising GATC sequence and therefore cuts the DNA at frequent intervals to generate a substantial number of differing gene fragments. The gene fragments generated from partial digestion with *Sau3A* were fractionated according to size by sucrose density centrifugation and those fragments of approximately 2,000 bp in size were then purified by dialysis and used in the ligation mix. Whilst the majority of *S. typhimurium* SL1344 DNA fragments used in the ligation reactions were approximately 2,000 bp in size (as observed by DNA gel electrophoresis (see Figure 3.17), the size of the fragments ranged from a minimum of 500 bp to a maximum of 3,000 bp. It was therefore not appropriate to use the more common insert: vector molar ratios since the inserts had a range of sizes. Instead it was more meaningful to identify the optimal insert: vector mass ratio.

Initially, to determine the amount of pZKC3 vector DNA to use in the ligation reactions, the saturation level for transformation using uncut pZKC3 vector needed to be determined (Garg *et al.*, 1999). A series of 5 x 1ml volumes of freshly prepared
electrocompetent DH5α cells were transformed with different quantities of vector DNA (as determined by spectrophotometry at 260 and 280nm) ranging from 0.1μg to 0.5μg. The results showed an increase in the number of transformants with an increase in the quantity of vector DNA. This was evident up to 0.4μg of vector DNA, which generated over $10^5$ transformants, after which no further increase in the number of transformants was observed with increasing quantities of vector DNA.

To determine the amount of *S. typhimurium* SL1344 insert DNA to use in the ligation reaction, a range of purified insert quantities from 150ng to 1.5μg (as determined by spectrophotometry) were mixed with 0.4μg of purified BamHI digested, dephosphorylated vector DNA. Freshly prepared competent *E. coli* DH5α cells were transformed by electroporation and the optimum quantity of insert DNA was determined by the number of colonies obtained following transformation (see Figure 3.11). In each case, the competency of DH5α cells was assessed by transforming them with uncut pZKC3 vector. The number of false positive transformants generated as a result of incomplete BamHI digestion of pZKC3 was assessed by transforming with BamHI digested pZKC3.

<table>
<thead>
<tr>
<th>Control Groups</th>
<th>Number of Transformants</th>
</tr>
</thead>
<tbody>
<tr>
<td>400ng Uncut Vector</td>
<td>&gt; $10^5$</td>
</tr>
<tr>
<td>400ng BamHI digested vector</td>
<td>= 12</td>
</tr>
<tr>
<td>400ng BamHI digested vector, re-ligated</td>
<td>~ $2x10^3$</td>
</tr>
</tbody>
</table>

*Table 3.3 Number of colonies obtained following transformation of 200μl of electrocompetent *E. coli* DH5α cells with different forms of the pZKC3 vector. In each case, immediately prior to ligation, the pZKC3 vector was purified from contaminating salts and enzymes by phenol/chloroform extraction.*

The results of the control groups in Table 3.3 indicate that the cells used in the experiment were reasonably competent since uncut pZKC3 vector DNA generated over $10^5$ transformants (as determined by a complete lawn of cells). Additionally, only 0.6% of these transformants resulted from the incomplete digestion of vector DNA as shown
by the number of colonies observed from transforming with BamHI digested pZKC3 vector.

![Graph showing the effect of mass of insert DNA on transformation efficiency.](image)

**Figure 3.11 Effect of the mass of insert on the efficiency of ligation and transformation.** For each ligation reaction, 0.4µg of purified pZKC3 vector previously digested with BamHI, and dephosphorylated with SAP was ligated with Sau3A digested S. typhimurium SL1344 chromosomal DNA, previously purified by dialysis. Values indicate the number of colonies counted following the transformation of 200µl of electrocompetent E. coli DH5α cells.

It is evident from Figure 3.11 that the quantity of *S. typhimurium* SL1344 insert DNA generating the most transformants following ligation with 0.4µg of pZKC3 DNA is between 500-700ng. Using the mid point of this range as the most efficient, the optimal insert: vector mass ratio was found to be 1.5:1. For all subsequent ligation reactions, an insert: vector mass ratio of 1.5:1 was therefore used.

Assuming only *S. typhimurium* SL1344 fragments of 2,000 bp were present in the ligation mixture, the more commonly used insert: vector molar ratios can be calculated, (see equation 4).
The molar ratio of insert DNA to vector DNA, $I: V$, is given by

$$I: V = \frac{M_i \times S_v}{M_v \times S_i}$$

(4)

where $M_i$ is the mass of insert, $M_v$ is the mass of vector, $S_v$ is the size of vector in kb and $S_i$ is the size of insert in kb. Using this equation, the insert: vector molar ratios for inserts of 2,000 bp were calculated to be 4.7:1, which is very close to the recommended insert: vector molar ratio of 5:1 provided by Invitrogen.

When comparing the number of colonies obtained from transforming *E. coli* DH5α cells with 0.4μg of re-ligated pZKC3 (see Table 3.3), to the maximum number of colonies obtained from transforming the same cells with pZKC3 ligated to *S. typhimurium* SL1344 DNA (see Figure 3.11), it was apparent that fewer transformants were generated from vector and insert ligation. This demonstrates that additional factors were effecting the efficiency of ligation and transformation, and that variables other than DNA purity and insert: vector mass ratios required optimisation in order to generate 2,000 colonies from each transformation event.

### 3.3.3 Effect of Transformation Method on the Efficiency of Transformation

To construct a representative EL, a large number of highly competent *E. coli* DH5α cells were required for transformation. Competent cells can be produced in a number of different ways according to the method of transformation. Two methods of transformation, electroporation and heat shock were compared. In brief, *E. coli* DH5α cells prepared for electroporation were initially grown to an OD of 0.5 at 600nm then washed repeatedly with chilled dH₂O. Electrocompetent cells were then used immediately, or alternatively stored in 10% glycerol at -80°C. *E. coli* DH5α cells prepared for heat shock transformation were similarly grown to an OD of 0.5 at 600nm,
following which, cells were pelleted and re-suspended in solutions containing CaCl$_2$, MnCl$_2$, RbCl$_2$ and 15% glycerol. Cells were then used immediately, or alternatively snap frozen on dry ice and stored at -80°C (see Chapter 2).

The purpose of this experiment was to determine the most efficient method of transformation as determined by the number of colonies observed following each type of transformation event. In addition, the experiment was designed to establish the effect of storage at -80°C on the level of cell competency and transformation efficiency, since reports in the literature had documented the detrimental effects of storing competent cells at -80°C prior to transformation (Michelsen, 1995). This was of particular importance for the construction of the entire *S. typhimurium* SL1344 EL, since competent cells needed to be produced in a single batch to ensure a uniformly high level of cell competency and aliquots of competent cells were required for use over a period of several days.

Electrocompetent and heat shock competent cells were prepared and immediately transformed accordingly with the vector pZKC3 in a number of different forms (see Table 3.4). To assess the level of cell competency, uncut pZKC3 vector prepared by CsCl gradient centrifugation and purified by dialysis was used to transform electrocompetent and heat shock competent *E. coli* DH5α cells (Table 3.4, Group A). In addition, pZKC3 vector digested with BamHI was used to transform competent cells to assess the number of transformants resulting from incomplete digestion of pZKC3 (Table 3.4, Group B). Electrocompetent and heat shock competent cells were also transformed with pZKC3 that had previously been digested with BamHI and re-ligated using T4 DNA ligase, and pZKC3 that had previously been digested with BamHI, dephosphorylated with SAP, and ligated with fragments of purified *S. typhimurium* SL1344 chromosomal DNA.
Table 3.4 Key for experimental groups shown in Figure 3.12. The pZKC3 vector DNA was produced by CsCl density gradient centrifugation and was purified by dialysis to remove all traces of contaminating CsCl salts. Where stated, restriction digestion of vector DNA was performed with BamHI and was dephosphorylated with SAP. The S. typhimurium SL1344 DNA was prepared by partial digestion with Sau3A and was fractionated by sucrose density gradient centrifugation. Fragments of S. typhimurium SL1344 were purified by dialysis before being ligated in the optimal ratios with pZKC3 DNA.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Nature of vector DNA prior to transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Uncut pZKC3</td>
</tr>
<tr>
<td>B</td>
<td>BamHI digested pZKC3</td>
</tr>
<tr>
<td>C</td>
<td>BamHI digested pZKC3, re-ligated</td>
</tr>
<tr>
<td>D</td>
<td>BamHI digested pZKC3, dephosphorylated with SAP and ligated with S. typhimurium SL1344 DNA</td>
</tr>
</tbody>
</table>

Figure 3.12 Comparison of transformation efficiency for electrocompetent and heat shock competent cells. Values indicate the number of colonies counted following the transformation of 200μl electrocompetent or 200μl of heat shock competent cells. Experimental group details are described in Table 3.4.

The results in Figure 3.12 show that both electrocompetent and heat shock competent cells were reasonably competent for this experiment, as both generated over $10^5$ colonies following transformation with uncut pZKC3 (as determined by the observation of a
complete lawn of cells). It is also evident from these results that the large number of colonies observed for experimental Groups C and D was due to vector re-ligation and vector and insert ligation respectively, since few colonies were observed as a result of incomplete digestion of pZKC3 with *BamHI* (Group B). It is notable however, that heat shock competent cells generated a greater number of transformants following transformation with pZKC3 previously digested with *BamHI* than electrocompetent cells. A possible explanation is that heat shock competent cells are capable of taking up more DNA during the transformation process than electrocompetent cells, increasing the likelihood of taking up incompletely digested pZKC3 vector.

From comparing the number of colonies observed following electroporation and heat shock transformation with re-ligated vector and vector ligated with *S. typhimurium* SL1344 DNA, it is evident that transformation by electroporation is marginally more efficient. For both Groups C and D, the number of colonies observed following transformation with electrocompetent cells was almost double the number of colonies observed following transformation with heat shock competent cells. This would indicate that transformation by electroporation is the most efficient method for constructing the entire EL for *S. typhimurium* SL1344.

It is also worthy to note that the number of colonies generated following transformation with pZKC3 ligated with *S. typhimurium* SL1344 is lower than the number of colonies generated from transformation with re-ligated pZKC3 vector. This is also observed in the experiments performed in section 3.3.2. One obvious difference between these ligation and transformation events is the dephosphorylation of *BamHI* digested pZKC3 prior to ligation, since dephosphorylated pZKC3 vector that was ligated with *S. typhimurium* SL1344 DNA generated fewer colonies after transformation than pZKC3 that was not dephosphorylated but just re-ligated. It is therefore possible that the dephosphorylation process is also effecting the efficiency of transformation, (see section 3.3.4).
Whilst the results in Figure 3.12 showed that transformation by electroporation was more efficient than transformation by heat shock, it was necessary to investigate how storage at -80°C affected the efficiency of transformation. Construction of the entire EL required in the region of 50 transformations, and it was not logistically possible to perform this number of transformations in one day with freshly prepared cells. It was therefore necessary to produce a single, large batch of competent cells (to ensure a uniform level of cell competency) and store the batch at -80°C until required.

Electrocompetent and heat shock competent cells produced from the previous experiment were stored for a period of 6 days at -80°C and then transformed with the same DNA that was used in the previous experiment (see Table 3.4), and the number of transformants generated were counted (see Table 3.5).

<table>
<thead>
<tr>
<th>Method of Transformation</th>
<th>Experimental Group</th>
<th>Number of Transformants Stored at -80°C</th>
<th>Not Stored at -80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrocompetence</td>
<td>A</td>
<td>1744</td>
<td>&gt; 100,000</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>316</td>
<td>3248</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>148</td>
<td>1736</td>
</tr>
<tr>
<td>Heat Shock</td>
<td>A</td>
<td>&gt; 100,000</td>
<td>&gt; 100,000</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>35</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1648</td>
<td>2296</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>1084</td>
<td>1324</td>
</tr>
</tbody>
</table>

Table 3.5 Effect of storage at -80°C on the efficiency of transformation. The values indicate the number of colonies counted following transformation of 200μl of electrocompetent cells and 200μl of heat shock competent cells used immediately following production and following 6 days storage at -80°C.

It was apparent from the results in Table 3.5, that in each case, the number of transformants observed following electroporation with cells stored at -80°C was substantially lower than the number observed from freshly prepared electrocompetent cells. There was however, a far smaller decrease in the number of colonies observed
following heat shock transformation with cells stored at -80°C compared to those cells used immediately following production. Moreover the number of transformants observed following heat shock transformation with cells stored at -80°C was substantially higher than the number of transformants observed following electroporation using cells that had similarly been stored at -80°C (see Figure 3.13).

![Figure 3.13 Comparison of transformation efficiency of electroporation and heat shock following competent cell storage at -80°C. The values indicate the number of colonies observed following the transformation of 200μl of electrocompetent and 200μl of heat shock competent E. coli DH5α cells previously stored at -80°C for 6 days and thawed on ice prior to use.](image)

It was therefore evident from these results that E. coli DH5α cells prepared for heat shock transformation retained the majority of their competency following a storage period at -80°C, whilst electrocompetent cells exhibited a substantial reduction in competency. Additionally, it was found that the number of cells required for heat shock transformation was considerably less than for electroporation and so could be produced in a single batch. This guaranteed the uniform competency of cells throughout the batch.
so the high transformation efficiency could be maintained for the construction of the entire EL.

Although the efficiency of heat shock transformation was not as high as for electroporation using cells that were freshly prepared on the day of use, it was essential that cells were stored at -80°C until required. Taking this into consideration, it was therefore decided that the method of transformation for constructing the EL of *S. typhimurium* SL1344 was via heat shock.

### 3.3.4 Effect of Shrimp Alkaline Phosphatase on the Efficiency of Transformation

The use of SAP to dephosphorylate linearised vector DNA prior to ligation is a common practice in molecular biology (Sambrook *et al*., 1989). Removing the terminal phosphate groups from linearised vector DNA greatly reduces the probability of the vector re-ligating to itself and generating transformants containing no inserts. However, the benefit of reducing vector re-ligation is partially offset by a reduction in ligation efficiency, since incomplete inactivation of SAP can cause the dephosphorylation of insert DNA, which prevents its ligation with the vector. The problem of inactivating phosphatase was partially reduced by using SAP as opposed to calf alkaline phosphatase, since the latter enzyme is particularly difficult to heat inactivate. However, as mentioned in section 3.3.1 and 3.3.3, dephosphorylation of vector DNA with SAP still appeared to have reduced the efficiency of transformation, since dephosphorylated vector ligated with insert DNA generated far fewer transformants than vector re-ligated to itself without dephosphorylation.

To increase overall efficiency of transformation, methods to increase ligation efficiency by avoiding the use of SAP were investigated. In each experiment, purified *S. typhimurium* SL1344 DNA was ligated with the pZKC3 vector previously digested with *Bam*HI. To minimise vector re-ligation and encourage insert DNA ligation, higher
vector mass ratios were used. For control purposes, *E. coli* DH5α cell competency was assessed by transforming electrocompetent cells with uncut pZKC3 vector (Table 3.6, Group A) and was shown to be reasonably competent for this experiment, generating in excess of $10^5$ colonies. In addition, the number of transformants generated from the incomplete digestion of pZKC3 was assessed by transforming electrocompetent cells with *BamHI* digested pZKC3 vector (Group B). Again, the low numbers observed for Group B indicates few false positives were contributing to transformant numbers in the experimental groups.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Nature of Vector DNA</th>
<th>Insert: Vector Mass Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Uncut pZKC3</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td><em>BamHI</em> digested pZKC3</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td><em>BamHI</em> digested pZKC3, then re-ligated</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td><em>BamHI</em> digested pZKC3, dephosphorylated then re-ligated</td>
<td>-</td>
</tr>
<tr>
<td>E</td>
<td><em>BamHI</em> digested pZKC3, dephosphorylated then ligated with <em>S. typhimurium</em> insert DNA</td>
<td>1.5:1</td>
</tr>
<tr>
<td>F</td>
<td><em>BamHI</em> digested pZKC3, ligated with <em>S. typhimurium</em> insert DNA</td>
<td>1.5:1</td>
</tr>
<tr>
<td>G</td>
<td><em>BamHI</em> digested pZKC3, dephosphorylated then ligated with <em>S. typhimurium</em> insert DNA</td>
<td>3:1</td>
</tr>
<tr>
<td>H</td>
<td><em>BamHI</em> digested pZKC3, ligated with <em>S. typhimurium</em> insert DNA</td>
<td>3:1</td>
</tr>
<tr>
<td>I</td>
<td><em>BamHI</em> digested pZKC3, dephosphorylated then ligated with <em>S. typhimurium</em> insert DNA</td>
<td>4.5:1</td>
</tr>
<tr>
<td>J</td>
<td><em>BamHI</em> digested pZKC3, ligated with <em>S. typhimurium</em> insert DNA</td>
<td>4.5:1</td>
</tr>
</tbody>
</table>

Table 3.6 Key to experimental groups in Figure 3.14. For each experimental group 0.4μg of pZKC3 vector DNA, previously digested with *BamHI* and dephosphorylated (where stated) was ligated to the appropriate amount (where stated) of *S. typhimurium* SL1344 insert DNA that had previously been digested with *Sau3A*, fractionated and purified by dialysis. The ligation mixture was then used to transform 200μl of electrocompetent cells.
Figure 3.14 Effect of vector dephosphorylation on the efficiency of ligation and transformation. The values indicate the number of colonies counted following electroporation of 200μl of E. coli DH5α electrocompetent cells. Details of the experimental groups are described in Table 3.6.

From the results of Groups E and F, G and H, I and J, in Figure 3.14, it would appear that treatment of the vector DNA with SAP reduced the efficiency of ligation and transformation. The number of transformants obtained from Groups F, H and J, which did not receive SAP treatment, was over double the number obtained from Groups E, G and I which had dephosphorylated vector in the ligation reaction. However, restriction analysis of the vector DNA taken from transformants in Group F, H and J, showed the high transformation numbers were due to vector DNA re-ligation rather than the incorporation of *S. typhimurium* insert DNA. Even with higher concentrations of *S. typhimurium* DNA in the ligation reaction, the percentage of recombinant clones was only 16%, indicating the vector DNA had preferentially re-ligated.

It was therefore concluded that whilst dephosphorylating the recipient vector DNA may have reduced the ligation efficiency, it was an essential process to minimise vector re-ligation and reduce the number of transformants containing no inserts in the library (see
Desphosphorylation of the pZKC3 vector with SAP was therefore performed prior to every ligation with *S. typhimurium* SL1344 insert DNA in the construction of the EL and any detrimental effects of SAP were reduced where possible by purifying the vector DNA prior to the ligation reaction.

### 3.3.5 Effect of DNA Ligase on the Efficiency of Transformation

Purifying both *S. typhimurium* SL1344 insert DNA and pZKC3 vector DNA, and optimising insert: vector mass ratios, were found to increase the overall efficiency of transformation, which was largely thought to be the result of maximising ligation efficiency (see Figure 3.15, Lane 3). To further increase the number of recombinant transformants following transformation, methods to directly improve transformation efficiency were investigated.

![Figure 3.15 Ethidium bromide-stained agarose gel of pZKC3 DNA ligation mixtures prior to transformation. Lane 1: BamHI digested pZKC3 vector. Lane 2: BamHI digested pZKC3 vector which had been dephosphorylated, purified by phenol/chloroform extraction and re-ligated. Lane 3: BamHI digested pZKC3 vector that has been dephosphorylated and purified by phenol/chloroform extraction and ligated with *S. typhimurium* SL1344 DNA fragments.](image)
Reports in the literature have documented that DNA ligase noticeably inhibits the transformation process (Ymer, 1991; Michelsen, 1995). It was therefore of interest to remove T4 DNA ligase prior to transformation and establish whether this process increased the number of colonies observed following transformation. Initial attempts to purify the recombinant vector following ligation by phenol/chloroform extraction were unsuccessful due to the amount of DNA lost in the process (see section 3.3.1). As an alternative method, the effect of heat inactivating the DNA ligase was analysed since it had been documented that heat inactivating T4 DNA ligase prior to transformation increased the number of transformants by 260-fold (Michelsen, 1995).

To establish the effect of heat inactivating T4 DNA ligase on the efficiency of transformation, the number of colonies obtained from transformations with ligation products that had been heat inactivated were compared to the number generated from ligation products receiving no heat treatment (see Table 3.7). For each experimental group, pZKC3 vector DNA was ligated with *S. typhimurium* SL1344 DNA and either heated to 65°C for 20 minutes to heat inactivate the T4 DNA ligase, or incubated on ice for 20 minutes. All ligation mixtures were cooled on ice for 10 minutes before being used to transform *E. coli* DH5α cells that had previously been stored at −80°C.

The experimental control Groups A and B generated >10^5 transformants and 23 transformants respectively. This indicated that the cells were reasonably competent for the investigation and that few transformants observed were likely to have resulted from incomplete digestion of pZKC3 vector DNA. The vertical scale in Figure 3.16 was chosen to illustrate differences between Groups C to H and does therefore not include Groups A and B.
<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>DNA used in Transformation</th>
<th>Heat Inactivated</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Uncut pZKC3 vector</td>
<td>No</td>
</tr>
<tr>
<td>B</td>
<td>BamHI digested pZKC3 vector</td>
<td>No</td>
</tr>
<tr>
<td>C</td>
<td><em>S. typhimurium</em> SL1344 DNA insert ligated with pZKC3 vector</td>
<td>No</td>
</tr>
<tr>
<td>D</td>
<td><em>S. typhimurium</em> SL1344 DNA insert ligated with pZKC3 vector</td>
<td>Yes</td>
</tr>
<tr>
<td>E</td>
<td><em>S. typhimurium</em> SL1344 DNA insert ligated with pZKC3 vector</td>
<td>No</td>
</tr>
<tr>
<td>F</td>
<td><em>S. typhimurium</em> SL1344 DNA insert ligated with pZKC3 vector</td>
<td>Yes</td>
</tr>
<tr>
<td>G</td>
<td><em>S. typhimurium</em> SL1344 DNA insert ligated with pZKC3 vector</td>
<td>No</td>
</tr>
<tr>
<td>H</td>
<td><em>S. typhimurium</em> SL1344 DNA insert ligated with pZKC3 vector</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 3.7 Key for experimental groups in Figure 3.16. Ligation reactions were performed using the optimal insert: vector mass ratio of 1.5:1 as determined in section 3.1.1. Additionally, all pZKC3 vector DNA used in the ligation reactions was digested with BamHI, dephosphorylated and purified by phenol/chloroform extraction prior to ligation. *S. typhimurium* insert DNA was purified by dialysis prior to ligation. A volume of 200μl of heat shock competent cells produced from a single batch were transformed with the ligation mixtures described above.

From the results in Figure 3.16, it is evident that heat inactivation of T4 DNA ligase prior to transformation greatly increased the number of transformants observed. The ligation mixtures for Groups D, F and H that were heated to 65°C prior to transformation generated on average a 2-fold increase in the number of transformants compared to Groups C, E and G which did not receive heat inactivation of the T4 DNA ligase. This indicates that active DNA ligase inhibits the transformation process to a degree and can explain many of the low numbers of transformants seen in the previous sections.

Moreover, these results showed that the number of transformants generated from transformation with ligated pZKC3 and *S. typhimurium* SL1344 DNA was in excess of the desirable figure of 2,000 previously established and therefore enabled the efficient construction of the EL.
Figure 3.16 Effect of DNA ligase activity on transformation efficiency. Values indicate the number of colonies counted following transformation of 200μl heat shock competent E. coli DH5α cells with ligation mixtures of pZKC3 vector and S. typhimurium SL1344 insert DNA. Groups C, E and G received no heat inactivation of DNA ligase prior to transformation, whilst Groups D, F and H received heat inactivation treatment at 65°C for 20 minutes.

3.3.6 S. typhimurium SL1344 Expression Library Construction

Since the variables had been optimised to obtain over 2,000 colonies per transformation, the EL for S. typhimurium SL1344 was constructed. The pZKC3 vector was prepared by CsCl gradient centrifugation (Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981) and was repeatedly dialysed against dH2O to remove traces of CsCl salt and ethidium bromide that may have interfered with future DNA manipulations. The pZKC3 vector was then digested with BamHI and dephosphorylated with SAP to remove terminal phosphate groups and prevent possible re-ligation. The digested and dephosphorylated pZKC3 vector was then purified from enzyme buffers and other contaminants by phenol/chloroform extraction and 0.4μg was used in the ligation reaction.

Chromosomal DNA was extracted from stationary phase S. typhimurium SL1344 by the protocol described by Silhavey et al., (1984). Chromosomal DNA was then partially
digested with *Sau3A* and was fractionated by sucrose density gradient centrifugation. Fragments of approximately 2,000 bp were identified by DNA agarose gel electrophoresis and were purified from contaminating sucrose and sarcosyl by dialysis. A quantity of 0.6μg of *S. typhimurium* SL1344 DNA was used in the ligation reaction with 0.4μg of pZKC3 vector DNA. Following the ligation reaction, the ligation mixture was subjected to a temperature of 65°C for a period of 20 minutes to inactive the T4 DNA ligase. The ligation mixture was then cooled on ice for 10 minutes before being used to transform 200μl of heat shock competent cells.

A total of 70 transformations were performed, each generating in excess of 2,000 colonies. The colonies obtained from each individual transformation were pooled together to make 1 sub-library. The entire EL therefore consisted of 70 different sub-libraries that were individually stored at -80°C.
3.4 Analysis of DNA Expression Library

The previous section described the requirements for a representative DNA expression library and discussed the optimisation of variables involved in constructing an EL for *S. typhimurium* SL1344. This section examines the *S. typhimurium* SL1344 EL by PCR and by sequencing analyses to determine whether the EL is representative of the *S. typhimurium* SL1344 genome. In addition sequencing data is used to identify a selection of genes encoded in the library. Genes encoded in the library that form fusion proteins with EGFP-HIS are isolated for expression studies in bacterial and mammalian cells and are used to determine whether the EL is functional.

3.4.1 PCR Analysis of Inserted *S. typhimurium* SL1344 DNA

The constructed DNA expression library was made up of 70 sub-libraries, each sub-library consisting of over 2,000 clones, making a total of over 140,000 clones. In order to assess the quality of the library, PCR analyses were performed on a total of 150 clones, taken equally from three different sub-libraries. Plasmid DNA was analysed by PCR using the primers ZKC3 INSERT F and ZKC3 INSERT R (Table 2.1). These primers were specifically designed to amplify *S. typhimurium* SL1344 chromosomal DNA that had ligated into the *Bam*HI restriction site in the vector pZKC3. DNA agarose gel electrophoresis was used to measure the number of recombinant clones and the approximate sizes of the inserts.

Of the 26 clones shown in Figure 3.17, only 3 clones (Lanes J, Q and W) did not contain *S. typhimurium* SL1344 DNA inserts. In total, analysis of 150 clones showed that 92% contained *S. typhimurium* SL1344 DNA fragments. In addition, the average insert size was approximately 2,000 bp as desired, but ranged from 500 bp to 3,000 bp.
Assuming that the random sample of 150 clones analysed was representative of the entire library of 140,000 colonies, the number of recombinant clones in the entire expression library was calculated to be 128,800.
3.4.2 Sequencing Analysis

Although the DNA expression library consisted of a large number of clones, not all of these contained DNA in the correct orientation or frame to form a fusion protein with the upstream EGFP-HIS. To fully evaluate this aspect of the library, 60 of the 150 clones analysed by PCR, were sequenced.

Sequence data was initially inserted into the National Center for Biotechnology Information (NCBI) database to identify the *Salmonella typhimurium* SL1344 gene fragments. The search results are shown in Table 3.8. Of the 60 clones sequenced, 54 matched to known sections of the *S. typhimurium* LT2 genome and one clone encoding the HCM1.221 gene matched the *S. typhi* CT18 genome. Interestingly, two clones were found to contain genes similar to those found in the P22 bacteriophage and a further two encoded genes that were found on possible virulence plasmids of strain SL1344. One clone however, was not matched to any known pathogen. Whilst it is possible this could be a gene exclusive to *S. typhimurium* SL1344, it is more likely that the sequencing process was not entirely correct.

The correct orientation and frame of each gene fraction was determined (see Table 3.9), using the annotation provided in the NCBI database. Some sequence data showed that 2 different gene fragments had been ligated into the one pZKC3 vector. For example, sequencing data from one clone (K19) identified both the *fliS* and the *fliT* genes, and similarly clone G22 encoded both the Gifsy-2 prophage major coat protein and the Gifsy-2 prophage minor coat protein genes. For each clone sequenced that identified 2 different gene fragments, each gene was found to have come from the same section of LT2. This indicated that the gene fragment incorporated into the vector had spanned 2 ORFs, rather than encoding 2 random genes. In these cases, both inserts were assigned appropriate frames.
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<th>Section of LT2</th>
<th>Base Match</th>
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Table 3.8 Identification of gene fragments encoded on plasmids taken from 60 clones in the S. typhimurium SL1344 EL. * Indicates gene fragments that have been inserted from the same sections of the LT2 genome.
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Table 3.9. Orientation and frame of 65 S. typhimurium SL1344 gene fractions identified by the sequence analysis of 60 clones taken equally from three different sub-libraries.

In order for S. typhimurium SL1344 gene fragments to successfully form a fusion protein with EGFP-HIS, and therefore be expressed, the DNA had to be in the forward orientation and in frame 2 and contain no translatable stop codons. The results in Table 3.9 show that 14% of the 65 sequences identified contain S. typhimurium SL1344 DNA in the correct orientation and frame to form fusion proteins with EGFP-HIS.

### 3.4.3 Expression in Prokaryotic Cells

A fundamental feature of DNA expression libraries is the expression of antigenic proteins encoded by foreign DNA ligated into the vector. It was important therefore to establish that S. typhimurium SL1344 gene fractions were translated efficiently and expressed as C-terminal fusion proteins to EGFP-HIS which were of the correct predicted size. Using the sequencing data, a collection of clones containing S. typhimurium SL1344 gene fractions in frame 2 and lacking stop codons within the ORF were further examined for protein expression. The clones chosen for characterisation were B26, B43 and K28 and were renamed pZKC5, pZKC6, and pZKC7 respectively (see Table 3.8).

Each of the three plasmids were transformed into E. coli BL21 and individual colonies were grown to an OD of 0.5 at 600nm in LB medium. At the desired OD, 0.1M of IPTG was added to the bacterial cultures and an induction profile was produced for each plasmid over a period of 3 hours (see Figures 3.18, 3.19 and 3.20).
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**Figure 3.18.** SDS-PAGE showing the induction profile for E. coli BL21 cells previously transformed with the pZKC5 vector encoding the ybbA gene fragment. Protein expression was examined at time 0 and then at 30 minute intervals for 3 hours following induction with IPTG.

<table>
<thead>
<tr>
<th>Molecular Weight kDa</th>
<th>Time in Minutes</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
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<td>66</td>
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**Figure 3.19.** SDS-PAGE showing the induction profile for E. coli BL21 cells previously transformed with the pZKC6 vector encoding the traD gene fragment. Protein expression was examined at time 0 and then at 30 minute intervals for 3 hours following induction with IPTG.
Whilst a clear band of protein induction is evident for pZKC5 at approximately 48-54kDa, induction bands for pZKC6 and pZKC7 are more difficult to determine from these profiles. As an alternative approach to circumvent this problem, proteins expressed from BL21 cells 180 minutes after induction with IPTG were purified by affinity chromatography using a nickel column and the purified proteins were then examined by Western blotting using a 1: 3,000 dilution of rabbit anti-EGFP antibody. Only S. typhimurium SL1344 proteins expressed in-frame with EGFP-HIS should be purified by the HIS tag and also detected by anti-EGFP antibody. Proteins encoded in vectors pZKC5, pZKC6 and pZKC7 were analysed by Western blotting and the relative sizes of each protein were compared to the EGFP-HIS fusion protein encoded in the parent vector, pZKC3 (see Figure 3.21).
Using a computer software package (Vector NTI, version 7), the predicted molecular weight of EGFP-HIS expressed from the pZKC3 vector was calculated to be 34kDa and this figure is in good agreement with the migratory position of the band detected for pZKC3 in Figure 3.21. Using the sequence data for pZKC5, pZKC6 and pZKC7, the predicted sizes of the expressed fusion proteins were 54.6 kDa, 60.4 kDa and 43.5 kDa respectively. The Western blot results in Figure 3.21, show bands of expressed fusion proteins for bacteria transformed with pZKC5 and pZKC7 that agree well with the predicted sizes of 54.6 kDa and 43.5 kDa respectively. However, the protein expressed by bacteria previously transformed with the vector pZKC6, generated a band at approximately 50 kDa, which was smaller than the expected size of 60.2 kDa. One possible explanation for this anomaly is that the expressed protein migrated faster through the polyacrylamide gel due to incomplete denaturation or retention of the protein’s secondary structure. This phenomenon is encountered when the protein is particularly hydrophobic such as for membrane proteins. Indeed a hydropathy plot...
produced from the sequence of the \textit{traD} gene showed the presence of several hydrophobic regions that may explain its unexpected migratory position.

To confirm that only \textit{S. typhimurium} SL1344 gene fractions in frame 2 would be correctly expressed as fusion proteins, several clones, B6, B38, B39 and K12 in which the DNA insert encodes the ORF in an alternative frame, were analysed for protein expression (see Table 3.8). Sequence analysis of these clones indicated that B6 and B38 encode ORF fragments in frame 1, whilst B39 and K12 encode ORF fragments in frame 3 (see Table 3.8).

<table>
<thead>
<tr>
<th>Clone</th>
<th>Hypothetical size if protein expressed (kDa)</th>
<th>Predicted size of protein expressed (kDa)</th>
<th>Actual size of protein expressed (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>41</td>
<td>34</td>
<td>~34</td>
</tr>
<tr>
<td>B38</td>
<td>52.5</td>
<td>35.6</td>
<td>~35</td>
</tr>
<tr>
<td>B39</td>
<td>52.9</td>
<td>35.3</td>
<td>~35</td>
</tr>
<tr>
<td>K12</td>
<td>61.5</td>
<td>35.7</td>
<td>~35</td>
</tr>
</tbody>
</table>

\textit{Table 3.10. Hypothetical size, predicted size and actual size of proteins expressed following induction of BL21 cells transformed with vectors purified from clones B6, B38, B39 and K12. Hypothetical size is the size of protein if expressed in frame with EGFP-HIS. The predicted size of protein is the size calculated if not expressed in frame with EGFP-HIS and the actual size of protein is the size determined from Western blot analysis.}

If clones B6 and B38 had partial ORF of \textit{S. typhimurium} SL.1344 genes fused in frame 2 with EGFP-HIS rather than frame 1, as determined from the sequencing data, the size of the proteins following induction would be approximately 41 kDa and 52.5 kDa respectively (see Table 3.10). Likewise, if clones B39 and K12 contained partial ORF of \textit{S. typhimurium} SL1344 genes fused in-frame with EGFP-HIS, the size of the encoded proteins following induction would be approximately 52.9 kDa and 61.5 kDa respectively. This was investigated by transforming \textit{E. coli} BL21 cells with the purified plasmids from clones B6, B38, B39 and K12, and analysing the size of the proteins expressed following induction with IPTG.
Figure 3.22 Western blot detection of affinity purified proteins expressed from clones B6 and B38 following induction with IPTG. Affinity purified EGFP-HIS induced from BL21 cells previously transformed with pZKC3 is included for size comparisons.

Figure 3.23 Western blot detection of affinity purified proteins expressed from clones K12 and B39 following induction with IPTG. Affinity purified EGFP-HIS and EGFP-HIS-YbbA, induced from BL21 cells previously transformed with pZKC3 and pZKC5 respectively, are included for size comparisons.
Following a period of 180 minutes after the induction of *E. coli* BL21 with IPTG, the expressed proteins were purified, as before, by affinity chromatography and analysed by Western blot using rabbit anti-EGFP antibody (see Figures 3.22 and 3.23).

The calculated size of EGFP-HIS expressed from pZKC3 is 34 kDa. Using the size of EGFP-HIS for comparisons, it is evident from Figure 3.22 that clones B6 and B38 express proteins of approximately 34 kDa and 35 kDa respectively. However, it was hypothesised that clones B6 and B38 would produce a protein of 41 kDa and 52.5 kDa respectively if in frame with EGFP-HIS. This would confirm that the *S. typhimurium* SL1344 gene fractions in B6 and B38 are not in frame 2 and only EGFP-HIS is being expressed. Moreover, the actual size of the proteins expressed from B6 and B38 are in agreement with the predicted fusion protein sizes calculated for *S. typhimurium* SL1344 fragments inserting in frame 1 (see Table 3.10). Similarly, clones K12 and B39 both express proteins of approximately 35 kDa in size as opposed to the hypothetical sizes of 52.9 kDa and 61.5 kDa respectively (see Figure 3.23 and Table 3.10). Again the actual size of the proteins expressed from K12 and B39 are in agreement with the predicted fusion protein sizes calculated for *S. typhimurium* SL1344 fragments inserting in frame 3. This confirms that the *S. typhimurium* SL1344 gene fractions encoded in clones K12 and B39 are not in frame with EGFP-HIS and verifies that only *S. typhimurium* SL1344 gene fractions inserting in frame 2 will be expressed.

### 3.4.4 Expression in Eukaryotic Cells

The effectiveness of DNA vaccines in animal models is largely dependent on the efficient expression of encoded proteins within mammalian cells. Whilst the fusion proteins encoded in pZKC5, pZKC6 and pZKC7 are known to be expressed in bacterial cells, it is vital that these gene fractions are also expressed in eukaryotic cells if they are to induce a protective immune response. To explore this situation, large scale plasmid preparations were prepared for clones pZKC5, pZKC6 and pZKC7, as described in
Chapter 2. Each plasmid was used to transfect a growing population of L cells in culture (Kundig et al., 1995). Since each vaccine construct was designed to express the egfp gene, independently of whether it produced a fusion protein, cells expressing this gene should appear green under a fluorescence microscope. However, this experiment assumed that L cells fluorescing green from EGFP-HIS expression, would also be producing the encoded S. typhimurium SL1344 gene fractions, based on bacterial expression studies.

The parent plasmid, pZKC3, was used as a positive control for EGFP-HIS expression (see Figure 3.24, Panel A), and the green fluorescence observed within the cells indicated that EGFP-HIS was expressed. Plasmids pZKC5, pZKC6 and pZKC7 were also observed to fluoresce green (see Figure 3.20 Panels B, C and D respectively) indicating that expression of EGFP-HIS fusion proteins had occurred.

The percentage of L cells transfected with pZKC5, pZKC6 and pZKC7 was between 5% and 10%, which was lower than the 40% transfection rate observed with pZKC3 under the same conditions (see Figure 3.24). The reasons for this are unclear, although it would appear that all the plasmids encoding EGFP-HIS fusions were limiting the transfection efficiency, since they were expressed in a lower proportion of cells than the EGFP-HIS alone. Although the transfection efficiency of pZKC5, pZKC6 and pZKC7 was reduced compared to pZKC3, the level of fluorescence produced from individual transfected cells was essentially as high as that observed for pZKC3, indicating that the EGFP-HIS fusions were expressed equally as well as EGFP-HIS in mammalian cells.
Figure 3.24 EGFP-HIS and EGFP-HIS-S. typhimurium SL1344 antigen fusion proteins expressed in vivo. Purified plasmid DNA was mixed with LipofectAMINE prior to incubation with adherent L cells. Panels show expression of EGFP from constructs (A) pZKC3, (B) pZKC5, (C) pZKC6 and (D) pZKC7. Panels A-D were visualised using a triple band filter (DAPI/FITC/TRITC), and images from blue (DAPI) and green (FITC) filters were overlaid here to show cell nuclei and EGFP fluorescence respectively.
3.5 Discussion

For the technique of ELI to be developed for identifying the protective antigens of a pathogen, an EL must be representative of the pathogen’s genome and must express the encoded foreign DNA. This Chapter identified a number of variables involved in constructing a representative EL and evaluated the importance of each variable for the efficient construction of a \textit{S. typhimurium} SL1344 EL. Furthermore, this chapter analysed the content of the EL by PCR and sequencing analyses and examined the functionality of the EL in both prokaryotic and eukaryotic cells.

3.5.1 Investigation of Variables Involved in the Efficient Construction of a \textit{S. typhimurium} SL1344 Expression Library

Construction of the \textit{S. typhimurium} SL1344 DNA EL involved several sequential stages. The efficiency of EL construction was determined by the efficiencies of each stage so it was therefore important to optimise each stage (Bottger, 1988).

Initial experiments investigated the effect of DNA purity on the ligation reaction and overall efficiency of transformation since contaminating enzymes, buffers and salts have been shown to decrease the number of transformants generated (Inoue \textit{et al.}, 1990; Maniatis \textit{et al.}, 1982). Indeed, the results demonstrated that the removal of contaminating restriction endonucleases, dephosphorylation enzymes and their appropriate buffers and salts from pZKC3 vector DNA by phenol/chloroform extraction prior to ligation, increased the number of colonies following transformation. The number of transformants generated could not however be increased further by similarly purifying the recombinant vector from T4 DNA ligase and the corresponding buffer immediately preceding transformation (Taketo, 1989). This was due to the loss of variable amounts of ligated DNA in the purification process, which prevented transformation from being sufficiently reproducible for library construction. Whilst phenol/chloroform extraction of
vector DNA prior to ligation produced slight increases in the number of transformants, a greater increase in the number of transformants was observed by purifying the insert *S. typhimurium* SL1344 DNA from sucrose and sarcosyl solutions prior to ligation. Due to the high losses of DNA experienced in phenol/chloroform extraction and the nature of the contaminating solutions, partially digested *S. typhimurium* SL1344 DNA was purified by dialysis against several changes of dH₂O. The results demonstrated a 3-fold increase in transformation efficiency when the insert DNA was purified prior to ligation and an overall 4-fold increase in transformation efficiency when both vector DNA and insert DNA was purified prior to ligation.

Following successful purification of insert and vector DNA, additional experiments investigated alternative variables involved in the ligation process that could be optimised. One variable known to have a significant role in the ligation reaction is the insert: vector molar ratio (Maniatis *et al.*, 1982). Since it was not appropriate to use insert: vector molar ratio due to the range of fragment sizes resulting from restriction endonuclease digestion of *S. typhimurium* SL1344 genomic DNA, the insert: vector mass ratio was determined.

Studies have shown a positive correlation between plasmid DNA concentration and the efficiency of transformation by electroporation. Increasing plasmid concentration results in a corresponding increase in transformation efficiency up to a point, beyond which, transformation efficiency declines (Taketo, 1988; Garg *et al.*, 1999). It was therefore desirable to identify the lowest quantity of plasmid DNA that generated the maximum number of transformants. The optimal quantity of vector DNA to use in transformation, and therefore use in the ligation mixture, was found to be 0.4µg under the conditions examined. The results demonstrated that the highest number of transformants could be obtained when *S. typhimurium* SL1344 insert DNA and pZKC3 vector DNA was ligated with an insert: vector mass ratio between 1.25:1 and 1.75:1. Taking the mid-point of this range, an insert: vector mass ratio of 1.5:1 was used for all subsequent ligation reactions. Whilst optimising the quantity of insert and vector in the ligation reaction, these results
also highlighted the extremely low number of transformants generated from transformation with the recombinant vector compared to the number of transformants generated following transformation with the uncut vector and re-ligated vector controls. This indicated, aside from DNA purity and insert: vector mass ratios, that other factors were influencing the efficiencies of ligation and transformation. Since most experiments had concentrated on the ligation of insert and vector DNA, attention was therefore turned towards increasing the efficiency of transformation.

One factor thought to have an effect on transformation efficiency was the method of transformation (Taketo, 1989; Zabarovsky and Winberg, 1990; Inoue et al., 1990). To construct an entire EL, a large number of highly competent *E. coli* DH5α cells were required. Additionally, it was vital that the level of cell competency was both high and consistent to ensure over 2,000 colonies were generated from every transformation. To identify the most efficient method of transformation, electroporation and heat shock transformation were compared and to ensure a consistent level of cell competency, *E. coli* DH5α cells required for the construction of the entire expression library were produced in a single batch. From the results in Figure 3.4, it is evident that electrocompetent cells were more efficient at transformation than cells prepared and transformed by heat shock, explaining why electroporation is the preferred method of transformation (Taketo, 1989; Zabarovsky and Winberg, 1990). However, the results in Table 3.5 indicate that under the conditions examined, the transformation efficiency of electrocompetent cells diminished following storage at —80°C. Indeed, these findings are in agreement with reports in the literature (Michelsen, 1995). In contrast, no major decline in efficiency was seen for cells that were prepared for transformation using heat shock, when stored at —80°C. Since the expression library had to be constructed over a period of several days, it was necessary to store cells at —80°C prior to transformation. Moreover, the ability to store cells at —80°C without a decline in transformation efficiency meant that the competency of cells could be assessed prior to library construction, ensuring that only highly competent cells were used in the transformations. Overall, transformation by thermal shock was used for the construction of the EL, since
it was more reproducible and provided a higher competency of cells than electroporation after storage at −80°C.

Having improved the purity of the insert and vector DNA and optimised the quantity of DNA used in the ligation reaction and the method of transformation, the average number of colonies obtained following a single transformation averaged around 1,200. To generate the approximate number of 2,000 colonies per transformation (as previously stated) it was necessary to increase the overall efficiency of transformation further. It was observed from previous experiments that dephosphorylating the vector DNA prior to ligation with the *S. typhimurium* SL1344 DNA was possibly reducing the number of transformants generated. It was therefore of interest to investigate whether the number of transformants generated would be increased if the vector was not dephosphorylated prior to ligation. Although the results showed the transformation efficiencies increased when vector DNA was not dephosphorylated, the reason was not due to more efficient insert DNA ligation, but was due to the vector DNA re-ligating. Even with the concentration of insert DNA in the ligation mix increased to encourage insert ligation, analyses showed only 16% of colonies were recombinant clones.

As described in Chapter 1, the critical issue in the application of ELI is its sensitivity. It was therefore vital that protective plasmids were not diluted beyond their threshold for inducing an immune response especially by non-recombinant plasmids. The ideal fraction of recombinant clones required for each transformation event was 90% and above. To help achieve this, all ligations involved in constructing the expression library were performed using dephosphorylated vector DNA, and the detrimental effects of using SAP were reduced by purifying the vector DNA by phenol/chloroform extraction prior to ligation with the *S. typhimurium* SL1344 DNA.

The final variable under investigation was the effect of DNA ligase on the efficiency of transformation, since its adverse effect on transformation had been documented (Ymer, 1991; Michelsen, 1995). Since purification of the recombinant vector DNA by
phenol/chloroform extraction following ligation was unsuccessful due to substantial DNA loss, the T4 DNA ligase was thermally inactivated prior to transformation. The results showed an approximate 2-fold increase in the transformation efficiency following thermal inactivation. By combining the thermal inactivation of DNA ligase with the previously optimised conditions, the number of transformants obtained from each transformation plate exceeded 2,000 colonies as required. A total of 70 transformations were performed, generating a *S. typhimurium* SL1344 EL comprising of 140,000 colonies.

3.5.2 Analysis of Expression Library

Ideally, an EL for a pathogenic bacterium should be representative. That is, the library should contain clones which are capable of expressing every gene of the pathogen of interest. Whilst the full identification of every gene in this expression library was beyond the scope of this work, it was possible to be 99% confident that the library was representative of the *S. typhimurium* SL1344 genome by ensuring three particular requirements were achieved (Clarke and Carbon, 1976). To determine if the *S. typhimurium* SL1344 DNA expression library was representative, a sample of the library was analysed by PCR and was sequenced.

Using the equation of Clarke and Carbon, the minimum number of clones required to construct a representative DNA library for *S. typhimurium* SL1344 in 2,000bp fragments is 11,200. To construct an expression library however, the *S. typhimurium* SL1344 DNA fragments must be cloned in the correct orientation and frame. Since statistically, only one clone in every six will have *S. typhimurium* SL1344 DNA fragments in the orientation and reading frame to allow expression, the number of clones required to ensure with 99% confidence that the entire *S. typhimurium* SL1344 genome had been represented, is 67,200. The library constructed for this work consisted of 140,000 independent clones and PCR analysis showed 92% of these clones contained fragments
of *S. typhimurium* SL1344 DNA. The library thus contained 128,800 recombinant clones of which the average insert size was found to be approximately 2,000 bp. Since one of the requirements for a DNA expression library to be representative was to consist of over 67,200 clones, PCR analyses can confirm that this prerequisite had been achieved.

However, the calculation for determining the number of clones required in the library for it to be representative is dependant on the *S. typhimurium* SL1344 DNA being randomly cleaved and ligated into the expression vector. The most random collection of genomic DNA fragments is produced by shearing the DNA. However, because the techniques for cloning sheared DNA are not very efficient, most libraries are constructed with a random collection of DNA fragments that have been produced by partial digestion with restriction enzymes (Seed *et al.*, 1982). Restriction enzymes that produce the most random collection of fragments are those that cleave DNA most frequently. For example, restriction enzymes that recognise 4-base sequences produce a more random collection of insert fragments than enzymes that recognise 6-base sequences. To therefore maximise the randomness of the *S. typhimurium* SL1344 fragments produced, *S. typhimurium* SL1344 DNA was partially digested with *Sau3A*, since it recognises the 4-base sequence GATC. In addition it has no bias in the selection of one restriction site over another (Seed *et al.*, 1982). This was particularly important for ensuring each gene was equally represented in each frame as the location of the *Sau3A* site within the gene will determine the frame in which the *S. typhimurium* SL1344 DNA fragment will be inserted into the compatible *BamHI* site in the pZKC3 expression vector.

To analyse whether the fragments of *S. typhimurium* SL1344 DNA had been randomly digested and ligated into the expression vector, a total of 60 clones were sequenced from 3 different sub-libraries. It was postulated that if the gene fractions analysed from each of the 60 clones sequenced were a random selection of different genes, and were ligated equally into each of the six possible orientations, this would suggest that gene fragments had been digested and ligated randomly.
Sequence analysis showed that all of the gene fractions were different, supporting the assumption of random ligation of inserts (see Table 3.8). In addition, the data showed that 14% of the 65 sequences identified contained *S. typhimurium* SL1344 DNA in the correct orientation and frame to form fusion proteins with EGFP-HIS in pZKC3. If the digestion and ligation of *S. typhimurium* SL1344 DNA was completely random, with equal chance of the DNA fragments inserting in either orientation and any frame, the expected fraction in the correct orientation and frame is 16.7%. The number of clones containing *S. typhimurium* SL1344 DNA in the correct orientation and frame is within one standard deviation of the value expected for an equally distributed population. While the results do not confirm with certainty that digestion and ligation are random, the difference from the expected value is not sufficient to say with high confidence that the processes exhibit bias. A larger sample size would exhibit a smaller distribution, and could reject the existence of bias with a higher degree of confidence. However, these results, together with the number of clones obtained would indicate that the EL constructed was representative of the *S. typhimurium* SL1344 genome according to requirements described by Clarke and Carbon (1976). In addition, this exact method of EL construction has been successfully performed for other pathogens such as *T. cruzi* (Alberti *et al.*, 1998).

The sequence analysis of 60 clones from the different sub-libraries also revealed a number of interesting features of the EL. Of the 66 sequences identified, 60 matched to known sequences of the *S. typhimurium* LT2 genome and 1 matched to a sequence in the *S. typhi* genome. Interestingly, 2 clones contained gene fragments that were similar to the gene sequences found in wildtype P22 enterobacteria phage. The sequence of the *S. typhimurium* serotype-converting bacteriophage P22 has recently been completed and putative proteins show relatedness to proteins from a great variety of other phages (Vander and Kropinski, 2000). More recently, a phage specifically released from strain SL1344 was identified as SopE (Figueroa-Bossi *et al.*, 2001), which may also have been sequenced in this DNA library, since the gene sequence for the SopE protein was identified but could not be matched with any section of the LT2 genome. SopE is one of
the 13 proteins identified which promotes bacterial internalisation into epithelial cells of the small intestine (Zhou and Galan, 2001). SopE is delivered via the SPI-1 type III secretion system (see Chapter 1) into the cytosol of the host cell and which through the activation of GTPases, induces actin polymerisation and membrane ruffling (Chapter 1; Zhou and Galan, 2001). SopE is therefore important in *Salmonella* pathogenicity and could be an important candidate in the EL.

Another sequence identified was that of the TraD protein which is found on the R64 plasmid (Furuya and Komano, 1991; Kim *et al.*, 1993). This was of particular interest since the entire sequence of TraD had been incorporated into the expression vector and was in frame with EGFP-HIS (see Figure 3.21).

```
MKLQYRIPLAIYSVWCIIAILLCKDGLYQMNIWTLVEILGMIALPLLVR
MKLQYRIPLAIYSVWCIIAILLCKDGLYQMNIWTLVEILGMIALPLLVR
PFFILLRIIFRKNFSKTKDMSDNHTVCVFSLALADTSSKKAIIKHFKQLE
PFFILLRIIFRKNFSKTKDMSDNHTVCVFSLALADTSSKKAIIKHFKQLE
LEKLPPLLRLQKKRIYMKSHLLTEARTQKLIQSLRRKGLVDVTAERRESA
LEKLPPLLRLQKKRIYMKSHLLTEARTQKLIQSLRRKGLVDVTAERRESA
MNSVMFRILIVSRILSQWKVPHIPRCEIVILTLCNDSSGNRE - 189
MNSVMFRILIVSRILSQWKVPHIPRCEIVILTLCNDSSGNRE - 189
```

*Figure 3. 25 Sequence of the TraD protein encoded on the R64 plasmid. Red letters indicate the protein sequence identified from sequence analysis of plasmid pZKC6.*

The R64 plasmid is a 122kb self-transmissible plasmid belonging to the incompatibility group I1 (IncI1) and has a similar mechanism to the F plasmids (IncF1) for conjugation (Komano *et al.*, 1990). Many homologues of genes in the tra operon of the F plasmid of *E. coli* have been identified in *S. typhimurium* LT2 and have been physically located on the 90kb virulence plasmid. Based on this correlation, it has been hypothesised that the F plasmid and the virulence plasmid might be one and the same (Ahmer *et al.*, 1999). Interestingly, whilst the virulence plasmid containing homologues of genes involved in conjugation and transfer is self-transmissible in *S. typhimurium* LT2, the virulence
plasmid of SL1344 is not self-transmissible (Ahmer et al., 1999). This would imply that the *S. typhimurium* SL1344 strain used in this thesis has the R64 plasmid in addition to the virulence plasmid.

There are several genes that are located within the transfer region of the R64 plasmid, including *traABCD*, *pilV*, shufflon, *rci*, *exc*, *nikAB* and *oriT*. The function of many of these genes has been described (Furuya and Komano, 1996). Whilst it has been demonstrated that the *traB* and *traC* genes are essential for conjugal transfer in liquid medium and on a solid surface and are both required for thin pilus formation (Kim et al., 1993), the function of *traD* in the R64 plasmid is not documented and does not appear essential for plasmid transfer (Furuya and Komano, 1996). However, it has been suggested that TraD homologs from other gram-negative plasmid transfer systems such as RP4 ([IncPα]), R388 ([IncW]), Ti and pSK41 mediate an interaction between the conjugal DNA metabolism proteins and the DNA transfer pore. Analysis of the TraD protein encoded on the R100 plasmid indicated the presence of three hydrophobic regions, of which two are located near the amino-terminal region (Yoshioka et al., 1990). This protein was also found to contain a possible ATP-binding consensus sequence at the amino-terminal region (Yoshioka et al., 1990). The presence of these three hydrophobic regions in TraD would support the theory that the TraD fusion protein encoded on pZKC6 was not fully denatured during SDS PAGE and would explain why it migrated faster in the gel (see later).

A total of 5 analysed sequences, identified genes found in the 2 temperate prophages Gifsy-1 and Gifsy-2 of the *S. typhimurium* LT2 strain. Many of the genes carried by these prophages contribute to *S. typhimurium* virulence (Figueroa-Bossi et al., 1999). For example, when *S. typhimurium* breaches the intestinal barrier, they sense and respond to the particular environment of the Peyer’s Patch (PP). This is important since the PP is the critical site for *S. typhimurium* replication. It has been shown that the *gipA* gene, carried by the Gifsy-1 phage, is specifically induced when bacteria colonise the small intestine of mice, and effects the survival of *S. typhimurium* in the PP (Stanley et
plasmid of SL1344 is not self-transmissible (Ahmer et al., 1999). This would imply that the S. typhimurium SL1344 strain used in this thesis has the R64 plasmid in addition to the virulence plasmid.

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A total of 5 analysed sequences, identified genes found in the 2 temperate prophages Gifsy-1 and Gifsy-2 of the S. typhimurium LT2 strain. Many of the genes carried by these prophages contribute to S. typhimurium virulence (Figueroa-Bossi et al., 1999). For example, when S. typhimurium breaches the intestinal barrier, they sense and respond to the particular environment of the Peyer’s Patch (PP). This is important since the PP is the critical site for S. typhimurium replication. It has been shown that the gipA gene, carried by the Gifsy-1 phage, is specifically induced when bacteria colonise the small intestine of mice, and effects the survival of S. typhimurium in the PP (Stanley et
The genes carried in these prophages that are important for *S. typhimurium* virulence are therefore ideal candidates to incorporate in DNA expression library (Figueroa-Bossi *et al.*, 1999; Figueroa-Bossi *et al.*, 2001). One such gene important in *S. typhimurium* virulence that has been identified in the EL is *sodC* (De Groot *et al.*, 1997; Fang *et al.*, 1999; Sly *et al.*, 2002; Langford *et al.*, 2002). The *sodC* gene encodes for Cu, Zn-superoxide dismutase, which is important in the protection of *S. typhimurium* from the phagocytic oxidative burst of the hosts defence mechanism (De Groot *et al.*, 1997; Vazquez-Torres *et al.*, 2000). *S. typhimurium* has three periplasmic Cu, Zn-superoxide dismutase enzymes, SodCI, SodCII and SodCIII (Sly *et al.*, 2002). Both SodCI and SodCII have been implicated in *S. typhimurium* virulence (De Groot *et al.*, 1997; Fang *et al.*, 1999). However, the role of SodCIII in pathogenesis has not been established, although its function is known to be inadequate for replacing the virulence function of SodCI (Figueroa-Bossi *et al.*, 2001). SodCI is encoded on the Gifsy-2 prophage and again highlights the importance of having genes from these prophages included in the EL (Figueroa-Bossi *et al.*, 1999).

It is important to note that one sequence did not match any gene sequence for any serovar of *Salmonella* stored in the NCBI database. Since the genome of *S. typhimurium* SL1344 has not been added to the NCBI database, it is possible that this sequence may be related to a protein that is specific to the strain SL1344, which has not yet been identified. Alternatively it may have resulted from errors in the sequencing process.

In addition to proteins encoded on virulence plasmids, the P22 bacteriophage and the Gifsy-1 and 2 prophages, sequence analysis of these 60 clones demonstrated a wide range of different genes from *S. typhimurium* SL1344 genomic DNA were incorporated into the EL. The proteins identified included several outer membrane proteins (OMPs), flagellin proteins, inner membrane proteins such as ABC transporters, periplasmic proteins and numerous cytoplasmic proteins. OMPs and flagella are likely to be some of the key antigens that induce immune responses, since being located on the bacterial surface they are likely to be recognised early in the infection process by the immune
system. Indeed, characterisation of a T cell response induced by attenuated *Salmonella* demonstrated that a significant fraction of CD4\(^+\) T cells responded to the flagella protein FliC *in vivo* and that FliC was sufficient to protect mice against virulent *Salmonella* challenge (McSorley *et al*., 2000). However, it is not known whether the OMPs encoded within the EL will be capable of folding correctly due to the absence of LPS and the interactions with the cell membrane. This would be particularly disadvantageous for the production of neutralising Ab, since B cells require structural epitopes for antigen recognition and subsequent proliferation. However, it is evident from studies by Lopez-Macias and colleagues that the structure of OmpC expressed in mammalian cells has little effect on total Ab production, since a DNA vaccine encoding the *S. typhi* OmpC protein induced significant levels of OmpC-specific Ab in mice (Lopez-Macias *et al*., 1996).

Having constructed a representative DNA expression library for *S. typhimurium* SL1344, it was essential to determine that the library was functional and that *S. typhimurium* SL1344 gene fragments were being expressed. The sequencing data indicated that only DNA fragments in frame 2 would be expressed as a C-terminal fusion protein to the upstream EGFP gene and HIS tag following ligation. Three clones containing *S. typhimurium* SL1344 DNA in-frame with EGFP-HIS, pZKC5, pZKC6 and pZKC7 were examined in more detail (see Table 3.8). Using the sequencing data, the predicted molecular weights for the fusion proteins expressed by the three clones were calculated and compared to the actual sizes of the proteins, as determined by SDS-PAGE. Whilst the protein expressed by pZKC6 appeared smaller than expected, both pZKC5 and pZKC7 expressed proteins that were consistent with the calculated molecular weights, indicating the efficient expression of the entire fusion protein in bacteria.

Analysis of the protein encoded in pZKC6 revealed an identical match to the entire TraD protein from the R64 plasmid involved in conjugation (Kim *et al*., 1993). A possible explanation for the anomaly with clone pZKC6 is that the protein expressed migrated
faster through the protein gel due to incomplete denaturing. This phenomenon is encountered when the protein is particularly hydrophobic, as is the case for membrane proteins. As previously described, homologs of TraD from other R plasmids have three hydrophobic regions, and the *traD* gene on the F plasmid is known to encode a cytoplasmic membrane protein (Jalajakumari and Manning, 1989). This would therefore support the theory that the TraD fusion protein encoded on pZKC6 was not fully denatured during SDS-PAGE, and thus explain why it appeared smaller on the gel than the size calculated from the DNA sequence obtained.

Since DNA vaccine technology is only applicable for use in mammalian cells, it was crucial that pZKC5, pZKC6 and pZKC7 expressed the encoded proteins in mammalian cells. Using EGFP as a fluorescent marker for expression, mouse L cells were transfected with pZKC5, pZKC6 and pZKC7 plasmid DNA and examined for fluorescence. Results showed each plasmid expressed EGFP and therefore also expressed the *S. typhimurium* SL1344 gene fragment downstream of the EGFP-HIS. It was however noticeable that only 5-10% of L cells were transfected with pZKC5, pZKC6 and pZKC7 compared to 40% transfected with pZKC3. Whilst the reasons for this are not entirely clear, a recent report into the *in vivo* protein expression of DNA vaccines encoding mycobacterial antigens fused with EGFP have shown significantly that EGFP alone was expressed in a higher proportion of cells than the antigen-EGFP fusion proteins (Quinn *et al.*, 2002). Moreover, there was a trend toward an inverse correlation between protein size and the proportion of cells with visible fluorescence, which was suggested to be the effect of plasmid and/or protein size (Quinn *et al.*, 2002). It is therefore possible a similar phenomenon is occurring in this experiment, since plasmids pZKC5, pZKC6 and pZKC7 all encode larger fusion proteins than pZKC3 which only encodes EGFP-HIS. Despite the variations in the efficiency of transfection, the level of fluorescence produced from individual transfected cells was essentially as high as that observed for pZKC3, indicating that the EGFP-HIS fusions were expressed equally as well as EGFP-HIS in mammalian cells and would similarly be expressed well in the mouse model.
An important consideration for the success of ELI is that the encoded *S. typhimurium* SL1344 gene fragments are translated efficiently. One factor known to affect the translation efficiency of bacterial genes in mammalian cells is the codon usage (Andersson and Kurland 1990). For example, codons that are common in bacterial cells, but which are seldom found in eukaryotes will affect protein accumulation, mRNA and plasmid stability (Zahn, 1996). In addition, codons for a specific amino acid in bacteria, may code for a different amino acid in mammalian cells and effect the primary structure of the encoded protein (Forman *et al.*, 1998). Indeed codon usage and translational efficiency have been reported to be the reasons for the failure of BALB/c mice immunised with a DNA vaccine encoding epitope 91-99 of listeriolysin O (LLO) to induce CTLs. BALB/c mice were only capable of inducing specific CTLs when immunised with the epitope LLO 91-99 in which the native codons were substituted with codons frequently found in highly expressed murine genes (Koide *et al.*, 2000). Whilst it is not possible to optimise the codon usage for this EL, a new and improved ELI technique has recently been described which allows for codon optimisation (Johnston *et al.*, 2002).

In summary this Chapter has described the optimal conditions for the reactions involved in constructing a *S. typhimurium* SL1344 EL and has demonstrated that the library is representative of the *S. typhimurium* SL1344 genome. In addition, analysis of the EL has identified a number of interesting genes from strain SL1344 including those on virulence plasmids and in Gifsy 1 and 2 prophages and P22 bacteriophages. The EL has also been demonstrated to express foreign fusion proteins in both prokaryotic and eukaryotic cells *in vitro* and would therefore be expected to express foreign antigens *in vivo*. 
Chapter 4

Immune Responses to DNA Immunisation
4.1 Introduction

Salmonella are ubiquitous pathogens found in humans and livestock, wild mammals, reptiles, birds and insects and are transmitted via contaminated food and water (Finlay and Falkow, 1989). \textit{S. typhimurium} is the most frequently isolated serovar world-wide and can infect a range of different hosts. The serovar \textit{S. typhi} however, is only capable of infecting humans. This may largely be due to the requirement of specific host interactions such as with the human cystic fibrosis transmembrane conductance regulator in the gut epithelium (Pier et al., 1998). In humans, \textit{S. typhimurium} is responsible for causing gastroenteritis, whereas \textit{S. typhi} is the causative agent of typhoid fever and systemic disease (Parry et al., 2002). It is however possible to study aspects of the natural \textit{S. typhi} infection in mice, since \textit{S. typhimurium} infection in susceptible mice causes a lethal systemic disease and so acts as a model for human typhoid fever (Lucas and Lee, 2000).

Considerable progress has been made over the past 10 years in researching the potential use of “naked” DNA for immunisation (Koide et al., 2000; Gurunathan et al., 2000). Plasmid DNA induces both humoral and cellular immune responses against encoded immunogenic proteins when injected through a wide range of routes in a wide range of hosts (see Chapter 1; Table 1.1; Lai and Bennett, 1998; Hasan et al., 1999; Gurunathan et al., 2000). Immune responses include the production of neutralising Ab and the stimulation and amplification of antigen specific T helper cells and antigen specific CTLs (Ulmer et al., 1993; Fynan et al., 1993). This process can be boosted by repeated injections of DNA (Liu et al., 1997). Since DNA vaccines are effective at inducing CD8$^+$ T cell responses, this immunisation strategy has considerable advantage over conventional protein-based vaccines, especially when targeting intracellular pathogens such as \textit{Salmonella enterica} for which a CTL response is considered necessary. Whilst attenuated vaccines and recombinant infectious vectors are also capable of activating a CTL response, DNA vaccines have no risk of reverting to a virulent form and causing
disease since the plasmids are typically constructed not to replicate in mammalian cells or produce infectious particles.

This work examines whether humoral and cellular immune responses to *S. typhimurium* SL1344 proteins are induced following immunisation with a *S. typhimurium* SL1344 DNA expression library. Furthermore it examines the effect of different DNA vaccine administration routes and the effect of host genetic background on the induction of immune responses. To date, no published literature has documented the induction of humoral and cellular immune responses following immunisation with a DNA vaccine encoding any *S. typhimurium* proteins. Additionally, few publications have directly compared the immune responses induced following two different routes of DNA immunisation, and little is known about the effects of mouse strain on the induction of immune responses by DNA immunisation.

This chapter aims to identify whether DNA vaccination with an expression library (EL) induces specific humoral and cellular immune responses. In addition the feasibility of expression library immunisation (ELI) for identifying novel antigens of *S. typhimurium* SL1344 is assessed. The chapter is divided into 3 main sections. The first section analyses and compares the immune responses generated following intramuscular (IM) and intradermal (ID) immunisation with the pZKC3 DNA construct encoding EGFP. By initially immunising with a uniform product, the route of administration and the process of immunisation can be assessed. The second section analyses the immunological responses induced by immunising with the EL of *S. typhimurium* SL1344, and the most effective route of immunisation is investigated. The final section compares the immunological responses generated following ID immunisation with the *S. typhimurium* SL1344 EL in mice that are genetically resistant to *S. typhimurium* infection and mice that are genetically susceptible.
4.1.1 Humoral Immunity

Humoral immunity involves the production of Ab from activated B cells, and the binding of these secreted Ab to antigen (Janeway and Travers, 1997). B cells are bone marrow derived cells which possess membrane-bound Ab on the surface that act as antigen specific receptors. B cells are not professional phagocytes, but can act as APCs since the membrane-bound Ab can be internalized, allowing it to be processed and presented to T cells in conjunction with MHC class II molecules. The interaction of T cells with antigen bound to B cells activates the T cells and results in the secretion of cytokines such as IL-4, IL-5 and IL-6. The cytokines in-turn induce the activation and proliferation of B cells and the generation of memory B cells. As a result of B cell stimulation, Ab molecules with the same antigen specificity as the membrane bound molecule are expressed in a secreted form. Once secreted, Ab can bind bacterial toxins and prevent their interaction with the appropriate targets. Additionally, specific types of Ab can bind to the surface of microorganisms and activate the complement system, which in-turn leads to the lysis of invading microorganisms. Furthermore, Ab can aid the antimicrobial functions of phagocytic cells (Gulig, 1996).

Naïve B cells that are exposed to antigen initially produce IgM Ab. IgM Ab have multiple antigen binding sites for complexing antigens and play an important role in the primary immune response. During the course of antigen stimulation, or following re-exposure to the same antigen, the type of Ab changes generally from IgM to IgG, a process termed isotype switching. In addition, the affinity of the Ab increases. These events form the secondary response and arise quicker than the primary immune response (Janeway and Travers, 1997).

The role of Ab in the development of immunity against most diseases has been studied extensively and methods for detection have been well documented (Janeway and Travers, 1997). For the immunological evaluation of the vaccines used in this thesis, Ab
induction and characterisation is performed by Western blotting and enzyme-linked immunosorbent assay (ELISA).

4.1.2 Cellular Immunity

During bacterial infection the major effector in cell-mediated immunity is the macrophage, especially where the bacterial pathogen is intracellular (Yrlid et al., 2001; Garcia-del Portillo, 2001; Eckmann and Kagnoff, 2001). Macrophages are regulated by CD4+ T cells via cytokines such as IFN-γ which act to increase their anti-microbial activity (Collins, 1974; Finlay and Falkow, 1989b; Kaufmann, 1993). IFN-γ enhances microbial killing mechanisms of the macrophage by elevating the rate of phagolysosomal fusion, and by increasing the expression of NADPH oxidase (Adams and Hamilton, 1984). In addition, IFN-γ stimulates an increase in the expression of MHC II molecules (Abbas et al., 1991) to enhance their antigen presentation capabilities. Once activated, macrophages release cytokines that activate T cells and stimulate their proliferation, further increasing the efficiency of the immune response.

Until 10 years ago, the immunologic evaluation of vaccines was largely dependant upon the measurement of Ab response in the serum of recipient animals. This approach was applicable for diseases prevented largely by humoral immunity, however it failed to detect the contributions made by cell-mediated immunity in providing protection against many infectious diseases. Salmonella is an intracellular pathogen, and as such requires the induction of both humoral and cellular immune responses for limiting systemic dissemination and promoting clearance following infection (Garmory et al., 2002). In addition to humoral responses, it was therefore necessary to assess the induction of cellular immune responses following vaccination.

The measurement of cell-mediated immunity can be accomplished by several methods (Reviewed Coe Clough and Roth, 1995). One of the first assays of cell-mediated
immune function was the delayed-type hypersensitivity (DTH) assay. Animals previously exposed to a pathogen, either through infection or vaccination, develop a localised swelling 48-72 hours after ID injection with antigens specific to that pathogen. Whilst DTH assays are economical to perform and do not require sophisticated laboratory equipment, they are being replaced by more popular lymphocyte proliferation assays. Cellular proliferation \textit{in vivo} is important for the expansion of reactive lymphocytes that are specific to a foreign antigen. Therefore animals that have developed cell-mediated immunity against a specific antigen have an increased number of lymphocytes that recognise the antigen. In proliferation assays, when lymphocytes that have been harvested from immunised or infected animals are cultured with the antigen of interest, lymphocytes will undergo cell division and secrete cytokines. The amount of proliferation can be assessed by the incorporation of radioactive (tritiated) thymidine, since actively dividing cells are producing DNA and will incorporate the thymidine into the new DNA molecules. However, proliferation assays do not indicate that protective immune responses have been induced since they do not provide any specific details of the effector function. It is therefore important to measure the nature and quantity of the cytokines released from proliferating lymphocytes. To assess the induction of cell-mediated immunity in response to vaccination in this thesis, lymphocyte proliferation assays are performed in conjunction with cytokine analyses by sandwich ELISA.

It is worthy to note that infection by Gram negative bacteria such as \textit{Salmonella}, results in the induction of humoral immunity that is independent of T cells. A large constituent of the bacterial cell wall is LPS, which directly induces B cell proliferation and Ab production through a mechanism not fully understood. Since both B cells and T cells were present in the lymph nodes removed for proliferation analysis, it was important to exclude all membrane proteins from the culture to ensure that only a T cell dependent proliferation occurred.
4.2 Immunological Responses Induced by Intradermal and Intramuscular Immunisation with a DNA Vaccine Encoding EGFP

DNA vaccines can be introduced into a host via injection, or through a number of carrier-mediated methods (Tang et al., 1992; Fynan et al., 1993; Sizemore et al., 1995; Haynes et al., 1996; Spier, 1996; Darji et al., 1997; Chapter 1). The different routes of DNA vaccine administration have been shown to influence the type and size of the immune response (Pertmer et al., 1996; Barry and Johnston, 1997; Donnelly et al., 1997b; Bohm et al., 1998; Kasinrerk et al., 2002). A variety of routes of DNA injection have been studied and include IM, ID, subcutaneous, intravenous, and intraperitoneal (Wolff et al., 1990; Raz et al., 1994; Fynan et al., 1993; Spier, 1996).

Following both IM and ID injection of plasmid DNA, the production of Ab and the activation of both MHC class I-restricted antigen-specific CTLs and MHC class II-restricted CD4+ T cells have been observed (Fynan et al., 1993; Raz et al., 1994; Ulmer et al., 1993; Manickan et al., 1995; Xiang and Ertl, 1995; Davis et al., 1995). In most models of DNA vaccines, there is a mixed Th1 and Th2 response. However, it is more often reported that a Type 1 response predominates, as demonstrated by the production of IFN-γ and IgG2a Ab (Raz et al., 1996; Davis et al., 1996b). IM injection of pure plasmid DNA has been the most studied method of immunisation and has been shown to generate good immune responses in animal models ranging from fish (Anderson et al., 1996) to chimpanzees (Davis et al., 1996a; Boyer et al., 1997). It has been suggested that ID injection is superior to IM injection since it directly transfects dendritic cells, the principle APCs involved in initiating the immune response (Corr et al., 1996; Davis et al., 1997). Indeed, some studies have shown ID immunisation generates protective immune responses where IM injections have failed (Hoffman et al., 1995; Gramzinski et al., 1997; Lodmell et al., 2000), however other investigations contradict this, showing that the ID route is less effective (Donnelly et al., 1997b). Thus neither route is conclusively favoured over the other for all immunisation strategies, with the immune
response varying according to factors such as the nature of the antigen expressed and the species under investigation.

This part of the present study therefore compares the ID and IM routes of injection by analysing the humoral and cellular immune responses generated following immunisation with the pZKC3 DNA vaccine vector encoding the egfp gene. EGFP expression has been confirmed in mammalian fibroblast cells transfected with the pZKC3 vector (see section 3.3.2) and should therefore be expressed in the mouse model. EGFP is a foreign protein to the mice used in this work, and would therefore be expected to generate immune responses following immunisation. The outcome of immunological responses generated from each method of vaccination can be used to determine the most effective administration route for egfp and indicate the best route of immunisation for subsequent DNA immunisations using the expression library. In order to carry out this study, it was firstly necessary to purify EGFP for immunological analysis.

4.2.1 Purification of EGFP for Immunological Analysis

In order to analyse humoral and cellular immune responses, it was necessary to purify the antigen EGFP. E. coli BL21 cells were transformed with the vector pTMB18 (Martin, 2003) and were induced with IPTG. The pTMB18 construct was based on the GST expression vector (pGEX-5X-1), but had the egfp gene from the vector pEGFP (Clontech) cloned in-frame and down stream of the ORF encoding GST. The size of GST-EGFP fusion expressed from the pTMB18 vector was calculated using computer software (Vector NTI version 7.1) to be approximately 59kDa.

Results of the purification process (see Chapter 2) are shown in Figure 4.1. Following induction (Figure 4.1A, Lane I) the cells were lysed and the expressed GST-EGFP fusion was purified by affinity chromatography using the immobilised cofactor glutathione and reduced glutathione (Figure 4.1A, E1-E5; Simons and Vander, 1981).
After the first purification process (Figure 4.1A), the GST-EGFP protein still appeared to be contaminated with several other proteins of approximately 30 kDa in size. The process of affinity chromatography was therefore repeated using the pooled elutes (E1-E2) from the first purification, which had been dialysed against PBS to remove the reduced glutathione. After the second purification process, eluted material (E3-E5) was dialysed against PBS and used for immunological analysis (Figure 4.1B). Whilst the GST-EGFP band at 59 kDa was more concentrated after the second purification, the faint protein bands of 30 kDa were still evident (Figure 4.1B, E3-5). These bands were thought to correspond to breakdown products of EGFP (30 kDa) and GST (29 kDa). To ensure these proteins were actually degradation products rather than contaminating proteins, a Western blot was performed using rabbit anti-GFP Ab. The Western blot (Figure 4.1C), clearly showed that the faint bands around 30 kDa were degradation products of GST-EGFP since they were detected by the anti-GFP Ab.

4.2.2 Immunisation Strategy of BALB/c Mice

BALB/c mice 8-12 weeks old were immunised either IM into each of the tibialis muscles, or ID into the lumbar region with 100μg of plasmid DNA suspended in 100μl of PBS (described in Chapter 2). A total of 25 mice were divided equally into 5 groups and each group was immunised with one of the following treatments: 100μg of plasmid pZKC3 ID (I.D pZKC3), 100μg plasmid pcDNA3 ID (I.D pcDNA3), 100μg plasmid pZKC3 IM (I.M pZKC3), 100μg plasmid pcDNA3 IM (I.M pcDNA3), and mice receiving no treatment (NT).
Figure 4.1 Purification of GST-EGFP, Coomassie stained protein gels and Western blot analysis. Figures indicate the 70SL molecular weight marker. Panel A: first purification process, whole cell lysate (WC), induced cell lysate (I), flow through (F), washes (W1, W2) and elutions (E1, E2). Panel B: second purification process, elutions from first purification process (E1-2), flow through (F) washes (W1 and W2) and final elutions (E3-E5). Panel C: Western blot of protein gel in panel B, using rabbit anti-GFP Ab.
Published literature commonly describes administering DNA vaccines in two or three separate doses (Spier, 1996). However, immunisation may fail when few injections are made, possibly due to the loss of DNA as a result of degradation by endonucleases found in the extracellular environment. To minimise failure, mice were immunised four times at two-weekly intervals. Blood samples were collected on days 7 and 12 post final boost for analysis by Western blot and ELISA. On day 56, groups of mice were then sacrificed for T cell proliferation analysis and cytokine production analysis.

4.2.3 Western Blot Analysis of Pooled Serum from Mice Immunised with a DNA Vaccine Encoding EGFP

Humoral immunity was analysed by detecting the presence of specific Ab. The two main methods used to characterise Ab responses to DNA immunisation in this work were Western blotting and enzyme-linked immunosorbent assay (ELISA). Western blotting determines the molecular mass of proteins detected by an Ab and ELISA determines the concentration, specificity and type of Ab produced (Coligan 1991; Janeway and Travers, 1997).

To determine whether DNA immunisation with the expression vector pZKC3 encoding EGFP was able to induce specific EGFP Ab production, Western blot analyses using pooled sera were performed for the 5 groups of immunised mice. A SDS-polyacrylamide gel (see Figure 4.2A) was loaded with BSA as a negative control and GST-EGFP. The gel was fixed with Coomassie Blue stain so that proteins electrophoresed on the gel could be visualised. Equivalent gels (see Figure 4.2B-F) were also electrophoresed, but were instead blotted on to nitrocellulose membrane. The membranes were then incubated with 1: 100 dilutions of the different pooled sera taken 12 days following the final immunisation.
Figure 4.2 Determination of the presence of EGFP-specific Ab in pooled sera taken from 5 groups of mice. Panel A shows a Coomassie stained protein gel whilst Panels B-F are equivalent Western Blots. Each gel was loaded with 10μg of BSA (Lane B), 5μg of EGFP (Lane E1) and 10μg of EGFP (Lane E2). Molecular weight markers (Lane W) of 66, 45, 36, 29, 24, 20.1 and 14.2 kDa were included on the gel in Panel A and molecular weight markers of 80, 60, 50, 40, 30 and 20 kDa were included on the gels in Panels B-F. Mice were immunised either ID with pZKC3 (Panel B) or pcDNA3 (Panel C) or IM with pZKC3 (Panel D) or pcDNA3 (Panel E), or received NT (Panel F). For each group, equal aliquots of serum were pooled from this assay.
The pooled sera from groups immunised either ID (Figure 4.2B) or IM (Figure 4.2D) with the vector pZKC3 encoding EGFP, show clear bands at 59 kDa and further bands at 30 kDa. Bands at 30 kDa have been observed previously during the purification of the GST-EGFP fusion (see section 4.1.1), and are due to the degradation of GST-EGFP into its constituent parts of 29 kDa and 30 kDa respectively. Figure 4.2C, E and F correspond to Western blots performed using the sera taken from mice immunised ID and IM with pcDNA3 (no insert controls) and mice receiving NT respectively. Since no specific EGFP Ab has been detected in these groups, it can be concluded that the specific EGFP Ab evident in the sera of mice immunised ID and IM with pZKC3 was due to successful DNA immunisation.

Interestingly, the bands in Figure 4.2B appear more intense than bands in Figure 4.2D. Since all Western blots were exposed to film for the same length of time, the stronger bands may imply the presence of more Ab in sera of mice receiving ID immunisation compared to IM immunisation, suggesting that ID immunisation is the more effective route. However, the relative concentrations and affinities of Ab present in the serum can only be analysed accurately by ELISA.

4.2.4 Analysis of EGFP-Specific Antibody by ELISA

To quantitatively assess the concentrations of EGFP-specific Ab in pooled sera and identify whether ID or IM injection was the more effective route of immunisation, ELISAs were performed for each group of mice. In addition, the type of humoral response was analysed, since a predominant Th1 response is more often reported in models of DNA immunisation than a Th2 response (Raz et al., 1996). Initially ELISAs were performed using pooled serum taken 12 days after the fourth immunisation to establish, by titration, an appropriate dilution for comparison between mouse groups. The chosen dilution was then used to compare the quantity and type of Ab in the individual mouse serum.
The results obtained from the ELISAs performed on pooled sera (see Figure 4.3), showed that there was substantially more EGFP-specific IgG Ab from mice immunised IM and ID with pZKC3 compared with mice injected with pcDNA3, and mice which received NT (see Figure 4.3A). This agrees with results of the Western blot analysis described in the previous section.

Results in Figure 4.3B, show there was very little EGFP-specific IgG1 Ab present in pooled sera taken from mice immunised both IM and ID with pZKC3. Additionally, there was no difference in the amount of EGFP-specific IgG1 Ab detected in the sera of mice immunised ID with pZKC3 compared to mice immunised ID with pcDNA3 or mice which received NT. In contrast to the levels of EGFP-specific IgG1, the amount of EGFP-specific IgG2a Ab detected in pooled serum samples was substantially higher for groups of mice receiving pZKC3 both IM and ID compared to those receiving either pcDNA3 IM or ID, and mice receiving NT. Moreover, the presence of IgG2a and the absence of IgG1 implies that there was a bias towards a Type 1 response (O’ Garra, 1998; Liew, 2002).

The combined ELISA results obtained using the pooled serum samples were analysed to obtain the dilution of serum that would provide the best discrimination between groups. A dilution of 1:32 (2^5) was therefore chosen for individual serum analyses. ELISAs were performed using individual serum taken from each mouse in each group and the results are summarised in Table 4.1.
Figure 4.3 Titration curves of EGFP-specific IgG (Panel A), IgG1 (Panel B) and IgG2a (Panel C) Ab. Pooled sera was collected from mice previously immunised either ID with pZKC3, or pcDNA3, or IM with pZKC3, or pcDNA3, and mice receiving NT (NT).
<table>
<thead>
<tr>
<th>Vaccine Treatment</th>
<th>Mean OD at 490nm ± SEM</th>
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<tr>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>pZKC3 ID</td>
<td>0.62 ± 0.06</td>
</tr>
<tr>
<td>pcDNA3 ID</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>pZKC3 IM</td>
<td>0.57 ± 0.08</td>
</tr>
<tr>
<td>pcDNA3 IM</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>NT</td>
<td>0.17 ± 0.04</td>
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Table 4.1 Ab levels detected in individual mice immunised ID with pZKC3 or pcDNA3, IM with pZKC3 or pcDNA3 and mice receiving NT. Values indicate the mean Optical Density (OD) at a 1: 32 dilution of serum for 4 or 5 mice in each group ± Standard Error of the Mean (SEM).

Statistical comparison of Ab levels were made by comparing mean OD values of mice receiving pZKC3 ID with mice receiving pcDNA3 ID by the Mann Whitney test. Similarly, mice receiving pZKC3 IM were compared to mice receiving pcDNA3 IM. The NT group functioned as an internal control for the entire investigation.

From the results (see Figure 4.4), it can be seen that mice immunised ID with pZKC3 had significantly more EGFP-specific IgG than the corresponding mice immunised ID with pcDNA3 (P = 0.02). Mice immunised ID with pZKC3 also had significantly more IgG2a than mice immunised ID with pcDNA3 (P = 0.02). There was however no significant difference between the amount of EGFP-specific IgG1 Ab between these two groups (P = 0.27). This confirms the induction of a Th1 response following ID immunisation with pZKC3.
The results in Figure 4.5 show that mice immunised IM with pZKC3 had significantly more EGFP-specific IgG than mice immunised IM with pcDNA3 \((P = 0.02)\) and similarly had significantly more IgG2a \((P = 0.02)\) than mice immunised IM with pcDNA3. Again, there was no significant difference between the amount of EGFP-specific IgG1 Ab detected in mice immunised IM with pZKC3 and mice immunised IM with pcDNA3 \((P = 0.67)\).
Figure 4.5 EGFP-specific IgG, IgG1 and IgG2a Ab levels detected following IM immunisation with pZKC3 or pcDNA3. Graph shows the OD values at a 1:32 dilution for 4 mice previously immunised IM with pZKC3 and 5 mice previously immunised with pcDNA3. Horizontal lines indicate the mean OD for each group.

Comparing the two routes of immunisation, the results demonstrate that there is no significant difference between levels of EGFP-specific IgG or IgG2a induced following immunisation with pZKC3 IM or ID (P = 0.77 and P = 1.00 respectively). Both routes of administration are therefore equally effective at inducing humoral responses in terms of Ab levels and isotype proportions following DNA immunisation.

The results from the Western blot and ELISA analyses demonstrate that EGFP is immunogenic and is capable of inducing humoral immunity when delivered in the form of a DNA vaccine both IM and ID. The OD values for EGFP-specific IgG2a Ab were 3.6 times and 3.4 times greater in the sera of mice immunised ID with pZKC3 and IM with pZKC3 respectively, compared to the background levels of IgG2a in the corresponding mice immunised with pcDNA3. The OD values for EGFP-specific IgG1 Ab were 1.4 times and 1.2 times greater in the sera of mice immunised ID with pZKC3
and IM with pZKC3 respectively, again compared to background levels. The greater increases in IgG2a Ab compared to IgG1 Ab confirms that the elicited response is biased towards a Th1 response. To verify the Th1 bias, cellular responses were investigated.

4.2.5 *In vitro* Analysis of T cell Responses to GST-EGFP Following DNA Immunisation

Cell-mediated immunity can be investigated by examining the number of T cells able to respond to antigen and their functional properties. The induction of specific cellular immune responses following DNA immunisation was assessed by analysing antigen-specific T cell proliferation. T cells taken from mice that have been immunised with antigen proliferate when they are exposed to that same antigen and APCs. T cells however do not proliferate when exposed to unrelated antigens to which they have not been immunised. The extent of proliferation was measured by the incorporation of $^3$H-thymidine into the DNA of actively dividing cells. Any antigen-specific proliferation was an indicator of specific CD4$^+$ T cell immunity.

Vaccinated mice and mice receiving NT were sacrificed 2 weeks after the fourth immunisation, and draining lymph nodes were collected and LNCs were cultured with cell culture medium (negative control), GST-EGFP (antigen), and Con A (positive control) as described in Chapter 2. Due to the magnitude of T cell proliferation when LNCs were incubated with Con A, proliferation data for Con A was not included in graphical form in Figure 4.6 but is shown in Table 4.2.

LNCs from mice immunised ID with pZKC3, and to a lesser extent, mice immunised IM with pZKC3 proliferated substantially when incubated with GST-EGFP (see Figure 4.6). However, mice immunised ID with pcDNA3, and again to a lesser extent, mice immunised IM with pcDNA3 also proliferated when incubated with this antigen.
Table 4.2 T cell proliferation results from mice immunised ID and IM with pZKC3 and pcDNA3, and mice receiving NT. Values indicate the means for 4 mice in each group ± SEM and the stimulation index (SI) ± SEM.

<table>
<thead>
<tr>
<th>Vaccine Treatment</th>
<th>T Cell Proliferation</th>
<th></th>
<th>SI ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average cpm x 10^3 ± SEM</td>
<td>GST-EGFP/Medium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>GST-EGFP</td>
<td>Con A</td>
</tr>
<tr>
<td>pZKC3 ID</td>
<td>0.56 ± 0.04</td>
<td>7.97 ± 0.34</td>
<td>57.17 ± 5.83</td>
</tr>
<tr>
<td>pcDNA3 ID</td>
<td>0.38 ± 0.01</td>
<td>3.22 ± 0.22</td>
<td>56.22 ± 3.15</td>
</tr>
<tr>
<td>pZKC3 IM</td>
<td>0.36 ± 0.07</td>
<td>5.60 ± 0.30</td>
<td>50.81 ± 9.52</td>
</tr>
<tr>
<td>pcDNA3 IM</td>
<td>0.32 ± 0.05</td>
<td>1.07 ± 0.15</td>
<td>52.06 ± 5.76</td>
</tr>
<tr>
<td>NT</td>
<td>0.25 ± 0.06</td>
<td>1.08 ± 0.05</td>
<td>65.21 ± 6.92</td>
</tr>
</tbody>
</table>

For each group of mice immunised or receiving NT, the proliferation of LNCs incubated with GST-EGFP was significantly different to the proliferation following incubation with medium alone ($P < 0.01$). This implies that a lymphocyte mitogen other than GST-EGFP was present within the antigen solution, causing a non-specific proliferation of LNCs from groups of mice receiving pcDNA3 and NT. One possibility was that LPS may have been present in the antigen solution since the GST-EGFP was purified from *E. coli* and LPS is a known B cell mitogen. However, repeated experiments using polymixin to inactivate LPS showed little reduction in the extent of the proliferative response, indicating that LPS was not contaminating the GST-EGFP antigen. In an attempt to characterise the nature of the proliferation in the GST-EGFP-stimulated cultures, cytokine analyses were performed (see section 4.1.4).
Figure 4.6 Proliferative responses of LNCs from mice immunised ID and IM with pZKC3, pcDNA3 and mice receiving NT. LNCs were incubated with either medium alone or GST-EGFP antigen. Graph shows the mean proliferation for 4 mice ± SEM.

To determine if there was a significant difference in the extent of proliferation between mice immunised IM and ID with pZKC3, and mice immunised IM and ID with pcDNA3, stimulation indices were calculated (see Figure 4.7). The stimulation index (SI) is the mean incorporation of $^3$H thymidine into cells stimulated with antigen divided by the mean incorporation in the absence of antigenic stimulation.

The SI for mice immunised ID and IM with pZKC3 is significantly greater than the SI for their respective pcDNA3 controls ($P = 0.03$ for both), indicating that immunisation with pZKC3 had induced a specific T cell response.
A significant increase in T cell proliferation was observed for mice immunised ID with pcDNA3 compared to the control group which received NT ($P = 0.03$) and was thought to be caused by non-specific proliferation. However, comparable groups of mice immunised IM showed no significant difference in the extent of proliferation ($P = 0.312$). This would suggest that the immunisation route affected the extent of the non-specific proliferation, or that there was some undefined ‘cage effect’.

4.2.6 Analysis of Cytokines Produced from GST-EGFP Stimulated T cells

CD4$^+$ T cell functions can be studied by measuring the type and amount of cytokines released following activation. Since different CD4$^+$ T cells release different types of cytokines, analysis of the cytokines produced following antigen proliferation can be used to determine the nature of the cell-mediated immune response, for example a Th1
or Th2 response. The method used in this thesis to detect cytokine production is a modification of ELISA termed sandwich ELISA. To determine the nature of the CD4+ T cell response to GST-EGFP in mice immunised by ID and IM injection with pZKC3, IFN-γ and IL-4 cytokine concentrations were analysed by sandwich ELISA. The production of IFN-γ indicates a Type 1 response, with a concomitant bias towards cell-mediated immunity, whilst the production of IL-4 indicates a Type 2 response. Concentrations of IFN-γ and IL-4 were calculated from standard curves produced by recombinant IFN-γ and IL-4.

![Figure 4.8 IFN-γ concentrations from T cell cultures stimulated with medium, GST-EGFP and Con A following ID or IM immunisation with pZKC3 or pcDNA3, or from mice receiving NT. IFN-γ values graphed are the means calculated for four mice in each group ± SEM.](image)

Analysis of the IFN-γ concentrations (see Figure 4.8) indicates that the T cells from mice immunised ID and IM with pZKC3 produced between 4 and 5 U/ml of IFN-γ when stimulated with GST-EGFP antigen. In contrast, no IFN-γ was detected for mice immunised ID or IM with pcDNA3. A small amount of IFN-γ was detected in antigen stimulated T cell cultures from mice receiving NT, but this level was not significantly
different from that seen with the T cell culture which was exposed to medium alone and can therefore be considered as background ($P > 0.5$). Since Con A is a T cell mitogen, a high level of IFN-γ production was expected, providing a positive control for the ELISA.

The results in Figure 4.9 show no IL-4 production in any of the T cell cultures stimulated with GST-EGFP antigen. Since Con A induced significant IL-4 secretion, the overall results suggest a bias towards a Th1 phenotype following immunisation with pZKC3. This agrees with the previous Ab isotype analysis.

The cytokine results also demonstrate that the obscure proliferative responses of mice immunised ID and IM with pcDNA3 are not the result of a T cell proliferative response,
since neither IFN-γ or IL-4 have been detected for these groups. The proliferative response must therefore be due to B cell proliferation, however the causative factor is unknown. Due to the non-specific proliferation observed in the T cell assays, it was not possible to conclude whether ID or IM immunisation was the most efficient route of DNA vaccine administration. Conclusions were particularly difficult to draw since there were no significant differences between the quantities of IFN-γ detected in the supernatants of LNCs taken from mice immunised ID with pZKC3 and those immunised IM with pZKC3 ($P > 0.99$). The results suggest that ID and IM routes of DNA immunisation are equally as effective at inducing cellular immune responses to EGFP encoded on the immunising plasmid. Further experiments were therefore required to determine the most effective route of DNA immunisation.
4.3 Comparison of Immunological Responses Induced by Intramuscular and Intradermal Immunisation with a S. typhimurium SL1344 Expression Library

The technique of expression library immunisation (ELI), as described in Chapter 1, was first applied to protect mice against *Mycoplasma pulmonis* by vaccination with either an entire DNA expression library consisting of 27,000 clones, or smaller sub-libraries each consisting of 3,000 clones (Barry *et al.*, 1995). Due to the sensitivity of ELI (see Chapter 1), only libraries with a complexity of up to 27,000 can be used efficiently to elicit a protective immune response, with smaller sub-libraries consisting of 3,000 clones generating stronger immune responses (Johnston and Barry, 1997). To ensure the sensitivity of ELI was not compromised for the work described in this section, only 7 sub-libraries, each consisting of 2,000 clones were used for immunisation, rather than the entire *S. typhimurium* SL1344 expression library. The complexity of the expression library used in this work was therefore 14,000 and is based on DNA from the pathogenic isolate SL1344 (Hoiseth and Stocker, 1981).

Whilst the technique of ELI can be used to screen the entire genome of a pathogen, ultimately leading to the identification of individual antigens for use in a vaccine, this goes beyond the scope of the work in this thesis. Instead the *S. typhimurium* SL1344 expression library (EL) was used to immunise mice so that humoral and cellular responses induced following DNA immunisation with multiple antigens could be analysed, and the feasibility of developing ELI for identifying individual antigens of *S. typhimurium* SL1344 could be assessed. Since previous work in section 4.1 showed that little difference existed between the immune responses generated following IM or ID immunisation, the work in this section has continued to examine the humoral and cellular responses generated following both immunisation approaches. All plasmid constructs constituting the EL contained the *egfp* gene, so in order to carry out this study it was firstly necessary to purify EGFP for immunological analysis.
4.3.1 Purification of EGFP for Immunological Analysis

The initial purification of EGFP for immunological analysis was performed by inducing GST-EGFP production in *E. coli* BL21 cells that had previously been transformed with the vector pTMB18. As previously stated, the pTMB18 construct is based on the GST expression vector pGEX-5X-1. The pGEX-5X-1 vector allows the expression of proteins, such as EGFP, as fusion proteins to GST. The *tac* promoter present on this vector is a strong promoter and can produce between 15-30% of the total cell protein after induction with IPTG (Baneyx, 1999). Expression from strong promoters can increase the tendency for recombinant proteins to aggregate (Cheng *et al*., 1981) and cytoplasmic GST fusion proteins have been reported to form inclusion bodies (Guan and Dixon, 1991; Cartwright *et al*., 1995). Indeed it was observed from previous purification attempts that GST-EGFP was forming inclusion bodies. Inclusion body formation has the advantage of protecting the recombinant protein against proteolysis (Enfors 1992), but increases the time and expense required to re-solubilise the recombinant protein. Strong denaturants such as guanidine hydrochloride and urea can be used to re-solubilise the aggregated protein but are disadvantageous in that they can chemically modify the recombinant protein (Murby *et al*., 1996).

Due to the difficulty in purifying sufficient quantities of GST-EGFP, additional EGFP antigen was therefore purified from *E. coli* BL21 that had been transformed with the expression vector pZKC3. The pZKC3 expression vector encodes the *egfp* gene fused to a HIS tag (see Chapter 3). Following induction with IPTG, EGFP-HIS was purified from *E. coli* BL21 cells by affinity chromatography using a nickel column.

The calculated size of EGFP-His is 34 kDa (Vector NTI version 7.1), and a band of this size can be seen in both the induced cell lysate (Figure 4.10A, Lane I) and the purified sample (Figure 4.10A, Lane E).
Figure 4.10 Coomassie stained protein gel showing the purification of EGFP-His (Panel A) and corresponding Western blot analysis (Panel B). Western blots were performed using rabbit anti-GFP Ab at a 1:3,000 dilution and were detected by chemiluminescence. Lanes correspond to un-induced whole cell lysate (WC), induced cell lysate (I), purified EGFP (E) and molecular weight marker (W).

A second gel with an identical loading pattern was examined by Western blot analysis (Figure 4.10B). Only bands at the expected size of 34 kDa were detected, despite the fact that samples contained additional contaminating proteins. Since the EGFP-His antigen was only for use in Western blot analyses, a higher level of purity was not considered necessary.

4.3.2 Immunisation Strategy of BALB/c Mice

BALB/c mice 8-12 weeks old were immunised either IM into the tibialis muscles, or ID into the lumbar region with plasmid DNA suspended in 100μl PBS (see section 2.2.6.1 and 2.2.6.2). A total of 32 mice were allocated into 4 equal groups and immunised IM with one of the following treatments: 100μg of *S. typhimurium* SL1344 EL (EL IM),
100μg plasmid pZKC3 (pZKC3 IM), 100μg plasmid pcDNA3 (pcDNA3 IM), and mice receiving NT (NT IM). A total of 20 mice were divided equally into 4 groups and immunised ID as follows: 100μg of *S. typhimurium* SL1344 expression library (EL ID), 100μg plasmid pZKC3 (pZKC3 ID), 100μg plasmid pcDNA3 (pcDNA3 ID), and mice receiving NT (NT ID). For mice injected IM, the treatment was given three times at two weekly intervals, whilst mice injected ID were immunised four times at two weekly intervals. Blood samples were collected on days 7 and 12 following the final immunisation for analysis by Western blotting and ELISA. Following a two-week period after the final immunisation, groups of mice were sacrificed for T cell proliferation analysis and cytokine analysis.

### 4.3.3 Western Blot Analysis of Pooled Serum from Mice Immunised with an EL

To determine whether IM DNA immunisation with a fraction of an *S. typhimurium* SL1344 expression library was able to induce specific Ab production, Western blot analyses were performed. Immunising with an EL is unbiased to membrane antigen (MA), so in order to observe if immunisation against MA had occurred, MA was included in the Western blot analyses.

A SDS-polyacrylamide gel (see Figure 4.11) was loaded with 10μg of BSA (Lane B), 100ng of GroEL (Lane G), 10μg of EGFP-His (Lane E), 12μg cytosolic antigen (CA), and 12μg membrane antigen (MA), and subjected to electrophoresis. Subsequently, the gel was stained with Coomassie Brilliant Blue in order to visualise the proteins in the gel (Figure 4.11A). Equivalent gels were blotted onto nitrocellulose membrane, then incubated with 1:200 dilutions of pooled sera, taken from groups of mice 12 days following the final immunisation (see Figure 4.11B-E).

IM immunisation with the EL generated strong antibodies to EGFP encoded within the expression vector (see Figure 4.11). This indicates that immunisation to the DNA
encoded in the vector had occurred and that EGFP fusions were being expressed in mice immunised with the EL. The Western blots also show two closely separated bands of cytosolic antigen (CA) around 66 kDa and several MA bands of approximately 65, 45, 36 and 27 kDa that are recognised by Ab in the pooled sera from mice immunised IM with the EL. The presence of EGFP specific Ab in the sera of mice immunised with pZKC3 also confirms EGFP is immunogenic and that the expression vector is functional.

The two bands of CA detected by specific Ab present in pooled sera from mice immunised with the EL, are also detected by CA specific Ab that were present in the serum from mice immunised with pZKC3. In addition, the pooled serum taken from mice immunised with pZKC3 appeared to contain GroEL specific Ab, as demonstrated by the detection of GroEL (see Figure 4.11C).

The nature of any previous immunological exposure to GroEL is uncertain, although the most likely explanation is that mice within this group have previously encountered a homologue of *S. typhimurium* SL1344 GroEL. The mice used in this work were not germ free, and would therefore have had large numbers of microorganisms colonising both their external and internal surfaces including the skin and mucosal lining of the intestinal tract. Many of these organisms are likely to contain homologues of GroEL, since it is a highly conserved protein and almost universal in its distribution. In fact, GroEL in *S. typhimurium* SL1344 has a 98% identity to the equivalent molecule in *E. coli*. Since *E. coli* forms a predominant part of the normal gut flora, and GroEL is an abundant cytosolic protein, it is quite possible that immunological memory to GroEL was provided by frequent cross-reactive re-stimulation, as postulated by Beverley (1990). Alternatively, *E. coli* may have been present on the skin of mice and have gained entry during the immunisation process, establishing an immunological response to GroEL and other proteins.
Another explanation for pre-existing immune responses to the *S. typhimurium* SL1344 antigen GroEL is that immunological stimulation by cross-reacting proteins in the mouse may have occurred. GroEL is highly conserved in both eukaryotes and prokaryotes and has been implicated as a causative agent in a number of autoimmune diseases. It is therefore possible that the humoral response to the *S. typhimurium* SL1344 GroEL protein has been generated from immune recognition of the mouse mitochondrial GroEL homologue.

The presence of GroEL-specific Ab in mice immunised with pZKC3 suggests that GroEL present in the CA should also be detected by Western blot, since GroEL is an abundant protein found in the cytosol of *S. typhimurium* SL1344. This would account for the CA band of approximately 65 kDa that is only detected by sera taken from mice immunised with pZKC3.

Whilst there is a modest Ab recognition of MA at approximately 38 kDa in all groups of pooled sera, the band is not as intense as that observed in mice immunised with the EL. In addition, the bands of MA at approximately 65 and 27 kDa are only very weakly recognised in mice receiving pZKC3 or NT. These background levels of Ab could however be explained by the frequent cross-reactive re-stimulation from normal organisms that colonise the intestinal tract or by the immunisation process, as discussed previously.

Overall, the Western blots indicate that IM immunisation with the EL induces Ab production to both CA and MA, since a greater number of protein bands are recognised, and the intensity of these bands is far greater, compared to the background levels observed in other groups.
Figure 4.11 Determination of the presence of BSA (B), GroEL (G), EGFP (E), cytosolic antigen (CA), or membrane antigen (MA) specific Ab in the serum of mice previously immunised IM with a fraction of a S. typhimurium SL1344 expression library (Panel B), pZKC3 (Panel C), pcDNA3 (Panel D), or from mice receiving NT (Panel E). The figure shows a Coomassie stained protein gel (Panel A) and Western blots analyses (Panels B-E). Numbers show the positions of molecular weight markers.
The Western blot results also confirm the presence of EGFP-specific Ab in the serum of mice immunised ID with the EL and verifies that immunisation has occurred (see Figure 4.12, Panel B). It is evident from comparing Western blots that the EGFP band detected by mice immunised ID is less intense than the EGFP band detected by mice immunised IM with the EL (see Figure 4.11). However, it is difficult to compare the amount of EGFP specific Ab directly since the EGFP antigen in lane E on each Western blot is of a different nature. In addition, Western blotting cannot be compared quantitatively between blots because factors such as the development time and exposure time may vary.

As observed for mice immunised IM, mice immunised ID with the EL possessed Ab against a number of cytosolic and membrane proteins which were only weakly detected, if at all, in all other groups of mice. This would suggest that ID immunisation with the EL has induced some level of Ab production to both cytosolic and membrane proteins. Again, a protein band of approximately 38 kDa is detected in both the CA and MA for all groups of mice (see Figure 4.12B-E, Lanes CA and MA). This would suggest that the immunising strategy could be enhancing the response of cross-reacting Ab that may have been present before any immunisations. Since pre-bleeds were not taken for these experiments, comparisons can only be made to the control group of mice receiving NT. As there is a background level of Ab that recognises both CA and MA in the control NT group, this suggests that cross-reactive Ab were present in the sera of all groups of mice before immunisation.

Despite the presence of cross-reacting Ab in all groups of mice, there would appear to be a modest increase in specific Ab above this background in mice immunised with the EL compared to all other groups.
Figure 4.12 Determination of the presence of BSA (B), GroEL (G), GST-EGFP (E), cytosolic antigen (CA), or membrane antigen (MA) specific Ab in the serum of mice previously immunised ID with a fraction of a S. typhimurium SL1344 expression library (Panel B), pZKC3 (Panel C), pcDNA3 (Panel D), or from mice receiving NT (Panel E). The figure shows a Coomassie stained protein gel (Panel A) and Western blots analyses (Panels B-E). Numbers show the positions of molecular weight markers.
4.3.4 Analysis of CA-Specific Antibody from Mice Immunised with an EL by ELISA

To assess the relative concentrations of CA-specific Ab in pooled sera and compare IM and ID routes of immunisation, ELISAs were performed for each group of mice immunised or receiving NT. Initially ELISAs were carried out using pooled serum taken 12 days following the final immunisation to establish an appropriate dilution for comparing Ab levels between different mouse groups (see Figures 4.15 and 4.16). This dilution was then used to compare the quantity and type of Ab present in the serum of each individual mouse. The dilution chosen for comparison was similarly 1:32 and the distribution of individual ODs for each group are summarised in Table 4.3. Using this data, attempts were made to assess which route of immunisation was the most effective at inducing humoral responses in BALB/c mice.

From the titration curves for the pooled sera of IM immunised mice (see Figure 4.13), it is evident that the group immunised IM with pZKC3 generated CA-specific IgG, IgG1 and IgG2a Ab. The most likely explanation for this aberrant result is that Ab specific for GroEL, which was detected in the Western blots of IM immunised mice, was also binding to GroEL within the mixture of CA. It is also evident from the titration curves for IgG1 (see Figures 4.13 and 4.14) and the results in Table 4.3, that JIM immunisation with the EL induced higher levels of CA specific IgG1 than ID immunisation with the same EL. Whilst this could be the consequence of the route of immunisation, it is most likely to be due to the fact that only 3 immunisations were performed IM, compared to 4 immunisations performed ID. In the case of the IM immunisation, the immune system would have had a shorter time period in which a bias in the direction of the induced immune response could have been established before the final bleed. Furthermore, by administering fewer IM injections, the innate immune system would have been exposed to less of the Th1 stimulatory sequences (CpG motifs) found in the DNA plasmid backbone so reducing the Th1 bias (Thalhammer et al., 2001).
Figure 4.13 Titration curves of CA-specific IgG (Panel A), IgG1 (Panel B) and IgG2a (Panel C) Ab. Pooled sera was collected from mice previously immunised IM 3 times with either the EL, pZKC3, or pcDNA3, and from mice receiving NT.
Figure 4.14 Titration curves of CA-specific IgG (Panel A), IgG1 (Panel B) and IgG2a (Panel C) Ab. Pooled sera was collected from mice previously immunised ID 4 times with either the EL, pZKC3, or pcDNA3, and from mice receiving NT.
Table 4.3 Ab levels detected in individual mice immunised IM or ID with either the EL, pZKC3, pcDNA3 or mice receiving NT. Values indicate the mean Optical Density (OD) at a 1: 32 dilution of serum for 6 mice in each group immunised IM ± Standard Error of the Mean (SEM), and 5 mice in each group immunised ID ± SEM.

<table>
<thead>
<tr>
<th>Vaccine Treatment</th>
<th>Mean OD at 490nm ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>EL IM</td>
<td>0.271 ± 0.011</td>
</tr>
<tr>
<td>pZKC3 IM</td>
<td>0.202 ± 0.009</td>
</tr>
<tr>
<td>pcDNA3 IM</td>
<td>0.092 ± 0.007</td>
</tr>
<tr>
<td>NT</td>
<td>0.091 ± 0.007</td>
</tr>
<tr>
<td>EL ID</td>
<td>0.266 ± 0.015</td>
</tr>
<tr>
<td>pZKC3 ID</td>
<td>0.092 ± 0.004</td>
</tr>
<tr>
<td>pcDNA3 ID</td>
<td>0.092 ± 0.006</td>
</tr>
<tr>
<td>NT</td>
<td>0.092 ± 0.006</td>
</tr>
</tbody>
</table>

As shown in Figure 4.15, mice immunised IM with the EL induced modest levels of CA-specific IgG and IgG2a Ab. However, the level of CA specific IgG was significantly higher in the sera of mice immunised with the EL compared to mice immunised with pZKC3, ($P = 0.01$) despite the aberrant OD results for pZKC3 immunised mice. Similarly, there was a significant increase in the level of CA specific IgG2a Ab in mice immunised with the EL compared to mice immunised with pZKC3 ($P = 0.01$). There was not however a significant increase in the levels of CA specific IgG1 Ab between these two groups ($P = 0.144$). Taking this into consideration, and the fact that there was almost a 2-fold increase in IgG2a specific Ab compared to no increase in IgG1 specific Ab, these results indicate the induction of a weak Th1 response.
The results in Figure 4.18 show that ID immunisation with the EL induced weak IgG and IgG2a specific Ab responses against CA. Despite the modest levels of Ab detected, there was a significant increase in total IgG and IgG2a between samples taken from mice immunised with the EL and mice immunised with pZKC3 ($P = 0.012$ for both). There was however, no significant difference in IgG1 Ab levels between these two groups ($P = 0.677$).

Since there was a significant 4-fold increase in specific IgG2a Ab and no significant increase in specific IgG1 Ab, these results imply ID immunisation with the EL (similar to IM immunisation) preferentially induced a Th1 response. This is also consistent with the results in section 4.1 whereby immunisation with DNA encoding EGFP preferentially induced a Th1 response.
Comparing the two routes of immunisation, it is clear that both IM and ID immunisation induced significant IgG and IgG2a Ab against unspecified antigens in the CA preparation compared to the control group pZKC3 and are both effective at inducing humoral responses. Whilst the levels of Ab induced were very modest for both routes of immunisation, it was evident that ID injection with the EL generated the greatest increase in CA-specific IgG and IgG2a Ab above the pZKC3 control compared to the equivalent IM injections. However, the reason for this was largely due to the presence of cross-reacting Ab found in the serum of mice immunised IM with pZKC3, which resulted in an increase in the control OD values and therefore minimised the difference between these mice and mice immunised IM with the EL. Despite this however, there was a significant difference in the levels of CA specific IgG2a detected in the sera of mice immunised IM with the EL and mice immunised ID with the EL ($P=0.0081$). There was however no significant difference in the levels of CA-specific IgG Ab in the
sera of mice immunised IM with EL and mice immunised ID with the EL \( (P = 0.784) \). This may indicate that ID immunisation is more effective than IM immunisation at inducing Type 1 humoral responses, although this is may be due to the consequence of immunising four times ID with the EL compared to three IM immunisations.

4.3.5 *In vitro* Analysis of T cell Responses to CA Following Immunisation with an EL

To determine whether cellular responses were induced following immunisation with a *S. typhimurium* SL1344 EL encoding multiple antigens, proliferative responses of T cells were analysed and cytokine production was investigated *in vitro*. Mice vaccinated with either the EL, pZKC3 or pcDNA3 and mice receiving NT were sacrificed 2 weeks after the final immunisation, and draining LNCs were collected and cultured with cell culture medium (negative control), CA, and Con A (positive control). Due to the magnitude of T cell proliferation when LNCs were incubated with Con A, proliferation data for Con A was not included in graphical form but is shown in tabular form (see Table 4.4).

The results in Table 4.4 highlight the variable nature of the proliferative response and demonstrate the need for the negative and positive controls within each group of mice. For example, mice immunised with the EL generated a lower level of proliferation when incubated with CA compared to groups immunised with the control pZKC3 and pcDNA3. However, comparing the level of proliferation following incubation with CA to incubation with medium alone (negative control), it is evident that only mice immunised with the EL generated a proliferative response that was double that of the negative control response.

LNCs taken from mice immunised IM with EL produced a significantly stronger specific T cell proliferation when incubated with cytosolic antigen compared to LNCs incubated with medium \( (P < 0.01) \). No significant difference in proliferation was
observed between cells incubated with cytosolic antigen and medium for mice immunised with pZKC3, pcDNA3 or mice receiving NT ($P > 0.1$). This was slightly surprising regarding mice immunised with pZKC3 since this group of mice appeared to have GroEL-specific Ab according to the ELISA results, and would therefore have been expected to have generated a small proliferative response to the GroEL present in the CA used in this T cell assay.

<table>
<thead>
<tr>
<th>Vaccine Treatment</th>
<th>T Cell Proliferation</th>
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<tbody>
<tr>
<td></td>
<td>Average cpm x $10^3$ ± SEM</td>
<td>SI ± SEM</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>CA</td>
</tr>
<tr>
<td>EL IM</td>
<td>0.10 ± 0.01</td>
<td>0.214 ± 0.04</td>
</tr>
<tr>
<td>pZKC3 IM</td>
<td>0.42 ± 0.22</td>
<td>0.426 ± 0.11</td>
</tr>
<tr>
<td>pcDNA3 IM</td>
<td>0.48 ± 0.12</td>
<td>0.741 ± 0.13</td>
</tr>
<tr>
<td>NT</td>
<td>0.10 ± 0.02</td>
<td>0.17 ± 0.04</td>
</tr>
</tbody>
</table>

Table 4.4 Proliferative responses of T cells incubated with medium, CA or Con A, from mice immunised IM with either the EL, pZKC3, pcDNA3 and from mice receiving NT. Values are the averages for 4 mice in each group ± SEM.

SIs were calculated for each group of mice immunised IM or receiving NT and are shown in Table 4.4 and Figure 4.17. Mice immunised IM with the EL produced a higher SI than mice immunised IM with either pZKC3 or pcDNA3, and mice receiving NT, indicating ELI could induce specific cellular responses. However, the difference between the SI for mice immunised IM with the EL and mice immunised IM with pZKC3, or pcDNA3 and mice receiving NT was not significant ($P = 0.312$, $P = 0.312$ and $P = 0.665$ respectively). This was largely due to the variation in the proliferative responses generated within the group of mice immunised with the EL, as two cultures gave strong proliferative responses with SIs of 3.1, whilst two cultures had weaker proliferative responses with SIs of 1.4. This also highlights the possibility that animals
may miss immunisation rounds when few injections are made as a result of DNA loss through degradation, since mice immunised IM only received 3 treatments.

Figure 4.17 Stimulation Index for 4 groups of mice receiving IM immunisations with either the EL, pZKC3 or pcDNA3 and for mice receiving NT. Graph shows the mean SI calculated for 4 mice ± SEM.

T cell analyses for mice immunised ID with DNA vaccine constructs are shown in Table 4.5 and indicate that mice immunised ID with the EL produced significantly stronger T cell proliferation when LNCs were incubated with cytosolic antigen compared to medium ($P < 0.001$). No significant difference in specific T cell proliferation was observed between LNCs incubated with medium and CA for mice previously immunised with pZKC3 ($P > 0.1$), pcDNA3 ($P > 0.06$) or receiving NT ($P > 0.07$).
Table 4.5 Proliferative responses of T cells incubated with medium, CA or Con A, from 4 groups of mice immunised ID with either the EL, pZKC3 or pcDNA3 and from mice receiving NT. Values are the averages for 4 mice in each group ± SEM.

Comparing the SI for each group of mice immunised (see Figure 4.18), it is evident that ID immunisation with the EL generated a stronger T cell proliferative response and higher SI. In contrast to IM immunisation, the difference between the SI calculated for mice immunised ID with the EL and mice immunised ID with pZKC3 or receiving NT was significantly different ($P = 0.047$ and $P = 0.030$ respectively). However, there was no significant difference between the SI calculated for ID and IM routes of immunisation ($P = 0.312$). This would suggest that both routes of immunisation were equally as effective at inducing cellular responses.
4.3.6 Analysis of Cytokines Produced from CA Stimulated T cells

To identify the nature of the cellular responses induced by IM and ID immunisation with the EL, IFN-γ and IL-4 cytokine concentrations were analysed by sandwich ELISA. Cytokine concentrations were calculated from standard curves of recombinant cytokines generated for each experiment. As previously mentioned, production of IFN-γ is an indicator of a Th1 response, whilst IL-4 indicates a Th2 response (O’ Garra, 1998; Liew, 2002).

The cytokine analysis shown in Figure 4.19, indicates that the LNCs of mice vaccinated IM with the EL have significantly higher levels of IFN-γ following stimulation with CA, compared to similarly stimulated groups immunised with pZKC3, pcDNA3 or receiving...
NT. These results agree with the small amount of specific T cell proliferation observed, and suggest a slightly stronger response than was revealed in the proliferation assay. No significant IL-4 responses were observed for any LNCs incubated with CA from any group of mice immunised or receiving NT. This confirms that the response induced in mice immunised IM with the EL was largely Type 1 in nature.

![Figure 4.19 Concentrations of IFN-γ from T cell cultures stimulated with medium, CA and Con A following IM immunisation with either the EL, pZKC3 or pcDNA3, or from mice receiving NT. IFN-γ values graphed are the means calculated for 4 mice in each group ± SEM.](image)

Following ID immunisation with the EL, IFN-γ levels from LNCs incubated with CA were also substantially increased. Whilst there was a low level of IFN-γ in all LNCs incubated with CA, the level produced in mice immunised with the EL was significantly higher than observed for mice immunised with pZKC3 (P < 0.001), pcDNA3 (P < 0.001) and mice receiving NT (P < 0.001).
Figure 4.20 IL-4 concentrations from T cell cultures stimulated with medium, antigen and Con A following IM immunisation with either the EL, pZKC3 or pcDNA3, or for mice receiving NT. IL-4 values are the means calculated for 4 mice in each group ± SEM.

The levels of IFN-γ detected in LNC cultures taken from mice immunised ID are slightly elevated in comparison to the levels of IFN-γ detected in LNC cultures taken from mice immunised IM. Since the levels of IFN-γ detected in LNC cultures stimulated with Con A (positive control) are comparable for both IM (see Figure 4.19) and ID immunisations, this would suggest that ID injection is mounting a stronger T cell response and maybe a more effective route of immunisation.
Figure 4.21 IFN-γ concentrations from T cell cultures stimulated with medium, antigen and Con A following ID immunisation with either the EL, pZKC3 or pcDNA3, or from mice receiving NT. IFN-γ values are the means calculated for 4 mice in each group ± SEM.

Very low levels of IL-4 were detected in cultures incubated with CA that were taken from mice immunised ID with both the EL and pZKC3. However, the quantities detected were not statistically different from the levels detected in cultures incubated with medium alone ($P > 0.05$ for pZKC3). These results again demonstrate that a Th1 response had been induced, which agrees with the predominance of the IgG2a Ab isotype detected previously.

Although these results demonstrated that ELI via ID and IM injection induced specific humoral and cellular responses, the sizes of the induced responses were only very modest. It was therefore of interest to observe whether the size of the induced immune
response to DNA immunisation was affected by the genetic background of the host strain and could be increased in CBA mice.

Figure 4.22 IL-4 concentrations from T cell cultures stimulated with medium, antigen and Con A following ID immunisation with either the EL, pZKC3 or pcDNA3, or from mice receiving NT. IL-4 values are the means calculated for 4 mice in each group ± SEM.
4.4 Investigation into the Immune Responses Generated in CBA Mice Following Intradermal Immunisation with a *S. typhimurium* SL1344 Expression Library

The resistance or susceptibility of inbred mouse strains to infection with intracellular pathogens including *S. typhimurium, Leishmania donovani* and *Mycobacterium bovis*, is controlled by the gene encoding the natural resistance associated macrophage protein (Vidal *et al.*, 1993; Vidal *et al.*, 1995). This protein was formerly known as Nramp 1 since it was only associated with macrophages. It has now been renamed Slc11A1 since it has also been found in neurons, pancreatic islets and the adrenal medulla (Blackwell *et al.*, 2001). Inbred mice such as BALB/c are susceptible to infection with *S. typhimurium* (Plant and Glynn, 1974; Hormaeche, 1979a; Hormaeche *et al.*, 1981) and the susceptibility has been associated with a single amino acid mutation in Slc11A1 (Malo *et al.*, 1994; Govoni *et al.*, 1996; Vidal *et al.*, 1996). CBA mice however are not susceptible to infection with *S. typhimurium* (Hormaeche, 1979b) and express the wild-type form of the Slc11A1 gene.

Previous experiments in this thesis involved immunising BALB/c mice with the EL of *S. typhimurium* SL1344 since this is the preferred model for analysing immune responses to vaccination against *Salmonella*. However, the success of DNA immunisation has been shown to depend on the mouse strain immunised (Doolan *et al.*, 1996; Tascon *et al.*, 1996; Tanghe *et al.*, 2000). There are many differences between BALB/c and CBA mice including their MHC haplotype (H-2^d^ versus H-2^k^ respectively) and their susceptibility to *S. typhimurium* infection. The Slc11A1 gene responsible for resistance to *S. typhimurium* infection exerts many of its pleiotropic effects through the regulation of macrophage activation (Lang *et al.*, 1997; Soo *et al.*, 1998; Cuellar-Mata *et al.*, 2002). Slc11A1 can influence macrophage function by regulating the expression of MHC class II molecules and the production of cytokines, all of which indirectly influence antigen processing and presentation (Lang *et al.*, 1997). Slc11A1 has also been shown to have a
direct effect on antigen processing, which is thought to be controlled by the regulation of protease activity in the late endosomal compartment (Lang et al., 1997). It was therefore of interest to analyse whether CBA mice (H-2\(^b\), with the wild-type Slc11A1 gene) could induce stronger immune responses following DNA immunisation with an EL of *S. typhimurium* SL1344 than BALB/c mice. This section presents results from CBA mice and compares them to results from BALB/c mice from the previous section so that differences in immunisation efficiencies between BALB/c and CBA mice can be identified.

4.4.1 Immunisation Strategy of CBA Mice

CBA mice 8-12 weeks old were immunised ID into the lumbar region with 100\(\mu\)g of plasmid DNA suspended in 100\(\mu\)l of PBS (see Chapter 2). A total of 20 mice were allocated into 4 equal groups and immunised ID as follows: 100\(\mu\)g of *S. typhimurium* SL1344 expression library (EL ID), 100\(\mu\)g of plasmid pZKC3 (pZKC3 ID), or 100\(\mu\)g of plasmid pcDNA3 (pcDNA3 ID). As an internal control for all experiments, the fourth group of mice received NT. Mice were immunised four times at 14 day intervals and blood samples were collected on days 7 and 12 after the final immunisation for analysis by Western blotting and ELISA. On day 14 after the final immunisation, groups of mice were sacrificed for T cell proliferation analysis and cytokine production analysis.

4.4.2 Western Blot Analysis of Pooled Serum from CBA Mice Immunised with an EL

To analyse the induction of humoral immune responses in CBA mice following immunisation with a *S. typhimurium* SL1344 EL, Western blot analyses were performed. SDS-polyacrylamide gels (see Figure 4.23) were loaded with 10\(\mu\)g of BSA (lane B), 100ng of GroEL (Lane G), 10\(\mu\)g of GST-EGFP (Lane E), 12\(\mu\)g cytosolic antigen (CA),
12\(\mu\)g membrane antigen (MA) and 5\(\mu\)g EGFP-YbbA (Lane P), and subjected to electrophoresis. Following electrophoresis, one gel was stained with Coomassie Brilliant Blue so that proteins in the gel could be visualised (see Figure 4.23A). Equivalent gels were blotted onto nitrocellulose membrane, and were incubated with 1: 400 dilutions of the different pooled sera taken 12 days after the final immunisation (see Figure 4.23B-E).

The Western blots in Figure 4.23 identify that specific EGFP Ab was present in the sera of mice immunised with the EL and pZKC3, indicating that immunisation had occurred. Immunisation is further confirmed by the detection of the EGFP-YbbA fusion protein, encoded on plasmid pZKC5 (Lane P). In addition, there is a strong induction of Ab specific to MA only in the sera of mice immunised ID with the EL. The protein bands detected in the MA are of similar sizes (approximately 27, 35, 40-50 and 55 kDa) to the protein bands detected using sera taken from BALB/c mice immunised ID with the EL (see Figures 4.14B and 4.23B, Lane MA). Whilst the MA band of 55 kDa is strongly detected in the sera of CBA mice immunised ID with the EL, it is also very weakly detected in the sera of CBA mice immunised ID with pZKC3 and mice receiving NT. This indicates a background level of Ab is present in all mice prior to immunisation, but MA specific Ab have also been induced from immunisation with the EL.

Similarly, a band of CA at approximately 36 kDa is detected by Ab present in the sera of CBA mice immunised ID with the EL (see Figure 4.23B, Lane CA). An extremely faint band of a similar size was present in the sera of mice receiving NT (see Figure 4.23E, Lane CA), however the band is substantially weaker than that observed in the group immunised with the EL. Additional CA bands detected in mice immunised with the EL are also detected to a similar extent in mice immunised with pZKC3, pcDNA3 and mice receiving NT. These additional bands indicate that there are low levels of cross-reacting Ab present in the sera of mice before immunisation.
Figure 4.23 Coomassie stained protein gel (Panel A), and Western blot analyses (Panels B-E) to determine the presence of BSA (B), GroEL (G), EGFP (E), cytosolic antigen (CA), membrane antigen (MA), and EGFP-YbbA (P) specific Ab. Pooled serum samples were taken from CBA mice previously immunised ID with either the EL (Panel B), pZKC3 (Panel C), or pcDNA3 (Panel D), and mice receiving NT (Panel E). Lane W indicates the molecular weight markers.
4.4.3 Analysis of CA-Specific Antibody in CBA Mice Immunised with an EL by ELISA

To assess the relative concentrations of CA-specific Ab, ELISAs were performed for each group of mice. Initially a titration was performed, using pooled serum taken 12 days after the fourth immunisation, to establish an appropriate dilution for the subsequent comparison of individual Ab levels between different mouse groups (see Figure 4.24). Based on this data, a dilution of 1: 32 was chosen for subsequent comparison of individual sera, which also allowed direct comparison with BALB/c mice in the previous section. The mean ODs obtained for mice from each group are summarised in Table 4.6. This data was analysed to determine whether ID immunisation with the EL significantly induced CA-specific Ab.

<table>
<thead>
<tr>
<th>Vaccine Treatment</th>
<th>Mean OD at 490nm ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>EL IM</td>
<td>0.28 ± 0.01</td>
</tr>
<tr>
<td>pZKC3 ID</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>pcDNA3 ID</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>NT</td>
<td>0.08 ± 0.01</td>
</tr>
</tbody>
</table>

Table 4.6 Ab levels detected in individual CBA mice immunised ID with either the EL, pZKC3, or pcDNA3 and mice receiving NT. Values indicate the mean OD at a 1: 32 dilution of serum for 5 mice in each group ± SEM.

Whilst there was only a very modest induction of CA specific Ab in mice immunised with the EL, there was a significant increase in IgG (P = 0.012) and IgG2a (P = 0.012) specific Ab compared to the amount of IgG and IgG2a Ab detected in mice immunised with pZKC3. There was however, no significant difference in the amount of IgG1 specific Ab between groups of mice immunised with the EL and mice immunised with pZKC3 (P = 0.676).
Figure 4.24 Titration curves for CA-specific IgG (Panel A), IgG1 (Panel B) and IgG2a (Panel C) Ab detected in pooled serum samples taken from mice immunised ID with the EL, pZKC3, or pcDNA3, and from mice receiving NT.
Figure 4.25 CA specific IgG, IgG1 and IgG2a Ab levels detected in CBA mice following ID immunisation with the EL or pZKC3. Graph shows the OD values at a 1:32 dilution of sera for 5 individual mice. Horizontal lines mark the mean OD for each group.

A 5-fold increase in CA-specific IgG2a from mice immunised with the EL compared to a 1.25-fold increase in CA-specific IgG1 above the equivalent pZKC3 controls demonstrates that a weak Th1 response has been induced by immunisation with the EL.

4.4.4 In vitro Analysis of T cell Responses to CA Following Immunisation with an EL

To assess the induction of cellular responses in CBA mice following ID immunisation with an EL, T cell proliferation assays were performed. Mice vaccinated with either the EL, pZKC3 or pcDNA3, and mice receiving NT were sacrificed 14 days after the fourth immunisation, and draining LNCs were collected and cultured with cell medium (negative control), CA, and Con A (positive control) as previously described (see
The results obtained from the proliferation assays are summarised in Table 4.7.

<table>
<thead>
<tr>
<th>Vaccine Treatment</th>
<th>T Cell Proliferation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average cpm x 10^3 ± SEM</td>
<td>SI ± SEM</td>
</tr>
<tr>
<td></td>
<td>Medium</td>
<td>CA</td>
</tr>
<tr>
<td>EL ID</td>
<td>3.12 ± 0.23</td>
<td>9.54 ± 1.44</td>
</tr>
<tr>
<td>pZKC3 ID</td>
<td>3.19 ± 0.14</td>
<td>3.35 ± 0.42</td>
</tr>
<tr>
<td>pcDNA3 ID</td>
<td>2.54 ± 0.23</td>
<td>2.94 ± 0.08</td>
</tr>
<tr>
<td>NT</td>
<td>2.27 ± 0.14</td>
<td>3.12 ± 0.15</td>
</tr>
</tbody>
</table>

Table 4.7 Proliferative responses of T cells incubated with medium, CA or Con A, from CBA mice immunised ID with either the EL, pZKC3, or pcDNA3 and mice receiving NT. Values are the averages for 4 mice in each group ± SEM.

A significant increase in proliferation was observed from LNCs incubated with CA that had been taken from mice immunised with the EL ($P < 0.001$). There was no significant difference in proliferation between LNCs incubated with CA as opposed to medium for mice immunised with pZKC3, and pcDNA3. There was however, a significant difference between the proliferative responses of LNCs incubated with CA and medium for mice receiving NT ($P < 0.01$). This was largely a consequence of the lower levels of proliferation recorded for LNCs incubated with medium alone, since the proliferation observed following incubation with CA was comparable to the equivalent pZKC3 and pcDNA3 controls and was significantly lower than the EL group.

The results in Figure 4.26 show that LNCs taken from CBA mice immunised ID with the EL underwent a significant increase in proliferation above the negative control when incubated with CA compared to LNCs taken from mice immunised with pZKC3 ($P = 0.03$) or pcDNA3 ($P = 0.03$), or mice which received NT ($P = 0.03$). This indicates that immunisation with the EL has induced a specific cellular response.
Figure 4.26 Graph showing the SIs for 4 groups of CBA mice previously immunised ID with either the EL, pZKC3 or pcDNA3, and from mice receiving NT. Graph shows the mean SI calculated for 4 mice ± SEM.

It is worthy of note that the SI calculated for CBA mice immunised ID with the EL is not significantly different to the SI calculated for BALB/c mice immunised with the EL ($P = 0.885$). The similar SIs indicate that both strains of mice are equally effective at inducing cellular responses following DNA immunisation with an EL.

### 4.4.5 Analysis of Cytokines Produced from CA Stimulated T cells

To characterise the nature of the T cell response induced in CBA mice by ID immunisation with the EL, IFN-γ and IL-4 cytokine concentrations were analysed by sandwich ELISA as previously described (see Chapter 2).
Analysis of the IFN-γ concentrations (see Figure 4.27) show a significant increase in the amount of IFN-γ in cultures of LNCs taken from mice immunised with the EL when incubated with CA compared to medium ($P < 0.001$). There were no significant increases in IFN-γ from LNCs taken from mice immunised with pZKC3 ($P > 0.2$), pcDNA3 ($P > 0.07$) or from mice receiving NT ($P > 0.1$) when incubated with CA compared to medium.

Analysis of the IL-4 concentrations (see Figure 4.28), showed no significant increases in the amount of IL-4 produced in LNC cultures incubated with CA, compared to medium for any of the groups of mice immunised or receiving NT.
Figure 4.28 Concentrations of IL-4 from T cell cultures stimulated with medium, CA and Con A following ID immunisation with either the EL, pZKC3 or pcDNA3, and from mice receiving NT. IL-4 values graphed are the means calculated for 4 mice in each group ± SEM.

The detection of significant levels of IFN-γ indicates specific T cell proliferation was induced to CA following immunisation with the EL, and that the cellular response was Type 1. This is in agreement with the induction of a humoral Th1 response.
4.5 Discussion

The work conducted in this chapter analysed three areas of DNA immunisation. Investigations were made into the ability of a DNA vaccine encoding single and multiple antigens to induce specific cellular and humoral responses in mice, the effect of different immunisation routes on the induction of immune responses, and the influence of host genetic strain on the outcome of the immune responses.

4.5.1 Immunological Responses Induced in BALB/c Mice Following DNA Immunisation with EGFP

Initial experiments aimed to identify whether specific humoral and cellular responses to EGFP could be induced in BALB/c mice and determine which route of immunisation was the most effective for inducing immune responses. Experiments were performed using the DNA vaccine construct pZKC3, which encoded the single foreign protein, EGFP.

Results showed that EGFP was immunogenic and that the DNA vaccine construct pZKC3 was capable of inducing specific humoral and cellular responses. Western blot analysis indicated the presence of anti-EGFP Ab in pooled sera of mice following four ID or IM immunisations with pZKC3. Mice immunised ID or IM with pcDNA3 or receiving NT did not generate any EGFP-specific Ab.

Analysis of Ab concentration and isotype showed the induction of specific IgG and IgG2a Ab in mice immunised both ID and IM with pZKC3. Negligible amounts of specific IgG1 were detected in mice immunised with pZKC3, and no Ab was detected for mice immunised with pcDNA3 or receiving NT. Through comparison of specific Ab isotypes, the ELISA results indicated that immune responses were biased towards a
Type 1 response as demonstrated by the ratio of EGFP-specific IgG2a to IgG1 (Abbas et al., 1996; Raz et al., 1996; O'Garra, 1998; Sallusto et al., 1998; Liew, 2002).

There was little difference between the amounts of specific Ab induced following ID and IM immunisation with pZKC3. However, the intensity of the Western blot for ID immunisation appeared more pronounced than for IM immunisation (see Figure 4.2B and D). The small increase in OD values for EGFP-specific IgG Ab taken from mice immunised ID, is in agreement with these Western blot results. However, the difference in IgG levels at the chosen dilution of 1:32 was not significant when compared to the IgG levels detected in mice immunised IM with pZKC3 ($P = 0.71$). Therefore, ID and IM routes of immunisation appear equally as effective at inducing humoral responses.

The pZKC3 vector was also capable of inducing specific T cell responses (see Figure 4.6), as observed by the significant proliferation of LNCs when incubated with GST-EGFP compared to medium alone ($P < 0.001$). However, there was also significant proliferation of LNCs from groups immunised with pcDNA3 ($P < 0.01$) and receiving NT ($P < 0.01$). From analysis of the cytokine data, the lack of either IFN-γ or IL-4 from groups of mice previously immunised with pcDNA3 or receiving NT, indicated the proliferation was not due to T cells, but rather B cells. The most likely contaminating B cell mitogen, LPS, was ruled out following repeated experiments using polymixin, as no reductions in the magnitude of proliferation were observed with increasing amounts of polymixin. Whilst the nature of the contaminating species was unknown, the source of contamination was most likely to be from the antigen preparation. It could therefore be assumed that this underlying proliferation had also occurred in LNC cultures taken from mice immunised with pZKC3, making it difficult to determine the extent of specific T cell proliferation. However, by assuming that the amount of non-specific proliferation in LNCs incubated with GST-EGFP was the same for each group of mice immunised or receiving NT, direct comparisons of SI could be made to determine if a specific T cell proliferation had occurred.
The results show a significant increase in the proliferation of LNCs incubated with GST-EGFP from mice immunised ID and IM with pZKC3 compared to mice immunised ID and IM with pcDNA3 ($P = 0.03$). This indicated that specific T cell proliferation had occurred in addition to the non-specific B cell proliferation. The T cell proliferation was further confirmed by cytokine analysis, as significant quantities of IFN-$\gamma$ were only detected in cultures of LNCs taken from mice immunised ID and IM with pZKC3. The absence of detectable levels of IL-4 in the same cultures also implied a bias towards a Type 1 cellular response, which agreed with the Ab isotype results.

Due to the non-specific proliferation observed in the T cell assays, it was not possible to conclude which route of immunisation was the most efficient at inducing specific cellular responses. Since there were no significant differences in the quantities of IFN-$\gamma$ detected in the supernatants of LNCs taken from mice immunised ID with pZKC3 and mice immunised IM with pZKC3 ($P > 0.99$), cytokine results could not determine the most effective route. Therefore it would appear that ID and IM routes of DNA immunisation are equally as effective at inducing cellular responses to EGFP encoded on the immunising plasmid.

Both IM and ID DNA immunisation has been shown to induce the production of Ab and the activation of both MHC class I restricted antigen-specific CTLs and MHC class II restricted CD4$^+$ T cells in a number of different studies (Fynan et al., 1993; Raz et al., 1994; Ulmer et al., 1993; Manickan et al., 1995; Xiang and Ertl, 1995; Davis et al., 1995). There is conflicting evidence over the most effective route for DNA immunisation. Studies have shown that ID immunisation generates protective immune responses which are not induced following IM immunisation with the same antigen (Hoffman et al., 1995; Forg et al., 1998; Lodmell et al., 2000). One explanation for this phenomenon has been suggested and relates to the fact that ID immunisation substantially increases the possibility of transfecting dendritic cells (Corr et al., 1996). Dendritic cells are the principle APCs involved in initiating immune responses following DNA immunisation (Raz et al., 1994; Doe et al., 1996; Corr et al., 1996; Iwasaki et al.,
1997; Morita and Takashima, 1999) and their concentrations are substantially higher in skin than muscle. However, other literature has reported that IM immunisation is the more effective route of DNA immunisation (Donnelly et al., 1997b; Flo et al., 2000). The results of this initial study have shown the induction of humoral and cellular responses following both IM and ID routes. However, the results have not demonstrated the superiority of one particular route over another at inducing specific humoral or cellular responses. Analysis of the humoral responses induced by DNA vaccination with glycoprotein D of Herpes simplex virus by Flo et al. (2000), support these findings, as similar levels and isotypes of specific Ab were detected following IM and ID immunisation. However, the analysis of cell-mediated immune responses showed that the IM route was overall superior (Flo et al., 2000). It has been reported that the nature of the antigen encoded within a DNA vaccine construct influences the outcome of the immune response. The outcome can be influenced by whether the antigen resides intracellularly, remains bound to the membrane or is secreted from the cell following expression (Davis, 1997; Cohen et al., 1998; Drew et al., 2000). It is therefore possible that the most effective route of immunisation is also linked to the type of antigen encoded within the DNA vaccine construct. In a further attempt to identify the most effective route of DNA immunisation, both IM and ID routes were used for investigating the immune responses induced following immunisation with a S. typhimurium SL1344 EL.

4.5.2 Immunological Responses Induced in BALB/c Mice Following DNA Immunisation with an EL

Section 4.3 aimed to determine whether immunisation with an EL of S. typhimurium SL1344 was capable of inducing specific humoral and cellular responses against an unspecified subset of bacterial antigens and to assess the feasibility of developing ELI for identifying novel antigens of Salmonella. In addition, comparisons between IM and ID immunisation were investigated.
The successful use of ELI has been reported for a number of pathogens including *Mycoplasma pulmonis* (Barry *et al.*, 1995), *Taenia crassiceps* (Manoutcharian *et al.*, 1998), *Trypanosoma cruzi* (Alberti *et al.*, 1998), *Cowdria ruminantium*, (Brayton *et al.*, 1998), *Leishmania major* (Piedrafita, *et al.*, 1999), *Helicobacter pylori*, (Lazowska *et al.*, 2000), and *Plasmodium chabaudi* (Smooker *et al.*, 2000). ELI is an attractive method to investigate vaccine antigens because it requires no prior knowledge of possible antigenic targets and has the potential to screen every gene in the genome of a pathogen. So far, the literature has only reported that DNA ELs can offer a degree of protection against pathogenic challenge. However, in theory it is possible to gradually sub-divide libraries conferring protection against challenge into smaller sub-libraries and gradually reduce the complexity of the library to individual protective clones. Indeed, Melby and colleagues (2000), have used the technique of ELI to identify a pool of five clones that induce protective immune responses against *Leishmania* challenge (Melby *et al.*, 2000).

Following immunisation with the *S. typhimurium* SL1344 EL, Western blot analyses showed an increased level of CA- and MA-specific Ab in the sera of mice immunised both IM and ID. It was also apparent from Western blot results that a low level of cross-reacting Ab were present in the sera of all groups of mice not previously immunised with the EL. Direct comparisons of Western blots between mice immunised IM and mice immunised ID suggested a stronger specific Ab response in mice immunised IM. This was confirmed by the higher OD values observed by ELISA, however, the levels of specific Ab were higher in all groups of mice receiving IM immunisation due to the high levels of cross-reacting Ab which bound to the CA coating on the plates.

Results of Ab ELISAs showed a modest but significant increase in CA specific IgG and IgG2a Ab in mice immunised IM with the EL compared to mice immunised with pcDNA3 ($P = 0.012$ for both IgG and IgG2a). Similarly, a modest, but significant
increase in CA specific IgG and IgG2a Ab were observed in the sera of mice immunised ID with the EL compared to mice immunised ID with pcDNA3 ($P = 0.012$ for both IgG and IgG2a). This indicates a weak humoral response was induced in both mice immunised IM and ID. From the ratios of IgG1 and IgG2a induced, it would appear that IM and ID immunisation with the EL preferentially induced a weak Th1 humoral response. Since the levels and isotypes of Ab detected as a result of immunisation were similar for both routes of immunisation, no one route appeared superior.

One explanation for observing only very low levels of Ab induction may be due to the nature of the EL. The EL used in these immunisations had a complexity of 14,000 plasmids. For each plasmid there is a minimum concentration that can elicit a humoral and cellular response. It has been reported that 100 times more plasmid was required to induce detectable humoral responses compared to that required to induce cellular responses (Johnston and Barry, 1997). The complexity of the EL used in these immunisations does not exceed the maximum complexity of 27,000 plasmids that was determined by Johnston and Barry for detecting a cellular response. However, the amount of each plasmid in the *S. typhimurium* SL1344 EL is marginally lower than the level required to detect a humoral response and may explain why only low levels of specific Ab were detected.

Significant increases in T cell proliferation were observed for LNCs incubated with CA compared to incubation with medium in mice immunised both IM ($P < 0.01$) and ID ($P < 0.001$) with the EL. No significant increases were observed for LNCs taken from mice immunised IM or ID with pZKC3, pcDNA3 or mice receiving NT for the same incubations. When comparing the SI of T cell responses for each route of immunisation, ID immunisation with the EL generated the higher SI ($3.11 \pm 0.48$, mean $\pm$ SEM) compared to IM immunisation ($2.4 \pm 0.55$). However, this difference was largely due to the sizable variations in T cell division following stimulation with CA that were observed for each mouse previously immunised IM with the EL. One explanation for
these low and varied levels of specific T cell proliferation between mice in the same group could be the result of missing immunisation rounds. When few injections are made, any DNA loss through degradation or through inaccuracies in administering the inoculum can significantly effect the success of immunisation. This is supported by reports demonstrating the difficulty of generating reproducible humoral and cellular responses following IM immunisation (Koide et al., 2000). Indeed it was reported that IM injection of a DNA vaccine encoding OVA induced a significant level of serum anti-OVA IgG in three of the six mice and only 2 mice of the 6 showed high specific CTL activity (Koide et al., 2000). In comparison, gene gun immunisation induced significant humoral and cellular responses in all mice (Koide et al., 2000). Since mice injected IM were only immunised 3 times compared to 4 ID immunisations, it is feasible that a similar phenomenon had occurred in this experiment. However, whilst the proliferative responses of LNCs taken from mice immunised IM with the EL were on average lower than for mice immunised ID, they also varied considerably so the difference in SI was found not to be significant ($P = 0.312$). Therefore, both routes would appear equally as effective at inducing a cellular response.

Cytokine analyses demonstrated significant increases in IFN-γ from cultures of LNCs incubated with CA from mice immunised both IM and ID with the EL compared to LNCs incubated with medium. There were no significant increases in IFN-γ from cells cultured with CA for mice previously immunised with pZKC3, pcDNA3 or for mice receiving NT. There was however, a higher level of IFN-γ (4.64 U/ml ± 0.16) from mice immunised ID compared to mice immunised IM (2.23 U/ml ± 0.844), but this corresponded to the increased level of T cell proliferation observed for the mice in these groups. No significant increases in IL-4 were detected in any cultures of LNCs previously stimulated with CA for any group of mice immunised or receiving NT. Significant increases in IFN-γ were detected in cultures of LNCs taken from mice immunised IM and ID with the EL, indicating a Th1 response was induced. A cellular Th1 response agrees with the humoral Type 1 response detected in mice immunised with the EL and agrees with the findings of Piedrafita et al. (1999) and Melby et al. (2000),
which also reported a predominant Th1 response following immunisation with an EL. Both routes of immunisation were again found to be equally effective in inducing humoral and cellular responses, but all subsequent immunisations were made ID since this route was simpler to administer effectively.

4.5.3 Immunological Responses Induced in CBA Mice Following DNA Immunisation with an EL

Several studies have shown that the success of DNA immunisation is dependent on the strain of mice used in the investigation (Tascon et al., 1996; Doolan et al., 1996; Ito et al., 2000), in particular the MHC haplotype of the host strain (Ito et al., 2000). Since only modest humoral and cellular responses were induced in BALB/c mice (H-2\textsuperscript{b}) following immunisation with an EL, it was proposed that CBA mice (H-2\textsuperscript{k}) may induce more substantial immune responses. In addition to the MHC haplotype difference between BALB/c mice and CBA mice, CBA mice are also resistant to S. typhimurium infection (Hormaeche, 1979a; Hormaeche, 1981). The resistance of CBA mice to Salmonella has been linked to the Slc11A1 allele (Blackwell et al., 1999). Slc11A1 has a number of pleiotropic effects and has been shown to regulate antigen processing and presentation through macrophage activation (Lang et al., 1997). It was therefore speculated that CBA mice expressing Slc11A1 would be able to process and present antigen more efficiently to cells of the immune system following immunisation and would possibly induce stronger cellular and humoral responses as a result. The aim of the work conducted in section 4.4 was to assess whether CBA mice were capable of inducing cellular and humoral responses following immunisation with an EL of S. typhimurium and whether mouse strain affected the size of the induced responses.

Western blot analyses demonstrated the presence of EGFP-specific Ab in the sera of mice immunised with the EL and with pZKC3, proving immunisation had been successful in CBA mice. It was not however possible to determine from these results
whether the response to EGFP was stronger in CBA mice than in BALB/c mice. The results also showed a noticeable increase in MA specific Ab in the sera of mice immunised ID with the EL compared to mice immunised with pZKC3, pcDNA3 or mice receiving NT. Whilst the bands of MA recognised on the Western blot were very distinct in CBA mice, there were no distinct bands of MA recognised in BALB/c mice, making it difficult to compare these results directly. Both strains of mice detected bands of CA of 66 kDa and 36 kDa following immunisation with the EL which were weakly detected in mice immunised with pZKC3, pcDNA3 or mice receiving NT.

Results of the Ab ELISA made it possible to compare the quantities of CA-specific Ab induced following immunisation with the EL between the two strains of mice. However, in both strains of mice, only very modest levels of IgG and IgG2a Ab were detected at a 1:32 dilution of sera. There were no significant differences in the levels of CA specific IgG and IgG2a Ab detected in BALB/c compared to CBA mice (P = 0.40 and P = 0.09 for IgG and IgG2a respectively), despite CBA mice having slightly more CA specific IgG2a Ab. Similarly, both strains of mice appeared to induce a Type 1 response as demonstrated by the ratio of IgG2a to IgG1. This would indicate that CBA mice are not more efficient than BALB/c mice at inducing a humoral response following immunisation with an EL of *S. typhimurium* SL1344.

T cell proliferation analyses showed that the SI calculated for CBA mice immunised with the EL was not significantly different from the SI calculated for similarly immunised BALB/c mice (P = 0.885). Whilst the extent of proliferation was modest for both strains of mice, there was a significant increase in T cell proliferation from CBA mice immunised with the EL compared to those immunised with pZKC3 (P = 0.03) or pcDNA3 (P = 0.03), and those receiving NT (P = 0.03). These results indicate that like BALB/c mice, CBA mice induce specific cellular responses following immunisation with an EL of *S. typhimurium* SL1344.
Analysis of cytokine results indicates that both CBA and BALB/c mice induced a Th1 response following ID immunisation with an EL. This was demonstrated by the significant increases in IFN-γ production ($P < 0.001$ for both CBA and BALB/c) and lack of IL-4 production from LNCs incubated with CA. The level of IFN-γ produced by LNCs from CBA mice following incubation with CA was marginally lower than the equivalent observed for BALB/c mice. However, the background levels of IFN-γ detected in all groups of BALB/c mice immunised were also noticeably higher.

The results demonstrate that CBA mice, like BALB/c, are capable of mounting Type 1 humoral and cellular responses following immunisation with a $S. typhimurium$ SL1344 EL. No significant difference in the magnitude of humoral or cellular responses was observed between CBA mice and BALB/c mice, indicating that contrary to reports, Slc11A1 does not exert an effect on antigen processing or presentation following DNA immunisation. However, it has recently been reported that the expression of Slc11A1 is induced by LPS or bacterial infection (Zang et al., 2000). Taking this into consideration, it may be that at basal levels of Slc11A1 expression, such as in mice immunised with a DNA vaccine, the pleiotropic effects of Slc11A1 on antigen processing and presentation can not be detected and that infection with $S. typhimurium$ SL1344 is required. These results would also indicate that mouse haplotype has not had an effect on the success of DNA immunisation, since both CBA (H-2^k) and BALB/c (H-2^d) induced similar humoral and cellular responses. However, to assess fully the effect of mouse haplotype on immune responses induced from DNA immunisation, a greater selection of mouse strains would be required.

In summary the results in this chapter have shown specific humoral and cellular responses can be induced from injecting mice both IM and ID with DNA encoding single or multiple antigens. Furthermore, the results have demonstrated a significant Th1 component in the responses induced following immunisation with the $S. typhimurium$ SL1344 EL, which in the context of $Salmonella$ infection is particularly advantageous. Comparisons between BALB/c and CBA mice have shown both to be equally as
effective at inducing humoral and cellular responses to DNA immunisation, and both preferentially induce a Th1 response. Since ELI has been shown to induce detectable immunity, it is feasible that this technique can be developed for identifying protective antigens of S. typhimurium SL1344.
Chapter 5

Investigation into the Immunogenicity of *S. typhimurium*

SL1344 Cytosolic Antigens
5.1 Introduction

5.1.1 Infection with *Salmonella*

*Salmonella* infections are a serious public health and veterinary problem in both developed and less developed countries (Mastroeni *et al.*, 2001; Parry *et al.*, 2002). *Salmonella* serovars are associated with human diseases that range from mild gastroenteritis to host-disseminated enteric fever. In 1994, there were 1.3 billion reported cases of acute gastroenteritis arising largely in developed countries from the ingestion of infected cattle, pigs, poultry and eggs. Moreover, in 1999, there were an estimated 33 million cases of *S. typhi* infection resulting in approximately 500,000 deaths largely in developing countries which include areas of Africa, Asia and South America where typhoid fever is endemic (Levine, 1999). Whilst many of the problems of *Salmonella* infection in both animals and humans have been overcome with the use of antibiotics, there is growing concern over the emergence of new multi-drug resistant strains (Glynn *et al.*, 1988; Mirza *et al.*, 1996; Murdoch *et al.*, 1998; Parry *et al.*, 2002). Vaccination is therefore an attractive alternative for the prevention of *Salmonella* infections and would benefit people living in or travelling within areas where typhoid fever is endemic. However, the efficacy of the vaccines currently available is not always sufficient (Mastroeni *et al.*, 2001).

5.1.2 Immune Responses to *Salmonella* Infection

The severity of *Salmonella* infection depends on a number of factors, namely the size of the ingested bacterial inoculum, the virulence of the infecting *Salmonella* serovar and the ability of the host to mobilise immune defenses to respond to the infection. Immune defenses to *Salmonella* infection require both innate and specific immunity (Abbas *et al.*, 1991; Weir and Stewart, 1993). Innate immunity provides an important antimicrobial defense to *Salmonella* infection and its importance is highlighted in BALB/c
mice with a non-functional Sc111A1 molecule in phagocytes (see Chapters 1 and 3; Blackwell and Searle, 1999). When analysing the role of specific immunity to *Salmonella* infection, Mastroeni and colleagues demonstrated that naïve BALB/c mice were only optimally protected against a virulent *S. typhimurium* challenge when both T cells and serum were donated from a susceptible mouse immunised with an attenuated strain of *S. typhimurium* (Mastroeni *et al.*, 1993). This would indicate that both humoral and cell-mediated immunity is required for host defense against *Salmonella* infection.

Humoral immunity involves the production of antibody from B cells, and the effector phase is mediated by the binding of secreted antibody to antigen. The importance of humoral immunity in resolving *Salmonella* infection is further demonstrated by the fact that protective immune responses can be generated from immunisation with the Vi polysaccharide vaccine developed for typhoid fever (Ivanoff *et al.*, 1994). In addition, it is known that people infected with *S. typhi* or immunised with attenuated vaccine derivatives, produce significant levels of IgM and IgG (Forest *et al.*, 1991; Tacket *et al.*, 1992). Whilst the majority of antibody produced is likely to have resulted from the stimulation of B cells with LPS, antibodies specific for bacterial surface components, including LPS and flagellin, and intracellular proteins have been detected in the serum of infected individuals (Brown and Hormaeche, 1989).

Cell-mediated immunity involves the destruction of intracellular pathogens by macrophages that have been activated by cytokines produced by Th1-like CD4+ T cells, and the destruction of infected cells by activated CD8+ CTLs. Most research indicates that the major effector of protective immunity to *S. enterica* infection is cell-mediated immunity. This is supported by the observation that immunity to *S. enterica* infection shows characteristics of a Th1 response (Nauciel and Espinasse-Maes 1992; Brett *et al.*, 1993; Vancott *et al.*, 1996; Mastroeni *et al.*, 1996). Cytokines associated with Th1 cells include IL-12, IFN-γ and TNF-α of which, IFN-γ is important in the activation of macrophages to produce reactive oxygen species and enzymes that kill phagocytosed bacteria. IFN-γ also stimulates the production of antibody isotypes, such as IgG2a in
mice, which activate complement and opsonise bacteria for phagocytosis (Kaufman, 1993; Romagnani, 1996).

Further evidence that cellular immunity is required for the resolution of *Salmonella* infection has been demonstrated by experiments using IFN-γ receptor knock out mice (Hess et al., 1996), mice treated with neutralising Ab to IL-12 (Mastroeni et al., 1998) and mice deficient in the expression of co-stimulatory molecules required for T cell activation (Mittrucker et al., 1999). In each case, mice were unable to resolve infection with an attenuated strain of *Salmonella*. Although the role for CD4+ T cells is considered essential for immunity against *Salmonella* in BALB/c mice, the importance of CD8+ CTLs is less clear, with some reports demonstrating the requirement of CD8+ T cells in clearing infection, and others reporting the opposite (Hess et al., 1996; Lo et al., 1999).

When designing a vaccine it is important to consider the type of immune response required by the host to clear the particular infection. In the case of *Salmonella* infection it is therefore important that a strong Th1 response is induced in BALB/c mice from vaccination.

5.1.3 Vaccines against *Salmonella* Infection

The first human vaccines developed against typhoid fever were based on killed whole *S. typhi* cells inactivated by heat, phenol or acetone (Hornick et al., 1970). However, these vaccines still only offered limited protection, largely because killed vaccines induce good humoral immune responses but insufficient Th1 cell responses (Collins, 1974). Furthermore the vaccines caused significant adverse reactions in many individuals (Levine, 1999). Alternative vaccines developed during the last 15 years for licensed use against typhoid fever include the Vi capsular polysaccharide vaccine and the live attenuated oral Ty21a vaccine. However, whilst both vaccines have been successfully used to immunise against typhoid fever, the results have been variable.
The Vi capsular polysaccharide of *S. typhi* was first identified almost 70 years ago (Felix and Pitt, 1934) and is both a virulence factor and a protective antigen (Robbins and Robbins, 1984). Immunisation with the Vi capsular polysaccharide has been shown to be immunogenic and stimulate protective Ab responses against typhoid fever (reviewed by Hessel *et al.*, 1999). However, whilst sub-unit vaccines based on the Vi capsular polysaccharide of *S. typhi* are safe and are currently licensed for human use, they only confer between 55% and 75% protection against typhoid fever (Klugman *et al.*, 1996). Moreover, the Vi capsular polysaccharide vaccines induce only short-lived Ab responses in children between the ages of 2 and 5 and do not elicit protective levels in children younger than 2 years. Additionally, reinjection after 2 years in adults restores the Vi Ab levels, but does not elicit a booster response (Kossaczka *et al.*, 1999). The further development of sub-unit vaccines has largely been ignored possibly due to the advances made in genomics and the potential superiority of live attenuated vaccines in inducing immunity (Mastroeni *et al.*, 1996, Garmory *et al.*, 2002). However, the Ty21a live attenuated oral vaccine is only modestly immunogenic and requires three to four initial doses (Levine *et al.*, 1999). Moreover, mutations that attenuate *S. typhi* are not fully defined, and whilst there have been no revertants isolated to date, revertants are an undesirable feature of the vaccine. Whilst numerous live attenuated vaccines are currently under investigation (Mastroeni *et al.*, 2001; Garmory *et al.*, 2002), the difficulty still remains of attenuating the organism to an appropriate level so as not to cause disease, but to persist long enough to induce a protective immune response. Due to the limitations of current subunit vaccines, alternative subunit vaccines for *S. typhi* should continue to be investigated.

The identity of many antigens responsible for protection in animals immunised with effective *Salmonella* vaccines remains unknown. However, low levels of protection against *Salmonella* infection have been induced by immunisation with surface components including porins, flagellins and polysaccharide fractions such as LPS (Tabaraie *et al.*, 1994; McSorley *et al.*, 2000; Mastroeni *et al.*, 2001). Investigations have not however identified whether any cytosolic antigens are capable of inducing
protective immune responses. Whilst studies have demonstrated that the single immunodominant heat shock protein Hsp65 of *Mycobacterium tuberculosis* provides a low level of protection against challenge (Tascon *et al.*, 1996; Huygen *et al.*, 1996; Lai and Bennett, 1998), the Hsp65 protein of *S. typhimurium* does not provide protection against challenge (Gallagher and Inchley, personal communication). One possible interpretation is that the immune responses induced by the single antigen vaccination were not strong enough to combat *Salmonella* challenge and that a collection of several key antigens may be required for immunisation and protection against *Salmonella*. Indeed, similar findings were reported for vaccination against *Schistosoma japonicum*, whereby individually administered defined antigens were unable to induce significant protection against *S. japonicum* challenge, but generated stronger protective responses when administered as a multivalent vaccine (Zang *et al.*, 2002).

It is likely that a sub-unit vaccine comprising of several key antigens would be safer and less reactogenic than killed whole cell vaccines. Moreover, with the correct adjuvant, subunit vaccines could induce strong Th1 responses and generate strong cellular immunity that is desirable for eliminating *Salmonella* infections. It was therefore of interest to determine whether a preparation of *S. typhimurium* SL1344 CA was capable of inducing protective immune responses in the mouse model of typhoid fever.

In a parallel set of experiments to those carried out in Chapter 4, the *S. typhimurium* cytosolic antigens (CA) used to examine the presence of memory T cells following DNA vaccination were used to investigate whether protective immune responses could be induced in BALB/c mice. This chapter provides details of the humoral and cellular responses induced following immunisation with *S. typhimurium* SL1344 CA formulated with the adjuvant DDA, and evaluates its protective capacity in mice when challenged with a virulent strain of *S. typhimurium* SL1344.
5.2 Methods for Immunological Analyses and Protection Studies

5.2.1 Immunisation Strategy of BALB/c Mice

The immunological effectiveness of vaccines prepared from purified protein fractions may be enhanced by their formulation. Many vaccines are formulated together with an adjuvant, which act to stimulate an increased humoral and/or cellular response to the co-administered antigen (Ellis, 2001). Adjuvants can bring about stimulatory effects by a number of different methods. Methods include retaining the antigen at the site of injection, acting as an irritant to promote a local inflammatory response and recruiting APCs such as macrophages to the area of the injection, improving interactions of the antigen with APCs or enhancing the interactions between APCs and the responding lymphocytes. Through these mechanisms, adjuvants are also capable of altering the nature of the immune response towards either a Th1 or Th2 response. The only widely licensed adjuvants for human use are aluminium salts such as hydroxide or phosphate which have been successfully used in vaccines for decades. However, aluminium salt adjuvants preferentially promote a Th2-type immune response which is not useful when cell-mediated immunity is required for protection.

For the elimination of intracellular bacteria such as Salmonella, it is preferable to induce both humoral and cell-mediated immune responses, which include the activation of CTLs. To achieve this, the nature of the induced immune response from vaccination against Salmonella would need to be of a Th1-type. The adjuvant dimethyl dioctadecyl ammoniumbromide (DDA) has been demonstrated to induce an efficient long-term immunity to tuberculosis and a strong Th1 response when combined with proteins secreted by M. tuberculosis (Lindblad et al., 1997). Based on these findings, the adjuvant DDA was combined with CA of S. typhimurium SL1344 for immunisation.

The CA of S. typhimurium SL1344 was prepared by lysing stationary phase S. typhimurium SL1344 cells and removing the membranous and LPS fractions by
centrifugation. The CA was then mixed with DDA previously heated to 80°C to allow micelle formation. Mice were injected subcutaneously in the lumbar region on days 0, 14 and 28. Each dose of 200μl was divided equally in two, so that 100μl was injected into the right and left sides of the spine. A total of 24 mice were allocated into 3 equal groups and were immunised as follows: CA and DDA (CA + DDA), DDA alone (DDA) or received no treatment (NT).

5.2.2 Analysis of Immune Responses

The induction of humoral and cellular responses in mice previously immunised with S. typhimurium SL1344 CA formulated with DDA adjuvant were analysed in vitro 12 days and 14 days after the final immunisation respectively. Humoral responses were analysed by Western blotting and ELISA using sera taken 12 days after the final immunisation. Western blots were performed in accordance with the methodology described in Chapter 2. Pooled serum samples were incubated with nitrocellulose membranes at a dilution of 1: 400. ELISAs were performed initially with pooled serum to obtain appropriate dilutions to compare individual serum samples. The dilutions of sera chosen for individual analyses were 1: 500 and 1: 1,000. Ab titres were calculated as the reciprocal dilution generating an OD value greater than the secondary Ab control plus 0.5. To test for differences in the median OD values for each group of mice, the Mann Whitney statistical test was applied. A $P \leq 0.05$ value was considered statistically significant.

Cellular responses were analysed by lymphocyte proliferation assays and cytokine ELISAs 14 days after the final immunisation (see Chapter 2). Draining LNCs were collected and dissociated to a single cell suspension. After adjustment of the concentration, LNCs were cultured in vitro with medium alone (negative control), CA (experimental) and Con A (positive control) in triplicate. After 3 days of culture, aliquots of supernatants were removed for IFN-γ and IL-4 cytokine analysis by sandwich ELISA, and the degree of proliferation was analysed by addition of tritiated
thymidine (see Chapter 2). Values for SIs were compared between groups using the Mann Whitney statistical test. A $P \leq 0.05$ value was considered to be statistically significant. Mean values for cytokine levels and T cell proliferations were compared using the Students two-tailed t-test and a $P \leq 0.05$ was considered to be statistically significant.

5.2.3 S. typhimurium SL1344 Protection Studies

A total of 45 BALB/c mice were allocated into 9 equal groups. A total of 6 groups were immunised with CA + DDA (as previously described) and 3 groups were injected with DDA alone. Mice were then challenged 7 days after the final immunisation with S. typhimurium SL1344, as described in Table 5.1, and were monitored for the following 30 days. All mice showing a significant level of distress were sacrificed and the day was recorded.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Group</th>
<th>Dose of S. typhimurium SL1344 (c.f.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA + DDA</td>
<td>E0</td>
<td>$1 \times 10^0 - 1 \times 10^1$</td>
</tr>
<tr>
<td></td>
<td>E1</td>
<td>$1 \times 10^1 - 1 \times 10^2$</td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td>$1 \times 10^2 - 1 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>E3</td>
<td>$1 \times 10^3 - 1 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td>E4</td>
<td>$1 \times 10^4 - 1 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td>E4</td>
<td>$1 \times 10^5 - 1 \times 10^6$</td>
</tr>
<tr>
<td>DDA</td>
<td>C0</td>
<td>$1 \times 10^0 - 1 \times 10^1$</td>
</tr>
<tr>
<td></td>
<td>C1</td>
<td>$1 \times 10^1 - 1 \times 10^2$</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>$1 \times 10^2 - 1 \times 10^3$</td>
</tr>
</tbody>
</table>

*Table 5.1. Immunisation and S. typhimurium SL1344 challenge strategy.*
5.3 Results of Immunological Analyses and Protection Studies

5.3.1 Western Blot Analysis of Pooled Serum Taken From Mice Immunised with CA of S. typhimurium SL1344

To determine whether immunisation with CA induced specific Ab production, Western blot analyses were performed. A SDS-polyacrylamide gel, (see Figure 5.1A) was loaded with 10μg of BSA (Lane B), 100ng of GroEL, (Lane G), 8μg CA, (Lane C1), 12μg CA (Lane C2) and 16μg CA (Lane C3) and was subjected to electrophoresis. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue so that proteins separated in the gel could be visualised. Equivalent gels were blotted onto nitro-cellulose membranes and incubated in the presence of 1: 400 dilutions of the different pooled sera (Figure 5.1B-D).

The pooled sera taken from mice 12 days after the final immunisation with CA and DDA show the presence of very strong CA specific Ab (Figure 5.1B). The size of CA detected ranged from 66 kDa through to 14 kDa, although a gap between 45 kDa and 25 kDa existed where no CA-specific Ab had been detected. A band of GroEL at 60 kDa was also recognised, indicating that GroEL-specific Ab had been induced in these mice. The BSA on the nitrocellulose membranes was not however detected, indicating that no non-specific Ab binding had occurred and that CA-specific Ab had been induced.

The sera from mice injected with the DDA alone (Figure 5.1C) and mice receiving NT (Figure 5.1D) showed no specific CA Ab, or Ab to GroEL or BSA. This indicates that immunising with CA + DDA has induced specific humoral responses to S. typhimurium SL1344 CA.
Figure 5.1 Determination of the presence of BSA (B), GroEL (G) and cytosolic antigen (CA) specific Ab in pooled serum of mice previously immunised with CA + DDA (Panel B), or DDA (Panel C), and from mice receiving NT (Panel D). The figure shows a Coomassie stained protein gel (Panel A), and Western blot analyses (Panels B-D). Molecular weight markers are shown on the Coomassie gel (Lane W) and are marked by numbers alongside the Western blots.
5.3.2 Analysis of CA-Specific Antibody by ELISA

To assess the relative concentrations of CA-specific Ab in the sera of mice immunised with CA + DDA, DDA alone and mice receiving NT, Ab levels were determined by ELISA. Initially ELISAs were performed to determine the Ab titre of the serum samples and to identify an appropriate dilution for comparing the amount and nature of Ab between the different groups of mice immunised (see Figure 5.2) Pooled serum samples were taken 12 days after the last immunisation.

As shown in Figure 5.2A, the CA preparation elicited substantial specific IgG Ab responses following immunisation with DDA which were not observed from mice injected with DDA alone or mice receiving NT. Similarly, substantial IgG1 (see Figure 5.2B) and IgG2a (see Figure 5.2C) Ab responses against CA were observed from mice immunised with CA + DDA, but not from mice injected with DDA alone or mice receiving NT. The titres of each Ab isotype from each group of mice immunised or receiving NT are summarised in Table 5.2.

<table>
<thead>
<tr>
<th>Vaccine Treatment</th>
<th>Antibody Subtype Titres</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>CA + DDA</td>
<td>16,384</td>
</tr>
<tr>
<td>DDA</td>
<td>16</td>
</tr>
<tr>
<td>NT</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 5.2 Ab sub-type titres for pooled serum samples taken from 3 groups of mice immunised with CA + DDA, DDA alone and from mice receiving NT. Titres were determined as the reciprocal dilution giving an OD greater than the secondary Ab control plus 0.5.
Figure 5.2 Titration curves of CA-specific IgG (Panel A), IgG1 (Panel B) and IgG2a (Panel C) Ab in pooled serum samples taken from 3 groups of mice immunised with CA + DDA, DDA alone and from mice receiving NT.
From the results shown in Figure 5.2, two dilutions of 1: 500 and 1: 1000 were chosen to compare individual mouse sera for each subtype of Ab.

<table>
<thead>
<tr>
<th>Vaccine Treatment</th>
<th>Mean OD values at 490nm ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 : 500 Dilution</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>CA + DDA</td>
<td>1.35 ± 0.06</td>
</tr>
<tr>
<td>DDA</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>NT</td>
<td>0.11 ± 0.01</td>
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</tbody>
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Table 5.3 Ab levels detected in individual mice immunised with CA + DDA, DDA alone and mice receiving NT. Values indicate the mean optical density (OD) at a 1: 500 and 1: 1000 dilution of serum for 6 mice in each group ± SEM.

As shown in Figure 5.3 and Table 5.3, there is a significant increase in the amount of IgG ($P = 0.005$), IgG1 ($P = 0.005$) and IgG2a ($P = 0.005$) Ab present in the sera of mice immunised with CA + DDA compared to mice receiving DDA alone at both 1: 500 and 1: 1000 dilutions. It is also apparent that mice immunised with CA + DDA developed a mixed Ab response, containing both IgG1 and IgG2a isotypes.

On the basis of these results, it can be predicted that T cells from mice immunised with CA + DDA would show a strong proliferative response when incubated in vitro with CA and APCs, and that cytokine analysis would reveal secretion of both IFN-γ and IL-4. In vitro T cell responses to CA are described in the next section.
Figure 5.3 Comparison of CA-specific IgG, IgG1 and IgG2a Ab detected at a 1: 500 and 1: 1000 dilution of serum from mice immunised with CA + DDA, and mice injected with DDA alone. Graph shows individual OD values for 6 mice with horizontal lines marking the means for each group.

5.3.3 In vitro Analysis of T cell Responses to CA Following Immunisation with CA + DDA

Mice injected with CA + DDA, DDA alone or mice receiving NT were sacrificed 2 weeks after the third immunisation. Draining lymph nodes were collected and dissociated to a single cell suspension before being cultured with medium alone, CA,
and Con A. Following 3 days incubation, aliquots of supernatant were removed for cytokine analysis (see below), and the extent of proliferation was analysed. The results obtained from the proliferation assays are summarised in Table 5.4.

<table>
<thead>
<tr>
<th>Vaccine Treatment</th>
<th>T Cell Proliferation</th>
<th>SI ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean cpm x 10^3 ± SEM</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>CA</td>
<td>Con A</td>
</tr>
<tr>
<td>CA + DDA</td>
<td>0.22 ± 0.06</td>
<td>3.11 ± 0.59</td>
</tr>
<tr>
<td>DDA</td>
<td>0.14 ± 0.03</td>
<td>0.22 ± 0.04</td>
</tr>
<tr>
<td>NT</td>
<td>0.12 ± 0.03</td>
<td>0.20 ± 0.03</td>
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</tbody>
</table>

Table 5.4 Proliferative responses of T cells incubated with medium, CA, or Con A, from mice immunised with CA + DDA, DDA alone and from mice receiving NT. Values are the means for 4 mice ± SEM. Proliferation was also expressed as a stimulation index (SI). SI was calculated as the mean [³H] thymidine incorporation of cells stimulated with CA/the mean incorporation in the absence of antigenic stimulation from the same animal.

A significant increase in proliferation was observed from LNCs incubated with CA that had been taken from mice immunised with CA + DDA compared to LNCs incubated with medium (P < 0.001). In contrast, there was no significant difference in the proliferative responses observed between LNCs incubated with CA and medium taken from mice immunised with DDA alone (P > 0.2) and mice receiving NT (P > 0.1). The variable nature of the proliferation results is highlighted again in this table, with background negative control values varying up to 2-fold between mice immunised with CA + DDA and mice injected with DDA or mice receiving NT. In each case however, the cells are perfectly functional as shown by the large proliferative responses obtained from the incubation of LNCs with Con A. The variations may instead be the result of slight discrepancies in cell counting and plating of LNCs taken from each mouse. To normalise the data and enable comparison of proliferative responses between groups of mice, standard indices (SIs) were calculated. Results of the SIs calculated for each group of mice are shown in Figure 5.4.
Figure 5.4 Stimulation Index for 3 groups of mice immunised with CA + DDA, DDA alone and for mice receiving NT. Graph shows the mean SI calculated for four mice ± SEM.

Calculation of the SI for each group of mice (see Table 5.4, Figure 5.4) confirms the strong proliferative response from LNCs incubated with CA following previous immunisation with CA + DDA. The proliferative response is significantly larger than the proliferation observed from LNCs incubated with CA from mice injected with DDA alone \((P = 0.01)\), and mice receiving NT \((P = 0.03)\). These results clearly indicate that immunisation with \textit{S. typhimurium} SL1344 CA together with the DDA adjuvant induces a substantial specific T cell response in BALB/c mice.

5.3.4 Analysis of Cytokines Produced from CA Stimulated T cells

To determine the nature of the CD4+ T cell immune response induced from immunisation with CA + DDA, levels of IFN-\(\gamma\) and IL-4 were analysed by sandwich
ELISA. As shown in Figure 5.5, the supernatants from LNCs cultures derived from mice immunised with CA + DDA contained high levels of IFN-γ. This contrasts with the almost undetectable quantities of IFN-γ in the supernatants of LNCs derived from mice injected with DDA alone, and mice receiving NT.

![Figure 5.5 Concentrations of IFN-γ from T cell cultures stimulated with medium alone, CA and Con A following immunisation with either CA + DDA, DDA alone, and from mice receiving NT. IFN-γ values graphed are the means calculated for 4 mice in each group ± SEM.](image)

The level of IFN-γ present in the supernatants of LNCs incubated with CA was significantly higher than the levels detected in the supernatants of LNCs incubated with medium alone \((P < 0.001)\). There were no significant differences between the levels of IFN-γ detected in the supernatants of LNCs incubated with CA compared to LNCs incubated with medium for both mice injected with DDA alone \((P > 0.5)\) and mice receiving NT. For all groups of mice, the levels of IFN-γ detected in the supernatants of LNCs cultured with Con A were similar between 4U/ml and 6U/ml, indicating that all LNCs were capable of producing IFN-γ. Moreover, the detected levels of IFN-γ were
comparable to those in the supernatants of LNCs cultured with Con A in previous experiments (see Chapter 4). The supernatants from LNC cultures derived from mice immunised with CA + DDA contained detectable levels of IL-4 (see Figure 5.6). Whilst the level of IL-4 detected in the supernatants of LNCs incubated with CA was significantly higher than the level of IL-4 detected in the supernatants of LNCs incubated with medium ($P < 0.001$), the level of IL-4 was low. There were no significant differences between the levels of IL-4 detected in the supernatants of LNCs incubated with CA compared to LNCs incubated with medium for both mice immunised with DDA alone and mice receiving NT ($P > 0.3$). Again, the levels of IL-4 detected in the supernatants of LNCs cultured with Con A indicate that all the cells were capable of producing IL-4 and the levels detected are comparable to the levels of IL-4 detected in comparable previous assays (see Chapter 4).

Figure 5.6 IL-4 concentrations from T cell cultures stimulated with medium alone, CA and Con A following immunisation with either CA + DDA, DDA alone, or mice receiving NT. IL-4 values graphed are the means calculated for 4 mice in each group ± SEM.
Comparing the level of IFN-γ produced by stimulated T cells to the level of IL-4, it is evident that the Th response to \textit{S. typhimurium} SL1344 CA and DDA was Th1. These results agree with the published results of Lindblad et al., (1997).

5.3.5 Protection of Mice Previously Immunised with CA + DDA from \textit{S. typhimurium} SL1344 Challenge

Since strong humoral and cellular responses were observed in BALB/c mice following immunisation with \textit{S. typhimurium} SL1344 CA formulated with DDA adjuvant, mice were challenged with virulent \textit{S. typhimurium} SL1344 to determine whether the immune responses induced were protective.

\textbf{Figure 5.7} Survival of BALB/c mice after immunisation with CA + DDA and challenge with \textit{S. typhimurium} SL1344. A total of five mice were challenged in each group. Challenge doses are shown in Table 5.1.
Using the immunisation strategy described previously, groups of mice were challenged with different doses of *S. typhimurium* SL1344 and the progression of infection was monitored over the following 30 days. The survival of mice immunised with CA + DDA is shown in Figure 5.7, and the survival of mice injected with DDA is shown in Figure 5.8.

The results in Figure 5.7 show that 4 out of the 5 mice immunised with CA + DDA survived challenge with $1 \times 10^0 - 1 \times 10^1$ c.f.u. *S. typhimurium* SL1344. Moreover, one mouse receiving CA + DDA survived at the higher challenge dose of $1 \times 10^1 - 1 \times 10^2$ c.f.u. From the results in Figure 5.8, no mice receiving DDA alone survived any challenge dose of *S. typhimurium* SL1344, indicating that immunisation with CA + DDA induced protective immune responses in BALB/c mice.

![Graph showing survival of BALB/c mice after injection with DDA and challenge with *S. typhimurium* SL1344. A total of five mice were challenged in each group. Challenge doses are shown in Table 5.1.](image)
Using the technique of Reed and Meunch, (1938) the LD$_{50}$ for mice immunised with CA + DDA was calculated to be $7 \times 10^1$ c.f.u., following challenge with *S. typhimurium* SL1344. This value is more than 70 times greater than the LD$_{50}$ level for mice injected with DDA alone after the equivalent challenge. This indicates that immunological memory to *S. typhimurium* had been established from immunising with CA + DDA and that the Th1 response (as shown by the secretion of dominant levels of antigen-specific IFN-γ in vitro and the presence of IgG2a Ab) was protective, albeit at a marginal level.
5.4 Discussion

The results in this Chapter have shown that CA of *S. typhimurium* SL1344 is capable of inducing strong humoral and cellular responses (see Table 5.5). Western blot analyses of pooled sera taken 12 days after the final immunisation showed a significant induction of CA-specific Ab (including GroEL-specific Ab) that were only present in the sera of mice previously immunised with CA + DDA. Induction of CA-specific Ab was confirmed with ELISA analyses which detected significantly high levels of IgG, IgG1 and IgG2a in mice immunised with CA + DDA. Due to differing affinities of the secondary IgG1 and IgG2a Ab for IgG1 and IgG2a respectively, the amounts of each isotype can not be compared directly with each other to determine the nature of the response. However, IFN-γ and IL-4 are important to direct Ab class switching for IgG2a and IgG1 respectively (O’Garra, 1998; Liew, 2002). The increase in IFN-γ was substantially larger than the increase in IL-4, indicating that the antigen specific T cells stimulated were predominantly Th1. These findings are consistent with reports in the literature, whereby DDA combined with secreted antigens of *M. tuberculosis* stimulated predominantly Th1 cells in mice, but also induced significant levels of IgG1 antibodies (Andersen, 1994; Lindblad *et al.*, 1997; Brandt *et al.*, 2000).

In addition to the strong humoral response, a strong cellular response to CA was detected *in vitro*. A substantial T cell specific proliferation was observed with LNCs taken from mice immunised with CA + DDA, which was not observed in mice previously injected with DDA alone, or mice receiving NT. From analysis of the cytokines present in the supernatants of the LNC cultures, the specific T cell proliferation observed in mice immunised with CA + DDA was a Type 1 response and was strongly polarised.
<table>
<thead>
<tr>
<th>Vaccine Treatment</th>
<th>Humoral</th>
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<tr>
<td></td>
<td>Western Blots</td>
<td>ELISA Antibody Titres</td>
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<tr>
<td></td>
<td>+ / -</td>
<td>IgG</td>
</tr>
<tr>
<td>CA + DDA</td>
<td>+++</td>
<td>16,384</td>
</tr>
<tr>
<td>DDA alone</td>
<td>-</td>
<td>16</td>
</tr>
<tr>
<td>NT</td>
<td>-</td>
<td>16</td>
</tr>
</tbody>
</table>

|  | Cellular |  |
|  | T Cell Proliferation | Cytokines |  |
|  | SI ± SEM | IFN-γ (U/ml) | IL-4 (pg/ml) |  |
| CA + DDA          | 15.89 ± 2.22 | 11.0 ± 1.55 | 78.8 ± 9.4 |  |
| DDA alone         | 2.01 ± 0.68 | 0.24 ± 0.14 | 0 |  |
| NT                | 2.01 ± 0.71 | 0 | 0 |  |

| Challenge | Survivors |  |
| Highest dose (c.f.u) | 1 at 1x10^{1-2} |  |
|            | None |  |

*Table 5.5 Summary of humoral and cellular immunity induced following immunisations with CA + DDA, DDA alone and mice receiving NT.*
Since stronger humoral and cellular responses were detected in mice immunised with CA + DDA compared to mice immunised with DNA in the previous Chapter, mice immunised with CA + DDA were challenged with a virulent strain of *Salmonella* to determine whether the induced immune responses were protective. The results of the protection study demonstrated that immunisation with CA + DDA increased the LD$_{50}$ of mice challenged with *S. typhimurium* SL1344 70 fold compared to the LD$_{50}$ of mice injected with DDA alone. This indicates that immunological memory to *S. typhimurium* SL1344 had been established from immunisation with CA + DDA.

The results of the protection studies with virulent *S. typhimurium* SL1344 showed that a low level of protection was induced by immunisation with CA + DDA, however the level of protection was far below the level generated from existing vaccines (Mastroeni *et al.*, 2001; Garmory *et al.*, 2002). More specifically, the level of protection to *S. typhimurium* SL1344 generated by immunisation with CA + DDA was lower than the protection induced in mice previously vaccinated with an AroA$^{-}$ attenuated strain. Both BALB/c and CBA mice immunised with an attenuated AroA- SL3261 strain derived from virulent SL1344 were protected against $1 \times 10^5$ c.f.u. compared to $1 \times 10^1$ c.f.u. for CA + DDA immunisation (Inchley, personal communication).

In addition, the results of the protection studies demonstrate that CA of *S. typhimurium* SL1344 are not sufficient alone to protect innately susceptible mice against IP challenge with virulent organisms even with a strong Th1 promoting adjuvant. This was similarly observed when alkali-treated *Salmonella*-LPS and a whole-cell bacterial soluble extract was shown to be insufficient in protecting innately susceptible mice against oral challenge with virulent organisms (Mastroeni *et al.*, 2001). It would however appear that innately resistant mice, such as CBA mice, could be protected with this vaccination strategy (Mastroeni *et al.*, 2001). This largely due to the fact that innately susceptible mice, such as BALB/c, require both cellular and humoral immune responses to clear infection, whereas innately resistant mice, such as CBA, require only humoral immunity to clear *Salmonella* infection. Since effective vaccines against *Salmonella* are reported to
require both specific cellular and humoral immune responses in humans, BALB/c mice are the recognised models for this work.

With a large number of different cytosolic antigens in the immunising vaccine, it is possible that the dilution effect may mask useful antigens (as was the case with the DNA EL) so that they are unable to generate sufficient immunological memory T and B cells. By identifying key cytosolic antigens, such as those required for virulence, and incorporating only these antigens in sufficient quantities into a vaccine with DDA, protective immunity may be increased. Several studies have been performed that characterise the pathogenicity of *Salmonella*, and have identified chromosomal genes and plasmid-derived genes that are essential for virulence (Jones and Falkow, 1996; Carlson and Jones, 1998). However, many of these include the invasion proteins such as SipB and SipC, which are delivered by the SPI-1 type III secretion system and are largely secreted or membrane bound. It is perhaps more likely that the moderate level of protection induced following immunisations with *S. typhimurium* SL1344 CA + DDA was due to the nature of the proteins in the vaccinating inoculum.

The proteins used to induce specific humoral and cellular responses in this study were all intracellular. During infection however, the first foreign proteins exposed to memory T and B cells would be those found extracellularly on the cell membrane or those secreted from the invading organism. Intracellular proteins would not be displayed to memory T and B cells generated from the immunisations until later, when the invading organism has been lysed and its intracellular contents released or displayed in association with MHC class I and II molecules. As a result, the time taken to recognise intracellular foreign antigens and to develop a secondary immune response may be long enough to permit virulent strains of *Salmonella* to replicate and disseminate and become established in the host. It may be that stronger protective immune responses can be induced from immunising with *Salmonella* antigens that are exposed to the immune system early during infection, such as those found extracellularly. Such a theory is supported by the findings of Strindelius and colleagues who have demonstrated that
extracellular antigens from *S. enteritidis* are capable of inducing effective immune responses in BALB/c mice against an oral challenge of $3 \times 10^4$ c.f.u. *S. enteritidis* (Strindelius *et al.*, 2002). Indeed, characterisation of a CD4$^+$ T cell response induced by attenuated *Salmonella* demonstrated that a significant fraction of CD4$^+$ T cells responded to the flagellar protein FliC *in vivo* (McSorley *et al.*, 2000). Moreover, responses to FliC were sufficient to protect C57BL/6 mice against infection with a virulent strain (McSorley *et al.*, 2000). Further evidence to support the theory that the cellular location of the immunising antigen is important for inducing protective responses includes the inability of the intracellular heat shock protein, GroEL, to induce protective immune responses in BALB/c mice against *S. typhimurium* SL1344 challenge (Gallagher and Inchley, personal communication). GroEL belongs to the heat shock family of proteins and has homologues in virtually all organisms (Kaufmann, 1990). GroEL is a chaperone that helps to repair misfolded proteins and has shown to be induced during interaction with macrophages, which correlates with its immunodominance (Buchneier and Heffron, 1990; Francis and Gallagher, 1993; Abshire and Neidhart, 1993). Despite this, GroEL does not protect mice from *S. typhimurium* SL1344 challenge.

Such findings demonstrate that the nature of the protein, whether intracellular, membrane bound or secreted, is important for establishing protective immune responses against *S. typhimurium* SL1344 infection. It may therefore be more effective if extracellular proteins including outer membrane proteins, porins and flagellin were used in the immunising inoculum instead of CA. Indeed, electrospray-mass spectroscopy analysis of the extracellular antigens that generated effective immunity against *S. enteritidis* challenge showed the amino acid sequences coincided with those of phase-1 flagellin and hook-associated protein 2 (Strindelius *et al.*, 2002).

The failure to demonstrate convincing protection may also relate to the *in vivo* expression of antigen used in the CA preparation. Only *S. typhimurium* SL1344 in stationary phase were used for generating the CA so only proteins that were
constituitively expressed would have been incorporated. *Salmonella* is a facultative intracellular pathogen that has developed a complex array of virulence mechanisms to promote its survival in the host, most importantly in the macrophage (Chapter 1; Finlay and Falkow, 1989; Abshire and Neidhardt, 1993; Schwan et al., 2000). The effectiveness of many live attenuated *Salmonella* vaccines to stimulate strong protective immunity has been attributed to the presence of immunogenic proteins encoded on virulence genes and their expression in response to the host environment (Kagaya et al., 1992). Due to the method of producing the CA for this study, it is unlikely that the cytosolic products of such virulence genes would be present in the CA preparation. As a result there would be no immunological memory cells specific for such proteins, thus reducing the effectiveness of this immunisation strategy. Instead, the immunisation strategy could be more effective if the CA was prepared from *S. typhimurium* SL1344 cultures taken at different stages of growth. Or more importantly, prepared from cultures previously exposed to a range of different external stimuli that mimics the *in vivo* environment during infection and induces the expression of key virulence genes. Such conditions might include exposure to oxidative stress, exposure to thermal stress, deprivation of nutrients or changes in acidity.

The results from this study have also confirmed the findings that DDA, when combined with bacterial antigens, induces strong Th1 responses (Lindblad et al., 1997). Since a Th1 response is required for clearing *Salmonella* infection, a combination of key surface antigens formulated with DDA may generate greater protective immunity in innately susceptible mice than CA + DDA used in this study. DDA has previously been used without toxic effects in human vaccination trials (Stanfield et al., 1973) and has been shown to induce efficient and long-term immunity to tuberculosis infection (Anderson, 1994). It is therefore feasible that a carefully selected protein-based subunit vaccine formulated with DDA could be a successful candidate for human use.

In summary, this Chapter has demonstrated that strong humoral and cellular responses to CA of *S. typhimurium* SL1344 can be induced in BALB/c mice following immunisation
with CA + DDA. Whilst the induced humoral response was mixed between a Type 1 and Type 2 response, as shown by significant levels of IgG1 and IgG2a, the cellular response was strongly polarised towards a Th1 response with the production of significant levels of IFN-γ. Taken together, these immune responses were capable of protecting BALB/c mice against virulent S. typhimurium SL1344 up to $1 \times 10^{12}$ c.f.u. Whilst the protection was only marginal, a 70-fold increase in LD$_{50}$ was identified. These results also highlight the complexity of Salmonella pathogenesis and the difficulty of identifying new protective vaccine strategies.
Chapter 6

Concluding Discussion
The species *S. enterica* is considered to cause approximately 1.3 billion incidences of gastroenteritis per year in the world (Ivanoff *et al.*, 1994). While the incidence of *S. enterica* related disease is particularly prevalent in developing countries, owing to faecal contamination of water supplies and limited health care, *Salmonella* infections are considered a serious medical and veterinary problem worldwide. Serovars such as *S. typhimurium* pose a great threat since they can infect a wide variety of animal hosts and adapt constantly to changing environments. The threat is further exacerbated by the emergence of antibiotic resistant strains. Vaccination is an effective tool for the prevention of *Salmonella* infections, however, the efficacy of the vaccines currently available are not always optimal.

The Vi polysaccharide sub-unit vaccine is currently licensed for human use, although it has variable success owing to its limited ability to induce cell-mediated immunity, and the fact that it is capable of inducing only short-lived antibody responses (Ivanoff *et al.*, 1994; Pang *et al.*, 1995; Kossaczka *et al.*, 1999). Little research has been conducted to identify alternative protective antigens of *Salmonella*, largely because efforts have been concentrated on developing attenuated *Salmonella* strains. Furthermore, despite the promise of DNA vaccination in animal models of other bacterial diseases (Barry *et al.*, 1995; Tascon *et al.*, 1996; Luke *et al.*, 1997; Alves *et al.*, 1998), very little research has been undertaken into the potential of DNA vaccines to induce protective immunity against *Salmonella* infections. In fact, only Lopez-Macias and colleagues have reported the construction of a DNA vaccine against *Salmonella* (Lopez-Macias *et al.*, 1996). This DNA vaccine construct encoded the outer membrane protein C (OMPC) of *S. typhi*, and was shown to induce specific antibody (as determined by ELISA and Western blot analyses) in BALB/c mice after four immunisations at 14 day intervals. The induction of cell-mediated immunity was not however assessed and no challenge with the virulent pathogen was performed to assess its protective capabilities.

The work in this thesis has investigated whether an EL could form the basis of a DNA vaccine against *Salmonella* by first constructing and analysing such a library, and then
examining its ability to induce humoral and cellular responses to cytosolic antigens in mice. It also contributes to the development of ELI as a technique for identifying key protective antigens of *Salmonella*. In addition, this thesis examines the ability of *S. typhimurium* SL1344 CA and DDA adjuvant to induce protective humoral and cellular immune responses.
6.1 DNA Vaccination with Multiple Antigens of S. typhimurium SL1344 from an Expression Library

DNA immunisation has recently emerged as a promising new approach to vaccination and protective responses have been demonstrated against several pathogens (Reviewed by Lai and Bennett, 1998; Hasan et al., 1999; Sharma and Khuller, 2001). Candidate DNA vaccines have been tested against a number of intracellular pathogens including HIV, rabies, hepatitis virus, Ebola virus, Influenza virus, Plasmodium spp., Leishmania major, Mycobacterium spp., Mycoplasma spp., and Schistosoma japonicum, but little research on DNA vaccines for Salmonella has been undertaken. One particular advantage of DNA vaccines is their ability to induce long term humoral and cell-mediated immunity without the risks associated with using live attenuated vaccines. However, for successful DNA vaccines, as with sub-unit vaccines, the key antigens of Salmonella must be determined so that the corresponding genes can be encoded in the vaccine vector. One possible method of identifying key antigens whilst using DNA immunisation is termed ELI (Johnston and Barry, 1997). Whilst ELI has been demonstrated for a number of pathogens, including M. pulmonis (Barry et al., 1995), M. hyopneumoniae (Moore et al., 2002), L. major (Piedrafita et al., 1999; Melby et al., 2000), and T. cruzi (Alberti et al., 1998), successful vaccination using ELI against any Salmonella species has not been previously reported.

The method of ELI involves making an expression library of a pathogen’s entire genome by incorporating DNA fragments into a suitable immunisation vector. The library is then reduced in stages into individually protective plasmids. For ELI to be most effective it is therefore critical that the libraries are representative of the entire pathogen’s genome, and that the pathogen DNA encoded in the vector can be successfully expressed. The construction of a DNA expression library for S. typhimurium SL1344 involved several sequential stages, all of which had to be optimised (see Chapter 3). For example, to ligate fragments of S. typhimurium genomic DNA, the optimal insert: vector mass ratio was found to be 1.5: 1 using a maximum of 400ng of digested vector DNA. Further
increases in the quantity of DNA in-turn reduced ligation efficiency as observed by the lower number of transformants. An insert: vector mass ratio was used as opposed to a molar ratio due to the ranges in size of the DNA fragments undergoing ligation.

The most efficient method for transforming *E. coli* DH5α cells in this study was identified to be via electroporation. This method has similarly been used very successfully to generate up to $10^9$ transformants per microgram of plasmid in *E. coli* DH5α cells (Taketo, 1989; Song *et al*., 1993), and K12 derivatives (Calvin and Hannawalt, 1988), and has been widely applied in molecular biology for the generation of plasmid cDNA libraries (Bottger, 1988). However, for the large quantity of highly competent cells required for the construction of an expression library, transformation was performed by heat shock using rubidium chloride saturated *E. coli* DH5α cells (Sambrook *et al*., 1989).

A number of additional factors have been reported to influence transformation efficiency including the growth phase of bacteria (Taketo, 1989; Szostkova *et al*., 1999), plasmid size (Siguret *et al*., 1994; Ohse *et al*., 1995), the duration of the transformation event (Hui, 1995), and the activation state of T4 DNA ligase (Ymer, 1991; Michelsen, 1995).

In constructing the expression library for this thesis, it was observed that transformation efficiency could be increased when vector DNA and insert DNA were purified prior to ligation. Dialysis was found to be the most efficient method for purifying both *S. typhimurium* SL1344 DNA following sucrose density gradient centrifugation and vector DNA following CsCl density gradient centrifugation. Attempts were made to purify the ligation products by phenol/chloroform extraction immediately before transformation to remove contaminating enzymes and salts, however this resulted in substantial losses in ligated DNA.

By examining the number of colonies generated from each transformation event, the dephosphorylation of pZKC3 vector DNA prior to ligation with *S. typhimurium* SL1344 insert DNA was thought to have an effect on transformation efficiency. In an attempt to
maximise the efficiency of ligation and transformation, linearised vector DNA was not treated with SAP prior to ligation, since dephosphorylating the vector appeared to decrease the number of transformants. However, it was concluded that SAP treatment of vector DNA was essential to prevent vector religation, since no amount of insert present in the ligation reaction could ensure insert ligation preferentially above vector religation. Furthermore the unwanted effects of SAP could be reduced by its removal prior to ligation by phenol/chloroform extraction.

When investigating the influence of T4 DNA ligase on the efficiency of transformation, it was postulated that removing T4 DNA ligase from the ligation products immediately prior to transformation would increase the transformation efficiency. However, since phenol/chloroform extraction of the ligation products was unsuccessful, an alternative method for increasing transformation efficiency was investigated. The heat inactivation of T4 DNA ligase prior to transformation was reported to increase transformation efficiency significantly with as much as a 260-fold increase in the number of transformants generated (Ymer, 1991; Michelsen, 1995). Indeed, these findings were further supported by this thesis since the heat inactivation of T4 DNA ligase prior to transformation increased the number of transformants generated almost 2-fold.

Using the optimised conditions stated above, each transformation generated over 2,000 colonies, which was the optimal number required for each sub-library. A total of 70 transformations were performed to construct a S. typhimurium SL1344 expression library consisting of approximately 140,000 colonies. Restriction endonuclease digestion of plasmid DNA taken from 150 transformed colonies estimated that 92% of the entire DNA expression library consisted of recombinant clones. Sequence analysis of 60 of these colonies showed 14% contained S. typhimurium SL1344 gene fragments in the correct orientation and frame to be expressed a fusion protein with EGFP-HIS. The proportions of each S. typhimurium SL1344 gene fragment inserted into the pZKC3 vector were close to the theoretically predicted values of 16.6%, suggesting that the gene fragments were digested and ligated randomly. Recombinant clones that did not express
the *S. typhimurium* SL1344 fragments were not removed from the library. The number of clones in the library and the random digestion and ligation of fragments, together confirm that the library constructed was representative of the *S. typhimurium* genome according to published requirements (Clarke and Carbon, 1976). Studies performed in prokaryotic cells with pZKC5, pZKC6 and pZKC7 demonstrated that foreign proteins forming a fusion protein with EGFP-HIS were expressed when induced with IPTG. Moreover, the sizes of the fusion proteins agreed with those calculated by the sequencing data. In addition, the fusion proteins were expressed when transfected into mammalian cells as determined by EGFP fluorescence. This demonstrated that a representative DNA expression library for *S. typhimurium* SL1344 had been constructed for the work in this thesis.

DNA vaccines have been demonstrated to induce strong humoral and cell-mediated immunity in a number of different animal models of disease, via a number of different immunisation routes (Reviewed Hasan *et al.*, 1999; Koide *et al.*, 2000; Gurunathan *et al.*, 2000; Sharma and Khuller, 2001). Whilst both IM and ID routes of DNA immunisation have been shown to induce cell-mediated and humoral immunity (Fynan *et al.*, 1993; Raz *et al.*, 1994; Ulmer *et al.*, 1993; Manickan *et al.*, 1995; Xiang and Ertl, 1995; Davis *et al.*, 1995), it has been reported that the size and nature of the immune response is affected by the route of administration and the type of antigen encoded in the DNA vaccine vector (Pertmer *et al.*, 1996; Barry and Johnston, 1997b; Davis, 1997; Felquate *et al.*, 1997; Gurunathan *et al.*, 2000). To establish whether the route of immunisation influenced the nature of the response to *Salmonella* antigens, IM and ID DNA immunisations were performed using the pZKC3 vector encoding the *egfp* gene.

The results demonstrated that EGFP was immunogenic and that the pZKC3 construct was capable of inducing both humoral and cellular immunity following both IM and ID administration. Humoral immunity was shown by the presence of EGFP specific antibody in both Western blot analyses and ELISAs. The ELISAs demonstrated a moderate induction of EGFP-specific antibody in mice immunised with pZKC3, with
significant increases in IgG and IgG2a compared to mice immunised with pcDNA3 and mice receiving NT. The level of IgG2a, compared to IgG1 suggested a Th1 component in the immune responses induced by IM and ID routes of administration. This was further confirmed by the significant increases in IFN-γ observed in the supernatants of LNCs incubated with GST-EGFP and the absence of IL-4. Due to the non-specific T cell proliferation in this experiment, it could not be determined whether IM or ID immunisation was the most effective route for T cell stimulation. However, it was evident that specific T cell proliferation had occurred as shown by the presence of IFN-γ, and that the nature of the induced response was predominantly Type 1.

Immunisation with the entire EL of S. typhimurium SL1344 was considered, however it was apparent from studies with M. pulmonis that lower protection was conferred when immunising with the total EL compared to smaller sub-libraries (Johnston and Barry, 1997). Likewise, sub-libraries of L. major have been observed to develop stronger protective effects than those induced by the entire library (Piedrafita et al., 1999). This highlighted the effect of plasmid dilution, whereby increasing the complexity of the EL diluted the few protective plasmids to the point of undetectable activity. The S. typhimurium EL was therefore divided into 10 smaller sub-libraries so that the complexity of each sub-library did not exceed that recommended by Johnston and Barry (1997).

Mice were immunised with a selected sub-library both IM and ID to assess whether immunisation with an expression library was capable of inducing specific cellular and humoral responses, and to determine whether the route of immunisation affected the level of the induced immune response. Despite the presence of significant background antibody, Western blot analyses showed an increased level of CA-specific and MA-specific antibodies in mice immunised both IM and ID. This was confirmed with ELISA results, although the level of CA-specific antibody induction was very modest. The ELISA results did however show that the induced immune response following both IM
and ID immunisation involved the stimulation of Th1 cells, due to the presence of IgG2a.

Immunisation with the EL also resulted in a small, but significant increase in T cell proliferation following the incubation of LNCs with the CA preparation, confirming the presence of memory T cells. T cell proliferation was accompanied by the production of significant amounts of IFN-γ, which also confirms the induction of a Th1 response. The type of T helper cell generated in response to DNA vaccines can be influenced by a number of factors (Pertmer et al., 1996; Davis, 1997; Feltquate et al., 1997). The most critical factor determining the nature of immune response is the type of cytokine that naïve cells are exposed to during antigenic stimulation (Xiang and Ertl, 1995; Chow et al., 1998; O'Garra, 1998). In most models of DNA vaccines there is a mixed Th1 and Th2 response, although a predominant Th1 response is more often reported as demonstrated by the production of IFN-γ and IgG2a (Raz et al., 1996; Carson and Raz, 1997). One explanation for the predominance of a Th1 response involves the adjuvant effects of unmethylated CpG dinucleotides that are found in microbial DNA (Reviewed by Thalhamer et al., 2001). It has been demonstrated that the presence of CpG motifs stimulate the innate immune system via Tlr-9 (Hemmi et al., 2000) to produce a series of immunomodulatory cytokines, including IL-12 and IFN-γ, which function as strong Th1 promoting adjuvants (Thalhamer et al., 2001; Yamamoto et al., 2002). It is possible that a mixed Th1 and Th2 response had occurred following the initial immunisations with the EL in this experiment (Davis, 1997). However, due to the very modest levels of antibodies and cytokines induced, only a predominant Type 1 response resulting from repeated injections with DNA containing CpG motifs may have been detected here (Raz et al., 1996; Barry and Johnston, 1997b). The effect of DNA dose on the nature of the immune response is most clearly demonstrated using the gene gun method of immunisation. DNA immunisation via the gene gun route induces a predominant Th2 response as demonstrated by the production of IL-4 and IgG1 antibodies (Hasan et al., 1999). Barry and Johnston suggested, and later proved, that this was because gene gun immunisation required far lower quantities of plasmid DNA than immunisation via
injection, which resulted in a lower stimulation of the innate immune system via CpG motifs (Barry and Johnston, 1997b). Indeed, increasing the amount of plasmid DNA delivered by gene gun was shown to increase the levels of IgG2a, indicative of a Th1 response (Barry and Johnston, 1997b).

Both IM and ID immunisations with the EL were equally successful at inducing humoral and cellular responses in this initial experiment, similar to the pZKC3 immunisations. However further immunisations were performed ID since this method was both easier and quicker.

Uniformly effective vaccines against Salmonella are thought to require both specific cellular and humoral responses in humans. BALB/c mice are genetically susceptible to Salmonella infections and require both cellular and humoral immune responses to clear infection (Mastroeni et al., 1993). Since effective vaccines against Salmonella are thought to require both specific cellular and humoral responses in humans, BALB/c mice are the preferred models for analysing immune responses to DNA immunisation. However, DNA vaccination against M. tuberculosis has demonstrated how mouse strain and possibly haplotype affect the success of DNA immunisation, with C57BL (H-2\(^b\)) but not BALB/c (H-2\(^d\)) mice being protected by IM DNA immunisation with antigen 85A against intravenous M. tuberculosis challenge (Tanghe et al., 2000). Similar effects of mouse strain on the success of DNA immunisation have also been reported (Tascon et al., 1996; Doolan et al., 1996; Ito et al., 2000). To investigate whether immune responses following immunisation with an EL of S. typhimurium SL1344 could be elevated using a different mouse strain, CBA (H-2\(^k\)) mice were immunised with the EL and the immune responses were analysed.

In the context of the present study, CBA mice are relatively resistant to S. typhimurium infection where BALB/c mice are prone to succumb to infection. This is in agreement with the susceptibility of BALB/c mice to other intracellular pathogens including Leishmania and Mycobacterium (Govoni et al., 1996; Lang et al., 1997; Govoni and
Gros, 1998; Gruenheid and Gros, 2000) and is known to be caused by a mutant allele of the Slc11A1 gene. Further differences between BALB/c and CBA mice have also been identified (Inchley, personal communication). For example, a number of S. typhimurium antigens have been detected by antibody found in the serum of CBA mice which have not been detected in the serum of BALB/c mice following immunisation with an aro A− attenuated strain (SL3261) and S. typhimurium SL1344 challenge. One explanation for this may be due to the presence of a functional Slc11A1 molecule in CBA mice. Slc11A1 has a number of pleiotropic effects that both directly and indirectly influence antigen processing and presentation and the induction of protective immune responses (Brown et al., 1997; Lang et al., 1997; Blackwell et al., 2001; Cuellar-Mata et al., 2002). BALB/c mice with a mutant form of the Slc11A1 gene have an inability to develop a DTH response to a Salmonella extract when immunised with attenuated Salmonella whilst CBA mice can elicit a significant DTH response (Hormaeche, 1979; Hormaeche et al., 1981). It was therefore of interest to investigate differences in humoral and cellular responses in CBA mice compared with BALB/c mice following ID immunisation with the same sub-library of S. typhimurium SL1344.

Western blot analyses showed that several distinct proteins in the MA preparation were recognised by antibody in the sera of immunised CBA mice. This was in marked contrast to the situation in BALB/c mice. Although it is possible that a greater concentration of a particular plasmid within the immunising inoculum was responsible for this effect, it was also possible that a lower dilution of key plasmids was necessary for CBA mice to elicit a humoral response. Comparing the presence of CA-specific antibody in the serum of CBA and BALB/c mice, Western blots showed that both CBA mice and BALB/c mice detected bands at approximately 50 kDa and 36 kDa, although the bands were more distinct with CBA serum. Interestingly, a higher band at 66 kDa was only recognised by antibodies present in the serum of BALB/c mice. However, the relative amounts of CA specific antibody detected by ELISA were very modest and were not significantly different from those induced in BALB/c mice. Similar to BALB/c mice, the presence of significant levels of IgG2a suggested a Th1 response.
A small but significant specific T cell proliferation was also induced in in vitro cultures of LNCs prepared from CBA mice immunised with the EL. Again, the level of the proliferation was not significantly different from that observed in BALB/c mice. Similarly, the cellular response induced was predominantly Type 1 as demonstrated by the presence of IFN-γ in culture supernatants and the absence of IL-4.

It has been reported in a number of experimental situations that BALB/c mice are Th2 "prone" including BCG vaccination (Huygen et al., 1992), Leishmania infection (Heinzel et al., 1989), and Mycobacterium infection (Brett and Ivanyi, 1990), and they acquire a more Th2 like phenotype under neutral conditions (Abbas et al., 1996). It has similarly been shown that C57BL/6 mice induce stronger Th1 responses than BALB/c mice toward M. tuberculosis antigen 85A following DNA immunisation, with BALB/c mice inducing a mixed Th1 and Th2 antibody response and lower titres of IFN-γ (Tanghe et al., 2000). Studies using pairs of BALB/c mice strains with wild-type Slcl1A1 versus Slc11A1 mutants have also shown enhanced IFN-γ producing Th1 responses in wild-type mice following M. bovis BCG infection (Kramnik et al., 1994).

The results from these experiments have not however demonstrated that BALB/c are Th2 prone or are less capable of inducing Th1 responses compared to CBA mice with a functional Slc11A1 protein. It would appear that the wild-type allele of Slc11A1 in CBA mice does not affect antigen processing and presentation following DNA immunisation. One possibility for not observing the pleiotropic effects of Slc11A1 is the absence of the infecting organism, S. typhimurium SL1344. It has been reported that Slc11A1 expression is increased following exposure to LPS (Zhang et al., 2000), and that LPS, has been observed to play a role in stimulating more efficient antigen processing and presentation (Lang et al., 1997). It would therefore appear that LPS increases Slc11A1 production which in-turn increases antigen processing and presentation by way of upregulating MHC II expression in macrophages and inducing cytokine production. Indeed, this is supported by other findings (Zhang et al., 2000) whereby bacterial
infection and proinflammatory mediators were found to induce Slc11A1 expression via the activation of mitogen-activated protein kinase pathways. Therefore without the bacterial infection, the effects of Slc11A1 on antigen processing and presentation are not observed.

It is perhaps more likely that any differences in antigen presentation and processing between BALB/c mice and CBA mice are the result of alternative genetic variations, such as the MHC haplotype (Ito et al., 2000). Indeed, it has been reported that immunisation of BALB/c mice with plasmid DNA encoding *P. yoelii* circumsporozoite protein was genetically restricted (Doolan et al., 1996). Similarly it has been demonstrated with an antigen of *M. tuberculosis*, that the genetic background of the host plays an important role in determining the outcome of vaccination (Tascon et al., 1996). This may also explain the differences observed between C57BL/6 and BALB/c mice following DNA immunisation with antigen 85A of *M. tuberculosis* (Tanghe et al., 2000). Whilst this is one explanation for differences in immune responses to DNA immunisation observed in different strains of mice, since no differences were observed between BALB/c and CBA in this work, it is evident that other factors are also involved. This is supported by the findings of Chen and colleagues, since immunisation with DNA encoding several key influenza antigens was found to be equally protective in BALB/c (H-2^d^), B10 (H-2^b^) and C3H (H-2^k^) strains of mice (Chen et al., 2001).

It is clear from the work in this thesis that DNA immunisation with a sub-library of *S. typhimurium* SL1344 was capable of inducing specific cellular and humoral responses. However, the specific immune responses induced were very modest, especially when compared to the specific immune responses induced following immunisation with CA + DDA (discussed later). There are a number of reasons that can explain the low levels of humoral and cellular responses.

The most likely explanation is that only a few antigenic proteins were encoded in the plasmid DNA purified from the 14,000 clones used for immunisation. Indeed, it has
been reported that sub-libraries differ substantially in their ability to induce protective cellular and humoral responses (Piedrafita et al., 1999; Melby et al., 2000), and it is on this basis that an entire library can be divided down sequentially into smaller sub-libraries. By taking the most protective sub-libraries and continually dividing these into smaller groups, a selection of protective antigens can be identified. It would therefore be important for developing ELI to test the remaining 9 sub-libraries of S. typhimurium SL1344 that have been constructed and compare the humoral and cellular responses induced by each one.

It is also important to consider that whilst both cytosolic and membrane bound proteins were encoded within the library, only the contribution of antigenic cytosolic proteins to induce humoral and cellular responses were being observed and may also explain the induction of only weak responses. This was due to the difficulty of purifying membrane proteins from the cell membrane and LPS (Petsch and Anspach, 2000). LPS is found in large quantities in the cell membranes of all Gram negative bacteria such as S. typhimurium SL1344 and is a strong T cell independent activator of B cells. It was therefore not possible to assess T cell specific proliferation in vitro with membrane proteins or identify IgG, IgG1 or IgG2a Ab specific for membrane proteins by ELISA.

One major problem associated with ELI is that only a fraction of each library can productively express the cloned fragments. The fragments of S. typhimurium DNA that are in the wrong orientation, wrong reading frame, or contain non coding DNA, will not express fusion proteins with EGFP-HIS and hence, are unable to induce an immune response. It has been suggested that only 10% of clones in random libraries will express a protein (Moore et al., 2002). As a result, 90% of DNA prepared from the S. typhimurium expression library is likely to be unproductive. This is highly significant since the dilution effect may mask useful clones that could induce strong immune responses, particularly humoral responses for which higher amounts of specific plasmid are required for antibody production (Johnston and Barry, 1997). Indeed, it has been reported on several occasions that immunisation with an entire EL has generated weaker
protective immune responses than immunising with a fraction of the EL (Johnston and Barry, 1997; Piedrafita et al., 1999). In addition, low concentrations of possible protective plasmids increases the difficulty of breaking down protective libraries to identify individual protective clones (Moore et al., 2002).

It is evident that the concentration of each plasmid is important in generating an immune response, as observed by immunising mice solely with the pZKC3 vector encoding the *egfp* gene. In this case, more EGFP specific IgG and IgG2a antibody was detected by ELISA, specific T cell proliferation was noticeably higher, and substantially more IFN-\(\gamma\) was detected compared to the responses induced from mice immunised with a mixture of up to 14,000 different plasmids. However, it has been demonstrated that immunisation with a DNA vaccine encoding a single antigen is not always capable of inducing protective immune responses when challenged with the virulent organism, and that immunisation with multiple antigens is more effective (Han et al., 1999; Kamath et al., 1999; Chen et al., 2001; Zhang et al., 2002).

The problem of having non-expressing clones diluting an EL can be overcome by cloning the *S. typhimurium* DNA upstream of EGFP-HIS. Clones that express a recombinant protein can be screened and isolated using anti-HIS antibody. By reducing the number of non-expressing plasmids in the immunising inoculum, the relative concentrations of potentially useful plasmids can be increased. Moreover, this increases the possibility of obtaining protective libraries and fewer rounds of screening are required to identify protective clones. By constructing an expression library of *Mycoplasma hyopneumoniae* in this manner, Moore and colleagues, (2002) have successfully used one library screen and two animal trials to progress from a library consisting of 20,000 clones to a group of 96 clones (Moore et al., 2002). Alternatively, cDNA expression libraries could be constructed so that any non-coding regions within the pathogen’s genome can be removed and their dilution effects minimised (Manoutcharian et al., 1998; Melby et al., 2000; Feng et al., 2002). In producing a cDNA EL of *Leishmania donovani*, Melby and colleagues, (2000), have fractionated
approximately 30,000 clones down to as few as five unique cDNAs that induced protection against parasitic challenge.

The construction method of the EL may also account for the moderate immune responses observed. Initially *S. typhimurium* SL1344 genomic DNA was partially digested with *Sau3A* before being cloned into a *BamHI* site in the expression vector. By partially digesting *S. typhimurium* genomic DNA with *Sau3A* the number of genes able to insert into the correct frame with EGFP-HIS is limited. As a result, a number of potential antigens may be missed which reduces the power of ELI and the likelihood of identifying individual protective clones.

One way of overcoming this limitation is to construct three separate expression vectors that have the *BamHI* site in three different frames (Piedrafita *et al.*, 1999). By constructing three separate libraries, all the possible antigens will be incorporated. Although there maybe three libraries and therefore three times the number of clones, by removing the 80-90% of the unproductive clones from each library (by the method previously mentioned) one smaller, more productive library containing all possible antigens will result. Indeed the method of constructing three separate libraries for each frame of insert has been adopted in the construction of a *Mycoplasma pulmonis* EL (Barry *et al.*, 1995) and a *L. major* EL (Piedrafita *et al.*, 1999). In the latter case, one of the three libraries comprising of 100,000 clones was reduced to 1,000 clones and was capable of inducing strong protective immune responses against *L. major* infection.

It has been well documented that the route of administration has a considerable effect on the outcome of an immune response (Donnelly *et al.*, 1997; Hasan *et al.*, 1999; Gurunathan *et al.*, 2000). The main method of delivery used for the majority DNA vaccine studies (see in Table 1.1) is by needle injection which includes IM or ID administration. This is largely because this technique of administration is inexpensive and is extensively practised. Particle bombardment by way of a gene gun is a very efficient way of administering DNA vaccines into the skin and has been reported to be
more efficient at inducing immunity than both IM and ID immunisation (Yamakami et al., 2001; Tanghe et al., 2000; Weiss et al., 2000; Han et al., 2000; Barry and Johnston, 1997b). It has been demonstrated using reporter genes that on average, IM and ID injections require delivery of 100 times the amount of luciferase plasmid to produce an equivalent amount of reporter gene expression as that produced by the gene gun (Barry and Johnston, 1997b). The difference in efficiency can largely be related to the fact that IM and ID injection places DNA extracellularly where the DNA can be rapidly degraded by nucleases (Lew et al., 1995). In contrast, the gene gun delivers DNA directly into the cell reducing the amount of DNA lost through degradation.

Whilst it would appear that immunising with a gene gun could overcome the dilution effect observed with the expression library constructed for S. typhimurium, immunisation with a gene gun is considerably more expensive than IM or ID injection. It is not only the initial cost of the gene gun that is high, every immunisation requires DNA to be coated onto microscopic gold beads for delivery. Moreover, immunising with a gene gun has been shown to preferentially induce a Th2 response over a Th1 response (Pertmer et al., 1996; Felquate et al., 1997; Kwissa et al., 2000; Weiss et al., 2000), which is not desirable for eliminating intracellular organisms such as Salmonella from the host.

It has however been demonstrated that the magnitude of and direction of the immune response can be modulated by the co-administration of plasmid encoded cytokines (Lewis et al., 1997). Immunisation strategies have included supplementing DNA vaccines with plasmids encoding cytokines such as IFN-γ, IL-2, IL-12 and GM-CSF (Chow et al., 1997; Chow et al., 1998; Sakai et al., 1999; Flo et al., 2000; Katae et al., 2002). For example, Chow and colleagues showed that co-delivery of IL-12 and IFN-γ encoding genes together with the hepatitis B virus (HBV) DNA vaccine significantly enhanced Th1 cells and increased the production of anti–HBV surface IgG2a antibodies, whilst also inhibiting a Th2 response. Additionally, it was demonstrated that the co-delivery of IL-4 with the HBV DNA vaccine in turn decreased Th1 responses and
resulted in an increase in Th2 responses (Chow et al., 1998). Similar responses have also been observed for DNA vaccines for *T. cruzi* (Katae et al., 2002), *P. falciparum* (Sakai, et al., 1999) and the herpes simplex virus (Flo et al., 2000).

Cytokine modulation of responses is achieved through the normal network interactions of these molecules. Macrophages that have been activated through Tlr-9 by CpG DNA secrete a panel of cytokines including TNF-α and IL-12 (Sun et al., 1998). The cytokines then potently stimulate NK cells, which provide the majority of IFN-γ before it is produced by activated T cells (Ballas et al., 1996). IFN-γ is the central cytokine for macrophage activation and has a number of different effects including the upregulation of MHC I and II molecules and the increased expression of molecules involved in antigen processing. IFN-γ also promotes Th1 cells by enhancing IL-12 production by macrophages and increasing the expression of IL-12 receptors on CD4+ T cells (Abbas et al., 1996; O’Garra, 1998; Liew, 2002). The effect of IL-12 in-turn, mediates the differentiation of Th1 cells by the activation of signal transducer and activator of transcription 4 (STAT4) (Abbas et al., 1996; Liew, 2002). The IFN-γ produced by Th1 cells then acts to suppress the development of Th2 cells and through a positive feedback system, acts to produce more IFN-γ. Conversely, IL-4, required for Th2 cell differentiation via activation of STAT6, is produced by activated Th2 cells and acts to suppress the development of Th1 cells (O’Garra, 1998; Liew, 2002). Both subsets of T helper cells are associated with distinct effector functions. A Th1 profile enhances the production of Th1 antibodies by B cells, such as IgG2a and activates phagocytic cells and NK cells. In addition, a Th1 response is required for the development of CTLs against viral and intracellular pathogens. The Th2 profile causes antibody class switching to IgG1 and IgE and leads to the activation of eosinophils and mast cells required for eliminating parasite infections (Allen and Maizels, 1997; Liew, 2002). It is therefore possible to co-administer genes encoding cytokines to both direct and enhance immune responses induced by the EL.
ELI is unbiased to membrane antigens. However, due to the limitations in analysing immune responses to membrane proteins, the effects of this component could not be thoroughly assessed. It would therefore be of interest to conduct challenge experiments with *S. typhimurium* SL1344 in mice previously immunised with the EL. However, taking into consideration the moderate humoral and cellular responses induced to CA following ELI, it was considered inappropriate to challenge mice with *S. typhimurium* SL1344 to analyse its protective capacity, since it was evident from preliminary findings that immunisation with CA + DDA generated significantly greater immune responses. However, with alterations to the method of expression library construction (as previously mentioned), the work in this thesis indicates that the technique of ELI can be developed to identify protective antigens of *S. typhimurium* SL1344.

The technique of ELI requires a substantial number of mice for assessing the protective responses of each sub-library and identifying a selection of protective antigens. To minimise the number of mice the expression library could alternatively be used to screen for antibodies in the serum of mice previously immunised or infected with *Salmonella*. This method has been successfully applied to a number of pathogens including *Trypanosoma brucei* (Radwanska *et al.*, 2000), *Helicobacter pylori* (Lazowska *et al.*, 2000), and *Plasmodium yoelii* (Burns *et al.*, 1999), and has even been used to identify immunogenic proteins of Tityus bahiensis and Tityus serrulatus (Kalapothakis *et al.*, 2001). Alternatively, the serum from mice immunised with the EL of *S. typhimurium* SL1344 can be used to screen a whole cell lysate of *S. typhimurium* SL1344 using 2D gel electrophoresis and standard Western blot techniques (Weldingh *et al.*, 1998). Proteins detected by Ab can then be identified by tryptic digestion and mass spectrometry. Recent improvements in the technique of ELI have also been investigated. Johnston and colleagues (2002), presented a “state-of-the-art” protocol for screening a genome for protective antigens. Using a sequenced genome, such as *S. typhimurium* LT2, each ORF can be amplified by PCR and introduced into a genetic immunisation vector, reducing the time taken to search for a specific antigen of interest by up to 20-fold compared to randomly digesting genomic DNA (Johnston *et al.*, 2002).
Alternatively, the ORFs can be chemically synthesised, which can allow the optimisation of codons for increased expression in a mammalian cells. The genes can then be displayed in a 3D array and all rows, columns and planes can be tested in mice. The best vaccine candidates inducing protective immune responses against pathogen challenge can then be isolated in one step in a process that is claimed to take 3-4 months (Johnston et al., 2002). It is therefore possible that as an increasing number of genomes are sequenced, the use of ELI as a discovery tool will increase significantly.
6.2 Subunit Vaccination with Multiple Antigens of *S. typhimurium* SL1344

It has been known for some time that protective immunity to *Salmonella* in the murine model can be conferred by immunisation with attenuated bacteria (Hobson, 1957). However, it is not known which antigens are responsible for inducing resistance and successful immunisation of susceptible mice with purified *Salmonella* proteins has not been widely reported. Whilst evaluating the feasibility of developing ELI as a potential solution to identifying protective antigens of *S. typhimurium* SL1344, preliminary investigations into immunising mice with CA of *S. typhimurium* SL1344 together with the adjuvant DDA showed that substantial humoral and cellular responses could be induced in susceptible BALB/c mice.

Western blot analyses indicated the presence of high levels of CA-specific antibody including GroEL-specific antibody, in the pooled sera of mice immunised with CA + DDA. This was confirmed with the ELISA analyses whereby high levels of IgG, IgG1 and IgG2a with end point titres of 16,384, >32,768 and 4096 respectively, were detected in mice immunised with the CA + DDA. From analyses of the individual serum samples, it was evident that a mixed antibody response had been induced as significant increases in IgG1 (P < 0.0051) and IgG2a (P < 0.0051) were observed in the sera of mice immunised with CA + DDA compared to mice immunised with DDA alone. However, from the analyses of cytokine production from stimulated T cells, it was evident that the immune response was biased towards a Th1 response, since substantial amounts of IFN-γ were produced coupled to low levels of IL-4. Once antigen stimulated T cells begin to differentiate along a particular pathway, the cytokines they produce amplify their growth and development and suppress the reciprocal pathway (Abbas *et al.*, 1996). It is therefore possible that the effects of IFN-γ were beginning to inhibit the Th2 response and IgG1 production, as both Th1 and Th2 responses were induced in the short term following immunisation or infection (Pearce and Reiner, 1995; Abbas *et al.*, 1996; O’Garra, 1998; Liew, 2002). The immune response only follows one particular pathway.
after successive immunisations or in the case of chronic disease (Pearce and Reiner, 1995; O’ Garra, 1998; Kersiek and Pamer, 1999; Raupach and Kaufman, 2001; Liew, 2002). In addition to the cytokine responses, a strong cellular response was also confirmed following immunisation with CA + DDA by high levels of specific T cell proliferation in vitro. The average SI for mice immunised with CA + DDA was 16, compared to 2 for mice immunised with DDA alone or mice receiving NT.

It was clear from these findings that both the humoral and cellular responses induced from CA + DDA immunisation were considerably greater than those induced from DNA immunisation. It was therefore of interest to investigate whether CA of *S. typhimurium* SL1344 together with the adjuvant DDA were capable of inducing protective immune responses when challenged with the virulent *S. typhimurium* strain, SL1344.

Challenge studies showed that mice immunised with CA + DDA were protected against up to $1 \times 10^1 - 1 \times 10^2$ c.f.u. This was noticeably higher than the figure for mice injected with DDA alone or receiving NT since all mice died at $1 \times 10^0$ c.f.u. This demonstrated that CA + DDA induced moderate protective immune responses in BALB/c mice. However, these responses were considerably lower than the protective responses to *S. typhimurium* C5 induced by Aro⁻ attenuated vaccines (Hormaeche, 1979; Harrison *et al.*, 1997).

By having a large number of different cytosolic antigens in one immunising inoculum it is possible that protective antigens may be masked, as observed with ELI. In effect, protective antigens may be too dilute to generate sufficient immunological memory T and B cells to protect against the challenge. In addition, it is possible that the immune response induced to the CA is not capable of protection because the CA of the live organisms are not presented early enough in the infection to re-stimulate memory cells to give a protective immune response before the infection becomes established. It would therefore be of interest to determine whether protection against *S. typhimurium* SL1344 could be induced using several key antigens expressed during the early phase of
infection. In addition it would of interest to observe whether an alternative route of immunisation and challenge would induce stronger levels of protection, for example, the specific induction of mucosal immunity.

The intestinal mucosa surface is the first site of contact with *Salmonella* organisms during natural infection (Jepson and Clark, 2001; Humphries *et al.*, 2001). It is therefore of great importance to develop strong mucosal as well as systemic immunity for protection against *Salmonella* in the environment (Sharma and Khuller, 2001). The major effector substance of mucosal immunity is secretory IgA, and immune cells activated at the mucosal site can migrate and produce IgA antibody both locally and distally. It has also been suggested that mucosal vaccines that can boost IgA output from the numerous lymphocytes located in the Peyer’s patches (primary site of *Salmonella* infection) may induce protection that is superior to that achieved with conventional IM administered vaccines (Healy, 1990).

This method of immunisation would be particularly advantageous for infections with virulent *Salmonella*, since virulent organisms are capable of replicating fast and disseminating quickly. By having strong mucosal immunity to *Salmonella* at the immediate site of entry, replication and dissemination can be reduced, preventing *Salmonella* from becoming fully established. Once established, *Salmonella* is difficult to eliminate from the host due to the many evasive methods it has developed to evade detection and killing by cells of the immune system, especially in innately susceptible mice (see Chapter 1). This may partially explain why immunisation subcutaneously with *S. typhimurium* CA and DDA generates only a modest protective immune response in BALB/c against systemic infection with the virulent *Salmonella* strain, SL1344, even though strong humoral and cellular responses had been induced. Indeed studies using extracellular antigens from *S. enteritidis* have been shown to induce effective immune responses in mice after oral vaccination, but not intramuscular (Strindelius *et al.*, 2002).
Immunisation via the mucosal route offers the advantage that both mucosal and systemic immunity could be stimulated (Czerkinsky et al., 1995; Ogra, 1999; McGhee et al., 1999). A number of different mucosal routes have also been studied for DNA vaccines, and have shown to induce strong mucosal immunity (Reviewed Hasan et al., 1999; Gurunathen et al., 2000). Mucosal immunisation is simple, safe and can be used for immunising large population groups. Additionally, it avoids the use of needles, reducing safety issues and costing less. One novel method of mucosal DNA delivery is via the use of microparticles (Barnes et al., 2000). DNA vaccines contained within biodegradable microparticles composed of polylactide-coglycolides have shown to induce both mucosal and systemic immune responses following oral administration (Jones et al., 1997), and intranasal immunisation (Vajdy and O’ Hagan, 2001). Investigations into using transgenic plants such as potatoes to deliver DNA vaccines orally have also been reported, and the induction of specific serum IgG and secretory IgA has been identified (Tacket et al., 1998).

Continuing from the work presented in this thesis, recommendations for future work are presented. A highly productive expression library could be produced to identify key antigens of S. typhimurium SL1344 through the technique of ELI. Key antigens could then be used in a DNA vaccine to assess the protective capacity following challenge with S. typhimurium SL1344. In addition, comparisons can be drawn between immunising orally verses ID to identify the extent to which mucosal immunity is important in protection against oral Salmonella infection. Alternatively, several key antigens identified from ELI, or antigens known to be exposed to the immune system early during the course of infection (such as proteins located on the surface of the bacterium) can be used in a subunit vaccine that is administered orally with the adjuvant DDA. This latter point may be of particular importance since the best characterised protein based subunit vaccinations against Salmonella have been comprised of outer membrane proteins such as OMPC, and have been shown to protect susceptible mice against challenge with virulent Salmonella (Tabaraie et al., 1994; Mastroeni et al., 2000).
Infection with *Salmonella* is a serious medical and economic issue for countries worldwide. The emergence of antibiotic resistant strains, particularly in endemic areas, makes antibiotic therapy impractical and highlights the urgent need to devise suitable prophylactic therapies to reduce the incidence of *Salmonella* infection. With constant advances in improving DNA vaccine efficacy in humans and large animals, it is feasible that a carefully selected DNA vaccine may be a key step to controlling *Salmonella* infection in the future.
6.3 Future of *Salmonella* DNA Vaccines

With subunit and live attenuated vaccines against *Salmonella* widely available, the motive for developing new approaches to vaccination against *S. typhi* may be questioned. Indeed, a similar question has been posed regarding the entire development of DNA vaccines (Leitner, 2001). However, the two current vaccines licensed for *S. typhi* (Vi capsular polysaccharide and Ty21a attenuated oral vaccine) have some practical problems including a limited immunogenicity and adverse side effects. Whilst there are a number of new attenuated *Salmonella* vaccines showing potential in clinical trials including CVD908-*htrA* (*aroC aroD htrA*), Ty800 (*phoP phoQ*) and χ4073 (*cya crp cdt*), there is always a risk of inadvertent infection when the bacterium is not sufficiently attenuated (Garmory *et al.*, 2002). Stronger attenuation would increase the safety of such vaccines, but does so at the expense of decreasing vaccine efficacy.

Alternatively, selecting antigenic proteins from *S. typhi* as the active compound of vaccines carries no risk of infection. However, immunising with protein antigens is disadvantageous since only the humoral branch of the immune system is effectively stimulated. Combining protein antigens with strong adjuvants such as DDA, may overcome this problem, but strong adjuvants can have undesirable side effects for humans.

Furthermore, DNA vaccines are capable of inducing both cellular and humoral responses, they are cheap and easy to construct and meet the requirements for applicability in developing countries where *S. typhi* is endemic. For all the above reasons, DNA vaccines against *Salmonella* should continue to be developed.
Chapter 7

References


Boyle, C., M. Morin, R. Webster, H. Robinson. 1996. Role of different lymphoid tissues in the initiation and maintenance of DNA-raised antibody responses to the influenza virus H1 glycoprotein. J. Virol. 70: 9074-9078.


plasmid DNA encoding Bet v 1, the major allergen of birch pollen. J. Allergy Clin. Immunol. 103: 107-113.


Kamath, A., T. Hanke, H. Briscoe, W. Britton. 1999. Co-immunisation with DNA vaccines expressing granulocyte-macrophage colony stimulating factor and


