Multivalent *Salmonella*-based vaccines: consequences of prior exposure to the vector strain

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ABSTRACT

Research into methods of vaccination against enteric diseases has demonstrated the advantages of using live attenuated *Salmonella* as carriers of foreign antigen. However, recent data from our laboratory have shown that prior exposure of mice to *Salmonella* can dramatically reduce serum antibody responses to foreign antigen subsequently delivered by the same strain. Since this finding contradicted an earlier report, the aim of this thesis was to further assess the significance of pre-existing anti-vector immunity as an impediment to the *Salmonella*-based vaccine strategy.

Attridge *et al.* (1997) found that mice initially infected with *Salmonella* Stanley ten weeks prior to the oral administration of recombinant (r) *S.* Stanley expressing *Escherichia coli* fimbrial antigen K88, showed greatly reduced serum IgG responses to K88 in comparison to control animals. In contrast, Bao and Clements (1991) primed mice with *aroA* attenuated *S.* Dublin and found enhanced serum IgG and mucosal IgA responses to the passenger antigen LT-B (the B subunit of *E. coli* heat labile toxin) subsequently delivered by the same vector. A comparison of experimental protocols revealed several variables which might influence the outcome of vector-priming, and the studies described here focus on two of these. First, the potential of the vector strain to colonise the gut associated lymphoid tissue (GALT). Second, the nature and location of the passenger antigen delivered by recombinant *Salmonella* to vector-primed mice.

Since previous attempts by this laboratory to measure intestinal responses to K88 have been unsuccessful, it was necessary to establish sampling techniques and develop ELISAs to allow reliable measurement of intestinal IgA responses. It was then possible to address the key issue of whether hyporesponsiveness to foreign antigen induced by vector-priming extends to the intestinal secretory IgA response. Control and vector (*S.* Stanley)-primed mice were immunised with r*S.* Stanley-K88. Both groups developed serum IgG anti-LPS responses, with those of primed mice being indicative of secondary exposure to the vector. In comparison to controls, serum anti-

K88 responses were completely abrogated by vector-priming. Significantly, it was possible for the first time to demonstrate hyporesponsiveness to K88 following vector-priming at the level of the intestinal IgA response.

To assess the importance of the vector strain's potential to colonise the GALT, an *aroA* mutation was introduced into *S.* Stanley by allelic exchange mutagenesis. Surprisingly, introduction of an *aroA* mutation had no effect on the GALT colonisation potential of *S.* Stanley. In contrast to mice primed with wt *S.* Stanley and then fed r*S.* Stanley-K88, priming with *aroA S.* Stanley allowed the development of intermediate serum IgG responses to K88. Primary and secondary anti-LPS responses measured in various experiments suggested that immunisation with the *aroA* construct induced weaker anti-vector responses than immunisation with wt *S.* Stanley, thereby reducing the effect of vector-priming.

An alternative approach to assessing the significance of vector GALT colonisation was to compare vector-priming in *Nramp1*$^{-/-}$ and *Nramp1*$^{+/+}$ hosts, as previous studies have shown that *Nramp1*$^{+/+}$ mice are better able to control (systemic) *Salmonella* infection. Moreover, it has been argued that *Nramp1*$^{+/+}$ mice represent a more appropriate model for studies of *Salmonella* infection in humans. Unexpectedly, preliminary studies questioned the significance of *Nramp1* in oral *Salmonella* infection, as GALT colonisation profiles of *S.* Stanley and *S.* Typhimurium were similar in *Nramp1*$^{+/+}$ and *Nramp1*$^{-/-}$ mice. *Nramp1*$^{+/+}$ mice primed with wt *S.* Stanley also failed to develop serum IgG or intestinal IgA responses to K88 when immunised with r*S.* Stanley-K88, confirming the significance of vector-priming.

Previous studies in this laboratory have used K88 as a passenger antigen, which is surface expressed. In contrast Bao and Clements (1991) used LT-B, which is localised in the periplasm and has been described as a strong mucosal immunogen and adjuvant. Several experiments were performed to ascertain the extent to which any observed hyporesponsiveness consequent upon vector-priming might be determined by the characteristics of the foreign antigen. The first was to use *S.* Stanley as a delivery vector for LT-B in vector-primed mice. In comparison to unprimed controls, hyporesponsiveness to LT-B was observed in the serum IgG and intestinal

IgA responses of vector-primed mice. Given that LT-B is considered to be a strong mucosal immunogen, these results provide confirmation of the significance of pre-existing anti-vector immunity.

As a corollary to this study, additional experiments to examine the importance of the passenger antigen were conducted using the vector strain and dosing regime of Bao and Clements (1991). The experiment conducted by Bao and Clements (1991) was repeated, confirming that antibody responses to LT-B delivered by r*Salmonella* were not affected by prior vector (*aroA S.* Dublin)-priming. However, when the passenger antigen was changed to K88, serum IgG and intestinal IgA responses were significantly impaired in vector-primed mice. These results showed that the nature of both the *Salmonella* vector strain and the foreign antigen are important determinants of the consequences of vector-priming.

Overall, experiments conducted in this thesis have demonstrated the adverse effects of vector-priming (i) at the level of both serum and intestinal antibody responses, (ii) with two different *Salmonella* vectors, (iii) with two different passenger antigens and (iv) in *Nramp1*$^{+/+}$ and *Nramp1*$^{-/-}$ hosts. Collectively these results strengthen concerns that pre-existing anti-vector immunity represents a serious threat to the *Salmonella*-based vaccine strategy.

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DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any University or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

I give consent to this copy of my thesis, when deposited into the University library, being made available for loan and photocopying.

Christofer John Vindurampulle, April 2002.

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ABBREVIATIONS

µg	microgram(s)
µl	microlitre(s)
A	adenine
Ap	ampicillin
APC	antigen presenting cell
ATP	adenosine 5'-triphosphate
ATR	acid tolerance response
BLAST	basic local alignment search tool
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
CBT	casamino acids, vitamin B1 and tryptophan
CFPS	clarified faecal pellet supernatant
cfu	colony forming unit
Cml	chloramphenicol
dATP	deoxy adenosine nucleoside triphosphate
dCTP	deoxy cytosine nucleoside triphosphate
dGTP	deoxy guanosine nucleoside triphosphate
dH_2O	distilled water
DHB	2,3-dihydroxybenzoic acid
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
dsDNA	double stranded DNA
DTT	diothiothreitol
dTTP	deoxy thymidine nucleoside triphosphate
dUTP	deoxy uridine nucleoside triphosphate
ECL	enhanced chemiluminescence
EDTA	ethylene-diamine-tetra-acetic-acid
EGTA	ethylene-glycol-bis-(β-amino-ethylether) N, N, N', N'-tetraacetic acid
fcs	foetal calf serum
g	gram(s)
G	guanine
g/l	gram(s) per litre
GM ± SD	geometric mean ± standard deviation
hr	hour(s)
i.n.	intranasal
i.p.	intraperitoneal
i.v.	intravenous
Ig	immunoglobulin
IL	interleukin
IPTG	isopropyl-β-D-thiogalacto-pyranoside
kb	kilobase pairs
kDa	kilodalton
klenow	klenow fragment of *E. coli* DNA polymerase I
KLH	keyhole limpet haemocyanin
Km	kanamycin
LB	luria broth

LD_{50}	lethal dose at which only 50% of the population survives
LPS	lipopolysaccharide
LRR	leucine rich repeats
LT	heat labile enterotoxin of *E. coli*
LT-B	B subunit of the heat-labile enterotoxin of *E. coli*
$M \pm SD$	arithmetic mean $\pm$ standard deviation
M	molar
mg	milligram
min	minute(s)
ml	millilitre(s)
mM	millimolar
MM	minimal medium
MOPS	4-Morpholinepropanesulfonic acid
MQ	milli Q water
mRNA	messenger RNA
ng	nanogram
no.	number
nt	nucleotide(s)
o/n	overnight
°C	degrees celsius
$OD_{410\text{-}620nm}$	OD_{410nm} minus OD_{620nm}
OD_{600nm}	optical density at 600 nm
oligo(s)	oligodeoxynucleotide primer(s)
ORF	open reading frame
P	probability
pABA	para-aminobenzoic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol-8000
pfu	plaque forming units
pg/ml	picograms per ml
^{R}	resistant
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
ssDNA	single stranded DNA
T	thymine
TEMED	N,N,N',N'-Tetramethyl-ethylenediamine
Th1	T helper cell type 1
Th2	T helper cell type 2
TLR	Toll like receptor
tg	transgenic
Tris	Tris (hydroxymethyl) amino methane
TSB	tryptic soy broth
TTBS	Tween-20 plus Tris-buffered saline
U	unit(s)
UV	ultraviolet
V	volts

v/v	volume per volume
W	watt
w/v	weight per volume
wt	wildtype
X-gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside
XLD	xylene, lactose and deoxycholate

CHAPTER ONE

Introduction

1.1 Salmonellae.

1.1.1 General description and nomenclature.

The bacterial genus *Salmonella* was first described by Lignières in 1900 and named after Dr. D. E. Salmon, an American bacteriologist. Members of this genus are Gram-negative, non-spore forming rods (0.7 - 1.5 x 2.0 - 5.0 μm in size), are motile due to peritrichous flagella (with the exception of the gallinarum-pullorum serotype), and belong to the family Enterobacteriaceae (Hook, 1990). *Salmonella* organisms can grow readily on simple media in aerobic or anaerobic conditions, and can be differentiated from other Enterobacteriaceae on the basis of certain biochemical reactions, including fermentation with specific sugars.

The nomenclature for the genus *Salmonella* has evolved from the initial one serotype-one species concept proposed on the basis of the serologic identification of O (somatic) and H (flagellar) antigens by Kauffmann (1954). There are currently more than 2,400 serovars (serotypes) of *Salmonella* (Popoff *et al.*, 2000) which comprise two species: *Salmonella enterica* (which is further subdivided into seven subspecies, designated I, II, IIIa, IIIb, IV, VI and VII) and *Salmonella bongori* (Le Minor and Popoff, 1987; Reeves *et al.*, 1989). Modifications to *Salmonella* nomenclature have arisen as a result of improvements in genetic analysis and comparative techniques. Although confusion exists for the correct method of reporting serovars, it is believed that the nomenclature system used by the Centers for Disease Control (CDC,

United States) adequately addresses the concerns and requirements of clinical and public health microbiologists; it is therefore used throughout this study. For the purpose of abbreviation, the name of each serovar will be written in a shortened form, for example *Salmonella enterica* subspecies *enterica* serovar Typhimurium will be written as *Salmonella* Typhimurium (or *S.* Typhimurium).

1.1.2 Host range and epidemiology.

Salmonella have been isolated from, and associated with disease in, a diverse range of species, including cattle, pigs, rodents, poultry and other birds, dogs, sheep, horses and donkeys, reptiles such as lizards and snakes, and higher mammalian species such as chimpanzees and humans (Bennett and Hook, 1959; Edwards, 1943; Sojka, 1975; Sojka *et al.*, 1986; Sojka *et al.*, 1983; Wray *et al.*, 1981). Some serovars, such as *S.* Typhimurium and *S.* Enteritidis, have a broad host range and cause disease in many species. Others serovars are host-adapted, and seldom cause disease in more than one species. Host-adaptation refers to the ability of a pathogen to circulate and cause disease within a particular host population (reviewed by Kingsley and Baumler, 2000; Uzzau *et al.*, 2000). *S.* Typhi, for example, only infects humans and is the causative agent of typhoid fever (enteric fever). *S.* Dublin and *S.* Choleraesuis are considered to be adapted to cattle and swine respectively, although they are able to cause disease in humans at a very low frequency, and are also virulent in mice (Sojka, 1970). Although the precise mechanism(s) of host adaptation are yet to be elucidated, *Salmonella* host-specific serovars have probably acquired numerous mechanisms which enable survival within the host with which they have evolved. Due to the complexity of the environment within host tissues, the consequences of such adaptation to a particular host may be an inability to infect other hosts which are distantly related.

Of the *Salmonella* serovars identified in cases of human infection, greater than 99% belong to subspecies *enterica* (subspecies I), with ~ 40% of these being *S.* Typhimurium and *S.* Enteritidis (Popoff *et al.*, 1997). The total number of reported cases of Salmonellosis in the USA for the year 2000 was almost 37,000 as determined by the CDC (~ 13 cases per 100,000 people), although this is believed to be severely underestimated due to under-reporting (Morbidity and Mortality Weekly Report 49:51 Table II). In Europe it has been estimated by the World Health Organisation (WHO) that there are 250 cases of Salmonellosis per 100,000 people each year (Weekly Epidemiological Report 75:53 - 60). On a global scale, it has been estimated that annually there are 16.6 million cases of typhoid fever, with nearly 600,000 deaths, and 1.3 billion cases of acute gastroenteritis/diarrhoea due to non-typhoidal salmonellosis, with 3 million deaths (Pang *et al.*, 1998). The rapid rise in multi-drug resistant strains of *Salmonella* species, due to the misuse of antibiotics by both medical professionals and livestock industries, will most likely result in an escalation in global mortality (Pang *et al.*, 1998; Rabsch *et al.*, 2001). *Salmonella* infections also represent a significant problem in the Western world and impose a major financial burden. Costs associated with hospitalisation and treatment of human patients are compounded by the economic impact of livestock infections (cattle and poultry) and the associated destruction of infected foods.

1.1.3 *Salmonella* pathogenesis.

1.1.3.1 Attachment to and invasion of the mucosal epithelium

S. Typhimurium infection in mice results in a similar disease to typhoid fever in humans, and as such is the accepted animal model for human typhoid (Eisenstein, 1999). Infection commences with the ingestion of virulent bacteria, followed by transversal of the highly acidic lumen of the stomach. The survival of *S.* Typhimurium in extreme acidic conditions has been shown to be influenced by a complex system called the acid tolerance response (ATR) (Foster

and Hall, 1990). The ATR in *S. typhimurium* was first demonstrated when it was shown that adapting bacteria to mild (pH 5.8) or moderate (pH 4.4) acidic conditions could enable the cells to survive at very low pH. Over 50 "acid shock proteins" are synthesised during the ATR process (Bearson *et al.*, 1997; Foster and Hall, 1990; Lee *et al.*, 1994), including iron regulatory protein Fur (which senses intracellular pH independently of its ability to sense iron (Foster and Hall, 1992; Hall and Foster, 1996), and the two component regulatory system PhoP/PhoQ which is involved in *Salmonella* invasion of eukaryotic cells and intracellular survival (further discussed in section 1.1.3.2) (Bearson *et al.*, 1998). The pathogenic significance of the ATR remains to be clearly demonstrated.

Once past the stomach, *Salmonella* penetrates the small intestinal mucosa by attaching to and invading specialised microfold cells (or M cells) which overlay the Peyer's patches (Neutra *et al.*, 1996). M cells are involved in sampling the intestinal milieu and delivering antigens to the underlying lymphoid tissue as part of the mucosal defence system (Siebers and Finlay, 1996). Attachment of *Salmonella* to M cells is mediated by chromosomally-encoded long polar fimbriae (*lpf*) (Baumler and Heffron, 1995). *lpf* mutants are unable to adhere to HEp-2 cells (human larynx-derived epithelial cells) *in vitro* (Baumler *et al.*, 1996b), and have a partly reduced capacity to adhere to murine Peyer's patches *in vivo* following oral inoculation (Baumler *et al.*, 1996a). This latter observation, in conjunction with evidence that *S.* Typhimurium LT2 contains at least eleven other fimbrial operons (including type 1, plasmid-encoded (*pef*) and thin aggregative fimbrial operons (curli)) (McClelland *et al.*, 2001), suggests that there is functional redundancy.

Invasion of M cells is mediated by a ~ 40 kilobase (kb) cluster of more than 35 genes termed *Salmonella* pathogenicity island 1 (SPI-1), located at 63 centisomes on the *S.* Typhimurium chromosome (recently reviewed by Brumell *et al.*, 1999; Darwin and Miller, 1999; Wallis and Galyov, 2000). Genes within this cluster can be divided into several groups: genes necessary for construction of a Type III secretion system (TTSS), genes encoding molecular

chaperones, genes encoding secreted effector proteins, and genes encoding SPI-1 regulators. The secretion of various effector proteins into the M cell cytoplasm by the TTSS, including SopE (distantly encoded near SPI-1 at 1 centisome), induces actin rearrangements and the characteristic membrane "ruffling" which facilitates bacterial internalisation (Hardt *et al.*, 1998a). SopE acts as a guanine nucleotide exchange factor for eukaryotic GTPases (Rac and Cdc42), converting them to their active GTP-bound forms which leads to membrane ruffling (Hardt *et al.*, 1998a; Hardt *et al.*, 1998b). Another protein secreted by this TTSS is the SPI-1 encoded effector protein SptP, which interacts with activated GTPases to convert them to their inactive forms, allowing the membrane ruffles to return to normal (Fu and Galan, 1998).

M cells are the main route of entry, and their invasion is a critical determinant of *S.* Typhimurium virulence (Penheiter *et al.*, 1997). Although SPI-1 is essential for M cell invasion (Baumler *et al.*, 1997; Penheiter *et al.*, 1997), *S.* Typhimurium carrying mutations in this locus retain invasive potential (albeit severely reduced) following oral administration and are able to cause systemic infection if injected into the peritoneal cavity (Galan and Curtiss, 1989). Studies by Vazquez-Torres *et al.* (1999) suggest that $CD18^{+}$-phagocytic cells survey, and initiate systemic immune responses to, gastrointestinal microbial antigens, and represent a second potential portal of entry for invasive *Salmonella*. This route does not appear to require involvement of SPI-1 genes. *S.* Typhimurium carrying mutations in *invA* (inactivating SPI-1 mediated invasion) and *lpf* (impairing targeting of Peyer's patches) failed to colonise murine GALT but were still able to reach the liver and spleen following oral administration. Consequently serum IgG, but not mucosal IgA, responses were generated.

S. Typhimurium is also able to adhere to and invade intestinal epithelial cells. This triggers an intense intestinal inflammatory response consisting mainly of neutrophils, which migrate across the epithelial lining of the intestine (McCormick *et al.*, 1993). Transepithelial signalling of neutrophils induces the production of interleukin (IL)-8 (a potent neutrophil chemotactic peptide) from the basolateral surface of the cells. In addition, bacteria may also

release a chemoattractant (McCormick *et al.*, 1995; McCormick *et al.*, 1998). Recent work suggests that this chemoattractant may be SopB (an inositol phosphate phosphatase encoded on SPI-5, centisome 20; Wood *et al.*, 1998), as infection with *S.* Dublin *sopB* mutants results in a 50% decrease in fluid secretion and neutrophil influx in calf ileal mucosa in comparison to the parent strain (Galyov *et al.*, 1997).

1.1.3.2 Intracellular survival and systemic infection.

Salmonella invasion via M cells leads to an interaction with the underlying lymphoid cells, in particular submucosal macrophages, dendritic cells and neutrophils, which represent the first line of cell-mediated defence (Clark *et al.*, 1994; Jones *et al.*, 1994; Sirard *et al.*, 1999). Although macrophages have long been regarded as the main target encountered by *Salmonella*, recent studies have shown that *S.* Typhimurium first associates with, and is internalised by, dendritic cells within the Peyer's patches of orally infected mice (Hopkins and Kraehenbuhl, 1997; Hopkins, 2000). Dendritic cells are the most potent antigen presenting cell (APC) for stimulating naïve T cells (Banchereau and Steinman, 1998), and therefore have been suggested to have an important role in the early induction of acquired immunity (Niedergang *et al.*, 2000). On the other hand, dendritic cells may also be a major niche for *Salmonella*, facilitating bacterial dissemination throughout the lymphatic and reticuloendothelial system as suggested by Sirard *et al.*, 1999. Studies in which *Listeria monocytogenes* (another intracellular bacterial pathogen) were inoculated into the intestines of rats are consistent with this, showing dendritic cells were involved in bacterial spread within the host (Pron *et al.*, 2001). More recently, Rescigno *et al.* (2001) have shown that dendritic cells are able to penetrate the tight junctions between epithelial cells and directly sample bacteria from the intestinal lumen (Rescigno *et al.*, 2001).

Salmonella serotypes that cause systemic infection are able to survive and proliferate within phagocytes due to a second pathogenicity island, SPI-2, located at 30.5 - 31 centisomes

on the *S.* Typhimurium chromosome (Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Ochman *et al.*, 1996). SPI-2 is ~ 25 kb in size and comprises 31 genes, some of which encode a second TTSS distinct from that encoded by SPI-1 (Hensel, 2000). Several genes within SPI-2 have been shown to be critical for *Salmonella* virulence (Klein and Jones, 2001; Ochman *et al.*, 1996; Shea *et al.*, 1999). Mutational analysis has shown that *spiC* encodes a protein that inhibits the fusion of *Salmonella*-infected phagosomes with either endosomes or lysosomes (Uchiya *et al.*, 1999). Similarly *sifA* has been shown to be essential in the maintenance of the *Salmonella*-containing vacuole, as *sifA* mutants are found within the cytosol of macrophages (Beuzon *et al.*, 2000; Brumell *et al.*, 2001; Salcedo *et al.*, 2001). *spiC* or *sifA* mutants retain an invasive phenotype.

Additional chromosomal loci encode enzymes that directly inactivate reactive oxygen and nitrogen species produced within the macrophage phagosome. Resistance to nitric oxide (NO) and related reactive nitrogen compounds is mediated partly by the NO-antagonist homocysteine; mutants in *metL* are unable to synthesise homocysteine and are hypersensitive to NO-compounds and more susceptible to killing within the macrophage (De Groote *et al.*, 1996). *S.* Typhimurium also produces two periplasmic superoxide dismutases that act to inactivate reactive oxygen species; mutation of either structural gene leads to decreased macrophage survival (Fang *et al.*, 1999).

S. Typhimurium transposon insertion mutants unable to survive within the macrophage have been studied in order to identify genes important for intracellular survival (Fields *et al.*, 1986). Various mutations affecting lipopolysaccharide (LPS) production or motility, or which imposed auxotrophy or complement sensitivity all resulted in an inability to survive within macrophages as well as a loss of virulence in the mouse model (further discussed in sections 1.1.3.3.3 and 1.2.1.4). Other attenuating mutations include genes involved in DNA repair (*recA* and *recBC*) (Buchmeier *et al.*, 1993), and the *htrA* virulence gene (involved in resistance to heat shock and oxidative stress, also known as *degP*) (Johnson *et al.*, 1991).

Macrophage survival has also been shown to be critically dependent on the function of a two-component regulatory system termed PhoP/PhoQ (Miller *et al.*, 1989). In this system, PhoQ is the membrane-spanning sensor that transfers a phosphate to the transcriptional regulator PhoP in response to environmental stimuli such as extracellular magnesium concentrations (Garcia Vescovi *et al.*, 1996) and acidity (Bearson *et al.*, 1998). The PhoP/PhoQ regulon co-ordinately controls expression of over 40 genes, inducing the expression of so-called PhoP-activated genes (*pags*) and repressing the expression of other genes, termed PhoP-repressed genes (*prgs*) (Miller *et al.*, 1989). The *pags* are expressed within the macrophage phagosome and are required for intracellular survival, whereas *prgs* are switched off in the phagosome and include components of the SPI-1 TTSS (Miller *et al.*, 1989; Pegues *et al.*, 1995). Both *phoP* null and *phoP* constitutive mutants are avirulent in the mouse model, implying that proper timing of both *pag* and *prg* expression is essential in the host (Miller *et al.*, 1989; Miller and Mekalanos, 1990).

Macrophages infected with *Salmonella* have been observed to undergo cell death (Brennan and Cookson, 2000; Hersh *et al.*, 1999; Jesenberger *et al.*, 2000; Monack *et al.*, 2000; Zhou *et al.*, 2000, and recently reviewed by Knodler and Finlay, 2001). The protein which triggers this response is SipB, which is encoded by a gene within SPI-1 and is secreted via the SPI-1 encoded TTSS. SipB interacts with the pro-apoptotic protease caspase-1 resulting in the commencement of programmed cell death and activation of pro-inflammatory cytokines IL-1β and IL18 (Hersh *et al.*, 1999; Navarre and Zychlinsky, 2000; Zychlinsky and Sansonetti, 1997). Purified SipB directly injected into the cytosol of macrophages results in cell death, although this can be prevented by blocking caspase-1 activity (Monack *et al.*, 2000). Jesenberger *et al.* (2000) showed macrophages deficient in caspase-1 still underwent *Salmonella*-induced death in an SPI-1 and SipB-dependent manner, but after four hours (as opposed to 30 min) following infection. In this instance, cell death was associated with caspase-2 activation.

There is some contention as to whether macrophage death results from apoptosis or necrosis. Rapid *Salmonella*-induced cell death is more representative of necrosis than apoptosis,

as apoptosis is not associated with inflammation whereas necrosis and activation of caspase-1 is (Brennan and Cookson, 2000; Platt *et al.*, 1998). From a bacterial viewpoint, it is most likely that necrosis and the subsequent proinflammatory cascade are required for bacterial uptake and carriage to the organs of the reticuloendothelial system. The reader is directed to a recent review by Knodler and Finlay (2001) for further discussion of this subject.

Recent work has shown that *Salmonella*-induced death of infected macrophages results in uptake and presentation of bacterial antigen in association with both MHC-I and MHC-II by bystander dendritic cells, but not by bystander macrophages (Yrlid and Wick, 2000). Infection of macrophages with a *Salmonella* mutant unable to induce cell death demonstrated that this process was critical for presentation of bacterial antigens by bystander dendritic cells. This suggests, from the viewpoint of the host, that cell death following *Salmonella* infection of macrophages may not be a quiescent death that allows the bacteria to avoid triggering the adaptive immune system. Rather, *Salmonella*-induced death of infected macrophages results in the generation of material containing bacterial antigens in a form that does not induce cell death in a subsequently phagocytosing cell (Yrlid and Wick, 2000).

1.1.3.3 Other *Salmonella* virulence determinants.

1.1.3.3.1 Additional pathogenicity islands.

Salmonella contain three other pathogenicity islands, SPI-3, SPI-4 and SPI-5, which have not been as extensively studied as SPI-1 and SPI-2, although each has been implicated in pathogenesis. The SPI-3 locus is located at 82 centisomes on the *S.* Typhimurium chromosome, and is ~ 17 kb in size. It contains at least ten open reading frames (ORFs) (Blanc Potard *et al.*, 1999), including the *mgtBC* locus which is activated via PhoP/PhoQ in conditions where magnesium ion concentrations are limiting (Garcia Vescovi *et al.*, 1996; Soncini and Groisman, 1996). *mgtB* encodes a magnesium ion transport protein, while *mgtC* encodes a protein of

unknown function, which has been shown to be required for long term survival in macrophages (Blanc Potard and Groisman, 1997; Moncrief and Maguire, 1998). The remaining ORFs have yet to be assigned functions.

The SPI-4 locus, first described by Wong *et al.* (1998), is ~ 25 kb in size and is located at 92 centisomes on the *S.* Typhimurium chromosome. It encodes 18 putative ORFs (designated A-R), three of which (ORF-C, ORF-D and ORF-R) show similarity to outer membrane proteins involved in the secretion of cytotoxic effectors from *Bordetella pertussis* (CyaE; Gross, 1995), *Serratia marcescens* (LipC; Akatsuka *et al.*, 1997), and *Pasturella haemolytica* (LktB; Strathdee and Lo, 1989). A small locus mapping within SPI-4 (*ims98*) was also found to be required for intra-macrophage survival (Baumler *et al.*, 1994).

The SPI-5 locus, first characterised in *S.* Dublin by Wood *et al.* (1998), is located at ~ 20 centisomes on the chromosome and is the smallest characterised pathogenicity island at only ~ 7.5 kb. In addition to from encoding the aforementioned SopB protein, this region was found to possess 4 additional ORFs. One ORF, designated *pipC*, is thought to encode a chaperone for SopB, as these proteins have significant homology to other effector/chaperone pairs found in *Shigella flexneri* (Allaoui *et al.*, 1993) and *S.* Typhimurium (Hong and Miller, 1998). Wood *et al.* (1998) showed that mutations in SPI-5 in *S.* Dublin resulted in reduced virulence for mice as assessed by fluid and neutrophil accumulation within bovine ileal loops. However, mutant bacteria were recovered in similar numbers from the livers and spleens following intraperitoneal (i.p.) or oral infection. These results suggest that the genes of SPI-5 are involved in the development of enteritis but not systemic disease.

1.1.3.3.2 Virulence plasmids.

A large (~ 100 kb) cryptic virulence plasmid (recently reviewed by Rotger and Casadesus, 1999) is found in serovars such as *S.* Typhimurium, *S.* Dublin, *S.* Enteritidis, *S.*

Choleraesuis, *S.* Gallinarum and *S.* Pullorum (Guiney *et al.*, 1995; Guiney *et al.*, 1994). Interestingly, *S.* Typhi does not carry a virulence plasmid, nor do all isolates of the aforementioned serovars. Although the role of the virulence plasmid in pathogenesis remains uncertain, experiments have shown a function for some plasmid encoded genes.

The predominant virulence factor on this plasmid is the highly conserved *spv* locus (*spvRABCD*) which appears to be required for systemic infection, although conflicting results have been reported. A strain of *S.* Typhimurium cured of the virulence plasmid showed undiminished GALT colonisation when fed to mice, but was unable to reach deeper tissues (Gulig and Curtiss, 1987). Libby *et al.* (1997) reported attenuation of both enteric and systemic disease with an *spvR* mutant of *S.* Dublin in calves, whereas other studies with this serovar found that the *spv* genes only affect systemic virulence in *S.* Dublin in the same host (Wallis *et al.*, 1995). More recently, a study by Matsui *et al.* (2001) demonstrated that *spvBC* can replace the entire *spv* locus, and indeed the entire virulence plasmid, with respect to establishing systemic infection in mice exposed to *S.* Typhimurium by the subcutaneous route. SpvB has been shown to belong to the same class of ADP-ribosylating enzymes as cholera and diphtheria toxins, using actin is the substrate for ADP-ribosylation. Site-directed mutagenesis demonstrated that the ADP-ribosylating activity of SpvB is essential for *Salmonella* virulence in mice (Lesnick *et al.*, 2001; Tezcan-Merdol *et al.*, 2001). Finally, evidence suggests that the *spv* genes promote dissemination of *S.* Typhimurium from the human intestine (Fierer *et al.*, 1992).

1.1.3.3.3 Lipopolysaccharide.

LPS consists of three components, the innermost being Lipid A, followed by core sugars, and finally the outermost O-antigen sugar repeats. At least 50 genes have been implicated in the biosynthesis and assembly of LPS (Mäkelä and Stocker, 1969; Schnaitman and Klena, 1993). Among these are genes which regulate polymerisation of O-subunits to achieve a non-random

chain length (preferred or modal length) within a given strain. Immune responses to LPS are directed against the highly immunogenic O-antigen component. Variation in the composition of the sugar repeat units (O-antigenic variation), and the flagellar proteins, forms the basis of the Kaufmann-White *Salmonella* serotyping scheme (Kauffmann, 1954). In addition, lysogenisation with bacteriophage can also modify the composition of O-antigen by providing novel sugar transferases; this has been associated with an increase in bacterial virulence (Nnalue *et al.*, 1990; Wollin *et al.*, 1987).

Lipid A is a hydrophobic glycophospholipid that anchors the entire LPS molecule into the outer leaflet of the outer membrane. It is the component primarily responsible for endotoxic shock, which results from extensive growth of Gram negative bacteria within the host, leading to overstimulation of the immune system (Morrison and Ryan, 1987), ultimately leading to multi-organ failure and death (Schletter *et al.*, 1995). Paradoxically, bacterial lysis brought about by antibiotic therapy can cause a massive release of LPS, compounding the problem (Leeson *et al.*, 1994).

Recently, much interest has been generated by the study of detoxified Lipid A mutants of *S.* Typhimurium. Khan *et al.* (1998) constructed a deletion-insertion in *waaN* (also known as *msbB*), a gene which encodes an enzyme that catalyses one of the two secondary acylation reactions that complete Lipid A biosynthesis. Mutant bacteria synthesised a full-length O-antigen-containing LPS molecule lacking only the expected secondary acyl chain and grew normally *in vitro*. The mutant stimulated weaker cytokine and inducible nitric oxide synthase (iNOS) responses both *in vivo* and *in vitro*, and showed reduced virulence following intravenous (i.v.) administration of BALB/c mice. Whereas animals given wild type (wt) bacteria died when counts reached 10^8 colony forming units (cfu) in livers and spleens, mice infected with mutant bacteria survived despite unprecedented bacterial burdens of ~10^9 cfu per organ. These results confirmed that death in the mouse typhoid model is directly related to the toxicity of Lipid A, and suggested that this may be mediated via proinflammatory cytokine and/or iNOS responses.

However, a re-evaluation of the *waaN* mutant by Watson *et al.* (2000) suggested that the reduction in *Salmonella* virulence may not be solely attributable to the modification of Lipid A, as a decrease in the net secretion of TTSS-dependent proteins was observed in the mutant. Moreover, Murray *et al.* (2001) suggested that the *waaN* mutant described by Khan *et al.* (1998) might carry a secondary cryptic mutation which allowed a normal growth phenotype. Further clarification of this area is required.

Lipid A can also be modified (*in vivo*) via enzymes regulated by the PhoP/PhoQ regulon (Guo *et al.*, 1997). Constitutive expression of PhoP results in the addition of aminoarabinose and 2-hydroxymyristate, as determined by mass spectrometry. PhoP null mutants were unable to make such modifications, were found to be less resistant to antimicrobial peptides, and their Lipid A was less stimulatory for macrophages (Guo *et al.*, 1997).

Studies comparing wt *S.* Typhimurium with isogenic LPS mutants have shown that GALT colonisation potential is proportional to LPS length (Nevola *et al.*, 1985; Roantree, 1967). Subsequent *in vitro* studies suggested that LPS deficient bacteria are less motile and therefore less able to penetrate deeply into the intestinal mucosa, a site where wt bacteria colonise and proliferate prior to the invasion of underlying tissues (Nevola *et al.*, 1987). Other experiments have confirmed that normal LPS expression can provide a sanctuary against non-specific host factors such as bile salts and antimicrobial peptides (refer to section 1.2.1.3), and is a permeability barrier to antibiotics (Groisman *et al.*, 1992; Miller *et al.*, 1990; Snyder and McIntosh, 2000; Sukupolvi and Vaara, 1989).

LPS is also able to activate complement, which is a complex system of serum proteins that perform a vital role in both innate and acquired immunity. Complement activation by LPS can occur via both alternative and classical pathways, which converge with the formation of the critical C3 convertase, leading to the deposition of C3b opsonin on the bacterial surface. This then leads to the generation of inflammatory peptides C3a and C5a, and ultimately to the formation of the lytic and proinflammatory membrane attack complex (MAC). Long chain LPS

can physically restrict access of the MAC to the bacterial membrane, as well as preventing the binding of C3b to conserved binding sites on the core sugars. In contrast to smooth (normal LPS expressing) *Salmonella*, rough LPS mutants (that have a complete core region, but lack O-antigen) are rapidly opsonised with C3b, leading to macrophage uptake and killing (Joiner, 1985; Joiner *et al.*, 1982).

LPS composition, however, appears to be the major determinant of serum resistance of salmonellae (Joiner, 1985). Related *S.* Typhimurium strains (constructed by transduction and recombination of various O-antigen biosynthetic genes) which differ only in the carbohydrate portion of their LPS also differ markedly in their virulence for mice (Makela *et al.*, 1973; Valtonen *et al.*, 1975; Valtonen, 1970; Valtonen and Makela, 1971). The rate of phagocytosis of such strains by the mouse macrophage cell line J774 was found to be inversely proportional to virulence (Liang Takasaki *et al.*, 1982), and results from differential activation of complement via the alternative pathway (Liang Takasaki *et al.*, 1983). Depleting mice of complement by pre-treatment with cobra venom factor showed that clearance of the least virulent *S.* Typhimurium strain was greatly reduced, whereas clearance of the more virulent strain was unaffected. These studies confirmed earlier observations that bacterial virulence was inversely proportional to the ability to activate complement, and showed the critical contribution of LPS structure.

Similarly, Grossman and Leive (1984) reported that purified *Salmonella* LPSs of different O-antigen composition (but containing core oligosaccharides and Lipid A) activated complement differentially. In a later experiment, Grossman *et al.* (1990) proved that O-antigen composition was solely responsible for the differential abilities of LPS to activate complement. In this instance, various *S.* Typhimurium-derived O-antigen octasaccharides (conjugated to a lipid carrier), when used to coat sheep erythrocytes, differentially activated complement (and consumed C3) in a manner similar to the native LPSs.

1.1.3.3.4 Flagella.

In vitro experiments by Jones *et al.* (1992) showed that non-flagellated *S.* Typhimurium mutants were impaired in their ability to invade HEp-2 cells, although these mutants were as invasive as the parent strain if centrifugation was used to bring the mutant in contact with the HEp-2 cells. This clearly distinguished the process of attachment and invasion, and indicated that the flagella may be important in the former, at least *in vitro*. Initial *in vivo* studies by Lockman and Curtiss (1990) showed that wt and non-flagellated mutants were equally virulent in mice when introduced by i.p. injection. Schmitt *et al.* (2001) demonstrated that mutations in *flhD* (the master flagellar regulator gene, which controls the expression of all products of the flagellar cascade including regulatory elements, flagellar elements and export machinery) did not affect the virulence of *S.* Typhimurium whether administered to mice orally or by i.p. injection.

In contrast, studies with the same *flhD* mutant indicated that flagella were essential for the induction of a complete inflammatory response in the bovine ligated ileal loop model (Schmitt *et al.*, 2001). Flagella of *S.* Typhimurium are known to be potent inducers of IL-8 which influences the recruitment of neutrophils (Gewirtz *et al.*, 2000; McCormick *et al.*, 1998). Experiments performed in the mouse typhoid model may measure aspects of virulence different from those critical for tissue culture invasion assays or for infection of ligated ileal loops. More experiments are required, which perhaps compare mutants in different serovars in different model systems, to better understand the role of flagella in pathogenesis and disease.

1.1.3.3.5 Enterotoxins.

Despite intensive study, the number, nature and pathogenic relevance of *Salmonella* toxins remains unclear. Presented in this section are a number of candidate proteins which have been identified as causing cellular and tissue damage.

A *S.* Typhimurium enterotoxin (Stn) which promotes fluid secretion in rabbit ileal loops has been isolated (Chary *et al.*, 1993). Stn is structurally distinct from other enterotoxins such as cholera toxin, although it displays a similar ADP-ribosylation function (Chopra *et al.*, 1994). A study by Prager *et al.* (1995) found that isolates of *Salmonella* serovars Typhi, Typhimurium, Enteritidis and Dublin all carried *stn*. However, of the genotypically *stn* positive isolates, only 45% were phenotypically positive, as indicated by cytotoxicity analysis of cell free supernatants. *S.* Typhimurium mutants unable to produce Stn show reduced virulence in mice (Chopra *et al.*, 1994), although this toxin appears to have no effect in bovine ileal loops when secreted from either *S.* Typhimurium or *S.* Dublin (Wallis *et al.*, 1999; Watson *et al.*, 1998).

A virulence plasmid encoded toxin has also been identified, which causes the lysis of murine macrophage monolayers *in vitro* after internalisation of *S.* Typhimurium, *S.* Dublin or *S.* Choleraesuis (Guilloteau *et al.*, 1996). *In vivo*, fewer peritoneal and splenic macrophages were recovered from mice infected with wt *S.* Dublin strains compared with plasmid-cured derivatives (Guilloteau *et al.*, 1996).

The destruction of murine M cells and penetration of adjacent enterocytes by invasive *S.* Typhimurium (Jones *et al.*, 1994) has also been attributed to the expression of a haemolysin encoded by *slyA* (Daniels *et al.*, 1996), which had earlier been implicated in survival within the macrophage (Libby *et al.*, 1994). A recent study by Lodge *et al.* (1999) examined the histotoxic effects of invasive *S.* Typhimurium strain GM3 in rabbit ileal loops. When sterile culture filtrates prepared from loops challenged with bacteria *in vivo* were transferred to fresh tissue *in vitro*, lesions were produced which were comparable to those seen following exposure to *Salmonella*. An *S.* Dublin isolate (3246) was found to produce similar lesions to *S.* Typhimurium GM3.

The demonstration of cellular and tissue damage has yet to be conclusively linked to the degree of enteropathogenicity displayed by *Salmonella* isolates. Further study is required that is considerate of the effects of using different animal models.

1.1.4 Disease state and host survival.

Depending on the immune status of the infected individual, non-typhoidal infections will usually result from the ingestion of ~ 10^5 - 10^6 cfu, whereas the establishment of typhoid fever requires ~ 10^6 - 10^9 cfu (Hook, 1990; Hornick, 1970). Symptoms of gastroenteritis caused by non-typhoidal salmonellosis normally appear within 6 - 24 hours and can last up to a week (Hook, 1990), and include nausea and vomiting which is then followed by abdominal pain and diarrhoea. Transient infections or gastroenteritis in humans are usually treated with symptomatic therapy including fluid and electrolyte replacement. In contrast, typhoid fever is a delayed systemic illness and incubation time ranges from one week to a month after infection. Clinical symptoms include high fever, abdominal pain, transient diarrhoea or constipation, nausea, vomiting, muscle aches. Patients suffering from bacteraemia or enteric fever may benefit from antibiotic treatment, although the increase in multi-drug resistant *Salmonella* species, and the danger of LPS release, hampers effective treatment.

The symptoms of typhoid can be induced in healthy volunteers by injection of isolated LPS, implicating this component of the bacterial cell wall in the establishment of endotoxic shock (Hornick, 1970). Isolated LPS associates with LPS binding protein present in human plasma and this complex is then recognized by CD14, a primary endotoxin receptor expressed on all macrophages (Wright *et al.*, 1990). Endotoxin-stimulated murine macrophages secrete a number of cytokines, particularly TNF-α, IL-1, IL-6, which are mediators in the development of endotoxic shock (Beutler *et al.*, 1985; Waage *et al.*, 1989; Waage and Espevik, 1988). The recent description of a *waaN* mutant of *S.* Typhimurium confirms the critical involvement of the Lipid A component in the induction of endotoxic shock in the mouse typhoid model (Khan *et al.*, 1998; also refer to section 1.1.3.3.3).

Host survival of infection by virulent *Salmonella* is determined by the ability to control bacterial growth and dissemination in the critical early period before the onset of acquired immunity. In mouse studies, the critical systemic load appears to be ~ 10^8 bacteria, in excess of which mice succumb to endotoxic shock. Bacterial burdens in non-lethal infections are restricted below this level, and the organisms can persist for several weeks (Eisenstein, 1999; Mäkelä and Hormaeche, 1997). During this time adaptive immune responses (further discussed in section 1.2.2) are generated, leading to bacterial clearance and long-term immunity to reinfection.

1.2 Host defence against *Salmonella* infection.

The host response to *Salmonella* infection can be divided into several elements - non-specific or innate immunity, acquired immunity and cytokine responses - which are individually examined below, and have recently been reviewed by Jones and Falkow (1996), Lalmanach and Lantier (1999), Mastroeni *et al.* (2001), and Mittrücker and Kaufmann (2000).

1.2.1 Innate immunity.

1.2.1.1 Toll-like receptors and epithelial cell responses to infection.

Lymphoid cells, particularly antigen presenting cells, are able to recognise and react to microbes via toll-like receptors (TLRs) (recently reviewed by Imler and Hoffmann, 2001). Named due to a high degree of homology to the *Drosophila* Toll protein, nine mammalian receptors have now been identified (Rock *et al.*, 1998). All members of the Toll family are membrane proteins with a single membrane-spanning domain, similar extracellular domains which include 18 - 31 leucine rich repeats (LRR), and similar cytoplasmic domains of ~ 200 amino acids which are homologous to the cytoplasmic domain of the IL-1 receptor. TLRs have

been shown to transduce signals through intracellular components shared by the IL-1 signalling machinery, ultimately leading to the activation of transcription nuclear factor (NF)-κB and proinflammatory cytokine production, stimulating lymphoid cell activation and recruitment (Burns *et al.*, 1998; Medzhitov *et al.*, 1998; Muzio *et al.*, 1998). The best characterised toll-like receptors, TLR2 and TLR4, are discussed briefly below.

CD14 has been recognised as the major receptor responsible for the effects of LPS on monocytes/macrophages and neutrophils (reviewed by Fenton and Golenbock, 1998). *tlr4* (initially termed *lps*) was discovered from experiments in mice that expressed normal CD14 but were naturally hyporesponsive to high doses of LPS, and were consequently highly susceptible to infection by Gram-negative (but not Gram-positive) bacteria (Qureshi *et al.*, 1999; Vogel, 1992). Subsequent mutation and transfection experiments have confirmed that the TLR4 receptor, in conjunction with co-receptor CD14, is necessary for sensitivity to LPS (Chow *et al.*, 1999; Hoshino *et al.*, 1999). Although the mechanism whereby TLR4 (and other TLRs) transmits signals is not fully understood, recent data have shown its interaction with an adapter protein MyD88, which also contains a LRR (Fitzgerald *et al.*, 2001; Schnare *et al.*, 2001). MyD88 forms complexes with other proteins (such as IRAK-2) to activate NF-κB, and MyD88-deficient mice are defective in the activation of antigen-specific T helper type 1 (Th1) but not Th2 immune responses, suggesting that distinct pathways of the innate immune system control activation of the two effector arms of adaptive immunity (Schnare *et al.*, 2001).

Experiments involving the transfection of human kidney cells with *tlr2* initially concluded that this receptor, which is normally recruited to macrophage phagosomes (Underhill *et al.*, 1999), was also involved in responses to LPS (Kirschning *et al.*, 1998; Yang *et al.*, 1998). However, Heine *et al.* (1999) demonstrated that hamster macrophage and ovary cells carrying a null mutation in *tlr2* were still responsive to LPS, and Tapping *et al.* (2000) suggested that impurities in commercially available LPS may have initially led to the incorrect assigning of *tlr2* function. Transfection experiments involving human kidney cell exposure to Gram-positive

bacteria *Staphylococcus aureus* and *Streptococcus pneumoniae*, and hamster ovary cell exposure to purified peptidoglycan and lipoteichoic acid, demonstrated that TLR2 was responsible for signalling and activation of NF-κB in response to such stimuli (Schwandner *et al.*, 1999; Yoshimura *et al.*, 1999). Recent transfection experiments have confirmed TLR2 is responsible for responding to bacterial lipoproteins, activation of NF-κB and reactive oxygen intermediates, and induction of host cell death (Aliprantis *et al.*, 2001; Birchler *et al.*, 2001). This suggests that TLR2 may have multiple roles in the response to bacterial pathogens. Other TLRs which have recently been characterised include TLR5 and TLR9, which have been shown to respond to unmethylated CpG nucleotides in bacterial DNA (Hemmi *et al.*, 2000) and flagellin (Hayashi *et al.*, 2001), respectively.

Epithelial cells are also able to respond to LPS, but via a mechanism independent of surface TLR activation. Of several epithelial cell lines tested, few expressed TLR4 and none expressed CD14 Cario *et al.* (2000). Interestingly, bacterial LPS directly injected into the cytosol of epithelial cells results in the activation of NF-κB (Philpott *et al.*, 2000). Subsequent experiments led to the identification of CARD4 (also known as Nod1), a cytosolic protein with a carboxy-terminal LRR domain that is thought to interact with LPS directly or as a protein complex with other unidentified molecules (Girardin *et al.*, 2001; Inohara *et al.*, 2001). Furthermore, the activation of NF-κB does not appear to involve the same intracellular cascade as that activated following TLR4 engagement (Girardin *et al.*, 2001).

1.2.1.2 Natural-resistance-associated-macrophage proteins.

Studies in inbred mice have allowed the identification of a single autosomal dominant gene called natural-resistance-associated-macrophage protein 1 (*Nramp1*; formerly *Ity/lsh/bcg*, Vidal *et al.*, 1993), which determines innate susceptibility to *Salmonella* infection (recently reviewed by Blackwell, 2001; Forbes and Gros, 2001; Gruenheid and Gros, 2000). Early work

showed that innately susceptible mice (*Nramp1*$^{-/-}$), administered virulent *Salmonella* by either i.v. or i.p. injection, succumbed to infection following administration of fewer than ten bacteria, whereas innately resistant mice (*Nramp1*$^{+/+}$) were able to survive a challenge dose of > 10^3 bacteria (Plant and Glynn, 1979; Plant and Glynn, 1982). *Nramp1*$^{+/+}$ macrophages were found to be better able to control the intracellular growth of *Salmonella in vitro*, though the rate of bacterial ingestion is not different to that of *Nramp1*$^{-/-}$ macrophages (van Dissel *et al.*, 1986).

Characterisation of Nramp1 has shown that the inability of innately susceptible mice to control infection is associated with a non-conservative glycine to aspartic acid substitution at position 169 (G169D) (Malo *et al.*, 1994). Nramp1 is an integral membrane protein, ~ 100 kDa in size, which is localised to the endosomal/lysosomal compartment and is rapidly recruited to the membrane of the particle-containing phagosome upon phagocytosis (Gruenheid *et al.*, 1997). The protein functions as a pH-dependent transporter of divalent cations, such as Mn^{2+} and Fe^{2+} (Jabado *et al.*, 2000), and it is proposed that depletion of these cations within the phagosomal compartment renders bacterial enzymes (such as superoxide dismutases) inactive, while concomitantly retarding bacterial growth (Supek *et al.*, 1996).

Nramp1 is expressed in the resident phagocytic cells of the spleen, liver and lungs, but is most abundant in circulating monocyte/macrophages and neutrophils (Lissner *et al.*, 1983; Vidal *et al.*, 1993). Experiments demonstrate that *Nramp1* mRNA in the RAW264.7 macrophage cell line is induced by treatment with bacterial LPS, interferon (IFN)-γ (further discussed in section 1.2.3), and proinflammatory stimuli (Govoni *et al.*, 1997).

1.2.1.3 Antimicrobial agents and peptides.

Antimicrobial agents and peptides are secreted by both neutrophils and macrophages (MacFarlane *et al.*, 1999; Vazquez-Torres and Fang, 2001), and form a significant part of the innate immune system since they constitute almost 7% of total protein within these cells. Over

40 have so far been discovered (Boman, 1995), including polymyxins (membrane permeability disrupting antibiotics) (Storm *et al.*, 1977), defensins and melittin (Vaara, 1992). Small cationic proteins, such as defensins and melittin, bind to and permeabilise the outer membrane of Gram-negative bacteria, reaching the inner cytoplasmic membrane and lysing the cell (Boman, 1995; Vaara, 1992). Interestingly, from the bacterial viewpoint, activation of the PhoP/PhoQ regulon can lead to LPS modification, increasing resistance to such anti-microbial peptides (Guo *et al.*, 1998). Mice depleted of neutrophils by the granulocyte-depleting monoclonal antibody RB6-8C5 are far more susceptible to *Salmonella* infections, and bacteria were found to grow to exceedingly high numbers in the livers and spleens, in comparison to untreated mice (Conlan, 1996; Conlan, 1997). In humans "specific granule deficiency" is a genetic disorder that results in a lack of defensins, and people with this condition are prone to severe and recurrent microbial infections (Lehrer *et al.*, 1993).

1.2.1.4 The role of complement in *Salmonella* infection.

As described in section 1.1.3.3.3, complement performs a vital role in both innate and acquired immunity. The complement cascade can be activated by *Salmonella* via the classical or alternative pathways, which leads to the opsonisation of bacteria with C3b, the generation of proinflammatory peptides C3a and C5a, and the formation of the MAC which is required for bacterial lysis.

Although LPS can efficiently activate complement, long molecules can physically restrict the insertion of the MAC into the bacterial cell membrane. In the mouse typhoid model, experiments have shown that an inverse relationship exists between complement fixation (as determined by O-antigen sugar composition) and virulence (Makela *et al.*, 1973; Valtonen *et al.*, 1975; Valtonen, 1970; Valtonen and Makela, 1971). The rate of bacterial uptake by macrophages *in vitro* also reflects the differential rate of complement activation by these

isogenic O-antigen polysaccharide variants (Liang Takasaki *et al.*, 1983; Liang Takasaki *et al.*, 1982; Saxen *et al.*, 1987; Valtonen, 1970). Moreover, destroying complement in mice by pre-treatment with cobra venom factor reduces the ability of the host to clear *S.* Typhimurium that express LPS which efficiently activate complement (Liang Takasaki *et al.*, 1983). This suggests that complement-dependent phagocytosis is an important host defence mechanism *in vivo*.

To further elucidate the role of the complement system in immunity to *Salmonella* Warren *et al.* (2002) recently investigated *S.* Typhimurium infection of *Nramp1*$^{+/+}$ mice (via i.p. or i.v. administration) deficient in C1q (*C1qa*$^{-/-}$), the first component of the classical pathway. A role for C1q in the opsonisation of salmonellae has already been suggested (Euteneuer *et al.*, 1985; Euteneuer *et al.*, 1986; Ryan *et al.*, 1989). However, in comparison to control animals, complement-deficient mice were found to be significantly more susceptible to *Salmonella* infection by either route of administration, as determined by assessing hepatic and splenic bacterial burdens at various time points. Bacteria were removed from the bloodstream less rapidly in complement-deficient mice than in control animals, but only after the first fifteen minutes following infection. By 24 hours, no bacteria were detected in the circulation in either experimental group. In both *in vitro* and *in vivo* comparisons, *C1qa*$^{-/-}$ macrophages were shown to have normal respiratory burst functions, nitric oxide production, and phagocytosing capabilities when compared to control macrophages. However, irrespective of the serum used for opsonisation, *in vitro* infection assays showed that *C1qa*$^{-/-}$ macrophages contained significantly greater numbers of bacteria. These results suggested, for the first time, an important role for C1q in protection against *Salmonella* infection *in vivo*. Furthermore, C1q may be involved in effector mechanisms by which phagocytes control the *Salmonella* replication, although additional studies are required to reveal these mechanisms.

1.2.2 Acquired immunity to *Salmonella*.

Studies of *S.* Typhimurium infection in mice have yielded conflicting reports with regards to the relative protective significance of humoral and cell-mediated immune responses. However, Eisenstein *et al.* (1984) reviewed the data and concluded that the *Nramp1* status of mouse strains used by various groups was a critical but unrecognised variable. This notion considerably clarified our understanding of the nature of protective immunity to *Salmonella.* Thus, in general, killed vaccines may be effective in protecting *Nramp1*$^{+/+}$ mice from (low challenge doses of) virulent *S.* Typhimurium, and transfer of immune serum between such animals is sufficient for the transfer of protection (Eisenstein *et al.*, 1984). In contrast, only live attenuated vaccines are protective in *Nramp1*$^{-/-}$ mouse strains. In such hosts the induction of cell-mediated immunity is essential, and passive transfer of immunity requires T cells and not serum (Mastroeni *et al.*, 1993c). Consistent with this interpretation of the literature, Michetti *et al.* (1992) showed that *in vivo* delivery of monoclonal secretory IgA directed against a carbohydrate epitope on the surface of *S.* Typhimurium was sufficient to confer protection against i.p. challenge in *Nramp1*$^{+/+}$ mice with the same strain.

Eisenstein (1998) also raised the question of whether humans are more like the innately resistant or innately susceptible mouse. Since parenteral administration of inactivated (heat-phenolised and acetone-inactivated) *S.* Typhi protects humans against challenge with virulent bacteria (DuPont *et al.*, 1971; Hornick *et al.*, 1970), it was suggested that humans are more like *Nramp1*$^{+/+}$ mice. This would therefore suggest that *Nramp1*$^{+/+}$ mice are more appropriate model for the study of human typhoid.

Early experiments involving antibody-mediated depletion or adoptive transfer of lymphoid cells demonstrated that T cells, and the cytokines which they secrete, are required for both recovery from primary infection and protective long term immunity (Chander *et al.*, 1986; Guilloteau *et al.*, 1993; Hochadel and Keller, 1977; Mastroeni *et al.*, 1993a; Mastroeni *et al.*, 1992; Nauciel, 1990). Most experiments have shown that $CD4^+$ T cells, as opposed to $CD8^+$ T cells, are more important for the control of *Salmonella* infection, and the induction of protection

by vaccination with attenuated *S.* Typhimurium (Hess *et al.*, 1996b; Mastroeni *et al.*, 1992; Nauciel, 1990; Pie *et al.*, 1997). As discussed below, the secretion of cytokines (particularly IFN-γ) by $CD4^+$ T helper cells is critical for the activation and anti-microbial activity of macrophages.

Evidence does exist to support the participation of $CD8^+$ T cells in immunity to *Salmonella*. Depletion of $CD8^+$ T cells reduces the ability to transfer protection against virulent *S.* Typhimurium (Mastroeni *et al.*, 1992; Nauciel, 1990). Work by Bao *et al.* (2000) demonstrated that oral administration of attenuated *S.* Typhimurium resulted in an increase in both intraepithelial and lamina propria $CD8^+$ T cells. $CD8^+$ T cells can differentiate into cytolytic T cells (CTLs), and several reports have described the generation of CTLs which lyse cells infected with *S.* Typhimurium (Aggarwal *et al.*, 1990; Pope and Kotlarski, 1994; Pope *et al.*, 1994; Weintraub *et al.*, 1997).

1.2.3 Cytokines induced in response to *Salmonella* infection.

Cytokines are key regulators of the host response to infection, and several have been implicated in the clearance of *Salmonella*. During early infection, cell wall components of *Salmonella*, particularly LPS, induce a massive inflammatory response in the surrounding tissue, resulting in the expression of inflammatory cytokines (particularly IFN-γ and tumour necrosis factor (TNF)-α), and effecting the activation and recruitment of lymphoid cells (Eckmann *et al.*, 1996; Jung *et al.*, 1995; McCormick *et al.*, 1998).

IFN-γ produced by T cells is responsible for the enhancement of macrophage anti-microbial activity, and is induced during early infection as shown by increased levels of IFN-γ mRNA in the gut-associated lymphoid tissue (GALT) and spleens of mice orally challenged with *S.* Typhimurium (Ramarathinam *et al.*, 1991). *In vivo* depletion of this cytokine by administration of antibodies dramatically increases the severity of *Salmonella* infection

(Muotiala, 1992; Muotiala and Makela, 1990). Furthermore, IFN-γ deficient mice are highly susceptible to both i.v. and oral challenge with strains which are readily controlled by normal mice (Bao *et al.*, 2000; Hess *et al.*, 1996b; VanCott *et al.*, 1998).

TNF-α production has been implicated in limiting the *in vivo* growth and systemic spread of *Salmonella* (Ables *et al.*, 2001). Gulig *et al.* (1997) showed that neutralising either IFN-γ or TNF-α resulted in increased splenic infection with *S.* Typhimurium following oral infection. Depletion of IFN-γ or TNF-α was also shown to exacerbate sublethal primary infection in *Nramp1*$^{+/+}$ mice, and secondary infection in vaccine protected *Nramp1*$^{-/-}$ mice, by virulent *S.* Typhimurium (Mastroeni *et al.*, 1992; Mastroeni *et al.*, 1993b).

Cytokines important for the induction and enhanced expression of IFN-γ include IL-12 and IL-18 (Mastroeni *et al.*, 1999; Mastroeni *et al.*, 1996). Macrophages and dendritic cells are major sources of these cytokines, with the expression of IL-12 upregulated by IFN-γ via positive feedback (Schaible *et al.*, 1999). IL-12 also directs the bias of the T cell response towards the Th1 cell type which is an important source of IFN-γ (Schaible *et al.*, 1999; Snapper and Paul, 1987). Numerous studies have demonstrated that infection of mice with *Salmonella* induces a Th1 biased response, characterised by the production of significant amounts of IFN-γ (Eckmann *et al.*, 1996; Matsui and Arai, 1992; Pie *et al.*, 1997; Ramarathinam *et al.*, 1991; Thatte *et al.*, 1993).

1.2.4. Influence of host genetics in *Salmonella* infection.

Studies have shown that immunity to *Salmonella* infection depends on the genetic background of the mouse strain used. An example already discussed is that of *Nramp1*, which is expressed during early *Salmonella* infection as part of the innate immune system. However, MHC-coded genes (*H-2* genes in mice) can also influence the immune response to *Salmonella* infection.

In principle, efficiency of antigen presentation (as determined by the ability to bind processed peptide in the binding cleft of the MHC molecule) relates to the expression of a particular MHC haplotype (defined as the total set of alleles of closely linked loci), which in turn influences lymphocyte stimulation and proliferation. Studies that compared *H-2* congenic mice infected with *S.* Typhimurium, showed that the expression of certain *H-2* haplotypes (particularly $H\text{-}2^a$, $H\text{-}2^k$ and $H\text{-}2^f$) was advantages in terms of the response to and clearance of *Salmonella* (Harrington and Hormaeche, 1986; Hormaeche *et al.*, 1985; Maskell and Hormaeche, 1986; Nauciel *et al.*, 1988). Moreover, the observed effects of *H-2* gene expression were independent of *Nramp1* expression (Harrington and Hormaeche, 1986; Nauciel *et al.*, 1988).

1.3 Vaccine studies.

Early volunteer studies with parenteral heat- or acetone-killed whole-cell *S.* Typhi vaccines showed that they were able to confer protection against typhoid (DuPont *et al.*, 1971; Gilman *et al.*, 1977; Hornick *et al.*, 1970; Levine *et al.*, 1976). However, this protective effect could be overcome if subjects ingested a large inoculum ($> 10^7$ cfu) of virulent bacteria (Hornick *et al.*, 1970), analogous to the limited protection conferred by killed *S.* Typhimurium vaccines in $Nramp1^{+/+}$ mice (Eisenstein *et al.*, 1984). Subsequent field trials which assessed the efficacy of these parenteral vaccines confirmed the observations made in volunteer studies (Levine, 1999). Nevertheless, attenuated *S.* Typhi oral vaccine Ty21a (Germanier and Fuer, 1975) and parenteral Vi capsular polysaccharide (Acharya *et al.*, 1987; Klugman *et al.*, 1987; Tacket *et al.*, 1986) have since replaced parenteral killed whole-cell vaccines as the recommended prophylaxis against typhoid fever.

Ty21a, one of the first attenuated *Salmonella* strains produced, was developed by treating the virulent wt *S.* Typhi Ty2 with the mutagen nitrosoguanidine (Germanier and Fuer, 1975). Initially, its attenuation was attributed to a mutation in *galE* (encoding a galactose epimerase

involved in LPS core and O-antigen sugar biosynthesis), but Hone *et al.* (1988b) later showed that *S.* Typhi Ty2 carrying a precise genetic mutation in *galE* was still capable of causing typhoid-like illness. This indicated that Ty21a carried other cryptic mutations, which remain unidentified. Oral vaccination with Ty21a induces both humoral (serum IgG, IgM and IgA and mucosal IgA) and cell-mediated immunity (Cancellieri and Fara, 1985; Forrest *et al.*, 1991; Kantele *et al.*, 1986; Kantele *et al.*, 1991; Kantele *et al.*, 1999; Murphy *et al.*, 1987; Tagliabue *et al.*, 1986). Although Ty21a is Vi-antigen negative and extremely slow growing, it is protective in humans (Levine *et al.*, 1987a) and is still being used in field trials. The most recent trial compared the delivery of the vaccine strain in either an enteric-coated capsule or liquid formulation, with the latter proving superior and conferring 77% protection over three years and 62% protection over seven years of follow up (Levine *et al.*, 1999). However, as in earlier trials, multiple doses of the vaccine were required to achieve this level of protection. Interestingly, a Vi^+ variant of Ty21a was unable to induce (serum or secretory) antibodies against Vi-antigen (Cryz *et al.*, 1989; Tacket *et al.*, 1991).

Purified Vi-antigen is thought to act solely by the induction of protective antibody. Virtually all *S.* Typhi strains that are isolated from patients with acute typhoid fever produce this antigen, which is encoded by two chromosomal loci (*viaA* and *viaB*) (Johnson and Baron, 1969). However, purified Vi behaves like a T-independent antigen, as antibody titres are not increased following the (parenteral) administration of booster vaccinations (Acharya *et al.*, 1987; Klugman *et al.*, 1987; Tacket *et al.*, 1986). Moreover, Vi-antigen is not as immunogenic in infants below two years of age, in which only transient responses develop.

Attempts to develop an improved vaccine against typhoid have focussed on deriving a better live attenuated vaccine, one which is genetically defined (and therefore acceptable to regulatory authorities), adequately immunogenic (induces protection after a single dose), and completely safe. Results of human clinical trials with other candidate *S.* Typhi vaccines (see below) have highlighted that a balance must be achieved between genetic attenuation and

immunogenicity. However, developing such vaccine candidates is made more difficult by the species specificity of *S.* Typhi. As mentioned previously, *S.* Typhimurium infection in mice is the accepted model for human typhoid. Although this model has provided invaluable information in the understanding of *Salmonella* pathogenicity (understanding the molecular aspects of pathogenicity islands for example), the information learned cannot be immediately applied to the understanding of human typhoid (a topic recently reviewed by Santos *et al.*, 2001). For example, mutations in *galE* render *S.* Typhimurium avirulent in mice (Germanier and Furer, 1971; Hone *et al.*, 1987), whereas a defined *galE S.* Typhi mutant is still virulent in humans (Hone *et al.*, 1988b). Furthermore, the presence of virulence factors encoded by the virulence plasmid in *S.* Typhimurium and their absence from *S.* Typhi (discussed in section 1.1.3.3.2), further complicates the interpretation of data obtained from the mouse model.

It is clear however that the strengths of the mouse model outweigh its disadvantages. For example, the model has allowed the screening and evaluation of a range of attenuating mutations in *S.* Typhimurium, with the aim of transferring these into *S.* Typhi. There is a mouse-based test for candidate *S.* Typhi vaccines which is required by regulatory authorities. This involves i.p. injection of mice with bacteria suspended in hog gastric mucin (Dougan *et al.*, 1987b; Hone *et al.*, 1988b). This model is highly contrived, and moreover a *galE S.* Typhi strain which was clearly attenuated in this model remained pathogenic in man (Hone *et al.*, 1988b). A recent innovation, which involves intranasal (i.n.) administration of r*S.* Typhi in mice, may constitute a practical breakthrough and expedite the design and testing of *S.* Typhi-based vaccines for use in humans. I.n. immunisation of mice with *S.* Typhi expressing tetanus toxin fragment C (FrgC) allowed, for the first time, the quantitation of strong serum anti-FrgC and anti-LPS antibody responses in comparison to oral immunisation with the same construct, which was only able to generate weak anti-vector responses (Galen *et al.*, 1997a). Furthermore, the former mice were protected against challenge with 100 times the 50% lethal dose of tetanus toxin.

Presently, based on phase 1 and 2 clinical trial results, three attenuated *S.* Typhi vaccine strains demonstrate an encouraging balance of immunogenicity and low reactogenicity. All are Vi-antigen negative and additionally carry attenuating mutations in various biosynthetic genes. These strains are CVD 908-*htrA* (which harbours mutations in *aroA*, *aroD* and *htrA*; refer sections 1.3.1.1 and 1.3.1.2) (Tacket *et al.*, 1997b; Tacket *et al.*, 2000), Ty800 (has a deleted *phoP/phoQ* regulon; refer to section 1.3.1.2) (Hohmann *et al.*, 1996a), and χ4073 (which harbours mutations in *cya*, *crp* and *cdt*; refer to section 1.3.1.2) (Curtiss and Kelly, 1987; Tacket *et al.*, 1997a).

The increasing incidence of non-typhoidal salmonellosis (reviewed by Rabsch *et al.*, 2001) indicates a need for live attenuated vaccines based on other *Salmonella* serovars. Such vaccine candidates have been tested in calves, horses, chickens and pigs with reasonable success (Cerquetti and Gherardi, 2000; Cooper *et al.*, 1994; Griffin and Barrow, 1993; Jones *et al.*, 1991; Lumsden and Wilkie, 1992; Lumsden *et al.*, 1991; Segall and Lindberg, 1993; Sheoran *et al.*, 2001; Villarreal-Ramos *et al.*, 1998; Zhang-Barber *et al.*, 1998). In hosts other than humans, vaccine development is much easier as the target species can be used to directly evaluate potential vaccine candidates.

Discussed below are biosynthetic, regulatory and virulence genes which have been mutated to construct live attenuated r*Salmonella.*

1.3.1 Evaluation of various mutations as attenuating markers for the development of live *Salmonella* vaccines.

1.3.1.1 Biosynthetic genes.

Hoiseth and Stocker (1981) were the first to evaluate *Salmonella* strains carrying precise deletions in genes involved in the biosynthesis of aromatic amino acids. Genes *aroA*, *aroC* and *aroD* encode enzymes which are essential for the synthesis of the chorismate intermediate

involved in the biosynthesis of aromatic amino acids, 2,3-dihydroxybenzoic acid (DHB) and *p*-aminobenzoic acid (pABA). DHB and pABA are precursors of enterochelin (iron chelator) and folic acid, respectively, and are not available in mammalian tissues. *Salmonella* carrying mutations in these genes are attenuated as they are limited in their ability to replicate and persist in cultured macrophages and host tissues, and are effective vaccines in several animal species (Fields *et al.*, 1986b; Hoiseth and Stocker, 1981; Stocker *et al.*, 1983).

Mutations in other biosynthetic pathway genes, such as *purA* and *purE* have also been constructed (McFarland and Stocker, 1987; O'Callaghan *et al.*, 1988). The enzymes encoded by *purA* and *purE* are necessary for purine synthesis. Studies of *purA* attenuated recombinant *S.* Typhimurium and *S.* Dublin have shown that these bacteria colonise the murine GALT poorly following oral immunisation, and elicit only weak serum and mucosal antibody responses against LPS and foreign antigen (Dunstan *et al.*, 1998; Sigwart *et al.*, 1989).

In humans, clinical trials of *purA aroA* attenuated *S.* Typhi strains 541Ty (Vi-antigen$^+$) and 543Ty (Vi-antigen$^-$) showed that these vectors were over attenuated and weakly immunogenic (Levine *et al.*, 1987b). In contrast, other *S.* Typhi strains have been shown to be under attenuated. An example of this is CVD 908, which is a derivative of *S.* Typhi Ty2 that carries deletions in *aroA* and *aroD* (Hone *et al.*, 1991). In phase 1 dose-response safety/immunogenicity trials a single dose of CVD 908 (of up to ~ $5x10^8$ cfu) was shown to be well tolerated and only excreted for three days after administration (Tacket *et al.*, 1992a; Tacket *et al.*, 1992b). However, 50% of subjects given higher doses (~ $5x10^7$ - $5x10^8$ cfu) developed bacteraemia four days after vaccination (Tacket *et al.*, 1992b). Although these infections were cleared from the bloodstream post day eight, infection beyond the GALT was considered a limitation for CVD 908 (further discussed in section 1.3.1.2).

A live attenuated *S.* Typhi strain (CVD 915) was constructed harbouring mutations in the *guaBA* operon (Wang *et al.*, 2001), which interferes with the biosynthesis of guanine nucleotides (Anderson *et al.*, 2000; Noriega *et al.*, 1996). Virulence comparisons using the hog gastric

mucin model showed that the 50% lethal dose (LD_{50}) of CVD 915 (7.7×10^7 cfu) was significantly higher than that of wild-type Ty2 (1.4×10^2 cfu) and was only slightly lower than that of Ty21a (1.9×10^8 cfu) (Wang *et al.*, 2001). Strong serum LPS and flagellar antibody responses were recorded in mice inoculated i.n. with CVD 915, which were higher than those elicited by Ty21a given by the same route. Moreover, splenocytes obtained from mice immunised i.n. with CVD 915 showed strong proliferation when stimulated with serovar Typhi antigens (flagella and whole-cell heat-inactivated *S.* Typhi). These encouraging preclinical data pave the way for phase 1 clinical trials in humans.

1.3.1.2 Regulatory and virulence genes.

An alternative method to attenuate *Salmonella* is to mutate genes involved with regulation and virulence. Live attenuated *S.* Typhi (Ty800) carrying a deleted *phoP/phoQ* regulon (section 1.1.3.2) was found to be safe in humans and quite immunogenic (Hohmann *et al.*, 1996a), while an *aroA phoP/phoQ S.* Typhi vaccine was over-attenuated and poorly immunogenic (Hohmann *et al.*, 1996b).

Mutations in the *cya* and *crp* genes have also successfully resulted in the attenuation of salmonellae. The *cya* gene encodes adenylate cyclase which is involved in the synthesis of cAMP, whereas *crp* encodes the cAMP receptor protein which complexes with cAMP to form the cAMP-CRP global regulatory protein complex. This complex in turn regulates expression of proteins involved in other biosynthetic processes, such as carbohydrate breakdown and transport, and the expression of cell surface outer membrane proteins, fimbriae and flagella (Curtiss *et al.*, 1989). *S.* Choleraesuis and *S.* Typhimurium strains carrying deletions of the *cya* and *crp* genes exhibit a diminished ability to reach the mesenteric lymph nodes and spleen following oral administration (Curtiss and Kelly, 1987; Kelly *et al.*, 1992). Mice immunised with these mutants are resistant to subsequent oral challenge with the respective wild type strains. Kelly *et al.*

(1992) also characterised an additional mutation in a region adjacent to *crp* in *S.* Choleraesuis, which restricted the ability of *cya crp* attenuated strains to colonise deep tissues, yet did not compromise vector immunogenicity. Termed *cdt* (colonisation of deep tissues), mutation of this gene was found to have the same beneficial effect in *cya crp* attenuated *S.* Typhi following oral administration in humans (Nardelli Haefliger *et al.*, 1996; Tacket *et al.*, 1997a). An inability to penetrate beyond the gut is obviously desirable for *Salmonella*-based vaccines.

Mutations in chromosomally encoded virulence genes, particularly *htrA*, have also allowed the construction of attenuated *Salmonella.* (Buchmeier *et al.*, 1993; Chatfield *et al.*, 1992c, Tacket *et al.*, 1997b; Tacket *et al.*, 2000). The *htrA* gene encodes a serine protease, and attenuation is thought to be due to an inability of the bacteria to function in harsh environments such as the gut, and intracellularly in macrophages. In mice, single-dose oral vaccination with *htrA* mutants as well as double *htrA aroA* mutants confers resistance against oral challenge with virulent salmonellae (Chatfield *et al.*, 1992c). Recently, a triple *aroC aroD htrA S.* Typhi mutant (CVD 908-*htrA*) was found to be safe in humans, and induce anti-LPS and anti-flagellar antibodies, as well as cell-mediated responses (lymphocyte proliferation and cytokine responses) (Tacket *et al.*, 1997b; Tacket *et al.*, 2000). More importantly, the triple mutant was shown to persist at lower levels beyond the gut mucosa than its progenitor, a double *aroC aroD S.* Typhi mutant called CVD 908 (discussed above in section 1.3.1.1).

1.3.2 Live attenuated *Salmonella* as delivery systems for heterologous antigens.

The ability of live attenuated *Salmonella* strains to induce both humoral and cell mediated immunity has prompted their use as carriers of genes derived from other organisms (Cardenas and Clements, 1992; Charles and Dougan, 1990; Chatfield *et al.*, 1989; Clements, 1987; Dougan, 1994; Dougan *et al.*, 1987a; Schodel, 1992; Schodel and Curtiss, 1995). Provided that such vectors retain the potential to protect against *Salmonella* infection, this

strategy allows construction of bi- or multi-valent vaccines, an attractive option for mass immunisation programmes in developing countries. Numerous studies have shown that oral immunisation of mice with r*Salmonella* induces bivalent protective immunity (Ascon *et al.*, 1998; Chatfield *et al.*, 1992b; Poirier *et al.*, 1988; Titball *et al.*, 1997). Furthermore, immunisation - either oral, i.n, vaginal or rectal - can lead to the generation of immunity at distal mucosal sites (Corthesy-Theulaz *et al.*, 1998; Gomez Duarte *et al.*, 1999; Gomez Duarte *et al.*, 1998; Nardelli Haefliger *et al.*, 1996; Nardelli Haefliger *et al.*, 1997; Pasetti *et al.*, 1999; Wu *et al.*, 1997), hence r*Salmonella*-based vaccines can be used against pathogens that infect various mucosal epithelia.

A major aim, with respect to the construction of multivalent r*Salmonella*-based vaccines, is to achieve stable expression of the heterologous antigen(s) in an immunogenic form at levels sufficient for priming the immune system against subsequent challenge. Controlled expression of antigen from multicopy plasmids is one method to maximise heterologous antigen synthesis in r*Salmonella*. However, multicopy plasmids, toxicity of the expressed protein(s) for the host cell, enzymatic degradation or incorrect folding of the expressed antigen(s), and the nature and the subcellular localisation of antigen can all affect the efficacy of r*Salmonella*-based vaccines. Discussed below are aspects of heterologous antigen expression that have been found to influence the immunogenicity of r*Salmonella*-based vaccines.

1.3.2.1 Plasmid expression systems.

Commercially available plasmids harbour different replicons which control plasmid copy number, from a few to over a hundred copies per cell. However, plasmid maintenance imposes a metabolic stress on the host bacteria due to the diversion of resources for plasmid replication, apart from the transcription and translation of the various plasmid-encoded proteins (including foreign antigen). Studies by Molina and Parker (1990) and Covone *et al.* (1998) have shown that

the use of high-copy number plasmids (which increase heterologous gene copy number and expression) can improve the immunogenicity of multivalent r*Salmonella*-based vaccines in mice. Nevertheless, increases in plasmid copy number can increase the metabolic stress on the bacterial host leading to decreased growth rates and plasmid loss (Boe *et al.*, 1987; Corchero and Villaverde, 1998; Glick, 1995). Lower copy number plasmids are advantageous in that plasmid stability is improved, which may also be a result of the reduction of heterologous antigen to non-toxic levels (Coulson *et al.*, 1994a; Coulson *et al.*, 1994b). Plasmid size has also been shown to affect the level of metabolic stress, with one study showing that the fitness of the bacterial host was shown to be inversely proportional to plasmid size (Cheah *et al.*, 1987).

Improvements in *in vivo* plasmid stability (and consequently heterologous antigen expression) can be achieved via a number of different mechanisms. One pertinent to the present study, is the use of "balanced-lethal" systems that ensure plasmid retention without the need for antibiotic selection. Plasmids which carry gene(s) encoding heterologous antigen can be modified to also carry biosynthetic markers instead of antibiotic resistance genes. The expression of the plasmid-borne biosynthetic gene compensates (*in trans*) for a chromosomal mutation in the same gene, which would normally prevent bacterial replication. The advantage over normal antibiotic-based expression plasmids, which may be lost *in vivo* due to the lack of selection pressure, is that bacteria are forced to retain a balanced-lethal plasmid for survival. Two examples of biosynthetic marker genes that have successfully been used to improve plasmid stability in r*Salmonella* are *asd* (Galan *et al.*, 1990; Nakayama *et al.*, 1988) and *thyA* (Morona *et al.*, 1991). *asd* encodes an enzyme common to the biosynthetic pathways of lysine, threonine and methionine, as well as diaminopimelic acid which is an essential constituent of the peptidoglycan component of the bacterial cell wall. *thyA* encodes a protein involved in DNA precursor biosynthesis, and mutants are non-pathogenic as they are incapable of synthesising DNA *in vivo* (Neuhard and Kelln, 1996). A recombinant plasmid expressing either $thyA^+$ or asd^+

is able to complement the ThyA$^-$ or Asd$^-$ phenotype of bacteria in which these genes have been deleted, thus encouraging plasmid retention.

An alternative method of improving plasmid stability has been recently demonstrated by Galen *et al.* (1999) using CVD 908-*htrA*, in which the balanced-lethal approach to plasmid maintenance was replaced by a mechanism involving post-segregational killing of plasmid-less daughter cells. The approach used was based on the naturally occurring *hok-sok* two component toxin-anti-toxin system, in which *hok* encodes a lethal pore-forming protein and *sok* mRNA binds to *hok* mRNA preventing translation (Gerdes *et al.*, 1997a; Gerdes *et al.*, 1997b). *sok* mRNA is highly susceptible to degradation by nucleases, hence continual transcription is required for cell viability. If the expression plasmid is lost from the bacteria, *hok* mRNA is translated leading to pore formation and cell death. Galen *et al.* (1999) further enhanced plasmid inheritance through the insertion of a naturally occurring plasmid partitioning function (*par*), which eliminates random segregation of plasmids in daughter progeny. In the absence of antibiotics, plasmids without either of these maintenance functions were lost from 85% of the bacterial population within 24 hours of *in vitro* culture . When both maintenance functions were present, ~ 95% of the population retained the expression plasmid for at least 24 hours and the amount of heterologous antigen expression was tripled.

1.3.2.2 Improving heterologous antigen gene expression.

Techniques for improving heterologous antigen expression include the use of *in vivo* regulated promoters, and gene integration into the bacterial chromosome. Promoter regulation allows low expression of antigen *in vitro* to reduce metabolic stress and minimise plasmid loss, while allowing maximal expression of antigen *in vivo*. One example is the *E. coli nirB* promoter, which is activated in anaerobic conditions (Oxer *et al.*, 1991) and has been successfully used to control expression of antigens derived from bacteria, parasites and viruses in r*Salmonella*

(Chatfield *et al.*, 1992a; Chen and Schifferli, 2001; Galen *et al.*, 1997b; Gomez-Duarte *et al.*, 1995; Huang *et al.*, 2000; Roberts *et al.*, 1999; Xu *et al.*, 1998). The promoter which controls expression of the PhoP-activated gene *pagC* is activated once bacteria are within macrophages, and has been successfully used to drive foreign antigen expression in attenuated *S.* Typhimurium (Hohmann *et al.*, 1995). Dunstan *et al.* (1999) compared *in vivo* regulated promoters *nirB*, *pagC*, and *katG* (also induced when *Salmonella* are within macrophages) for the control of foreign protein expression in an *aroA aroD* attenuated *S.* Typhimurium vector. The *in vitro* expression of β-galactosidase was most efficient when under the control of the *pagC* promoter (in comparison to *nirB* and *katG*); β-galactosidase activity was at its lowest in bacteria grown in non-inducing (aerobic) conditions, and at its highest in inducing (anaerobic) culture conditions. Similarly, luciferase activity *in vivo* was highest when under the control of the *pagC* promoter, as detected by assaying luminescence of Peyer's patch homogenates. Furthermore, mice immunised with *aroA aroD S.* Typhimurium expressing tetanus toxin fragment C (FrgC) under the control of *pagC* mounted the highest anti-toxin serum antibody responses.

A recent study by Bumann (2001) investigated the effect of regulated versus constitutive foreign antigen expression on the colonisation potential and antigen-specific $CD4^+$ T cell responses induced by *aroA S.* Typhimurium expressing green fluorescent protein (GFP) fused to ovalbumin (OVA). Various inducible promoters were selected for the *in vitro* and *in vivo* regulated expression of GFP-OVA. Mice were orally immunised with *aroA S.* Typhimurium harbouring the various promoter constructs, and bacteria harvested five days later from Peyer's patches for the quantitation of GFP-OVA production by two-colour flow cytometry. Consistent with the findings of Dunstan *et al.* (1999), *aroA S.* Typhimurium harbouring pP_{pagC}GFP-OVA induced the greatest amount of GFP-OVA per cell *in vivo*, while having the lowest *in vitro* activity (when grown under non-inducing aerobic conditions). This construct also induced the most potent ovalbumin-specific cellular immune responses. These results clearly highlight the advantage of *in vivo* regulated antigen expression.

Alternatively, in order to reduce the toxic effects of foreign antigen overexpression and/or to overcome plasmid stability problems, foreign genes can be integrated into the chromosome. Hone *et al.* (1988a) developed a system whereby genes encoding foreign antigen could be incorporated into the chromosome (via suicide vector mediated recombination) next to the histidine (*his*) biosynthesis operon of *Salmonella.* This system was successfully applied to construct r*Salmonella* expressing *E. coli* fimbrial antigen K88 from the chromosome. The resulting construct was immunogenic in mice, and showed much enhanced retention of the foreign gene, compared with a construct in which the same gene was plasmid derived (Hone *et al.*, 1988a). Strugnell *et al.* (1990) conducted an experiment in which the expression of FrgC in *S.* Typhimurium was chromosomally derived. However, mice exposed to this recombinant construct failed to develop anti-toxin immune responses due to lower levels of antigen expression. A possible way to avoid this problem may be to integrate into the chromosome several copies of the foreign gene of interest, under the control of strong promoters.

1.3.2.3 Intracellular localisation versus surface expression or secretion of heterologous antigen.

Numerous expression plasmids are available for the production of foreign antigen(s) in *Salmonella.* Several studies have shown that the localisation of the foreign antigen is a significant determinant of the immunogenicity of recombinant bacterial vaccines. Expression of foreign antigen intracellularly in r*Salmonella* can result in the induction of protective immunity when such constructs are used to immunise mice (Corthesy-Theulaz *et al.*, 1998; Huang *et al.*, 2001; Leary *et al.*, 1997). However, Haddad *et al.* (1995) demonstrated that expression of a malarial protein on the surface of *aroA S.* Typhimurium was as immunogenic as periplasmically expressed antigen, even though surface expression levels were 10 - 100 times lower than periplasmic levels. Charbit *et al.* (1997) also showed that serum IgG responses, following

injection of mice i.v. with *aroA S.* Typhimurium constructs expressing lambda phage receptor (LamB) or maltose binding protein (MalE), were greater when these proteins were exported beyond the cytoplasm.

Surface expression of proteins in both *E. coli* and *Salmonella* has been achieved using the ice-nucleation protein (Inp) derived from *Pseudomonas syringae* (Jung *et al.*, 1998a; Jung *et al.*, 1998b; Kim and Yoo, 1999; Kwak *et al.*, 1999; Lee *et al.*, 2000). Inp contains three domains: an N-terminal unique region (membrane anchored), a central repeating domain (CRD), and a C-terminal unique region. The Inp structure can tolerate replacement of the CRD by foreign polypeptides less than 60 amino acids in size, as shown by Lee *et al.* (2000) who used this approach to express epitopes from hepatitis B surface antigen and hepatitis C core protein on the surface of Ty21a. I.n. immunisation or i.p. injection of mice with the multivalent vector construct induced serum antibody responses that were significantly higher than those induced by a construct in which the viral antigens were expressed intracellularly.

In addition to surface display, several groups have shown that secretion of heterologous antigens from r*Salmonella* vectors enhances immunogenicity. Hess *et al.* (1996a) demonstrated that immunisation of mice with r*Salmonella* expressing cytoplasmically localised listeriolysin did not protect mice against lethal challenge with *Listeria monocytogenes*. In contrast, fusion of listeriolysin with a truncated *E. coli* haemolysin (HlyA) resulted in extracellular secretion of the foreign antigen (in the presence of co-expressed *E. coli* haemolysin export machinery), and mice immunised with r*Salmonella* expressing this construct were protected against lethal challenge with *L. monocytogenes*. Similarly, Russmann *et al.* (1998) demonstrated that mice immunised with r*Salmonella* secreting an immunodominant epitope of the murine lymphocytic choriomeningitis virus (LCMV) through the TTSS apparatus were protected against intracerebral challenge with virulent LCMV. In contrast, mice immunised with TTSS deficient r*Salmonella*, in which the epitope was retained intracellularly, were not protected. Gentschev *et al.* (1998) also showed that secretion, as opposed to intracellular expression, of p67 sporozoite antigen of

Theileria parva from *aroA S.* Dublin provided better protection in calves against parasitic challenge.

1.4 Consequences of pre-existing anti-vector immunity for the efficacy of live attenuated *Salmonella*-based vaccines.

The main focus of this thesis is to examine the significance of pre-existing anti-vector immunity in the context of using attenuated *Salmonella* strains as carriers for the delivery of foreign antigens. At the commencement of this thesis, there were only two studies that had directly addressed this issue, and these had provided conflicting results. Because of its potential implications for the use of *Salmonella*-based multivalent vaccines, it was deemed important to perform additional studies to further address the significance of pre-existing anti-vector immunity. Other reports have since been published and will be considered in the final chapter of this thesis together with results from the present study.

In 1991, Bao and Clements reported that antibody responses generated in mice by a r*Salmonella*-based vaccine expressing foreign antigen were unaffected by prior exposure to either the homologous or a heterologous (but cross-reacting) vector strain. Groups of BALB/c mice were orally primed with either *aroA his S.* Dublin (SL1438; serotype O-1,9,12) or *aroA his S.* Typhimurium (SL1479; O-1,4,5,12), with additional mice set aside as unimmunised controls. At various intervals after primary immunisation, vector-primed and control mice were orally dosed with strain EL23 (SL1438 expressing the B subunit of the heat labile toxin (LT-B) of enterotoxigenic *E. coli* (ETEC)). Serum and intestinal washout samples were prepared at various intervals to monitor serum IgG and mucosal IgA anti-LT-B and anti-LPS responses in the various groups. Prior exposure to the homologous *Salmonella* serotype (SL1438) actually potentiated the subsequent antibody response to LT-B upon challenge with EL23, more so than prior exposure to the heterologous serotype (SL1479). This potentiation was positively

correlated with antibody responses directed against the LPS of the priming strain; these responses were not significantly different in animals primed with either homologous (SL1438) or heterologous (SL1479) *Salmonella* serotypes.

In contrast to Bao and Clements, Attridge *et al.* (1997) reported that pre-existing anti-vector immunity could result in hyporesponsiveness to heterologous antigen subsequently presented by the same vector. In this study, groups of BALB/c mice were orally primed with *Salmonella* Stanley (*S.* Stanley; O-1,4,5,12), with additional mice set aside as unimmunised controls. *S.* Stanley is naturally attenuated in mice and persists in the Peyer's patches for several weeks. At various intervals after primary immunisation, sub-groups of vector-primed and control mice were orally dosed with r*S.* Stanley expressing the *E. coli* fimbrial protein K88. Serum was prepared at various time points to monitor anti-LPS and anti-K88 responses. The serum anti-K88 responses in mice which had been vector-primed ten weeks earlier were completely inhibited in comparison to controls which developed sustained primary responses. Mice primed either four or twenty weeks prior to immunisation with r*S.* Stanley-K88 also showed reduced responses to K88, but the inhibition was not as consistent in these groups. Regardless of the interval between vector-priming and administration of r*S.* Stanley-K88, primed mice showed secondary responses to vector LPS, whereas control mice developed primary anti-LPS responses. An attempt was made to measure mucosal IgA responses, but inconsistent results suggested that the procedure used for collecting intestinal samples was unreliable. In comparing the protocols of the two studies described above, there are two variables which stand out as having the potential to at least partly explain the discordant findings made.

1.4.1 Comparison of experimental protocols: significance of the vector strain.

The first major difference between these two studies was that different *Salmonella* vectors, probably with different GALT colonising potential, were used. SL1438 harbours

mutations in *his* and *aroA*. As described above (section 1.3.1.1) mutants in *aroA* are unable to synthesise intermediates DHB and pABA in the aromatic biosynthesis pathway. While histidine is available within host tissues, DHB and pABA are not, hence *aroA* mutants are attenuated as they are limited in their ability to replicate within the host. To compensate for the (presumed) inability of their *aroA* vector to persist in the GALT, Bao and Clements (1991) primed and boosted mice with two doses of ~ 10^{10} cfu spaced four days apart.

In contrast, wt *S.* Stanley used as a vector by Attridge *et al.* (1997) is naturally attenuated for mice, but colonises the Peyer's patches for at least four weeks following a single dose of ~ 10^9 cfu. This extended GALT colonisation may have induced stronger anti-vector immune responses, perhaps explaining the observed hyporesponsiveness to K88 (further discussed in section 1.4.3) (Attridge *et al.*, 1997). Consistent with this explanation is the finding that lowering the priming dose of *S.* Stanley from ~ 10^9 to ~ 10^6 cfu resulted in an undetectable level of GALT colonisation and essentially prevented the development of hyporesponsiveness to K88.

1.4.2 Comparison of experimental protocols: significance of the foreign antigen.

The second difference in the two aforementioned studies was the nature and location of the heterologous antigens. The foreign antigen used by Bao and Clements was LT-B, which has been demonstrated to be a strong mucosal immunogen and adjuvant (de Haan *et al.*, 1998; Richards *et al.*, 2001; Verweij *et al.*, 1998). Briefly, the LT holotoxin is a multimeric protein consisting of a single enzymatic A subunit of 28 kDa (LT-A) and a pentamer of five identical B subunits of 11.6 kDa (Gill *et al.*, 1981). In *E. coli*, pre-LT-A and pre-LT-B immature peptides are formed in the cytoplasm and transported into the periplasm to form A and B subunits, which are then assembled into the holotoxin (Hofstra and Witholt, 1985). LT remains in the periplasm of ETEC strains (Hirst *et al.*, 1984), but is released from the cell upon exposure to factors likely to be present in the intestine (Hunt and Hardy, 1991). The B-subunit pentamer mediates the

specific binding of the holotoxin to ganglioside GM_1 present on the brush border membranes of the intestinal epithelium (Merritt *et al.*, 1994). The formation of the pentameric structure and binding of host monoganglioside is crucial for the adjuvanticity and immunogenicity of the holotoxin (Nashar *et al.*, 1996). Following binding, holotoxin is internalised into vesicles that are transported retrogradally to the Golgi complex (Bastiaens *et al.*, 1996; Majoul *et al.*, 1996). The holotoxin is then disassociated, and the A subunit catalyses the ADP-ribosylation of the stimulatory GTP-binding protein of the adenylate cyclase enzyme complex on the basolateral surface of the epithelial cells, resulting in an increase in cyclic AMP production (Cassel and Selinger, 1977). This leads to the inhibition of absorption of sodium chloride and increased water efflux from intestinal crypt cells, thereby producing watery diarrhoea.

Attridge *et al.* (1997) utilised the *E. coli* fimbrial protein K88 (also known as F4) as a heterologous antigen, which is a colonisation factor originally characterised in porcine ETEC infections (Moseley *et al.*, 1986; Orskov and Orskov, 1966; and recently reviewed by Van den Broeck *et al.*, 2000). Ten genes, designated *faeA-J*, have been identified for regulation and biosynthesis of K88. FaeA and FaeB are specifically involved in the regulation of expression of the assembly genes consisting of *faeC-J* (Huisman *et al.*, 1994; Huisman and de Graaf, 1995; Mooi *et al.*, 1979; Shipley *et al.*, 1978). FaeG is the 27 kDa major subunit which encodes the adhesin responsible for binding Galα(1-3)Gal residues on unknown host cell receptors (Shipley *et al.*, 1981; Willemsen and de Graaf, 1992). The genes for *faeC*, *faeF*, *faeH*, *faeI* and *faeJ* all encode minor pilin subunits (Bakker *et al.*, 1992; Oudega *et al.*, 1989). Mutational analysis and immunoelectron microscopy have shown that these minor pilins are present at either the tip (FaeC) or at regular intervals throughout the length of the K88 fibrilla (FaeF, FaeH and FaeI) (Mooi *et al.*, 1984; Mooi *et al.*, 1982; Oudega *et al.*, 1989). The *faeD* and *faeE* genes encode the 81.7 kDa outer membrane assembly protein and the 24.8 kDa chaperone protein, respectively (Bakker *et al.*, 1991; Mol *et al.*, 1994; Mooi *et al.*, 1986). The FaeE periplasmic chaperone stabilises the major subunit FaeG, but also FaeH, FaeI and FaeF (Mooi *et al.*, 1983).

In contrast to the likely release of LT-B from r*Salmonella* upon contact with intestinal factors, Attridge *et al.* (1997) showed that K88 is surface expressed and is therefore likely to remain cell associated *in vivo*. Although a pivotal colonisation factor of porcine ETEC, there is no evidence that K88 (as expressed by r*Salmonella*) mediates attachment to murine gut epithelial cells, nor does it possess the properties of a mucosal adjuvant. These differences in heterologous antigen nature and location could be significant, depending on the mechanism(s) responsible for the hyporesponsiveness to foreign antigen presented by r*Salmonella* in vector-primed mice.

1.4.3 Mechanisms of hyporesponsiveness to heterologous antigen presented by r*Salmonella* in vector-primed mice.

Attridge *et al.* (1997) proposed two explanations for the hyporesponsiveness to K88 observed in vector-primed mice, and these are not mutually exclusive. First, vector pre-immunisation may elicit immune responses which subsequently curtail the colonisation and growth of the r*Salmonella* construct, thereby reducing primary immune responses against the passenger antigen. Second, vector pre-immunisation may lead to the expansion of B cell clones reactive to epitopes associated with the vector. Following the subsequent administration of the r*Salmonella* construct, primed B cells would function as antigen presenting cells for T cells, thus biasing the response in favour of vector epitopes. This latter explanation was proposed by analogy with the phenomenon of epitopic suppression.

Epitopic suppression was first described by Herzenberg and Tokuhisa (1980) who observed that mice previously immunised with a protein carrier subsequently developed poor responses to hapten when boosted with a haptenated form of the same carrier. In their experiment, mice were primed with keyhole limpet haemocyanin (KLH) and subsequently exposed to KLH- dinitrophenol (DNP). In comparison to unimmunised controls, KLH-primed mice developed weaker serum IgG responses to DNP. Initially epitopic suppression was thought

to reflect the activity of suppressor T cells, but work by Schutze *et al.* (1989) suggested an alternative explanation. This group proposed that carrier priming induces the clonal expansion of carrier-specific B cells which have the potential to act as APCs; upon subsequent exposure to haptenated carrier, these expanded clones of carrier-specific B cells bind and present the antigen, reducing its availability to B cells potentially reactive to hapten. In support of this notion, increasing the degree of haptenation, or prior priming with the same hapten on an unrelated carrier, abrogated epitopic suppression. A later study by the same group showed that *in vivo* elimination of $CD4^+$ T cells prevented the induction but not the expression of carrier-induced epitopic suppression. In contrast to the consequences of $CD4^+$ T cell depletion, *in vivo* depletion of $CD8^+$ T cells - the phenotype of suppressor T cells - had no effect on the establishment of carrier-induced epitopic suppression (Leclerc *et al.*, 1990). The depletion of $CD4^+$ T cells at the stage of carrier (KLH) priming prevented the induction of a memory response to KLH, since $CD4^+$ T cells were essential for memory B cell development. The elimination of $CD4^+$ T cells after carrier immunisation did not abolish the secondary anti-carrier response following exposure to haptenated-carrier, and did not prevent the development of epitopic suppression.

A further study of carrier-induced epitopic suppression was recently published by Renjifo *et al.* (1998). Here, it was hypothesised that epitopic suppression may result from defective hapten presentation by carrier-specific B cells following capture of the hapten-carrier conjugate. In contrast to carrier-primed mice subsequently injected with conjugate, hyporesponsiveness to the hapten was prevented if carrier-primed mice were instead injected with conjugate-primed dendritic cells (used as efficient APCs) together with additional soluble conjugate and IL-12 (which promotes T cell activation and differentiation). Targeting the conjugate on dendritic cells led to the generation of hapten-specific serum IgG responses approaching these observed in unprimed controls.

The phenomenon of epitopic suppression is not restricted to hapten-carrier studies, but has also been demonstrated with recombinant vaccinia virus expressing a herpes simplex virus

(HSV) derived glycoprotein (gD) (Rooney *et al.*, 1988). Mice primed (i.v.) with vaccinia virus generated less antibodies to gD than controls when subsequently exposed to the recombinant construct two to three months later. Moreover, primed mice were less protected against challenge with HSV than unvaccinated animals. In humans, subjects primed with a tetanus toxoid vaccine and subsequently exposed to a tetanus toxoid-malarial peptide conjugate vaccine showed that the consequences of priming were less significant in subjects who originally received a low dose of tetanus toxoid (Di John *et al.*, 1989). This observation was consistent with those of Leclerc *et al.* (1990) who also examined the effects of differential priming doses of KLH on the subsequent development of epitopic suppression in mice subsequently exposed to KLH-TNP. It was therefore suggested that the phenomenon of epitopic suppression may occur in humans.

The experiment conducted by Attridge *et al.* (1997) showed results analogous to those observed in the carrier priming studies described above. Epitopic suppression was suggested as a possible mechanism for the hyporesponsiveness to K88 due to several observations: (i) the hyporesponsiveness to K88 seen in vector-primed animals was accompanied by secondary responses to vector O-antigens; (ii) reducing the priming dose of wt *S.* Stanley from ~ 10^9 to ~ 10^6 cfu abolished the hyporesponsiveness to K88; (iii) boosting with *Salmonella* Strasbourg (serotype O-9,46) expressing K88 circumvented the hyporesponsiveness observed following boosting with r*S.* Stanley-K88. These findings are all consistent with a mechanism of epitopic suppression.

1.5 Thesis aims.

Given the current focus on the r*Salmonella* vaccine strategy, any reduction of vaccine immunogenicity resulting from vector-priming might preclude the use of some recombinant constructs currently being tested in clinical trials. Hence, the overall goal of this thesis is to

evaluate the potential significance of the findings of Attridge *et al.* (1997). Based on a comparison between protocols used in that study and in the report of Bao and Clements (1991), the following aims will be addressed:

1. To develop sampling techniques and ELISAs that allow reliable measurement of the secretory IgA responses of the murine GALT. This will then allow the measurement of intestinal IgA responses to foreign antigens delivered to vector-primed mice by r*Salmonella*.

2. To evaluate the significance of the GALT colonising potential of *Salmonella* vectors as a determinant of the significance of vector-priming. The primary approach here will be to introduce an *aroA* mutation into *S.* Stanley. The mutant will then be compared to wt *S.* Stanley, both in terms of GALT colonisation and potential to induce a state of hyporesponsiveness to foreign antigen as a consequence of vector-priming.

3. To evaluate the significance of the foreign antigen delivered by r*Salmonella* as a determinant of immune responses obtained in vector-primed mice. r*S.* Stanley and *aroA S.* Dublin (SL1438) strains will be used as vectors for the delivery of either LT-B or K88 to vector-primed mice. Any differences in immune responses would provide an insight into the importance of the foreign antigen as a determinant of immunogenicity in vector-primed mice.

CHAPTER TWO

Materials and Methods

2.1 Chemicals and reagents.

All chemicals used were AnalaR grade. Triton X-100, phenol, glucose, para-aminobenzoic acid (pABA), tryptophan, aneurine hydrochloride (vitamin B1), chloroform, sodium dodecyl sulphate (SDS), ethanol, methanol, propan-2-ol (isopropanol), perchloric acid, glycerol, sucrose, ammonium sulphate, potassium acetate, glacial acetic acid, and Coomassie Brilliant Blue G250 were from BDH Chemicals. 2,3-dihydroxybenzoic acid (DHB), thymine, serine, histidine, ethidium bromide, D(+)-galactose, monoganglioside-GM1 from bovine brain, magnesium chloride, Tris (Trisma base) and Tween-20 were obtained from Sigma. Bovine serum albumin (BSA; fraction V) was from ICN. Calcium chloride, ethylene-diamine-tetra-acetic-acid disodium salt (EDTA), ethylene-glycol-bis-(β-amino-ethylether) N, N, N', N'-tetraacetic acid (EGTA), sodium chloride, and sodium hydroxide were from Ajax chemicals, NSW, Australia.

Deoxyribonucleoside triphosphates (dATP, dCTP, dGTP and dTTP), 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), isopropyl-β-D-thiogalacto-pyranoside (IPTG) and glycine were purchased from Roche. Mineral oil was Primol 352 (Esso). Antibiotics ampicillin (Ap), kanamycin sulphate (Km), chloramphenicol (Cml), trimethoprim (Tm) and tetracycline

(Tc) were purchased from Sigma. All other anti-microbial agents (dyes and detergents) were purchased from Glaxo, Calbiochem, Sigma Chemical Co., or BDH Chemicals Ltd.

Electrophoresis grade reagents and their suppliers were: acrylamide and ammonium persulphate (Bio-Rad), ultra pure N,N'-methylene bis-acrylamide and urea (BRL); high gelling temperature agarose (Seakem); sodium dodecyl sulphate (SDS) and N,N,N',N'-Tetramethyl-ethylenediamine (TEMED) (Sigma).

2.2 Bacterial strains and plasmids.

The *Escherichia coli* and *Salmonella* strains used in this study are listed in Tables 2.1 and 2.2 respectively. Plasmids used in this study are listed in Table 2.3.

2.3 Growth and diagnostic media.

Bacteria were generally grown in 10 ml broth cultures (containing antibiotics if necessary) in 25 ml McCartney bottles, with shaking at 37°C. Luria-Bertani broth (LB) is composed of Bacto-tryptone (Difco; 10 g/l), Bacto-yeast extract (Difco; 5 g/l) and NaCl (5 g/l), and was the most frequently used growth medium for *E. coli* and *Salmonella* strains. CBT (casamino acids, vitamin B1, tryptophan), a defined medium for the growth of Salmonellae, is composed of M9 salts (K_2HPO_4, 7 g/l; KH_2PO_4, 3 g/l; $(NH_4)_2SO_4$, 1 g/l (pH7.5)), 0.5% (w/v) casamino acids (Difco), 0.5% (w/v) glucose, 0.01% (w/v) $MgSO_4.7H_2O$, 1 μg/ml vitamin B1, and 10 μg/ml tryptophan. M9 minimal medium (MM) is CBT medium without casamino acids and tryptophan. Solidified media required the addition of Agar (BBL technical grade; 15 g/l). Tryptic Soy Broth (TSB) and XLD (xylene, lactose and deoxycholate) medium were obtained as powders from Difco and Oxoid respectively and reconstituted according to the manufacturers' instructions.

Table 2.1 *E. coli* strains used in this study.

Name	Description	Source/Reference
DH5α	F$^-$, *endA*1, *hsdR*17 [r_k^- m_k^+], *supE*44, *thi*1, *recA*1, *gyrA* [Nal^R], *relA*1 Δ(*lac*IZYA-*arg*F), U169, *deoR* [ϕ 80*dlac*Δ[*lacZ*] M15]	Bethesda Research Laboratories
S17-1	*pro*, *hsdR*, RP4-2-Tc::Mu, Km::*Tn7* used for conjugal transfer of plasmids	U. Priefer[a]
CVSA6	DH5 α + pCVSA1	this study, section 4.2.3.1
CVSA7	DH5 α + pCVSA2	this study, section 4.2.3.1
CVSA8	DH5 α + pCVSA3	this study, section 4.2.3.1
CVSA9	DH5 α + pCVSA4	this study, section 4.2.3.2
CVSA10	DH5 α + pCVSA5	this study, section 4.2.3.2
CVSA11	DH5α + pCVSA6	this study, section 4.2.3.3
CVSA12	S17-1+ pCVSA6	this study, section 4.2.3.3
CVSA16	DH5 α + pCVSA7	this study, section 4.2.4
CVSA27	DH5α + pCVSA8	this study, section 5.2.6.1
CVSA30	DH5 α + pCVSA10	this study, section 5.2.5.1

[a] Max Plank-Institut fur Biologie, Tübingen, FRG.

Table 2.2 *Salmonella* strains used in this study.

Name	Description	Source/Reference
S. Stanley	O-4,5,12	Laboratory Collection
thyA S. Stanley	*thyA* negative *S.* Stanley isolated following growth on trimethoprim	Attridge *et al* (1997)
EX2000	Typhimurium LT2 *tryC2*, *metA22*, *Hl-bnml*, *H2-enx*, *fla-66*, *rpsL12*, *xyl-404met*, *E55-l*, *hsdSA29*, *ilv-452*, *hsdSB121*, *leu-3121*, *galE856* Intermediate strain for the transfer of plasmids from *E. coli* to *Salmonella*	R. Morona[a]
S. Typhimurium C5	wildtype	Laboratory Collection
EX143	*S.* Typhimurium LT2 LB5010 (Bullas and Ryu, 1983) made thymine dependent (*thyA*), *metA22*, *metE551*, *trpD2*, *ilv452*, *hsdLT6*, *hsdSA29*, *strA120*, *galE* Intermediate strain for the transfer of $thyA^+$ balanced-lethal plasmids from *E. coli* to *Salmonella*	R. Morona
PE951	Is SL3261 *aroA* DEL407 (clean excision mutant of *aroA*554::*Tn*10 ($SerC^+$) by Bochner selection) made *galE* Source of the *aroA* deletion mutation for bacteriophage mediated transduction	Bruce Stocker[b]
PE952	Is SL5242 (*S.* Typhimurium LT2 *aroA*[*serC*]1121::*Tn*10) Tc^R, made *galE* Source of the transposon-interrupted *aroA* gene for bacteriophage mediated transduction	Bruce Stocker
SL1438	*aroA S.* Dublin (O-9,12)	John Clements[c]
EL23	pJC217 in SL1438	John Clements
CVSA1, 2	*S.* Stanley made *galE* by growth on 2-deoxygalactose; clones 1, 2	this study, section 4.2.1

Table 2.2 continued.

CVSA3, 4, 5	CVSA1 *aroA*[*serC*]1121::*Tn*10, Tc^R; clones 1, 2, 3	this study, section 4.2.2
CVSA13	*S.* Stanley + pCVSA6	this study, section 4.2.3.3
CVSA14, 15	*S.* Stanley made *aroA* using allelic exchange mutagenesis, Km^R; clones 1, 2	this study, section 4.2.3.3
CVSA17	EX2000 + pCVSA7	this study, section 4.2.4
CVSA18	EX2000 + pWSK30	this study, section 4.2.4
CVSA19	CVSA14 + pCVSA7	this study, section 4.2.4
CVSA20	SL1438+ pCVSA7	this study, section 4.2.4
CVSA21	CVSA14 + pWSK30	this study, section 4.2.4
CVSA22	SL1438 + pWSK30	this study, section 4.2.4
CVSA23	*S.* Stanley + pWSK30	this study, section 4.2.4
CVSA24	*S.* Stanley + pJC217	this study, section 5.2.6.1
CVSA25	EX2000 + pFM205	this study, section 5.2.4
CVSA26	SL1438 + pFM205	this study, section 5.2.4
CVSA29	EX143+ pCVSA10	this study, section 5.2.5.1
CVSA31	*thyA S.* Stanley + pCVSA10	this study, section 5.2.5.1
CVSA32	EX143+ pEVX46	this study, section 5.2.5.1
CVSA33	*thyA S.* Stanley + pEVX46	this study, section 5.2.5.1
CVSA34	EX2000+ pCVSA9	this study, section 5.2.6.1
CVSA35	*thyA S.* Stanley + pCVSA9	this study, section 5.2.6.1

[a] University of Adelaide, Adelaide, South Australia.

[b] Stanford University School of Medicine, Stanford, California.

[c] Tulane University Health Science Center, New Orleans, Louisiana

Table 2.3 Cloning vectors and plasmid constructs used in this study.

Plasmid	Description	Source/Reference
pGEM-T Easy®	PCR cloning vector; Ap^R	Promega
pBluescript-SK^+	Cloning vector; Ap^R	Stratagene
pCACTUS-*mob*	Suicide vector with temperature sensitive replicon and *mob* region; Cml^R	Laboratory Collection, C. Clark
pWKS30	Low copy number cloning vector; Ap^R	Wang and Kushner (1991)
pUC18K	Source of the *apha-3* non-polar Km resistance cartridge; Ap^R	Ménard *et al.* (1993)
pJC217	pUC8 containing a 0.8 kb *Hind*III fragment encoding LT-B from a human isolate of *E. coli*; Ap^R	Clements and El-Morshidy (1984)
pFM205	pBR322 with a 6.7 kb *Sau*3AI fragment encoding K88ab biosynthesis inserted into the *Hind*III and *Eco*RI sites; Ap^R	Mooi *et al.* (1979)
pBTAH	pBR322 with the 1.2 kb $thyA^+$ gene from *E. coli* K-12 cloned into the *Hind*III site; $thyA^+$	
pEVX46	*Nae*I deletion derivative of plasmid pBTAH; $thyA^+$	Morona *et al.* (1994)
pEVX49	pBTAH with a 6.7 kb *Sau3*A1 fragment encoding K88ab biosynthesis inserted into the *Sca*I site Vector was subsequently treated with *Nae*I to destroy gene encoding Tc resistance; $thyA^+$	Morona *et al.* (1994)
pCVSA1, 3	pGEM-T Easy®+ *aroA* PCR product amplified from *S.* Stanley; *aroA* is in the direction of the *lacZ* promoter; clones 1,3; Ap^R	this study, section 4.2.4
pCVSA2	pGEM-T Easy®+ *aroA* PCR product amplified from *S.* Stanley; *aroA* is in the direction of the T7 promoter; Ap^R	this study, section 4.2.4
pCVSA4, 5	pCVSA1 with the *apha-3* cartridge inserted into the *Eco*RV site of the *aroA* gene; clones 1, 2; Ap^R	this study, section 4.2.3.2

Table 2.3 continued.

pCVSA6	pCACTUS*mob* with *Pst*I/*Sph*I *aroA*::*apha-3* fragment from pCVSA1 inserted into the *Nsi*I/*Sph*I sites of pCACTUS*mob*; Cml^R Km^R	this study, section 4.2.3.3
pCVSA7	pWSK30 with *Eco*RI *aroA* fragment from pCVSA1 inserted into the *Eco*RI site; *aroA* in the direction of the *lacZ* promoter; Ap^R	this study, section 4.2.4
pCVSA8	pBluescript SK with *thyA* gene excised from pEVX46 via *Hind*III digest and inserted into the *Hind*III site; Ap^R	this study, section 5.2.6.1
pCVSA9	pJC217 with *thyA* gene excised from pCVSA8 via *Sma*I/*Hinc*II digestion and inserted into the *Ssp*I site; Ap^R	this study, section 5.2.6.1
pCVSA10	pEVX49 treated with *Sma*I to create Δ*faeD* derivative; $thyA^+$	this study, section 5.2.5.1

Antibiotics were added as required into liquid or solid media at the following final concentrations: Ap, 100 μg/ml; Km, 50 μg/ml; Cml, 25 ug/ml; Tc 8 μg/ml. Tm, for the selection of thymine auxotrophs, was present in solid media at a final concentration of 50 μg/ml.

Aromatic amino acid supplements (DHB and pABA) were made as 20 mg/ml stocks in ethanol, and diluted to a final concentration of 20 μg/ml in liquid or solid media. 2-deoxygalactose (Sigma), was added to solid medium at a final concentration of 1 μg/ml, and allowed selection of bacteria with mutations in genes involved in metabolism of galactose. Sucrose was added to salt-free LB agar at a final concentration of 60 g/l (prior to autoclaving) for the selection of sucrose resistant intermediates in allelic exchange mutagenesis.

X-gal was dissolved in dimethyl formamide at a final concentration of 20 mg/ml; addition of 1 ml of this solution, plus 1ml of IPTG (23.7 mg/ml dissolved in Milli Q water), to 500 ml molten LB agar produced blue-white detection plates for the selection of bacteria containing functional β-galactosidase.

2.4 Maintenance of Bacterial Strains

Bacterial strains were stored as suspensions of freshly grown bacteria (harvested from solid media) in glycerol (32% v/v) - peptone (0.6% w/v) at - 70°C. In order to provide fresh culture, a loop full of the glycerol suspension was streaked onto an agar plate (with antibiotic where appropriate) and incubated overnight; resulting colonies were inoculated into growth medium.

Reserve stocks of bacterial strains were maintained as lyophilised cultures, stored *in vacuo* in sealed glass ampoules. When required, an ampoule was opened and its contents suspended in several drops of the appropriate broth (containing antibiotics if necessary). A loopful of this suspension was streaked onto solid media and incubated for 18 hr at the

appropriate growth temperature. If the colony form was uniform, the bacterial growth was harvested using a sterile loop and used to prepare a fresh glycerol stock.

2.5 Preparation of chemically competent cells and transformation.

Bacteria were grown overnight (o/n) in LB (with antibiotics if necessary), subcultured 1:20 into fresh LB and incubated to an OD_{600nm} of ~ 0.6. Cultures were cooled on ice for 5 min, pelleted at 4°C in a bench centrifuge (Minor MSE bench centrifuge, 10 min, 4,500 rpm) and resuspended in 10 ml of ice-cold solution α (30 mM KAc, 100 mM KCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol). The bacteria were spun again and resuspended in 1 ml of ice-cold solution β (10 mM 4-Morpholinepropanesulfonic acid (MOPS; Roche), 75 mM $CaCl_2$, 10 mM KCl, 15% glycerol) and left on ice for 2 hr. This suspension was aliquotted (100 µl) into 1.5 ml reaction tubes and either transformed immediately, or snap frozen on dry ice and stored at -70°C.

For transformation, competent cells (100 µl) were thawed on ice, mixed with DNA and left on ice for 10 min. The cell/DNA mixture was heat shocked at 42°C for 3 min then cooled on ice for a further 10 min. This was followed by the addition of 500 µl broth and incubation with aeration for 1 hr at 37°C. Aliquots of the suspension were then plated onto selection plates, either directly or after recovery of all bacteria by centrifugation (Heraeus Biofuge 15, 14,000 rpm, 1 min). Transformation controls were routinely included - a negative control in which no DNA was added to the competent cells, and a positive control in which the unmodified cloning vector was added. These confirmed the sterility, antibiotic sensitivity, and competence of the bacteria used.

2.6 Preparation of electrocompetent cells and electroporation.

Bacteria were subcultured 1:20 from o/n broths grown to OD_{600nm} of ~ 0.6. Cultures were cooled on ice for 5 min, pelleted at 4°C (Minor MSE bench centrifuge, 10 min, 4,500 rpm) and washed twice in 10 ml of ice cold 10% (v/v) glycerol. (Recipient strains carrying a *thyA* mutation were grown with exogenous thymine, and so were washed an additional two times.) Finally the bacteria were resuspended in 1 ml of ice cold 10% (v/v) glycerol, with aliquots (100 µl) stored in 1.5 ml reaction tubes at - 70°C.

For electroporation, cells were thawed on ice, mixed with the plasmid DNA of interest (< 0.5 µg for maximum efficiency) and immediately transferred to a pre-chilled Gene Pulser™ cuvette (0.2 cm electrode gap, Bio-Rad). (If electroporation was to be performed with DNA from a ligation reaction, the ligation mix was first dialysed for 30 min by pipetting it onto a 0.025 µm 13 mm nitrocellulose filter (Millipore) suspended in a petri dish containing 20 mls of distilled water.) Cells were pulsed at 2.5 kV in a Bio-rad Gene pulser (25 µF, 200 Ω) resulting in time constants of 4.5 - 4.7 msec. Immediately after electroporation, cells were washed out of the cuvette with 500 µl of LB (CBT for *thyA* bacteria receiving a complementing $thyA^+$ plasmid) and transferred to a sterile tube. After incubation at 37°C for 60 - 90 min, bacteria were plated on appropriate selection medium. Bacteria electroporated with low copy number plasmids were concentrated by centrifugation prior to plating.

2.7 Conjugation.

Recipient and donor cells were grown overnight in LB (with appropriate antibiotics), recovered by centrifugation (IEC Centra 4X centrifuge, 4,000 rpm for 10 min) and resuspended in 10 ml of LB (without antibiotics). Donor cells were mixed with recipient cells at a ratio of 1:10, and the mix centrifuged and resuspended in 1ml of fresh LB. The bacteria were applied to

0.2 μm filter discs (Type HA, Millipore) placed on LB agar (without antibiotics) and incubated at 37°C for 6 hours. Bacteria were recovered from the filters by vortexing in 1 ml of LB in a 25 ml McCartney bottle prior to plating various dilutions of the suspension onto appropriate selection plates.

2.8 Isolation, analysis and manipulation of DNA.

2.8.1 Chromosomal DNA isolation.

Cells from an o/n culture were pelleted in a bench centrifuge for 6 min at 5,000 rpm (Centra4X, IEC) and washed with 10 ml of TES (50 mM Tris HCl pH 8.0, 5 mM EDTA, 50 mM NaCl) buffer. Bacteria were resuspended in 2 ml of a cold solution of 25% (w/v) sucrose in 50 mM Tris-HCl, pH 8.0, to which was added 1 ml of a freshly made lysozyme (Sigma) solution (10 mg/ml in 0.25 M EDTA pH 8.0). The mixture was left to stand on ice for 20 min (with occasional swirling), then 0.75 ml of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and 0.25 ml of lysis solution (5% (w/v) Sarkosyl, 50 mM Tris-HCl pH 8.0, 62.5 mM EDTA) was added, followed by 3 mg of solid pronase. The mixture was swirled gently, left at 56°C for 1 hr (or until clear), then extracted three times with 4 ml TE-saturated phenol (pH 7.5); these extractions were performed in McCartney bottles and involved rolling the bottles briskly between the hands until the contents were well mixed, followed by centrifugation at 2,500 rpm for 6 min. After phenol extraction, the aqueous phase was recovered and extracted twice with 4 ml of ether in the same manner. Chromosomal preparations were precipitated with 2 - 3 volumes (~ 8 ml) of ice cold 100% ethanol. The bottles were again rolled briskly between the hands until chromosomal precipitate appeared and the rest of the solution was clear. Chromosomal DNA was removed with a Pasteur pipette (heat sealed and shaped into a hook), washed in 1 ml ice cold 70% (v/v) ethanol, and stored in 1 ml of Milli Q water at 4°C.

2.8.2 Plasmid DNA isolation.

Plasmid DNA for both cloning and sequencing was prepared by the following method. 1.5 - 10 mls of o/n culture (depending on plasmid copy number) was centrifuged (2 min, 14,000 rpm, Eppendorf 5417R centrifuge, room temperature) and the pellet resuspended in 300 µl of Solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.1 µg/ml RNase A (Roche)). After incubation at room temperature for 5 min bacteria were lysed by adding 300 µl of Solution II (0.2 M NaOH, 1% (w/v) SDS), followed by further incubation at room temperature for 5 min. Protein, chromosomal DNA and high molecular weight RNA were precipitated by the addition of 300 µl of Solution III (5 M KAc, pH 4.8) and incubation for 10 min on ice. The cellular debris was pelleted (10 min, 14,000 rpm at 4°C) and the supernatant transferred to a fresh 1.5 ml reaction tube. Chloroform (400 µl) was added to extract any remaining protein and cell debris, the tube was mixed by inversion for 30 sec and then centrifuged for 3 min at room temperature. The upper aqueous layer was transferred to a fresh 1.5 ml reaction tube, ice-cold isopropanol (650 µl) was added and the sample incubated on ice for 10 min. Plasmid DNA was pelleted (14,000 rpm, 5 min, 4°C), washed with 300 µl of 70 % (v/v) ethanol and dried at 65°C for 10 min. Pellets were resuspended in 30 µl of sterile Milli Q water and stored at - 20°C until required.

2.8.3 DNA quantitation.

The concentration of DNA in chromosomal or plasmid preparations was determined by measurement of absorption at 260 nm. An OD_{260nm} of 1.0 was assumed to be equal to 50 µg DNA/ml dsDNA.

2.8.4 Digestion of DNA with restriction enzymes.

Restriction enzymes (endonucleases) were purchased from either Roche, New England Biolabs or Progen, and used with reaction buffers as recommended by the manufacturer. 0.1 - 0.5 μg of plasmid DNA was incubated with 2 units of restriction enzyme in a final volume of 20 μl, at 37°C for 2 hr. For digestion of > 1 μg of plasmid DNA, 4 - 8 units of restriction enzyme was used in a final volume of 30 - 50 μl, with o/n incubation at 37°C. Restriction digests of chromosomal DNA for Southern analysis were performed using 8 units of restriction enzyme with 1 - 5 μg of DNA in a final volume of 50 μl, incubated for 4 hr at 37°C. Digestion reactions were terminated by heating for 20 min at either 65°C or 85°C depending on the enzyme used.

2.8.5 Agarose gel electrophoresis.

Electrophoresis of uncut or digested chromosomal or plasmid DNA was carried out at room temperature on horizontal, 0.8%, 1% or 1.5% (w/v) agarose gels (Seakem HGT) made in either TBE buffer (67 mM Tris base, 22 mM boric acid and 2 mM EDTA, final pH 8.8) or TAE buffer (40 mM Tris acetate, 2 mM EDTA). Samples, containing a 1:10 volume of tracking dye (15% (w/v) Ficoll (Pharmacia), 0.1% (w/v) bromophenol blue (BDH Chemicals), 0.1 mg/ml RNase A), were loaded into lanes in gels which routinely contained appropriate molecular weight marker samples. Gels were electrophoresed at 100 - 120 V for 1 - 3 hr and then stained in distilled water containing 2 μg/ml of ethidium bromide. After de-staining in distilled water, DNA bands were visualised using an ultraviolet transilluminator (UVP Inc.), and photographed using a Tracktel gel documentation video imager and thermal printer (Mitsubishi).

Digested DNA fragments required for cloning procedures were extracted from 1% TAE-agarose gels using a QIAquick gel extraction kit (QIAGEN) as described by the manufacturer.

Extracted DNA was combined with cleaved vector DNA (treated with Klenow fragment and/or SAP if necessary) and ligated as described below.

2.8.6 Calculation of restriction fragment size.

The sizes of restriction enzyme fragments were calculated by comparing their relative mobilities with those of *Eco*RI-digested *Bacillus subtilis* bacteriophage SPP1 DNA (Ratcliff *et al.*, 1979). SPP1 fragment sizes (kilobases, kb) were: 8.5, 7.35, 6.1, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16, 0.98, 0.72, 0.48, 0.36 and 0.09. Estimation of larger fragment sizes involved comparing relative mobility with a combination of *Bgl*II-digested bacteriophage Lambda DNA and *Eco*RI-digested *Bacillus subtilis* bacteriophage SPP1 DNA. The sizes (kb) of the Lambda fragments were: 22.01, 13.29, and 9.68.

2.8.7 End-filling with Klenow fragment

DNA with cohesive ends was end-filled using Klenow fragment of *E. coli* DNA polymerase 1 (Roche) to create blunt ended fragment. 1 - 2 μg of DNA, 2 units of Klenow, 2 μl of 10x Klenow buffer (100 mM Tris-HCl (pH 8.0) and 100 mM $MgCl_2$) and 1 μl of each deoxynucleoside triphosphate (dNTP) were combined in a final volume of 20 μl and incubated for 15 min at 37°C. DNA was then extracted twice with phenol/chloroform in a total volume of 100 μl and precipitated in 2 volumes of 100% ethanol and one-tenth volume of 3 M sodium acetate for 30 min at - 70°C. Pellets were washed in 1 ml of 70% (v/v) ethanol, dried *in vacuo* and resuspended in a total volume of 20 μl in TE buffer.

2.8.8 Shrimp alkaline phosphatase treatment of DNA.

Dephosphorylation of restriction endonuclease digested vector DNA was carried out using shrimp alkaline phosphatase (SAP; Roche). Digested DNA (1 - 2 μg) was added to a mixture containing 2 units of SAP, 2 μl of dephosphorylation buffer (50 nm Tris-HCl, 0.1 mM EDTA, pH 8.5) and sterile Milli Q water to a total of 20 μl. The mixture was incubated for 15 min at 37°C for cohesive DNA ends, or 1 hr at 37°C for blunt DNA ends. The SAP was inactivated at 65°C for 15 min, and dephosphorylated DNA used directly in a ligation reaction, or stored at - 20°C until further use.

2.8.9 DNA ligation procedure.

Ligation reactions were performed in buffer A (20 mM Tris-HCl, 10 mM $MgCl_2$, 0.6 mM ATP and 10 mM dithiothretol (DTT; Sigma)) for ligation of fragments with cohesive ends, or buffer B (1 mM Tris, pH 7.5, 1 M $MgCl_2$, 50% polyethylene glycol (w/v), 0.1 M ATP and 1 M DTT) for blunt end ligations. Reactions were performed using 1 - 2 units of T4 DNA ligase (New England Biolabs) in volumes of 20 μl, and incubated at 4°C for 16 hr. A molar ratio of approximately 1:3 vector to insert DNA was routinely used for cohesive end ligations, whereas a ratio of 1:1 was used for blunt end ligations.

Ligated DNA was used directly for transformation of *E. coli* strains, and blue/white colour selection was used where possible. After isolation, plasmids were screened for the correct insert either by restriction endonuclease digestion of isolated plasmid or by PCR screening of small-scale lysates (see below).

2.9 Polymerase Chain Reaction (PCR) protocol.

2.9.1 Standard PCR reaction.

Oligodeoxynucleotide (oligo) primers used in this study were (5' - 3'): M13 forward (GGAAACAGCTATGACCATG; binds to pBluescript-SK$^+$ from nt 826-808); M13 reverse (GTAAAACGACGGCCAGT; binds to pBluescript-SK$^+$ from nt 599-616); #2934 (GAGAGTTGAGTTTCATG; binds to *aroA* from nt -14-3, start codon underlined); #2936 (AGAAGACTTAGGCAGGCG; binds to *aroA* from nt 1279-1298, stop codon underlined). These were purchased from Geneworks in a lyophilised form and reconstituted with Milli Q water prior to use. The protocol used for PCR is that described by Delidow (1997) for the generation of PCR products with cohesive ends. The PCR was performed in reaction tubes (0.5 ml, Perkin Elmer Cetus) in a total of 50 µl containing Taq buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$), 2 mM final concentration of each deoxyribonucleoside triphosphate (dNTP), 100 pmol of each oligo primer, 200 ng of plasmid template or genomic DNA and 2 U of Taq polymerase (Perkin Elmer Cetus). The mixture was overlaid with a drop of Primol 352 mineral oil (Esso) and subjected to 25 cycles of amplification (95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min per 1 kb of DNA template to be amplified, followed by a final extension at 72°C for 5 min) using a DNA thermal cycler (Perkin Elmer Cetus). Following amplification, the mixture was carefully removed from under the oil and 10 µl of reaction product analysed on a 1% agarose gel. The remainder was purified using a QIAquick PCR purification kit (QIAGEN) according to the manufacturer's instructions and stored at 4°C until required.

2.9.2 Cloning of PCR products.

Purified PCR products were generally cloned initially into the pGEM-T Easy® vector (Promega). A typical ligation mixture comprised pGEM-T Easy® vector (1 µl), T4 DNA ligase

10x buffer (1 μl), T4 DNA ligase (1 μl), PCR product (at a volume designed to give a molar ratio of 3:1 insert:vector) and Milli Q water (to 10 μl). After overnight ligation at 4°C, the mixture was transformed into DH5α and plated onto blue-white selection plates containing Ap to select for vector with insert (white colonies).

2.9.3 PCR screening.

PCR was sometimes used to screen putative mutant or transformant colonies. Small scale cell lysates were prepared by resuspending a single colony in 70 μl of sterile Milli Q water followed by boiling for 5 min. After the mixture had cooled to room temperature, 1 - 2 μl (for screening of chromosomal mutations) or 5 - 10 μl (for plasmid screening) was used in a standard PCR reaction using the appropriate oligonucleotides. 10 μl of the PCR product was electrophoresed on 1% agarose gels to ascertain the size of the amplified DNA.

2.10 DNA sequencing.

2.10.1 Preparation of DNA for big dye-labelled terminator sequencing.

Plasmid DNA or PCR products (the latter purified using the appropriate Qiagen kit) were templates for big dye-labelled terminator sequencing using kits supplied by Applied Biosystems. Thin walled tubes (0.5 ml, Gene Amp, Perkin Elmer) containing 1 - 2 μg of template DNA and 3.2 pmol of primer were made up to a final volume of 20 μl with 8 μl of pre-mix (Applied Biosystems) and Milli Q water. Reactions were overlaid with mineral oil and subjected to 25 cycles of PCR (96°C 30 sec; 50°C 15 sec; 60°C 4 min) in a DNA thermal cycler (Perkin Elmer). PCR product was precipitated by incubating for 2 hr at - 20°C in the presence of 2 μl of 3 M sodium acetate and 50 μl of ice cold 100% ethanol. DNA was recovered by centrifugation at

15,000 rpm for 15 min at 4°C before washing with 70% (v/v) ethanol and drying *in vacuo* or at 65°C for 6 min.

2.10.2 DNA sequencing and data analysis.

The dried DNA pellets were resuspended in 4.5 µl of loading buffer (83% deionised formamide (BDH Chemicals), 8.3 mM EDTA, pH 8.0) and heated to 95°C for 2 min. Samples were electrophoresed on a 6% polyacrylamide-8M urea gel in an Applied Biosystems 373A or 377 DNA sequencer at the Molecular Pathology Sequencing Laboratory, Institute of Medical and Veterinary Science, Adelaide. Raw sequence data were analysed using Applied Biosystems Seq Ed Program Version 6.0.

DNA sequence data were further analysed using the DNA and protein analysis programs DNASIS and PROSIS (Hitachi Software). Multiple sequence alignments were carried out using CLUSTAL W (Higgins and Sharp, 1988). Hydropathy plots were generated by the Kyte and Doolittle program in PROSIS (Kyte and Doolittle, 1982) and aligned using PROFILEGRAPH (Hofmann, 1990). DNA and amino acid sequence homologies were detected using the basic local alignment search tool BLAST (Altschul *et al.*, 1990).

2.11 Southern DNA hybridisation.

2.11.1 Preparation of Digoxigenin (DIG)-labelled DNA probes via PCR.

DNA Probes for Southern hybridisation were prepared as follows. Chromosomal or plasmid DNA (0.05 µg) was used as template in a PCR reaction mix containing PCR buffer (1.5 mM $MgCl_2$, 10 mM Tris pH 8.4, 50 mM KCl), 20 µM dATP, 20 µM dCTP, 20 µM dGTP, 19 µM dTTP, 1 µM DIG-11-dUTP, 100 pmol of each oligo primer, 2 U Taq polymerase and sterile

Milli Q water to a total volume of 50 µl. An unlabelled control reaction was set up at the same time. PCR products from the labelling and control reactions were compared on a 1% agarose gel; as DIG-11-dUTP is larger than dTTP (which it replaces at random in the amplified PCR product), a probe was considered labelled if it migrated more slowly than the control amplicon.

The DIG-labelled product was precipitated with 6 µl 4 M LiCl and 100 µl of cold ethanol at - 20°C for 2 hr. After centrifugation (15 min, 14,000 rpm, Eppendorf 5417R centrifuge, 4°C) the probe was washed with 200 µl 70% (v/v) ethanol and dried at 65°C for 15 min. The probe was resuspended in 50 µl of sterile Milli Q water, and tested for dUTP-DIG incorporation by dispensing 1 µl onto Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech) and using colorimetric detection as described below (section 2.11.3). Successfully labelled probes were stored at - 20°C. Before use, the probe was thawed, heated at 100°C for 10 min, then diluted (~1:200) into alternative hybridisation fluid (5 x SSPE (Sambrook *et al.*, 1989), 7% (w/v) SDS, 1% (w/v) skim milk powder, 50% (v/v) formamide, 250 mg/ml herring sperm DNA).

2.11.2 Electrophoretic separation, Southern transfer and probe hybridisation.

Digested chromosomal DNA samples were electrophoresed overnight at 10 V in 0.8% (w/v) agarose gels in TAE buffer. The gel was then treated with several changes of denaturing solution (1.5 M NaCl, 0.5 M NaOH; 150 mls per change) over a period of 1 hr at room temperature, followed by immersion in neutralising solution (1 M Tris-HCl pH 8.0, 1.5 M NaCl; in 150 mls) for an additional hour. DNA was then transferred from the gel to Hybond N^+ nylon membrane (Amersham Pharmacia Biotech) by overnight transfer at room temperature using capillary action as described by Sambrook *et al.* (1989), using 10 x SSC (1.5 M NaCl, 0.15 M Na citrate, pH 7.0) as the transfer buffer. DNA was fixed to the membrane by immersion in 0.4 M NaOH for 20 min, after which the membrane was washed briefly in 5 x SSC and treated with 20 ml of alternate hybridisation fluid for 2 hr at 42°C. The membrane was then placed in

alternate hybridisation fluid containing DIG-labelled probe, and incubated for ~ 18 hr at 42°C. High stringency washes were performed by washing the membrane twice (5 min at room temperature) with 2 x SSC containing 0.1% (w/v) SDS, followed by washes (2 x 15 min at 65°C) with 0.2 x SSC containing 0.1% (w/v) SDS.

2.11.3 Colorimetric Detection of bound DIG-labelled probe.

Hybridised filters were washed briefly in Buffer 1 (0.1 M Tris pH 7.5, 0.15 M NaCl) and blocked by incubation in 5% (w/v) skim milk in Buffer 1 (60 min at room temperature with agitation). An alkaline phosphatase (AP)-conjugated anti-DIG antibody (Roche), diluted 1:5,000 in 5% (w/v) skim milk in Buffer 1, was then added to the filter, and incubation continued at room temperature for at least 30 min. Unbound AP-conjugate was removed by washing (twice for 10 min at room temperature) in Buffer 1. The filter was then incubated in Buffer 2 (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5) for a minimum of 10 min before detection of probe by exposure to 10 ml of freshly prepared colour solution (45 µl of nitroblue-toluidine (75 mg/ml, Roche) and 35 µl of the substrate 5-bromo-4-chloro-3-indolylphosphate (50 mg/ml, Roche) in 10 ml of Buffer 3). The filter was developed in the dark without agitation, and the reaction stopped by removing the colour solution and adding 100 ml of TE buffer.

2.12 Sodium dodecyl sulphate -polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed on 5% stacking, and 10% or 15% separating polyacrylamide gels using a modification of the procedure described by Lugtenberg *et al.* (1975). Samples were boiled for 5 min in SDS-sample buffer (25 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, and 15% (w/v) bromophenol blue) prior to loading, and electrophoresed through the gel at 200 V for 2 - 3 hr. Gels were stained o/n at room temperature,

with gentle agitation in 100 mls of staining solution (5 ml 0.9% (w/v) Coomassie Brilliant Blue G250, 10 ml perchloric acid and 85 ml of distilled water). Gels were de-stained with several changes of 5% (v/v) acetic acid, with agitation o/n at room temperature or for 1 - 2 hr with intermittent heating (1 min in a 700 W microwave oven).

Size markers (Pharmacia) were α-lactalbumin (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa) and phosphorylase B (94 kDa). Gels destined for transfer to nitrocellulose for immunoblotting included pre-stained molecular mass standards (New England Biolabs) comprising triosephosphate isomerase (32.5 kDa), aldolase (47.5 kDa), glutamic dehydrogenase (62 kDa), MBP-paramyosin (83 kDa) and MBP-β-galactosidase (175 kDa).

2.13 Western immunoblot analysis.

After separation by SDS-PAGE, proteins were transferred from the gel matrix to nitrocellulose (Schleicher and Schuell) in a Trans-blot cell (Bio-Rad, 200 mA for 2 hr), using a transfer buffer comprising 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 5% (v/v) methanol (Towbin *et al.*, 1979). Following transfer, the nitrocellulose membrane was incubated for 1 hr at room temperature in 5% (w/v) skim milk powder in TTBS (0.05% (v/v) Tween-20, 20 mM Tris-HCl, pH 7.4, 0.9% (w/v) NaCl) to block non-specific binding sites. The membrane was then incubated with primary antibody - either rabbit anti-K88 or rabbit anti-LT-B (both used at a 1:20,000 dilution in TTBS) - overnight at room temperature with gentle agitation. Unbound antibody was removed by washing the filter (3 x 10 min) in TTBS before incubation with horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Kirkegaard and Perry Laboratories Inc.; diluted 1:10,000 in TTBS) for 2 hours at room temperature. Prior to detection, the filter was washed (5 x 5 min) with TBS (20 mM Tris-HCl, pH 7.4, 0.9% (w/v) NaCl). For enhanced chemiluminescence (ECL) detection, the membrane was soaked in chemiluminescence blotting

substrate (POD; Roche) for 1 min at room temperature in a transparent plastic bag. The membrane was then exposed to X-ray film (Cronex) in a film cassette (Kodak) for 30 sec - 10 min and the film developed in D19 developer (Kodak) for 2 - 5 min before being washed and fixed for 5 min in negative fixer (Kodak).

2.14 Bacteriophage techniques.

2.14.1 Bacteriophage propagation and titration.

Donor strains for bacteriophage propagation were subcultured 1:20 from o/n LB cultures and grown at 37°C to early exponential phase. 100 µl of bacterial culture was mixed with 100 µl of bacteriophage stock (see below) and 100 µl of MC salts solution (100 mM $MgSO_4$ and 5 mM $CaCl_2$) and left to stand at 37°C for 10 min. Soft agar (1.5 ml LB and 1.5ml molten LB agar, mixed and held at 50°C) was then added and the mixture poured onto pre-warmed LB agar plates and allowed to set. Plates were incubated at 37°C for 6 hr, or until the appearance of the plates suggested bacterial lysis had occurred (in comparison to plates lacking bacteriophage). The soft agar layer was scraped off with a sterile spatula, and transferred to a McCartney bottle containing 20 ml of LB. One drop of chloroform was then added, followed by vigorous vortexing for ~ 1 - 2 min, to lyse any remaining bacteria. Bottles were spun at ~ 4,000 rpm for 10 min (IEC Centra 4X centrifuge) to sediment the soft agar, and the supernatant collected as a bacteriophage stock. Where possible, bacteriophage stocks were prepared within a few days of the transduction event.

Plaque assays were performed to determine bacteriophage concentration using the above procedure. This involved incubating aliquots of the indicator strain with several dilutions of the bacteriophage stock (10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}), before addition of soft agar and transfer to LB plates. Resulting plaques were counted on an illuminated colony counter (Selby Anax). Bacteriophage stocks that were estimated to contain ~ $5x10^9$ plaque forming units (pfu)/ml were

considered to be suitable for transduction; stocks below this concentration were subjected to additional rounds of propagation.

2.14.2 Bacteriophage sensitivity tests.

S. Stanley was tested for sensitivity to P22 (laboratory collection), P1 *dam rev* (Professor B. Stocker, Stanford University, CA), Felix O (FO; laboratory collection) or C21 (laboratory collection) bacteriophage as follows. An o/n LB culture was diluted 1:20 and grown to OD_{650nm} ~ 0.5 - 0.6. A loopful of suspension was streaked across the surface of an agar plate (with antibiotics as appropriate). This was then cross-streaked (in one pass at right angles) with the various bacteriophage stocks and the plates incubated at 37°C overnight. A zone of lysis was indicative of bacteriophage sensitivity.

2.14.3 Bacteriophage transduction.

Bacteriophage transduction is one method by which a mutation in *aroA* could be introduced into the chromosome of *S.* Stanley. The strategy would require two separate transduction events with bacteriophage P1 *dam rev* (a derivative of P1 able to package a greater amount of DNA within bacteriophage head particles). The first involves the replacement of the wt *aroA* gene with an *aroA*(*serC*)::*Tn*10 transposon interruption found in donor strain PE952. Positive transductants would be dependent on aromatic and serine supplements, and could be selected by their expression of tetracycline resistance (*aroA*, *serC* and Tc^R). A second P1 *dam rev* transduction event would then involve replacement of the *aroA*(*serC*)::*Tn*10 transposon with a mutant (C-terminal deletion) *aroA* gene (found in donor strain PE951), and selection for the loss of tetracycline resistance. Several transduction methods were used in attempts to create the

intermediate *aroA*(*serC*)::*Tn*10 transposon mutant in *S.* Stanley, using phage stock harvested from the donor strain PE952.

2.14.3.1 Method 1

S. Stanley was grown o/n in LB, sub-cultured 1:20 into fresh LB, and grown for a further 4 - 5 hr. Bacteria were then harvested by centrifugation and the pellet resuspended in 2 ml LB. After the addition of 100 µl of 1 M $MgSO_4$, 10 µl of 1 M $CaCl_2$ and 100 µl of bacteriophage stock, the suspension was left standing at 37°C for 10 min (a control suspension lacking bacteriophage was also set up). EGTA (100 µl of 10 mM) was added and the mixture incubated for a further 10 min at 37°C, followed by centrifugation and resuspension of pellets in 1 ml LB; 100 µl aliquots were plated onto appropriate selection medium and incubated for 18 - 24 hr at 37°C.

2.14.3.2 Method 2

A second transduction method was adapted from a publication by Cerquetti and Hooke (1993). Equal volumes (400 µl) of bacteria (early log phase, ~ 10^8 cfu/ml in LB) and bacteriophage (titre of 10^7 pfu/ml) were mixed and left standing at 37°C for 10 min (a control suspension lacked bacteriophage). The mixture was then placed on ice to inhibit phage replication and (at least three) 100 µl aliquots were separately added to 10 ml of melted LB agar at 45°C and poured into a sterile petri dish. The plates were incubated at 37°C o/n before being overlaid with 10 ml of LB agar (containing the appropriate antibiotic) and incubated for an additional 24 hr. Only bacteria that had undergone a successful transduction event should express antibiotic resistance and grow into the top overlay. Such colonies were removed using a Pasteur pipette, and the agar plugs incubated in LB (containing antibiotic) at 37°C o/n.

2.14.3.3 Method 3

S. Stanley was grown o/n in LB, diluted 1:20 into fresh medium and grown for 4 - 5 hr at 37°C. Bacteria were recovered by centrifugation and resuspended in 10 mls of 10 mM $MgSO_4$. Bacteriophage (10^7 - 10^8 pfu in 2 - 20 µl) were added to 50 µl aliquots of bacteria and the suspensions left standing at 37°C for 10 min. 1 ml of LB containing 10 mM of Na citrate was then added, and the mixture incubated at 37°C for 1 hr with vigorous aeration. Bacteria were then centrifuged, washed in 1 ml of 10 mM $MgSO_4$ and resuspended in 50 µl of the same solution for plating on LB agar containing antibiotic.

2.14.4 Selection of putative *aroA* deletion mutants.

Tetracycline resistant *S.* Stanley were putative *aroA*(*serC*)::*Tn*10 transductants. These were then subjected to a second round of transduction to replace the transposon-interrupted *aroA* gene with a more stable *aroA* deletion mutation (*aroA* DEL407) using bacteriophage propagated on the donor strain PE951. Following transduction, bacteria were plated on Bochner medium (with aromatic supplements) to inhibit the growth of Tc^R colonies (Bochner *et al.*, 1980). The medium was prepared as follows: 15 g agar, 10 g tryptone, 5 g yeast extract, 10 g of NaCl, 2 g of glucose, 0.05 g of chlortetracycline hydrochloride, 10 g $NaH_2PO_4.H_2O$ and distilled water (to 1 l) were mixed and autoclaved. Once cooled to 56°C, 6 ml of fusaric acid (2 mg/ml (w/v) stock), and 5 ml of 20 mM $ZnCl_2$ were added and plates poured. Tc^s colonies were re-streaked and stored in glycerols at - 70°C.

2.15 Immunological methods.

2.15.1 Preparation of bacteria and mice for immunisation.

All BALB/c or CBA mice were female, and aged 9 - 10 weeks at the commencement of experiments unless otherwise stated. Bacteria to be fed to mice were grown, from single colonies, o/n at 37°C with aeration in either CBT (for all *S.* Stanley-based strains) or LB (for *aroA S.* Dublin-based strains), with supplements and/or antibiotics as required. Bacteria were pelleted (10 min, 8,000 rpm, Beckman JA-20 rotor) and washed in fresh broth (containing antibiotics if applicable), prior to being subcultured (1:10) into 300 ml of broth (CBT for *S.* Stanley-based strains, and TSB for *aroA S.* Dublin-based strains, after Bao and Clements (1991)) in a 5 L conical flask. After incubation at 37°C for 4 hr (to late log phase), bacteria were pelleted (15 min, 8,000 rpm, Beckman JA-10 rotor), washed with 20 mls of PBS, and re-pelleted. Once excess supernatant had been drained, pellets were resuspended in PBS to give ~ $3x10^{11}$ cfu/ml (checked retrospectively by viable counting). Mice that were to be fed 10^{10} cfu were orally immunised with 30 µl of this bacterial suspension, whereas mice fed 10^{9} cfu were orally immunised with 30 µl of a 1:10 PBS dilution of this suspension. Mice were starved at least 4 hr prior to, and 45 min following oral immunisation. Animals were fed 35 µl of 10% (w/v) sodium bicarbonate 5 - 10 minutes before receiving bacteria to neutralise stomach acidity.

S. Typhimurium C5 for i.p. injection was grown, from a single colony, o/n at 37°C with aeration in CBT broth. Bacteria were then subcultured 1:20 into 20 ml of fresh CBT and grown in a 100 ml conical flask with aeration at 37°C for 3 hr (mid to late log phase). The bacterial suspension was diluted in PBS to give ~ 400 cfu/ml as determined by measuring cell culture optical density with a Biorad Smartspec 3000 spectrophotometer and making appropriate dilutions (actual dose given was determined retrospectively by viable counting). Mice were then immunised by i.p. injection with 0.1 ml of this suspension using a 1 ml syringe (Becton Dickinson) fitted with a 13 mm 26G needle (Becton Dickinson).

2.15.2 Collection and processing of blood, tissues and faecal pellets.

In order to prepare serum samples, mice were anaesthetised with diethyl ether and bled via the retro-orbital plexus with a sterile Pasteur pipette. The blood samples, collected in sterile tubes, were left at 37°C for 90 min to facilitate clot formation. After centrifugation (5 min, 2,000 rpm, Heraeus Biofuge 15) the sera were transferred to fresh sterile tubes and stored at - 20°C.

Peyer's patches, excised from the small intestine of orally-immunised mice, were collected in 2 mls of PBS and homogenised with an Ultra-Turrax (Janke and Kunkel, IKA-Labortechnik) tissue homogeniser fitted with a Thyristor Regler speed regulator (Janke and Kunkel, IKA-Labortechnik, model TR50; setting 6 for 10 secs). Mouse spleens were collected from mice infected by i.p. injection and were homogenised in 3 mls of PBS in the same manner. Appropriate dilutions of homogenates were plated on selective XLD medium (containing antibiotics if necessary) and incubated at 37°C o/n; resulting colonies were counted to estimate bacterial burdens.

Fresh mouse faecal pellets (initially 4 pellets per ml) were collected on ice in reaction tubes pre-blocked (o/n 4°C) with 1% BSA-PBS. Preliminary experiments (section 3.2.1) compared three different collection buffers: buffer I was 0.1% (w/v) BSA in PBS (Dr. G. Deuce, personal communication); buffer II was 30 mM Tris-HCl pH8.8, 0.9% NaCl, 50 mM EDTA, and 1% (w/v) Tween 20 (Dr. G. Russell-Jones, personal communication); buffer III was PBS containing 0.1 mg/ml Soybean trypsin inhibitor (Sigma), 1% (w/v) BSA, 25 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma; 100 mM stock made in 100% ethanol) (Butterton *et al.*, 1996). Pellets were homogenised with blunted Pasteur pipettes and left at 4°C for either 4 hours or o/n. Samples were then spun at 14,000 rpm at 4°C (Eppendorf 5417R centrifuge), and clarified supernatants split into two aliquots in fresh (pre-blocked) tubes and stored at - 20°C.

Buffer III was selected for routine use, and it was also decided to routinely use a 4 hr incubation period for sample preparation. The immunoglobulin content of each faecal pellet

sample was subsequently increased by changing the collection protocol to 9 pellets in 600 µl of buffer III. Finally, the addition of glycerol to the supernatants (to 50% (v/v)) was found to improve sample storage at - 20°C (see section 3.2.3).

2.16.3 Enzyme linked immunosorbent assay (ELISA) procedures.

2.16.3.1 Titration of antibodies to K88.

K88 pili had been isolated and purified prior to the commencement of this study using a procedure described by Hone *et al.* (1988). This preparation, at a concentration of 1 µg/ml in TSA buffer (Tris-Saline-Azide; 132 mM NaCl, 25 mM Tris-HCl pH 7.5, 0.05% Na azide and Milli Q water), was used to sensitise ELISA trays (Nunc, Polysorp F96; 100 µl/well) o/n at 4°C. The following day, the sensitising solution was discarded and the trays washed three times with wash buffer (0.1% (v/v) Triton X-100, 4.9 mM Tris, 150.6 mM NaCl in Milli Q water, pH 7.6). After banging the trays dry, wells were blocked with 200µl of blocking solution for ~ 90 min at 37°C. At the commencement of this study, the blocking solution consisted of 0.02% (w/v) BSA in buffer (0.18 M NaCl, 12.5 mM tri-ethanolamine pH 7.6, 0.05% (v/v) Tween 20, 0.1% (w/v) sodium azide and Milli Q water). Experiments to be described (section 3.2.2) compared several blocking proteins, and resulted in 1% (w/v) BSA in PBS being selected for routine use.

The blocking solution was discarded and residual liquid removed from wells by banging the trays dry. Test samples were initially diluted 1:3 (clarified faecal pellet supernatants (CFPSs)) or 1:20 (sera) and thereafter subjected to five-fold serial dilutions (by transferring 25 µl into 100 µl) in blocking solution. The top row in each tray served as negative control wells (being sensitised with K88 but then incubated with blocking solution instead of sample) and was subsequently used to blank the microplate reader. Each tray also contained one row set aside for the titration of a standard anti-K88 serum (or CFPS) to check assay performance.

Trays were incubated with test samples for 4 hr at 37°C and then washed three times in wash buffer. Secondary antibody for the detection of IgG in serum samples was AP-conjugated goat anti-mouse IgG (Kirkegaard and Perry Laboratories Inc.); 100 µl of a 1:50,000 dilution in blocking solution was dispensed into each well and the trays left o/n at 4°C. After three washes in wash buffer, 100 µl substrate solution was added to each well. This was prepared by dissolving Sigma 104® alkaline phosphatase substrate tablets in assay buffer (1M diethanolamine, 0.05% (w/v) sodium azide, 5 mM $MgCl_2$ and Milli Q water, pH 9.8; one tablet per 5 mls of buffer). After incubating at 37°C for 3 hr, optical density (OD) was measured using either a Molecular Dynamics Biolumin 960 or a Labsystems Multiskan Ascent ELISA plate reader, with a primary filter setting of 410 nm and a secondary filter setting of 620 nm. OD readings were adjusted by subtracting secondary filter and averaged blank readings from the primary filter reading, and sample titres were determined as the reciprocal dilution yielding an endpoint of $OD_{410\text{-}620nm}$ 0.15.

The detection of IgA in CFPS samples required a two layered detection system, the first being biotin-conjugated goat anti-mouse IgA (Sigma) diluted 1:5,000 in blocking solution (100 µl per well) and incubated o/n at 4°C. After washing, trays were incubated at 30°C for 90 min with an avidin-peroxidase conjugate (Extravidin®, Sigma, 1:3,500 dilution, 100 µl per well). Trays were washed again, and incubated for 3 hr at 30°C with Sigma Fast™ o-phenylenediamine dihydrochloride (OPD) substrate tablets (Sigma) dissolved in distilled water (as recommended by the manufacturer; 100 µl per well). Colour development was stopped by the addition of 50 µl 3 M HCl per well, and OD measured using the aforementioned plate readers with a primary filter setting of 485 nm. After subtracting the averaged blank readings, titre values were determined as the reciprocal CFPS dilution yielding an endpoint of OD_{485nm} 0.15.

2.16.3.2 Titration of antibodies to LPS.

The measurement of anti-LPS responses by this laboratory has traditionally involved sensitising ELISA trays with 5 μg/ml methylated BSA-treated LPS (MeBSA-LPS) in TSA. This reagent was prepared by adding 0.1 mls of MeBSA (Sigma; 5 mg/ml stock dissolved in water) per ml of stock LPS solution (5 mg/ml) and incubating with stirring for 30 min at room temperature. In other laboratories however, an alternative method of sensitisation is used, which involves incubating trays with 20μg/ml LPS in carbonate buffer (3.2 mM Na_2CO_3, 6.7 mM $NaHCO_3$, 0.5 μM $MgCl_2$, pH 9.6). These methods were compared in a preliminary experiment described in section 3.2.4.

Purified, lyophilised LPS isolated from *S.* Typhimurium (O-4,5,12; the same O-antigen serotype as *S.* Stanley) and *S.* Enteritidis (O-9,12; the same O-antigen serotype as *S.* Dublin) were obtained from Sigma. These were held at 4°C as 20 mg/ml stocks in sterile Milli Q water, and diluted into carbonate buffer or treated with MeBSA as above. Trays were sensitised o/n at 4°C, washed and blocked with 200μl of 1% (w/v) BSA-PBS per well. From this point, the protocol for the titration of antibodies to K88 was followed.

2.16.3.3 Titration of antibodies to LT-B by GM_1 ELISA.

Antibodies to LT-B were titrated in a GM_1 ELISA (Holmgren *et al.*, 1975), which exploits the natural affinity of LT-B for GM_1 gangliosides. ELISA trays were sensitised (o/n at 4°C) with GM_1 monoganglioside (from bovine brain, Sigma; 2 μg/ml diluted in TSA, 100 μl per well). The following day trays were washed and blocked as described above, then incubated for 4 hr at 37°C with 1 μg/ml of purified LT-B (kindly provided by Dr. T. Duthy, University of Adelaide), diluted in blocking solution and dispensed at 100 μl per well. Trays were washed and incubated for a further 4 hr with primary antibody (serum or CFPS samples which were diluted

and serially titrated in blocking buffer). Negative control wells were sensitised with GM1 and LT-B but received no source of primary antibody; positive control wells were similarly sensitised and incubated with a known positive anti-LT-B sample. Trays were washed and incubated with secondary antibody (AP-conjugated goat anti-mouse IgG, or biotin-labelled goat anti-mouse IgA and HRP-conjugated avidin) and developed as described above.

2.16.3.4 Estimating total IgA content of CFPS samples.

ELISA trays were sensitised o/n at 4°C with 5 μg/ml goat anti-mouse IgA (Kirkegaard and Perry Laboratories Inc.) diluted in TSA buffer (100 μl/well). Trays were washed and blocked as described above. CFPS samples were titrated in duplicate in four-fold falling dilutions (beginning at 1:100) in blocking solution. A standard curve was included in each tray and was constructed using purified mouse myeloma IgA (MOPC 315, kindly provided by Dr. P. Ey, University of Adelaide); this was titrated in duplicate in three-fold falling dilutions from a starting concentration of 1.6 μg/ml. Trays were then left at 37°C for 4 hr, washed, and incubated with the abovementioned biotin-labelled and avidin-peroxidase conjugates. Trays were developed at 30°C using OPD substrate tablets and the reaction stopped after 30 min by the addition of 50 μl of 3 M HCl per well. OD_{485nm} values where adjusted by subtracting an averaged blank reading from all wells. The IgA concentration of each sample was determined by selecting an OD value which fell within the linear range of the standard curve, estimating IgA concentration and multiplying by the appropriate dilution factor.

2.16.3.5 ELISA Inhibition Assay (EIA)

2.16.3.5.1 Preparation of samples for estimation of LT-B production.

Estimation of LT-B release from the periplasm of cultured bacteria was done using a method modified from that published by Hunt and Hardy (1991). O/n LB cultures of the strains of interest were subcultured 1:20 into 20 mls fresh medium containing conalbumin (iron chelator, Sigma) at 450 μg/ml and grown to an OD_{600nm} ~ 0.6 (additional subcultures into medium without conalbumin were set up as negative controls). At this stage trypsin (100 μg/ml), and bile salts (to the following concentrations) were added to mimic the environment of the human small intestine: 1.4 mM sodium glycholate; 0.7 mM sodium deoxycholate; 1.2 mM sodium glycochenodeoxycholate; 2.8 mM sodium taurocholate; 2.4 mM sodium taurochenodeoxycholate; 1.4 mM sodium taurodeoxycholate. Negative control cultures did not receive trypsin or bile salts. Samples (1 ml) were taken from cultures at hourly intervals and centrifuged (4°C, 15,000 rpm, Eppendorf 5417R centrifuge). Supernatants were transferred to clean tubes, and soybean trypsin inhibitor (to 120 μg/ml) and PMSF (to 0.25 mM) added to inhibit protein degradation. Cell pellets were resuspended in 1 ml of a solution containing the same protease inhibitors (50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 0.25 mM PMSF, 120 μg/ml soybean trypsin inhibitor) and subjected to sonication on ice (Branson Sonifier cell disruptor fitted with a microtip; 3 pulses at 1 min per pulse, output control of 3). Samples were stored at - 20°C until assayed by ELISA inhibition assay (EIA).

2.16.3.5.2 Preparation of samples for estimation of K88 production.

Bacteria to be tested were harvested from CBT plates (to prevent damage to or loss of pili by vigorous aeration and repeated centrifugation) and standardised to ~ $2x10^{10}$ cfu/ml in PBS using a Biorad Smartspec 3000 spectrophotometer. Cells (1 ml) were pelleted (8,000 rpm,

Heraeus Biofuge 15) and killed by carefully resuspending in 1 ml of 1% formalin-PBS and incubating standing at 37°C for 60 - 90 min. Bacteria were centrifuged, washed once in PBS, and resuspended in 1 ml of PBS.

2.16.3.5.3 Preparation of antiserum for estimation of K88 production.

Rabbit anti-K88 serum (prepared prior to this study; refer to Hone *et al.*, 1988) was absorbed with live *S.* Stanley (containing an appropriate control plasmid) to eliminate potential cross reactivity to vector-associated proteins. An overnight 50 ml CBT culture of *S.* Stanley was pelleted (10 min, 8,000 rpm, Centra4X IEC bench centrifuge) and resuspended in 10 ml of serum (containing of 0.02% (w/v) sodium azide). Successive absorptions with fresh bacteria were carried out by incubating the serum either at 37°C for 4 hr or o/n at 4°C. After each absorption, the bacteria were removed by centrifugation (10 min, 8,000 rpm, Centra4X IEC bench centrifuge) and the serum used to resuspend the next bacterial pellet. The process was repeated 8 times, and after the final absorption the serum was passed through a 0.2 μm Millipore filter and stored at 4°C.

2.16.3.5.4 EIA protocol.

EIAs were performed following a method adapted from Hone *et al.* (1988). ELISA trays were sensitised with K88 or LT-B as described above. A standard curve was included on each tray, and was prepared by initially diluting purified protein (either K88 or LT-B) to 20 μg/ml in blocking solution. Then 62.5 μl of this solution was dispensed into the first well (in each of duplicate rows) and serially diluted five-fold (12.5 μl in 50μl blocking solution) across the tray. In other rows, the test samples (formalin-inactivated bacterial suspensions for K88, cell sonicates or supernatants for LT-B) were serially diluted (also in duplicate) three-fold (25 μl in 50 μl

blocking solution) across the tray. Primary antibody (absorbed rabbit anti-K88 diluted 1:10,000, or rabbit anti-LT-B diluted 1:20,000 (provided by Dr. T. Duthy, University of Adelaide)) was diluted in blocking buffer and 50 µl dispensed into all wells (except a negative control row which was incubated with 100 µl of blocking solution). A positive control row (lacking inhibitor) contained 50 µl of primary antibody and 50 µl of blocking solution.

Trays were mixed briefly on a tray shaker (Flow Laboratories TiterTek; 15 sec; 800 rpm) and incubated at 37°C for 4 hr. Following washing, 100 µl of AP-conjugated goat anti-rabbit IgG (diluted 1:50,000) was dispensed into all wells and the trays incubated at 4°C o/n. The trays were then washed and developed as described above (section 2.16.3.1), with OD values adjusted as described previously ($OD_{410\text{-}620nm}$). The concentration (µg/ml) of protein per sample was calculated from OD values that fell within the linear range of the standard curve; the protein concentration corresponding to the OD value being multiplied by the appropriate dilution factor. Samples that returned low $OD_{410\text{-}620nm}$ values below the linear range of the standard curve were considered to be negative (no protein detectable). The sensitivity of the assay was determined by calculating the amount of purified K88 or LT-B that would produce an OD equivalent to 50% of the OD of the averaged positive control wells (ie. 0.5 x the averaged $OD_{410\text{-}620nm}$ of the wells in the positive control row). K88 concentrations were expressed as µg per 10^{10} formalin-inactivated bacteria. LT-B concentrations within cell sonicates or supernatants were expressed as µg/ml, and standardised by dividing this value by the OD_{600nm} of the bacterial culture at the point of sampling.

2.16.3.6 Estimation of serum IgG_1 and IgG_{2a} antibodies by ELISA.

For some studies it was of interest to gain some insight into the Th1/Th2 bias of serum antibody responses by determining the ratio of IgG_{2a} to IgG_1 antibodies. In order to avoid any bias in detection of these subclasses, preliminary ELISAs were performed (section 3.2.5) to

compare the efficiency of their respective immunoconjugates. ELISA trays were sensitised (o/n 4°C) with 5 μg/ml (in TSA; 100 μl per well) of either purified goat anti-mouse IgG_1 or IgG_{2a} antibody (Rockland Immunochemicals). The following day, the trays were washed, and purified IgG_1 or IgG_{2a} (kindly provided by Dr. P. Ey, University of Adelaide) added at a final concentration of 5 μg/ml in blocking buffer (100 μl per well). Negative control wells were included which were incubated with washing buffer instead of immunoglobulin. Trays were left at 37°C for 4 hr prior to being washed. AP-labelled goat anti-mouse IgG_1 and IgG_{2a} immunoconjugates (Rockland Immunochemicals) were then titrated in duplicate, beginning from 1:300 dilutions with falling three-fold dilutions. Trays were left o/n at 4°C, washed and developed as described above.

This comparison revealed that the two immunoconjugates detected antibodies of homologous isotype with similar efficiency and identified 1:20,000 as a suitable working dilution for each. Additional capture ELISAs were performed to confirm the specificity of each immunoconjugate, by performing parallel titrations and comparing the recognition of immunoglobulin of homologous or heterologous isotype. No significant recognition of the heterologous isotype ($OD_{410\text{-}620nm} < 0.15$) was observed with either immunoconjugate when these were tested at dilution of 1:20,000.

2.17 Graphing and statistical analysis.

Graphs, histograms, ELISA endpoint titres and total IgA concentrations, were plotted and calculated using GraphPad Prism (GraphPad Software, Inc.). Probability values were determined using the Student's t-TEST (TTEST) function in Microsoft Excel (Microsoft Corporation). Data were $\log_{10}$ transformed prior to these calculations unless otherwise indicated.

CHAPTER THREE

Measurement of the secretory IgA response of the murine GALT.

3.1 Introduction

Previous studies in this laboratory showed that serum antibody responses to a foreign antigen (K88) being delivered by r*Salmonella* could be abrogated by prior exposure to the vector strain (Attridge *et al.*, 1997). Attempts to measure intestinal responses to K88 were unsuccessful however, leaving unresolved the key issue of whether the state of hyporesponsiveness induced by vector-priming extends to the intestinal mucosal IgA response. The aim of the experiments described in this chapter was to establish sampling protocols and ELISAs to allow this question to be addressed.

3.2 Results

3.2.1 Comparison of collection buffers for the preparation of clarified faecal pellet supernatants.

Earlier attempts in this laboratory to measure murine gut IgA responses to K88 were performed primarily on intestinal washout samples collected by excising and flushing the entire small intestine with PBS. After vortexing and centrifugation, the clarified supernatant was

titrated across duplicate rows of an ELISA tray; the wells of one row had been sensitised with K88 while the other row (unsensitised, but subsequently incubated with blocking solution containing BSA) served as a control to assess the specificity of IgA binding. Although colour development suggested the binding of IgA antibodies in K88-sensitised wells, significant but variable colour development was also observed in control wells, suggesting that either the sampling procedure or the ELISA protocol was unreliable (Attridge *et al.*, 1997).

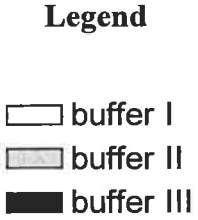
At the commencement of these studies, a review of the literature indicated that several groups were successfully using fresh faecal pellets as a source of material for intestinal IgA response measurement. Based on these reports, a protocol was adopted for the preparation of clarified faecal pellet supernatant (CFPS) samples (section 2.15.2). An initial experiment was conducted to compare the collection buffers used by various groups, with particular interest in the efficiency of IgA extraction.

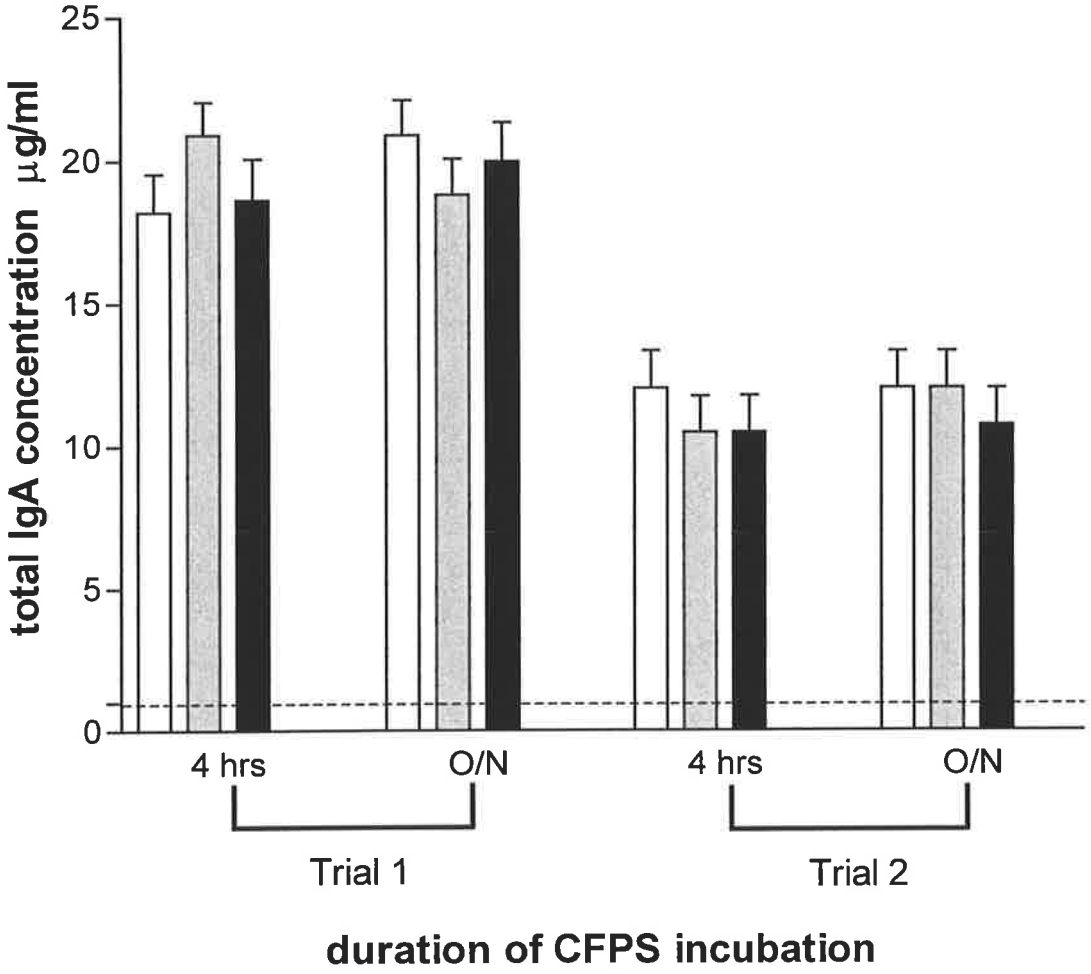
Twelve faecal pellets were collected from each of nine unimmunised BALB/c males (twelve weeks of age) on two separate trials. Four faecal pellets from each animal were dispensed into 1 ml of collection buffers I, II or III (section 2.15.2). Pellets were dispersed with a blunted Pasteur pipette, vortexed vigorously, and left on ice for four hours. At this time the samples were thoroughly mixed again and half removed for preparation of CFPSs. The remainder were incubated at 4°C o/n and additional CFPSs prepared the next day. ELISAs were performed for each trial, and the IgA content of the various samples is shown in Figure 3.1.

The assessment of intestinal IgA responses would normally require the titration of each sample in two separate ELISAs, one to determine the specific antibody titre, and the other to estimate total IgA content. This allows the activity to be expressed as an ELISA titre per μg of total IgA, thereby correcting for variability in immunoglobulin content between samples. In the present experiment, only the total IgA content was determined as the mice had not been immunised. As shown in Figure 3.1, the IgA content of CFPSs within each trial was not significantly affected by the collection buffer used, nor did the incubation period influence the

Figure 3.1 Comparison of faecal pellet collection buffers.

CFPS samples were prepared in buffers I, II or III as described in the text; data from two separate trials are shown. Histograms show the total IgA concentration (μg/ml) of samples prepared using either four hour or o/n incubation (arithmetic mean (M) ± standard deviation (SD); $n = 9$), and the dashed line represents the limit of detection at a titre of 1.




25
20
15
10
5
0
total IgA concentration µg/ml
4 hrs
O/N
4 hrs
O/N
Trial 1
Trial 2
duration of CFPS incubation

efficiency of IgA recovery. The significant variation in the immunoglobulin content of the samples between each trial exemplified the need to express antibody titres per unit weight of IgA.

Since these results did not eliminate the possibility that a proportion of the IgA present in the CFPS samples was degraded, two additional experiments were designed to examine whether collection buffers, which each contain protease inhibitors, differentially overcame the degradative potential of intestinal proteases.

The first experiment assessed the potential of the various CFPS samples to degrade antigen bound to wells of sensitised ELISA trays, as an indirect indication of protease activity. CFPS samples were incubated in duplicate in K88-sensitised ELISA wells while similar control wells were incubated in collection buffer alone (or in PBS). After a period of four hours at 37°C, the conditions routinely used for binding of primary antibody in our ELISAs, the trays were washed, and a pooled mouse serum known to contain IgG antibodies to K88 was titrated serially across all rows of the tray. The trays were left for a further four hours at 37°C, following which the specific ELISA protocol was followed, to compare the level of binding in the various rows. As shown in Figure 3.2 A, there appeared to be no reduction in anti-K88 ELISA titre following the incubation of various CFPSs in sensitised wells.

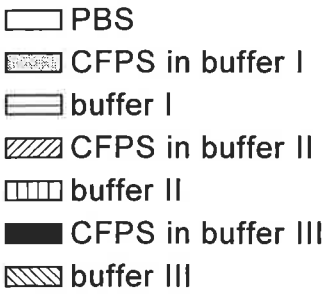
The second experiment was designed to assess the extent of antibody degradation during sample collection and preparation. Tubes containing the various collection buffers were seeded with a 1:10 dilution of a mouse antiserum known to contain IgG antibody to K88, prior to faecal pellet collection (seeded buffer controls were also set aside at this point). CFPSs were subsequently prepared after incubation at 4°C for four hours or o/n. IgG is more susceptible to degradation than secretory IgA, therefore providing a more sensitive indicator of CFPS proteolytic activity. These "spiked" samples were then serially titrated in duplicate in rows sensitised with K88; a control titration involved mouse antiserum diluted 1:10 into PBS. The tray was left for four hours at 37°C, following which the specific ELISA protocol was followed.

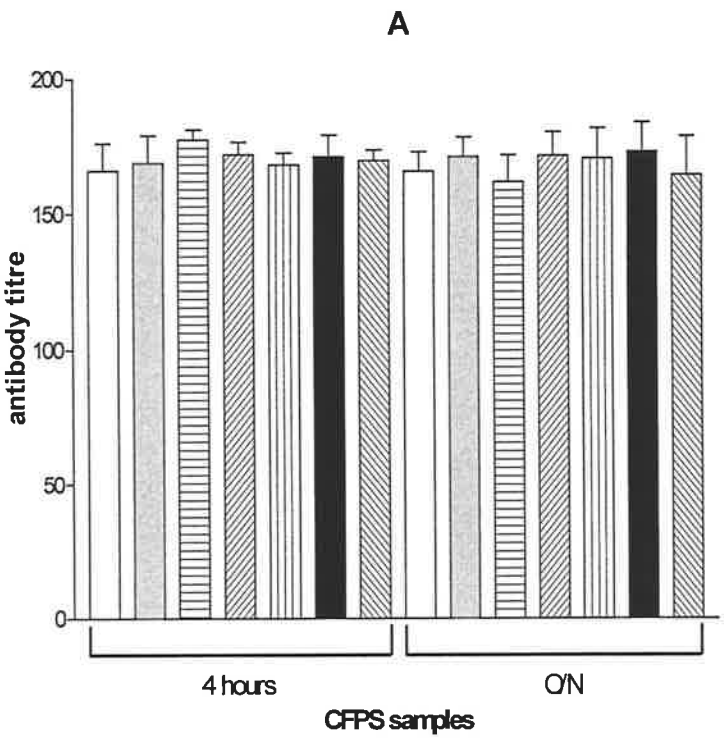
Figure 3.2 Assessment of CFPS protease activity.

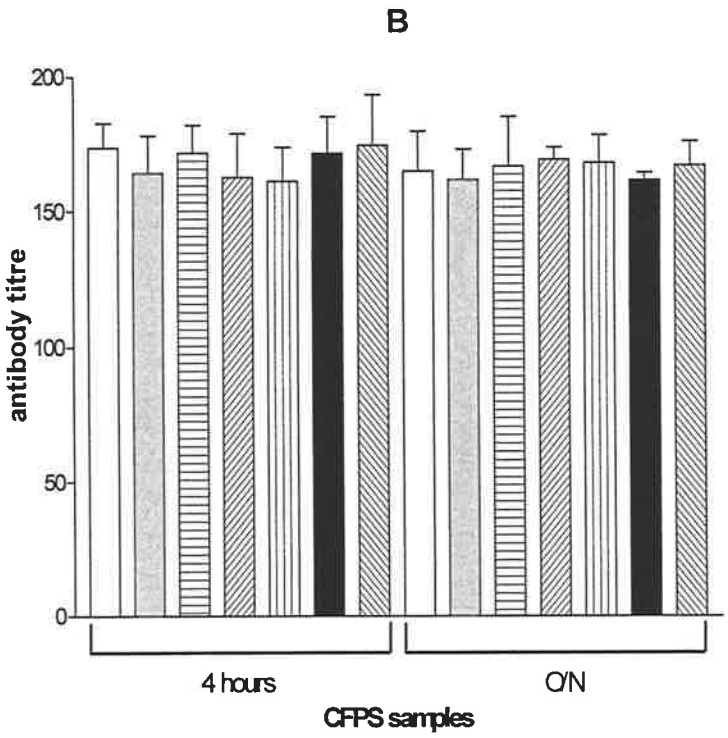
CFPS samples collected in buffers I, II or III following either four hour or o/n incubation were tested for possible protease activity as described in the text. Panel **A** shows the result of an experiment in which K88-sensitised ELISA wells were pre-incubated with CFPS (prepared following incubation at 4°C for four hours or o/n) or buffer controls, prior to titration of anti-K88 serum to assess removal of the sensitising antigen. Histograms show titre of antiserum in ELISA wells after various treatments (M ± SD of duplicate titrations).

Panel **B** shows the effect of seeding CFPS (prepared as described in text, and incubated at 4°C for four hours or o/n) or buffer solution controls with anti-K88 serum. Histograms show anti-K88 titres of spiked samples (M ± SD of duplicate titrations).

Legend




A
200
150
100
50
0
antibody titre
4 hours
O/N
CFPS samples


B
200
150
100
50
0
antibody titre
4 hours
O/N
CFPS samples

As shown in Figure 3.2 B, the antibody titre was not reduced during the CFPS collection procedure.

It would have been expected that if proteases present within the CFPSs had an impact on either the K88 sensitising antigen or on the seeded IgG, a decrease in antibody titre would have resulted. No such effects were observed however, and neither approach revealed any of the collection buffers to be significantly better than the others. Since buffer III contained two (rather than one) non-specific protease inhibitors and EDTA (iron chelator), it was chosen for the collection of CFPSs in all subsequent experiments. It was also decided to routinely use a four hour incubation period for CFPS preparation.

3.2.2 Comparison of ELISA blocking proteins.

Previous attempts at measuring murine gut IgA anti-K88 responses in this laboratory were hampered by the observation of non-specific colour development in control ELISA wells when compared to the corresponding K88-sensitised wells. It was suspected that this "non-specific" binding might actually reflect specific recognition of BSA used in the ELISA blocking solution which contains 0.2 mg/ml BSA (section 2.16.3.1). Hence solutions were prepared containing other blocking proteins - either keyhole limpet haemocyanin (KLH; 0.2 mg/ml), casein hydrolysate (0.2 or 10 mg/ml), or foetal calf serum (FCS; 1% or 5% (v/v)).

In order to test the various blocking solutions, it was necessary to generate CFPS samples with IgA activity against a known antigen. LT-B is known to be a strong mucosal immunogen, hence mice were immunised according to a protocol found to generate gut IgA to this antigen (Professor J. D. Clements, personal communication). A group of nine BALB/c mice was orally immunised with 100 μg of LT-B on days 0 and 7. To increase the immunoglobulin content of CFPSs, it was decided to collect nine pellets from each mouse in 600 μl of buffer III. CFPSs collected on days 20 and 24 were combined to yield a volume sufficient for numerous replicate titrations. The pooled sample was then assayed for IgA antibodies to LT-B in a GM_1 ELISA

(section 2.16.3.3). Parallel titrations were performed using the various blocking solutions (each tested in duplicate) in both LT-B sensitised and control wells.

As shown in Figure 3.3, the use of blocking solution containing 0.2 mg/ml casein hydrolysate resulted in the highest raw and adjusted titres, the latter being significantly higher than that obtained with the original 0.2 mg/ml BSA blocking solution (*, $P < 0.05$). Interestingly, while wells blocked with 10 mg/ml casein hydrolysate showed the greatest reduction in non-specific binding (**, $P < 0.01$ compared with the original 0.2 mg/ml BSA solution), they also displayed a reduced specific anti-LT-B titre, suggesting that casein used at this concentration was interfering with antibody-antigen reactions. It seemed possible that the "non-specific binding" previously encountered in this laboratory may be due to inefficient blocking of ELISA wells, rather than specific recognition of BSA present in the wells following blocking. A second experiment was performed to compare 0.2 mg/ml casein hydrolysate and 10 mg/ml BSA as blocking agents.

The pooled CFPS sample generated above, containing IgA antibody to LT-B, was titrated in duplicate in LT-B sensitised and control wells, using both blocking solutions. As shown in Figure 3.4 A, and diagrammatically in Figure 3.4 B, 10 mg/ml BSA-PBS completely abolished non-specific binding (negative samples were given a titre of 1 even though they did not produce a significant absorbance reading). The corrected antibody titre resulting from blocking with 10 mg/ml BSA-PBS was significantly greater than that achieved using 0.2mg/ml casein (**, $P < 0.01$). As a consequence, 10 mg/ml BSA-PBS was chosen as the blocking solution for all subsequent ELISAs.

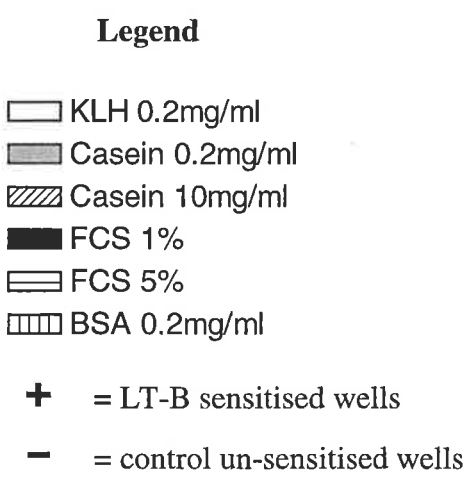
3.2.3 Use of glycerol to improve sample storage.

The long term nature of the proposed immunisation experiments (up to 20 weeks) made it necessary to store CFPS samples for extended periods. During an analysis of samples collected

Figure 3.3 Comparison of ELISA blocking proteins.

Figure shows results from a comparison of proteins used in the ELISA blocking solution. Keyhole limpet haemocyanin (KLH), casein hydrolysate and foetal calf serum (FCS) were used at the concentrations indicated. A CFPS sample (pooled from nine mice) positive for IgA anti-LT-B was titrated in duplicate in LT-B sensitised (+) and unsensitised (-) wells in a specific GM_1 ELISA. Histograms show raw anti-LT-B titres using different blocking solutions; below are shown the adjusted titres obtained by subtracting the ELISA titre in control rows from that obtained in rows sensitised with LT-B. Points of significance are indicated by '*' ($P < 0.05$), or '**' ($P < 0.01$), and the dashed line represents the limit of detection at a titre of 1.

NB: As the same pooled CFPS sample was used for all titrations, it was not necessary to adjust titres in relation to IgA content.



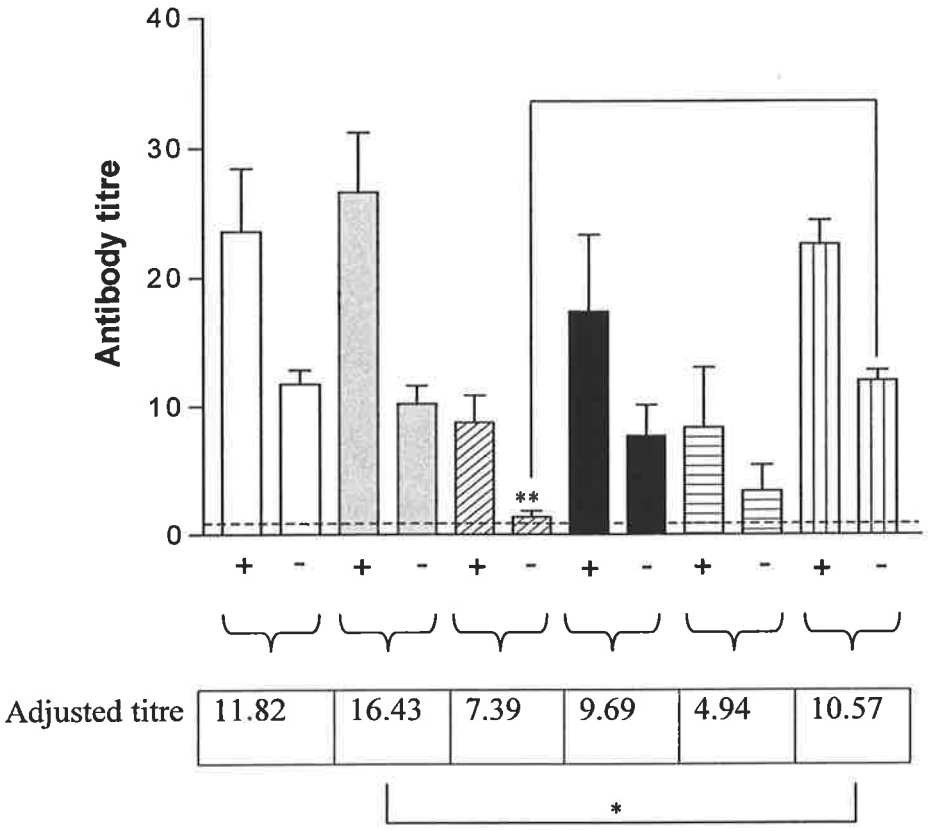
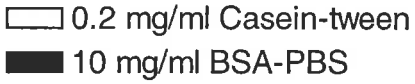

40
30
20
10
0
Antibody titre
**
+ - + - + - + - + - + -
Adjusted titre
11.82
16.43
7.39
9.69
4.94
10.57
*

Figure 3.4 Comparison of ELISA blocking proteins: 0.2mg/ml casein hydrolysate versus 10 mg/ml BSA-PBS.

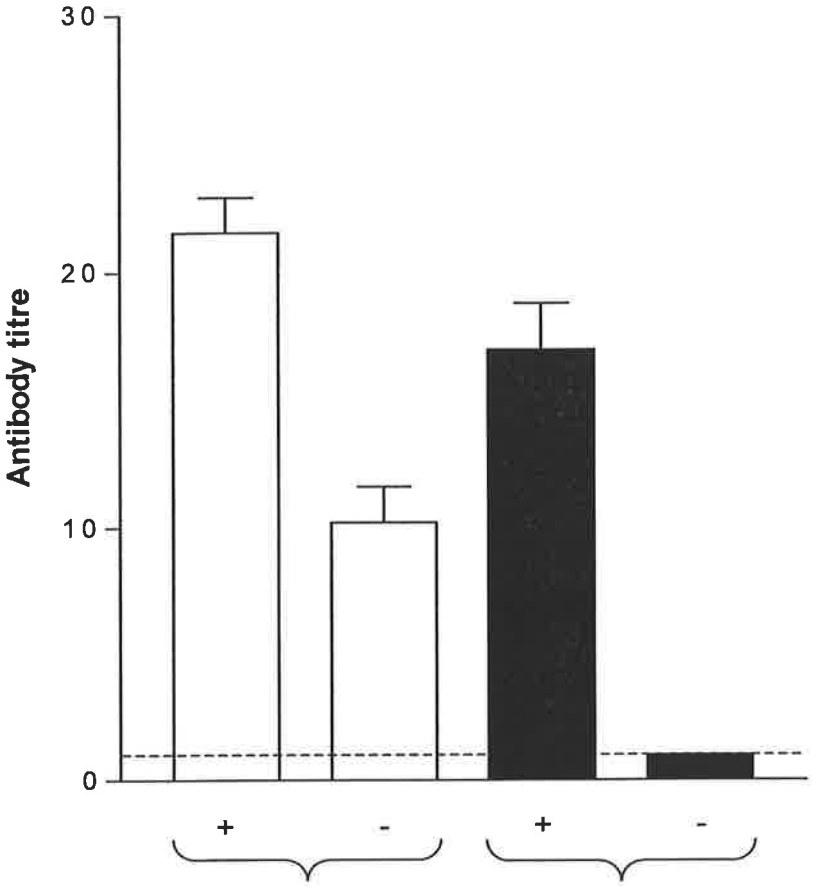
A positive anti-LT-B CFPS (pooled from nine mice) was tested in duplicate in both LT-B sensitised (+) and unsensitised (-) wells with both blocking solutions. Histogram (**A**) shows raw anti-LT-B titres (M $\pm$ SD) in the presence and absence of antigen; below are shown adjusted anti-LT-B titres. The dashed line represents the limit of detection at a titre of 1. There was a significant difference in the corrected ELISA titre (denoted as '**'; $P < 0.01$). The elimination of non-specific binding activity using 10 mg/ml BSA-PBS is diagrammatically illustrated in panel **B**, where the intensity of shading reflects the relative absorbance seen in the presence (+) and absence (-) of LT-B.

NB: CFPSs were titrated in three-fold falling dilutions, beginning at 1:3 in the first well.

Legend

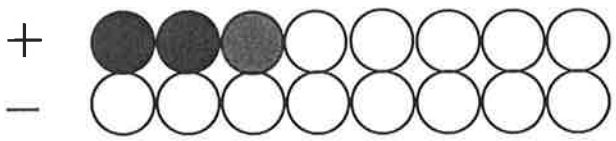


A



adjusted titre	11.37	15.98**
±SD	0.88	1.82

B



prior to the commencement of this study, a dramatic decrease in sample titre was observed when the same CFPSs were assayed on two occasions about a month apart. These samples had been stored at - 70°C in PBS, suggesting that cycles of freeze-thawing in this buffer may have caused sample degeneration. Hence, an experiment was designed to optimise storage conditions in an attempt to prolong sample integrity.

Two groups of ten BALB/c mice were orally immunised with either *aroA S.* Dublin (SL1438) or *aroA S.* Dublin expressing LT-B (EL23), with each mouse receiving two doses of 10^{10} cfu on days 0 and 4. This immunisation regime has been previously shown to induce intestinal IgA antibodies to LT-B (Bao and Clements, 1991). On days 26 and 46, nine faecal pellets were collected from each mouse in 600 µl of buffer III; at each time point the resulting pooled CFPS sample was divided into four aliquots, for storage at - 20°C or - 70°C in the presence or absence of 50% (v/v) glycerol. Samples were later analysed by ELISA (post day 60). Preliminary investigation had shown that addition of glycerol to CFPS samples did not interfere with binding of antibody to antigen: using the capture ELISA for detection of total IgA (section 2.16.3.4), standard curves set up with myeloma IgA in the presence and absence of glycerol were very similar (data not shown but see below).

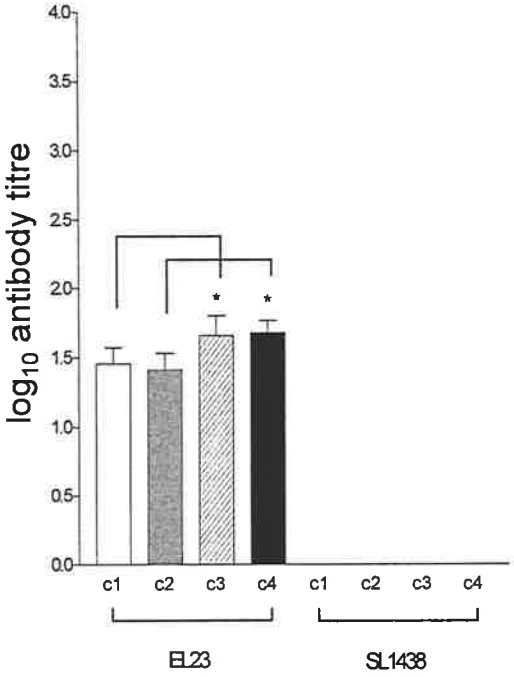
Figure 3.5 shows data obtained from the comparison of the CFPS samples collected on day 26; samples collected on day 46 yielded similar results (data not shown). ELISAs were conducted using the improved blocking conditions (10 mg/ml BSA-PBS). Immunisation with EL23 elicited intestinal IgA antibodies to LT-B, as reported previously (Bao and Clements, 1991). There was no significant difference in specific anti-LT-B titres between CFPS aliquots stored at - 20°C or - 70°C. However, titres were significantly greater in the presence of glycerol (*, $P < 0.05$ for c1 vs c3 and for c2 vs c4, Figure 3.5 A). As expected, no anti-LT-B response could be detected in SL1438 immunised mice; these samples were assigned a titre of 1 during the calculation of standardised ELISA titres (Figure 3.5 C). Differences in standardised titres of CFPS samples collected from the two groups of mice reflected differences in specific IgA titres

Figure 3.5 Comparison of CFPS Storage Conditions

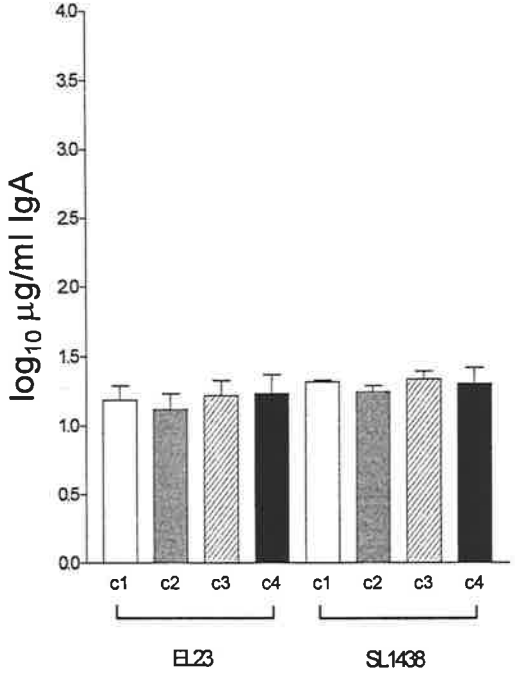
CFPS samples were collected in buffer III and aliquots stored at - 70°C or - 20°C in the presence or absence of 50% (v/v) glycerol. Histograms show specific anti-LT-B titres (**A**) and total IgA content (**B**) of samples collected 26 days after oral immunisation with EL23 or SL1438 (geometric mean (GM) ± SD of ELISA titres from duplicate dilution series). ELISAs were performed post day 60. Panel **C** shows standardised anti-LT-B titres, with specific ELISA titres being divided by the IgA content. Points of significant difference are indicated by '*' ($P < 0.05$).

Legend

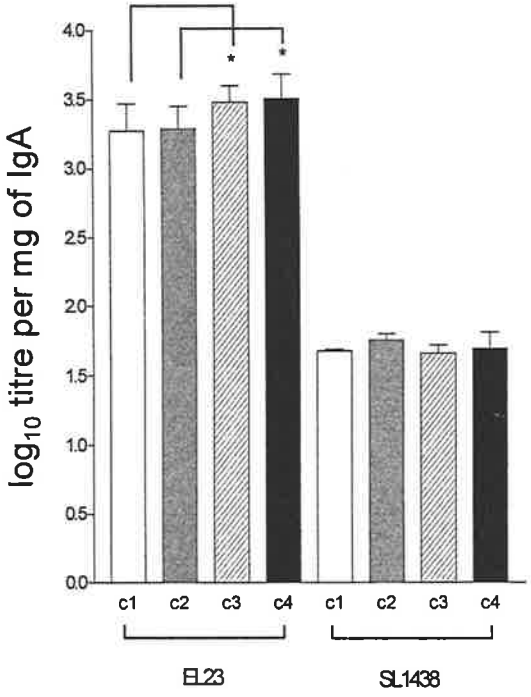
c1= stored at - 70°C
c2= stored at - 20°C
c3= stored at - 70°C with 50% (v/v) glycerol
c4= stored at - 20°C with 50% (v/v) glycerol


log10 antibody titre
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
c1 c2 c3 c4 c1 c2 c3 c4
EL23
SL1438

A


log10 µg/ml IgA
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
c1 c2 c3 c4 c1 c2 c3 c4
EL23
SL1438

B


log10 titre per mg of IgA
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
c1 c2 c3 c4 c1 c2 c3 c4
EL23
SL1438

C

and not variations in the estimates of total IgA (Figure 3.5 B), which were unaffected by the presence of glycerol.

The results from this experiment showed that the addition of glycerol improved sample activity following thawing, suggesting reduced antibody degradation. However, to eliminate the need for repetitive freeze-thawing, CFPS samples collected in future immunisation experiments were separated into duplicate aliquots prior to freezing.

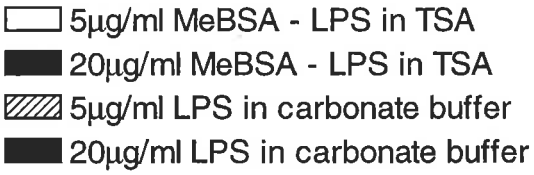
3.2.4 ELISA for the detection of antibodies to LPS.

The measurement of anti-LPS responses by this laboratory has traditionally involved sensitising ELISA trays with 5 μg/ml methylated BSA-treated LPS (MeBSA-LPS) in TSA. In other laboratories however, an alternative method is used, which involves sensitising with 20 μg/ml LPS in carbonate buffer pH 9.6 (section 2.16.3.2). An experiment was therefore conducted to compare these two methods for their efficiency of detection of IgG or IgA antibodies to LPS.

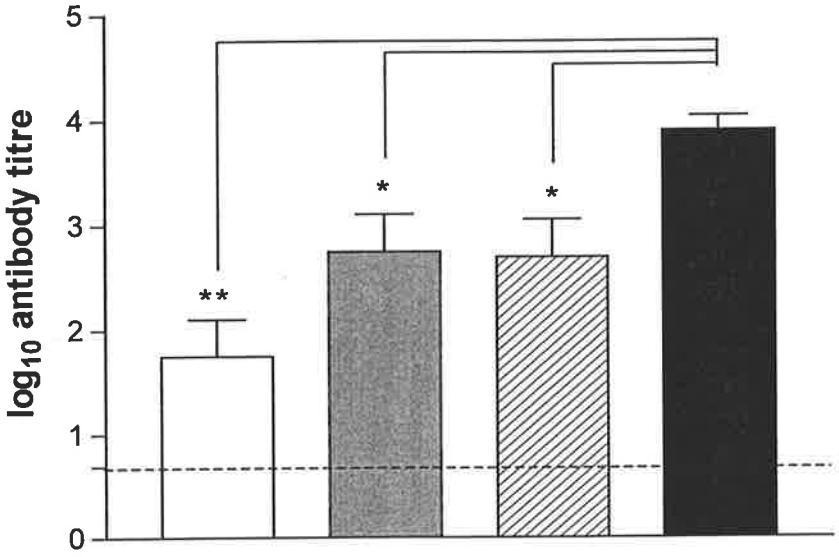
Serum and CFPS samples from mice orally immunised with *S.* Stanley were separately pooled and assayed for anti-LPS activity by performing parallel titrations (in duplicate) in rows sensitised with either MeBSA-LPS in TSA or LPS in carbonate buffer (each at either 5 μg/ml or 20 μg/ml). As shown in Figure 3.6, sensitising ELISA wells with 20 μg/ml of *S.* Typhimurium LPS in carbonate buffer resulted in significantly greater antibody titres for both serum IgG and intestinal IgA. This protocol was therefore used in all subsequent ELISA titrations of antibodies to LPS.

Figure 3.6 Detection of antibodies to LPS by ELISA.

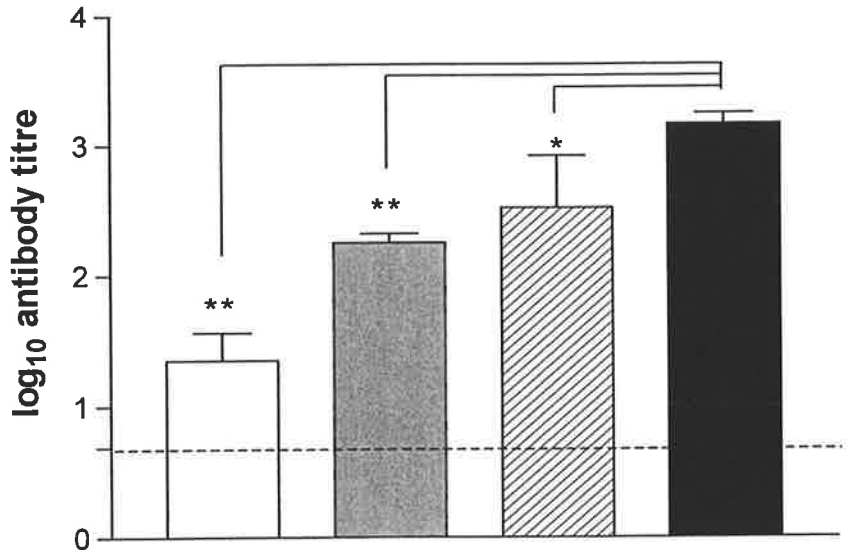
Comparative $\log_{10}$ ELISA titres (GM $\pm$ SD of duplicate titrations) of pooled serum (**A**) and CFPS (**B**) samples, following titration in wells sensitised with LPS according to four different protocols. Points of significance are denoted by '*' ($P < 0.05$) and '**' ($P < 0.01$), and the dashed lines represent the limit of detection at a titre of 5.



A



B



3.2.5 Determination of IgG_1 and IgG_{2a} antibody titres by ELISA.

Measurement of the ratio of IgG subclasses, particularly the IgG_1:IgG_{2a} isotype ratio, can provide an insight to the Th1/Th2 bias of an immune response. This was of interest in relation to several studies to be described in later chapters and so appropriate immunoconjugates were obtained (section 2.16.3.6). To ensure that these would detect IgG subclasses with similar efficiency - thereby avoiding any biasing of the IgG_1:IgG_{2a} ratio - preliminary capture ELISAs were performed to compare the activity of the two secondary antibodies, as outlined in section 2.16.3.6.

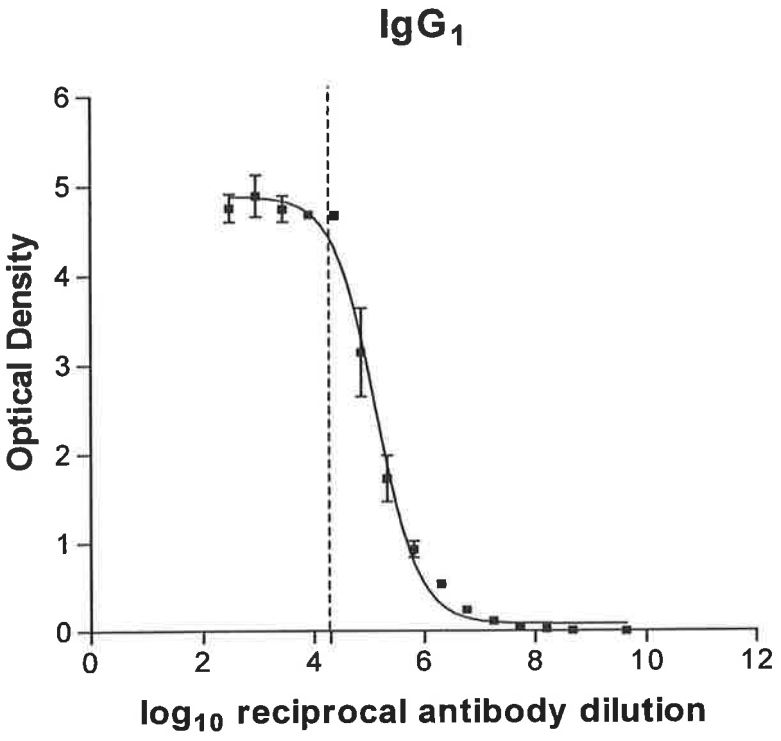
Figure 3.7 shows the standard curves generated for each immunoconjugate. The similarity of these titrations suggested that the immunoconjugates detected their respective isotypes with comparable sensitivity. A final dilution of 1:20,000 was chosen for each immunoconjugate, since the efficiency of detection decreased significantly at higher dilutions (Figure 3.7). Other titrations confirmed the specificity of these immunoconjugates (see section 2.16.3.6).

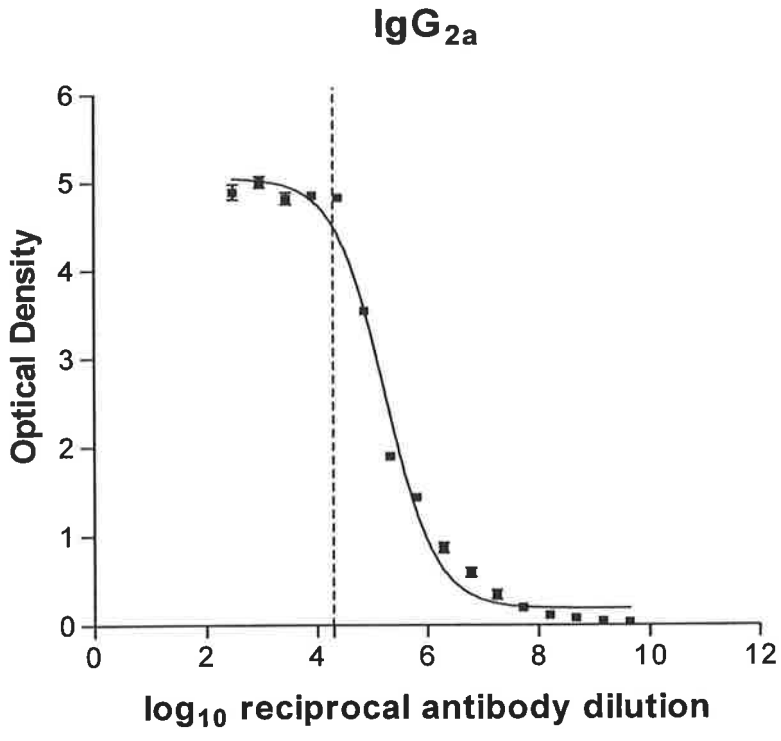
3.3 Discussion.

Previous attempts at measuring murine intestinal IgA responses in this laboratory have been unsuccessful. To address this issue, an investigation was performed to compare several protocols for the preparation and storage of supernatants from murine faecal pellet samples. A comparison of collection buffers found no significant differences in recoveries of IgA. Further tests showed that CFPSs prepared using the various buffers did not erode the antigen used to sensitise ELISA wells, nor were the buffers differentiated on the basis of likely degradation of immunoglobulin. The latter was assessed by monitoring the activity of IgG antibodies added to the various CFPSs, as this isotype is more susceptible to proteolysis than the IgA antibodies of interest. Since none of these experiments indicated any of the collection buffers to be superior,

Figure 3.7 Titration of IgG_1 and IgG_{2a} immunoconjugates.

Graphs show standard curves generated from capture ELISAs performed to compare the sensitivities of mouse IgG_1 or IgG_{2a} immunoconjugates as described in text. Dashed lines represent the dilutions of secondary antibody selected for future use.


IgG1
Optical Density
0
1
2
3
4
5
6
0
2
4
6
8
10
12
log10 reciprocal antibody dilution


IgG2a
Optical Density
0
1
2
3
4
5
6
0
2
4
6
8
10
12
log10 reciprocal antibody dilution

the buffer containing two protease inhibitors and an iron chelating agent was selected for routine use.

The key achievement described in this chapter was the demonstration that inadequate ELISA blocking conditions were responsible for the background colour development problem previously observed when measuring murine intestinal IgA responses in this laboratory. A comparison of various blocking proteins indicated that the non-specific colour development seen in control wells was not due to the recognition of BSA as first thought, but rather reflected an incomplete blocking of protein binding sites. Increasing the BSA concentration in the blocking solution significantly reduced background antibody binding in control wells to a negligible level. This was despite the fact that, in order to increase the IgA concentration within CFPSs, it was decided to collect more faecal pellets in a smaller volume of collection buffer.

Due to the long term nature of the proposed immunisation experiments (up to 20 weeks), it was necessary to examine CFPS storage conditions. Initial experiments suggested that IgA antibodies within CFPS samples prepared in PBS had lost activity during successive cycles of freeze-thawing. Glycerol was therefore added to the collection buffer to improve sample storage conditions. CFPSs prepared in the presence, as opposed to the absence, of glycerol had significantly improved specific IgA ELISA titres upon thawing and analysis of samples. As a further precaution, samples were separated into duplicate aliquots prior to freezing to eliminate the need for repetitive freeze-thawing.

This chapter also describes improvements to the ELISA protocol used in the measurement of antibody responses to LPS, which resulted in a dramatic increase in assay sensitivity. Finally, ELISAs for the detection of IgG subclasses were also established.

CHAPTER FOUR

The significance of pre-existing anti-vector immunity: impact of the GALT colonising potential of the *Salmonella* vector.

4.1 Introduction

In 1997, Attridge *et al.* proposed that the significance of pre-existing immunity was dependent upon the extent to which the vector strain could colonise the GALT. This proposal was, in part, based on the results published by Bao and Clements (1991), where mice previously primed with *aroA S.* Dublin (SL1438) were not compromised in their ability to subsequently respond to a bivalent *aroA S.* Dublin-LTB vaccine. In this instance, the priming bacteria were attenuated by a mutation in *aroA* which is thought to limit persistence in host tissues, presumably resulting in the induction of weaker primary anti-vector responses. In contrast, Attridge *et al.* (1997) primed mice with wt *S.* Stanley, which is naturally attenuated in mice and can survive in Peyer's patches for greater than forty days. In comparison to controls, these primed mice were hyporesponsive to K88 following oral administration of r*S.* Stanley-K88. Additional results published by Attridge *et al.* (1997) showed that by reducing the priming dose of *S.* Stanley from $\sim 10^9$ to $\sim 10^6$ cfu, colonisation of the Peyer's patches was reduced to undetectable levels, and subsequent hyporesponsiveness to K88 was not observed.

Two approaches were therefore adopted to further examine the possibility that the significance of pre-existing anti-vector immunity is determined (at least in part) by the extent to which the vector strain colonises the GALT during primary infection. Based on a comparison of the studies conducted by Bao and Clements (1991) and Attridge *et al.* (1997), an obvious approach involved introducing an *aroA* mutation into *S.* Stanley. If the expected curtailment of GALT colonisation potential resulted in weaker immune responses to *S.* Stanley, the consequences of vector-priming with the mutant could subsequently be determined. Initial sections describing the results of this chapter demonstrate attempts to construct an *aroA S.* Stanley mutant using the transduction procedure pioneered by Hoiseth and Stocker (1981) and used to construct the SL1438 vector used by Bao and Clements (1991). This approach, however, proved unsuccessful and consequently the mutant was constructed using allelic exchange mutagenesis.

A second approach compared the significance of vector-priming in mice innately susceptible (*Nramp1*$^{-/-}$) or resistant (*Nramp1*$^{+/+}$) to *Salmonella* infection. *Nramp1* (formerly *Ity/lsh/bcg*) gene expression has been implicated in control of virulent *Salmonella* replication in cells of the reticuloendothelial system (Plant and Glynn, 1979). Following intravenous or intraperitoneal injection of virulent Salmonellae, innately resistant mice show an enhanced capacity to control intracellular bacterial growth and consequently are able to survive infection with low challenge doses (in contrast to innately susceptible mice). Both reports mentioned above were performed using BALB/c mice, which are *Nramp1*$^{-/-}$. It was therefore of interest to define the pattern of GALT colonisation by *S.* Stanley in *Nramp1*$^{+/+}$ mice. If expression of *Nramp1* limits bacterial replication in the GALT, this would then prompt the assessment of the significance of vector-priming in such mice. In the context of this chapter, this would provide a second approach for evaluating the importance of GALT colonisation potential as a determinant of the impact of vector-priming.

4.2 Results

4.2.1. Attempted construction of *aroA S.* Stanley by transduction.

In order to best compare experiments conducted by Bao and Clements (1991) and Attridge *et al.* (1997), an attempt was made to construct an *aroA* mutation in *S.* Stanley which was equivalent to that found in SL1438, the *aroA S.* Dublin vector used in the former study. The *aroA* mutation in SL1438 was created by transducing a tetracycline-interrupted *aroA* gene (*aroA*554::*Tn*10) from *S.* Typhimurium LT2 (Smith *et al.*, 1984), followed by the forced imprecise excision of *Tn*10 by plating bacteria on Bochner medium (which inhibits the growth of tetracycline resistant bacteria).

The first stage in creating an *aroA S.* Stanley mutant was to determine which bacteriophage were able to infect this strain and therefore potentially transduce the mutation from *S.* Typhimurium *aroA* to *S.* Stanley. Bacteriophage P1 and P22 are both well characterised in their infection of *E. coli* and *S.* Typhimurium; both are generalised transducers requiring rough or smooth LPS respectively for adsorption (Sternberg and Maurer, 1991). Although *S.* Stanley and *S.* Typhimurium share the same O-antigen serotype (O-4,5,12), P22 was found to infect *S.* Typhimurium but not *S.* Stanley (not shown). Before being able to test P1 transduction into *S.* Stanley, it would be necessary to change its LPS from smooth to rough.

This was achieved by generating a *galE* mutation in *S.* Stanley by isolating colonies capable of growing on minimal medium containing 0.1% (w/v) 2-deoxygalactose (Nnalue and Stocker, 1986). In wt salmonellae, the metabolism of 2-deoxygalactose results in the production of a toxic bi-product which causes cell death. Strains carrying mutations in the galactose metabolism pathway - namely in genes *galE*, *galU* and *galETK* - are able to grow in the presence of 2-deoxygalactose. All of these mutants can be distinguished from wt as they display a rough

LPS phenotype, but only *galE* mutants are able to revert to a smooth phenotype in the presence of galactose Nnalue and Stocker (1986).

Twenty 2-deoxygalactose resistant colonies were isolated and tested for their abilities to revert from a rough to a smooth LPS phenotype by growth on blood agar containing 5.5 mM galactose. Their phenotypes were defined using indicator bacteriophage FO and C21 (section 2.14.2); FO is able to adsorb and cause plaques on smooth *Salmonella*, whereas C21 only adsorbs and causes plaques on *Salmonella* with rough LPS. Only two of the twenty mutants exhibited resistance to FO when grown in the presence of galactose. These putative *galE* mutants were chosen as recipients for the P1-mediated transduction of the *aroA* mutation, and were designated CVSA1 and CVSA2 respectively.

4.2.2 Attempted transduction of the *aroA* mutation into *S.* Stanley.

The construction of an *aroA* mutation in *S.* Stanley via transduction was split into two stages, prior to the reversion of the *galE* mutation. The first involved the transfer of the tetracycline-interrupted *aroA* gene (designated *aroA*(*serC*)1121::*Tn10*) from PE952 into CVSA1 and CVSA2 using bacteriophage P1, selecting for tetracycline (Tc) resistance. The second step would then require P1-mediated transduction to replace the Tn*10*-interrupted *aroA* gene with an *aroA* deletion mutation (designated *aroA* DEL407 and residing in PE951). Exchange of the Tn*10* *aroA* insertion with the deletion mutation would be selected by growth on Bochner medium, to inhibit the growth of tetracycline resistant bacteria. This strategy would produce a stable non-reverting *aroA* vector equivalent to the SL1438 vector used by Bao and Clements (1991).

Initial attempts at the first stage of the procedure, which included a number of variations in the transduction protocol (described in section 2.14.3) were unsuccessful. Inexplicably, after a period of approximately three months, the original method for transduction was repeated and

was successful in producing three isolates (CVSA3 - 5) exhibiting tetracycline resistance. However, attempts at the second transduction procedure also proved unsuccessful. At this stage it was decided to create a chromosomal interruption in *aroA* via allelic exchange mutagenesis.

4.2.3 Derivation of an *aroA* mutant of *S.* Stanley via allelic exchange mutagenesis.

4.2.3.1 Amplification and sequencing of the *S.* Stanley *aroA* gene via PCR.

The sequence of the *S.* Typhimurium *aroA* gene (accession number Y10355) was retrieved using the online Entrez nucleotide database provided by the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/entrez/). Oligo primers (#2934 and #2936, section 2.9.1) were designed from this sequence and PCR was employed to amplify the *aroA* gene from the *S.* Stanley chromosome, producing a 1.2 kb fragment.

Three 1.2kb PCR products, derived from separate reactions using the *S.* Stanley chromosome as a template, were ligated into pGEM-T Easy®. The resulting plasmids (pCVSA1 - 3) were transformed into *E. coli* strain DH5α producing strains CVSA6 - 8. Following sequencing with M13 forward (M13F) and reverse (M13R) primers, the compiled consensus sequence from these PCR products (Appendix Figure A.1) was shown to have 98% sequence homology to the *aroA* genes of *S.* Typhimurium, *S.* Typhi, and *S.* Gallinarum following submission to the NCBI Blastn database (Altschul *et al.*, 1990). CLUSTALW alignment (Higgins and Sharp, 1988), and hydrophobicity comparison (Kyte and Doolittle, 1982) of the predicted *aroA* proteins of the same strains also suggested greater than 98% homology (Appendix Figure A.2).

4.2.3.2 Interruption of the *S.* Stanley *aroA* gene.

The interruption of the *S.* Stanley *aroA* gene was achieved by introducing the *apha-3* non-polar Km resistance cartridge (Ménard *et al.*, 1993) into a unique *Eco*RV restriction site

within *aroA*. The *apha-3* cartridge does not contain a promoter or transcriptional terminator, hence expression of Km resistance is dependent on transcription of the gene targeted for interruption.

Figure 4.1 A is a diagrammatic representation of the construction of the *aroA*::*apha-3* gene interruption. Plasmid pCVSA1 (from CVSA6) contains the *aroA* gene in the direction of the *lacZ* promoter in pGEM-T Easy®. pCVSA1 was subjected to *Eco*RV digestion, followed by treatment with shrimp alkaline phosphatase (SAP) to prevent vector recircularisation during ligation. The *apha-3* cartridge was excised from pUC18K (Ménard *et al.*, 1993) by *Sma*I-digestion and purified following gel electrophoresis (described in section 2.8.5). The *Sma*I *apha-3* insert was then ligated into the *Eco*RV-digested *aroA* gene, destroying the *Sma*I and *Eco*RV sites; this ligation mix was transformed into DH5α with selection for Km resistance. Plasmid DNA was extracted from putative transformants and treated with *Nsi*I and *Eco*RV (found within *apha-3*) to confirm the orientation of the cartridge with respect to the *aroA* gene. Plasmids pCVSA4 (in CVSA9) and pCVSA5 (in CVSA10), were then selected for storage. PCR was employed to confirm interruption of the *aroA* gene as shown in Figure 4.1B.

4.2.3.3 Construction of a chromosomal *aroA* mutation via allelic exchange mutagenesis.

The plasmid pCACTUS*mob* (Table 2.3) is a suicide vector which has been used extensively in this laboratory. It carries a Cml resistance gene, and a temperature sensitive replicon which is inoperative at 42°C but supports plasmid replication at 30°C. It also carries a *mob* region required for plasmid transfer via conjugative pili, and the *sacB* gene from *Bacillus subtilis*, which produces toxic sugar polymers when bacteria are grown in the presence of sucrose (Kaniga *et al.*, 1991).

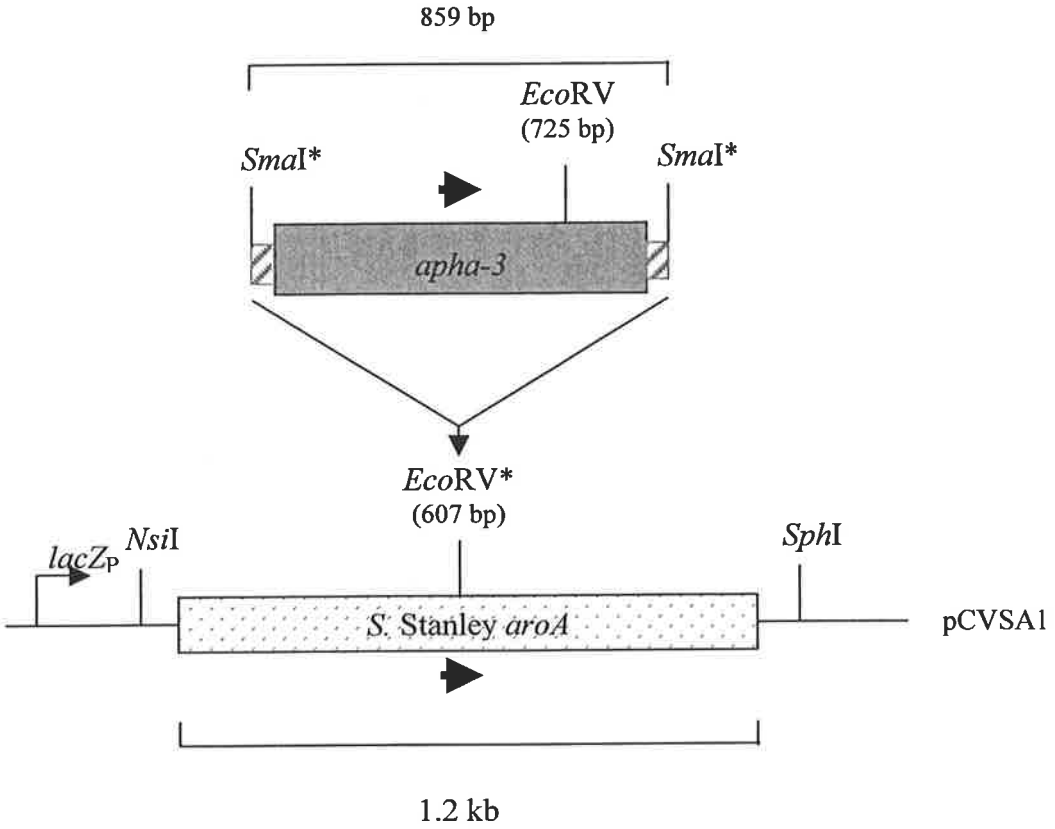
Figure 4.2 is a diagrammatic representation of the insertion of the *aroA*::*apha-3* construct into pCACTUS*mob*. pCVSA4 was digested with *Nsi*I and *Sph*I, liberating the 2 kb *aroA*::*apha-3* insert, which was purified following agarose gel electrophoresis. Meanwhile, pCACTUS*mob*

Figure 4.1 Construction of the *aroA*::*apha-3* gene interruption.

The *apha-3* cartridge was purified from pUC18K following *Sma*I digestion and inserted into the *Eco*RV site of the *S.* Stanley *aroA* gene in pCVSA1, creating pCVSA4. Both *Sma*I and *Eco*RV sites were destroyed in this process as indicated by '*'. *Nsi*I and *EcoRV* digestion confirmed insert orientation (**A**). PCR was employed to confirm interruption of the *aroA* gene (**B**). Lane 1, predicted 1.2 kb *aroA* fragment from wt *S.* Stanley; lane 2, 1.2 kb *aroA* fragment amplified from pCVSA1; Lane 3, predicted 2 kb fragment derived from pCVSA4.

Lane marked SPP1 represents *Eco*RI digested *Bacillus subtilis* bacteriophage SPP1 DNA. Fragment sizes (kb) are: 8.5; 7.35; 6.10; 4.84; 3.59; 2.81; 1.95; 1.86; 1.51; 1.39; 1.16; 0.98; 0.72; 0.48

A


859 bp
EcoRV
(725 bp)
SmaI*
SmaI*
apha-3
EcoRV*
(607 bp)
lacZP
NsiI
SphI
S. Stanley aroA
pCVSA1
1.2 kb

B

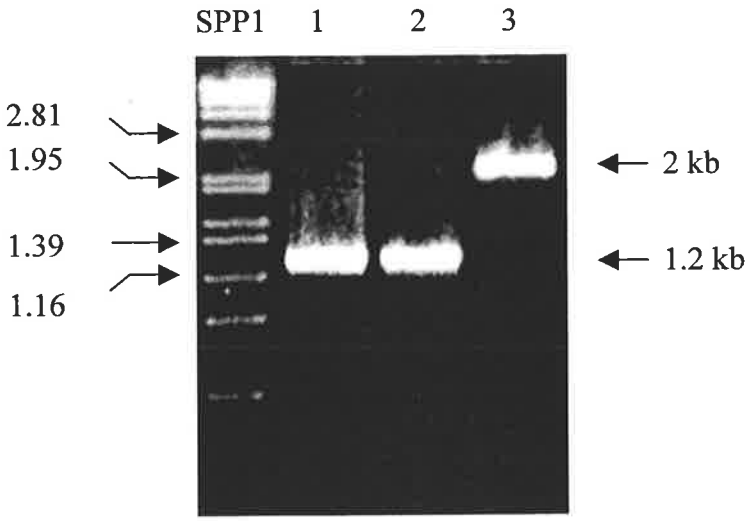
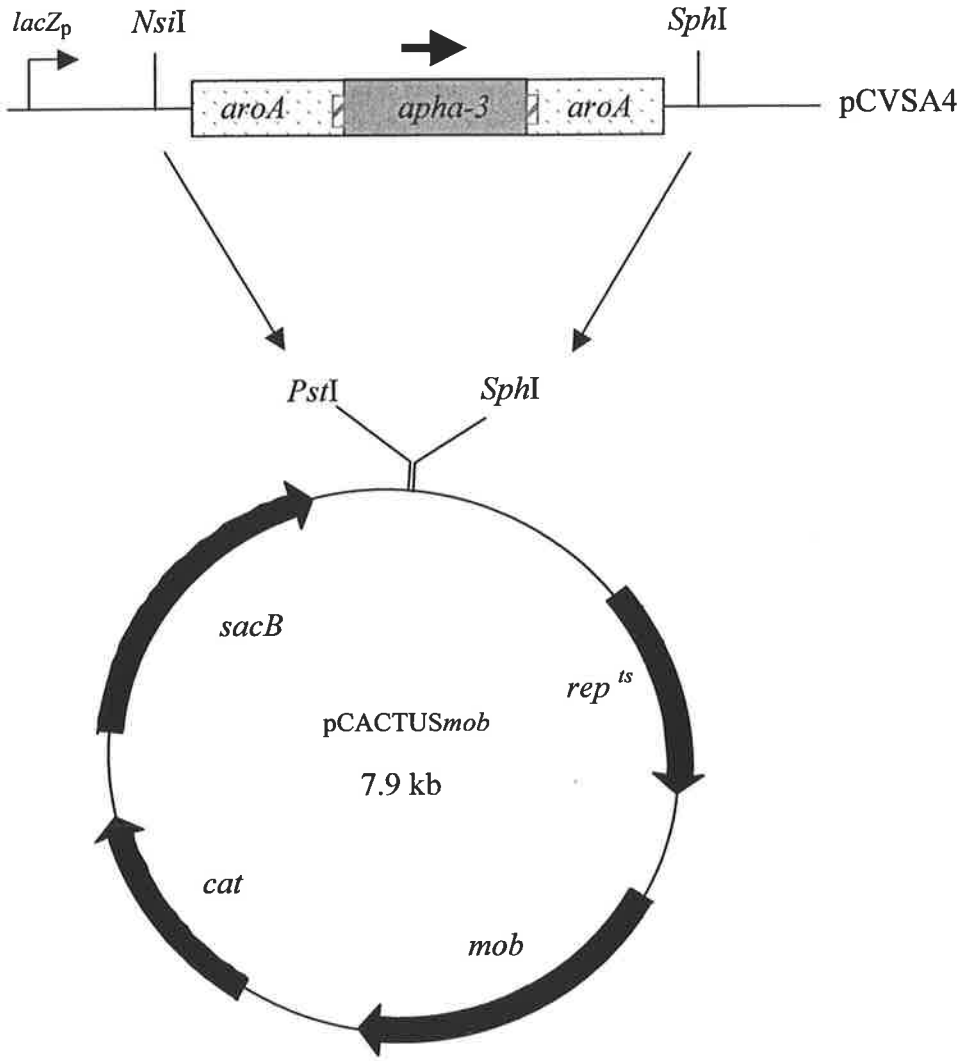

SPP1
1
2
3
2.81
1.95
1.39
1.16
2 kb
1.2 kb

Figure 4.2 Insertion of the *aroA*::*apha-3* construct into pCACTUS*mob*.

pCVSA4 was digested with *Nsi*I and *Sph*I, liberating the 2 kb *aroA*::*apha-3* insert. Following gel extraction and purification, this insert was ligated into pCACTUS*mob* which had been digested with *Pst*I and *Sph*I, forming pCVSA6.


lacZp
NsiI
SphI
aroA
apha-3
aroA
pCVSA4
PstI
SphI
sacB
rep ts
pCACTUSmob
7.9 kb
cat
mob

was digested with *Pst*I and *Sph*I (*Nsi*I and *Pst*I yield compatible cohesive ends) and then treated with SAP. Insert and vector were ligated and the mixture electroporated into DH5α with growth at 30°C on Luria agar containing antibiotics Km and Cml (only plasmids which had undergone a successful ligation could confer resistance to both antibiotics). Plasmids isolated from the resulting colonies were subjected to PCR with primers #2934 and #2936 in order to confirm the presence of the *aroA*::*apha-3* interruption (not shown); plasmid pCVSA6 (in CVSA11) was identified for storage.

S17-1 is an *E. coli* strain which carries the chromosomally located *tra* region necessary for plasmid mobilisation. Plasmid pCVSA6 was transformed into S17-1, forming CVSA12, which was then used as a donor strain for the conjugal transfer of pCVSA6 to *S.* Stanley (forming CVSA13). Recipients were selected at 30°C on minimal medium (to select against the auxotrophic donor) containing Km and Cml (to prevent growth of untransformed recipients). Following single colony purification, slide agglutination with O-4,5,12 antiserum confirmed that recipients expressing Km and Cml resistance were *S.* Stanley. One such colony (CVSA13) was selected for o/n LB culture (at 30°C with Km) followed by plating on LB agar containing Km and incubation o/n at 42°C (these conditions select for bacteria in which plasmid integration has occurred, as pCACTUS*mob* cannot replicate at this temperature). Several of the resulting colonies were separately cultured overnight at 30°C in LB containing Km (without Cml), allowing for the resolution of co-integrates. Serial dilutions of these cultures were plated on (salt-free) LB agar containing Km and sucrose (section 2.3), selecting against the presence of *sacB* (unresolved co-integrates). Resulting colonies were replica-patched on Luria agar containing either Km, Km and sucrose, or Cml. Colonies with a Km^R Cml^S $sucrose^R$ phenotype were compatible with the desired allelic exchange.

To confirm the presence of a chromosomal *aroA* mutation, genomic DNA was extracted from two putative *S.* Stanley *aroA* mutants, CVSA14 and CVSA15. After digestion with *Pst*I, chromosomal DNA was analysed by Southern hybridisation, using *aroA* and *apha-3* DIG-

labelled gene probes. The *aroA* gene lies on a 6.1 kb *Pst*I fragment, as detected in *S.* Stanley and *S.* Typhimurium (Figure 4.3A, lanes 3 and 4). Hybridisation of the *aroA* gene probe was also visible in lanes containing *aroA* and *aroA*::*apha-3* PCR products (Figure 4.3A, lanes 6 and 5) which were included as positive controls. A *Pst*I fragment of approximately 7 kb was detected in CVSA14 and CVSA15 using both *aroA* and *apha-3* gene probes (Figure 4.3A and 4.3B, lanes 1 and 2). Together these blots confirmed that the *aroA* gene had been interrupted by the *apha-3* cartridge. As expected, the *apha-3* gene probe did not bind to *S.* Stanley or *S.* Typhimurium chromosomal DNA (Figure 4.3B lanes 3 and 4) or to the PCR-derived *aroA* amplicon (Figure 4.3B, lane 6).

4.2.4 *In vitro* complementation of the *aroA* mutation in *S.* Stanley.

It was important to ascertain whether the *aroA S.* Stanley mutant could be restored to wt by provision of a functional *aroA* gene *in trans*. This would eliminate the possibility that a second mutation had fortuitously arisen during mutant construction, as has previously been reported for *aroA S.* Typhimurium (Lockman and Curtiss, 1990).

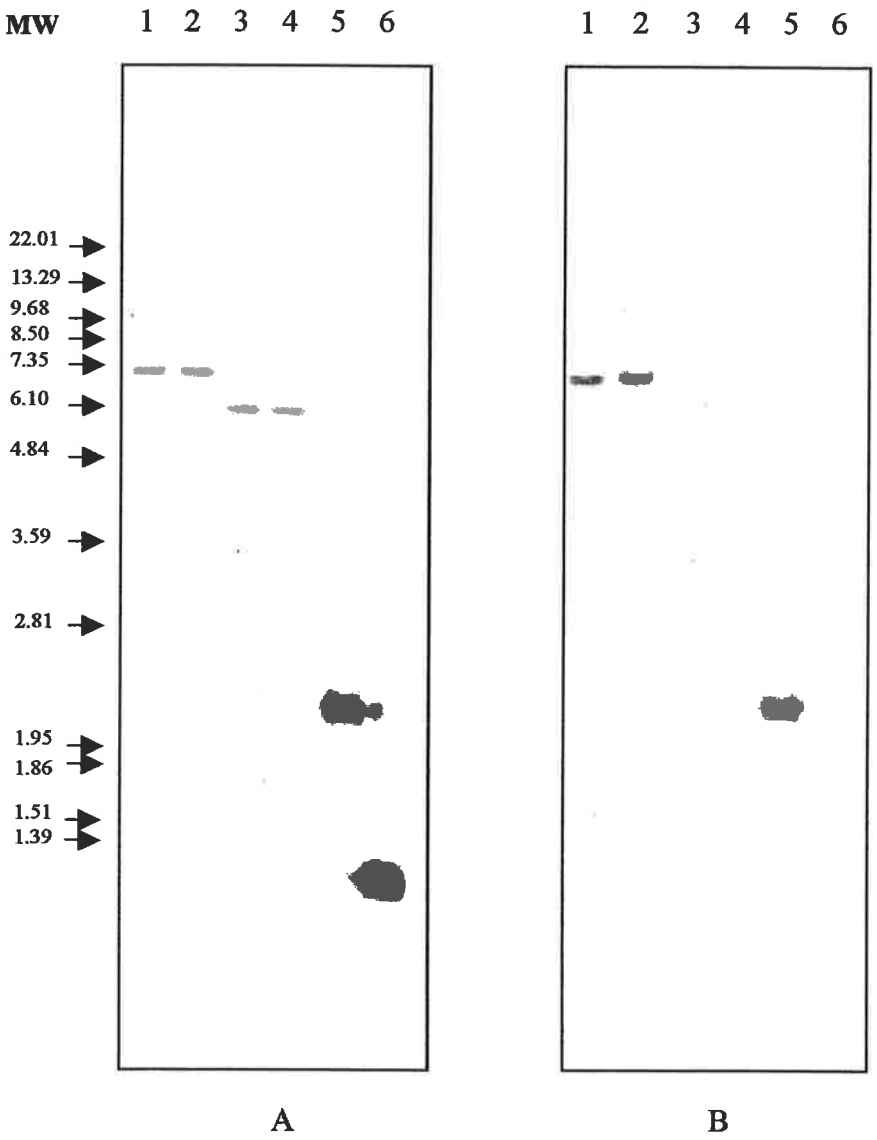
Complementation analysis would require the construction of a low copy number plasmid containing a minimal *aroA* fragment. The *aroA* gene was therefore excised from pCVSA1 by *Eco*RI digestion, exploiting the *Eco*RI sites flanking inserts introduced into the cloning vector pGEM-T Easy®, and ligated into the *Eco*RI site of pWSK30. Following transformation into DH5α, and selection on blue-white selection medium (section 2.3), plasmid DNA was extracted from white colonies and subjected to PCR analysis. A plasmid with the *aroA* gene orientation appropriate for expression from the *lacZ* promoter was isolated and termed pCVSA7 (stored in CVSA16).

EX2000 is a *Salmonella* strain which is restriction negative but modification positive, and acts as an intermediate in the transfer of plasmid DNA from *E. coli* to *Salmonella* spp. Plasmids pCVSA7 and pWSK30 were electroporated into and subsequently re-isolated from EX2000.

Figure 4.3 Southern analysis of putative *S.* Stanley *aroA* mutants.

Genomic DNA preparations from *S.* Stanley, *S.* Typhimurium and putative *aroA S.* Stanley mutants were subjected to *Pst*I digestion, followed by electrophoresis and Southern analysis. Panels A and B show hybridisation patterns with DIG-labelled gene probes for *aroA* and *apha-3* respectively. Lanes: 1, CVSA14; 2, CVSA15; 3, *S.* Typhimurium C5; 4, wt *S.* Stanley; 5, *aroA*::*apha-3* PCR product amplified from pCVSA4; 6, *aroA* PCR product amplified from pCVSA1.

Lane marked MW represents a combination of *Bgl*II digested bacteriophage Lambda DNA and *Eco*RI digested *Bacillus subtllis* bacteriophage SPP1 DNA. Fragment sizes (kb) are: 22.01, 13.29, 9.68, 8.5; 7.35; 6.10; 4.84; 3.59; 2.81; 1.95; 1.86; 1.51; 1.39; 1.16; 0.98; 0.72; 0.48.


MW
1 2 3 4 5 6
1 2 3 4 5 6
22.01
13.29
9.68
8.50
7.35
6.10
4.84
3.59
2.81
1.95
1.86
1.51
1.39
A
B

Each plasmid was then electroporated into *aroA S.* Stanley and also into SL1438 (the *aroA S.* Dublin vector used by Bao and Clements (1991)), creating strains CVSA19 - 22 (Table 4.1). It was now possible to determine whether the minimal *aroA* fragment was sufficient to overcome the auxotrophy imposed by *aroA* mutations in both vectors.

CVSA19 - 22 were streaked onto Ap-containing MM in the presence or absence of aromatic amino acid supplements DHB and pABA. The wt *S.* Stanley strain, after transformation with pWSK30, was included as a control. The uncomplemented *aroA S.* Stanley mutant did not grow on unsupplemented MM but did grow in the presence of DHB and pABA (not shown), arguing against the presence of a second, cryptic auxotrophy. The presence of pCVSA7, but not of the plasmid vector pWSK30, was sufficient to allow growth on unsupplemented MM (Table 4.1). This confirmed that the auxotrophy results from the introduced *aroA* mutation, eliminating the possibility of a second, cryptic mutation affecting this biosynthetic pathway.

The *aroA S.* Dublin is also a histidine auxotroph, but even after allowing for this additional requirement, strains CVSA20 and CVSA22 were still not able to grow on either minimal or supplemented MM (Table 4.1). These strains were also tested on CBT medium (a defined medium rich in amino acids, section 2.3) in the presence or absence of supplements, but again no growth was observed (not shown). As *aroA S.* Dublin is able to grow on luria agar, these results suggested that there was another biosynthetic mutation(s) present within this strain.

Interestingly CVSA19 and CVSA20 grew with different colony morphologies on XLD agar. XLD is a selective medium which is normally used for the isolation of Salmonellae and Shigellae from clinical specimens and foods (section 2.3). Most Salmonellae grown on this medium produce red colonies with black centres, although Salmonellae which do not produce hydrogen sulphide (eg. *Salmonella enterica* var. Paratyphi A) produce uniformly red colonies. It had been observed that *aroA* mutants of *Salmonella* also produce uniformly red colonies when grown on XLD agar, and so it was of interest to ascertain the phenotype of the complemented

Table 4.1 Growth comparison of complemented *aroA* strains on minimal medium.

		GROWTH ON MM	
STRAIN	**DESCRIPTION**	**- suppl.**	**+ suppl.**
CVSA19	*aroA S.* Stanley transformed with pCVSA7 containing a minimal *aroA* fragment.	+	+
CVSA20	*aroA S.* Dublin transformed with pCVSA7 containing a minimal *aroA* fragment.	-	-
CVSA21	*aroA S.* Stanley transformed with pWSK30	-	+
CVSA22	*aroA S.* Dublin transformed with pWSK30	-	-
CVSA23	wt *S.* Stanley transformed with pWSK30	+	+

+ = growth observed

- = no growth observed

Transformed *aroA* mutants were grown on MM in the absence (-suppl.) or presence (+suppl.) of supplements DHB and pABA. Medium used for culture of CVSA20 and CVSA22 additionally contained 0.01% (w/v) histidine.

aroA strains. Of the strains listed in Table 4.1, only CVSA19 and CVSA23 produced red colonies with black centres, further strengthening the link between a functional aromatic biosynthetic pathway and the presence of the black colour within the colonies. The failure of the complemented *aroA S.* Dublin strain CVSA20 to produce red colonies with black centres reinforced the suspicion that another biosynthetic mutation(s) may be present in this strain.

4.2.5 *In vivo* characterisation, and attempted complementation, of *aroA S.* Stanley.

An experiment was performed to examine the impact of an *aroA* mutation on the *in vivo* behaviour of *S.* Stanley, and to determine whether the anticipated reduction in GALT-colonising potential could be overcome by expression of a minimal *aroA* fragment.

BALB/c mice were separated into five groups as shown in Figure 4.4 (Table I). Consistent with the dosing regimes used by both Attridge *et al.* (1997) and Bao and Clements (1991), mice in groups 2 and 3 were fed *aroA S.* Stanley with either one dose of ~ 10^9 cfu or two doses of ~ 10^{10} cfu (on days 0 and 4). Groups 4 and 5 were included to ascertain whether the *aroA* mutation could be complemented by pCVSA7 *in vivo*. At various times after immunisation, Peyer's patches were excised from the small intestine, homogenised, and plated onto XLD medium. Homogenates recovered from mice in groups 2 and 3 were also plated on XLD with Km (to ascertain the *in vivo* stability of the *aroA* mutation), while those from mice in groups 4 and 5 were also plated on XLD with Km and Ap (to assess the level of plasmid stability).

Wt *S.* Stanley colonised the Peyer's patches at levels comparable to those previously seen in this laboratory (Attridge *et al.*, 1997), reaching a maximum burden of ~ 10^4 cfu around days 5 to 8, and still present in each of the eight mice sacrificed at days 34 or 40. The *aroA* mutant (CVSA14) was recovered in surprisingly high numbers from mice given a single dose of ~ 10^9 cfu. The recoveries of mutant bacteria were significantly lower than the mean recoveries of wt bacteria on days 5, 8 ($P < 0.05$ for both time points), 27 and 40 ($P < 0.01$ for both time points),

Figure 4.4 Peyer's patch colonising potential of *aroA* mutants.

Groups of BALB/c mice were immunised with wt or *aroA S.* Stanley as shown in Table I below. Bacterial recoveries from Peyer's patches were determined from four (groups 1 - 3) or three (groups 4, 5) mice per timepoint (panel **A**); data from groups 4 and 5 are not plotted but are shown in Table 4.2. Colonisation by *aroA S.* Dublin was determined in a later experiment, following the same dosing regimes (Table II) and using four mice per timepoint (panel **B**). In both figures, histograms show $\log_{10}$ bacterial burden (GM + SD) in the Peyer's patches, calculated from colonies recovered from plating tissue homogenates on XLD medium. Dashed lines show the limit of detection (20 bacteria).

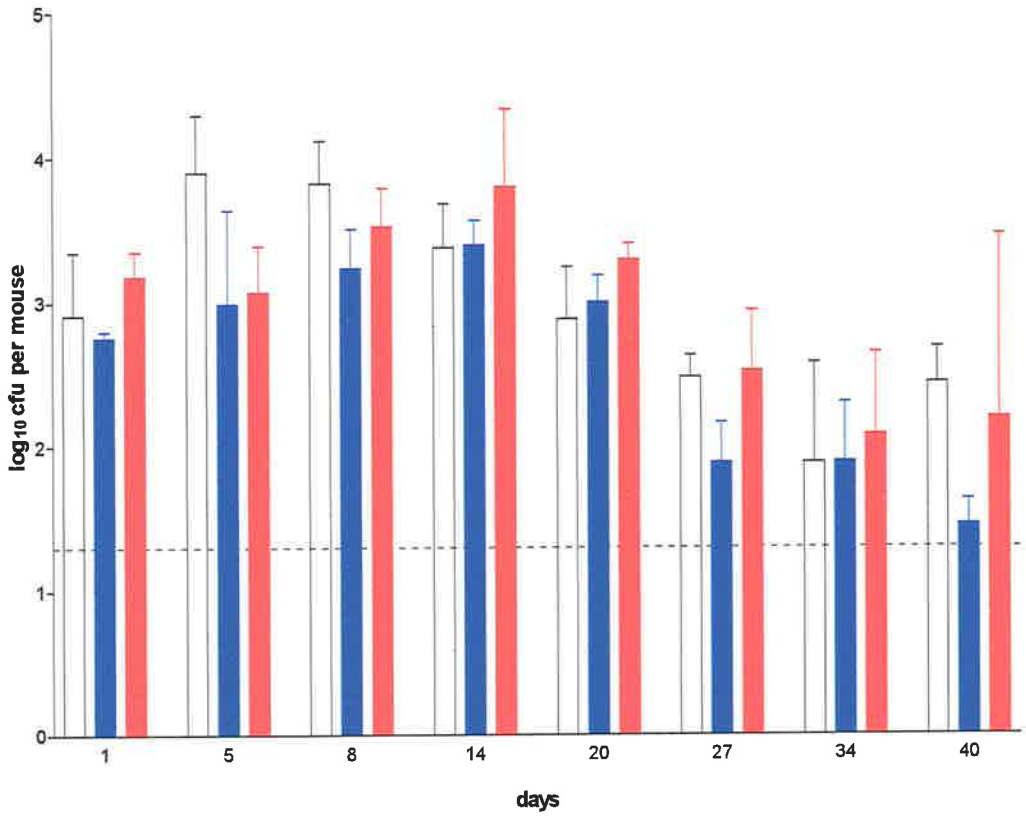
(Table I)

GROUP	Nº OF MICE	BACTERIAL STRAIN	DOSE (CFU)
1	35	wt *S.* Stanley	$1.1x10^9$ on day 0
2	35	*aroA S.* Stanley (CVSA14)	$1.3x10^9$ on day 0
3	35	*aroA S.* Stanley (CVSA14)	$1.5x10^{10}$ on day 0 $1.2x10^{10}$ on day 4
4	9	*aroA S.* Stanley complemented with *aroA in trans* (CVSA19)	$8.7x10^8$ on day 0
5	9	*aroA S.* Stanley transformed with pWSK30 (CVSA21)	$8.1x10^8$ on day 0

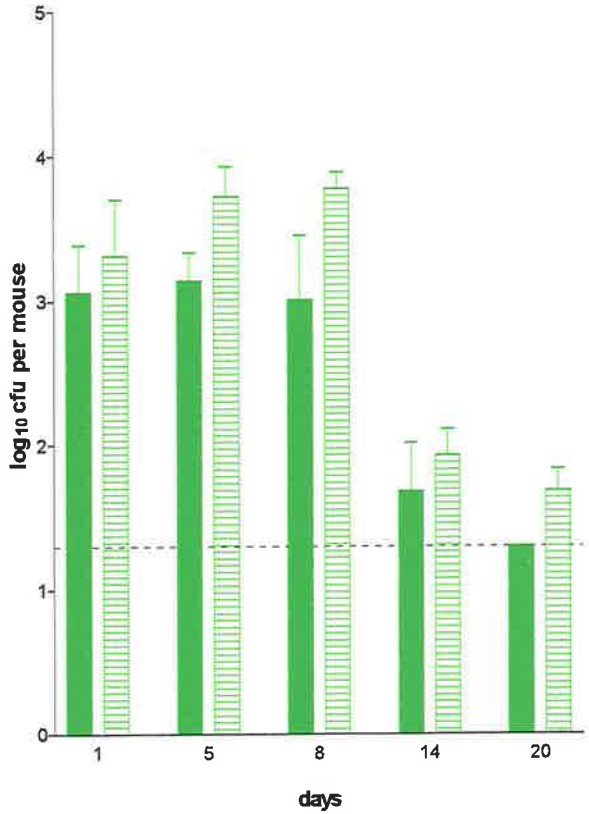
(Table II)

6	25	*aroA S.* Dublin (SL1438)	$1.2x10^9$ on day 0
7	25	*aroA S.* Dublin (SL1438)	$9.4x10^9$ on day 0 $9.5x10^9$ on day 4

A


log10 cfu per mouse
0
1
2
3
4
5
1
5
8
14
20
27
34
40
days

B


log10 cfu per mouse
0
1
2
3
4
5
1
5
8
14
20
days

but recoveries were not significantly different at the other four time points (Figure 4.4). Using the alternative dosing schedule of Bao and Clements, slightly greater numbers of *aroA S.* Stanley were recovered - these were significantly higher than the mean recovery of bacteria from group 2 on days 1 ($P < 0.01$), 20 and 27 ($P < 0.05$ at both time points). Overall, bacterial recoveries from mice in groups 1 and 3 were very similar. Nevertheless, the introduction of the *aroA* mutation clearly attenuated *S.* Stanley; none of the mice in group 3 died, whereas similar doses of wt *S.* Stanley would be fatal (Attridge, unpublished). For both groups 2 and 3, replicate aliquots of homogenate plated on XLD $\pm$ Km confirmed that the *apha-3* cartridge was stably maintained (data not shown).

The unexpected similarity of the colonisation patterns of wt and *aroA S.* Stanley made it difficult to demonstrate the *in vivo* significance of the plasmid-borne $aroA^+$ gene in the complemented mutant (CVSA19, group 4). However, bacterial recoveries on day 5 suggested that the complementing gene was functional and able to overcome the effect of the chromosomal *aroA* mutation (Table 4.2). Thus, recoveries of CVSA19 and wt bacteria (group 1) were similar at this time point, and significantly higher than the recoveries of the uncomplemented mutant (CVSA14 fed to both groups 2 and 3; $P < 0.01$ for both CVSA19 and wt *S.* Stanley) or the control strain carrying pWSK30 (CVSA21 fed to group 5; $P < 0.01$ for CVSA19, and $P < 0.05$ for wt *S.* Stanley). The relative stabilities of pCVSA7 (~ 100% retention) and pWSK30 (< 4% retention) in the *aroA* mutant were also consistent with *in vivo* $aroA^+$ expression (Table 4.2).

The high recoveries of the *aroA S.* Stanley prompted an examination of the GALT-colonising potential of the *aroA S.* Dublin (SL1438) strain used by Bao and Clements (1991), which has not been reported previously. Two groups of mice were fed SL1438 using the dosing regimes used with *aroA S.* Stanley, to define and compare the colonisation profile of this mutant (Figure 4.4, Table II). Bacteria were recovered from the Peyer's patches as described above. To assist comparison, the data from this experiment are also presented in Figure 4.4. Bacteria fed to mice in groups 6 and 7 exhibited similar colonisation patterns to those in groups 2 and 3,

Table 4.2 Complementation of *aroA S.* Stanley *in vivo*.

GROUPS	log_{10} CFU day 1		log_{10} CFU day 5		log_{10} CFU day 8	
	+ antibiotics	- antibiotics	+ antibiotics	- antibiotics	+ antibiotics	- antibiotics
1	na	2.9 ± 0.4	na	3.9 ± 0.2	na	3.8 ± 0.2
2	2.8 ± 0.0	2.7 ± 0.1	3.0 ± 0.6	3.0 ± 0.5	3.2 ± 0.3	3.2 ± 0.2
3	3.2 ± 0.2	3.2 ± 0.1	3.1 ± 0.3	3.0 ± 0.4	3.5 ± 0.3	3.6 ± 0.4
4	3.2 ± 0.3	3.2 ± 0.3	4.5 ± 0.1	4.5 ± 0.1	3.3 ± 0.0	3.3 ± 0.0
5	< 1.3	2.7 ± 0.7	< 1.3	3.1 ± 0.2	< 1.3	3.0 ± 0.1

Mice were fed bacteria according to Table 1 in Figure 4.4, and log_{10} bacterial burdens (GM ± SD) determined by plating Peyer's patch homogenates on XLD medium with (+) or without (-) antibiotics (± Km for groups 2 and 3; ± Km Ap for groups 4 and 5). The limit of detection was 20 bacteria, where no bacteria were detected, the sample was assigned a value of 20 for the purposes of statistical analysis.

respectively, until day 8. From this point on, bacteria were cleared more quickly from mice in groups 6 and 7, such that recoveries of the *S.* Dublin mutant at day 14 were similar to those of the *S.* Stanley mutant at day 34.

4.2.6 Immune responses to recombinant *Salmonella* in vector-primed mice

4.2.6.1 Responses in mice primed with wt or *aroA S.* Stanley.

Despite the unexpected GALT-colonising potential of the *aroA S.* Stanley mutant, an experiment was set up to compare the consequences of priming mice with ~ 10^9 cfu of either wt or *aroA* bacteria, with respect to the capacity of such animals to subsequently respond to a foreign antigen orally delivered by the same vector. Groups of mice were primed with wt or *aroA S.* Stanley on day - 70, while controls received $NaHCO_3$ only. A fourth group was given the *aroA* mutant according to the dosing schedule used by Bao and Clements, involving two doses of ~ 10^{10} cfu on days - 70 and - 66 (Figure 4.5). On day 0 all mice were orally immunised with r*S.* Stanley-K88, following which serum and CFPS samples were collected for the assessment of antibody responses to K88 and vector LPS. It was of particular interest to determine whether the hyporesponsiveness to K88 previously described in *S.* Stanley-primed mice extended to the intestinal response.

Serum anti-K88 and anti-LPS responses in (wt) vector-primed and control mice (groups 1 and 4) were comparable to those seen previously (Attridge *et al.*, 1997). Control mice developed a sustained primary serum IgG response to K88 following immunisation with r*S.* Stanley-K88, whereas this response was completely abrogated by vector-priming (Figure 4.5 Panel I, A). Significantly, a similar pattern of antibody responses was observed in the GALT. Although the intestinal IgA response to K88 seen in control mice was less dramatic and more variable than the serum IgG response, this too was completely inhibited by prior exposure to the vector. The IgA responses in control and vector-primed mice were significantly different on days 39, 52 ($P <$

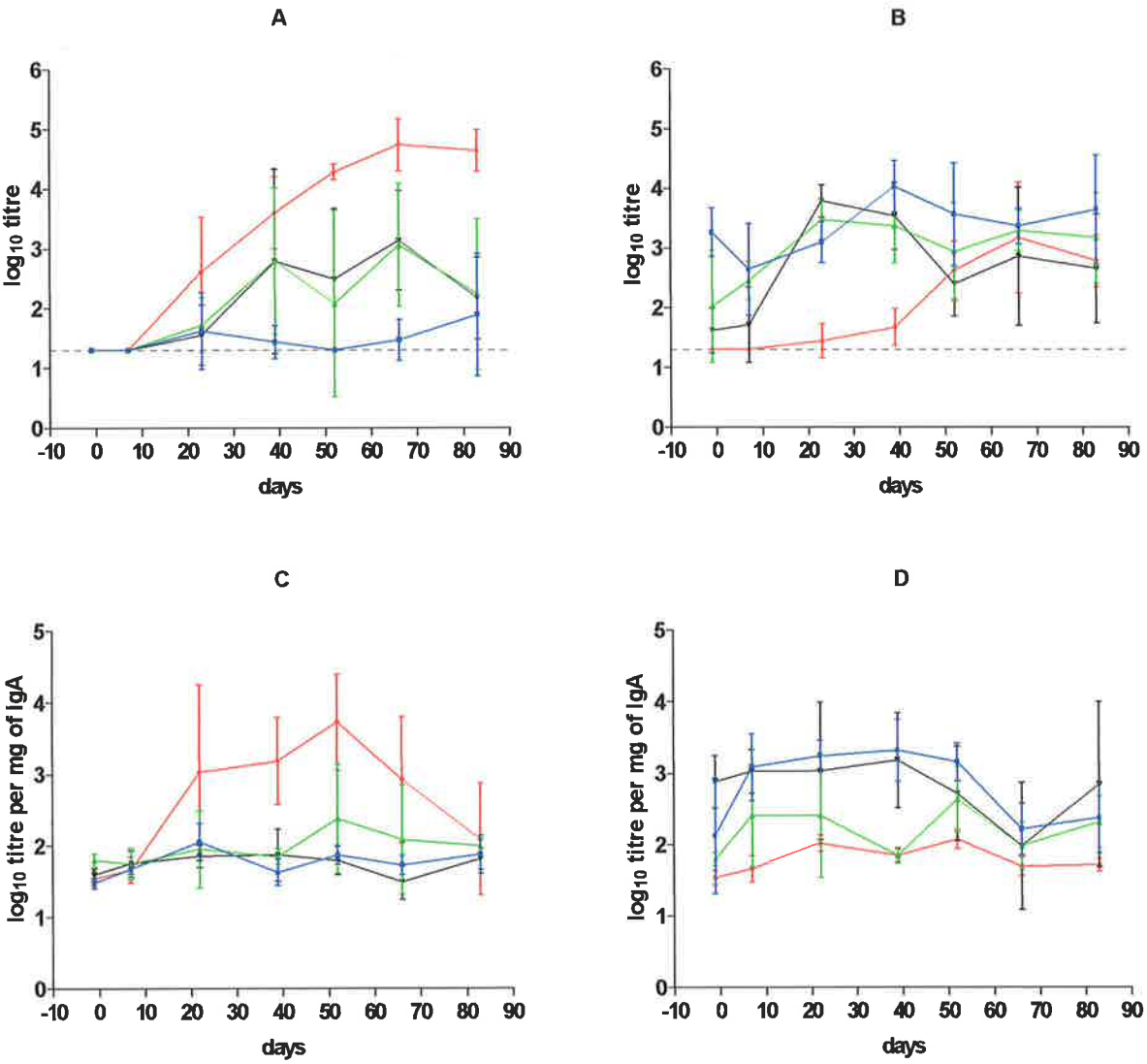
Figure 4.5 Comparison of wt and *aroA S.* Stanley priming in mice.

Panel I: Groups of BALB/c mice were orally primed with wt or *aroA S.* Stanley as described in the table below. Ten weeks later, all mice were orally dosed with 1.68 x10^9 r*S.* Stanley-K88, and serum IgG (**A**, **B**) and intestinal IgA (**C**, **D**) responses to K88 (**A**, **C**) and LPS (**B**, **D**) monitored by ELISA from blood and CFPS samples (alternate subsets of four mice were sampled at successive timepoints). Serum responses are represented as $\log_{10}$ ELISA titres (GM $\pm$ SD; n = 4), whilst gut IgA responses are expressed as $\log_{10}$ ELISA titres (GM $\pm$ SD; n = 4) per mg of IgA. Dashed line on graphs shows the limit of detection of serum ELISAs at a titre of 20.

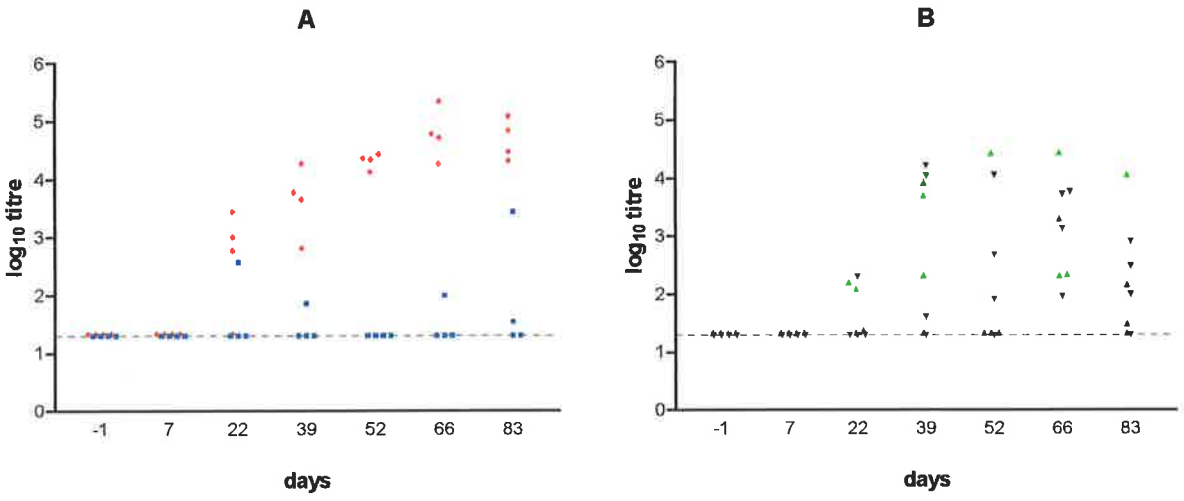
Panel II: Serum anti-K88 responses from panel I are represented in scatter plots. Figure A shows the responses of wt *S.* Stanley-primed and unprimed mice, whereas Figure B shows the responses of *aroA S.* Stanley-primed mice. Dashed line on graphs shows the limit of detection of serum ELISAs at a titre of 20.

GROUP	**VECTOR PRIMING**	
	STRAIN	**DOSE (CFU)**
1 —■—	wt *S.* Stanley	1.5x10^9 on day - 70
2 —▲—	*aroA S.* Stanley (CVSA14)	1.7x10^9 on day - 70
3 —▼—	*aroA S.* Stanley (CVSA14)	1.9x10^{10} on day - 70 9.6x10^9 on day - 66
4 —◆—	-	-

I


A
B
C
D
log10 titre
log10 titre per mg of IgA
days
-10 0 10 20 30 40 50 60 70 80 90
0 1 2 3 4 5 6

II


A
B
log10 titre
days
-1 7 22 39 52 66 83
0 1 2 3 4 5 6

0.01) and 66 ($P < 0.05$). This is among the first demonstrations of hyporesponsiveness consequent upon vector-priming at the level of the GALT; certainly the > ten-fold reduction in IgA antibody titre is the most significant reported to date.

Mice primed with either one or two doses of *aroA S.* Stanley developed partial serum anti-K88 responses (Figure 4.5 Panel I, A). Despite the variability of responses within these groups (see below), the mean antibody titre was significantly lower than that seen in control mice on days 52, 66 and 83 ($P < 0.05$, $P < 0.05$, and $P < 0.01$ respectively for both groups). Anti-K88 responses following priming with *aroA S.* Stanley were, however, significantly greater than those of mice primed with wt *S.* Stanley on days 39 ($P < 0.05$), 52 ($P < 0.05$) and 66 ($P_{group\ 2} < 0.05$ and $P_{group\ 3} < 0.01$). Figure 4.5, Panel II, shows individual serum anti-K88 responses of mice in the various groups. Control mice developed uniformly high titres of antibody to K88, particularly from day 52 onwards, when each mouse had an ELISA titre > 10^4 (Figure 4.5, Panel II A). In contrast, priming with wt *S.* Stanley reliably blocked the induction of antibody to K88; in general these mice had no detectable serum IgG of this specificity. Against these extremes, the responses of mice primed with *aroA S.* Stanley can be assessed. Of the sixteen sera collected on days 52 and 66 from different mice primed with *aroA S.* Stanley, five had titres $\geq$ 5000, representing a 250-fold increase above background and typical of responses seen in unprimed controls; six other mice showed serum titres $\leq$ 200, typical of the responses seen in animals primed with wt *S.* Stanley; and five mice showed intermediate titres.

Evidence of priming was indicated by elevated serum and intestinal anti-LPS antibody levels on day - 1 in mice pre-immunised with either wt or *aroA S.* Stanley, and by the occurrence of secondary responses, particularly in serum (Figure 4.5 B and D). Although the latter were similar in strength among animals primed with one low or two high doses of *aroA S.* Stanley, this did not appear to be the case in relation to anti-LPS responses in the gut. Priming with two doses of the auxotrophic vector resulted in higher IgA titres upon exposure to r*S.* Stanley-K88 ($P < 0.05$ on days 7 and 35). This raised the possibility that, despite the similarity in Peyer's patch

burdens seen with the two immunisation regimes (Figure 4.4 A), mice given two doses of the *aroA S.* Stanley might have been more effectively primed. This therefore prompted an examination of the primary anti-LPS responses generated by the two schedules used for vector-priming.

4.2.6.2 Anti-LPS responses induced by oral immunisation with wt or *aroA S.* Stanley.

Three groups of mice were immunised with wt or *aroA S.* Stanley using the same dosing regimes employed in the experiment described above (Figure 4.6, Table I)). As a comparison, a fourth group was immunised with two doses of ~ 10^{10} cfu of SL1438 (*aroA S.* Dublin) spaced four days apart. Serum and CFPS samples were collected at various time points, and anti-LPS responses measured by ELISA as shown in Figure 4.6 A and B, respectively.

Mice fed wt *S.* Stanley produced serum anti-LPS responses that were significantly greater than those fed *aroA S.* Stanley at either one dose of 10^9 cfu ($P < 0.01$ on days 46 and 70) or two doses of 10^{10} cfu spaced 4 days apart ($P < 0.05$ on days 46 and 70). Mice primed with two high doses of the *aroA* mutant developed weak anti-LPS responses which were only five-fold greater than background at day 70, whereas responses in mice primed with one low dose of bacteria were barely detectable. Intestinal IgA responses to LPS were weak and did not differ. Thus the data from this experiment did not reveal any major difference in anti-vector responses between the two *aroA* immunisation protocols. However, in the context of the experiment conducted in section 4.2.6.1, these results suggest that the intermediate nature of the serum anti-K88 responses seen in mice primed with *aroA S.* Stanley reflects the induction of weaker anti-vector immune responses following vector-priming.

Despite its more rapid clearance from the Peyer's patches (Figure 4.4 B), mice orally dosed with *aroA S.* Dublin induced stronger serum and mucosal anti-LPS responses than those elicited by wt *S.* Stanley on days 26 and 46 ($P < 0.05$ for serum responses, and $P < 0.01$ for

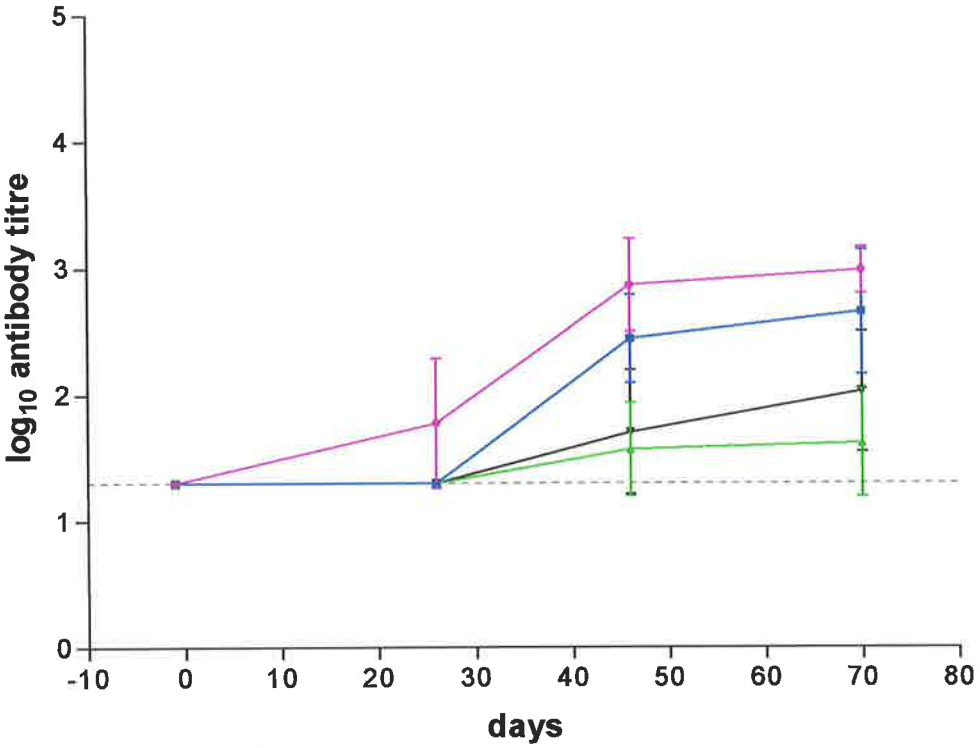
Figure 4.6 Anti-LPS responses induced by oral immunisation with wt or *aroA S.* Stanley, or with *aroA S.* Dublin.

Groups of mice were immunised as shown in the table below. Serum IgG (**A**) and CFPS IgA (**B**) responses to LPS are shown as $\log_{10}$ ELISA titres (GM $\pm$ SD; $n = 5$); in the case of the intestinal response, titres were standardised per mg of IgA. Dashed line on graphs shows the limit of serum ELISAs at a titre of 20.

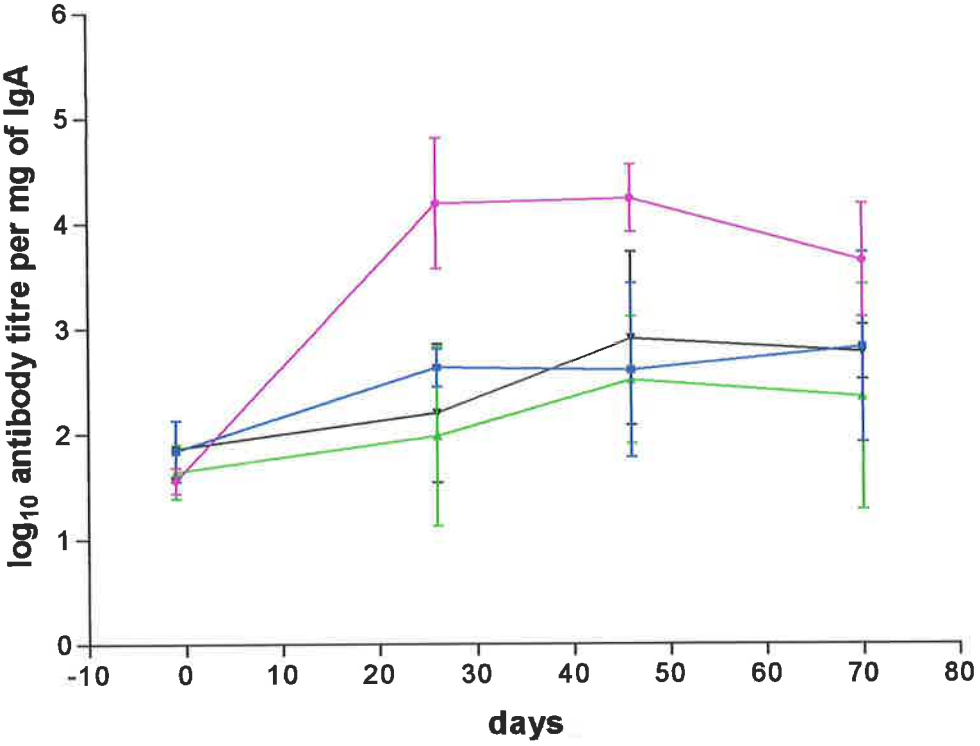
Table I

GROUP	STRAIN	DOSE (cfu)
1 —■—	wt *S.* Stanley	1.1×10^9 on day 0
2 —▲—	*aroA S.* Stanley (CVSA14)	1.3×10^9 on day 0
3 —▼—	*aroA S.* Stanley (CVSA14)	1.6×10^{10} on day 0 1.1×10^{10} on day 4
4 —●—	*aroA S.* Dublin (SL1438)	1.1×10^{10} on day 0 1.0×10^{10} on day 4

A


log10 antibody titre
0
1
2
3
4
5
-10
0
10
20
30
40
50
60
70
80
days

B


log10 antibody titre per mg of IgA
0
1
2
3
4
5
6
-10
0
10
20
30
40
50
60
70
80
days

mucosal responses for both time points). Bao and Clements (1991) also reported strong primary anti-vector (*aroA S.* Dublin) responses in mice which were subsequently shown to respond to LT-B following boosting with EL23 (*aroA S.* Dublin-LT-B). With this vector, the development of strong anti-LPS responses following primary exposure clearly does not compromise the subsequent delivery of a foreign antigen (also see section 5.2.4).

4.2.6.3 Does K88 expression by *S.* Stanley modify the IgG isotype profile of the serum anti-LPS response?

By convention, oral immunisation with *Salmonella* vectors induces $CD4^+$ T cell responses by IFN-γ-dominated pathways both to the vector and passenger antigen, resulting in serum IgG_{2a} and modest mucosal IgA antibody responses (Mastroeni *et al.*, 1992; Okahashi *et al.*, 1996; Ramarathinam *et al.*, 1993; VanCott *et al.*, 1996; Wu *et al.*, 1997; Wu *et al.*, 1995). Recently, oral immunisation of mice with *aroA S.* Typhimurium expressing ETEC colonisation factor antigen I (CFA/1) has been shown to modify cytokine production, from being Th2 biased during the early phase of infection, to a Th1 bias later in infection (Pascual *et al.*, 1999). It was therefore of interest to determine whether the expression of K88 (also a colonisation factor) by r*S.* Stanley would influence the serum IgG subclass profile (an indirect measurement of the balance between cell mediated and humoral immunity) of the anti-LPS response. Accordingly, sera collected for the experiment described in section 4.2.6.1 were titrated by ELISA for IgG_1 and IgG_{2a} antibodies specific for LPS. Sera were prepared from mice which had been primed with ~ 10^9 cfu of wt *S.* Stanley; others were prepared from control mice (immunised with $NaHCO_3$ at day -70) subsequently exposed to r*S.* Stanley-K88. The ratios of IgG_{2a}:IgG_1 antibodies within each serum set are shown in Figure 4.7. There were no significant differences in the subclass profiles of the antibody responses, which all showed a slight bias towards the IgG_{2a} subclass.

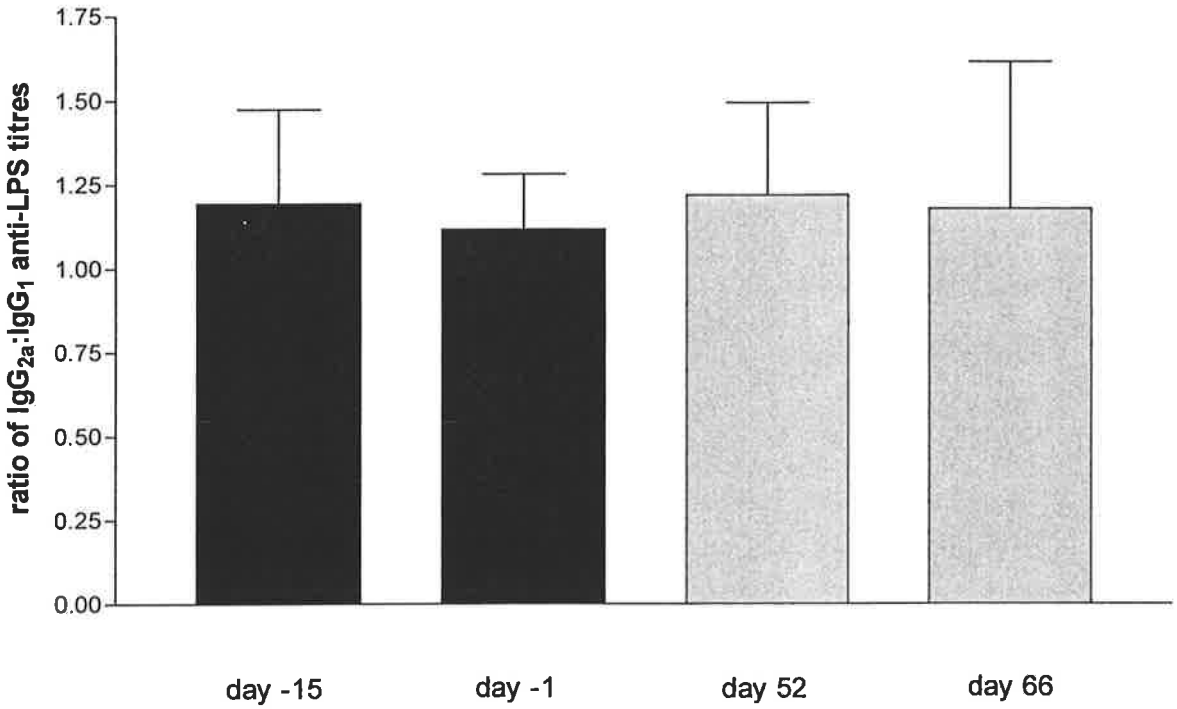
Figure 4.7 Effect of K88 expression on the IgG subclass profile of the anti-LPS response to *S.* Stanley.

Sera collected from mice used in the experiment described in section 4.2.6.1 were titrated in anti-LPS ELISAs using conjugates specific for IgG_1 and IgG_{2a} antibodies (section 2.16.3.6). Sera obtained 55 and 69 days after priming with wt *S.* Stanley (-15 and -1 days prior to exposure to r*S.* Stanley-K88, Figure 4.5), and others collected from unprimed controls 52 and 66 days after administration of r*S.* Stanley-K88, were selected for analysis. Histograms show the ratio of IgG_1:IgG_{2a} anti-LPS ELISA titres (M $\pm$ SD; $n = 4$) at each timepoint.

Legend

mice primed with wt *S.* Stanley

controls exposed to r*S.* Stanley-K88


ratio of IgG_{2a}:IgG_1 anti-LPS titres
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
day -15
day -1
day 52
day 66

4.2.7 Significance of vector-priming: relevance of host *Nramp1* status.

4.2.7.1 GALT colonisation by *S.* Stanley in *Nramp1*$^{-/-}$ and *Nramp1*$^{+/+}$ hosts.

It was of interest to compare the GALT colonisation potential of *S.* Stanley in mice innately susceptible or resistant to *Salmonella* infection. As discussed at the start of this chapter, significantly different colonisation profiles would allow an alternative approach to assessing the significance of vector-priming, with respect to subsequent delivery of foreign antigen by r*Salmonella.*

Groups of BALB/c (*Nramp1*$^{-/-}$) and CBA (*Nramp1*$^{+/+}$) mice were orally dosed with ~ 10^9 cfu of wt *S.* Stanley, and Peyer's patches were collected at various intervals and plated on XLD medium to determine bacterial recoveries. Similar numbers of bacteria were recovered from both mouse strains at each of the first three time points, with mean burdens of ~ 10^4 cfu on days 5 and 8 (Figure 4.8). Thereafter fewer *S.* Stanley were recovered from the *Nramp1*$^{+/+}$ hosts, the differences being significant at three of the four time points from days 14 to 34. This result was unexpected and prompted an experiment to confirm the *Nramp1* status of the mice being used.

Groups of CBA and BALB/c mice were injected i.p. with ~ 40 cfu of the highly virulent C5 strain of *S.* Typhimurium. Spleens were removed at various time points, and bacterial loads determined by plating homogenates on XLD medium (Figure 4.9). BALB/c mice were unable to control the C5 infection and were sampled more frequently as a result. Only four (of the remaining thirteen) BALB/c mice were still alive on day 7; these animals had splenic burdens of ~ 10^8 cfu at this time. In contrast, bacterial replication in the spleens of CBA mice was significantly lower on day 4, and reached a peak burden of < 10^6 on day 12 (Figure 4.9). Thereafter the infection was cleared, and no mouse of this strain died. This experiment confirmed that there was a difference in *Nramp1* gene expression in the BALB/c and CBA mice

Figure 4.8 Peyer's patch colonisation by wt *S.* Stanley in CBA and BALB/c mice.

BALB/c and CBA mice were orally dosed with 1.11×10^9 cfu of wt *S.* Stanley. At various timepoints, Peyer's patches were harvested to determine bacterial burdens, which are shown as $\log_{10}$ cfu (GM $\pm$ SD; n = 4). Dashed line represents the limit of detection (20 bacteria). Recoveries from the mouse strains were significantly different at some timepoints as indicated by '*' ($P<0.05$), or '**' ($P<0.01$).

Legend

-▫- CBA

—▪— BALB/c

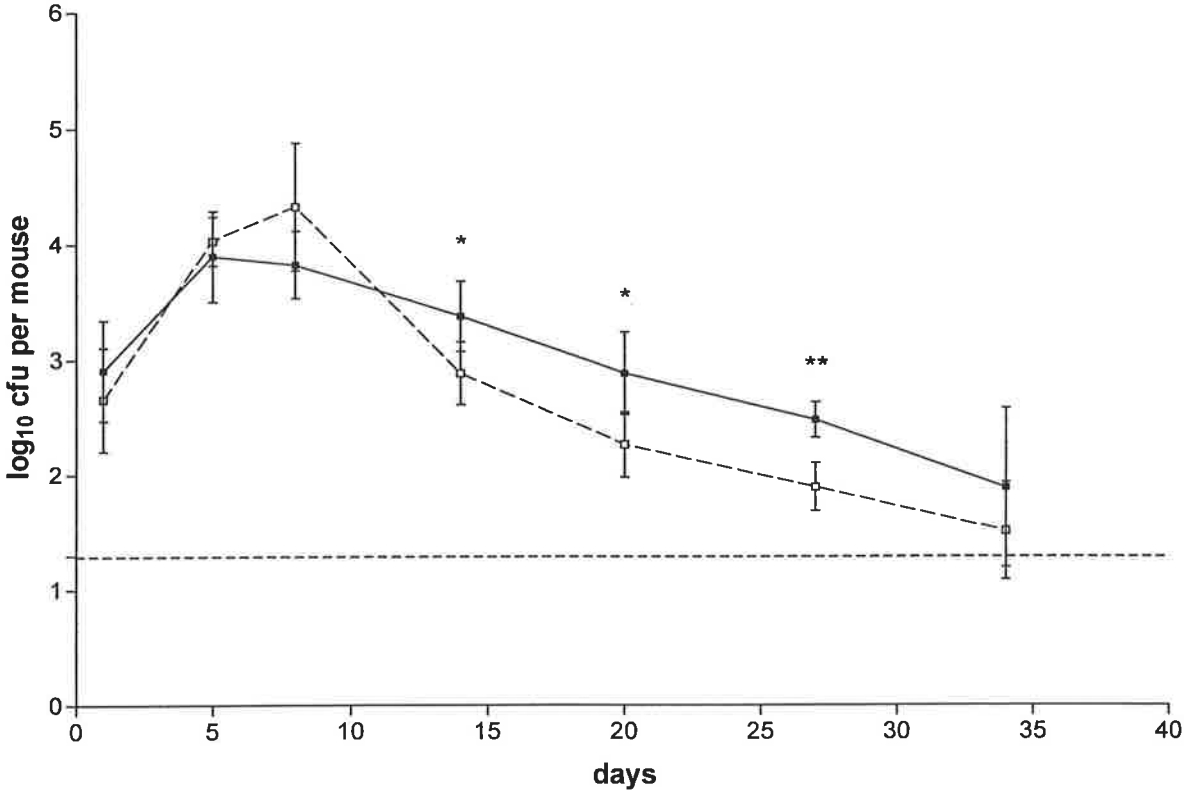

log10 cfu per mouse
6
5
4
3
2
1
0
0
5
10
15
20
25
30
35
40
days
*
*
**

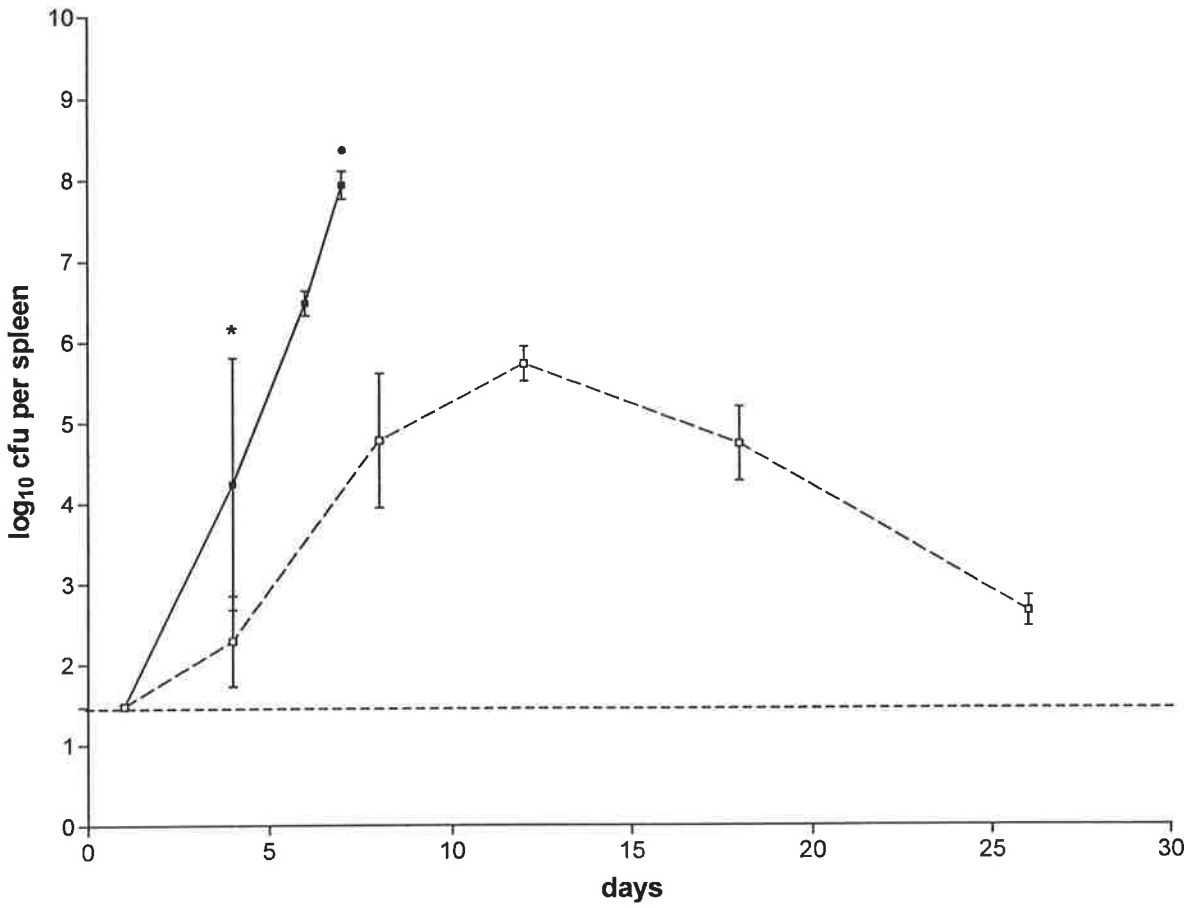
Figure 4.9 Confirmation of the *Nramp1* status of CBA and BALB/c mice.

CBA and BALB/c mice were injected i.p. with 40 cfu of *S.* Typhimurium C5. Graph represents the $\log_{10}$ cfu per spleen (GM $\pm$ SD; n = 4). All but four of the BALB/c mice had died upon inspection on day 7, as indicated by '●'. Dashed line represents the limit of detection (30 bacteria) and statistically different bacterial burdens are denoted by * ($P < 0.05$).

Legend

–▫– CBA

—▪— BALB/c


10
9
8
7
6
5
4
3
2
1
0
log10 cfu per spleen
*
•
0
5
10
15
20
25
30
days

used, with the latter strain being able to control the growth of virulent *Salmonella* introduced systemically.

4.2.7.2 GALT colonisation by *S.* Typhimurium C5 in *Nramp1*$^{-/-}$ and *Nramp1*$^{+/+}$ hosts.

Oral infection of BALB/c and CBA mice with wt *S.* Stanley had resulted in Peyer's patch colonisation profiles which were surprisingly similar. It was of interest to ascertain whether an *Nramp1*-related difference in colonisation may be apparent if mice were orally infected with the highly virulent C5 strain. Groups of CBA and BALB/c mice were orally dosed with ~ 10^8 cfu of *S.* Typhimurium C5. At various times after immunisation, Peyer's patches were harvested, homogenised and plated onto XLD to estimate bacterial burdens.

Again, there was no difference in the Peyer's patch colonisation profiles seen in BALB/c or CBA mice (Figure 4.10). However, the last four BALB/c mice died as a result of infection before day 7, whereas the remaining CBA mice showed no visible signs of illness at this time, consistent with the difference in *Nramp1* status. Although these results were contrary to expectations, it was considered important to assess the impact of vector-priming on the oral immunogenicity of r*Salmonella* constructs in *Nramp1*$^{+/+}$ hosts.

4.2.7.3 Significance of vector-priming in *Nramp1*$^{+/+}$ hosts.

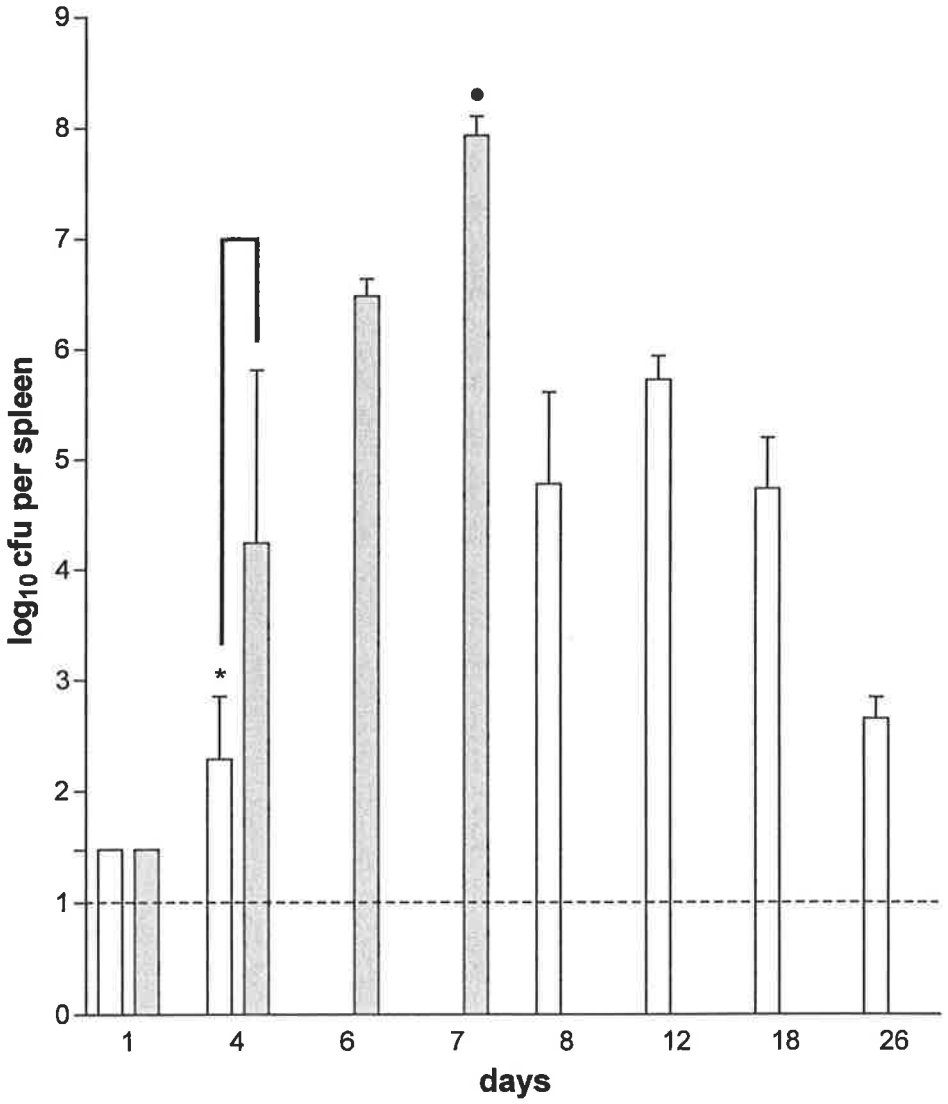
Groups of BALB/c and CBA mice were orally dosed with ~ 10^9 cfu of wt *S.* Stanley, with additional mice kept aside as controls. After eight days, five mice from each group were sacrificed to estimate bacterial Peyer's patch burdens. Consistent with data shown in Figure 4.8, bacterial burdens were equivalent in CBA and BALB/c mice at ~ 10^4 cfu per mouse (not shown). Ten weeks after primary infection, all animals were dosed with ~ 10^9 cfu r*S.* Stanley-K88, following which serum and CFPS samples were collected for the determination of anti-K88 and anti-LPS antibody responses.

Figure 4.10 Peyer's patch colonisation of CBA and BALB/c mice following oral challenge with *S.* Typhimurium C5.

BALB/c and CBA mice were orally dosed with $1.1x10^8$ cfu of *S.* Typhimurium C5. Peyer's patches burdens are shown as log_{10} bacteria (GM $\pm$ SD; $n = 4$). Dashed line represents the limit of detection (20 bacteria). The last four BALB/c mice died before day 7 as indicated by '●'.

Legend

CBA
BALB/c


9
8
7
6
5
4
3
2
1
0
log10 cfu per spleen
*
●
1
4
6
7
8
12
18
26
days

Control and vector-primed BALB/c mice developed serum anti-K88 (Figure 4.11 A) and anti-LPS (Figure 4.11 B) responses comparable to those observed previously. A similar pattern of responses was seen in control and vector-primed CBA mice; again the serum anti-K88 response was completely abrogated as a consequence of prior exposure to the vector strain. The primary serum response to K88 in naïve animals was significantly lower in CBA than in BALB/c mice on days 28 ($P < 0.05$), 46 and 69 ($P < 0.01$ at both time points).

The state of hyporesponsiveness to K88 extended to the mucosal IgA responses in both mouse strains (Figure 4.11 C). However, the mucosal anti-K88 response in control CBA mice was only modest, and consequently the differences between the (corrected) antibody titres of control and vector-primed mice did not achieve statistical significance. As with the serum IgG response, the intestinal IgA response was significantly weaker in CBA then in BALB/c mice on days 14, 28 ($P < 0.01$ at both time points), 46 and 69 ($P < 0.05$ at both time points).

Evidence of vector-priming was indicated by elevated serum (Figure 4.11 B) and mucosal (Figure 4.11 D) anti-LPS responses, in both CBA and BALB/c mice. In contrast, control groups developed primary anti-LPS responses. The similarity of the anti-LPS responses in CBA and BALB/c mice, compared to the observed differences in anti-K88 responses, suggested that the recognition/processing of K88 expressed by r*S.* Stanley may be less efficient in CBA mice. Nevertheless, the significance of vector-priming was evident irrespective of *Nramp1* status.

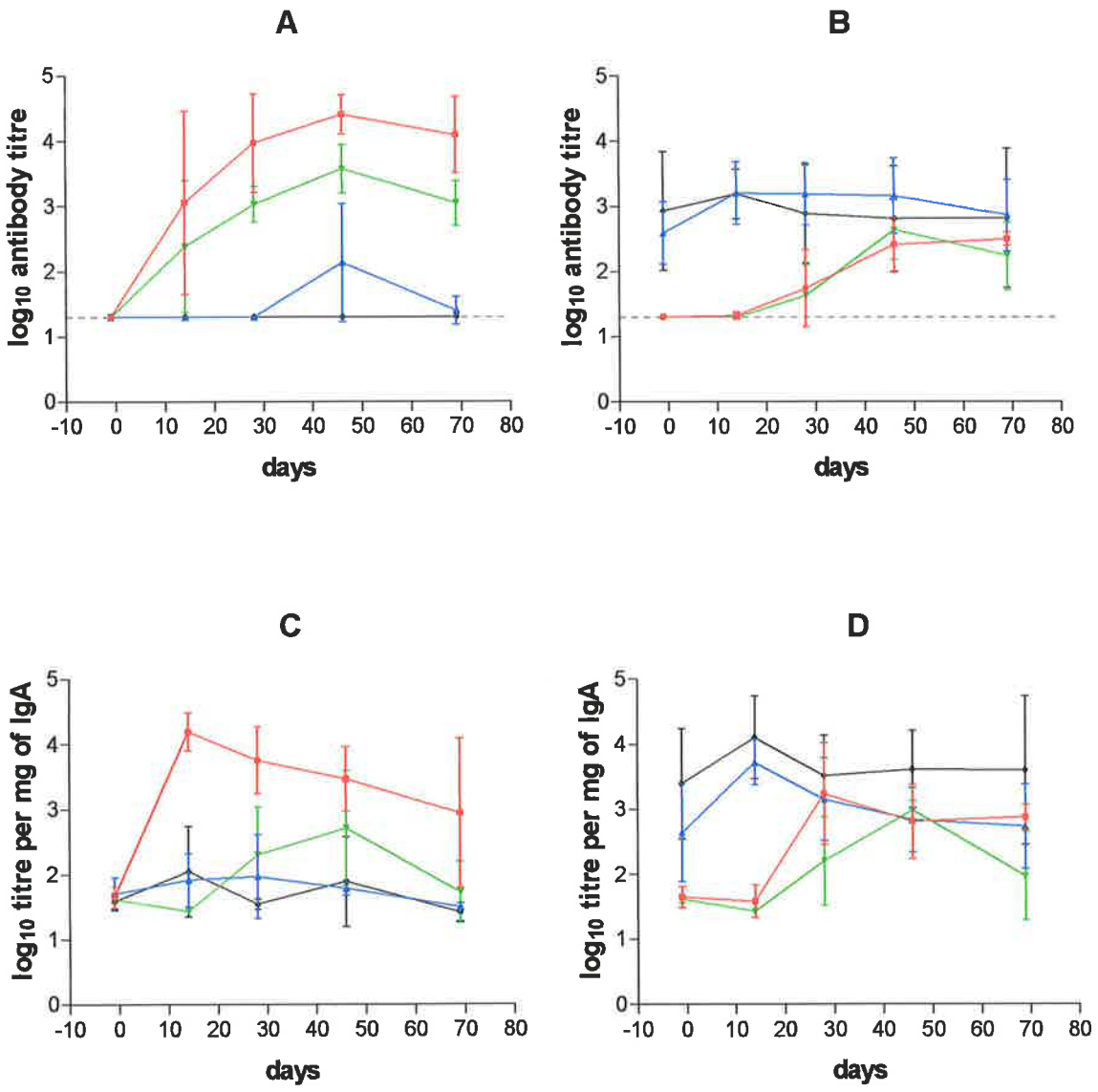
4.2.7.4 Significance of mouse strain background for IgG isotype of anti-LPS responses.

It was of interest to compare the IgG isotype profiles of the serum anti-LPS responses of BALB/c and CBA mice following immunisation with either wt *S.* Stanley or r*S.* Stanley-K88. Serum samples collected in the preceding experiment were used to obtain preliminary data on this point. Sera obtained 49 and 69 days after priming with wt *S.* Stanley, and those collected

Figure 4.11 The significance of vector priming in *Nramp1*$^{+/+}$ mice.

Groups of CBA and BALB/c mice were orally dosed with wt *S.* Stanley or $NaHCO_3$ (controls) as detailed below. Five mice from groups 1 and 3 were sacrificed on day 8. Remaining animals were fed $1.3x10^9$ cfu of *S.* Stanley-K88 on day 70. Serum (**A**, **B**) and CFPS (**C**, **D**) samples were collected (from alternate subsets of mice) for the determination of anti-K88 (**A**, **C**) and anti-LPS (**B**, **D**) responses by ELISA. Graphs represent $\log_{10}$ ELISA titres (GM $\pm$ SD; $n = 5$); in the case of the mucosal IgA response, titres were standardised per mg of IgA. Dashed line on graphs represent the limit of detection of serum ELISAs at a titre of 20.

GROUP	MOUSE STRAIN	NUMBER OF MICE	VECTOR PRIMING	
			STRAIN	DOSE (cfu)
1 —▲—	BALB/c	16	wt *S.* Stanley	$1.11x10^9$
2 —■—	BALB/c	11	$NaHCO_3$	-
3 —◆—	CBA	16	wt *S.* Stanley	$1.11x10^9$
4 —▼—	CBA	11	$NaHCO_3$	-


A
B
C
D
log10 antibody titre
log10 titre per mg of IgA
days
-10 0 10 20 30 40 50 60 70 80
0 1 2 3 4 5

from unprimed controls 49 and 69 days after administration of r*S.* Stanley-K88, were selected for analysis.

As shown in Figure 4.12, anti-LPS IgG subclass ratios in sera taken from BALB/c mice dosed with either wt or r*S.* Stanley-K88 were not significantly different, which supported earlier data presented in Figure 4.7. Similarly, the IgG_{2a}:IgG_1 antibody ratios determined from sera collected from CBA mice fed either wt or r*S.* Stanley-K88 were also not significantly different. However, in comparing sera collected from the two mouse strains, the anti-LPS responses seen in BALB/c mice showed a slight IgG_{2a} bias, and those of CBA mice an IgG_1 bias; the difference between the responses of the two mouse strains were statistically significant on three of the four time points (*, $P < 0.05$, Figure 4.12). This result suggested that CBA mice respond to *S.* Stanley in a manner different to BALB/c mice, although this might be unrelated to *Nramp1* status (see below).

4.3 Discussion.

Attridge *et al.* (1997) showed that pre-existing anti-vector immunity could render mice unresponsive to foreign antigen subsequently delivered by r*Salmonella.* This outcome, which contrasted the results of Bao and Clements (1991), was suggested to be due (at least in part) to a more sustained colonisation of the GALT by their *S.* Stanley vector strain. Therefore, an attempt was made in this chapter to assess the significance of the vector's GALT colonising potential by introducing a chromosomal mutation into the *aroA* gene of *S.* Stanley.

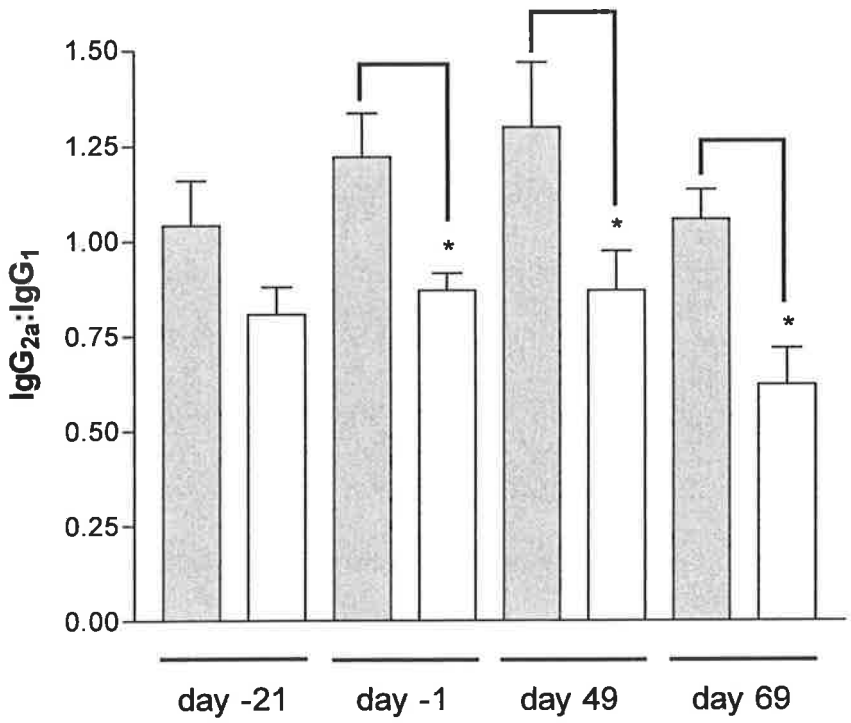
Initially, attempts were made to introduce an *aroA* mutation into *S.* Stanley by bacteriophage-mediated transduction. These attempts proved unsuccessful, and thus an alternative strategy was pursued. An interrupted *aroA* gene was created by insertion of the Km^R cartridge *apha-3* (*aroA*::*apha-3*), and this construct cloned into suicide vector pCACTUS*mob*. Allelic exchange mutagenesis was performed to introduce the *aroA*::*apha-3* construct into the *S.*

Figure 4.12 Significance of mouse strain background for IgG isotype profile of serum anti-LPS responses.

Sera from BALB/c and CBA mice immunised with wt *S.* Stanley and r*S.* Stanley-K88 were analysed by ELISA to compare the IgG isotype profiles of the anti-LPS responses. Sera obtained 49 and 69 days after priming with wt *S.* Stanley (- 21 and - 1 days prior to exposure to r*S.* Stanley-K88, respectively), and those collected from unprimed controls 49 and 69 days after administration of r*S.* Stanley-K88 were selected for analysis. Histograms show the ratio of IgG_1:IgG_{2a} ELISA titres (GM $\pm$ SD; n = 5), and points of significant difference are indicated by '*' ($P < 0.05$)

Legend

CBA
BALB/c


1.50
1.25
1.00
0.75
0.50
0.25
0.00
IgG2a:IgG1
*
*
*
day -21
day -1
day 49
day 69

Stanley chromosome, which was subsequently confirmed by Southern analysis. *In vitro* tests showed that the *aroA S.* Stanley could only grow on supplemented MM, unless transformed with a plasmid expressing a minimal *aroA* fragment (pCVSA7). Surprisingly, the *aroA S.* Dublin strain used by Bao and Clements (1991) failed to grow on supplemented MM, and could not be complemented when transformed with pCVSA7, suggesting that this strain contains cryptic mutations in addition to *aroA*.

An experiment was performed to assess murine Peyer's patch colonisation profiles following the oral administration of *aroA S.* Stanley, using the dosing regimes employed by both Bao and Clements (1991) and Attridge *et al.* (1997). The *aroA* mutant was clearly attenuated as oral administration of two doses of 10^{10} cfu (spaced four days apart) caused no obvious ill effects, whereas wt *S.* Stanley is lethal if given in doses greater than ~ $2x10^9$ cfu (Attridge, unpublished). Unexpectedly however, GALT colonisation by the *aroA* mutant was similar to that seen with wt *S.* Stanley, particularly in mice given two doses according to the protocol of Bao and Clements (1991). Regardless of the dosing regime, *aroA* bacteria were able to persist in the Peyer's patches for the (forty day) duration of the experiment. Complementation of *aroA S.* Stanley with pCVSA7 was also demonstrated *in vivo*. In a limited experiment, the complemented mutant reached colonisation levels equivalent to wt *S.* Stanley on day 5 (Table 4.2). Indirect evidence for *in vivo* expression of $aroA^+$ from pCVSA7 came from a comparison of plasmid retention rates; viable counts in the presence and absence of antibiotics showed that ~ 100% of bacteria retained pCVSA7, whereas $\leq$ 4% retained the control plasmid (pWSK30) devoid of the *aroA* gene.

Despite the unexpected GALT-colonising potential of the *aroA S.* Stanley mutant, an experiment was conducted comparing serum and antibody responses induced by r*S.* Stanley-K88 in mice primed with either *aroA* or wt *S.* Stanley. Mice primed with wt *S.* Stanley displayed a hyporesponsiveness to K88 in comparison to unimmunised controls, as seen previously by Attridge *et al.* (1997). Notably, this hyporesponsiveness extended to the intestinal IgA response

(Figure 4.5 C), which has previously been unmeasurable in this laboratory. This is among the first demonstrations of hyporesponsiveness consequent upon vector-priming at the level of the GALT (see section 6.2.1), and therefore strengthens concerns about the use of r*Salmonella* vaccines in individuals previously exposed to *Salmonella*.

Mice primed with either one or two doses of *aroA S.* Stanley showed highly variable serum anti-K88 responses when boosted with r*S.* Stanley-K88. Scatter plot analysis of these responses showed that a small proportion of mice generated intermediate anti-K88 responses, whereas the rest responded in a manner similar to either wt or unprimed animals (Figure 4.5). This may reflect highly variable GALT colonisation in individual animals, however it was not possible to link day -1 sera to those prepared post administration of r*S.* Stanley-K88. A later experiment compared primary anti-LPS responses in mice immunised with one or two doses of *aroA S.* Stanley, or the wt vector strain. This revealed that the *aroA* mutant induced weaker anti-vector immunity, especially when administered at a single low dose; mice primed with two high doses of the *aroA* mutant developed weak anti-LPS responses, whereas responses in mice primed with one low dose of bacteria were barely detectable (Figure 4.6). Intestinal IgA responses to LPS were weak and did not differ. In the original experiment, mice primed with one low dose of the *aroA* vector failed to generate a strong secondary intestinal IgA response to LPS following exposure to r*S.* Stanley-K88, unlike animals primed with two high doses of the same strain, or with wt *S.* Stanley. Together, these data suggest that the introduction of the *aroA* mutation has resulted in weaker anti-vector immunity, allowing *aroA S.* Stanley-primed mice to mount intermediate K88 responses upon boosting with r*S.* Stanley-K88. This would be consistent with the earlier finding that mice primed with ~ 10^6 cfu of wt *S.* Stanley (instead of ~ 10^9 cfu) were able to develop anti-K88 responses when subsequently exposed to r*S.* Stanley-K88 (Attridge *et al.*, 1997).

In comparison to *aroA S.* Stanley, *aroA S.* Dublin colonised the Peyer's patches for a significantly shorter period of time (Figure 4.4). The inability of *aroA S.* Dublin to grow on

supplemented minimal medium, and its failure to be complemented *in vitro* by pCVSA7, suggested that other cryptic mutations are present within this strain which possibly contribute to its more rapid clearance from the Peyer's patches. Despite its comparatively transient colonisation of the GALT, the *aroA S.* Dublin mutant nevertheless induced stronger serum IgG and intestinal IgA anti-LPS responses than its *S.* Stanley counterpart. Nevertheless, Bao and Clements (1991) found that priming with the former did not compromise the immunogenicity of LT-B subsequently delivered by the same vector (also see section 5.2.4). It is possible that *S.* Stanley and *S.* Dublin differ in their potential to induce proinflammatory responses in the GALT. In the case of *aroA S.* Dublin, induction of a greater proinflammatory response may allow responses to foreign antigen in vector-primed mice. However, the intrinsic characteristics of the foreign antigen being delivered by the r*Salmonella* construct must also be considered, and is addressed in the next chapter.

The experiments with the *aroA* mutants of *S.* Stanley and *S.* Dublin suggest that there is no clear correlation between persistence of bacteria in the Peyer's patches, the magnitude of subsequent anti-vector responses and the potential to respond to a foreign antigen subsequently delivered by the same vector. Other reports have provided data relevant to this issue. Dunstan *et al.* (1998) compared the immunogenicity of various attenuated *S.* Typhimurium expressing a peptide derived from tetanus toxin (FrgC). R*Salmonella* persistence in the Peyer's patches of infected animals (which varied due to the nature of the vector attenuation) was found to be proportional to the induction of serum antibody responses to both vector LPS and foreign antigen, *in vitro* IFN-γ production and T cell proliferative responses to FrgC. In contrast, Benyacoub *et al.* (1999) showed that the *in vivo* persistence of r*Salmonella* following i.n. administration did not correlate with the strength of specific antibody responses induced against the foreign antigen (hepatitis B core antigen). Bacteria were recovered in significantly different numbers from the lungs, Peyer's patches, spleens and cervical lymph nodes depending on the nature of the vector attenuation. The conflicting results of these two studies may be explained by

differences in *Salmonella* attenuation, the nature of the foreign antigen, and the route of vector administration. These points of difference are further addressed in the following chapters.

Although *aroA* attenuated *Salmonella* were originally thought to be unlikely to persist *in vivo*, studies by VanCott *et al.* (1998) and Dunstan *et al.* (1998) have also demonstrated that *aroA S.* Typhimurium can persist for a significant duration in the murine GALT. The former investigated the immune responses of normal and IFN-γ knockout mice orally immunised with *aroA* and *phoP* attenuated *S.* Typhimurium; surprisingly, mice given the *aroA* vector were unable to control bacterial replication and died. Dunstan *et al.* (1998) showed that *aroA S.* Typhimurium expressing FrgC was able to persist in the Peyer's patches and spleens of mice for at least 21 days post oral immunisation. (In neither study however, were the Peyer's patch colonisation profiles of the *aroA* mutants directly compared with those of their wt parent strains.) Stocker (2000) has suggested that the *in vivo* persistence of *aroA* attenuated salmonellae may be due to a leeching of DHB and pABA precursors from mouse chow, allowing partial compensation of their auxotrophy.

In a preliminary assessment, sera from a vector-priming experiment were retrospectively examined for IgG_1 and IgG_{2a} antibodies directed against vector LPS to ascertain whether K88 expression had affected the isotype balance. This was prompted by a recent study which investigated the oral immunisation of mice with *aroA S.* Typhimurium expressing a different ETEC colonisation factor, CFA/1. Whereas the control vector induced a Th1-biased cytokine response, the expression of CFA/1 altered the Th1/Th2 balance during the early phase of infection towards a Th2-bias (Pascual *et al.*, 1999). Interestingly, a recent report by Chen and Schifferli (2001) showed that mice orally immunised with r*S.* Typhimurium expressing chimeric fimbriae developed differential serum IgG_1:IgG_{2a} ratios depending on the *in vivo* inducible promoter (either *nirB* or *pagC*) used to control fimbrial expression. Consistent with other reports (Mastroeni *et al.*, 1992; Okahashi *et al.*, 1996; Ramarathinam *et al.*, 1993; VanCott *et al.*, 1996; Wu *et al.*, 1997; Wu *et al.*, 1995) was our finding that immunisation with r*S.* Stanley induced an

IgG_{2a}-biased antibody response directed against vector LPS. The expression of K88 did not appear to have an effect on serum IgG isotype bias. However, in view of the studies by Pascual *et al.* (1999) and Chen and Schifferli (2001), and to properly investigate the impact of K88 on serum IgG responses to *S.* Stanley, a more controlled experiment is required which would include the preparation of sera at earlier time points as well as the measurement of Th1 and Th2 specific cytokines.

Most studies of *Salmonella* infection have been performed in innately susceptible BALB/c mice. As previously discussed (section 1.3) however, Eisenstein (1999) has suggested that innately resistant (*Nramp1*$^{+/+}$) mice might represent a more appropriate model system, in terms of gathering data relevant to the development of vaccines against *S.* Typhi infection in humans. In the context of this thesis, it was thought that if *Nramp1*$^{+/+}$ animals could better control the growth of *Salmonella* in GALT, they may offer a different way of assessing the significance of vector strain GALT colonisation with respect to the subsequent delivery of foreign antigen by r*Salmonella*. Early time points in Peyer's patch colonisation profiles of mice orally dosed with wt *S.* Stanley showed that *Nramp1* expression had no effect on bacterial burdens. This was unexpected given that cells which express *Nramp1* (particularly macrophages and dendritic cells) are present in the GALT. Intra-peritoneal administration of virulent *S.* Typhimurium C5, and the assessment of splenic bacterial burdens, confirmed the *Nramp1* status of the mice being used. In a later experiment, the highly virulent *S.* Typhimurium strain C5 was given orally to *Nramp1*$^{-/-}$ (BALB/c) and *Nramp1*$^{+/+}$ (CBA) mice, but again the Peyer's patch colonisation profiles showed no difference in bacterial load. Despite this, *Nramp1*$^{+/+}$ mice were still healthy when sacrificed on day 7, whereas *Nramp1*$^{-/-}$ mice had all died by this time point. Although splenic bacterial burdens were not assessed in this experiment, this suggested a delayed growth of *S.* Typhimurium C5 in the reticuloendothelial system of *Nramp1*$^{+/+}$ mice, consistent with data obtained in the earlier experiment involving i.p. challenge with the C5 strain. Experiments described in this chapter suggest that Nramp1 is not a significant innate

defence against GALT colonisation in the critical early period of *Salmonella* infection, and that it may act exclusively to limit systemic infection. This interpretation may partly explain why Dunstan *et al.* (2001) were not able to find an association between NRAMP1 polymorphisms in man and susceptibility to *S.* Typhi, which infects via the oral route.

Further comparison of innately susceptible and resistant mice showed that *Nramp1* expression did not influence the development of hyporesponsiveness to foreign antigen following vector-priming. Vector-primed CBA mice failed to develop serum IgG and intestinal IgA antibodies to K88 following exposure to r*S.* Stanley-K88, while developing secondary antibody responses to vector LPS equivalent to those in *Nramp1*$^{-/-}$ mice (Figure 4.11). This finding strengthens concerns that the efficacy of r*Salmonella* vaccines might be severely reduced in recipients living in areas where *Salmonella* infections are prevalent. Surprisingly, unprimed *Nramp1*$^{+/+}$ hosts exposed to r*S.* Stanley-K88 developed weaker serum responses to K88, while developing primary anti-vector responses equivalent to those in *Nramp1*$^{-/-}$ mice. A retrospective but uncontrolled comparison of serum anti-LPS IgG isotypes suggests that *Nramp1*$^{+/+}$ mice might respond differently to r*S.* Stanley-K88. Whereas IgG isotype ratios in BALB/c mice indicated a slight Th1 bias (Figures 4.7 and 4.12), responses in CBA mice were Th2 biased; this difference was significant at the 5% level, but is not necessarily a consequence of *Nramp1* status as discussed below.

The nature of the primary serum IgG responses against vector LPS and K88 observed in naïve BALB/c and CBA mice is consistent with the results published by Fayolle *et al.* (1994). In their study, serum IgG antibody responses were compared after i.p. immunisation of naïve *Nramp1* congenic mice with *aroA S.* Typhimurium expressing maltose binding protein (MalE). Although antibody responses to vector LPS were equivalent in all strains, responses to the foreign antigen in *Nramp1*$^{+/+}$ mice were significantly lower than those in *Nramp1*$^{-/-}$ animals (but see below). Experiments in this chapter confirm the effect of *Nramp1* expression on systemic infection with virulent *Salmonella* (section 4.2.7.1); it is not surprising a more rapid clearance of

bacteria by *Nramp1*$^{+/+}$ mice is associated with weaker responses to passenger antigens. Differences in immune responses generated by mice congenic for *Nramp1* have also been observed by Soo *et al.* (1998). *Nramp1*$^{+/+}$ mice injected i.v. with *aroA aroD S.* Typhimurium expressing FrgC mounted a Th1-biased (IL-2 and IFN-γ) response against the foreign antigen, whereas similarly vaccinated *Nramp1*$^{-/-}$ mice mounted a Th2-biased (IgE and IL-4) response. Using an *aroA aroD S.* Typhimurium construct expressing a leishmanial antigen, i.v. vaccination of *Nramp1*$^{+/+}$ mice conferred protection against footpad challenge with *Leishmania major*, whereas similarly vaccinated *Nramp1*$^{-/-}$ mice showed exacerbated lesion growth following challenge. Given our inability to demonstrate any impact of *Nramp1* status on GALT colonisation by r*Salmonella*, it remains to be determined whether the results of the experiments by Fayolle *et al.* (1994) and Soo *et al.* (1998) would differ if r*Salmonella* were administered by the oral route.

In addition to *Nramp1*, genes of the *H-2* complex can influence immune responses to *Salmonella*, as well as processing and presentation of passenger antigen (Hormaeche *et al.*, 1985; Nauciel, 1990; Nauciel *et al.*, 1988). Moreover, it has been suggested that *H-2* gene expression and the development of acquired immune responses, rather than *Nramp1* expression as a determinant of innate immunity, is more critical for the response to r*Salmonella*. Fayolle *et al.* (1994) initially found that *Nramp1*$^{+/+}$ mice immunised (i.p.) with r*Salmonella* failed to mount serum responses against the foreign antigen, whereas similarly immunised *Nramp1*$^{-/-}$ mice developed strong serum responses. However, i.p. administration of the same construct to *H-2* (*Nramp1*$^{-/-}$) congenic mice revealed that responses to the foreign antigen were dictated by the expression of certain *H-2* haplotypes, rather than *Nramp1* status. Interestingly, improving the level of foreign antigen expression within r*Salmonella* overcame differences due to *H-2* gene expression between mice initially classified as either strong or weak responders. A subsequent study by the same group extended these observations to proliferative T cells responses; strong

Th1 $CD4^+$ cell responses against the foreign antigen were also dependent on *H-2* haplotype (Lo-Man *et al.*, 1996).

A recent study by Koesling *et al.* (2001) has further addressed the influence of *H-2* gene expression on r*Salmonella* immunogenicity in mice, but following oral administration. *Nramp1*$^{-/-}$, H-2^d (BALB/c) and H-2^b (C57BL/6) mice were orally immunised with *aroA S.* Typhimurium expressing *Helicobacter pylori*-derived antigens (urease subunits A and B), and subsequently challenged with *H. pylori* at various time points. Vaccination conferred long-lasting immunity in both mouse strains. However, protection in C57BL/6 mice was still observed at 54 weeks while not all vaccinated BALB/c mice were immune when challenged at this time. Interestingly, vaccination with r*Salmonella* induced a greater serum IgG_{2a} anti-urease response in C57BL/6 mice than in BALB/c mice. Moreover, immune responses to vector LPS and foreign antigen were also found to be significantly greater in the former mouse strain. However, it should be noted that the mouse strains used were not *H-2* congenic, hence limiting the significance of the results.

BALB/c mice express the H-2^d haplotype, whereas CBA mice express H-2^k. In the context of the observations made by the abovementioned groups, the differences in *H-2* gene expression in mouse strains used in this chapter may explain the reduced serum anti-K88 responses observed in the latter mouse strain. However, in order to better assess the significance of Nramp1 and H-2 in vector-primed mice, further tests are required which utilise congenic BALB/c mice that differ in *Nramp1* gene expression, but express the same *H-2* haplotype. These experiments may also include the measurement of Th1 specific cytokines such as IFN-γ and IL-12.

CHAPTER FIVE

The significance of pre-existing anti-vector immunity: importance of the foreign antigen subsequently presented by r*Salmonella*.

5.1 Introduction

The experiments described in this chapter assess the importance of the foreign antigen delivered by r*Salmonella* as a determinant of the immune responses elicited in vector-primed mice. Attridge *et al.* (1997) reported that vector-primed mice were compromised in their ability to respond to K88 presented by r*S.* Stanley, whereas Bao and Clements (1991) found that vector-primed mice were able to generate normal responses to LT-B presented by *aroA S.* Dublin.

LT-B has been demonstrated to be both a potent mucosal immunogen and adjuvant (de Haan *et al.*, 1998; Richards *et al.*, 2001; Verweij *et al.*, 1998). Its ability to form pentamers and bind host monoganglioside, present on all cells of the mucosal epithelium, appears to be crucial for these properties (Nashar *et al.*, 1996). Although K88 normally functions as a colonisation factor essential for ETEC infection in pigs (Moseley *et al.*, 1986; Orskov and Orskov, 1966; and recently reviewed by Van den Broeck *et al.*, 2000), there is no evidence that it mediates attachment to murine intestinal epithelial cells, nor that it can act as an adjuvant.

In addition to these intrinsic differences, the location of the two proteins differs in the r*Salmonella* constructs used in the studies mentioned above. K88 pili are surface expressed in the *S.* Stanley construct used by Attridge *et al.* (1997) and is therefore likely to remain cell

associated *in vivo*. In contrast, LT remains primarily cell associated, but *in vitro* studies have shown that it can be released from the periplasm of *E. coli* upon contact with physiological concentrations of intestinal factors such as bile salts and proteolytic enzymes (Hunt and Hardy, 1991); analogous *in vivo* release of holotoxin is obviously essential for establishment of disease. In the context of the present studies, release of LT-B *in vivo* may be significant, depending on the mechanism(s) responsible for hyporesponsiveness seen in vector-primed mice. Potentially, the release of LT-B from the recombinant vector might circumvent epitopic suppression by reducing sequestration of antigen by vector-primed B cells (as discussed in section 1.4.3).

Several strategies were devised to assess the importance of the nature and localisation of the foreign antigen as a determinant of the significance of vector-priming. First, LT-B would be assessed as a foreign antigen in *S.* Stanley using the protocol of Attridge *et al.* (1997). Immune responses generated by r*S.* Stanley-LT-B in vector-primed mice would be compared with those seen after administration of r*S.* Stanley-K88. Second, and in line with this reasoning, an *aroA S.* Dublin strain which expresses K88 would be constructed, and evaluated using the experimental protocol of Bao and Clements (1991).

A recent report suggested a different approach to evaluating the significance of foreign antigen localisation within r*Salmonella*. Chen and Schifferli (2000) compared the immunogenicities of two r*Salmonella* constructs carrying genes encoding production and export of the major subunit of the *E. coli* 987P pilus. In one construct the (*E. coli*) gene encoding the outer membrane usher protein had been mutated, resulting in accumulation of subunits within the periplasm. Mice orally immunised with r*Salmonella* expressing normal 987P pili generated serum IgG and intestinal IgA anti-pilus responses ~ 10 fold ($P < 0.01$) greater than those fed the construct carrying the mutation in the usher protein.

The r*S.* Stanley-K88 construct used by Attridge *et al.* (1997) carries pEVX49 (Morona *et al.*, 1994) containing a 6.7 kb fragment derived from pFM205 (Mooi *et al.*, 1982). This fragment encodes a truncated operon of six genes (*faeC-faeH*) providing functional K88 pilus

formation. FaeG is the 27 kDa major structural subunit which functions to mediate attachment to the brush borders of porcine villous enterocytes. The *faeD* gene encodes a 81.7 kDa outer membrane assembly protein; *faeD* mutants do not produce surface pili, but accumulate subunits in the periplasm. Such localisation of FaeG would mirror the subcellular localisation of LT-B, and so FaeG may similarly be released from the cell *in vivo* upon contact with intestinal factors. Depending on the significance of antigen localisation, it might then be possible to elicit anti-K88 responses in vector-primed mice using a Δ*faeD* construct. This study is the third to be described in the present chapter.

The final study represents an initial examination of the potential significance of the plasmid construct responsible for foreign antigen expression within r*Salmonella*. This is another point of divergence between the protocols used by Bao and Clements (1991) and Attridge *et al.* (1997). The former used an Ap-based vector (pBR322) to express LT-B, whereas the latter used a balanced-lethal (*thyA*) expression system to produce K88. The latter approach was developed to increase plasmid stability *in vivo*, where antibiotic selection is impractical, and might result in a more sustained presentation of foreign antigen. In order to better compare experimental protocols, it was decided to construct a balanced-lethal vector which expresses LT-B.

5.2 Results

5.2.1 Construction of *S.* Stanley expressing LT-B.

Plasmid pJC217 contains an origin of replication derived from pBR322 (intermediate to high copy number) and expresses LT-B under the control of the *lacZ* gene promoter/operator (Clements and El-Morshidy, 1984). This plasmid was isolated from EL23 and introduced into *S.* Stanley via electroporation (forming CVSA24). Immunoblotting analysis suggested that CVSA24 and EL23 produced similar levels of LT-B following growth *in vitro* (not shown, but see Figure 5.1).

5.2.2 *In vitro* release of LT-B from r*Salmonella* vectors.

An experiment, adapted from a publication by Hunt and Hardy (1991), was performed to compare LT-B release from EL23 and CVSA24. Cultures were incubated (in duplicate) in the absence (controls) or presence of physiological concentrations of bile salts and conalbumin (section 2.16.3.5.1), in an attempt to gauge the effects of intestinal factors on the release of LT-B. Supernatants and sonicated cell pellets were prepared from samples taken at hourly intervals and LT-B production quantified by EIA (section 2.16.3.5.4).

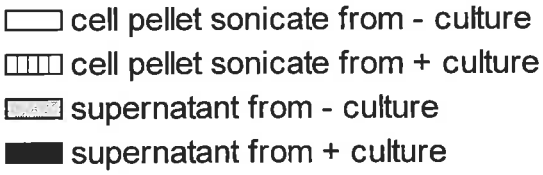
Figure 5.1 shows the distribution of LT-B in supernatant and pellet samples taken three hours after the addition of intestinal factors; similar patterns were observed at earlier time points. Bacteria cultured with bile salts and conalbumin secreted three-fold more LT-B ($P < 0.05$) into the external medium. The concentration of LT-B in the supernatant of such cultures was much too high to be explained on the basis of enhanced release from the periplasm, since levels of cell-associated LT-B were very low and unaffected by the presence of intestinal factors. (It is assumed that sonication had been effective in releasing LT-B from the cell pellet fraction.) This suggests that periplasmically retained LT-B is normally rapidly degraded, but that in the presence of bile salts and conalbumin LT-B secretion is accelerated. In the present context however, the key point is that the two r*Salmonella* constructs behaved similarly, and produced and secreted similar levels of LT-B in the presence or absence of intestinal factors.

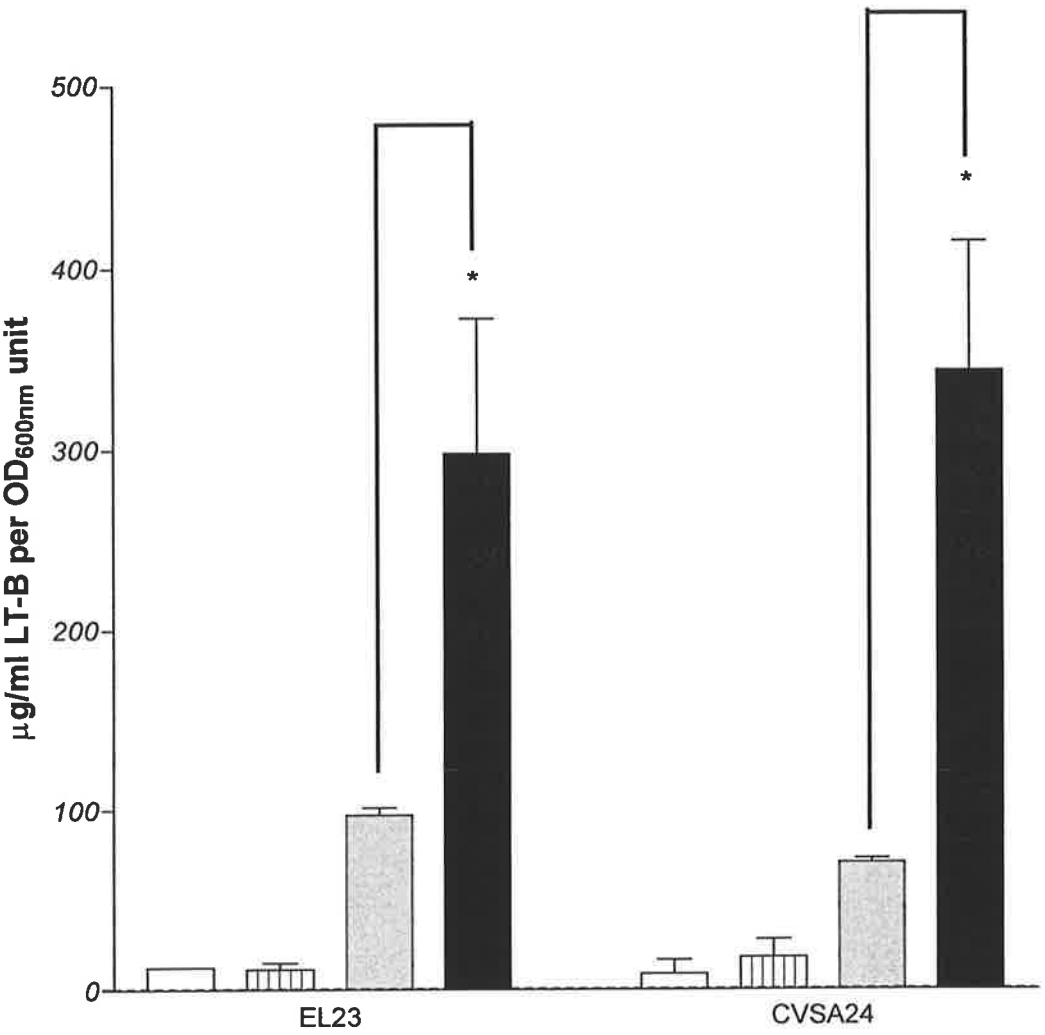
5.2.3 Immunogenicity of r*S.* Stanley-LT-B in vector-primed mice.

An experiment was designed to assess the immunogenicity of r*S.* Stanley-LT-B in mice previously primed with wt *S.* Stanley. Mice were primed on day - 70 with ~ 10^9 cfu of wt *S.* Stanley, while controls received $NaHCO_3$ only; ten weeks later both groups were fed ~ 10^9 cfu

Figure 5.1 Measurement of LT-B release from r*Salmonella*.

Supernatants and cell pellets isolated from CVSA24 and EL23 cultures grown for 3 hours in the absence (-) or presence (+) of intestinal factors were examined by EIA for the presence of LT-B. Histograms show the concentration of LT-B (μg/ml; M ± SD; $n = 2$), standardised by dividing by the culture OD_{600nm}. '*' represents a significant difference ($P < 0.05$) in supernatant LT-B concentrations following the addition of intestinal factors. The dashed line represents the sensitivity of the assay at a level of 0.6 μg/ml of purified LT-B.




500
400
300
200
100
0
μg/ml LT-B per OD600nm unit
*
*
EL23
CVSA24

r*S.* Stanley-LT-B (CVSA24). Serum and CFPS samples were prepared at various intervals for the determination of anti-LT-B and anti-LPS responses.

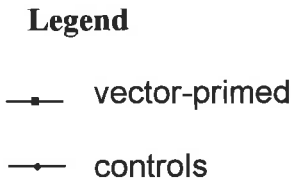
Control mice developed a strong primary serum anti-LT-B response following exposure to r*S.* Stanley-LT-B (Figure 5.2 A). In comparison, vector-primed mice developed weaker serum anti-LT-B responses, which were significantly different from those in the control group on days 29, 42 ('**', $P < 0.01$ at both time points) and 56 ('*', $P < 0.05$). The variability of the anti-LT-B serum responses in vector-primed mice is apparent from a scatter plot analysis (Figure 5.2, Panel II A). On days 29, 42, 56 and 70 four of the five vector-primed mice had the lowest serum anti-LT-B titres of the ten mice sampled. On days 56 and 70 however, the fifth mouse in this group showed the highest antibody titre, explaining the large standard deviations obtained. A later experiment which included these same groups (section 5.2.6.2) confirmed the significance of this hyporesponsiveness to LT-B in vector-primed mice.

The intestinal IgA anti-LT-B response seen in control mice was less pronounced than the serum response, peaking at ~ twenty-fold increase above background (Figure 5.2 Panel I C, and Panel II B). The response seen in vector-primed mice was significantly impaired on days 42, 56 and 70 (**, $P < 0.01$ at all three time points), further highlighting the significance of pre-existing immunity at the level of the GALT.

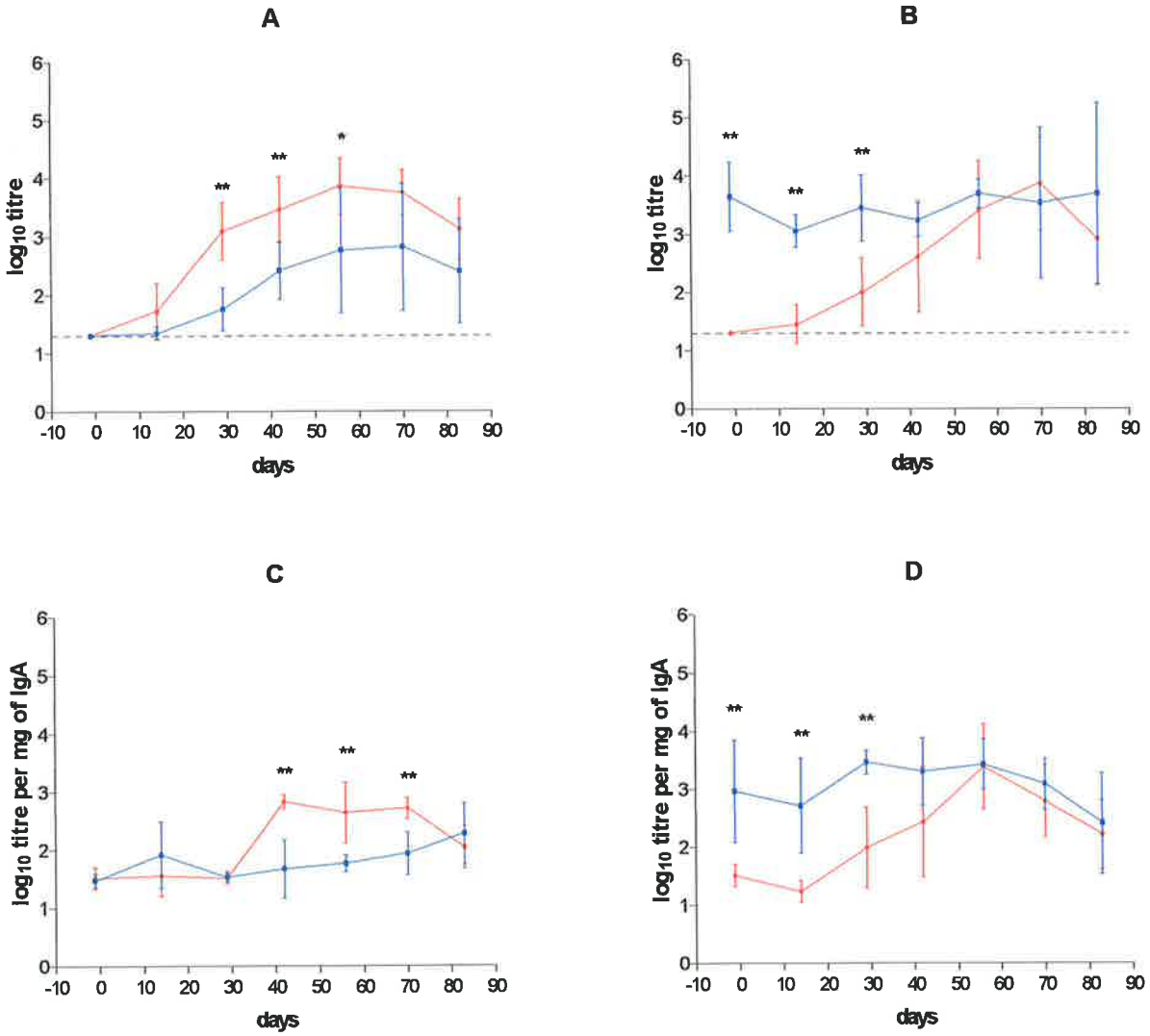
Serum and CFPS anti-LPS responses were comparable to those seen in earlier experiments and confirmed the efficacy of vector-priming (Figure 5.2 Panel I B and D). A study by Chen and Schifferli (2000) prompted an assessment of whether the delivery of LT-B would influence the IgG isotype balance of the serum anti-LPS response. Accordingly, selected sera from the above experiment were titrated in ELISAs using immunoconjugates specific for IgG_1 and IgG_{2a}. No significant difference in IgG isotype ratios was found between sera collected from mice immunised with *S.* Stanley (days -15 or -1) or r*S.* Stanley-LT-B (days 56 and 70) (not shown).

Figure 5.2 Immunogenicity of r*S.* Stanley-LT-B in vector-primed mice

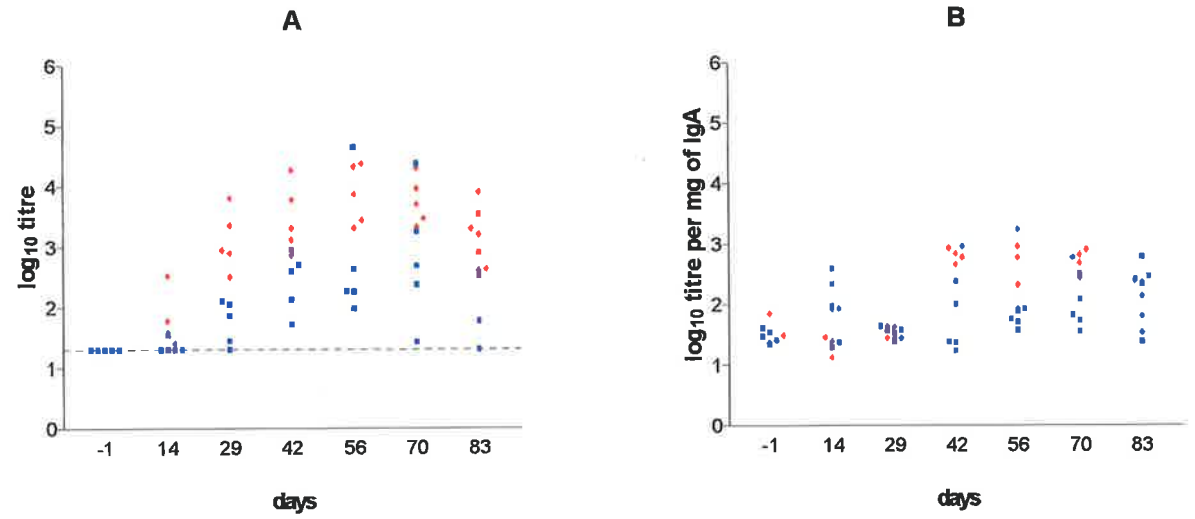
BALB/c mice were orally dosed with either $1.2x10^9$ cfu of wt *S.* Stanley or $NaHCO_3$ on day - 70; ten weeks later both groups received $1.3x10^9$ cfu r*S.* Stanley-LT-B. Panel I shows serum IgG (**A**, **B**) and intestinal IgA (**C**, **D**) responses to LT-B (**A**, **C**) and LPS (**B**, **D**), determined by ELISA on serum and CFPS samples (alternate subsets of five mice were sampled at successive timepoints). Serum responses are represented as $\log_{10}$ ELISA titres (GM $\pm$ SD; $n = 5$), while gut IgA responses are $\log_{10}$ ELISA titres per mg of IgA (GM $\pm$ SD; $n = 5$). Dashed lines on graphs represent the limit of detection of serum IgG ELISAs at a titre of 20. Points of significance are shown by '*' ($P < 0.05$) and '**' ($P < 0.01$). Panel II shows scatter plots of serum IgG (**A**) and intestinal IgA (**B**) responses to LT-B.



I


A
B
C
D
log10 titre
log10 titre per mg of IgA
days
**
*

II


A
B
log10 titre
log10 titre per mg of IgA
days

These data show that vector-priming according to the protocol used by Attridge *et al.* (1997) elicits a state of hyporesponsiveness which is demonstrable even with a potent mucosal immunogen. In contrast, Bao and Clements (1991) had reported that vector-primed mice were not compromised in their ability to respond to LTB delivered by an *aroA S.* Dublin vector. In view of these results, it was decided to repeat their experiment but extend the study to include delivery of K88 as an alternative foreign antigen.

5.2.4 Delivery of LT-B or K88 by *aroA S.* Dublin in vector-primed mice.

To complement the experiment described above, it was decided to adopt the vector strain and immunisation regime of Bao and Clements (1991), and compare the responses to LT-B and K88 in mice primed with *aroA S.* Dublin. It was first necessary to construct a derivative of this vector which expressed K88. Plasmid pFM205 (Mooi *et al.*, 1979) carries a truncated K88 pilus biosynthetic operon which is constitutively expressed from a vector-derived Tc promoter; from this was derived the *thyA*-based plasmid pEVX49 used in the experiments conducted by Attridge *et al.* (1997). Plasmid pFM205 was transferred via the r- m+ intermediate strain EX2000 into *aroA S.* Dublin (SL1438) by electroporation. Slide agglutination and immunoblot analysis confirmed the expression of K88 pili by this construct (not shown), which was termed CVSA26. Since pJC217 (which specifies LT-B production in EL23) and pFM205 are both pBR322 based, plasmid copy number was assumed to be equivalent; both plasmids also carry Ap^R, allowing selection for plasmid retention *in vitro* but not *in vivo*.

Two groups of BALB/c mice were orally primed with SL1438, delivering two doses of $\sim 10^{10}$ cfu at days - 70 and - 66 according to the protocol of Bao and Clements (1991), with additional mice set aside as controls. At days 0 and 4, paired groups of unimmunised and primed mice were boosted with $\sim 10^{10}$ cfu of either CVSA26 or EL23. Serum and CFPS samples were

collected at various time points and anti-LT-B, anti-K88 and anti-LPS responses monitored by ELISA.

Serum and mucosal anti-LT-B responses (Figure 5.3 A1 and A3) in primed mice were equivalent to (if not greater than) those in control mice, which confirmed the results of Bao and Clements (1991). In contrast, serum and mucosal anti-K88 responses (Figure 5.3 B1 and B3) were significantly reduced in vector-primed mice. The mean serum response was consistently ten-fold lower in primed mice (**, $P < 0.01$ on day26; *, $P < 0.05$ on days 48 and 70), while the intestinal response was equivalent to those in control mice if not slightly reduced (*, $P < 0.05$ on day 26). Serum and mucosal anti-LPS profiles (Figures 5.3 A2, A4, B2 and B4) confirmed effective vector-priming in mice initially dosed with SL1438.

5.2.4.1 Does expression of K88 or LT-B by *aroA S.* Dublin modify the IgG isotype profile of the serum anti-LPS response?

An assessment was made as to whether the expression of K88 or LT-B by SL1438 influenced the serum IgG subclass profile of the anti-LPS response. This was examined by titrating sera collected in the previous experiment in ELISAs detecting IgG_1 and IgG_{2a} antibodies specific for LPS. Samples prepared from vector-primed mice (day -1) were compared to samples prepared from control animals subsequently dosed with either EL23 or CVSA26 (days 48 and 70). The ratios of IgG_2:IgG_{1a} antibodies within each serum set were not significantly different (not shown), and were similar to those seen in Figure 4.7.

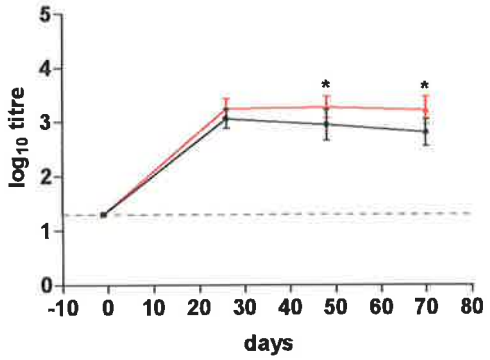
Figure 5.3 Immune responses to r*Salmonella* in mice primed with *aroA S.* Dublin.

Two groups of BALB/c mice were orally primed with SL1438 (*aroA S.* Dublin; $1.4x10^{10}$ cfu on day - 70 and $1.3x10^{10}$ cfu on day – 66), with other mice set aside as unimmunised controls. At days 0 and 4, paired groups of unimmunised and primed mice were boosted with either EL23 (*aroA S.* Dublin-LT-B; $9.5x10^{9}$ followed by $1.6x10^{10}$ cfu; Figures **A1-A4**) or CVSA26 (*aroA S.* Dublin-K88; $9.5x10^{9}$ followed by $2.0x10^{10}$ cfu; Figures **B1 - B4**). Graphs **A1** and **A3** show serum IgG and CFPS IgA responses to K88, whereas graphs **B1** and **B3** show serum IgG and CFPS IgA responses to LT-B. Graphs **A2/B2** and **A4/B4** show serum IgG and CFPS IgA anti-LPS responses respectively. Serum responses are represented as $\log_{10}$ ELISA titres (GM $\pm$ SD; n = 5), while gut IgA responses are $\log_{10}$ ELISA titres per mg of IgA (GM $\pm$ SD; n = 5). Dashed lines on graphs show the limit of detection of serum ELISAs at a titre of 20, and points of significance are indicated by '*' ($P < 0.05$) or ** ($P < 0.01$).

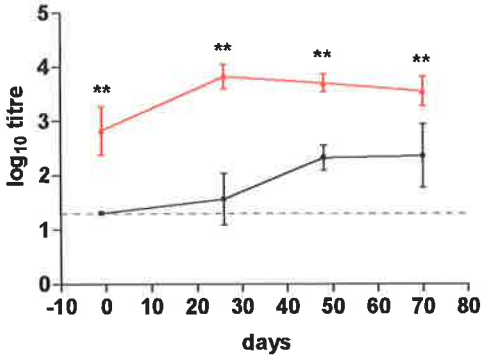
Legend

—■— control mice
—▲— vector-primed mice

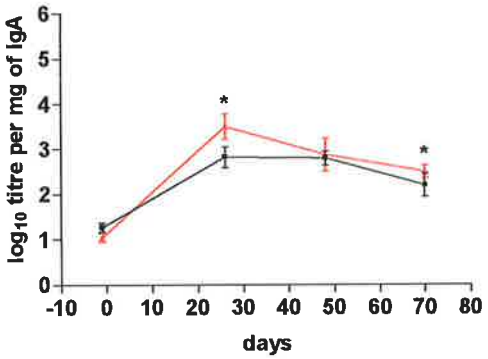
A1



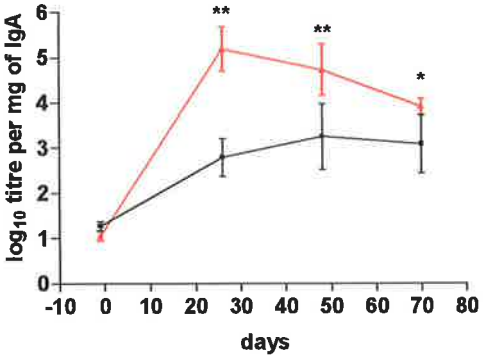
A2



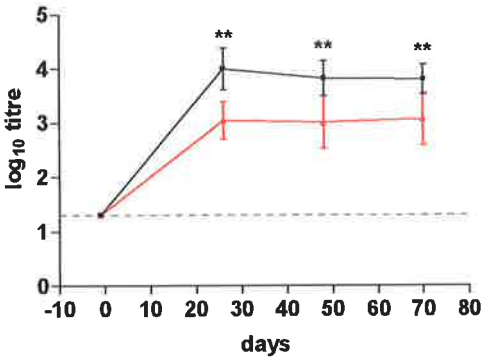
A3



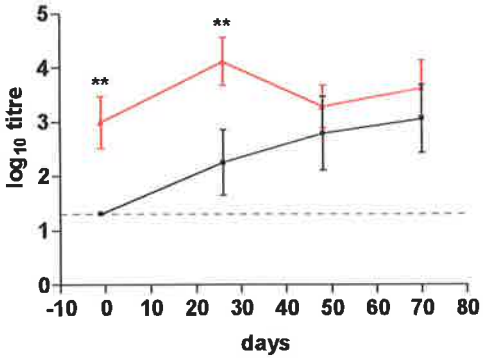
A4



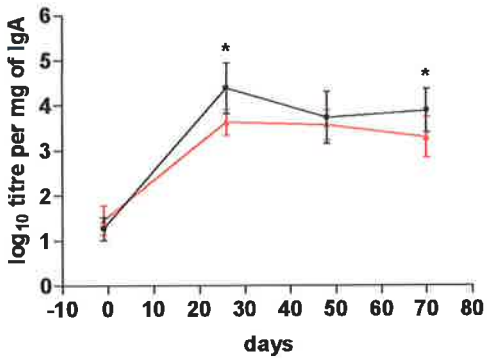
B1



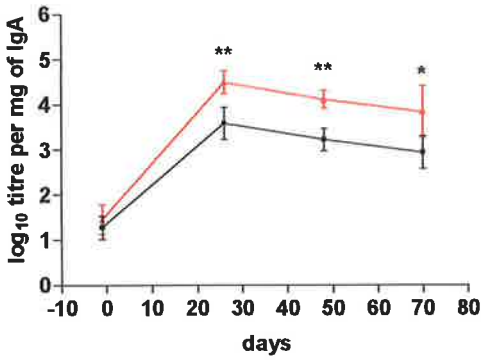
B2



B3



B4



5.2.5 Significance of antigen localisation within r*Salmonella*.

5.2.5.1 Construction and characterisation of a *faeD* deletion mutation.

As discussed in the introduction to this chapter, one way of evaluating the significance of antigen localisation within r*Salmonella* would be to introduce a *faeD* mutation into the K88 operon present on plasmid pEVX49. Pilus subunits would be expected to accumulate within the periplasm of this construct, and be released from the cell following contact with intestinal factors.

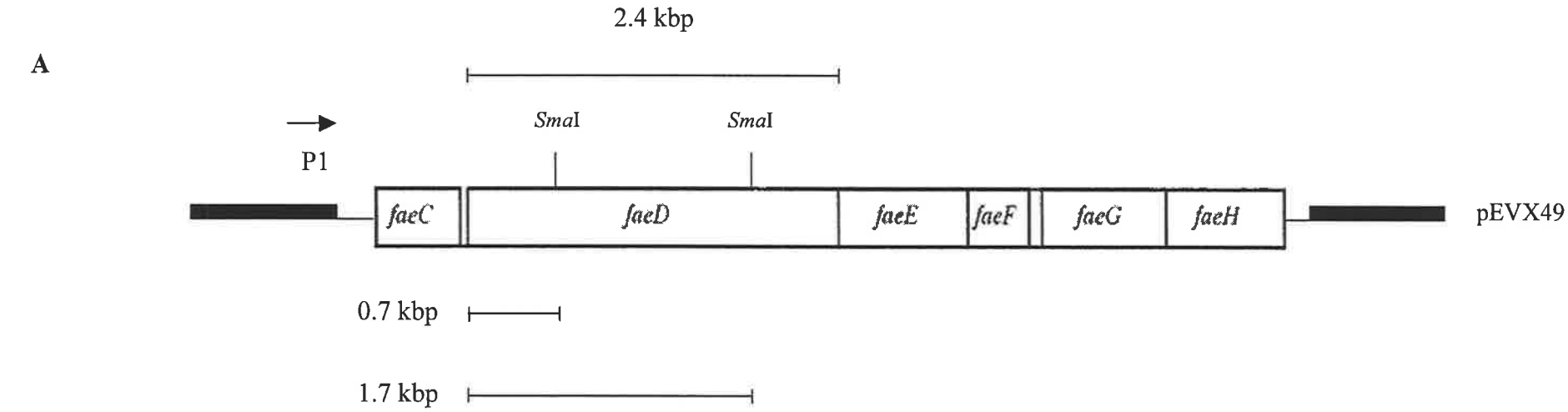
Examination of the sequence of the K88 operon (Mooi *et al.*, 1986) revealed two *Sma*I restriction sites within *faeD*, allowing the construction of an in-frame deletion mutation (termed Δ*faeD*; Figure 5.4 A). Following *Sma*I digestion, fragment purification and re-ligation, plasmid pCVSA10 was isolated as a Δ*faeD* derivative of pEVX49. This plasmid was transferred via the *thyA* r- m+ strain EX143 to *thyA S.* Stanley (creating CVSA31); the latter is the progenitor of the r*S.* Stanley-K88 construct used in earlier experiments. At the same time, a control strain (CVSA33) was constructed by electroporating pEVX46 (also passaged through EX143) into *thyA S.* Stanley. pEVX46 represents a control for pEVX49, as it is structurally identical but does not carry the genes associated with K88 pilus biosynthesis.

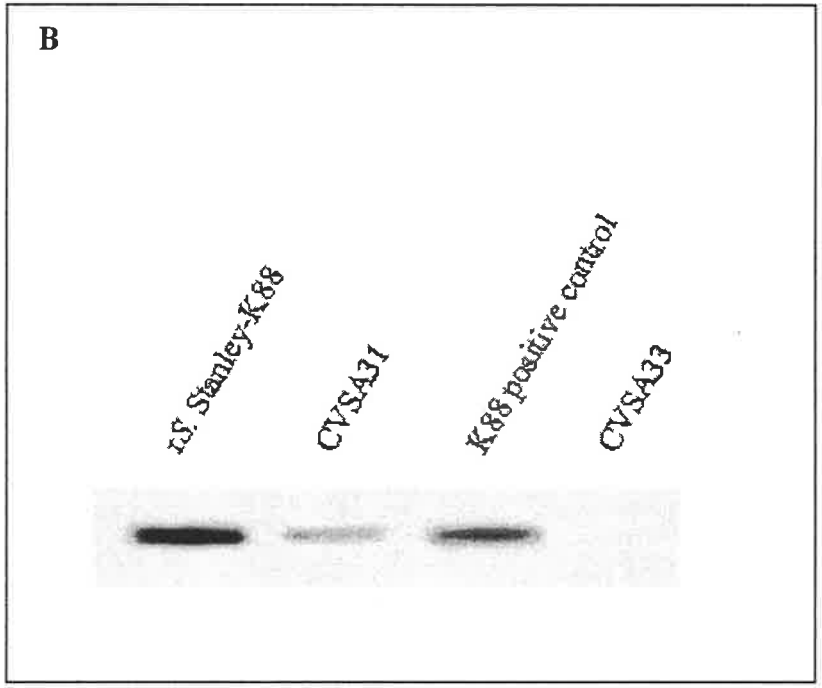
Immunoblot analysis confirmed that the K88 pilin subunit (FaeG) was expressed in CVSA31, but not in CVSA33 (Figure 5.4 B). Notably, FaeG expression in the former strain appeared to be significantly lower than that in r*S.* Stanley-K88. A similar observation was reported previously by Chen and Schifferli (2000) in the examination of an outer membrane usher mutant (*fasD*) in *E. coli* pilus 987P biosynthesis. They proposed that retention of the major pilin subunit (FasA) in the periplasm of the usher mutant led to its proteolytic degradation; this may explain the lower level of FaeG expression in CVSA31.

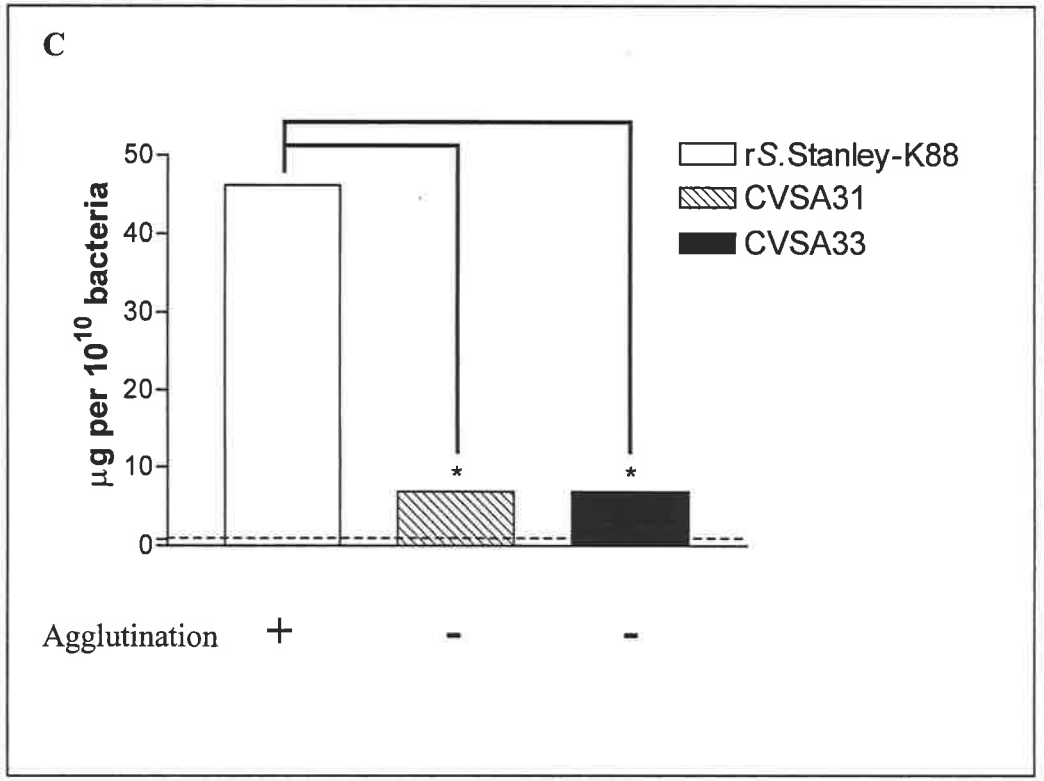
EIA analysis confirmed that surface pilus expression was dramatically inhibited (if not abolished) in CVSA31 (Figure 5.4 C). Although the polyclonal serum used in both Western and

Figure 5.4 Construction and characterisation of r*S.* Stanley harbouring a plasmid-borne K88 operon carrying a Δ*faeD* mutation.

Plasmid pEVX49 was digested with *Sma*I (which cuts at 0.7 and 1.7 kb in *faeD*) and religated creating pCVSA10 (**A**). The thick and thin black lines represent vector and cloned DNA respectively. P1 is a constitutive vector-derived promoter that controls K88 fimbrial expression. Western analysis (**B**) was performed on r*S.* Stanley-K88, CVSA31 (Δ*faeD*), and CVSA33 (negative control); ~ 10^9 bacteria were loaded, together with 10 μl (~ 0.6 μg) purified K88 as a positive control (**B**). Results of an EIA analysis are shown in panel **C.** Histograms show the the amount of K88 associated with 10^{10} bacteria (mean of duplicate titrations; $n = 2$). The dashed line represents the sensitivity of the assay at a level of 0.8 μg of K88 per 10^{10} bacteria. Finally, slide agglutination using an absorbed polyclonal rabbit anti-K88 serum (as used in the immunoblot and EIA) was used to confirm expression of surface pili (panel C; + = agglutination positive, - = agglutination negative).


A
2.4 kbp
SmaI
SmaI
P1
faeC
faeD
faeE
faeF
faeG
faeH
pEVX49
0.7 kbp
1.7 kbp


B
rS. Stanley-K88
CVSA31
K88 positive control
CVSA33


C
µg per 10^10 bacteria
50
40
30
20
10
0
rS.Stanley-K88
CVSA31
CVSA33
*
*
Agglutination
+
-
-

EIA analyses had been subjected to numerous rounds of absorption (section 2.16.3.5.3) to remove antibodies not specific for K88 pili, weak and equivalent inhibition was observed by both CVSA31 and CVSA33 in the EIA analysis. As the latter is a control lacking the genes necessary for K88 pilus production, this background was attributed to the presence of antibodies cross reactive to proteins associated with the vector strain and was therefore not considered to be significant. Only r*S.* Stanley-K88 could be agglutinated by the absorbed anti-K88 serum.

5.2.5.2 Immunogenicity of r*S.* Stanley carrying a K88 *ΔfaeD* operon.

Before conducting any vector-priming experiments it was necessary to determine whether CVSA31 could stimulate an anti-K88 response in mice when administered orally. Groups of BALB/c mice were immunised with ~ 10^9 cfu of either r*S.* Stanley-K88, CVSA31 or CVSA33; serum samples were prepared at various time points for the determination of anti-K88 and anti-LPS responses by ELISA.

Mice in all three groups developed similar primary serum anti-LPS responses (Figure 5.5 B) which were weak and delayed. However, only mice fed r*S.* Stanley-K88 developed a serum anti-K88 response (Figure 5.5 A). Proteolytic degradation of FaeG in CVSA31, and/or the periplasmic localisation of the protein, evidently resulted in a dramatic impact on its immunogenicity. This result precluded further experiments that were to have compared the immunogenicity of CVSA31 and r*S.* Stanley-K88 in vector-primed mice.

5.2.6 Comparison of antigen delivery systems in r*Salmonella.*

5.2.6.1 Construction of a *thyA*-based LT-B expression plasmid.

Plasmid pJC217 was used by Bao and Clements (1991) to specify LT-B production in strain EL23. It carries a gene encoding Ap resistance and is therefore maintained within a

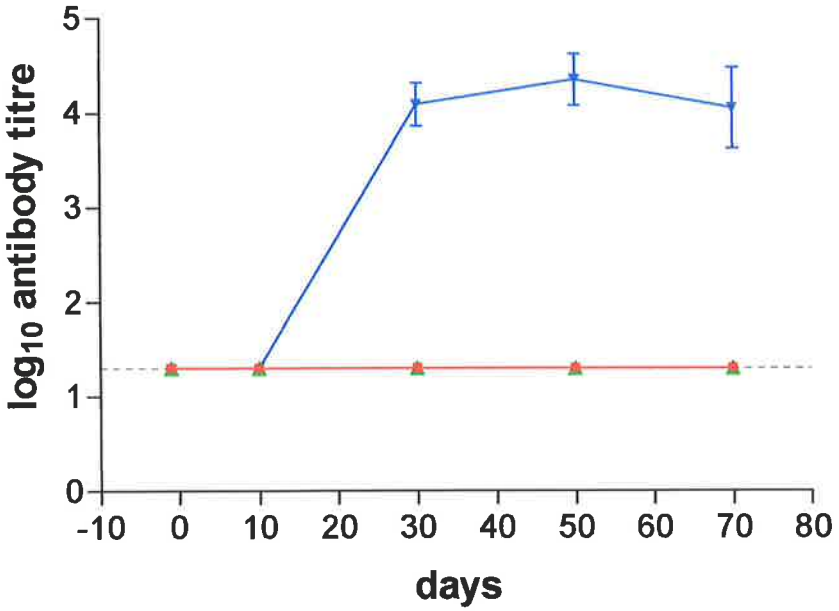
Figure 5.5 Immunogenicity of r*S.* Stanley expressing K88 Δ*faeD*.

Three groups of BALB/c mice were immunised with either CVSA31 ($9.2x10^8$ cfu), CVSA33 ($1.1x10^9$ cfu) or r*S.* Stanley-K88 ($1.6x10^9$ cfu). Serum was prepared at various timepoints, and IgG anti-K88 (**A**) and anti-LPS (**B**) responses determined by ELISA. Serum responses are represented as $\log_{10}$ ELISA titres (GM $\pm$ SD; n = 5), and the dashed line represents the sensitivity of the ELISAs at a titre of 20.

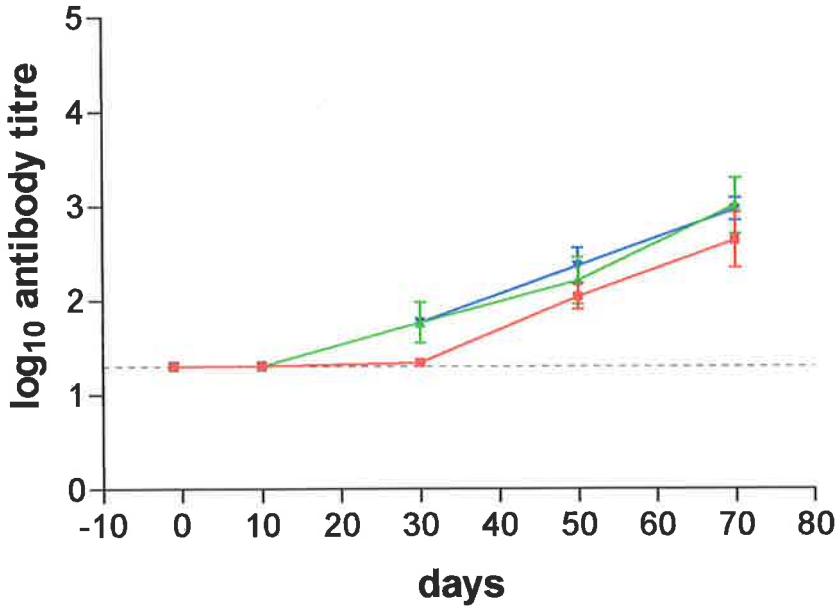
Legend

—▪— CVSA31
—▲— CVSA33
—▪— rS. Stanley-K88

A


log10 antibody titre
5
4
3
2
1
0
-10
0
10
20
30
40
50
60
70
80
days

B


log10 antibody titre
5
4
3
2
1
0
-10
0
10
20
30
40
50
60
70
80
days

bacterial population via the addition of antibiotic to the culture medium. The level of pJC217 retention *in vivo* in the absence of such selection pressure has not been reported. In the experiments conducted by Attridge *et al.* (1997), a balanced-lethal host-vector system was employed to ensure plasmid retention, and hence K88 fimbrial expression, *in vivo*. To assess the possible impact of plasmid stability in these studies, and the extent to which this might underlie the different observations recorded in the two laboratories, a balanced-lethal version of pJC217 was constructed.

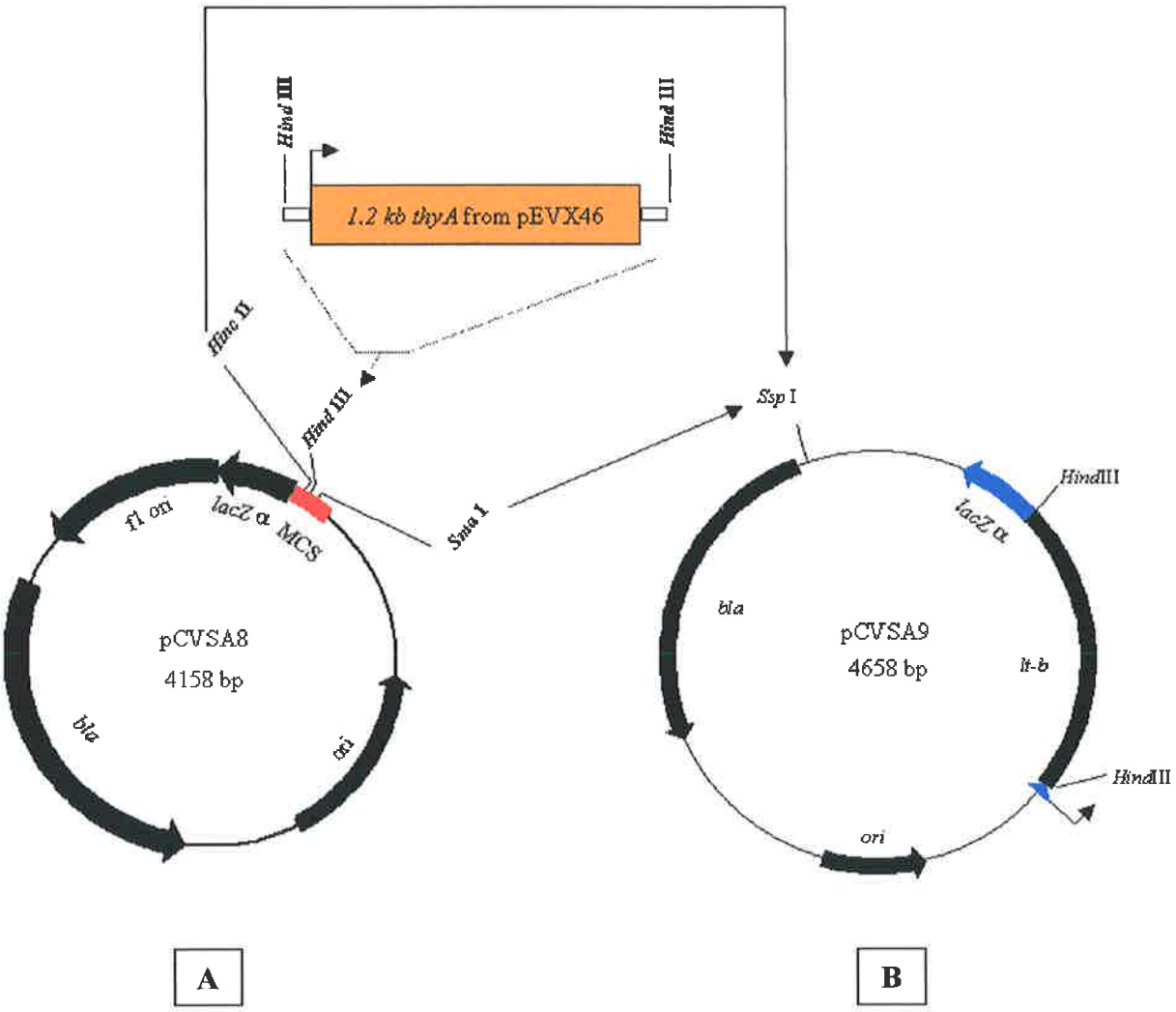
The 1.2kb *thyA* gene (with its own promoter) was gel purified from *Hind*III-digested pEVX46 (Morona *et al.*, 1991) and subsequently ligated into *Hind*III-digested pBluescript SK (Figure 5.6 A). The resulting plasmid (pCVSA8) was digested with *Hinc*II and *Sma*I liberating the 1.2kb *thyA* gene, which was then ligated into the *Ssp*I site of pJC217 forming pCVSA9 (Figure 5.6 B). *Ssp*I resides next to the Ap gene in pJC217, hence insertion of the *thyA* gene at this site allowed for the expression of both selectable markers. Plasmid pCVSA9 was subsequently electroporated via the r- m+ intermediate strain EX143 into *thyA S.* Stanley. The resulting strain, CVSA35, was tested to examine production and secretion of LT-B *in vitro*. Immunoblotting and EIA analysis showed that it was similar to both EL23 and CVSA24 in these respects (data not shown).

5.2.6.2 Impact of antigen delivery systems on immunogenicity of r*Salmonella* in vector-primed mice.

An experiment was designed to examine the importance of the antigen delivery system in the context of inducing immune responses to r*Salmonella* in vector-primed mice. Groups of BALB/c mice were orally dosed at day - 70 with ~ 10^9 cfu of wt *S.* Stanley, while other mice were set aside as controls. On day 0 all mice were boosted with ~ 10^9 cfu of r*S.* Stanley-LT-B; either CVSA24 (carrying pJC217) or CVSA35 (carrying pCVSA9). Serum and CFPS samples

Figure 5.6 Construction of the *thyA*-based LT-B expression plasmid pCVSA9.

The *thyA* gene from pEVX46 was isolated by *Hind*III digestion and ligated into *Hind*III-digested pBluescript SK(+) creating pCVSA8 (**A**). pCVSA8 was then digested with *Hinc*II and *Sma*I liberating a fragment carrying the *thyA* gene. This was gel purified and ligated into *Ssp*I-digested pJC217, creating pCVSA9 (**B**).


Hind III
Hind III
1.2 kb thyA from pEVX46
Hinc II
Hind III
Ssp I
Sma I
f1 ori
lacZ α
MCS
pCVSA8
4158 bp
bla
ori
lacZ α
HindIII
bla
pCVSA9
4658 bp
lt-b
HindIII
ori

A **B**

were subsequently prepared at various time points for the determination of anti-LPS and anti-LT-B antibody responses by ELISA, with results shown in Figure 5.7.

Control mice dosed with CVSA24 developed serum anti-LT-B responses which were significantly greater than those seen in vector-primed mice on days 26, 46 and 70 (Figure 5.7 A; $P < 0.01$ at each time point). These results were similar to those seen in an earlier experiment (Figure 5.2). However, this difference was less apparent in the mucosal anti-LT-B responses (Figure 5.7 C); unprimed control mice developed a less pronounced response than that recorded earlier (Figure 5.2 C), and this was only significantly greater than that seen in vector-primed mice at one time point (day 70, $P < 0.05$).

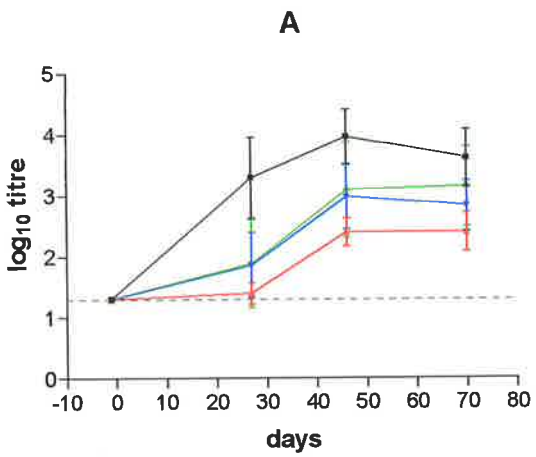
A different result was obtained when control and vector-primed mice were immunised with CVSA35, as both groups developed comparable serum and mucosal anti-LT-B responses (Figure 5.7 A and C). Serum anti-LT-B responses in control mice subsequently exposed to CVSA35 were significantly lower than those following exposure to CVSA24 (days 27, 46 and 70; $P < 0.01$ at all time points). However in vector-primed mice, the anti-LT-B responses following CVSA35 immunisation were significantly greater than those to CVSA24 (days 46 and 70; $P < 0.05$ for both time points). No such differences were apparent in the gut IgA responses. Thus the consequences of vector-priming, at least in terms of serum responses, are clearly influenced by the nature of the plasmid construct specifying synthesis of LT-B.

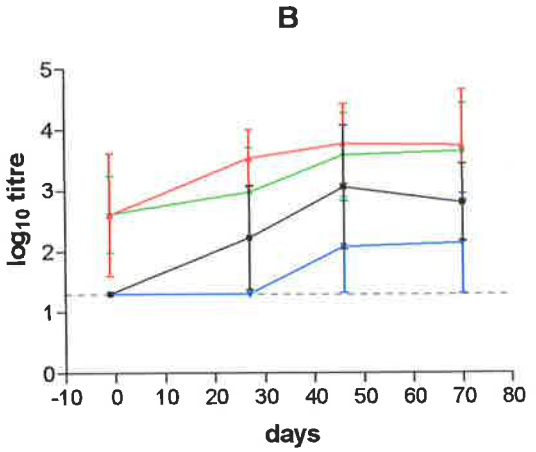
Serum and mucosal anti-LPS responses in control and primed mice subsequently dosed with CVSA24 (Figures 5.7 B and D) were comparable to those seen previously (Figure 5.2). Vector-primed mice immunised with CVSA35 generated secondary serum IgG and intestinal IgA anti-LPS responses which were similar to those of mice fed CVSA24 (Figure 5.7 B and D). However, primary serum IgG and intestinal IgA anti-LPS responses in control mice fed CVSA35 appeared weaker, though the differences observed were not significantly different were not significantly different.

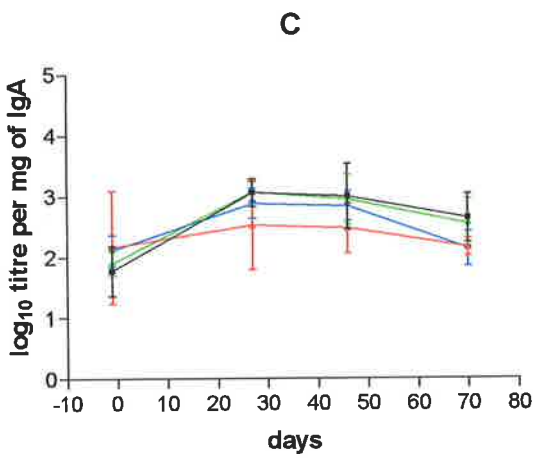
Figure 5.7 Importance of antigen delivery system for induction of immune responses to r*Salmonella* in vector-primed mice.

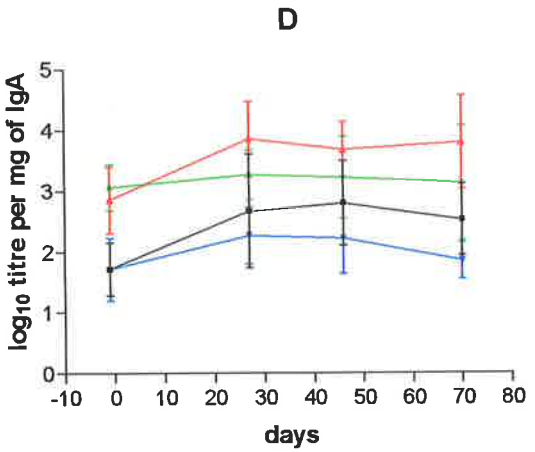
Four groups of BALB/c mice were immunised with either $NaHCO_3$ or wt *S.* Stanley at day - 70, and boosted with either CVSA24 (wt *S.* Stanley containing pJC217) or CVSA35 (*thyA S.* Stanley containing pCVSA9) ten weeks later, as shown in the table below. Serum (**A**, **B**) and CFPS (**C**, **D**) samples were prepared at various timepoints, and anti-LT-B (**A**, **C**) and anti-LPS (**B**, **D**) responses monitored by ELISA. Serum responses are represented as $\log_{10}$ ELISA titres (GM $\pm$ SD; $n = 5$), while gut IgA responses are $\log_{10}$ ELISA titres per mg of IgA (GM $\pm$ SD; $n = 5$). Dashed lines on graphs represent the limit of detection of serum IgG ELISAs at a titre of 20.

GROUP	VECTOR PRIMING (DAY - 70)		BOOST (DAY 0)	
	STRAIN	DOSE (CFU)	STRAIN	DOSE (CFU)
1	wt *S.* Stanley	1.2×10^9	CVSA24	9.7×10^8
2	-	-		
3	wt *S.* Stanley	1.2×10^9	CVSA35	1.1×10^9
4	-	-		


A
log10 titre
5
4
3
2
1
0
-10 0 10 20 30 40 50 60 70 80
days


B
log10 titre
5
4
3
2
1
0
-10 0 10 20 30 40 50 60 70 80
days


C
log10 titre per mg of IgA
5
4
3
2
1
0
-10 0 10 20 30 40 50 60 70 80
days


D
log10 titre per mg of IgA
5
4
3
2
1
0
-10 0 10 20 30 40 50 60 70 80
days

These results have two significant implications. Firstly, they show the significance of the antigen delivery construct with respect to the immunogenicity of r*Salmonella* in vector-primed mice. Secondly, in comparison to anti-K88 responses evoked by r*S.* Stanley-K88 in vector-primed mice, this experiment confirms the significance of the foreign antigen.

5.3 Discussion.

Work in this chapter has assessed the significance of antigen nature and location in the context of responses to heterologous antigen delivered by r*Salmonella* in vector-primed mice. Several different approaches were taken, the first involving the introduction of the plasmid pJC217 (which specifies LT-B expression in EL23; Bao and Clements, 1991) into *S.* Stanley. Comparison of the immunogenicity of r*S.* Stanley-LT-B (CVSA24) in control and vector-primed mice with that of the r*S.* Stanley-K88 construct used in previous experiments would give an initial indication of the significance of the foreign antigen.

Before commencing mouse immunisations, an *in vitro* experiment was conducted based on that performed by Hunt and Hardy (1991), to compare production and release of LT-B by EL23 and CVSA24. Incubating both r*Salmonella* constructs in the presence and absence of intestinal factors revealed similar patterns of LT-B distribution within sonicated cell pellets and culture supernatant extracts. Whereas Hunt and Hardy (1991) found no LT released from ETEC in the absence of intestinal factors, Figure 5.2 shows that $\geq$ 80% of the LT-B produced by EL23 and CVSA24 was released into the supernatant under the same conditions. There are at least two possible explanations for this difference, which are not mutually exclusive. Firstly, ETEC carry a single (chromosomal) copy of the LT operon, whereas r*Salmonella* express multiple plasmid-borne copies. Secondly, different culture conditions were used in our experiment. Hunt and Hardy (1991) used minimal-low phosphate medium, but EL23 does not grow when plated on supplemented minimal (section 4.2.4) or CBT medium (not shown), and so it was necessary to

use a rich culture medium such as LB. An increased gene copy number, in conjunction with a richer culture medium, may have promoted greater LT-B production by the r*Salmonella* constructs. The overexpression of LT-B to potentially toxic levels (see below) may have contributed to its leakage from the periplasm even in the absence of intestinal factors, and/or caused cell death and the release of LT-B into the supernatant.

Although EL23 and CVSA24 were found to release LT-B to similar extents *in vitro*, culturing in LB precluded an examination of any effect of the *aroA* mutation on LT-B production by the former construct. It is possible that the *aroA* mutation would impede the production of large amounts of foreign protein *in vivo* due to the limited supply of aromatic amino acid precursors. The unexpected GALT colonisation potential of *aroA* mutants would tend to argue against this however. One way to assess the effect of *aroA* on antigen presentation by r*Salmonella* would be to compare Peyer's patch colonisation profiles, plasmid retention rates, and anti-LT-B responses induced by oral administration of wt or *aroA S.* Stanley harbouring pJC217 (or pCVSA9). If GALT colonisation and plasmid retention rates were similar, any reduction in anti-LT-B responses following immunisation with *aroA* attenuated constructs would indirectly imply that *aroA* auxotrophy can impede foreign antigen biosynthesis *in vivo*.

The immunogenicity of CVSA24 (r*S.* Stanley-LT-B) was assessed in control and vector-primed mice (section 5.2.3). The latter developed only weak serum anti-LT-B responses upon exposure to CVSA24, and failed to develop intestinal IgA responses (Figure 5.2 and 5.7). This experiment provided additional evidence supporting the significance of vector-priming, even when the foreign antigen delivered by r*Salmonella* is a potent mucosal immunogen.

In contrast to these results, Bao and Clements (1991) reported that vector-primed mice were not compromised in their ability to respond to LT-B delivered by an *aroA S.* Dublin vector. It was therefore decided to repeat their experiment but extend the study to include delivery of K88 as an alternative foreign antigen. This experiment would then provide a complementary assessment of the significance of the nature and localisation of the foreign antigen within

r*Salmonella*. *aroA S.* Dublin-primed and control mice were subsequently exposed to either EL23 or CVSA26 (*aroA S.* Dublin-K88), following which serum IgG and intestinal IgA responses to LPS and foreign antigen were measured. Anti-LT-B responses confirmed the observations of Bao and Clements (1991), as no inhibition of antibody responses was observed in vector-primed mice. In contrast, primed mice subsequently exposed to CVSA26 displayed inhibited serum IgG (Figure 5.3 B1) and mucosal IgA (Figure 5.3 B3) responses to K88. These results confirm the importance of foreign antigen, particularly since the plasmid constructs used to express LT-B and K88 from *aroA S.* Dublin are so alike.

In comparison to earlier vector-priming experiments involving priming and boosting with *S.* Stanley-based constructs (Figure 4.5), these results also highlight the significance of the *Salmonella* vector. When r*S.* Stanley constructs were used, immune responses to K88 were completely abolished, and those to LT-B significantly reduced, as a consequence of vector-priming. With constructs based on the *aroA S.* Dublin vector however, vector-priming only partially reduced anti-K88 responses and had no effect on responsiveness to LT-B. Evidently, the impact of vector-priming on delivery of foreign antigen by r*Salmonella* is less significant with the latter vector strain.

Despite the reduced Peyer's patch colonisation potential of *aroA S.* Dublin in comparison to *aroA S.* Stanley (see Figure 4.4), the former construct was observed to induce strong serum and intestinal anti-LPS responses in naïve mice (see Figure 4.6). Additional results presented in this chapter show ~ ten-fold greater secondary intestinal anti-LPS responses in *aroA S.* Dublin-primed mice subsequently exposed to EL23 or CVSA26 (Figure 5.3 A4 and B4), in comparison to those of wt *S.* Stanley-primed mice subsequently exposed to r*S.* Stanley-K88 (Figure 4.5 B) or CVSA24 (Figure 5.2 D). This provides further evidence that *aroA S.* Dublin may induce greater proinflammatory responses in the GALT (as mentioned in 4.2.6.2). In the context of vector-priming, the induction of a greater proinflammatory response in the GALT following immunisation with r*aroA S.* Dublin may increase the exposure of the recombinant construct to

naïve cells of the immune system. Additional studies are required which, for example, compare immune responses in groups of wt *S.* Stanley-primed mice subsequently exposed to either r*S.* Stanley-K88, or a mixed inoculum of r*S.* Stanley-K88 and *aroA S.* Dublin; in the latter situation, any responsiveness to K88 would imply that the inflammatory potential of the vector may overcome the effects of vector-priming.

Pascual *et al.* (1999) showed that expression of CFA/1 by r*Salmonella* induced a biphasic T helper cell response in orally immunised mice in contrast to the consistent Th1 bias seem with the control vector. It was therefore of interest to determine whether foreign antigen expression would influence the IgG isotype balance of antibody responses to vector LPS. There was no significant difference in the ratio of IgG isotypes when serum anti-LPS responses in vector-primed mice were compared to those in control mice subsequently exposed to either CVSA24 (section 5.2.3), EL23 or CVSA26 (section 5.2.4.1); all mice developed an IgG_{2a} biased anti-LPS response equivalent to those shown previously in section 4.2.6.3. Thus the ratio of serum IgG isotypes did not appear to be affected by the expression of either foreign antigen. However, a more controlled experiment is required which would include the preparation of sera at earlier time points as well as the measurement of Th1 and Th2 specific cytokines.

Another method of assessing the significance of foreign antigen localisation within r*Salmonella* involved construction of a mutation in the outer membrane usher (Δ*faeD*) of the K88 biosynthetic operon. Mutation of *faeD* and the resultant localisation of the major pilin subunit (FaeG) in the periplasm would be analogous to the expression of LT-B in *aroA S.* Dublin. In contrast to surface K88 pili which would remain cell associated, the putative release of periplasmic FaeG upon contact with intestinal factors, may promote its interaction with naïve lymphoid cells. This might then circumvent the hyporesponsiveness normally associated with the delivery of K88 by r*S.* Stanley in vector-primed mice (see section 1.4.3).

Unfortunately, although the mutation of Δ*faeD* resulted in the localisation of FaeG in the periplasm as demonstrated by immunoblot and EIA (Figure 5.4), mice immunised with r*S.*

Stanley-Δ*faeD* K88 failed to develop serum and mucosal responses against FaeG. It was therefore not possible to evaluate the immunogenicity of this construct in vector-primed mice. Chen and Schifferli (2000) studied a similar usher mutation in the *E. coli* 987P pilus biosynthetic operon expressed in r*S.* Typhimurium, and suggested localisation of the major pilin subunit in the periplasm promoted its degradation by intracellular proteases. In that study however, the usher mutant retained the potential to elicit (reduced) immune responses to the pilin subunit.

The final experiment described in this chapter represented an initial examination of the significance of the plasmid expression systems which direct foreign antigen expression in r*Salmonella*. Bao and Clements (1991) utilised an Ap^R plasmid (pJC217) which specified LT-B expression from *aroA S.* Dublin. In contrast, Attridge *et al.* (1997) utilised a balanced-lethal plasmid (pEVX49) to specify K88 expression from *S.* Stanley. In order to compare plasmid expression systems used by the aforementioned groups, plasmid pJC217 was modified by insertion of the *E. coli thyA* gene (creating pCVSA9), such that it could be maintained by *thyA*-based balanced lethal retention (as with pEVX49) rather than antibiotic selection. Plasmid pCVSA9 was introduced via a r- m+ *Salmonella* intermediate into *thyA S.* Stanley, which was the progenitor of the r*S.* Stanley-K88 construct used in earlier experiments. Control and vector-primed mice subsequently exposed to r*S.* Stanley harbouring $thyA^+$-modified pJC217 (CVSA35) developed equivalent serum anti-LT-B responses (Figure 5.7). These contrasted the differential anti-LT-B responses in control and vector-primed mice subsequently exposed to r*S.* Stanley harbouring pJC217 (CVSA24). Clearly, this demonstrates the impact of different plasmid expression systems.

Control mice mounted stronger serum responses to CVSA24 (carrying pJC217) than CVSA35 (carrying pCVSA9) while vector-primed mice show the opposite pattern. Interestingly, intestinal IgA anti-LT-B responses in all mice sampled were not significantly different regardless of the r*Salmonella* construct used. It is therefore difficult to explain the differential patterns of serum IgG responses observed with the two constructs, and this experiment is currently being

repeated. A recent experiment examined the relative *in vivo* plasmid retention rates of the two constructs following oral immunisation of naïve mice. Plating on CBT medium ± Ap revealed that CVSA24 retained pJC217 for only one day *in vivo*, whereas CVSA35 retained pCVSA9 for at least five days. It is therefore possible that the equivalent serum responses to LT-B in control and vector-primed mice exposed to CVSA35 were due to improved plasmid retention. This would imply that the duration of exposure to foreign antigen presented by r*Salmonella* may be significant with respect to the effects of vector-priming. Surprisingly, the recovery of both recombinant constructs from the Peyer's patches on day five were well below the recovery of constructs harbouring control plasmids. This suggested that the expression of LT-B in both CVSA24 and CVSA35 was detrimental for cell survival *in vivo*. In order to further address the significance of the plasmid expression systems, additional experiments are required which compare immune responses induced by *S.* Stanley harbouring either *thyA*$^+$- (pEVX49) or Ap-based (pFM205) plasmids specifying K88 biosynthesis.

Cardenas and Clements (1993) showed that mice orally immunised with r*S.* Typhimurium mounted a better response to LT-B when foreign antigen expression was derived from a multicopy plasmid, as opposed to a single chromosomal gene. This occurred even though, in the former construct, the plasmid was lost from the vector strain by 24 hours post inoculation. A subsequent study by the same group showed that oral immunisation with viable and non-viable (UV-killed) EL23 induced equivalent serum and mucosal anti-LT-B responses in naïve mice (Cardenas *et al.*, 1994); these responses were consistent with those found in earlier studies (Bao and Clements, 1991; Clements *et al.*, 1986). It was therefore suggested that strain persistence and viability were not requirements for the effective delivery of foreign antigen by r*Salmonella*. Although preliminary, results from the final experiment in this chapter suggest that persistence of r*Salmonella* vectors, and the plasmid encoding foreign antigen harboured within, are important for the generation of immune responses to the recombinant construct in vector-primed mice.

CHAPTER SIX

Discussion

6.1 Overview.

The main focus of this thesis has been to examine the significance of pre-existing anti-vector immunity in the context of using attenuated *Salmonella* strains as carriers for the delivery of foreign antigens. Prior to this study, two groups (Attridge *et al.*, 1997; Bao and Clements, 1991) had addressed this potential problem, and obtained conflicting results. In comparing these two studies, factors which might critically influence the establishment of hyporesponsiveness consequent upon vector-priming were identified. These were: (i) the significance of the *Salmonella* vector strain - in particular, its propensity to colonise the GALT; (ii) the nature and subcellular localisation of the foreign antigen; (iii) the plasmid expression system used to specify foreign antigen production from r*Salmonella*.

Experiments were designed to assess the importance of each of these factors. Significantly, the results demonstrated the adverse effects of vector-priming at the level of both serum and intestinal antibody responses, with two different *Salmonella* vectors, with two different passenger antigens, and in *Nramp1*$^{+/+}$ and *Nramp1*$^{-/-}$ hosts. Collectively, these results provided important new information furthering our understanding of this phenomenon, while strengthening concerns that pre-existing anti-vector immunity represents a serious threat to the *Salmonella*-based vaccine strategy.

It was initially thought that the significance of pre-existing immunity was dependent upon the extent to which the vector strain could colonise the GALT. To address this possibility, an *aroA* mutation was introduced into *S.* Stanley, and the mutant compared to the parent strain in its ability to colonise the GALT. The *aroA* mutant was clearly attenuated as it could be orally administered to mice in doses which would normally be fatal if the wt parent was used. Unexpectedly however, the introduction of an *aroA* mutation had no effect on the colonisation potential of *S.* Stanley. In contrast to the complete hyporesponsiveness displayed in mice primed with wt *S.* Stanley, mice primed with the *aroA* mutant subsequently developed variable serum IgG responses to K88 following exposure to r*S.* Stanley-K88. A later experiment suggested (at least in terms of the serum anti-LPS response) that the introduction of the *aroA* mutation resulted in weaker anti-vector immunity, allowing *aroA S.* Stanley-primed mice to mount intermediate K88 responses upon boosting with r*S.* Stanley-K88. This would be consistent with previous findings (Attridge *et al.*, 1997).

In comparison to *aroA S.* Stanley, *aroA S.* Dublin colonised the murine GALT for a shorter period of time, yet induced stronger serum and intestinal anti-LPS responses in naïve mice. Since Bao and Clements (1991) had shown that mice primed with *aroA S.* Dublin were able to respond to LT-B subsequently presented by the same vector, this suggested that the level of anti-vector immunity induced by primary immunisation may not be a determinant of the significance of vector-priming. *S.* Stanley and *S.* Dublin may differ in their potential to induce proinflammatory responses in the GALT; induction of a greater such response by the latter strain may promote responses to foreign antigen in vector-primed mice.

The passenger antigens used by Attridge *et al.* (1997) and Bao and Clements (1991) differed both in their intrinsic immunogenicities and locations within the *Salmonella* vector. Experiments designed to assess the importance of the foreign antigen as a determinant of the significance of vector-priming compared the outcome of using both antigens with the vector strains and immunisation protocols followed in the two laboratories. In mice primed with *S.*

Stanley and boosted with r*S.* Stanley, immune responses to the passenger antigen were severely depressed (LT-B) or completely abolished (K88). When similar experiments were performed using the *aroA S.* Dublin vector, responses to K88 were significantly inhibited (K88) or unimpaired (LT-B). These findings highlight the relevance of the passenger antigen presented by r*Salmonella* to vector-primed mice. Futhermore, the comparative difficulty in demonstrating hyporesponsiveness to foreign antigen in studies with the *aroA S.* Dublin vector further emphasises the significance of the *Salmonella* vector.

Final experiments in this thesis examined the significance of the plasmid expression system used to specify foreign antigen production from r*Salmonella.* The plasmid used by Bao and Clements (1991) to specify LT-B production was modified such that it could be retained (*in vivo*) within r*Salmonella* without the requirement for antibiotic selection. The results showed that differences in plasmid expression systems used to specify foreign antigen production from r*Salmonella* can influence the significance of vector-priming, representing another important finding.

6.2 Other studies relevant to the issue of pre-existing anti-vector immunity.

Since the commencement of this thesis, others studies have been published which have also examined the effects of pre-existing anti-vector immunity on the immunogenicity of r*Salmonella* in mice. These are described below in the context of the findings of this thesis.

Whittle and Verma (1997) conducted a study in which mice were primed (i.p.) with *aroA S.* Dublin (SL5930) three to six months prior to the administration of the same strain expressing an epitope (24 amino acids) of the Murray Valley encephalitis virus envelope protein (MVE-E). SL5930 is flagellin-negative, but carries a complementing plasmid that allows the expression of functional flagella that provide motility (Newton *et al.*, 1989; Newton *et al.*, 1991). Modifications to this plasmid allow the insertion of epitope-specifying oligonucleotides (such as

that encoding MVE-E) into the gene encoding the major flagellar subunit (*fliC*) for the expression of chimeric flagellins. Vector-primed mice subsequently exposed to SL5930 expressing chimeric flagella developed enhanced serum antibody titres to the MVE-E epitope.

This finding is not necessarily inconsistent with present and past results from our laboratory, if one considers epitopic suppression as a mechanism for hyporesponsiveness consequent upon vector-priming. According to this theory, vector-priming with SL5930 would have expanded clones of B cells specific for both LPS and flagellar antigens. Upon subsequent exposure to the same strain expressing chimeric flagella, B cells of either specificity would act as APCs facilitating the processing and presentation of vector antigens, including flagellin and the associated MVE-E epitope. In other words, there would appear to be no likelihood of sequestration of recombinant bacteria by vector-primed B cells, to the detriment of cells potentially reactive to MVE-E; the passenger antigen was part of the flagella to which B cells had previously been primed. Moreover, an *aroA S.* Dublin vector was used in the experiment of Whittle and Verma (1997), and our data (section 5.2.4) suggest that it is more difficult to demonstrate adverse consequences of vector-priming with a strain of this serotype. It is possible that the potential to induce a strong proinflammatory response would have the effect of promoting the recognition of MVE-E by naïve lymphoid cells in vector-primed mice. Finally, given the fact that each flagellin subunit contained the MVE-E epitope, the resulting high level of epitope expression may have decreased the likelihood of suppression of a potential response to this antigen. This is suggested by the finding of Schutze *et al.* (1989) who showed that increasing the degree of substitution of hapten on a protein carrier blocked the expression of epitopic suppression in carrier-primed mice.

Roberts *et al.* (1999) investigated the significance of pre-existing anti-vector immunity in mice orally primed with homologous (*aroA aroD S.* Typhimurium) or heterologous (*aroA aroD S.* Dublin) *Salmonella* serotypes. Mice primed with the homologous vector strain exhibited diminished serum (IgG, IgA and IgM) and intestinal IgA antibody responses against FrgC

subsequently delivered by *aroA aroD S.* Typhimurium six weeks later. Interestingly, priming with a strain of heterologous serotype had a negative effect on serum, but not intestinal, anti-FrgC responses in vector-primed mice; the degree of inhibition of the former was not as severe as that observed in mice primed with the homologous strain.

A significant aspect of the study by Roberts *et al.* (1999) was a comparison of *in vivo* inducible promoters *htrA* (P_{htrA}) and *nirB* (P_{nirB}), which were used to control the expression of FrgC from *aroA aroD S.* Typhimurium. Unprimed mice developed greater anti-FrgC serum IgG titres when exposed to a construct expressing FrgC under the control of P_{htrA} as opposed to P_{nirB}, suggesting that P_{htrA} was more effective for the expression of FrgC *in vivo*. As a measure of vaccine efficacy, mice which had been vector-primed and then boosted with r*Salmonella* were challenged with 0.01 μg of tetanus toxin (50 LD_{50} doses) 53 days after the second immunisation. In line with higher antibody titres, boosting with the construct in which FrgC expression was under the control of P_{htrA} provided superior protection regardless of whether mice had been primed with a strain of homologous or heterologous serotype. Control (unprimed) mice immunised with either FrgC-expressing construct were completely protected.

The results published by Roberts *et al.* (1999) support the findings of Attridge *et al.* (1997) and this thesis. Pre-existing anti-vector immunity (to strains of homologous or heterologous serotype) was shown to diminish r*Salmonella* vaccine efficacy. The impact of vector-priming was shown to be dependent upon the efficiency of the promoter used to control foreign antigen expression. Roberts *et al.* (1999) discussed their findings in relation to the report of Bao and Clements (1991), and suggested that differences in the nature and subcellular localisation of the passenger antigens (FrgC and LT-B) were more likely causes of the conflicting findings made, rather than variation in immunisation protocols or *Salmonella* vectors.

Kohler *et al.* (2000b) assessed the effect of pre-existing anti-vector immunity on the immune responses induced in mice following exposure to *cya crp* attenuated *S.* Typhimurium (χ4072) expressing the *Porphyromonas gingivalis* haemagglutinin HagB. In comparison to

unprimed controls, vector-primed mice exhibited reduced serum IgG anti-HagB responses following χ4072-HagB administration at both seven and fourteen weeks post priming. Vector-priming also significantly reduced the salivary IgA anti-HagB responses in mice, but only in mice boosted seven weeks after priming. This is interesting considering Attridge *et al.* (1997) reported that the adverse effects of vector-priming were demonstrable following intervals of four, ten or 26 weeks between priming and boosting. However, a different *Salmonella* vector, foreign antigen, and dosing regime was used. Overall, the findings of Kohler *et al.* (2000b) are in agreement with those of Attridge *et al.* (1997) and this thesis, emphasising the significance of vector-priming.

6.3 Studies which have investigated recall responses to r*Salmonella*.

Two studies which have recently been published have investigated long term recall responses to r*Salmonella*. Although neither directly assessed the impact of vector-priming, their findings are relevant to the consideration of epitopic suppression as a mechanism responsible for hyporesponsiveness consequent upon vector-priming. Kohler *et al.* (2000a) orally immunised mice with χ4072 expressing HagB, and boosted with the same construct following the same dosing regime 52 weeks later. This interval between primary and secondary immunisation was chosen to represent long-term memory since it approximates half the lifespan of a BALB/c mouse. Antibody (serum, salivary and vaginal) responses to HagB were elevated as a result of boosting. These results are significant considering that, in a previous study, prior immunity to the χ4072 vector was shown to inhibit serum IgG responses to HagB when delivered by the same strain (Kohler *et al.*, 2000b; see above in section 6.2). A similar observation was published recently by Jespersgaard *et al.* (2001) in which the i.n immunisation of mice with r*Salmonella* primed the animals for secondary responses to the passenger antigen following boosting with the same construct. The authors of this latter study claimed that "pre-existing immunity to the

Salmonella vector did not inhibit recall responses", even though mice were primed and boosted with the same construct.

The data presented in these two studies are not inconsistent with those presented in this thesis. Primary immunisation with r*Salmonella* would result in the expansion of B cell clones specific for the foreign antigen, as well as antigens associated with the vector strain. This would prevent epitopic suppression of the immune response to the passenger antigen at the time of secondary immunisation. This interpretation is consistent with the analysis of the study by Whittle and Verma (1997) (see section 6.2), which is not surprising given the similar experimental protocols involved.

6.4 Current studies and future directions.

6.4.1 Consideration of animal models.

Ultimately, it will not be possible to determine the effect(s) of pre-existing anti-vector immunity in humans until similar vector-priming experiments are done in volunteers. In the meantime, continuing studies in animal models will hopefully provide sufficient pre-clinical data to optimise the efficacy of r*Salmonella*-based vaccines.

The adverse consequences of vector-priming are more clearly demonstrated using the naturally attenuated *S.* Stanley; perhaps this model represents the 'worst-case scenario'. If strategies to circumvent hyporesponsiveness consequent upon priming with this vector can be identified, one would have more confidence that these might be successful in other situations.

6.4.2 Is hyporesponsiveness consequent to vector-priming due to epitopic suppression?

Experiments in this thesis have shown that the outcome of vector-priming studies in mice is dependent on the characteristics of both the *Salmonella* vector and foreign antigen. It will be

important to extend the results of this study to the measurement of cellular immunity (T cell proliferation for example) and the secretion of cytokines. Measurement of T cell (particularly CTL) responses may be more relevant to the induction of protection against, for example, infection with viruses. Most importantly, it will be essential to ascertain the mechanism(s) underlying the hyporesponsiveness to foreign antigen which can occur following vector-priming. Such knowledge will be invaluable in the design of strategies to overcome the problem.

As discussed above, our data and those of others are consistent with the phenomenon of epitopic suppression, and further studies are required to confirm or refute this hypothesis. For example, if epitopic suppression rather than a primed GALT (see section 1.4.3) is responsible for the negative impact of vector-priming, it should be possible to introduce r*Salmonella* parenterally and still observe hyporesponsiveness. In addition, by analogy with the findings of Leclerc *et al.* (1990) and Schutze *et al.* (1989) (see section 1.4.3), it should be possible to circumvent the effects of vector-priming by first expanding B cell clones specific for foreign antigen. These experiments are currently underway.

6.4.3 Strategies which may circumvent the adverse effects of vector-priming.

If epitopic suppression is indeed the mechanism by which hyporesponsiveness to foreign antigen occurs following vector-priming, it may be possible to circumvent this phenomenon by using various strategies, applied alone or in combination. One strategy may be to secrete the foreign antigen from the r*Salmonella* vector. An attempt to assess the importance of foreign antigen localisation proved unsuccessful (see section 5.2.5). However, one could use the *E. coli* haemolysin export system to secrete a FaeG-HlyA hybrid protein from the cell, as demonstrated with the secretion of listeriolysin (Hess *et al.*, 1996). An alternative to this approach may be to use r*Salmonella* as a delivery vehicle for a eukaryotic expression plasmid containing *faeG*. Darji *et al.* (2000) showed that the oral delivery of a eukaryotic expression plasmid (carrying a

virulence genes derived from *Listeria monocytogenes*) by *aroA S.* Typhimurium was able to induce protection against challenge. Turnes *et al.* (1999) demonstrated anti-FaeG serum antibody responses following the intramuscular injection of mice and pigs with a eukaryotic expression vector specifying FaeG production. Therefore, it may be possible to use r*S.* Stanley as a delivery vehicle for a eukaryotic plasmid encoding FaeG. It will be interesting to determine the effects of vector-priming on plasmid transfer from r*Salmonella* to host cells, or whether the expression of epitopic suppression is affected by 'delivery' of foreign antigen by this method.

Another strategy to circumvent the effects of vector-priming may be to utilise *in vivo* regulated promoters. The potential of this approach, particularly in the context of vector-priming, has most clearly been demonstrated by Roberts *et al.* (1999) as described above. In the context of the results discussed in section 5.2.6, regulating LT-B expression by use of (for example) P_{htrA} may improve the GALT colonisation potential of the r*S.* Stanley construct by reducing apparent toxicity resulting from constitutive LT-B synthesis.

Attridge *et al.* (1997) examined the delivery of K88 to vector-primed mice using a different *Salmonella* vector of heterologous O-antigen serotype. Mice orally primed with wt *S.* Stanley, which were subsequently exposed to r*S.* Strasbourg-K88 via the same route, were able to develop serum anti-K88 responses, largely (but not completely) circumventing the hyporesponsiveness previously observed with vector-priming. Although it was not possible to measure secretory IgA responses in this laboratory at that time, these results suggested that the effects of vector-priming could be circumvented using a construct based on a *Salmonella* vector of heterologous O-antigen serotype. This experimental approach needs to be refined however, and ideally should utilise an isogenic O-antigen variant of *S.* Stanley as the vector for foreign antigen in *S.* Stanley-primed mice (see Attridge *et al.*, 1997). Whether developing vaccines for human or veterinary use, the availability of a suite of isogenic O-antigen variants would allow selection of the one most appropriate for a given target population. Hone *et al.* (1994) has already described the construction of isogenic O-antigen variants of *S.* Typhi.

Experiments conducted with *aroA S.* Dublin in this thesis suggest its ability to induce a strong proinflammatory response in the GALT may be a partial explanation for the failure of Bao and Clements (1991) to observe any hyporesponsiveness consequent upon vector-priming. It may be possible to otherwise modulate immune responses to r*Salmonella* in vector-primed individuals by the co-administration of various immunomodulatory agents. For example, the oral co-administration of a detoxified variant of *E. coli* LT toxin (LT_{R192G}) with *aroA S.* Typhimurium expressing the major subunit of CFA/1 increased murine serum and intestinal responses to the CFA/1 by three-fold (Guillobel *et al.*, 2000). Other studies have shown that *Salmonella* can be successfully engineered to express cytokines which are active *in vivo* (al-Ramadi *et al.*, 2001; Carrier *et al.*, 1992; Denich *et al.*, 1993; Dunstan *et al.*, 1996; Xu *et al.*, 1998). Furthermore, immunisation with such constructs can improve resistance to i.p. challenge with virulent *Salmonella* (al-Ramadi *et al.*, 2001), or footpad challenge with *Leishmania major* (Xu *et al.*, 1998). The choice of cytokine to express in r*Salmonella* will depend on the bias in immune response which is most appropriate to confer protection against the pathogen of interest.

Modulation of immune responses may also be achieved by using different *in vivo* promoters as recently demonstrated by Roberts *et al.* (1999) (see above in section 6.3) and Chen and Schifferli (2001). In the latter study, mice orally immunised with r*S.* Typhimurium expressing chimeric fimbriae developed differential serum IgG_1:IgG_{2a} ratios depending on the *in vivo* inducible promoter (either *nirB* or *pagC*) used to control fimbrial expression. As with cytokine expression, the choice of promoter used to express foreign antigen in r*Salmonella* will vary depending on the desired bias in immune response.

A final strategy by which pre-existing immunity may be circumvented could be via the administration of r*Salmonella* by a parenteral route. It remains to be determined whether the route of administration of r*Salmonella* would influence the subsequent immune responses generated against the passenger antigen in vector-primed individuals. Experiments are required

in which mice primed by the oral route are subsequently exposed to r*Salmonella* by the i.n. route, for example.

6.5 Concluding remarks.

This study has sought to evaluate the potential significance of the findings published by Attridge *et al.* (1997). In order to do this, a comparison was made between protocols used by the aforementioned study and those followed by Bao and Clements (1991). Hyporesponsiveness consequent upon vector-priming has be shown to extend to intestinal responses, and is demonstrable in mice regardless of *Nramp1* genotype. The impact of vector-priming appears to be dependent on the characteristics of both the *Salmonella* vector and the foreign antigen which it delivers, as demonstrated in this thesis using two different *Salmonella* vectors and two different passenger antigens. Collectively, these findings strengthen concerns that prior exposure to *Salmonella* might seriously compromise the immunogenicity of *Salmonella*-based multivalent vaccines and therefore threaten mass immunisation programmes in developing countries.

APPENDIX A

Figure A.1 Consensus sequence of the *S.* Stanley *aroA* gene.

PCR products from three separate reactions were isolated and ligated into pGEM-T Easy® producing plasmids pCVSA1 to pCVSA3. M13 forward and reverse primers were used in big-dye terminator sequencing reactions (see section 2.10) to generate the consensus sequence of the *S.* Stanley *aroA* gene.

```
   1  ATG GAA TCC CTG ACG TTA CAA CCC ATC GCG CGG GTC GAT GGC GCC ATT   48
   1    M   E   S   L   T   L   Q   P   I   A   R   V   D   G   A   I   16

  49  AAT TTA CCT GGC TCC AAA AGT GTT TCA AAC CGT GCT TTG CTC CTG GCG   96
  17    N   L   P   G   S   K   S   V   S   N   R   A   L   L   L   A   32

  97  GCT TTA GCT TGT GGT AAA ACC GTT CTG ACG AAT CTG CTG GAT AGC GAT  144
  33    A   L   A   C   G   K   T   V   L   T   N   L   L   D   S   D   48

 145  GAC GTC CGC CAT ATG CTC AAT GCC CTG AGC GCG TTG GGG ATC AAT TAC  192
  49    D   V   R   H   M   L   N   A   L   S   A   L   G   I   N   Y   64

 193  ACC CTT TCT GCC GAT CGC ACC CGC TGT GAC ATT ACG GGT AAT GGC GGC  240
  65    T   L   S   A   D   R   T   R   C   D   I   T   G   N   G   G   80

 241  CCA TTA CGC GCG TCA GGC GCT CTG GAA CTG TTT CTC GGT AAT GCC GGA  288
  81    P   L   R   A   S   G   A   L   E   L   F   L   G   N   A   G   96

 289  ACC GCG ATG CGT CCG TTA GCG GCA GCG CTA TGT CTG GGG CAA AAT GAG  336
  97    T   A   M   R   P   L   A   A   A   L   C   L   G   Q   N   E  112

 337  ATA GTG TTA ATC GGC GAA CCG CGT ATG AAA GAG CGT CCG ATA GGC CAT  384
 113    I   V   L   I   G   E   P   R   M   K   E   R   P   I   G   H  128

 385  CTG GTC GAT TCG CTG CGT CAG GGT GGG GCG AAT ATT GAT TAC CTG GAG  432
 129    L   V   D   S   L   R   Q   G   G   A   N   I   D   Y   L   E  144

 433  CAG GAA AAC TAT CCG CCC CTG CGT CTG CGC GGC GGT TTT ATC GGC GGC  480
 145    Q   E   N   Y   P   P   L   R   L   R   G   G   F   I   G   G  160

 481  GAC ATT GAG GTT GAT GGT AGC GTT TCC AGC CAG TTC CTG ACC GCT CTG  528
 161    D   I   E   V   D   G   S   V   S   S   Q   F   L   T   A   L  176

 529  CTG ATG ACG GCG CCG CTG GCG CCT GAA GAC ACA ATT ATT CGC GTT AAA  576
 177    L   M   T   A   P   L   A   P   E   D   T   I   I   R   V   K  192

 577  GGC GAA CTG GTA TCA AAA CCT TAC ATC GAT ATC ACG CTA AAT TTA ATG  624
 193    G   E   L   V   S   K   P   Y   I   D   I   T   L   N   L   M  208

 625  AAA ACC TTT GGC GTG GAG ATA GCG AAC CAC CAC TAC CAA CAA TTT GTC  672
 209    K   T   F   G   V   E   I   A   N   H   H   Y   Q   Q   F   V  224

 673  GTG AAG GGC GGT CAA CAG TAT CAC TCT CCG GGT CGC TAT CTG GTC GAG  720
 225    V   K   G   G   Q   Q   Y   H   S   P   G   R   Y   L   V   E  240

 721  GGC GAT GCC TCG TCA GCG TCC TAT TTT CTC GCC GCC GGG GCG ATA AAA  768
 241    G   D   A   S   S   A   S   Y   F   L   A   A   G   A   I   K  256

 769  GGC GGC ACG GTA AAA GTG ACC GGA ATT GGC CGC AAA AGT ATG CAG GGC  816
 257    G   G   T   V   K   V   T   G   I   G   R   K   S   M   Q   G  272

 817  GAT ATT CGT TTT GCC GAT GTG CTG GAG AAA ATG GGC GCG ACC ATT ACC  864
 273    D   I   R   F   A   D   V   L   E   K   M   G   A   T   I   T  288

 865  TGG GGC GAT GAT TTT ATT GCC TGC ACG CGC GGT GAA TTG CAC GCC ATA  912
 289    W   G   D   D   F   I   A   C   T   R   G   E   L   H   A   I  304

 913  GAT ATG GAT ATG AAC CAT ATT CCG GAT GCG GCG ATG ACG ATT GCC ACC  960
 305    D   M   D   M   N   H   I   P   D   A   A   M   T   I   A   T  320

 961  ACG GCG CTG TTT GCG AAA GGA ACC ACG ACG TTG CGC AAT ATT TAT AAC 1008
 321    T   A   L   F   A   K   G   T   T   T   L   R   N   I   Y   N  336

1009  TGG CGA GTG AAA GAA ACC GAT CGC CTG TTC GCG ATG GCG ACC GAG CTA 1056
 337    W   R   V   K   E   T   D   R   L   F   A   M   A   T   E   L  352
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1057  CGT AAA GTG GGC GCT GAA GTC GAA GAA GGG CAC GAC TAT ATT CGT ATC 1104
 353   R   K   V   G   A   E   V   E   E   G   H   D   Y   I   R   I  368

1105  ACG CCG CCG GCG AAG CTC CAA CAC GCG GAT ATT GGC ACG TAC AAC GAC 1152
 369   T   P   P   A   K   L   Q   H   A   D   I   G   T   Y   N   D  384

1153  CAC CGT ATG GCG ATG TGC TTC TCA CTG GTC GCA CTG TCC GAT ACG CCA 1200
 385   H   R   M   A   M   C   F   S   L   V   A   L   S   D   T   P  400

1201  GTT ACG ATC CTG GAC CCT AAA TGT ACC GCA AAA ACG TTC CCT GAT TAT 1248
 401   V   T   I   L   D   P   K   C   T   A   K   T   F   P   D   Y  416

1249  TTA GAA CAA CTG GCG CGA ATG AGT ACG CCT GCC TAA                 1291
 417   L   E   Q   L   A   R   M   S   T   P   A   *                 430
```

Figure A.2 Clustal W alignment of predicted translation products of *aroA* genes from different *Salmonella* serovars.

Figure represents the alignment of the translated *aroA* gene sequences of *S.* Stanley ($AroA_{SS}$), *S.* Gallinarium ($AroA_{SG}$), *S.* Typhi ($AroA_{ST}$) and *S.* Typhimurium ($AroA_{SM}$) determined using the default settings of the program CLUSTAL W (Higgins and Sharp, 1988). '*' represents identical residues, and '.' represents similar residues.

$AroA_{ss}$	MESLTLQPIARVDGAINLPGSKSVSNRALLLAALACGKTVLTNLLDSDDVRHMLNALSAL
$AroA_{SG}$	MESLTLQPIARVDGAINLPGSKSVSNRALLLAALACGKTVLTNLLDSDDVRHMLNALSAL
$AroA_{ST}$	MESLTLQPIARVDGAINLPGSKSVSNRALLLAALACGKTVLTNLLDSDDVRHMLNALSAL
$AroA_{SM}$	MESLTLQPIARVDGAINLPGSKSVSNRALLLAALPCGKTALTNLLDSDDVRHMLNALSAL
	********** **** **********
$AroA_{ss}$	GINYTLSADRTRCDITGNGGPLRASGALELFLGNAGTAMRPLAAALCLGQNEIVLIGEPR
$AroA_{SG}$	GINYTLSADRTRCDITGNGGPLRAPGALELFLGNAGTAMRPLAAALCLGQNEIVLTGEPR
$AroA_{ST}$	GINYTLSADRTRCDITGNGGPLRASGTLELFLGNAGTAMRPLAAALCLGQNEIVLTGEPR
$AroA_{SM}$	GINYTLSADRTRCDITGNGGALRAPGALELFLGNAGTAMRPLAAALCLGQNEIVLTGEPR
	********** *** *.********** ****
$AroA_{ss}$	MKERPIGHLVDSLRQGGANIDYLEQENYPPLRLRGGFIGGDIEVDGSVSSQFLTALLMTA
$AroA_{SG}$	MKERPIGHLVDSLRQGGANIDYLEQENYPPLRLRGGFIGGDIEVDGSVSSQFLTALLMTA
$AroA_{ST}$	MKERPIGHLVDSLRQGGANIDYLEQENYPPLRLRGGFIGGDIEVDGSVSSQFLTALLMTA
$AroA_{SM}$	MKERPIGHLVDSLRQGGANIDYLEQENYPPLRLRGGFTGGDIEVDGSVSSQFLTALLMTA
	********** **********
$AroA_{ss}$	PLAPEDTIIRVKGELVSKPYIDITLNLMKTFGVEIANHHYQQFVVKGGQQYHSPGRYLVE
$AroA_{SG}$	PLAPKDTIIRVKGELVSKPYIDITLNLMKTFGVEIANHHYQQFVVKGGQQYHSPGRYLVE
$AroA_{ST}$	PLAPEDTIIRVKGELVSKPYIDITLNLMKTFGVEIANHHYQQFVVKGGQQYHSPGRYLVE
$AroA_{SM}$	PLAPKDTIIRVKGELVSKPYIDITLNLMKTFGVEIANHHYQQFVVKGGQQYHSPGRYLVE
	**** **********
$AroA_{ss}$	GDASSASYFLAAGAIKGGTVKVTGIGRKSMQGDIRFADVLEKMGATITWGDDFIACTRGE
$AroA_{SG}$	GDASSASYFLAAGAIKGGTVKVTGIGRKSMQGDIRFADVLEKMGATITWGDDFIACTRGE
$AroA_{ST}$	GDASSASYFLAAGGIKGGTVKVTGIGGKSMQGDIRFADVLHKMGATITWGDDFIACTRGE

$AroA_{SM}$ GDASSASYFLAAGAIKGGTVKVTGIGRKSMQGDIRFADVLEKMGATITWGDDFIACTRGE

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

$AroA_{ss}$ LHAIDMDMNHIPDAAMTIATTALFAKGTTTLRNIYNWRVKETDRLFAMATELRKVGAEVE

$AroA_{SG}$ LHAIDMDMNHIPDAAMTIATTALFAKGTTTLRNIYNWRVKETDRLFAMATELRKVGAEVE

$AroA_{ST}$ LHAIDMDMNHIPDAAMTIATTALFAKGTTTLRNIYNWRVKETDRLFAMATELRKVGAEVE

$AroA_{SM}$ LHAIDMDMNHIPDAAMTIATTALFAKGTTTLRNIYNWRVKETDRLFAMATELRKVGAEVE

****

$AroA_{ss}$ EGHDYIRITPPAKLQHADIGTYNDHRMAMCFSLVALSDTPVTILDPKCTAKTFPDYLEQL

$AroA_{SG}$ EGHDYIRITPPAKLQHADIGTYNDHRMAMCFSLVALSDTPVTILDPKCTAKTFPDYFEQL

$AroA_{ST}$ EGHDYIRITPPAKLQHADIGTYNDHRMAMCFSLVALSDTPVTILDPKCTAKTFPDYFEQL

$AroA_{SM}$ EGHDYIRITPPAKLQHADIGTYNDHRMAMCFSLVALSDTPVTILDPKCTAKTFPDYFEQL

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$AroA_{ss}$ ARMSTPA

$AroA_{SG}$ ARMSTPA

$AroA_{ST}$ ARMSTPA

$AroA_{SM}$ ARMSTPA

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