Characterisation of a secreted immunogenic protein, phase-1 flagellin (FliC) of *Salmonella enterica* subspecies *enterica* Brandenburg

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Abstract

Cell-envelope associated and secreted proteins of *Salmonella* are integral for host-pathogen interactions, and for the induction of protective immune responses. An array of exported proteins of *S.* Brandenburg was identified through constructing an expression library using alkaline phosphatase gene technology. A partial digest of *S.* Brandenburg strain S59 was cloned into the vector pJEM11, and expressed in *E. coli.* The DNA inserts from randomly selected alkaline phosphatase positive clones were sequenced, and the sequences were analysed using public databases to find the ones that may play a role in host immune cell activation. The phase-1 flagellin (*fliC*) gene identified from an alkaline phosphatase positive phenotype was chosen for further studies. The complete nucleic acid sequence of the *fliC* gene was obtained by PCR amplification. The complete ORF, part of the variable region (V456) and region IV (V4) of the *fliC* gene were cloned into the pET14b vector for the expression of N-terminal histidine-tagged fusion proteins. The proteins were purified through metal affinity chromatography, and were evaluated for their humoral immunogenic properties by Western blotting with sera collected from 81 sheep naturally infected with *S.* Brandenburg. All 81 naturally infected sheep had IgG antibodies against recombinant FliC, V456, and V4 proteins. Furthermore, Western blotting of sera from 6 Salvexin^{TM}+B-vaccinated sheep (Trial 2004) had IgG antibodies against the 3 recombinant proteins. Whole blood cells of vaccinated sheep did not show interferon-gamma production upon stimulation with recombinant FliC and V456 proteins. Western blotting of sera from sheep vaccinated with Salvexin^{TM} and Salvexin^{TM}+B (Trial 1999), and those from rabbits vaccinated with *S.* Brandenburg, *S.* Hindmarsh and *S.* Typhimurium suggested that recombinant V4 contains epitopes specific for *S.* Brandenburg. Therefore, V4 was used to develop a novel indirect enzyme-linked immunosorbent assay (ELISA) for the detection of serum IgG antibodies in *S.* Brandenburg infected sheep. The ELISA showed a specificity of 100%, and a sensitivity of 93.8%. Furthermore, a new PCR assay was developed targeting *rfbJ*(B) gene in a single reaction, and genes *invA*, *fliC* and *fljB* in a multiplex reaction for the identification of *S.* Brandenburg from pure cultures. The sensitivity and specificity of the PCR assay was calculated to be 100%.

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g	acceleration due to gravity
PhoA	alkaline phosphatase
AP	alkaline phosphatase
APS	ammonium persulfate
AFLP	amplified fragment length polymorphism
APC	antigen presenting cell
AP-PCR	arbitrarily primed–PCR
bp	base pair(s)
BLAST	basic local alignment search tool
BSA	bovine serum albumin
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BCIP/NBT	5-bromo-4-chloro-3-Indolyl phosphate/nitroblue tetrazolium
BPW	buffered peptone water
CIAP	calf intestinal alkaline phosphatase
CO_2	carbon dioxide
Cat. No.	Catalogue number
Cat. Nos.	Catalogue numbers
CMI	cell-mediated immune
ConA	concanavalin A
CTL	cytotoxic T-cell
^oC	degrees Celsius
DC	dendritic cell
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
DAB	3,3'-Diaminobenzidine
Na_2HPO_4	disodium hydrogen phosphate
ESR	Environmental Science and Research Limited
ELISA	enzyme-linked immunosorbent assay
EDTA	ethylenediamine tetraacetic acid
H	flagellar
FAFLP	fluorescent amplified-fragment length polymorphism
Kan^r	gene conferring kanamycin resistance
GSP	general secretory pathway
HACCP	Hazard Analysis and Critical Control Point
h	hour(s)
HCl	hydrochloric acid
H_2S	hydrogen sulphide
ID	identity
IMAC	Immobilized metal affinity chromatography
IgA	immunoglobulin A
IgD	immunoglobulin D
IgG	immunoglobulin G
IgM	immunoglobulin M
IFN-γ	Interferon-gamma
IL	interleukin
IAC	internal amplification control
IPTG	isopropyl-β-δ-thiogalactopyranoside

kb	kilo base pair(s)
kDa	kilodalton
LPS	lipopolysaccharide
LSP	lipoprotein signal peptide
L	litre(s)
LB	Luria-Bertani
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MHC	major histocompatibility complex
Tm	melting temperature
MLN	mesentric lymph nodes
mRNA	messenger RNA
µg	microgram(s)
µl	microlitre(s)
µm	micrometre(s)
µM	micromolar
mg	milligram(s)
ml	millilitre(s)
mm	millimitre(s)
mM	millimolar
min	minutes
M	molar
KH_2PO_4	monobasic potassium phosphate
MAb	monoclonal antibody
ng	nanogram(s)
nm	nanometre(s)
nM	nanomolar
NCBI	National Centre for Biotechnology
NK	natural killer
NKT	natural killer T
NZ	New Zealand
TEMED	N-N-N-N-tetramethylene diamine
No.	number
ORF	open reading frame
OD	optical density
OMP	outer membrane protein
*p*NPP	para-nitrophenyl phosphate
PAMP	pathogen-associated molecular patterns
PCR-SSCP	PCR-single-strand conformation polymorphism
%	percent
PBMC	peripheral blood mononuclear cells
FliC	phase-1 flagellin
FljB	phase-2 flagellin
PBS	phosphate-buffered saline
pM	picomolar
PCR	polymerase chain reaction
PMN	polymorphonuclear leukocytes
PMB	polymyxin B sulfate
PVDF	polyvinylidene difluoride

KCl	potassium chloride
PFGE	pulsed-field gel electrophoresis
RAPD-PCR	randomly amplified polymorphic DNA-PCR
RNS	reactive nitrogen species
ROS	reactive oxygen species
rep-PCR	repetitive-element PCR
rpm	revolutions per minute
RNA	ribonucleic acid
RBS	ribosomal binding site
rRNA	ribosomal RNA
SB	*Salmonella* Brandenburg
SCV	*Salmonella* containing vacuoles
SPI	*Salmonella* pathogenicity island
sec	seconds
s-IgA	secretory-IgA
SD	Shine-Dalgarno
SPase	signal peptidase
SP	signal peptide
SRP	signal recognition particle
SI	similarity
$NaHCo_3$	sodium bicarbonate
Na_2CO_3	sodium carbonate
NaCl	sodium chloride
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
O	somatic
spp.	species
SD(s)	standard deviation(s)
SSP	standard signal peptide
subsp.	subspecies
SOC	super optimal broth
TLR	toll-like receptor
TMH	transmembrane helix
TSI	triple sugar iron
TAE	tris-acetate-EDTA
TBS	tris-buffered saline
TTBS	tris-buffered saline (TBS) containing 0.1% Tween-20
TE	tris-HCl-EDTA
TNF-α	tumour necrosis factor-alpha
T1SS	type I secretory system
T2SS	type II secretory system
T3SS	type III secretory system
T4SS	type IV secretory system
T5SS	type V secretory system
UV	ultraviolet
Vi	virulent
V	Volts
v/v	volume/volume
w/v	weight/volume
WHO	World Health Organization

XLD	xylose-lysine--deoxycholate

Amino acids

A	alanine
E	glutamic acid
H	histidine
L	leucine
P	proline
S	serine
V	valine
C	cysteine
F	phenylalanine
I	isoleucine
M	methionine
Q	glutamine
T	threonine
Y	tyrosine
D	aspartic acid
G	glycine
K	lysine
N	asparagine
W	tryptophan

Nucleotides

A	adenosine
C	cytidine
G	guanosine
T	thymidine

Nucleotide triphosphates

ATP	adenosine triphosphate
CTP	cytidine triphosphate
GTP	guanosine triphosphate
TTP	thymidine triphosphate

Deoxyribonucleotide triphosphates

dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate

Chapter 1 Literature review

1.1 Taxonomy of *Salmonella*

Salmonella species are Gram-negative, non-sporing straight rods belonging to the family *Enterobacteriaceae*. They are usually motile with peritrichous flagellae, and are facultatively anaerobic intracellular pathogens of humans and animals. *Salmonella* are ubiquitous in nature, and are found in soil, water, plants, fruits, vegetables, grains, trees, insects and animals. The bacterial genus *Salmonella* is divided into two species, *Salmonella bongori* (*S. bongori*) and *Salmonella enterica* (*S. enterica*). *S. enterica* is subdivided into six subspecies: I, II, IIIa, IIIb, IV and VI which are known as *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salame*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica* respectively (Popoff & Le Minor, 1997). Each subspecies is classified into serotypes (serovars) based on cell wall lipopolysaccharide 'O' and flagellar 'H' antigens they carry, in accordance with the Kaufmann-White scheme (Popoff & Le Minor, 1991). Each serotype is designated by an antigenic formula, a series of numbers and letters that refer to the antigens it carries. Serotypes belonging to *S. enterica* subsp. *enterica* are also known by a name, usually related to the geographical location where each was first isolated. This name is written after the genus or subspecies in Roman letters (not italicised) and the first letter is a capital letter (Popoff *et al.*, 2003). One example is *Salmonella* Brandenburg (*S.* Brandenburg) that can also be described as *Salmonella enterica* serovar Brandenburg or *Salmonella enterica* subsp. *enterica* serovar Brandenburg. Serotypes belonging to other subspecies are designated by their antigenic formulae, following the subspecies name. The antigenic formulae of *Salmonella* serotypes are described in the Kaufmann-White scheme, which is updated by the WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France (Centres for Disease Control and Prevention, 2002). To date, 2523 serotypes have been documented (Popoff *et al.*, 2003). Of the six *S. enterica* subspecies, *S. enterica* subsp. *enterica* includes more than 60% of the serotypes, and is responsible for 99% of the cases of salmonellosis in humans and domestic animals (Chan *et al.*, 2003; Porwollick *et al.*, 2002). All other *S. enterica* subspecies, and the species *S. bongori* are mostly restricted to cold-blooded hosts (Porwollik & McClelland, 2003) and will not be discussed further.

1.2 Host range of *Salmonella*

The genus *Salmonella* contains serotypes that are closely related genetically but which differ in their host range (Bäumler *et al.,* 1998). *Salmonella* serotypes can be classified into two groups based on their adaptation to hosts (Uzzau *et al.,* 2000). One group contains broad-host-range or non-host adapted serotypes such as *S.* Typhimurium and *S.* Enteritidis that have the ability to cause disease and persist in a number of different hosts. These serotypes usually cause a self-limiting gastroenteritis in a broad range of unrelated host species or a systemic disease in a wide range of host animals (van Immerseel *et al.*, 2005). The second group includes host-restricted or highly host-adapted serotypes that infect only a very narrow range of hosts. They are most infectious in one host species, but can cause disease in few other species (Montagne *et al.,* 2001). They have the ability to cause systemic disease associated with high mortality in their respective reservoir hosts (Rabsch *et al.,* 2002). Host-adapted serotypes include *S.* Dublin (bovine), *S.* Abortusovis (ovine), *S.* Choleraesuis (porcine), *S.* Pullorum (poultry) and *S.* Gallinarum (poultry) that cause bacteraemia, abortions, pig paratyphoid, pullorum disease and fowl typhoid respectively (Montagne *et al.,* 2001; Porwollick & McClelland, 2003). It is speculated that *Salmonella* serovar-host specificity is due to specific interactions between *Salmonella* and the host immune system (Barrow *et al.,* 1994; Pascopella *et al.,* 1995).

1.3 Infectious causes of ovine abortion

Infectious ovine abortion is caused by bacteria, viruses, some fungal species and protozoa (I'lhan *et al.*, 2005). The common agents include *Chlamydophila abortus*, *Toxoplasma gondii*, *Brucella melitensis*, *Salmonella* Abortusovis, *Listeria monocytogenes*, *Campylobacter fetus* and *Coxiella burnetii* (West, 2002; West *et al.*, 2004, 2006). In addition to *S.* Abortusovis, other *Salmonella* serotypes including Dublin, Typhimurium, Agona, Arizona, Ruiru, Derby, Oranienburg, Hindmarsh, Reading and Montevideo have been reported to cause abortions in sheep (Baker *et al.*, 1971; Clark *et al.*, 2002; Findlay, 1973; Long *et al.,* 1978; Spence & Westwood, 1978). In New Zealand, until 1995, 80% of ovine abortions were known to be caused by *Campylobacter fetus* subsp. *fetus* and *Toxoplasma gondii.* In 1996, *S.* Brandenburg

was identified as another major cause of abortion in sheep (Bailey, 1997). Pathogens and infectious diseases such as *Brucella ovis*, *Fusobacterium* spp., *Bacillus* spp., Border disease virus, mycotic infections, listeriosis, yersiniosis and leptospirosis are minor causes of abortions (West *et al.*, 2004, 2006). Other ovine-abortion causing pathogens such as *Chlamydophila abortus*, *Coxiella burnetii*, *Salmonella* Abortusovis, *Brucella melitensis*, Bluetongue virus, Wesselsbron disease virus and Akabane disease virus are absent in New Zealand (West *et al.*, 2006).

1.4 *Salmonella* Brandenburg in sheep

1.4.1 History

In 1949, Salisbury identified *S.* Typhimurium, the first *Salmonella* serotype isolated from New Zealand sheep (Salisbury, 1958). Other *Salmonella* serotypes isolated from sheep in New Zealand include Adelaide, Anatum, Bovismorbificans, Brandenburg, Derby, Dublin, Eastbourne, Enteritidis, Havana, Heidelberg, Hindmarsh, Infantis, Kottbus, Lillie, London, Mbandaka, Newington, Oranienburg, Orion, Potsdam, Saintpaul, Senftenberg and Tennessee (Clark *et al.*, 1999, 2002; ESR LABLink 2000, 2001, 2002, 2003; ESR non-human *Salmonella* report 2003, 2004, 2005, 2006; Kane, 1979). In New Zealand, *S.* Brandenburg was first isolated from a sewage swab in 1966 and was later isolated from cases of human gastroenteritis (Clark *et al.*, 2004). In sheep, the organism was first isolated in 1996 from an aborted ovine foetus in a Merino flock in mid-Canterbury in the South Island (Bailey, 1997; Clark *et al.*, 2003, 2004). On this farm, thirty ewes aborted and a number died with an acute necrotizing metritis. In 1997, *S.* Brandenburg was isolated from a further 17 farms in Canterbury and one farm in Southland. In 1998, it was isolated from 55 farms in Otago in addition to 31 in Canterbury and 67 in Southland (Table 1.1). Since then, *S.* Brandenburg has continued to be an important cause of ovine abortions and ewe deaths in the Canterbury, Otago and Southland regions in the South Island (Clark, 2000; Clark *et al*, 2004; Li *et al.*, 2005; Roe, 1999; Smart, 2000). The number of sheep affected by *S.* Brandenburg increased from 1996 to 2000, followed by a decline until 2003. From 2004 to 2006, the number of affected animals increased again (Table 1.2). Pulsed-field gel electrophoresis (PFGE) has shown that the *S.* Brandenburg strain responsible for sheep

abortions since 1996 is genetically distinct from the strains isolated from other animals and humans prior to 1996, and has remarkable genetic stability both geographically and temporally (Baker *et al.*, 2007). The same PFGE pattern as that of the 1996 epidemic strain was evident in sheep yard dust isolates typed in 2000, with one exception. The PFGE pattern of this exceptional isolate differed from that of the epidemic strain by the absence of one high molecular weight band, and the presence of three additional lower molecular weight bands. Aborted foetuses and sheep yard dust samples collected in 2001 from the farm that had the variant strain, and neighbouring farms showed only the presence of the epidemic *S.* Brandenburg strain. This indicates that the variant strain may be less virulent or less viable than the epidemic strain. The appearance of the variant strain may be due to a random genetic event caused by a genetic mutation, insertion or deletion of a DNA sequence. This was the first evidence of a change in the *S.* Brandenburg sheep abortion epidemic strain (Clark *et al.*, 2003).

Table 1.1. Isolations of *S.* Brandenburg from sheep farms in South Island (Canterbury, Otago and Southland) from 1996–2006

Year	No. of farms with *S.* Brandenburg			
	Canterbury	Otago	Southland	Total
1996	1	0	0	1
1997	17	0	1	18
1998	31	55	67	153
1999	45	71	162	278
2000	36	62	233	331
2001	8	21	187	216
2002	6	20	84	110
2003	6	11	40	57
2004	8	75[a]		83
2005	25	133[a]		158
2006	17	130[a]		147

[a]Combined figures for Otago and Southland

(Clark, 2001b; Clark *et al.*, 2004; ESR LABLink, 2000; Smart, personal communication, 2006).

Table 1.2. Number of *S.* Brandenburg isolations from sheep from 1996–2006

Year	No. of sheep identified with SB	% SB cases out of total ovine salmonellosis
1996	1	0.5
1997	30	11
1998	172	52
1999	403	71
2000	545	84
2001	344	77
2002	174	58
2003	95	55
2004	153	57
2005	216	64
2006	230	58

SB, *Salmonella* Brandenburg

(ESR LABLink 2000, 2001, 2002, 2003; ESR non-human *Salmonella* report 2003, 2004, 2005, 2006; Nicol, personal communication, 2007).

1.4.2 Economical impact of *Salmonella* Brandenburg infections in sheep

In New Zealand, *S.* Brandenburg has emerged as a serious animal and human health issue for the sheep industry, with important implications for product safety and market access. *S.* Brandenburg infection has a substantial impact both on animal productivity and on public health as animal handlers and veterinarians are at risk from this zoonotic agent. Major losses to the farmer are from abortions and ewe deaths. Once an outbreak starts, spread continues via contact with other sheep and aborted lambs. On average, about 5% of ewes abort with 50% of these ewes subsequently dying. This results in significant economic losses to farmers. It has been estimated that the loss is approximately NZ$ 10,000 per 100 ewes that abort (Smart, 2000).

Salmonella are among the most important food-borne hazards on a worldwide basis, and *Salmonella* performance criteria in foodstuffs are increasingly being applied as import standards by different countries (Clark *et al.*, 2002). Sheep are one of the principal animal species carrying *Salmonella* in New Zealand, accounting for 32%, 33%, 21%, 21%, 16%, 24%, 22% and 28% of the animal isolates reported to the Institute of Environmental Science and Research Ltd. (ESR; Porirua, Wellington, NZ) in 1999, 2000, 2001, 2002, 2003, 2004, 2005 and 2006 respectively (ESR LABLink 2000, 2001, 2002, 2003; ESR non-human *Salmonella* report 2003, 2004, 2005, 2006). Infected animals that do not develop salmonellosis, and recovered animals, may become carriers of *Salmonella* and serve as sources of infection for humans and other animals. During slaughter, intestinal contents contaminate the surfaces of carcasses, which can lead to extensive contamination of meat and meat products during processing (Hadley *et al.*, 1997). Therefore, *Salmonella* infections lead to restricted access to the export market and serious public health issues (Clark *et al.*, 2002).

1.4.3 Transmission and predisposing factors

Aborting ewes contaminate the environment through aborted foetuses, placentae, vaginal discharges and faeces (Clark, 2001b). Sheep that have recovered from clinical disease may become carriers, and excrete the organisms in faeces. *S.* Brandenburg has been isolated from the faeces of ewes that have aborted for up to 6 months (Clark *et al.*, 2004) and can remain viable in the environment for up to 3 months (Clark, 1999; Clark *et al.*, 2000). Sheep acquire infection through ingestion and possibly via inhalation of the pathogen in aborted foetuses, placentae, uterine discharges, contaminated pasture, feed, water and sheep yard dust (Clark, 2001a, 2001b; Clark *et al.*, 1999, 2004; Kerslake & Perkins, 2006). Contaminated vehicles and foot wear, and birds scavenging on infected carcasses and aborted foetuses, play an important role in spreading the infection. Scavenging birds such as black-backed gulls have been shown to carry the organism (Clark *et al.*, 1999). Wild birds, scavenging animals such as dogs, vehicles, fomites and humans can all transmit the organisms mechanically (Clark, 2000; Clark *et al.*, 2004). Factors that increase the risk of *S.* Brandenburg infection include high fecundity flocks, presence of other diseases and stress caused by management practices including high stocking rates, strip grazing and back fencing, starvation, yarding, shearing, dipping, drought and movement of sheep. Disease is

most common in winter and spring (Clark *et al.*, 2002, 2004; Kerslake & Perkins, 2006; Smart, 2000).

1.4.4 Clinical signs and pathological lesions of *Salmonella* Brandenburg infections

Ovine salmonellosis manifests either as acute infections causing septicaemia, gastroenteritis and abortions, or as subacute infections. The main manifestation of *S.* Brandenburg infection in sheep is abortion. Mostly, the disease is seen in twin- and triplet-bearing ewes and occurs in late pregnancy starting around 80 days with a peak between 100 and 120 days of gestation (Boxall *et al.*, 1999, Clark, 2000; Clark *et al.*, 2004). The prevalence is 6% in two-tooth and 3% in mixed-aged ewes (Smart, 2000). Abortions peak 4-6 weeks before lambing. When an outbreak occurs in a farm for the first time, it results in an abortion rate of up to 20% (5% average), and a mortality rate of 10-100% (50% average) in aborting ewes (Clark *et al.*, 2004; Smart, 2000). Subsequently, both abortion and death rates decline in the following years (Clark, 2001a). On an affected farm, the disease is seen for about 16-20 days, with a peak around 8-10 days after identification of the first case (Kerslake & Perkins, 2006). The reported number of deaths of non-pregnant sheep is low (Clark *et al.*, 2004). Foetuses often die and decompose inside ewes before being aborted. Some ewes die a few days after abortions and some may die without aborting. Other signs include lethargy, loss of appetite, depression, droopy ears, dullness, fever and occasional severe diarrhoea (Bailey, 1997; Clark, 1999; Clark *et al.*, 2000). Affected sheep show lesions of abomasitis, enteritis, metritis, placentitis and septicaemia. There is reddening of the abomasal and small intestinal mucosa (Bailey, 1997). Lesions such as adhesions of fallopian tubes, pyometra, and uterine fibrous tags that could affect conception rates have been observed (Clark *et al.*, 2004). Aborted foetuses have a cooked and bruised appearance, and show lesions including oedematous subcutaneous tissue, swollen liver, bloody-putrid stomach contents, and lung lesions consistent with pneumonitis. Cotyledons are pale and oedematous (Bailey, 1997; Clark *et al.*, 2004; Roe, 1999).

1.4.5 Control and prevention of *Salmonella* Brandenburg infections

Treatment of *S.* Brandenburg-infected animals with antibiotics reduces ewe mortality. The organism has been shown to be sensitive to ampicillin, cephalothin, enrofloxacin, streptomycin, tetracycline and trimethoprim, and resistant to lincomycin and penicillin (Clark *et al.*, 2004). Control of *S.* Brandenburg depends on good management practices and vaccination (Clark *et al.*, 2004; Fenwick *et al.*, 2000). In New Zealand, Salvexin™ vaccine (Schering-Plough Animal Health Ltd., Upper Hutt, Wellington, NZ) has been available since the mid 1980's. This killed vaccine included the 3 most common serotypes of *Salmonella* in New Zealand (*S.* Typhimurium, *S.* Hindmarsh and *S.* Bovismorbificans). The somatic O-antigens of *S.* Typhimurium and *S.* Brandenburg are [1], 4, [5], 12, [27] and [1], 4, [5], 12 respectively. Since, both these organisms share some somatic antigens, a mouse challenge study with Salvexin™ vaccine was carried out by Schering-Plough Animal Health Ltd., in order to study the efficacy of the vaccine against *S.* Brandenburg. The results showed some cross protection against *S.* Brandenburg and veterinarians started using this vaccine in 1997 to prevent *S.* Brandenburg infection (Marchant, 1999, 2000). Since, Salvexin™ did not appear to reduce the disease in sheep, a sheep challenge experiment was carried out in 1999. In this study, 3 groups of sheep were used. One group of animals acted as unvaccinated controls, one group was vaccinated with Salvexin™ and the third group received Salvexin™+B. Salvexin™+B is a killed vaccine that includes *S.* Brandenburg antigens in addition to the 3 serotypes that were present in Salvexin™. The study showed that the onset of abortions in Salvexin™+B-vaccinated group was delayed with respect to the other two groups, and the company began to add *S.* Brandenburg antigens to the commercial vaccine in 2000 (Marchant, 2000). Vaccination of sheep with Salvexin™+B since its use in 2000 has resulted in a reduction of abortions and ewe deaths. However, disease is still seen on some farms where vaccine is used (Clark *et al.*, 2004; Marchant *et al.*, 2002). The recommended vaccination program consists of two injections given to breeding ewes during early pregnancy. The second injection needs to be given 4 to 6 weeks after the primary one, and 2 to 3 weeks before the high-risk period. A booster is recommended in the following year prior to the high-risk period (Marchant, 1999, 2000). A recent study to compare the efficacy of the Salvexin™+B vaccine, a live attenuated *S.* Typhimurium vaccine with mutations in *cya* and *crp* genes, and a subunit vaccine with crude cell walls of *S.* Brandenburg showed

that none of the vaccines protected sheep against abortion and ewe deaths, following nasal and oral challenge with *S.* Brandenburg. However, vaccination reduced the number of ewes shedding *S.* Brandenburg (Li *et al.*, 2005).

1.5 *Salmonella* Brandenburg in other domestic animal species

Prior to 1996, *S.* Brandenburg was rarely isolated from animals other than sheep in New Zealand (Kerslake & Perkins, 2006). With the emergence of *S.* Brandenburg as an important pathogen in sheep in 1996, the serovar started to be isolated from other domestic animals including horses, cattle, goats, pigs, cats and dogs. The organism has been isolated from horses with diarrhoea and foal death, cattle with diarrhoea, reduced milk yield and abortions, and goats with diarrhoea. Furthermore, *S.* Brandenburg has been isolated from dogs with diarrhoea, abortions and stillbirths, and pigs with sudden death (Bailey, 1997; Clark, 2000, 2001a; Clark *et al.*, 2000, 2004).

1.6 *Salmonella* Brandenburg in humans

In humans, non-typhoidal salmonellosis is caused by more than 2000 *Salmonella* serotypes (Tauxe, 1991). Humans are infected mainly by consumption of animal products containing pathogens that are not thoroughly cooked leading to food poisoning (Hobbs & Roberts, 1993). In New Zealand and other parts of the world, the most common serotypes causing non-typhoidal salmonellosis are *S.* Typhimurium and *S.* Enteritidis (Hjartardóttir *et al.*, 2002). Recently, *S.* Brandenburg was identified as another important non-typhoidal human pathogen in New Zealand (Clarke & Tomlinson, 2004). In 1930, *S.* Brandenburg was first identified in a human with gastroenteritis in the county of Brandenburg in Berlin, Germany (Wright *et al.*, 1998). Since then, it has been associated with a number of gastroenteritis outbreaks, in England in 1963, Switzerland in 1992, and in Japan in 2001. These cases were due to consumption of contaminated pork and other meat products (Baquar *et al.*, 1994; Hamada & Tsuji, 2001; Jones *et al.*, 1964). In addition to gastroenteritis, *S.* Brandenburg also has been isolated from a number of systemic infections from different parts of the world (Bjorkman *et al.*, 2002; Chiovato *et al.*, 1993). In New Zealand, *S.* Brandenburg was first isolated from a human in 1985. Subsequently, the

first case of human *S.* Brandenburg bacteraemia, and the first extra-intestinal invasive disease due to *S.* Brandenburg were identified in 1995 and 1999 respectively (Clarke & Tomlinson, 2004; Wright *et al.*, 1998). Table 1.3 shows the number of human *S.* Brandenburg infections in New Zealand from 1985-2006.

Table 1.3. Number of human cases of *S.* Brandenburg in New Zealand from 1985-2006

Year	No. of SB cases	% SB cases out of total human salmonellosis	Year	No. of SB cases	% SB cases out of total human salmonellosis
1985	28	2.3	1996	27	2.0
1986	11	0.9	1997	39	2.8
1987	12	1.1	1998	164	7.4
1988	10	0.8	1999	176	7.6
1989	7	0.4	2000	184	9.3
1990	26	1.5	2001	137	5.3
1991	2	0.2	2002	85	4.1
1992	6	0.5	2003	55	3.4
1993	7	0.5	2004	86	7.0
1994	33	2.0	2005	68	4.7
1995	31	2.0	2006	55	3.9

SB, *Salmonella* Brandenburg

(ESR human *Salmonella* report 2003, 2004, 2005, 2006; ESR LABLink, 2000, 2001, 2002, 2003; Smart, 2000; Wright *et al.*, 1998).

Clinical signs in infected humans can last up to 6 weeks and include abdominal pain, localised pain, diarrhoea, vomiting and fever (Baker *et al.*, 2007; Clark *et al.*, 2004; Clarke & Tomlinson, 2004). Symptoms may be more severe in young, old, and immunologically compromised people, and pregnant women. Three children between six months and eight years with symptoms of meningitis, osteomyelitis and arthritis in

the hip due to *S.* Brandenburg were admitted to Southland Hospital during 1999 and 2002 (Clarke & Tomlinson, 2004). Infection in humans is seasonal, with cases peaking in Spring (September to November inclusive) during the lambing and calving seasons (Clark *et al.*, 2004; Clarke & Tomlinson, 2004; Fenwick *et al*, 2000; Smart, 2000). Cases are concentrated in the southern half of the South Island, especially in Southland, Otago, South Canterbury, and Canterbury health districts (Baker *et al.*, 2007; ESR human *Salmonella* report 2003, 2004, 2005, 2006; ESR LABLink 2000, 2001, 2002, 2003). PFGE of DNA from humans, aborted lambs, sheep, cattle, dogs and black-backed gulls collected after 1996 showed a single molecular type suggesting a single stable clonal strain (Clark *et al.*, 1999, 2004). However, the PFGE profile of this strain was significantly different from 13 of these obtained from 115 human and non-human isolates collected between 1 January 1990 and 31 March 1995 (Clark *et al.*, 2004; Wright *et al.*, 1998). Most of the humans affected are ones who are exposed to infected animals and a contaminated environment. These include veterinarians, veterinary workers, sheep and cattle farmers, farm workers, and their family members (Clark, 2001a; Clark *et al.*, 2004). Therefore, people who handle infected animals and aborted foetuses need to follow strict hygienic measures including the use of disposable gloves and protective clothing, regular washing of hands especially after contact with farm animals and equipment, before eating, smoking or touching the mouth, changing of clothes immediately after contact with farm animals and equipment, and proper cleaning and disinfection of contaminated foot wear, equipment and vehicles. In addition, proper disposal of aborted foetuses, dead animals, and control of scavengers need to be carried out to reduce the contamination of farming environment (Baker *et al.*, 2007; Clark *et al.*, 2004). To date, human cases due to consumption of contaminated animal products have not been reported in New Zealand (Clark *et al.*, 2004).

1.7 Protein secretory systems in bacteria

1.7.1 Potential significance of secreted proteins

All organisms, including bacteria, localize a fraction of all their proteins partially or completely outside of the cytoplasm (Fisher & DeLisa, 2004). These secreted proteins

are used for synthesis of bacterial structures such as the cell envelope, metabolism, and interaction with host cells to cause disease (Schmidt & Hensel, 2004). Several studies have shown that the majority of bacterial virulence factors are secreted proteins (Finlay & Falkow, 1997). They are of particular importance for the development of diagnostic tests and vaccines because they are often immunogenic. Therefore, elucidation of protein secretion in *S.* Brandenburg could provide insights into virulence factors, and provide a source of proteins with potential for the development of diagnostic tests and vaccines.

1.7.2 Protein secretory systems in Gram-negative bacteria

The envelope of Gram-negative bacteria is composed of an inner cytoplasmic membrane and an outer membrane with a peptidoglycan (murein) layer, and a periplasmic space in between them (Fisher & DeLisa, 2004). Gram-negative bacteria use three basic pathways to secrete proteins. These are: (i) pathways that translocate polypeptides across the cytoplasmic membrane (ii) pathways that are required for crossing the outer membrane from the periplasm (iii) pathways to translocate unfolded proteins directly from the cytoplasm to the outside or into the host cell cytoplasm. Pathways that translocate polypeptides across the cytoplasmic membrane include general secretory, YidC and twin-arginine (Tat) pathways. While the second pathway includes type II, some of type IV and type V systems, the third one includes types I, III, and majority of IV systems (Holland, 2004). Protein translocation requires targeting signals on proteins to allow recognition by the targeting receptors, and the presence of membrane-bound transport structures known as translocons with a cavity through which proteins can pass without compromising the general permeability barrier of the membrane. Usually, but not always, transport signals are located at the N-termini of the secreted proteins (Sargent *et al.*, 2006; Schnell & Hebert, 2003).

1.7.3 Signal sequences

Many proteins that are exported from the cytoplasm contain an additional 15-30 amino acid stretch at their amino terminus known as the signal sequence or signal peptide (SP) (Tuteja, 2005). These are responsible for the transport of proteins through the membrane (Pugsley, 1993), and the classical SP (Figure 1.1) has three regions: a

hydrophilic N-region (1-5 residues) that is polar and positively charged due to one or two basic residues (arginine and/or lysine), a hydrophobic H-region (9-15 residues) that forms a membrane-spanning α-helix, and the neutral polar C-region (3-7 residues). The N- and H- regions are required for translocation, and the C-region contains the cleavage site that is recognized by the signal peptidases (SPases) (von Heijne, 1990). Signal peptidases are membrane-bound serine proteases with the catalytic domain located on the periplasmic surface of the inner membrane (Tuteja, 2005). Most SPs are cleaved off during or immediately after the completion of translocation through the inner membrane (Stephenson, 2005). This cleavage is necessary for the release of mature proteins from the membrane so that they can be transported to the periplasm, outer membrane or outside the cell (Tuteja, 2005). There are 4 types of signal peptides: Type I (SP I), type II (SP II), Tat, and type IV (Holland, 2004). The most common is SP I which is cleaved by SPase I (LepB in *E. coli*) (von Heijne, 1989). The (-3, -1) rule states that the residues at positions –3 and –1 (relative to the cleavage site) must be small and neutral for cleavage to occur correctly. This area is often referred to as Ala-X-Ala cleavage site due to the presence of alanine in positions –3 and –1 relative to the cleavage site. Alternatively, there may be glycine or serine in position –1, and valine, serine or threonine in position -3 (Stephenson, 2005; Tuteja, 2005). The SP II is present in lipoproteins, and is cleaved by SPase II, also called lipoprotein SPase (LSPase). Both SP I, and SP II have a similar N-region, but the H-region of SP II is shorter than that of SP I. Furthermore, SP I has a polar C-region before the cleavage site. The C-region of SP II contains a conserved region of four amino acids around the cleavage site known as the lipobox followed by a cysteine, Leu-Ala- (Gly, Ala)↓Cys. The most conserved amino acids in the lipobox are a leucine in position –2, and a glycine or an alanine in position –1 (von Heijne, 1989). The SP II is cleaved off immediately upstream of the cysteine residue. In Gram-negative bacteria, the cysteine residue of the lipoprotein is attached to either the inner or the outer membrane via a lipid anchor, and a single amino acid in position +2 is believed to determine the final destination of the lipoprotein (Juncker *et al.*, 2003; Sargent *et al.*, 2006). The Tat SP has an N-H-C structure similar to a classical signal sequence. However, there are a number of differences between them. The Tat SP contains an SRRxFLK motif in the boundary of N- and H-regions with almost invariant twin-arginine (RR) residues. The H-regions of Tat SP are less hydrophobic than those of classical SP. The Tat SP often

contains basic residues such as proline in the C-region whereas such amino acids are almost never found at the equivalent position of the classical SP. Tat signals are longer than classical signals, in some cases reaching up to 58 amino acids in length (Palmer & Berks, 2003). The positive charge in the C-region prevents mistargeting Tat signal peptides to the Sec pathway. Tat signal peptide C-regions contain cleavage sites similar to those of classical signals, and are recognized by SPase I (Sargent *et al.*, 2006). Type IV signal peptides are short (approximately six residues) without an N-H-C structure. They are cleaved by prepilin peptidase downstream of a glycine residue that precedes a long N-terminal stretch of hydrophobic amino acids in the mature protein. A GFTLIE motif is often seen in type IV signal peptides (LaPointe & Taylor, 2000; Lewenza *et al.*, 2005). While, most of the membrane proteins have uncleavable N-terminal signal sequences that remain as transmembrane segments, others have cleavable signal sequences with additional stop-transfer sequences that anchor the proteins in the membrane. A stop-transfer sequence usually consists of a hydrophobic segment of at least 20 amino acid residues, which allows stable membrane integration of the protein (Clemons Jr *et al.*, 2004; Facey & Kuhn, 2004). While SignalP (Bendtsen *et al.*, 2004) and LipoP (Juncker *et al.*, 2003) programs predict SP I, and SP II signals, TatP program predicts Tat signals (Bendtsen *et al.*, 2005). TMHMM, HMMTOP, MEMSTAT, PHDhtm, TopPred and Phobius programs identify and predict the topology of inner membrane proteins (Käll *et al.*, 2004; Rapp *et al.*, 2006).

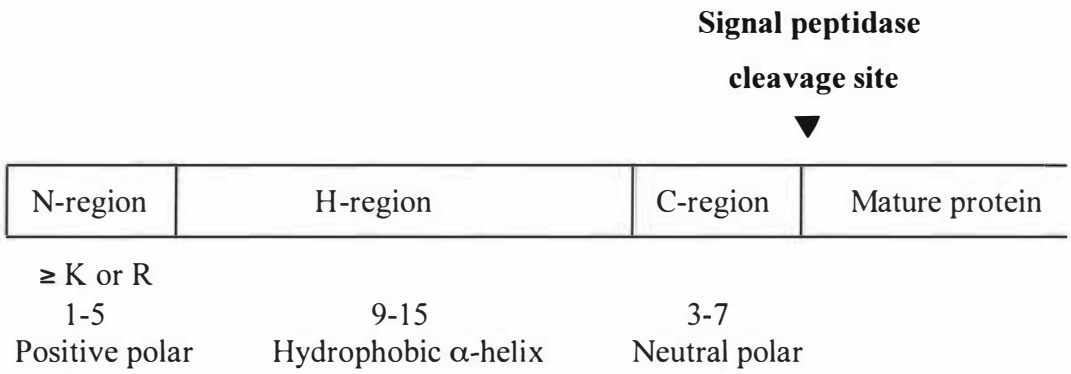

Figure 1.1. Schematic diagram showing a classical signal peptide.

1.7.4 General secretory pathway (GSP)

In bacteria, most proteins are exported across the inner membrane through the general secretory pathway (GSP), which is also known as the Sec pathway (Stephenson, 2005). The Sec pathway recognizes substrates through N-terminal SP I and SP II (Clemons Jr *et al.*, 2004). The Sec pathway transports newly synthesized unfolded precursor polypeptides (pre-proteins), and is mediated by a membrane protein complex called Sec translocase. In *E. coli*, the Sec translocase is made up of three integral membrane proteins (SecY, SecE, SecG) that form a heterotrimeric SecYEG complex, SecD, SecF, YajC forming heterotrimeric SecDF-YajC complex, and membrane-associated ATPase SecA. The core of the translocon is a channel formed by SecY and SecE. SecA plays a role in SP recognition, targeting, and ATP hydrolysis to provide energy for polypeptide translocation (Hand *et al.*, 2006; Mori & Ito, 2001; Schnell & Hebert, 2003). The SecYEG complex, along with SecA is the minimum required for protein translocation. The SecDF-YajC complex is believed to play a role in membrane cycling of SecA, clearing of misfolded proteins, proper assembly of the translocon, and release of pre-proteins from the translocase upon completion of translocation (Nouwen *et al.*, 2005; Stephenson, 2005). Three of the major protein secretory pathways of Gram-negative bacteria are Sec-dependent. These are substrates of type II, IV (some), and V. Substrates of types I, III, and IV (some) are Sec-independent (Büttner & Bonas, 2002). Pre-proteins that are ready to reach the Sec translocase do so in two ways: through the SecB chaperone in post-translational translocation of less hydrophobic proteins, and through the signal recognition particle (SRP) in co-translational translocation of highly hydrophobic, integral membrane proteins or lipoproteins (Holland, 2004; Zhou & Xu, 2005).

1.7.4.1 SecB chaperone-directed Sec pathway

SecB is a cytoplasmic chaperone that does not recognize the signal sequence, but recognizes the mature part of the protein targeted for secretion (Henderson *et al.*, 2004). A minimal motif for Sec binding consists of a stretch of nine amino acids consisting of mainly charged and aromatic amino acids (Stephenson, 2005). In the cytoplasm, SecB binds to the pre-proteins after they exit from the ribosome translation site to maintain it in the unfolded and unaggregated state. Subsequently, pre-protein-

bound SecB associates with SecA, which in turn binds to the SecA binding site made up of SecY and SecE (Mori & Ito, 2001). SecB-SecA binding changes the conformation of the polypeptide binding sites in the SecB, mediating the transfer of pre-protein from SecB to SecA (Zhou & Xu, 2005). The channel opens across the membrane, and protein translocation takes place upon ATP hydrolysis (Figure 1.2) (Collinson, 2005; Stephenson, 2005).

1.7.4.2 SRP-directed Sec pathway

In bacteria, most inner membrane proteins are co-translationally targeted to the inner membrane by signal recognition particle (SRP), and its membrane-associated receptor (SR). The SRP of *E. coli* is a ribonucleoprotein containing a 4.5 S RNA and a single protein known as *F fh* that is a GTPase. The *F fh* recognizes and binds to the highly hydrophobic H-region signal sequence of the protein as it emerges from the ribosome, and associates with the membrane-bound SR (*Fts Y*) through a GTP-dependent mechanism. The interaction between *F th* and *Fts Y* stimulates GTP hydrolysis that releases the ribosome-associated polypeptide complex from SRP and transfers it to the SecYEG translocon (Figure 1.3). The signal peptide gets inserted into the channel. While hydrophilic amino acid stretches are translocated across the membrane, hydrophobic transmembrane segments get embedded in the lipid bilayer (Clemons Jr *et al.*, 2004; Doudna & Batey, 2004; Egea *et al.*, 2005; Halic & Beckmann, 2005; Luirink & Sinning, 2004; Schnell & Hebert, 2003). The translocation of membrane proteins stops upon the entry of a transmembrane portion into the translocon. The transmembrane domain acts as a stop-transfer signal or a signal-anchor sequence that is recognized by the translocon. This initiates a series of events that result in the lateral opening of the translocation channel exposing the transmembrane domain into the lipid bilayer. The hydrophobic region gets into the bilayer and the translocon channel closes (Schnell & Hebert, 2003). The hydrophilic segments of membrane proteins are located either in the cytoplasm or periplasm (Clemons Jr *et al.*, 2004; Facey & Kuhn, 2004). A classical signal sequence translocates the carboxy-terminal segment adjacent to it into the periplasmic space, and this process usually depends on the Sec system (Mori & Ito, 2001). In addition to inner membrane proteins, some soluble proteins with highly hydrophobic signal peptides are also translocated through the SRP-directed Sec pathway (Stephenson, 2005; van der Does *et al.*, 2003).

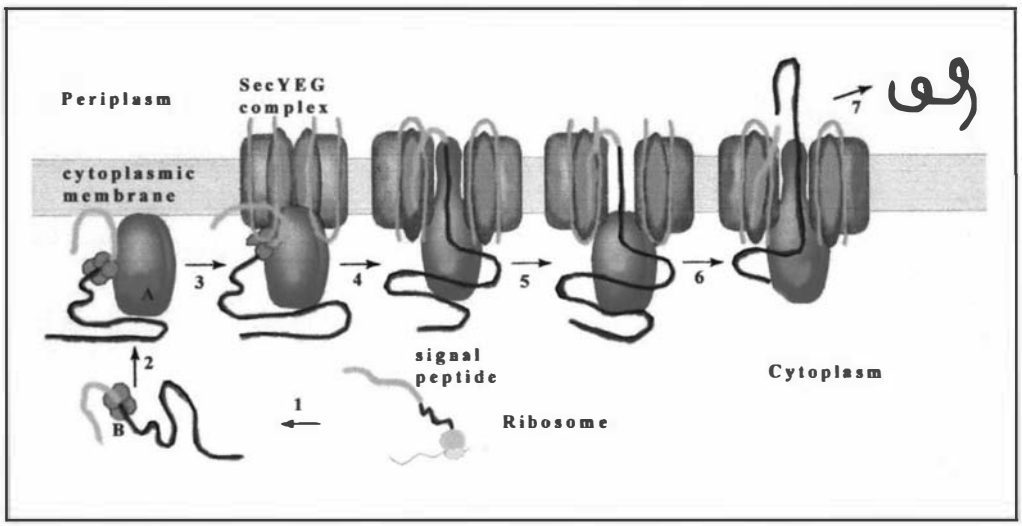

Figure 1.2. SecB chaperone-directed Sec pathway. A, SecA; B, SecB. The pre-protein is indicated by black, and the signal peptide by pink. Steps 1-3 targeting, 4 and 5 initiation, 6 continuation, 7 completion of translocation. Adapted from Mori & Ito, 2001. See text for details.

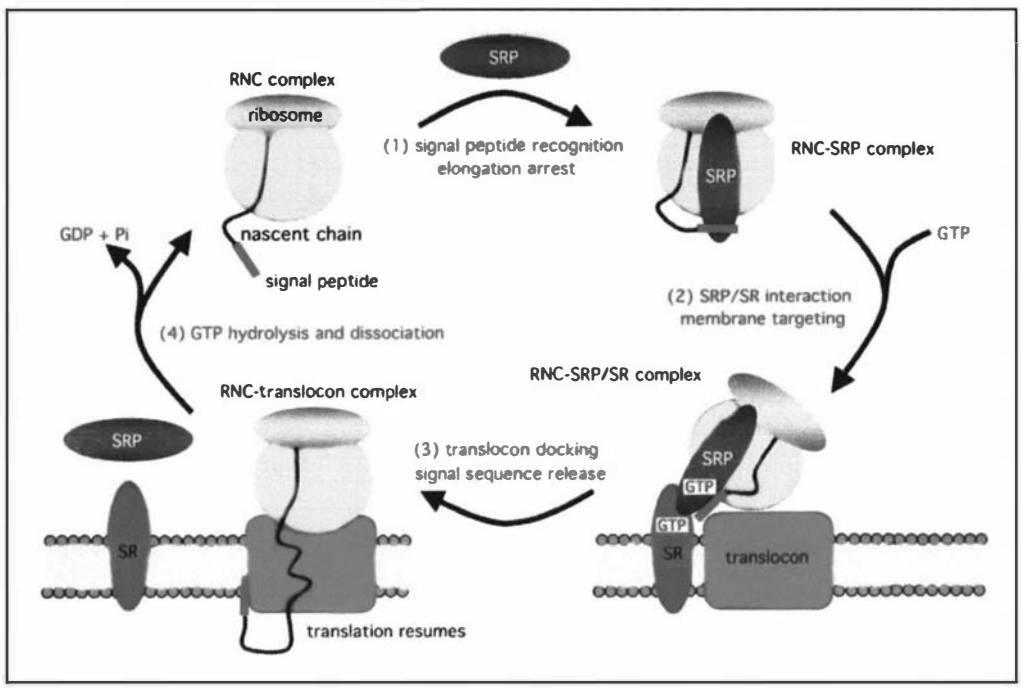

Figure 1.3. SRP-directed co-translational targeting of inner membrane proteins. SRP, signal recognition particle; SR, membrane-associated receptor for SRP; RNC, ribosome-nascent chain complex; GTP, guanidine triphosphate. Adapted from Egea *et al.*, 2005. See text for details.

1.7.5 YidC pathway

The YidC pathway is an evolutionarily conserved system in which the membrane protein YidC is responsible for the insertion of some proteins into the inner membrane in both Sec-dependent (SRP-directed) and Sec-independent pathways. YidC interacts with hydrophobic regions of its substrates and stimulates membrane insertion. YidC is required for the membrane insertion of the F_1F_0 ATP synthase subunit C and influences the assembly of the whole F complex. Furthermore, YidC plays a role in the folding and assembly of multi-subunit protein complexes (Baker, 2005; De Gier & Luirink, 2001; Pohlschröder *et al.*, 2005). In *E. coli*, YidC is found to be associated with both SecYEG and SecDF-YajC complexes (Hand *et al.*, 2006; Nouwen *et al.*, 2005; Stephenson, 2005). A signal-anchor sequence is similar to a non-cleavable signal sequence and assumes an N-in, C-out transmembrane orientation. Membrane proteins of this orientation use SecYEG pathway for their integration with the involvement of YidC. The orientation of a transmembrane segment is determined by the positive-inside rule orienting the positively charged side of the hydrophobic segment towards the cytoplasm. The hydrophobic region with a positive charge on the carboxy-terminal side can assume the N-out, C-in orientation, translocating the amino-terminally adjacent region to the periplasmic side. This type of translocation and integration depends on YidC (Mori & Ito, 2001).

1.7.6 Twin-arginine translocation (Tat)

The Tat system is located in the cytoplasmic membrane, and has been identified in bacteria only recently (Dilks *et al.*, 2003; Palmer & Berks, 2003). In contrast to Sec substrates, Tat substrates need to be in a folded conformation for translocation. The Tat translocation requires three integral membrane proteins TatA, TatB, and TatC. The TatBC complex binds to the substrate upon recognition of the twin-arginine residues in the signal sequence (Pohlschröder *et al.*, 2005; Sargent *et al.*, 2006). The TatA multimers form a ring within membranes and are likely to form a channel through which substrates pass (Pohlschröder *et al.*, 2005). The transmembrane proton motive force provides energy for Tat translocation. The Tat system is responsible for the translocation of most of the cofactor-containing redox proteins such as dimethyl sulfoxide reductase chain A (DmsA), trimethylamine N-oxide reductase (TorA), and

some non-redox proteins such as amidases that are involved in cell wall biosynthesis, phospholipases, and alkaline phosphatase D (PhoD). Some Tat substrates are membrane proteins, and the mechanism of their translocation is still unknown (Palmer & Berks, 2003; Sargent *et al.*, 2006).

1.7.7 Type I secretory system

The type I secretory system (T1SS, TOSS) (Figure 1.4) is found in a number of Gram-negative bacteria (Saier Jr, 2004). This system is Sec-independent, and allows the secretion of proteins from the cytoplasm to the extracellular milieu in one step, without a periplasmic intermediate. Proteins secreted through the T1SS vary in size from 11-kDa to 178-kDa. Examples include lipase (LipA) of *Serratia marcescens*, cyclolysin (CyaA) of *Bordetella pertussis*, haemolysin A (HlyA) of *Escherichia coli*, and RtxA toxin of *Vibrio cholerae*. The T1SS is composed of three proteins that span the cell envelope: an inner membrane ATP-binding cassette (ABC) protein with ATPase activity, periplasmic adaptor or membrane fusion protein (MFP) that is anchored to the inner membrane, and a specific outer membrane protein (OMP) (Delepelaire, 2004; Schmidt & Hensel, 2004). Usually, the ABC protein recognizes the protein through the uncleaved C-terminal secretion signal of 30-80 residues. Exceptions are colicin V of *E. coli* and haemoprotein HasA of *Serratia marcescens* with cleavable N-terminal signals. It has been suggested that the C-terminal secretion signals contain three features in the final 50 residues: an amphipathic helix, a cluster of charged residues, and a weakly hydrophobic terminal sequence rich in hydroxylated residues (Koronakis, 2003). The specific OMPs include TolC for HlyA, and CyaE for CyaA (Delepelaire, 2004). *S.* Typhimurium and *S.* Typhi possess T1SSs that are encoded by *Salmonella* pathogenicity islands (SPI) 4 and 9 respectively. They are speculated to play a role in toxin secretion (Schmidt & Hensel, 2004).

1.7.8 Two-step type II secretory system

Gram-negative bacteria utilize the two-step type II secretory system (T2SS) (Figure 1.4) for the transportation of folded proteins across the outer membrane. This system involves a separate step of transport of proteins with N-terminal signal sequences

across the inner membrane through the Sec-dependent GSP prior to transport across the cell envelope. Therefore, T2SS is also known as the main terminal branch (MTB) of the Sec-dependent GSP. Recent studies have shown that, in addition to the Sec pathway, proteins also reach the periplasm by the Tat pathway for secretion through the T2SS (Filloux, 2004). After transport across the cytoplasmic membrane into the periplasm through the Sec or Tat pathways, the signal sequence of the protein is cleaved off, and folding of Sec substrates takes place. In the periplasm, proteins may undergo further modifications, such as disulfide bond formation or subunit assembly before they are translocated through the T2SS that is composed of 12-16 proteins known as GSP proteins (Henderson *et al.*, 2004; Sandkvist, 2001). The signals that target the proteins to the T2SS have not yet been identified (Henderson *et al.*, 2004). It is believed that recognition and outer membrane translocation of the secreted proteins occur once they have folded into a secretion-competent conformation (Scott & Sandkvist, 2003). The T2SS is responsible for the secretion of extracellular degradative enzymes and toxins by Gram-negative bacteria. These include amylase and protease of *Aeromonas hydrophila*, elastase, exotoxin A and phospholipase C secreted by *Pseudomonas aeruginosa,* and cholera toxin of *Vibrio cholerae* (Hueck, 1998; Sandkvist, 2001).

1.7.9 Type III secretory systems

The type III secretory systems (T3SS, TTSS) are of two types, non-flagellar and flagellar. The substrates of the T3SS differ from other secretion pathways by the absence of both cleavable signal sequences and periplasmic intermediates (Blocker *et al.,* 2003). DNA sequence comparisons and structural analysis indicate a close relationship between the non-flagellar and the flagellar T3SS suggesting a common origin of the two (Galán & Collmer, 1999).

1.7.9.1 Non-flagellar T3SS

A number of Gram-negative pathogenic bacteria harbour complex, Sec-independent type III secretion systems known as "injectisomes" (Hueck, 1998; Wallis & Galyov, 2000). The non-flagellar T3SS (Figure 1.4) is utilized by many pathogens including

Salmonella enterica, Escherichia coli, *Shigella flexneri*, and *Yersinia* spp. to cause disease (Kimbrough & Miller, 2002; Mota *et al.*, 2005, Stuber *et al.*, 2003; Zaharik *et al.*, 2002). While pathogenicity island-encoded T3SS is found in *S. enterica* and *E. coli*, plasmid-encoded T3SS is present in *Yersinia* and *Shigella* (Schmidt & Hensel, 2004). Two T3SSs encoded by SPI-1 and SPI-2 of *S. enterica* translocate virulence-associated proteins from bacterial cytoplasm into the host cell cytoplasm across eukaryotic cell membrane and vacuolar membrane respectively (Pallen *et al.*, 2005; Troisfontaines & Cornelis, 2005; Zaharik *et al.*, 2002). Inside the eukaryotic cells, these proteins are known as effectors (Feldman & Cornelis, 2003). The translocated effectors interfere with basic host cell functions such as signal transduction, cytoskeletal architecture, membrane trafficking, and cytokine gene expression (Kimbrough & Miller, 2002; Marcus *et al.*, 2000; Mota *et al.*, 2005). Some T3SSs including T3SS encoded by SPI-1 of *Salmonella* are triggered when bacteria come in contact with the host cell, and this secretion is known as contact-dependent. All T3SSs are not contact-dependent, and some effectors are also secreted into the external milieu rather than into the host cell cytoplasm (Henderson *et al.*, 2004; Sorg *et al.*, 2005). Furthermore, some laboratory growth conditions can activate T3SS (Galán 1998). The assembly of T3SS requires approximately 25 proteins. It has a cylindrical base spanning inner and outer membranes linked to a hollow, elongated needle-like structure that forms a direct channel between the bacterial and host cell cytoplasm. Proteins are translocated through this channel in one step (Mota *et al.*, 2005). The *invA* gene that is located on the SPI-1 of *Salmonella* encodes InvA, a structural component of the SP-1 encoded T3SS. It is essential for the invasion of host cells (Browne *et al.*, 2002; Collazo & Galán, 1997; Wallis & Galyov, 2000). The *invA* gene is present in all invasive strains of *Salmonella* (Galán, 1996), and absent from closely related genera such as *Escherichia* (Bäumler *et al.*, 1998). The mechanism of recognition and targeting substrates to the T3SS is still unknown (Plano *et al.*, 2003). Some believe that the signal is present in the 5' mRNA, while others believe that it is present in the N-terminal 20 amino acids (Henderson *et al.*, 2004; Page & Parsot, 2002; Ruiz-Albert *et al.*, 2003). A recent study showed that the secretion of effector SopE is through an N-terminal amino acid signal, and not through an mRNA signal (Karavolos *et al.*, 2005). Secretion of some, but not all T3SS substrates requires cytoplasmic chaperones (Mota *et al.*, 2005).

1.7.9.2 Flagellar T3SS

The basal body of the flagellum houses a flagellum-specific type III export apparatus that shows homology with T3SS (Kirov, 2003). However, the flagellar T3SS is not contact-dependent, and does not translocate proteins into eukaryotic cells (Blocker *et al.*, 2003). The flagellar T3SS is involved in the transport of external flagellar proteins (rod, hook, filament, and proteins associated with these structures) that are located beyond the cytoplasmic membrane through the central channel to the distal end of the growing flagellum. The ATPase FliI provides energy by ATP hydrolysis (Macnab, 2003; Végh *et al.*, 2006; Yonekura *et al.*, 2002). The recognition signal of flagellar T3SS substrates is believed to be within its N-terminal region (Hirano *et al.*, 2003; Macnab, 2004). The flagellar T3SS has recognized and exported a 183-residue N-terminal fragment of the 497-residue flagellin of *E. coli* (Kuwajima *et al.*, 1989). A recent study showed that a 22-residue long (N-terminal amino acids 26--47) *Salmonella* phase-2 flagellin contains the recognition signal for the flagellar T3SS (Végh *et al.*, 2006). Export of substrates is facilitated by cytoplasmic substrate-specific chaperones, which bind to the C-terminal regions of their substrates in the cytoplasm, and protect them from degradation (Thomas *et al.*, 2004). The general chaperone FliJ is necessary for export of many substrates (McMurry *et al.*, 2004).

1.7.10 Type IV secretory systems

Secretion systems that are ancestrally related to bacterial conjugation systems are known as type IV secretory systems (T4SS) (Figure 1.4). These systems are responsible for transport of DNA from bacteria to other bacteria or eukaryotic cells, for exchange of DNA with the extracellular medium, and for transport of virulence factors into the extracellular medium or directly into eukaryotic host cells (Backert & Meyer, 2006; Nagai & Roy, 2003). When DNA is transported, it is associated with one or more proteins (Christie & Vogel, 2000). The best-studied T4SS belongs to *Agrobacterium tumefaciens* that translocates oncogenic nucleoproteins into plant cells inducing tumours (Schmidt & Hensel, 2004). The T4SS of *A. tumefaciens* contains a coupling protein VirD4 and, 11 VirB (VirB1-VirB11) proteins that form the structure spanning both inner and outer membranes, and the extracellular pilus (Backert &

Meyer, 2006). It is believed that the coupling protein VirD4 recognizes proteins for transport, and aids in the translocation. While the transport of nucleoproteins of *A. tumefaciens* occurs in a single continuous step, that of some proteins including pertussis toxin of *Bordetella pertussis* occurs in two steps. In the latter, proteins containing N-terminal signal sequences are first exported to the periplasm via the Sec-dependent pathway followed by the transport through T4SS (Holland, 2004). Examples of T4SS virulent proteins include pertussis toxin (PT) of *Bordetella pertussis*, CagA protein of *Helicobacter pylori*, and DotA and RalF proteins of *Legionella pneumophila* (Backert & Meyer, 2006; Cascales & Christie, 2003; Nagai & Roy, 2003).

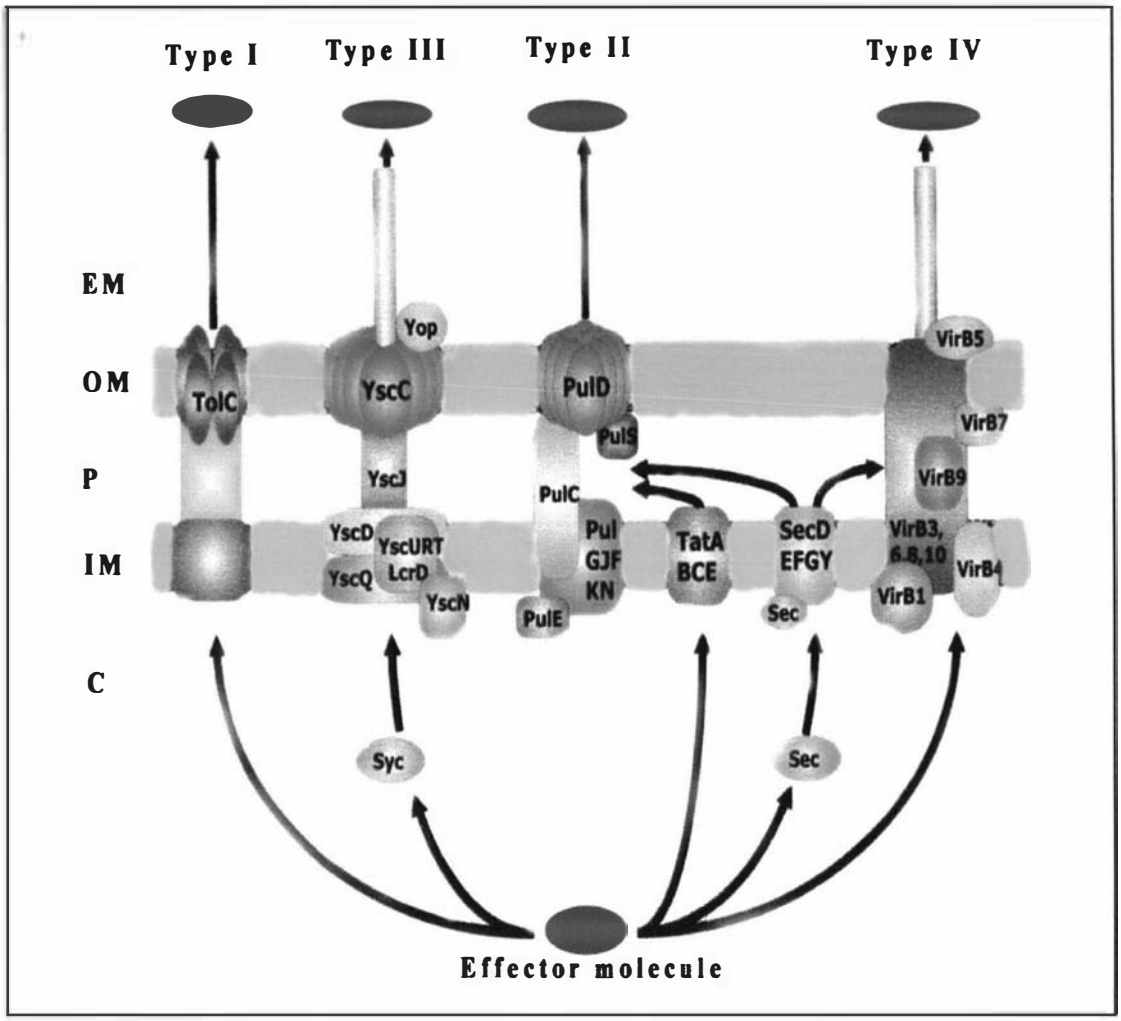

Figure 1.4. Schematic diagram showing types I, II, III and IV protein secretory systems in Gram-negative bacteria. EM, extracellular milieu; OM, outer membrane; P, periplasm; IM, inner membrane; C, cytoplasm. Adapted from Henderson *et al.*, 2004. See text for details.

1.7.11 Type V secretory systems

The type V secretory systems (T5SS) export proteins including proteases, toxins, adhesins and invasins. Substrates of T5SS include ShdA (involved in adherence) and ApeE (esterase) of *S. enterica*, and VacA (vacuolating cytotoxin) of *Helicobacter pylori*. Pathogenicity islands including SPI-3 of *S. enterica,* LPA of pathogenic *E. coli*, and SHI-1 of *Shigella flexneri* encode T5SSs (Henderson *et al.*, 2004; Schmidt & Hensel, 2004). The N-terminal SP of T5SS substrates have an extra amino acid stretch of approximately 25 residues upstream of the amino terminus of the classical SP (Jacob-Dubuisson *et al.*, 2001). The T5SSs are of 3 types: autotransporter secretion pathway (Va), two-partner secretion pathway (Vb), and Vc (AT-2). In the Va system, the entire transport system and the substrate protein are synthesized as a single large polyprotein containing 3 domains: an N-terminal SP, a passenger domain, and a C-terminal transporter domain that contains a linker region and a β-barrel. The N-terminal SP directs the secretion of the polyprotein across the inner membrane via the Sec system in to the periplasm. After proteolytic cleavage of the SP, the β-barrel of the transporter domain forms a β-barrel pore in the outer membrane, and the passenger domain passes through this pore. Finally, proteolytic cleavage allows the release of the passenger domain into the extracellular space (Henderson *et al.*, 2004; Schmidt & Hensel, 2004). In the two-partner pathway, the passenger domain with the SP and the protein forming the β-barrel are synthesized as two separate proteins. After the passenger protein is secreted across the inner membrane by the Sec pathway, it inserts into the pore formed by its partner protein and passes through this pore to the extracellular environment. The passenger protein folds at the cell surface as it is translocated through the transporter domain (Jacob-Dubuisson *et al.*, 2001). The Vc system includes the members of the oligomeric coiled-coil adhesins that have been described as a subfamily of surface-attached oligomeric auto transporters. The linker region of the transporter forms a β-barrel pore consisting 12 β-strands after trimerization and confers a lollipop-like structure on the proteins displayed on the cell surface (Henderson *et al.*, 2004). Figure 1.5 shows the 3 classes of type V secretory systems.

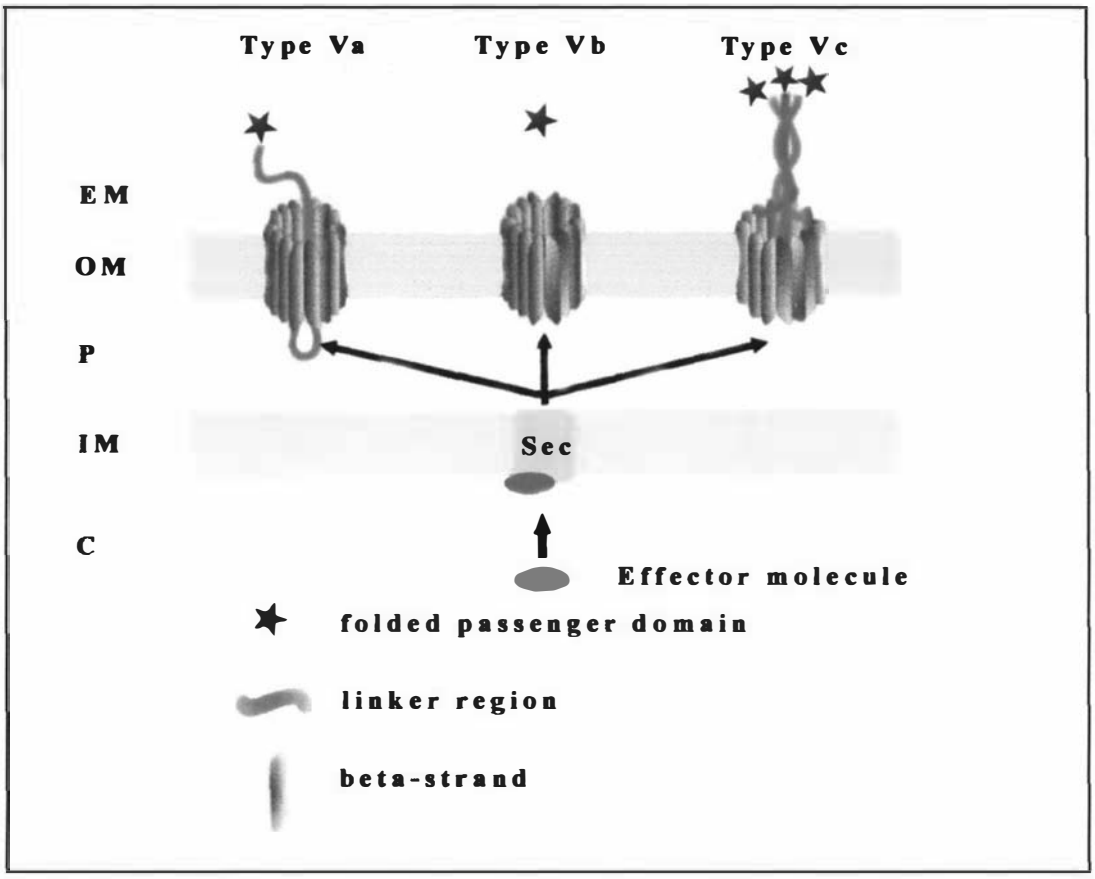

Figure 1.5. Schematic diagram showing type V secretory systems. EM, extracellular milieu; OM, outer membrane; P, periplasm; IM, inner membrane; C, cytoplasm. Adapted from Henderson *et al.*, 2004. See text for details.

1.8 Virulence factors of *Salmonella*

The ability of *Salmonella* to cause disease and the severity of salmonellosis depend on the virulence of the strain, and host factors. Virulence is determined by factors such as flagellae, fimbriae, endotoxins, exotoxins, virulence plasmids, Vi antigen, and pathogenicity islands (van Asten & van Dijk, 2005). At least 4% of the *Salmonella* genome (about 200 genes) is involved in virulence (Beuzon & Holden, 2001; Haimovich & Venkatesan, 2006).

1.8.1 Flagellae

1.8.1.1 Structure and assembly

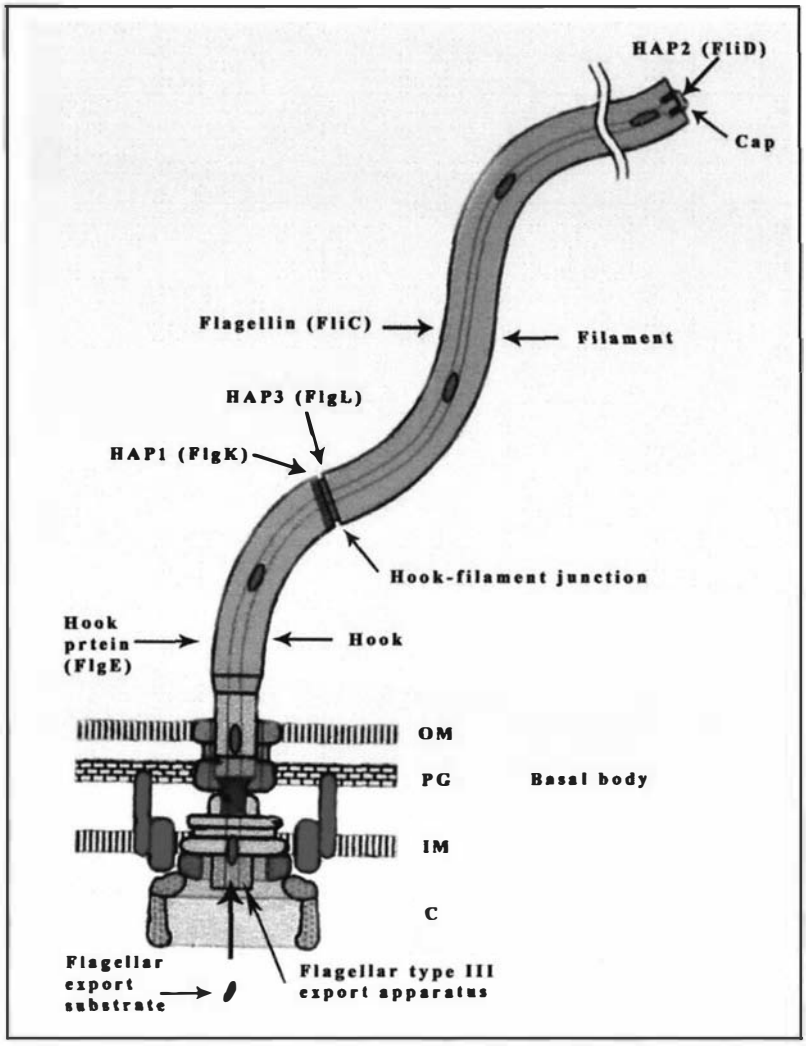

Figure 1.6. Schematic diagram showing the components of the bacterial flagellum. OM, outer membrane; PG, peptidoglycan layer; IM, inner membrane; C, cytoplasm. Adapted from Yonekura *et al.*, 2002. See text for details.

Flagellae are sub cellular organelles that originate in the cell membrane and extend 15-20 μm from the cell surface (Silverman & Simon, 1977). *S.* Typhimurium has approximately 6 to 10 flagellae that are peritrichously arranged around the cell. The bacterial flagellum (Figure 1.6) contains a continuous interior channel of about 3 nm, and is composed of three main parts: the basal body and a motor, the flexible hook (universal joint), and the filament (corkscrew-like propeller) (McMurry *et al.*, 2004). The basal body crosses both cytoplasmic and outer membranes. The hook and the

filament form the extracellular structure. The assembly of the flagellum starts from the formation of the membrane-supramembrane (MS) ring in the cytoplasmic membrane. Then it proceeds in inward, outward and lateral directions. The inward assembly involves the formation of the cytoplasmic (C) ring in the cytoplasm. The flagellar type III protein export apparatus is then formed within the C-ring. The lateral assembly involves the MotA/MotB complex in the inner membrane. The outward assembly of the flagellar axial structure including rod, hook, hook-filament junction, filament and filament cap is the major event in the formation of the flagellum in that order. The rod is connected to the FliF ring proximally and to the hook distally. The hook is formed through the assembly of FlgE. FlgD forms a capping complex at the distal end of the hook until it grows up to a length of about 55 nm. FlgD cap falls off and is replaced by FlgK, and then by FlgL and FliD in that order (Yonekura *et al.*, 2002).

The filament is an approximately 10-15 μm long tubular structure made up of 11 chains or pro-filaments. It is a polymer of approximately 20,000 subunits of either phase-1 (FliC) or phase-2 (FljB) flagellin (Bonifield & Hughes, 2003; Le Moigne *et al.*, 2006), and assembles in about 10 minutes. The growth of filament occurs at the distal end. Flagellin subunits that are synthesized in the cell travel through the central channel of the hook to its distal end and then are polymerised sequentially. Flagellin monomers may transit partially unfolded through the channel to its tip, where they refold and insert into the growing filament (Blocker *et al.*, 2003). The cap of the filament (FliD) stays attached to the distal end during filament growth. It is believed that the cap prevents leakage of flagellin (Yonekura *et al.*, 2002). Although most of the secreted flagellin is used for the assembly of the flagellum, flagellin can also accumulate in the extracellular medium due to leakage (Gómez-Gómez & Boller, 2002). Flagellin has a strong tendency to form the filament, but polymerisation occurs only when the monomer concentration reaches critical concentration. FliI provides the energy to keep the flagellin concentration above the critical concentration through ATP hydrolysis (Yonekura *et al.*, 2002). In *Salmonella*, flagellin (FliC and FljB) is a 55-kDa monomer encoded by *fliC* and *fljB* genes (Zeng *et al.*, 2003). Flagellae require approximately 8% of the total *Salmonella* protein for its construction (Cookson & Bevan, 1997).

1.8.1.2 Role of flagellae in virulence

Flagellae are organelles of locomotion (swimming and swarming motilities), and have multiple roles in pathogenesis (Kirov, 2003). Flagellae mediate tactic behaviour, moving towards favorable or away from unfavorable conditions such as extreme pH or saline concentrations. Motility combined with chemotaxis enables pathogens to reach target mucosal tissues (Ramos *et al.*, 2004; Salazar-Gonzalez & McSorley, 2005; Yonekura *et al.*, 2002). Furthermore, flagellae contribute to pathogenesis through facilitating adhesion to epithelial cells, colonization, invasion, and biofilm formation (Rumbo *et al.*, 2006; Salazar-Gonzalez & McSorley, 2005). Flagellae may play a role in the secretion or production of exo-polysaccharide present in biofilms (Prouty *et al.*, 2002). In diphasic serotypes, FliC and FljB are alternatively expressed by a phase variation mechanism in a manner, that only either FliC or FljB is expressed at a time in a single organism (Bonifield & Hughes, 2003; Sojka *et al.*, 2001). Prior colonization with a different flagellate strain of *S. enterica* that resulted in an immune response can provide cross-immunity to other flagellated strains. Therefore, phase variation provides a mechanism for evasion of cross-immunity in this species that can facilitate colonization (Salaün *et al.*, 2003).

Salmonella flagellin plays a major role in mucosal and systemic inflammation. It is the major factor that activates the expression of pro-inflammatory genes including tumour necrosis factor-α (TNF-α), Interleukin (IL)-1, IL-6 and IL-8 in *Salmonella* infections (Eaves-Pyles *et al.*, 2001; Zeng *et al.*, 2003). The pathogen-associated molecular patterns (PAMPs) including flagellin present on *Salmonella* are sensed by pattern-recognition receptors (PRRs) known as Toll-like receptors (TLRs) of host cells (Gordon, 2002; Rochon & Römling, 2006; Underhill, 2003). The TLR5 is present on epithelial cells, monocytes, immature dendritic cells (DC), natural killer cells and T lymphocytes, and is the ligand for flagellin (Alaniz *et al.*, 2006; Bachmann *et al.*, 2006; Salazar-Gonzalez & McSorley, 2005). Upon adherence of *Salmonella* organisms to the intestinal epithelium, flagellin monomers translocate through the epithelial cells, and bind to the TLR5 expressed exclusively on the baso-lateral surface of intestinal epithelial cells. This binding induces epithelial cells to produce the chemoattractant chemokine IL-8 (CXCL8). IL-8 expression is a marker and a mediator of mucosal

inflammation in the intestine. IL-8 attracts polymorphonuclear leukocytes (PMNs) from circulation into the sub-mucosa within hours of infection (Gewirtz *et al.*, 2001a, 2001b; Watson *et al.*, 1995). These PMNs can transmigrate, via bacteria-induced chemoattractants, to the luminal surface where they trigger fluid secretion leading to *Salmonella*-induced enteritis and diarrhoea. This innate immune response, even though undesirable, is essential for the clearance of bacteria to prevent the systemic spread (Zeng *et al.*, 2003). IL-8 production is markedly diminished in the absence of flagellin (Huang *et al.*, 2004). Low levels of TNF-α are protective, whereas increased production of this cytokine is associated with damage to the small intestinal epithelium (Ciacci-Woolwine *et al.*, 1999). Translocation of *Salmonella* into the mesenteric lymphatics and the systemic circulation further activates the transcription of pro-inflammatory genes including TNF-α, IL-1 and IL-6 in mononuclear cells via flagellin-TLR5 binding (Gewirtz *et al.*, 2001a, 2001b; Honko & Mizel, 2004; McSorley *et al.*, 2002). Elevated levels of these cytokines result in systemic inflammation, pathological lesions (Akira & Takeda, 2004: Ramos *et al.*, 2004; Zeng *et al.*, 2003) and shock. High doses of flagellin induce shock characterized by hypotension, reduced vascular contractility, organ injury and death (Eaves-Pyles *et al.*, 2001).

1.8.2 Other virulence factors

Salmonella pathogenicity islands (SPI) are large gene cassettes within the *Salmonella* chromosome that encode factors responsible for bacterial virulence. Most *S. enterica* virulence factors are encoded by chromosomal genes, and many of these are located within pathogenicity islands (Schmidt & Hensel, 2004). To date, twelve SPIs termed SPI-1 to SPI-12 have been described (Hensel, 2004). While SPI-1 genes are necessary for the invasion of host cells (M cells and epithelial cells) (Marcus *et al.*, 2000; Zhou & Galán, 2001), SPI-2 genes are necessary for systemic infection (Amavisit *et al.*, 2003; Knodler & Steele-Mortimer, 2003). SPI-3 is required for the survival within macrophages and growth in low Mg^{2+} conditions, and SPI-4 is believed to be required for survival in macrophages (Soto *et al.*, 2006). Some SPI-4 gene products show a high similarity to CyaE, LipC, and LktB, which are the components of a T1SS for the secretion of toxins of RTX toxin family (Welch, 1991). It is believed that SPI-4 encodes a T1SS involved in toxin secretion and a cytotoxin that results in apoptosis of

macrophages (Wong *et al.*, 1998). SPI-5 encodes the effector protein SopB that plays a role in fluid loss and diarrhoea (Zhang *et al.*, 2003). SPI-6 contains a *saf* gene cluster encoding fimbriae, *pagN* encoding invasin and several genes of unknown functions. SPI-7 is specific for *Salmonella* serotypes Typhi, Paratyphi C and Dublin, and encodes Vi antigen, SPI-1 effector SopE and type IV pili. While, functions of SPI-8 and SPI-9 are unknown, SPI-10 encodes for Sef fimbriae. *Salmonella* genomic island 1 (SGI-1) encodes for antibiotic resistance genes, and high pathogenicity island (HPI) encodes for high-affinity iron uptake pathways (Hensel, 2004).

Fimbriae or pili are filamentous, hair-like surface organelles, and are polymers of fimbrin or pilin (Collinson *et al.*, 1996). *Salmonella* contains a number of different fimbriae including *agf, fim, lpf, sef* and *pef* (Bäumler *et al.*, 1997a) that are involved in adhesion and invasion of the mucous membranes (Jepson & Clark, 2001; Stanley *et al.*, 2000; van Asten & van Dijk, 2005). A very few strains of *Salmonella* such as *S.* Typhi, *S.* Paratyphi C, and a few strains of *S.* Dublin contain Vi polysaccharide on the outer membrane. This increases resistance to oxidative killing, reduces secretion of TNF-α, increases infectivity, and thus the severity of disease (Wain *et al.*, 2005). High molecular weight plasmids known as *Salmonella* virulence plasmids are present in a small number of *Salmonella* serotypes including Typhimurium, Choleraesuis, Dublin, Enteritidis, Gallinarum, Pullorum, and Abortusovis (Rychlik *et al.*, 2006). Virulence plasmids contain a highly conserved *Salmonella plasmid virulence* locus (*spvABCDR*) that is important for intracellular bacterial growth and systemic infection (Haneda *et al.*, 2001; Hochmann *et al.*, 2006). SpvB is an actin-ribosylating toxin (Lesnick & Guiney, 2001). Depending on the serovar, plasmids harbour a few other genes that encode virulence factors. These include the *pef* fimbriae involved in bacterial adherence to intestinal epithelial cells, the *rck* that encodes an OMP involved in resistance to complement, *Mig-5* (macrophage-inducible gene for a carbonic anhydrase) and *srgA* (putative disulphide bond oxidoreductase (Haneda *et al.*, 2001; Rychlik *et al.*, 2006).

Lipopolysaccharide (LPS, endotoxin) is a major surface-exposed structural component of the outer membrane of Gram-negative bacteria. It shows a large spectrum of biological activities, which are both beneficial and toxic (Ernst *et al.*, 2001; Link *et al.*,

2004). LPS is composed of three regions: the O-polysaccharide chain (O-antigen), the oligosaccharide core, and the lipid A moiety (Ernst *et al.*, 2001; Raetz & Whitfield, 2002). The length of O-antigen determines the clearance of *Salmonella* by complement (Cirillo *et al.*, 1996), and influences the uptake of *Salmonella* by macrophages (Murray *et al.*, 2006). The lipid A moiety binds to the TLR4 present on host cells such as macrophages and DCs inducing secretion of pro-inflammatory cytokines including TNF-α, IL-1, and IL-6 (Basset *et al.*, 2003; Ernst *et al.*, 2001). Cytokines, when in excess lead to endotoxic shock and death (Ernst *et al.*, 2001; Nomura *et al.*, 2000). In addition to endotoxins, studies have reported the presence of two *Salmonella* exotoxins, namely cytotoxin and enterotoxin (van Asten & van Djik, 2005). However, in most cases, these factors have not been purified and their role in *Salmonella* infections has not been studied (Darwin & Miller, 1999). Cytolysin A (ClyA) is a haemolytic and pore-forming cytotoxin encoded by *clyA* gene in *S.* Typhi and *S.* Paratyphi A. ClyA cytotoxin is conserved in typhoid serovars while it is absent in many other serovars, including Typhimurium (Oscarsson *et al.*, 2002; Wyborn *et al.*, 2004).

1.9 Pathogenesis of salmonellosis

The most common route of *Salmonella* infection is through ingestion of the organism (Bao *et al.*, 2000; Eckmann & Kagnoff, 2001). However, infection can also occur through contamination of the conjunctivae and inhalation (Pardon *et al.*, 1990). After ingestion, a proportion of *Salmonella* organisms survives the low pH of the stomach and reaches the small intestine (Mastroeni, 2002; Mastroeni & Sheppard, 2004). Subsequently, organisms initiate infection of the host by inducing their uptake into non-phagocytic M cells and enterocytes. The most common route is through M cells, which overlie Peyer's patches (Jepson & Clark, 2001). Infection of intestinal epithelial cells results in cell destruction, loss of absorptive surface, ulceration, and sloughing of the epithelium (Jepson & Clark, 2001; Wallis & Galyov, 2000). Epithelial damage provides *Salmonella* easy access to subepithelial tissues, resulting in unrestricted bacterial invasion (Jepson & Clark, 2001). A number of factors, including increased enterocyte fluid secretion, inflammatory response, and decreased epithelial barrier function are responsible for intestinal pathology (Huang *et al.*, 2004). As described previously, flagellin and LPS of *Salmonella* activate secretion of cytokines that initiate an intestinal inflammatory response leading to enteritis and diarrhoea (Gewirtz *et al.*,

2001a, 2001b; Zeng *et al.*, 2003). Uptake of *Salmonella* by M cells and enterocytes is followed by entry into phagocytes (Warren *et al.*, 2002; Yrlid *et al.*, 2001). *Salmonella* can survive and replicate in both phagocytic and non-phagocytic cells. Immediately following invasion, individual *Salmonella* organisms are found within *Salmonella*-containing vacuoles (SCV) (Knodler & Steele-Mortimer, 2003). They replicate within these vacuoles throughout the infection thereby being protected from antibodies and cell-impermeable antibiotics (Sirard *et al.*, 1999). *Salmonella* travel to distant sites within mononuclear phagocytes such as macrophages and DCs (Popiel & Turnbull, 1985). From the intestine, *Salmonella* spread to mesenteric lymph nodes (MLN), and then to visceral tissues including liver and spleen where they are present mainly in macrophages (Eckmann & Kagnoff, 2001; McSorley & Jenkins, 2000). Later in the infection, *Salmonella* are seen in granulomas that are rich in macrophages (Mastroeni, 2002). The outcome of infection depends on bacterial and host factors including infecting serovar, infective dose, route of infection, age, and the ability of the host to mount an adequate inflammatory and immune response (Eckmann & Kagnoff, 2001; Mastroeni & Sheppard, 2004; Soloski & Metcalf, 2001).

1.10 Immune responses to *Salmonella*

Both innate (non-specific) and adaptive (specific) immune responses are important to protect the host against *Salmonella*. The establishment of adaptive immunity is not rapid enough to eradicate pathogens as it involves cell proliferation, gene activation and protein synthesis. Innate immunity provides rapid defence mechanisms to prevent or limit an infection (Werling & Jungi, 2003). However, an acquired immune response comprising both cellular and humoral components is essential for effective elimination of *Salmonella* (Strindelius *et al.*, 2004a).

1.10.1 Innate immunity

The innate immune system includes the physical barrier function of the epidermis and mucosa that prevents penetration of the organism; physiological functions such as ciliary action, mucus secretion, motility and desquamation; and bactericidal activities of antimicrobial proteins or peptides such as defensins, lysozymes and lactoferrin.

These mechanisms act at extra-epithelial and epithelial levels to defend against the bacterial invasion (Basset *et al.*, 2003; Wick, 2004). Other components of the innate immune system include epithelial cells, plasma proteins such as complement that opsonise microbes, and effector cells. The effector cells include phagocytic cells (DCs, neutrophils and macrophages) and natural killer T-cells (Fierer, 2001; Ugrinovic *et al.*, 2003; Wick, 2004). *Salmonella* that evade the primary defence mechanisms at extra-epithelial and epithelial levels will adhere to the intestinal epithelial cells, and become internalised (Jepson & Clark, 2001; Strindelius *et al.*, 2004b). Innate immune responses induced at this level trigger a variety of responses. *Salmonella* internalised by phagocytes results in activation of antimicrobial killing mechanisms such as the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), up-regulation of co-stimulatory molecules on antigen-presenting cells (APC), maturation of naïve DCs, and stimulation of the production of inflammatory cytokines and chemokines that activate other immune cells and initiate the development of adaptive immunity (Basset *et al.*, 2003; Underhill, 2003). As described previously, the inflammatory response elicited in the intestine is necessary to limit the spread of the infection. In addition to IL-8, flagellin also induces intestinal epithelial cells to produce chemokine CCL20 (LARC, MIP-3α, exodus). This molecule is a chemoattractant for the migration of DCs into the subepithelial dome region of Peyer's patches, and is the ligand of CCR6 receptor present on immature DCs. DCs attracted to the affected area mediate phagocytosis of *Salmonellae*. As described previously, flagellin and LPS induce the secretion of TNF-α that acts as a recruitment factor for both macrophages and neutrophils (Cummings *et al.*, 2005; Li & Cherayil, 2003; Wick, 2004). The chemokine MCP-1 is released by intestinal cells and attracts macrophages (Sierro *et al.*, 2001).

Upon phagocytosis, both macrophages and neutrophils kill *Salmonella* by producing toxic ROS and RNS (Fierer, 2001; Vazquez-Torres & Fang, 2001). ROS including bactericidal superoxide and hydrogen peroxide are produced through the respiratory burst NADPH phagocytic oxidase (phox) (Fierer, 2001; Linehan & Holden, 2003; Vazquez-Torres & Fang, 2001). In vitro, *Salmonella* can evade killing by ROS through inhibiting the localization of NADPH oxidase to the phagosome. SPI-2 gene products inhibit the formation of NADPH oxidase by preventing the transport of components

necessary for its assembly (Foster *et al.*, 2003; Vazquez-Torres *et al.*, 2000). Nitric oxide synthase (iNOS, encoded by NOS2 gene) catalyzes the reaction that leads to the release of RNS (Linehan & Holden, 2003). Lysosomal enzymes and defensins also contribute to macrophage-dependent killing of *Salmonella* (Groisman *et al.*, 1992). PMNs contain azurophil, and gelatinase granules that contain proteases, antimicrobial proteins, and enzymes. Proteases such as neutrophil elastase and cathepsin G degrade bacterial proteins including virulence factors (Mayer-Scholl *et al.*, 2004). Cytokines TNF-α, IFN-γ, IL-12 and IL-18 are important in the innate responses against salmonellosis (Eckmann & Kagnoff, 2001; Raupach *et al.*, 2003; Vasquez-Torress *et al.*, 2001). Both TNF-α and IFN-γ activate macrophages to enhance killing of intracellular *Salmonella* (Basset *et al.*, 2003; Vazquez-Torres *et al.*, 2001). Macrophages and neutrophils are the main sources of TNF-α during the early stages of *Salmonella* infection (Wick, 2004). DCs are another source of TNF-α (Rescigno *et al.*, 2002). Both activated macrophages and DCs release IL-12, which activates natural killer (NK) cells and natural killer T (NKT) cells. While, NK cells are cytotoxic and are sources of both TNF-α and IFN-γ (Basset *et al.*, 2003), NKT cells are one of the early sources of IFN-γ (Brigl *et al.*, 2003). IFN-γ is a cytokine that enhances the phagocytic oxidative killing of *Salmonella* at the level of NADPH phagocytic oxidase-producing genes (Condino-Neto & Newburger, 2000). IFN-γ reduces the establishment of infection in macrophages by stimulating an enhanced phagocytic oxidase response leading to an increased and prolonged oxidative burst. In addition, IFN-γ decreases the time between invasion and the induction of oxidative burst. It increases the expression of IFNγ-receptor-α that increases the efficiency of IFN-γ utilization. Uninfected macrophages respond to low levels of IFN-γ that occurs early in infection to produce ROS (Foster *et al.*, 2003).

1.10.2 Adaptive immunity

The early, innate immune response is important for the development of an effective adaptive immune response (Medzhitov & Janeway Jr, 2000). Adaptive immune responses are initiated when T- and B-lymphocytes recognize foreign antigens expressed on APCs (Cuadros *et al.*, 2004). Both cell-mediated and humoral immune responses (systemic and mucosal) that are conferred by T- and B-lymphocytes

respectively are necessary to clear a *Salmonella* infection completely. Since, *Salmonella* is an intracellular pathogen, cell-mediated immune (CMI) responses are the most important for its clearance (Kotton & Hohmann, 2004; Mittrücker & Kaufmann, 2000). Clearance of bacteria from the tissues can result in long-lasting specific immunity to rechallenge. Resistance to re-infection with virulent *Salmonella* requires CD4+-dependent T-helper 1 (Th1) memory cells, CD8+ T-cells and anti-*Salmonella* antibodies (Mastroeni *et al.*, 1993, 2000a, 2000b).

1.10.2.1 T-cell mediated responses

The induction of acquired, predominantly T-cell mediated immune responses is essential for the clearance of *Salmonella* through activation of phagocytic cell defences and direct killing of infected host cells (Hess *et al.*, 1996; Mastroeni *et al.*, 1993). Both CD4+ and CD8+ T-lymphocytes, with the contribution of TNF-α and IFN-γ are needed for a complete acquired resistance to *Salmonella* (Mastroeni *et al.*, 1993). T-cell responses are seen against antigens including flagellae, fimbriae, OMPs and LPS (Mastroeni & Ménager, 2003). Mice containing defects that affect cellular immunity including T-cell receptor, major histocompatibility complex (MHC)-II and IFN-γ receptor knockouts are incapable of clearing *Salmonella* infections (McSorley & Jenkins, 2000). T-cell activation begins with the recognition of peptides bound to MHC molecules presented on an APC. DCs and macrophages are the main APCs (Hopkins *et al.*, 2000). DCs present antigens to naïve T-cells during the recognition phase to initiate T-cell responses while macrophages present antigens to differentiated (effector) T-cells during the effector phase of CMI (Rescigno *et al.*, 2001; Yrlid & Wick, 2000). Macrophages and immature DCs present in the subepithelial dome region of Peyer's patches phagocytose *Salmonella* that are transported through M cells. Furthermore, subepithelial phagocytes are able to capture *Salmonella* antigens transported across the follicle-associated epithelium (FAE) (Banchereau & Steinman, 1998;Banchereau *et al.*, 2000). Immature DCs are the most efficient APCs that have the ability to phagocytose, process and present antigens, migrate from peripheral tissues to lymphoid organs, and induce a primary immune response (Banchereau & Steinman, 1998; Banchereau *et al.*, 2000; Clark *et al.*, 2001). They have significant surface expression of MHC-I and MHC-II, and express low to intermediate levels of co-stimulatory molecules such as CD80 (B7-1), CD86 (B7-2), CD40 and CD54 (ICAM-

1). Inflammatory cytokines such as TNF-α and IL-1, produced by monocytes and macrophages present in the lamina propria in response to flagellin, induce the maturation of DCs (Sierro *et al.,* 2001; Sundquist *et al.,* 2004). It is unknown whether flagellin induces DC maturation directly via the TLR5 expressed on the DCs or indirectly through the cytokines produced by other cell types (Didierlaurent *et al.*, 2004; Means *et al.*, 2003). DC maturation is characterised by loss of adhesiveness, down-regulation of antigen-capture capacity, and up-regulation of antigen presentation. Furthermore DC maturation shows an up-regulation of co-stimulatory molecules such as B7, ICAM-1, IL-12, and CCR7 chemokine receptor. The CCR7 receptor is specific for chemokines produced in the T-cell areas of lymph nodes and mediates migration of activated DCs to the draining lymph nodes. Mature DCs have the ability to activate naïve T-cells present in the lymph nodes (Vazquez-Torres *et al.*, 1999). A recent study showed that *Salmonella*-infected DCs show an increased secretion of chemokine CCL22 that attracts T-cells and enhances contact between DCs and mature T-cells through CCR4 (Fu *et al.,* 2005).

It has been shown that both CD4+ and CD8+ T-cells are important for resistance against *Salmonella* infections (Mastroeni *et al.*, 2000a, 2000b). APCs present *Salmonella*-derived antigens on MHC-I and MHC-II to CD8+ cytotoxic (CTL) and CD4+ helper T-cells respectively (Svensson & Wick 1999; Yrlid *et al.*, 2001). DCs can present *Salmonella* antigens either directly or indirectly. They directly present antigens to T-cells upon phagocytic processing of *Salmonella. Salmonella* can be cytotoxic to infected macrophages leading to apoptotic death. This makes macrophages unable to present antigens to T-cells (Yrlid & Wick 2000). DCs have the ability to act as bystander APCs through processing and presenting phagocytosed apoptotic material containing *Salmonella* antigens. Therefore, antigen presentation by non-infected bystander cells provides a mechanism to present *Salmonella* that has killed infected cells (Sundquist *et al.*, 2004). MHC-I and MHC-II molecules on APCs present peptides derived from proteins synthesized in the cell (endogenous) and phagocytosed proteins (exogenous) respectively. Cytoplasmic proteasomes process endogenous antigens, which are then transported to endoplasmic reticulum for MHC-I binding. Since *Salmonella* reside in phagosomes in macrophages and DCs, it is not clear how antigens gain access to the cytoplasm for the presentation on MHC-I. It is

believed that antigens leak into the cytoplasm through the SCV membranes (Soloski & Metcalf, 2001). These will then be processed and presented on the surface with MHC-I. Furthermore, bystander DCs seem to be important for the cross-presentation of *Salmonella* antigens obtained from infected apoptotic cells with MHC-I to activate CD8+ T-cells (Yrlid *et al.*, 2001; Winau *et al.*, 2004). The MHC-II molecules bind antigenic peptides from exogenous antigens such as internalised bacteria within the late endosomes/lysosomes where they are processed by lysosomal enzymes. Lipids are presented to T-cells with CD1 molecules that are primarily present on DCs and some B-cells (Soloski & Metcalf, 2001).

Signals needed for T-cell activation are: interaction of MHC antigen-peptide complex with the T-cell receptors, presence of adhesion molecules that increase APC-T-cell interactions, and secondary signals generated by the co-stimulatory molecules (CD80, CD86, CD40) and cytokines (IL-1) (Geppert *et al.*, 1990). Activated CD4+ and CD8+ T-cells release IL-2, which is responsible for the proliferation and differentiation of T-cells into effector and memory cells. There are two subsets of CD4+ helper T-cells, namely Th1 and Th2, that induce cell-mediated and antibody responses respectively (Basset *et al.*, 2003). Th1- and Th2-cells produce predominantly IFN-γ and IL-4 respectively (Das *et al.*, 2001; Medina *et al.*, 2000). The two main cytokines that have an effect on the direction of acquired response towards Th1 and Th2 are, IL-12 and IL-4 respectively. The innate immune system influences the type of Th response elicited. The sources of IL-12 and IL-4 originate from the innate immune system, with macrophages and DCs producing IL-12, and basophils and mast cells producing IL-4. While the main cytokines produced by Th1-cells are IL-1, IL-2, IFN-γ and TNF-α, those produced by Th2-cells are IL-4, IL-5, IL-6, IL-10 and IL-13 (Basset *et al.*, 2003). The cytokines IFN-γ, and IFN-γ inducing cytokines such as IL-12, IL-18 and IL-15 play an important role in the CMI responses against *Salmonella* (Eckmann & Kagnoff, 2001; Mittrücker & Kaufmann, 2000; Mizuno *et al.*, 2003). IL-12 induces Th1 differentiation and IFN-γ production from Th1 and NK cells. IL-12 together with IL-18 strongly induces IFN-γ production by Th1-cells (Dinarello, 1999; Mizuno *et al.*, 2003). IL-15 is produced by monocytes/macrophages, and induces proliferation of Th1-cells resulting in IFN-γ production (Mizuno *et al.*, 2003) that in turn inhibits the differentiation of Th2-cells (Das *et al.*, 2001). Low levels of IFN-γ come from NK

cells early in infection, while high levels of IFN-γ come from activated Th1-cells later in infection (Kaufmann, 1993). IFN-γ, together with TNF-α, activates macrophages to kill *Salmonella*-infected cells (Mittrüker *et al.*, 2002; van de Vosse & Ottenhoff, 2006). Furthermore, IFN-γ increases the expression of MHC-II on macrophages, which in turn increases antigen presentation to T-cells. Thus, the induction of IFN-γ producing CD4+ T-cells is critical for the resolution of infection (Hess *et al.*, 1996; McSorley & Jenkins, 2000). In an IFN-γ gene knockout mouse model, host immunity was impaired in IFN-$\gamma^{-/-}$ mice following *S.* Typhimurium challenge despite elevated antibody responses in both mucosal and systemic compartments (Bao *et al.*, 2000). IFN-γ and IL-12 are crucial for host resistance to *Salmonella* infection in humans. Deficiencies in IFN-γ receptors and IL-12 p40 subunit predispose humans to salmonellosis (Mastroeni & Ménager, 2003; van de Vosse & Ottenhoff, 2006).

CD8+ T-cells participate in protection against *Salmonella* (Winau *et al.*, 2004). Beta2 microglobulin (β2m)-/- mice deficient in CD8+ T-cells were found to be more susceptible to infection with *S.* Typhimurium, and exhibited impaired protection when they were challenged with a virulent strain (Lo *et al.*, 1999). Mechanisms underlying *Salmonella*-specific CD8+ T-cell effector functions are poorly understood. Because *Salmonella* is a facultative intracellular pathogen, CTL-mediated lysis of infected cells via perforin could be one of the mechanisms likely to contribute to clearing infections, by releasing bacteria from their protective habitat and thus rendering them accessible to activated macrophages and specific antibodies (Pasetti *et al.*, 2002). Furthermore, activated CD8+ T-cells release granulysin that directly kills intracellular *Salmonella*, and produce cytokines such as IFN-γ to activate macrophages (Mittrücker *et al*, 2002; Winau *et al.*, 2004). *Salmonella* infections induce T-cell activation, with 20-30% of CD4+ and CD8+ T-cells producing IFN-γ 3 to 4 weeks after infection (Wong and Pamer, 2003). The CMI responses can be measured by assays including IFN-γ, IL-12, delayed type hypersensitivity (DTH), and lymphocyte proliferation assays (Babu *et al.*, 2003; Le Moigne *et al.*, 2006).

1.10.2.2 B-cell responses

Mucosal immunoglobulin (Ig) A and serum IgG antibodies produced by plasma cells improve protection against *Salmonella* infections (Mittrücker *et al.*, 2000; Spreng *et al.*, 2006). Antibodies accelerate the clearance of secondary infections through effector functions that occur extracellularly (McSorley & Jenkins, 2000). They bind to the surface of infecting organisms to prevent them from attaching to and invading host cells, to enhance their engulfment and killing by phagocytes, and to activate complement-mediated killing (McGhee *et al.*, 1992; Michetti *et al.*, 1992; Lehmann *et al.*, 2006). Humoral immune responses are initiated by the recognition of antigens by B-lymphocytes in the mucosal lymphoid tissues for ingested and inhaled antigens. Antigens bind to the membrane IgM and IgD receptors on naïve B-cells, which leads to the activation of cells following T-cell cooperation to proliferate and differentiate into plasma cells and memory cells. Cross-linking of antigen receptors (IgM, IgD) on B-cells by the antigen activates them. Protein antigens that do not have the ability to cross-link antigen receptors need to be internalised, processed and presented with MHC-II molecules to CD4+ T-cells. IL-4 is needed for the proliferation of Th2 population. Th2-cells stimulate B-cell proliferation, and differentiation into plasma cells and memory B-cells, isotype switching and affinity maturation through co-stimulatory molecules and cytokines (Cosgrove *et al.*, 1991). Activated B-cells proliferate and differentiate further on their route through regional lymph nodes before they reach mucosae via peripheral blood. At these sites, they complete their terminal differentiation into plasma cells and secrete dimeric IgA. The latter crosses the epithelium and is released into external secretions as secretory-IgA (s-IgA) (Wijburg *et al.*, 2006). Mucosal humoral immune responses play an important role in the host defence against *Salmonella* (Iankov *et al.*, 2004). Secretory-IgA can block bacterial adhesion to epithelial cells through agglutination of bacteria, which are then entrapped in mucus, and cleared by peristalsis. It is also possible that opsonisation with antibodies leads to more effective uptake and destruction of bacteria by phagocytes in gut-associated lymphoid tissues (Mittrücker *et al.*, 2000; Strindelius *et al.*, 2002).

Mucosal and serum antibody responses are seen against *Salmonella* components including LPS, flagellae, fimbriae, OMPs and Vi antigens (Bergman *et al.*, 2005; Ochoa-Repáráz *et al.*, 2005). Serum antibody responses can be seen as early as 1-week

post-infection and can persist for 10 weeks or more in poultry, pigs, cattle and sheep (Hassan *et al.*, 1991). Serum IgM appears first, followed by IgG and IgA (Withanage *et al.*, 2005). The IgM and IgA levels decline, while IgG levels can persist for extended periods (Hassan *et al.*, 1991). Cytokines produced affect the antibody isotype produced by B-cells. Th1-cell cytokine IFN-γ results in high levels of IgG2a and IgG3 subclasses, while Th2-cell cytokine IL-4 results in those of IgG1 subclass and IgA (Finkelman *et al.*, 1988; vanCott *et al.*, 1998). Serum antibodies can pass into egg yolk (Skov *et al.*, 2002), colostrum (Nielsen *et al.*, 2004) and to milk (Kranker *et al.*, 2003; Veling *et al.*, 2000). Serum antibody responses to different *Salmonella* species show cross-reactivity to LPS (Barrow, 1992). Cross-reactivity is less pronounced in responses against flagellin, although some degree of cross-reaction does occur (Thorns *et al.*, 1996; Yap *et al.*, 2001). In serum, LPS antibodies persist longer than flagellar antibodies (Dalby *et al.*, 2005). Reinfection results in a rapid, enhanced antibody response (Hassan *et al.*, 1991). In addition to antibody production, B-cells are involved in immune responses including antigen presentation and production of cytokines (Mittrücker *et al.*, 2000; Ugrinovic *et al.*, 2003). By the production of cytokines such as IFN-γ, B-cells in the gut-associated lymphoid tissue (GALT) can activate phagocytes and induce bactericidal mechanisms in these cells (Yoshimoto *et al.*, 1997). Naïve B-cells have no ability to take in, process and present the whole *Salmonella* organism to T-cells (Ugrinovic *et al.*, 2003). B-cells can acquire antigen from DCs after synapse formation (Batista *et al.*, 2001; Dustin & Dustin, 2001). Therefore, it is possible that naïve B-cells acquire *Salmonella* antigens from other APCs and present them to T-cells. *Salmonella*-activated B-cells express high levels of MHC and co-stimulatory molecules including CD86, and present *Salmonella* antigens to CD4+ T-cells. Furthermore, binding of B-cell receptor (antibodies) with antigen and CD40 with CD154 (CD40 ligand) expressed on activated T-cells rapidly induces the expression of CD86 on B-cells, which in turn further increases the T-cell responses (Ugrinovic *et al.*, 2003).

1.11 Vaccination against salmonellosis

Vaccination against salmonellosis is an effective tool for the prevention of disease. Currently available vaccines against salmonellosis can be divided into three groups:

whole-cell killed, live attenuated, and subunit vaccines. Whole-cell killed vaccines contain bacterial cells inactivated with heat, acetone or formalin (Mastroeni & Ménager, 2003). Currently available killed *Salmonella* vaccines include Salenvac© against *S.* Enteritidis in poultry, Salmovin against *S.* Abortusovis in sheep, and Salvexin™+B against serotypes Typhimurium, Bovismorbificans, Hindmarsh and Brandenburg in sheep (Marchant *et al.*, 2002; Woodward *et al.*, 2002). Live attenuated vaccines are prepared by introducing mutations in known virulent genes. These include *aroA, aroC, aroD* (aromatic amino acid biosynthesis), *htrA* (heat shock proteins), *aroA htrA* double mutants, *purA, purE* (purine metabolism enzymes), *ompR*, *crp/cya* (regulatory proteins) and *phoP/phoQ* (two-component regulator systems) (Hess *et al.*, 1996; Mastroeni *et al.*, 1998; Sinha *et al.*, 1997). Currently licensed Typhi oral vaccine Ty21a has an unrecognized mutation that was achieved by chemical mutagenesis (Cao *et al.*, 1992; Garmory *et al.*, 2002; Tacket *et al.*, 2000). Humans immunized with live oral typhoid vaccines have circulating IgG, IgM, and IgA antibodies and cellular immunity as well as s-IgA antibodies against *S.* Typhi (Michetti *et al.*, 1992). STM1 is an *aroA*- mutant of *S.* Typhimurium vaccine that is commercially available for use in the livestock industry including poultry (Bachtiar *et al.*, 2003). *S.* Gallinarum 9R is a commercially available live vaccine against fowl typhoid. A recent study has shown that mortality of vaccinated chickens 21-days post challenge was 0-5% when compared to control group with a mortality of 95-100% (Lee *et al.*, 2005). A single dose of live *aro*-mutant *S.* Typhimurium has been shown to reduce mortality and the prevalence of diarrhoea in sheep due to *S.* Typhimurium (Begg *et al.*, 1990). Calves vaccinated with a *S.* Typhimurium strain lacking the DNA adenine methylase (*Dam*) gene showed a significant attenuation of clinical disease, and a reduction in both faecal shedding and colonization of the mesenteric lymph nodes against challenge with a heterologous *S.* Dublin challenge (Mohler *et al.*, 2006).

Live attenuated *Salmonella* vaccines generally confer better protection than killed vaccines, because the former stimulates both cell-mediated and humoral immunity at both mucosal and distant sites (Mastroeni *et al.*, 2001; Norimatsu *et al.*, 2004; van Immerseel *et al.*, 2005). Killed vaccines induce an IL-4 dominated Th2-type response with high levels of antibodies of the IgG1 isotype, poor CMI responses and low IgA levels (Deitemeyer, 2004; Mastroeni & Ménager, 2003). Live attenuated vaccines

induce strong and sustained Th1-type responses, with the production of IL-12, TNF-α, IFN-γ and high levels of specific antibodies of the IgG2a isotype in animal models (Lehmann *et al.*, 2006; Mittrücker & Kauffmann, 2000; Pasetti *et al.*, 2002; Salerno-Goncalves *et al.*, 2003). The increased protection elicited by live vaccines is most likely a result of the combination of antigen expression and biasing the immune response towards Th1-type responses (Norimatsu *et al.*, 2004). However, there are some commercial inactivated *Salmonella* vaccines that are shown to be protective. This protective role may be due to specific antibodies and/or adjuvants that induce a sufficient unspecific CMI response (Lehmann *et al.*, 2006).

Subunit vaccines contain purified bacterial components. They are safer to use than live attenuated vaccines, since the latter in some instances revert to virulence causing disease (Okamura *et al.*, 2003). Subunit vaccines containing OMPs, porins, fimbriae, flagellin, and cell extracts have been tested in experimental animal models including poultry, mice, rabbits and calves (Chaturvedi *et al.*, 1994; De Buck *et al.*, 2005; Li *et al.*, 2004; Meenakshi *et al.*, 1999; Ochoa-Repárárz *et al.*, 2005; Strindelius *et al.*, 2004a, 2004b; Thukral *et al.*, 1998; Udhayakumar and Muthukkaruppan 1987). Immunization of chickens with OMPs has been shown to reduce caecal colonization, and shedding of *S.* Enteriditis in poultry (Khan *et al.*, 2003; Meenakshi *et al.*, 1999). Furthermore, OMPs of *S.* Gallinarum in chickens have been shown to be more protective than a formalin-killed whole-cell bacterin (Bouzoubaa *et al.*, 1987). Mucosal immunization of chickens with liposome-associated fimbrial antigens has been shown to elicit protective immunity against *S.* Enteriditis (Li *et al.*, 2004). Laying hens immunized with type I fimbriae showed a reduction of reproductive organ colonization and egg contamination with *S.* Enteriditis (De Buck *et al.*, 2005). Another recent study showed that histidine-tagged *Salmonella* fimbriae SafB/D recombinant protein induced protective immune responses in BALB/C mice against *S.* Enteritidis (Strindelius *et al.*, 2004b). Mucosal immunization of C3H/HeJ mice with flagellin showed a lower degree of infection than the control group upon challenge with *S.* Enteritidis. Immunized mice showed the production of IFN-γ from splenocytes, high levels of s-IgA, and a relatively low ratio of IgG1/(IgG2a+IgG2b) (Strindelius *et al.*, 2004a). A recent study showed that an antigenic extract enriched in surface antigens were protective in mice against *S.* Enteritidis. Immunized mice had higher levels of

IgG2a antibodies than IgG1, in agreement with the levels of IFN-γ and IL-4 released from spleen cells indicating a bias towards Th1 immune responses (Ochoa-Repáráz *et al.*, 2005). Okamura and his co-workers (2003) showed that the CMI responses of chicken vaccinated with a crude protein extract and OMPs of *S.* Enteritidis were mainly against flagella. Responses were determined by lymphocyte proliferation and the production of Th1-type cytokine IL-12, the central mediator of CMI (Okamura *et al.*, 2003). A recent challenge study in sheep immunized with *S.* Brandenburg crude cell wall fraction did not show significant protection against ewe deaths. However, the subunit vaccine resulted in a reduction in the number of ewes shedding the organism (Li *et al.*, 2005).

1.12 Diagnosis of salmonellosis

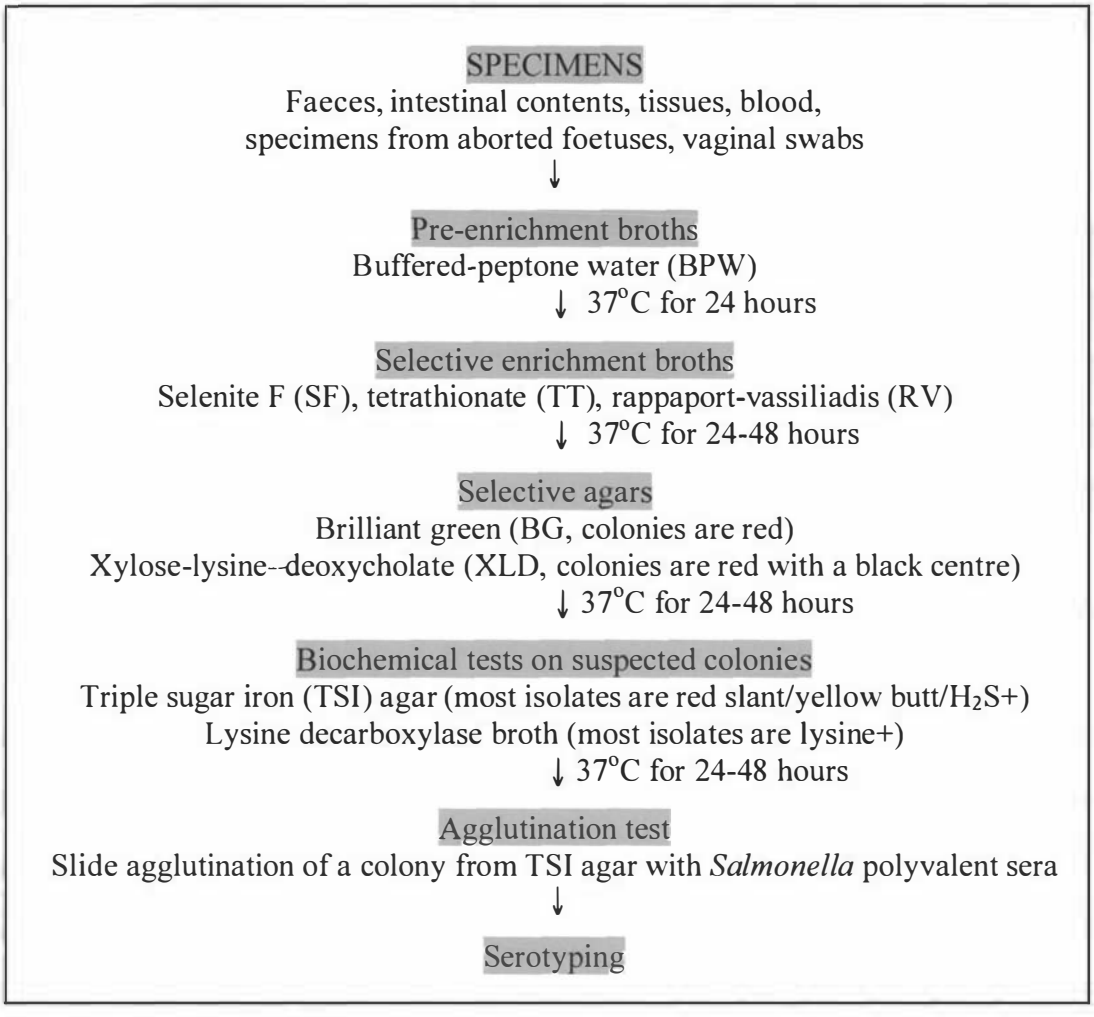

Figure 1.7. Schematic diagram showing the steps in isolation and identification of *Salmonella*.

1.12.1 Isolation and identification

Salmonellosis is diagnosed by isolation and identification of the organism (Figure 1.7). This procedure consists of four stages: pre-enrichment, selective enrichment, isolation on selective agars, and confirmation of the isolate by biochemical tests and serotyping (Duncanson *et al.*, 2003; Manzano *et al.*, 1998). Usually, each step requires 16-48 hours, taking approximately 5-7 days for completion (Maciorowski *et al.*, 2006; Schrank *et al.*, 2001). Depending on the sample type, some stages can be avoided. Samples suspected of having a large number of organisms for example faeces, cloacal swabs, tissues such as intestines, liver, spleen, mesenteric lymph nodes, heart blood, vaginal swabs, placentae, and tissues from aborted foetuses can be inoculated into selective enrichment media or selective agars. Samples including animal feed, eggs and environmental samples contain a low number of organisms that require all four stages of isolation. In sheep, samples including placentae and foetal stomach contents from cases of abortions are used to isolate and identify *S.* Brandenburg. Serotyping specifically identifies serovars on the basis of their somatic (O) and flagellar (H) antigens. The lipopolysaccharide O-antigen, together with phase-1 flagellar (H1) and phase-2 flagellar (H2) antigens, forms the basis for *Salmonella* serotyping. Each serotype has a unique combination of O, H1 and H2 antigens (Popoff & Le Minor, 2001). Initially, serotyping is carried out with polyvalent O- and H-antisera in separate slide agglutination tests. Subsequently, more agglutination tests are carried out to determine the serotype, using serovar-specific O- and H-typing antisera that react with groups of related antigens or a single antigen. Currently, fifty-four O-antigens and 114 H-antigens are used for *Salmonella* serotyping. In different combinations these result in 2523 serotypes (Popoff *et al.*, 2003). The antigenic formula of each serotype (O: H1: H2) is shown in the order of O, H1 and H2 antigens. Examples are [1], 4, [5], 12, [27]: 1, v: e, n, z15 (*S.* Brandenburg) and [1], 4, [5], 12: i: 1, 2 (*S.* Typhimurium) (http://www.salmonella.amg.gda.pl/salmonella-serowary.php).

1.12.1.1 O-antigen

The O-antigen is the outermost component of LPS, and is a polymer of the O-subunit. Each O-subunit is an oligosaccharide that is generally composed of between two and six sugars (Wang *et al.*, 2002). O-antigens are highly variable among *Salmonella*

serotypes and this feature has been used to classify them into serogroups or groups. The majority of isolates from humans and livestock belong to serogoups B, C, D and E (Kingsley & Bäumler, 2000). The O-antigen specificity is based on the composition and arrangement of sugars in the O-subunit, linkages between the O-subunits, and the addition of branch sugars and modifying side groups (Fitzgerald *et al.*, 2003). The O-groups are designated by the primary O-factors that are associated with the group. For example, all the serotypes of Group B contain O-antigen 4, and are designated as Group O: 4. The O-4 antigen is the major O-antigenic determinant of group B. Additional O-antigens are associated with some O-groups, and are often variably present or variably expressed. When multiple O-factors are present, they are listed sequentially and separated by commas (Centres for Disease Control and Prevention, 2002). An example is *S.* Abortusovis belonging to B group and containing O-antigens 4, 12 which is written as O: 4, 12 (http://www.salmonella.amg.gda.pl/salmonella-serowary.php). Variable O-factors are shown in parentheses. The variable regions of *rfb* genes are responsible for encoding enzymes that give the O-antigen specificity of a serogroup (Luk *et al.*, 1993).

1.12.1.2 H-antigen

The diphasic *Salmonella* serotypes contain two types of H-antigens known as H1 and H2 that are encoded by two non-allelic structural genes *fliC* and *fljB* respectively (Kutsukake *et al.*, 2006; Sojka *et al.*, 2001; Zeng *et al.*, 2003). Flagellin monomers of the flagellar filament are composed of conserved amino- (regions I, II) and carboxy-terminal domains (region VIII), and a central variable domain (regions III, IV, V, VI and VII) (de Vries *et al.*, 1998; van Asten *et al.*, 1995; Wei & Joys, 1985). Monomers fold into a hairpin-like conformation, with the conserved N- and C-terminal domains forming the internal walls of the filament, whilst the central variable domain forms the outside of the filament (Winstanley & Morgan, 1997). The N- and C-terminal domains of flagellin form packed α-helix structures, which constitute D0 and D1 domains, positioned in the filament core. The variable region is exposed as a β-sheet folded structure (D2 and D3 domains) on the filament outer surface (Ramos *et al.*, 2004). The central variable domain that varies in both amino acid sequence and size is responsible for flagellar antigenic variability among serotypes (van Asten *et al.*, 1995; Winstanley & Morgan, 1997). Some of the H-antigens are composed of a single antigenic factor

(b, c, d, i, r), while others are composed of multiple antigenic factors (l, v; l, w; e, h; e, n, x; e, n, z15; 1, 2) (Centres for Disease Control and Prevention, 2002). To date, 63 and 37 antigenic factors have been identified for phase–1 and phase–2 flagellin respectively (Dauga *et al.*, 1998). Different combinations of these 100 antigenic factors are responsible for 114 flagellar antigens. Flagellar antigens that are immunologically related are known as complexes. For example, the L complex includes all H-antigen types that contain antigenic factor l (l, v; l, w; l, z13), E complex includes antigenic factor e (e, n, x; e, n, z15; e, n, x, z15), and G complex includes antigenic factor g (g, m; f, g) (Centres for Disease Control and Prevention, 2002; McQuiston *et al.*, 2004). Studies have shown that regions IV and VI are hypervariable, and region V is moderately variable (Joys & Schödel, 1991). Wei and Joys (1985) showed that predicted amino acid sequence of region IV showed less than 32% homology for any pair-wise comparison of four different flagellins used in their study. Studies have shown that major serotype-specific flagellin antigenic determinants are located in the region IV (de Vries *et al.,* 1998; Newton *et al.,* 1991).

In diphasic serotypes, the two phases of flagellin are alternatively expressed by a phase variation mechanism in a manner, that only either phase-1 or phase-2 flagella is expressed at a time in a single organism (Bonifield & Hughes, 2003; Sojka *et al.*, 2001). This is due to the reversible inversion of a DNA segment known as the H segment that contains the *fljBA* promoter for *fljB* gene. The *fljBA* operon contains genes *hin*, *fljB* and *fljA* that encode Hin invertase, phase-2 flagellin and repressor for the unlinked *fliC* gene respectively. The H segment is flanked by the recombination sites *hixL* and *hixR*. The Hin invertase together with the recombination enhancer proteins Fis (factor for inversion stimulation) and HU, mediates the reversible recombination reaction between the *hix* sites, resulting in the inversion of the H segment. In one orientation, the promoter results in the transcription of *fljB* and *fljA* genes. Phase-2 flagellin is expressed and *fliC* is repressed. In the other orientation, *fljB* and *fljA* are not expressed, leading to the expression of phase-1 flagellin (Bonifield & Hughes, 2003; Yamamoto & Kutsukake, 2006) (Figure 1.8). A recent study by Kutsukake and his co-workers (2006) showed the presence of another DNA invertase (fin invertase) that is involved in phase variation in *S.* Typhimurium. Due to phase reversion, only one H-antigen is detected in some instances (Bonifield & Hughes, 2003; Sojka *et al.*, 2001). When this happens, time-consuming phase reversal is

necessary to identify both phases of flagellin during serotyping. This is done by inoculating the isolate onto the top of a tube of phase reversal media. This media is semisolid and contains antisera to the H-antigen that has already been identified. Organisms expressing the previously detected H-antigen are immobilized by the added antisera and grow only at the top of the tube. Organisms expressing the second H-antigen are able to move away from the top of tube, and grow throughout the tube. The second H-antigen is then determined using organisms recovered from the bottom of the phase reversal media. In some instances both H-antigens can be detected in a single culture eliminating the need for phase reversal, especially for older strains or for isolates that have been passed multiple times (Centres for Disease Control and Prevention, 2002).

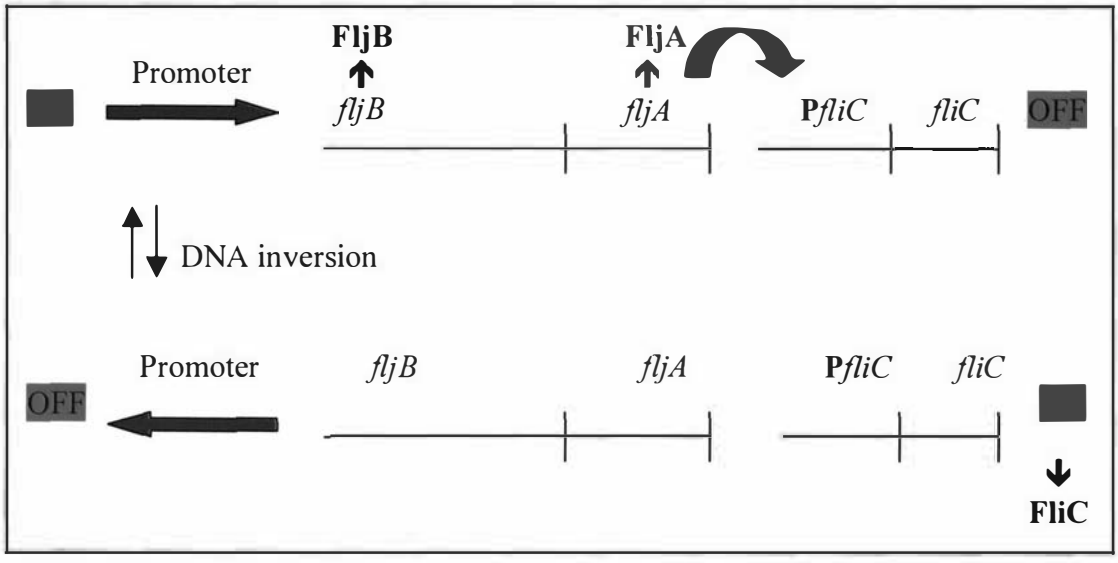

Figure 1.8. Schematic diagram showing phase variation of *Salmonella* phase-1 and phase-2 flagellin proteins. *fliC*, gene encoding phase-1 flagellin; FliC, phase 1-flagellin; *fljB*, gene encoding phase-2 flagellin; FljB, phase-2 flagellin; *fljA,* gene encoding repressor for phase-1 flagellin; FljA, repressor for phase-1 flagellin; P, promoter for phase-1 flagellin. Adapted from Bonifield & Hughes, 2003.

1.12.2 Other diagnostic methods

Table 1.4. Genes amplified by PCR for the detection of *Salmonella* genus

Target gene	References
16S *rDNA*	Chiang *et al.*, 2006; Iida *et al.*, 1993; Lin & Tsen, 1996
SPI-1 invasion gene *hilA* (HilA is a transcriptional activator regulating SPI-1 encoded T3SS)	Guo *et al.*, 2000; Pathmanathan *et al.*, 2003
Enterotoxin gene *stn*	Makino *et al.*, 1999
Invasion gene *invA* (InvA is a structural component of SPI-1 encoded T3SS)	Arnold *et al.*, 2004; Chiu & Ou, 1996; Ferreti *et al.*, 2001; Guy *et al.*, 2006; Iijima *et al.*, 2004; Rahn *et al.*, 1992; van Nierop *et al.*, 2005
Invasion genes *invE/invA*	Schrank *et al.*, 2001; Stone *et al.*, 1994
Repetitive DNA fragment	Jitrapakdee *et al.*, 1995
Random fragment	Aabo *et al.*, 1993; Myint *et al.*, 2006
Invasion gene *iagA*	Bej *et al.*, 1994; Schindler *et al.*, 2002
Insertion sequence IS*200*	Cano *et al.*, 1993
Fur-regulated gene *iroB*	Bäumler *et al.*, 1997b
Thin aggregative fimbriae gene *agfA*	Doran *et al.*, 1993
Histidine transport operon	Cohen *et al.*, 1993
Junction between *sipB* and *sipC* genes	Carlson *et al.*, 1999
Gene encoding outer membrane protein C	Alvarez *et al.*, 2004; Amavisit *et al.*, 2001; Kwang *et al.*, 1996
Chromosomal origin of replication *oriC*	Espinoza-Medina *et al.*, 2006; Widjojoatmodjo *et al.*, 1991
Fimbrial gene *fimA*	Naravaneni & Jamil, 2005
Fimbrial gene *fimC*	Piknova *et al.*, 2005
Integration host factor gene *himA*	Brands *et al.*, 2005; Chen *et al.*, 2000
Internal transcribed spacer region of 16S-23S rRNA gene *Its*	Chiu *et al.*, 2005; Park *et al.*, 2006
prgK gene involved in invasion (PrgK is a structural component of SPI-1 encoded T3SS)	Farrel *et al.*, 2005
ttr sequence	Delibato *et al.*, 2006; Malorny *et al.*, 2004
Quorum sensing gene *sdiA*	Halatsi *et al.*, 2006

Bacteriological methods for the identification of *Salmonella* are time-consuming, laborious, and yield false negative results when the initial number of *Salmonella* is low in the sample (D'Souza & Jaykus, 2003; Naravaneni & Jamil, 2005). To circumvent these problems, alternative methods are used to supplement bacteriological findings. However, isolation and identification procedures are still the "gold standard". The alternative methods include electrical conductance and impedance tests, immunological methods, heterogeneous immuno-electrochemical methods, DNA hybridisation and PCR assays (Cherrington & Huis in't Veld, 1993; Yang *et al.*, 1998). Several laboratories have developed PCR-based assays for the identification of *Salmonella* at the genus level (Table 1.4). The PCR is very useful for the detection of *Salmonellae*, especially when they are at low concentrations, as in samples from carrier animals without clinical signs or when the organisms are not viable (Cohen *et al.*, 1994; Rossen *et al.*, 1992).

A number of assays to identify the *Salmonella* isolate at the serotype level have also been published. Ribotyping and IS*200*-based methods are among them. Ribotyping is a ribosomal-RNA (*rrn*) gene restriction pattern. Chromosomal DNA is restriction-digested, gel-electrophoresed and transferred to a membrane for hybridisation with 16S and 23S rRNA-specific probes (Stull *et al.*, 1988). This detects variations in the copy number and location of rRNA loci in restriction endonuclease-cleaved chromosomal DNA (Altwegg *et al.*, 1989). Some serotypes have a serotype-specific pattern while others do not (Millemann *et al.*, 2000). IS*200* typing is based on hybridisation of digested genomic DNA with an IS*200* probe. In *Salmonella*, IS*200* is a 708 base pair (bp) sequence that varies in the number of copies both between serotypes and within the same serotype (range from 1-25) (Gibert *et al.*, 1990; Stanley & Saunders, 1996). The discriminatory power depends on the copy number of the element (Baquar *et al.*, 1993). Both ribotyping and IS*200* typing are complex techniques requiring a number of crucial steps that are beyond the technical ability of many diagnostic laboratories (Lagatolla *et al.*, 1996).

Other DNA-based assays, including pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) (Aarts *et al.*, 1998), PCR-ribotyping (Lagatolla *et al.*, 1996), repetitive-element PCR (rep-PCR) (Rasschaert *et al.*, 2005) and randomly amplified polymorphic DNA-PCR (RAPD-PCR) (Betancor *et al.*, 2004) can be used to

differentiate *Salmonella* serotypes. PFGE involves the digestion of total genomic DNA with a rare-cutting restriction enzyme such as *Xba* I to yield between 5 and 30 fragments, and analysis of the relatively small number of large fragments. In PFGE, the field is pulsed by alternating the direction of the electrical current between two field orientations for a predetermined time period (Liebana *et al.*, 2001). The disadvantages of this technique include time-consumption, and relatively high costs for the required equipment and consumables. Protocols allowing completion of analysis within one day have been described (Gautom, 1997). An international database with PFGE profiles of more than 25,000 *Salmonella* isolates is available to facilitate the early recognition of outbreaks (Lukinmaa *et al.*, 2004). AFLP includes the digestion of genomic DNA with two endonucleases, ligation of suitable adapters and subsequent PCR amplification (Aarts *et al.*, 1998). AFLP is less labour-intensive and less difficult to adapt for automation than PFGE. In fluorescent amplified-fragment length polymorphism (FAFLP), the PCR products are labelled with fluorescent tags and analysed using an automated sequencer. With the introduction of new multi-capillary instruments, FAFLP can be scaled to a very high throughput (Lindstedt *et al.*, 2000). Rep-PCR utilizes primers complementary to naturally occurring, highly conserved, non-coding, repetitive sequences that are present in multiple copies in most Gram-negative bacteria. Repetitive elements include repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), BOX and polytrinucleotide sequences (GTG) (Rasschaert *et al.*, 2005). While some studies showed that Rep-PCR produced a serotype-specific pattern (van Lith & Aarts, 1994), other studies did not (Burr *et al.*, 1998; Millemann *et al.*, 1996). RAPD-PCR and arbitrarily primed–PCR (AP-PCR) are based on the amplification of unknown sequences with short (10-20 bp) random primers (Welch & McClelland, 1990, Williams *et al.*, 1990). Since 1995, RAPD-PCR has been used to differentiate either *Salmonella* serotypes or isolates within a single serotype (Betancor *et al.*, 2004). RAPD-PCR is a rapid technique, but the variable reproducibility among laboratories is a disadvantage (Meunier & Grimont, 1993). Nair and his co-workers (2002) showed that PCR-single-strand conformation polymorphism (PCR-SSCP) could be used to differentiate serotypes. They observed a unique SSCP profile for each of the 10 *Salmonella* serotypes used in the study, using the nucleotide variation in the *groEL* gene, which encodes a heat shock protein. In PCR-SSCP analysis, amplified double-stranded DNA is denatured to single-stranded DNA and subjected to non-denaturing polyacrylamide gel electrophoresis. The mobility of the

single-stranded DNA in the gel depends on its length and secondary structure, as determined by nucleotide sequence (Nair *et al.*, 2002). PCR ribotyping is based on the amplification of the spacer sequences between 16S and 23S genes in the rRNA genes. The polymorphism of intergenic spacer regions can be used to identify *Salmonella* at the level of the serotype. It is a simple technique with good discriminatory power (Lagatolla *et al.*, 1996). All these methods have one or more drawbacks, including insufficient discrimination, poor reproducibility between laboratories, and difficulties with the comparison and accumulation of results obtained by different laboratories.

1.12.3 Serological methods

Although a serological test is as not definitive as a bacteriological test, it can provide good evidence of salmonellosis (Chart *et al.*, 1990). Both clinically and sub-clinically infected animals are responsible for environmental contamination. Detection of carriers by bacteriological methods is unpredictable due to the intermittent nature of shedding, necessitating frequent testing of samples. Serological testing gives an indication of the *Salmonella* status of the animal because it detects circulating antibodies regardless of when the animal was infected. In contrast, the detection of *Salmonella* in faecal samples indicates that animals are shedding the organism. However, although bacteriological isolation is more specific, it is insensitive (Grafanakis *et al.*, 2001; Kranker *et al.*, 2003). Serum IgG levels are usually persistent in spite of intermittent shedding of *Salmonella* by infected animals (Nielsen *et al.*, 2004). Furthermore, when dealing with a large number of samples, a serological test is faster and cheaper to perform than bacteriology (Funk *et al.*, 2005). It has been recommended that serological methods for *Salmonella* diagnosis should be used with caution due to the following factors. Some animals with a positive serological response may no longer be infected with *Salmonella*. Animals that are serologically positive may have stopped excreting salmonellae although circulating immunoglobulin concentrations may remain high (Kranker *et al.*, 2003; Nielsen *et al.*, 2004). Furthermore, animals that are actively excreting salmonellae may be serologically negative (Hoorfar *et al.*, 1996). Serologically negative animals may result from a recent infection resulting in excretion before immunoglobulin production is detectable (Veling *et al.*, 2000). Newborn animals are immunologically immature and do not respond serologically to the somatic, LPS antigens until 2-3 weeks of age. Some

animal species, including cattle, may be unresponsive until about 3 months of age, leading to false negative results (Nielsen *et al.*, 2004; Veling *et al.*, 2000). However, they do produce a serological response to flagellar antigens (Office International des Epizootics, 2000). Furthermore, maternally derived antibodies from colostrum may cause false positive reactions (Nielsen *et al.*, 2004). Following *Salmonella* infections, immunoglobulin concentrations remain elevated for 2-3 months. The effect of antibiotic therapy on the serological responses is unclear. Diagnostic serology may be more useful than culture if antibiotics are used. Depending on the antigen and test used, serological cross-reactions between different serovars may occur. Serological tests should be used to identify infected flocks or herds rather than individual animals (Office International des Epizootics, 2000).

Serological tests for salmonellosis include agglutination, complement fixation, immunoblotting and ELISAs. Slide agglutination tests have been used for the rapid diagnosis and elimination of carriers of *S.* Pullorum and *S.* Gallinarum in poultry (Jones *et al.*, 2000). The disadvantages of the assay include cross-reactions, and the dependence of the results on antigen quality and operator skills (Gordon & Brander, 1942). Serum agglutination tests (SAT) with O- and H-antigens of different serovars in different animal species have been described. Standard sera are necessary for quality control of SAT antigen preparations (Jones *et al.*, 2000). Immunoblotting is a sensitive test and reactions to individual antigens can be obtained. This is a time-consuming technique making it unsuitable for the analysis of a large number of samples. ELISA tests are sensitive, specific, rapid and easy assays that can be used for screening flocks and herds. ELISA tests based on LPS (Funk *et al.*, 2005), flagellae (de Vries *et al.*, 1998; Veling *et al.*, 2000), fimbriae (Hoofar *et al.*, 1996), OMPs (Secundino *et al.*, 2006), whole cell extracts (Berthelot-Hérault *et al.*, 2003) and Vi antigen (Ferry *et al.*, 2004) have been described. In addition to serum (Nielsen *et al.*, 2004), other sources such as meat juice extracted from a piece of a carcass (Davies *et al.*, 2003; Korsak *et al.*, 2006), egg yolk (Mizumoto *et al.*, 2004; Skov *et al.*, 2002) and milk (Hoorfar *et al.*, 1995) have been used in different ELISAs. ELISA tests are commonly used to detect antibodies against *Salmonella* in pig herds in Germany and Denmark. *Salmonella* surveillance programs in Denmark use meat juice as the source of antibody to identify *Salmonella*-infected pig herds (Nielsen *et al.*, 1995). Furthermore, Danish surveillance programs use milk and serum samples in ELISAs to screen cattle for salmonellosis

(Nielsen *et al.*, 2004). ELISA tests are more sensitive than agglutination tests for serological diagnosis of salmonellosis (Veling *et al.*, 2000; Yap *et al.*, 2001). ELISA tests are readily adapted to automation and hence to large-scale testing programmes (Veling *et al.*, 2000).

1.13 Objectives of the Thesis

Bacterial pathogens secrete proteins that are either cell envelope-associated, or are secreted in to the extracellular milieu. These proteins play a role in cell adherence, cell invasion, and immune cell activation (Kornacki & Oliver, 1998). Therefore, these proteins are being targeted for the development of vaccines and diagnostic tests (Lewenza *et al.*, 2005). The secreted proteins of *Salmonella* include fimbrial and flagellar proteins, OMPs, porins and proteases (Cookson & Bevan, 1997). In addition to these, a number of proteins that are involved in *Salmonella* pathogenesis are encoded by *Salmonella* pathogenicity islands, and are secreted through T1SS, T2SS and T3SS (Pallen *et al.*, 2005; Schmidt & Hensel, 2004). However, there is no information on the secreted proteins of *S.* Brandenburg to date. Therefore, my aim was to identify protein antigens secreted by *S.* Brandenburg, with a view to identifying those that induce an immune response. To date, standard control methods based on hygiene have not limited the transmission of *S.* Brandenburg, nor has the current *Salmonella* vaccine eliminated abortion outbreaks. Therefore, it is very important to identify the immunogenic proteins of this organism. This information could then be used to develop an improved vaccine and specific diagnostic tests. Diagnostic tools depend on detecting genes and products of bacteria or antibodies that are specific for a particular pathogen.

As described previously, exported proteins that contain cleavable signal sequences are transported across the inner membrane into the periplasm, the outer membrane, or into the extracellular milieu. One of the commonly used genetic assays for identifying exported proteins containing N-terminal signal sequences uses fusions to alkaline phosphatase, encoded by the *E. coli phoA* gene (Lim *et al.*, 1995; Mintz & Fives-Taylor, 1999; Ward *et al.*, 2001). This system identifies proteins that pass through the inner membrane differentiating non-cytoplasmic from cytoplasmic proteins.

The main objectives of this study were to:

1. Prepare a *Salmonella* Brandenburg *phoA* genomic library in the plasmid vector pJEM11, and express it in *E. coli*.
2. Sequence DNA isolated from PhoA-positive recombinant *E. coli* colonies, and identify genes that encode potential immunogenic proteins through a database search.
3. Express selected *S*. Brandenburg genes in *E. coli*, and purify the resulting recombinant proteins.
4. Evaluate the immunogenic properties of selected purified proteins using sera from sheep naturally infected with *S.* Brandenburg, and those vaccinated with Salvexin™ and Salvexin™+B vaccines.
5. Develop a PCR and an ELISA as diagnostic tests to identify *S.* Brandenburg infection in sheep.

Chapter 2 General materials and methods

2.1 Bacterial strains and plasmids

2.1.1 Bacterial strains

The bacterial strains used in this study are shown in Tables 2.1, 2.2, and 2.3. *Salmonella* strains Potsdam and Livingstone listed in Table 2.2 were purchased as freeze-dried cultures from the New Zealand Reference Culture Collection, Medical Section, Institute of Environmental Science and Research Ltd., Porirua, Wellington, NZ. Bacterial strains designated 'S --" were obtained as glycerol stocks from the Institute of Veterinary, Animal and Biomedical Sciences (IVABS), Massey University, Palmerston North, NZ. These strains had been isolated from clinical and environmental material from 1997-2002. *S.* Brandenburg isolates had been recovered during the outbreak in this period and had been sent to IVABS for molecular characterisation. Strains designated 'MU --" were obtained as freeze-dried cultures from the Institute of Molecular Biosciences (IMBS) of Massey University, Palmerston North, NZ. Genomic DNA samples listed in Table 2.3, were kindly provided by Professor Peter. R. Reeves of the Department of Veterinary Microbiology, Sydney University, Australia (Wang *et al.,* 2002).

Table 2.1. Commercial bacterial strains used in this study

Strain	Genotype/Phenotype	Source/Reference
E. coli DH10B	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 *deo*R Δ *lac*X74 *rec*A1 *end*A1 *ara*D139 Δ (*ara, leu*) 7697 *gal*U *gal*K λ⁻ *rps*L *nup*G	Life Technologies, Carlsbad, CA, USA Sambrook *et al.,* 1989
E. coli BL21 (DE3)	F⁻ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm*	Life Technologies, USA Sambrook *et al.,* 1989

Table 2.2. Other bacterial strains used in this study

Strain	Reference number	Source
S. Brandenburg	S13	Porcine intestines, 1997
S. Brandenburg	S59	Schering-Plough Animal Health Ltd., Upper Hutt, Wellington, NZ
S. Brandenburg	S73	Black-backed gull intestines, 1999
S. Brandenburg	S74	Black-backed gull intestines, 1999
S. Brandenburg	S75	Canine urinary tract, 1999
S. Brandenburg	S76	Ovine faeces, 1999
S. Brandenburg	S79	Ovine faeces, 1999
S. Brandenburg	S82	Bovine isolate, 1999
S. Brandenburg	S84	Avian clinical isolate, 1999
S. Brandenburg	S85	Bovine isolate, 1999
S. Brandenburg	S103	Ovine foetal stomach contents, 1999
S. Brandenburg	S104	Ovine foetal stomach contents, 1999
S. Brandenburg	S119	Sheep yard dust, 2000
S. Brandenburg	S124	Sheep yard dust, 2000
S. Brandenburg	S126	Sheep yard dust, 2000
S. Brandenburg	S134	Sheep yard dust, 2000
S. Brandenburg	S196	Isolate from meat, 2001
S. Brandenburg	S199	Isolate from meat, 2001
S. Brandenburg	S201	Isolate from meat, 2001
S. Brandenburg	S207	Porcine faeces, 2001
S. Brandenburg	S229	Ovine uterus, 2001
S. Brandenburg	S233	Ovine uterus, 2001
S. Brandenburg	S247	Ovine uterus, 2001
S. Brandenburg	S252	Ovine uterus, 2001
S. Brandenburg	S293	Sheep yard dust, 2001
S. Brandenburg	S342	Porcine clinical isolate, 2002
S. Typhimurium	S40	Bovine faeces, 1998

Table 2.2. Continued

S. Typhimurium	S47	Ovine intestines, 1998
S. Typhimurium	S50	Ovine intestines, 1998
S. Typhimurium	S160	Sparrow gut contents, 2000
S. Derby	S20	Sea lion lymph node, 1998
S. Derby	S26	Sea lion isolate, 1998
S. Derby	S139	Human isolate, 2000
S. Derby	S144	Feed, 2000
S. Agona	S61	Ovine isolate, 1999
S. Agona	S63	Poultry carcass, 1999
S. Agona	S67	Poultry carcass. 1999
S. Agona	S68	Poultry carcass, 1999
S. Saintpaul	S151	Canine faeces, 2000
S. Liverpool	MU609	Unknown
S. Infantis	MU623	Unknown
S. Singapore	MU605	Unknown
S. Newport	S19	Sea lion intestines, 1998
S. Newport	S140	Porcine isolate, 2000
S. Newport	S143	Human isolate, 2000
S. Newport	S349	Sea lion tonsils, 2002
S. Hindmarsh	S8	Unknown, 1996
S. Hindmarsh	S11	Ovine intestines, 1997
S. Hindmarsh	S48	Ovine intestines, 1998
S. Hindmarsh	S60	Ovine gut contents, 1999
S. Gallinarum	MU123	Unknown
S. Enteritidis	S25	Sea lion isolate, 1998
S. Enteritidis	S208	Human isolate, 2001
S. Enteritidis	S209	Seal heart blood, 2001
S. Havana	MU603	Unknown
S. Cerro	S22	Sea lion isolate, 1998
S. Cerro	S27	Sea lion isolate, 1998

Table 2.2. Continued

S. Cerro	S137	Human isolate, 2000
S. Cerro	S141	Porcine isolate, 2000
S. Anatum	MU604	Unknown
S. Newington	MU122	Unknown
S. Potsdam	1117	NZ isolate NHI 75/1253
S. Livingstone	1201	NZ isolate NHI 75/2347
E. coli	MU89A	Unknown
Klebsiella aerogenes	MU84	Unknown
Klebsiella edwardsii	MU179	Unknown
Proteus mirabilis	MU181	Unknown
Shigella flexneri	MU180	Unknown

Table 2.3. Genomic DNA used in this study

Salmonella serovar	Reference number
Azteca	M 1996
Bredeney	M 1490
Jos	M 2038
Budapest	M 1491
Abortusovis	M 1975
Wien	M 1995
Ball	M 2023
Gloucester	M 2051
Mono	M 2096
Togo	M 2113

2.1.2 Plasmids

The plasmids used in this study are shown in Table 2.4. The plasmid vector pJEM11 was kindly provided by Professor Brigette Gicquel from the Pasteur Institute, Paris, France.

Table 2.4. Plasmids used in this study

Plasmid	Description	Source/Reference
pJEM11	*E.coli*-mycobacterial shuttle vector containing a truncated *phoA* gene, Kan^r	Lim *et al.*, 1995
pET14b	Expression vector containing an N-terminal histidine tag	Novagen, Madison, WI, USA
pET14b-*fliC*	pET14b containing *fliC*	This study
pET14b-*V456*	pET14b containing regions IV, V and VI of *fliC*	This study
pET14b-*V4*	pET14b containing region IV of *fliC*	This study

2.2 Growth and storage of bacterial cultures

2.2.1 Media

Blood, xylose-lysine–deoxycholate (XLD), MacConkey, triple sugar iron (TSI) and urea agars, and buffered peptone water (BPW), selenite F, tetrathionate and lysine decarboxylase broths were purchased from Fort Richard Laboratories Ltd. (Otahuhu, Auckland, NZ), and stored at 4°C until used. Luria-Bertani (LB) agar was prepared by the addition of 1% agar (Cat. No. 0140-01, Difco, Detroit, MI, USA) to LB broth (1%[w/v] tryptone (Cat. No. 0123-17-3, Difco), 0.5% [w/v] yeast extract (Cat. No. 0127-17-9, Difco), 1% [w/v] NaCl) before sterilization. All media were sterilized by autoclaving at 121°C and 15 psi for 20 min. Sterilized media were cooled to 55°C before supplementing with appropriate antibiotics/supplements (Table 2.5). Stock solutions of antibiotics, including carbenicillin (Cat. No. C1389, Sigma, St. Louis, MO,

USA) and kanamycin (Cat. No. 11815-024, Life Technologies), and the supplement 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Cat. No. B6149, Sigma) were prepared in sterile distilled water and filter sterilized using 0.22 μm sterile, disposable syringe filters (Cat. No. 4652, Pall Gelman Sciences, Ann Arbor, MI, USA). They were aseptically aliquoted into microcentrifuge tubes, and stored at –20°C. Transformed *E. coli* cells were recovered using super optimal broth (SOC medium {20% [w/v] tryptone, 5.5% [w/v] yeast extract, 10% [v/v] 1 M NaCl, 10% [v/v] 1 M KCl, 10% [v/v] 2 M glucose, 10% [v/v] 2 M Mg++}). The broth was autoclaved and cooled to 55°C before supplementing with glucose and Mg solutions that were filtered through 0.22 μm syringe filters.

Table 2.5. Antibiotics and supplements used in media

Antibiotic/Supplement	Stock concentration (mg/ml)	Final concentration (μg/ml)
Kanamycin	20	30
Carbenicillin	50	50
BCIP	40	25

2.2.2 Growth of bacteria

Salmonella isolates were routinely grown on blood, XLD and MacConkey agars at 37°C for 18-24 h under aerobic conditions. The freeze-dried cultures of *Salmonella* and other bacterial isolates were resuspended in 0.5 ml of nutrient broth, and a few drops of suspension were placed on an agar plate and incubated as above. From frozen glycerol stocks, a few microliters were inoculated and grown on agar plates. For *Salmonella* biochemical tests, colonies were grown on TSI agar, urea agar and lysine decarboxylase broth at 37°C for 24-48 h. *E. coli* strains, including recombinants, were grown on LB agar and/or in LB broth containing appropriate antibiotics and/or supplements at 37°C under aerobic conditions. Liquid cultures were grown in tubes or flasks with a volume of at least 4 times that of the culture volume, and were shaken at approximately 225 rpm on an Innova 4000 incubator shaker (New Brunswick Scientific Company, Inc., Edison, NJ, USA) to ensure sufficient aeration.

2.2.3 Measurement of cell density of cultures

Cell densities of recombinant *E. coli* liquid cultures were calculated from the OD values of cultures at 600 nm (OD_{600}). The double beam spectrophotometer (Helios α, Unicam, UK) was calibrated to zero at 600 nm using 2 blank polystyrene cuvettes (Global Science & Technology Ltd., Auckland, NZ) each containing 1 ml of sterile LB broth. Routinely, 100 µl of culture and 900 µl of sterile LB broth were mixed carefully to avoid bubbles in a cuvette. One of the blank cuvettes was replaced with the one containing 1:10 diluted culture, and the OD_{600} reading was recorded. All samples were analysed in duplicate.

2.2.4 Storage of cultures

Bacterial cultures were kept for short periods (up to 1 week) at 4°C. For longer-term storage, cultures of recombinant *E. coli*, *Salmonella* and other bacteria were stored as glycerol stocks at –70°C. Bacteria were streaked onto an appropriate agar plate and incubated overnight at 37°C. For the storage of *Salmonella* and other bacterial isolates, several loopfulls of bacterial cells from overnight agar plates were mixed in 1 ml of 15% glycerol in trypticase soy broth in cryovials (Nalgene Nunc International, Roskilde, Denmark). For recombinant *E. coli*, a single colony was picked from the agar plate either with a sterile toothpick or a pipette tip, and inoculated into 5 ml of LB broth. When the recombinant *E. coli* cultures reached 0.6-0.8 at OD_{600}, 800 µl of liquid culture was mixed with 200 µl of sterile glycerol (20% v/v) in a cryovial by inversion or a brief vortex. The cryovials were then stored at –70°C.

2.3 DNA extractions

2.3.1 Extraction of genomic DNA

2.3.1.1 Boiling method

For most PCR reactions, genomic DNA was obtained by boiling a suspension of bacteria. Each strain was grown on an agar plate overnight at 37°C. A single colony

was resuspended in 20 µl of distilled water, and heated at 100°C for 10 min. It was immediately cooled on ice for 5 min, and centrifuged at 13,000 × g at 4°C for 10 min. The supernatant containing DNA was stored at –20°C until used.

2.3.1.2 Phenol-chloroform method

For preparation of the *S.* Brandenburg genomic library, genomic DNA was extracted using a phenol-chloroform method. A loopful of *Salmonella* culture from a frozen glycerol stock was streaked onto a blood agar plate. After an overnight incubation at 37°C, a single colony was grown in 6 ml of LB broth. The overnight culture was centrifuged at 2,000 rpm for 10 min, and the cell pellet was resuspended in 2 ml of TE buffer pH 8.0 (10 mm Tris.Cl, 5 mm ethylenediamine tetraacetic acid [EDTA]). The cell suspension was transferred into a microcentrifuge tube, and centrifuged at 12,000 × g for 10 min. The pellet was resuspended in 1 ml of TE buffer pH 8.0 containing 10 µl of 50 mg/ml lysozyme stock (Cat. No. 1-243-004, Roche, Mannheim, Germany) and incubated at 37°C for 1 h. Fifty microlitres of 10% sodium dodecyl sulfate (SDS) was added to the tube and the mixture was vortexed. After addition of 10 µl of 20 mg/ml proteinase K (Cat. No. 745-723, Roche), the samples were vortexed again and incubated at 55°C for 2 h. Genomic DNA was isolated with two phenol-chloroform-isoamyl alcohol (25:24:1, v/v; Cat. No. 15593-031, Life Technologies) extractions to remove contaminating proteins. Briefly, 1 ml of phenol-chloroform-isoamyl alcohol was added to the suspension, vortexed and centrifuged at 13,000 × g for 10 min. The upper aqueous phase was removed to a clean microcentrifuge tube and phenol-chloroform extraction was repeated one more time. The upper aqueous phase (approximately 1 ml) was then aliquoted into two microcentrifuge tubes. Subsequently, ethanol precipitation was performed to remove salts from the preparation. One hundred microlitres (0.1 volume) of 3 M sodium acetate pH 5.0, and 1 ml (2 volumes) of 100% ice-cold ethanol were added to each tube, vortexed and incubated at –20°C for at least 30 min. The tubes were centrifuged at 13,000 × g for 30 min at 4°C. After removing the supernatant, the pellet was washed by mixing with 1 ml (2 volumes) of 70% ice-cold ethanol and centrifuging at 13,000 × g for 5 min at 4°C. The resulting DNA pellet was vacuum dried using an SC 100 Speed Vac (Savant Instruments Inc., Holbrook, NY, USA) and resuspended in 100 µl of sterile distilled

water. Where RNA removal was desired, 90 µl of isolated genomic DNA was incubated with 10 µl of 10 mg/ml RNase (Cat. No. 109-142, Roche) at 37°C for 1 h. Subsequently, DNA was isolated with two phenol-chloroform-isoamyl alcohol extractions and ethanol precipitation as above. The DNA pellets were dried, resuspended in sterile water and stored at –20°C.

2.3.2 Extraction of plasmid DNA from *E. coli*

Plasmids were extracted from *E. coli* using the QIAprep Spin Miniprep Kit (Cat. No. 27104, Qiagen, Hilden, Germany). Briefly, *E. coli* carrying the desired plasmid was grown in 5 ml of LB broth overnight at 37°C with vigorous shaking. Two to five millilitres of overnight culture was micro-centrifuged at 14,000 rpm for 5 min, and the harvested cell pellet was used for the isolation of plasmids according to the manufacturer's instructions. Plasmid DNA was eluted with 50 µl of EB buffer (10 mM Tris·Cl, pH 8.5) supplied with the kit, and was stored at –20°C until needed.

2.4 DNA techniques

2.4.1 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out to separate DNA fragments based on their size. Agarose (Cat. No. 15510-027, Life Technologies) solutions of 0.7 to 1% (w/v) were prepared in 1 × TAE buffer pH 8.0 (40 mM Tris, 20 mM acetic acid, 2 mM EDTA), heated in a microwave, and cooled to 55°C before pouring gels. DNA samples were mixed with 0.2 volumes of 6 × DNA loading buffer (0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol, 30% [v/v] glycerol). Samples were loaded onto solidified gels in parallel with 1-kb Plus DNA Ladder marker (Cat. No. 10787-018, Life Technologies). Electrophoresis was carried out in a horizontal gel electrophoresis unit (Bio-Rad, Hercules, CA, USA) containing 1 × TAE buffer pH 8.0 at 80-100V (Bio-Rad Power Pac). Gels were stained in distilled water containing 0.5 µg ethidium bromide (Cat. No. E1510, Sigma) per ml for 15-30 min, destained in water for 10 min, and visualised under UV light using the Quantity One® software (Bio-Rad) from the Bio-Rad Gel Doc 2000 imaging system.

2.4.2 Extraction of DNA from agarose gels

DNA digests and PCR products were loaded onto an agarose gel. After electrophoresis, gels were stained with ethidium bromide, and bands of the expected sizes were excised under UV light with a clean scalpel blade. DNA was extracted either immediately or the following day after storing gel slices at –20°C. DNA was extracted using a QIAquick Gel Extraction Kit (Cat. No. 28704, Qiagen) according to the manufacturer's instructions and eluted in 30-50 µl of EB buffer (10 mM Tris.CI, pH 8.5) supplied with the kit. Samples were stored at –20°C.

2.4.3 DNA quantitation

DNA concentrations were measured using GeneQuant (Amersham Pharmacia Biotech, Uppsala, Sweden) and a spectrophotometer at 260 nm. Routinely, 1:20 diluted samples were used. When, readings less than 0.1 were obtained, more concentrated samples of DNA were used. All samples were analysed in duplicate. At 260 nm, one OD corresponds to 50 µg/ml of DNA. Thus, concentration of DNA is calculated according to the following formula:

DNA concentration (µg/ml) = Absorbance at OD_{260} × 50 × Dilution factor.

In addition, readings were taken at 280 nm to check the purity of samples by calculating the ratio of OD_{260}/OD_{280}.

2.4.4 Polymerase chain reaction (PCR)

2.4.4.1 Primer design

PCR was used to amplify single genes and portions of selected genes. Primers were designed to contain 20-40 nucleotides with a GC content between 40 and 60, and an absence of self-complementary hairpins. Approximate melting temperature (Tm) was calculated using the formula Tm = 4(G + C) + 2(A + T)°C. For cloning, restriction enzyme sites with a few extra nucleotides were added onto the 5′ end. Custom primers

were synthesized in desalted form by Life Technologies. They were resuspended in sterile distilled water to a final concentration of 100 μM, and stored at –20°C. The volume of water needed to resuspend the primers was calculated according to the following formula. EM is the molar extinction coefficient.

$$\text{Volume of water (ml)} = \frac{\text{Total OD (Absorbance at 260 nm)}}{[(\text{EM}) \times \text{Molar concentration required}]}$$

2.4.4.2 PCR conditions

Each PCR reaction included the DNA template, two primers, nucleotides, $MgCl_2$, DNA polymerase and buffer. Genomic DNA or PCR products were used as the template for amplification. Primers were used at a final concentration of 400 nM. More than 2 primers were included in multiplex PCR reactions. A working solution of deoxynucleotide triphosphate (dNTP) mix containing 10 mM dATP, dCTP, dGTP and dTTP (each) was prepared by mixing 10 μl of dATP, dCTP, dGTP, and dTTP (each with a starting concentration of 100 mM; Cat. No. 1-969-064, Roche) with 60 μl of sterile distilled water. Working solutions were stored at –20°C. The PCR reaction contained dNTP at a final concentration of 100 μM. Most PCR reactions gave a specific band with 1.5 mM $MgCl_2$ or 1.5 mM $MgSO_4$. For others, the optimum concentration of Mg ions was determined by carrying out reactions containing 1, 1.5 and 2 mM of the Mg salt. High fidelity DNA polymerases including *PfuTurbo*® DNA polymerase (Cat. No. 600250, Stratagene, La Jolla, CA, USA) and Platinum® *Taq* DNA polymerase (Cat. No. 10966-018, Life Technologies) were used. Reactions were carried out in 0.2 ml thin-walled PCR tubes (Life Technologies) in a total volume of 50 μl. A negative control with genomic DNA replaced with sterile water was included in each PCR assay. Amplifications were achieved in 25-30 cycles using a GeneAmp Model 9600 PCR thermocycler (Perkin-Elmer Cetus, Norwalk, CT, USA). Prior to the first cycle, DNA was denatured at 95°C for 5 min. Subsequently, each cycle consisted of denaturation at 95°C for 30-60 sec, followed by annealing and elongation. The annealing temperature was selected depending on the Tm of primers and was usually 5°C below the lowest Tm of the pair of primers. Annealing was routinely performed for 30-60 sec. Routinely, elongation was carried out at 72°C and the extension time

was usually 1 min per 1-kb of the DNA template. Subsequently, a final elongation was performed at 72°C for 5-10 min. Following amplification, PCR products were size fractionated on an agarose gel. When only one band was present, it was cleaned with a QIAquick PCR Purification Kit (Cat. No. 28104, Qiagen), and used for sequencing and restriction endonuclease digestion for cloning. When more than one band was present, the desired band was gel purified. Amplification reaction products were stored at 4°C for short-term and at –20°C for long-term storage.

2.5 Cloning

2.5.1 Restriction endonuclease digestions

Routinely, PCR products and plasmid vectors were digested with restriction endonucleases in a total volume of 50 μl containing 5-10 units of enzyme with buffers supplied by the manufacturer at recommended temperature. One unit of enzyme is defined as the amount of enzyme that will digest 1 μg of DNA in 1 h at the recommended temperature. Subsequently, digests were cleaned using QIAquick PCR Purification Kits (Qiagen) according to the manufacturer's instructions. Digested plasmid vector was then treated with calf intestinal alkaline phosphatase (CIAP; Cat. No. 713-203, Roche) to prevent self-ligation of the sticky ends. One unit of CIAP is the enzyme activity that hydrolyses 1 μM of 4-nitrophenyl phosphate in 1 min at 37°C. The reaction was carried out in a reaction volume of 50-100 μl containing CIAP and 1 × dephosphorylation buffer at 37°C for 1-2 h. CIAP was then inactivated by adding 0.1 volume of 200 mM EDTA, and heating at 65°C for 10 min. Subsequently, the reaction was cleaned with QIAquick PCR Purification Kit (Qiagen) for the complete removal of CIAP. Efficiency of phosphatase treatment was tested through transforming *E. coli* with ligation reactions of the dephosphorylated vector. Undigested and digested (without dephosphorylation) vectors were used for comparison.

2.5.2 Ligation reactions

For ligation reactions, plasmid vectors and inserts were digested with one or two different restriction endonucleases to produce complementary protruding termini compatible between vector and insert. Routinely, ligation reactions between the restriction endonuclease-digested plasmid vector and insert were carried out in a total volume of 10-20 µl containing 1 unit of T4 DNA ligase (Cat. No. 481-220, Roche) and supplied 1 × ligase buffer for approximately 16 h at 16°C. Usually 20-100 ng of vector, and vector: insert ratios of 1: 3 and 1: 5 were used. Amounts of vector and insert used for ligation reactions were calculated using the following formula:

$$\text{Amount of Insert (ng)} = \frac{\text{Amount of Vector (ng)} \times \text{Insert Size (kb)}}{\text{Vector Size (kb)}} \times \text{Insert : Vector Ratio}$$

2.6 Transformation of *E. coli* and plating

2.6.1 Electroporation

Electrocompetent *E. coli* cells (ElectroMAX™ DH10B™; Cat. No. 18290-015, Life Technologies) were transformed with ligation reactions or plasmids by electroporation. Ligation reactions and plasmids were dialysed by adding them to the centre of 0.025 µm pore filter discs (Cat. No. VSWP 01300, Millipore Corporation, Bedford, MA, USA) placed in petri dishes containing sterile distilled water for 10-20 min. This procedure removes salts, which otherwise affect electroporation while pulsing. Routinely, 50 µl of electrocompetent cells and 10-50 ng of purified plasmid, or 100 ng of ligation mix were added into an ice-chilled 0.1 cm electroporation cuvette (Cat. No. 165-2089, Bio-Rad). Electroporation was carried out using a Bio-Rad Gene Pulser apparatus set to 1.8 kV, 25 µF capacitance and 200 ohms resistance. Cells were recovered by immediately adding 1 ml of room temperature SOC medium to the cuvette. Transformed cells were incubated at 37°C for 1 h before plating on LB plates containing appropriate antibiotics. The plates were incubated overnight at 37°C.

2.6.2 Heat shock

Chemically competent *E. coli* (One Shot® BL21(DE3); Cat. No. C6000-03, Life Technologies) cells were transformed with 10-20 ng of plasmid or 10-100 ng ligation reaction product by heat shock. In a pre-cooled microcentrifuge tube, 50 µl of ice-thawed competent cells and DNA were mixed gently by tapping and incubated on ice for 30 min. The mixture was then incubated for 30 sec at 42°C and immediately placed on ice. After addition of 250 µl of pre-warmed SOC medium, the transformation reaction was incubated for 1 h at 37°C with shaking at 225 rpm. The contents were plated on LB plates containing appropriate antibiotics, and incubated overnight at 37°C.

2.7 DNA sequencing

PCR products and inserts cloned into plasmid vectors were sequenced. PCR products were gel purified prior to sequencing. For each sequencing reaction, 12 µl each of 0.8 pM/µl primer, and 30 ng/µl PCR product or 200-500 ng/µl plasmid were submitted to the DNA Analysis Service (Massey University, Palmerston North, NZ) for automated sequencing. The sequencing reactions were carried out with an ABI Prism® BigDye™ Terminator v3.1 Sequencing Ready Reaction Kit either in an ABI Prism® 377 DNA Sequencer or an ABI 3730 Capillary Sequencer (Applied Biosystems, Foster City, CA, USA). The reaction kit uses dye-labelled dideoxynucleotides in which a specific fluorescent dye covalently attaches to each base. Products were visualised as fluorescent colour-coded bands collected by the ABI Prism® Data Collection System and interpreted by the ABI Prism DNA Sequencing Analysis Computer Software.

2.8 Protein preparations

2.8.1 Preparation of *E. coli* cell lysates

Each selected recombinant *E. coli* clone from the pJEM11 genomic library was grown in 5 ml of LB broth supplemented with kanamycin at 37°C. One millilitre of an overnight culture was inoculated into 100 ml (1:100 dilution) of LB and incubated at 37°C with shaking at 225 rpm. When cultures reached the mid-log phase, cells were

harvested by centrifuging at 3,000 × g for 15 min. The cell pellet was washed twice, and resuspended in 10 ml of TE buffer pH 8.0. Cells were sonicated on ice for 5 × 30 sec using a Vibra-Cell VCX-500 sonicator (Sonics and Materials, Danbury, CT, USA) set at 55% amplitude with a 13 mm diameter probe. The cell lysate was centrifuged at 10,000 × g for 30 min at 4°C. The supernatant was aliquoted and stored at –20°C.

2.8.2 Expression of recombinant proteins

For protein expression, recombinant *E. coli* colonies were grown on LB agar containing 50 μg/ml carbenicillin, and a single colony was inoculated into 5 ml LB broth. The culture was incubated at 37°C at 225 rpm until it reached mid-log phase ($OD_{600\ nm}$= 0.5). The culture was stored at 4°C overnight for convenience, and the procedure was continued on the following day. Cells were harvested by centrifuging at 3,500 × g for 30 min at 4°C. The pellet was resuspended in 5 ml of LB, and used to inoculate 500 ml of LB (1:100 dilution of culture) that was incubated at 37°C while being shaken at 225 rpm on an Innova 4000 incubator shaker. When the culture reached mid log phase, isopropyl-β-δ-thiogalactopyranoside (IPTG; Cat. No. 724-815, Roche) was added to a final concentration of 1 mM to induce over-expression of recombinant protein. The culture was incubated at 37°C at 225 rpm for a further 3 h. One millilitre of the culture was removed pre- and 1, 2, 3 hours post-induction, and stored on ice until used. At the end of 3-hour induction, the culture was chilled on ice for 15 min, split into 250 ml centrifuge bottles (Cat. Nos. 3120-0250, 3122-0250, Nalgene Nunc International), and pelleted by centrifugation in a Sorvall® GSA rotor (Thermo Fisher Scientific, Waltham, MA, USA) at 5,000 × g for 30 min at 4°C using a Sorvall® RC-5 Plus centrifuge (Thermo Fisher Scientific). Each pellet was resuspended in 0.25 ml culture volume of ice-cold 20 mM Tris-HCl pH 8.0, and centrifuged as above. The pellets were stored at –70°C. Protein expression was analysed using 1 ml cultures removed pre- and post-induction. The volumes of culture giving 0.5 OD_{600} were pelleted at 13,000 × g for 2 min. Each cell pellet was resuspended in 20 μl of 2 × sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (0.125 M Tris-HCl pH 6.8, 4% [w/v] SDS, 20% [v/v] glycerol, 10% [v/v] β-mercaptoethanol, 0.2% [w/v] bromophenol blue), and heated at 95°C for 10 min. The samples were then analysed by SDS-PAGE.

2.8.2.1 Solubility of recombinant proteins

To determine the solubility, 100 ml of an induced culture was centrifuged at 5,000 × g for 30 min at 4°C in a Sorvall® GSA rotor. The cell pellet was resuspended in 10 ml of ice-cold phosphate-buffered saline pH 7.4 (PBS; 140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) supplemented with Complete™ EDTA-free Protease Inhibitor Cocktail (Cat. No. 11-836-170-001, Roche). The suspension was sonicated and pelleted at 10,000 × g for 15 min at 4°C. The supernatant was kept on ice. The pellet was washed twice with 2 ml of ice-cold PBS pH 7.4, and was resuspended in 3.75 ml of 1% SDS by vigorous vortexing. The supernatant and the resuspended pellet were mixed separately with an equal volume of 2 × SDS-PAGE loading buffer, and heated at 95°C for 10 min. The samples were analysed by SDS-PAGE.

2.8.3 Protein purification

2.8.3.1 Preparation of recombinant E. coli cell lysates

Cell lysates of recombinant *E. coli* cultures were prepared from the pellets prepared as described in Section 2.8.2. A two-hundred and fifty millilitre cell pellet was thawed on ice, and resuspended in 20 ml of chilled extraction/wash buffer pH 7.0 (50 mM sodium phosphate, 300 mM NaCl) supplemented with 750 µl of 100 mg/ml lysozyme and Complete™ EDTA-free Protease Inhibitor Cocktail (Roche) at the concentration recommended by the manufacturer. The suspension was incubated at room temperature for 30 min, and transferred to a 50 ml polypropylene centrifuge tube. It was subjected to sonication as described in Section 2.8.1. The cell lysate was pelleted by centrifugation at 10,000 × g for 30 min at 4°C, and the supernatant was used for protein purification.

2.8.3.2 Immobilized metal affinity chromatography (IMAC)

IMAC is a protein purification system that utilizes a metal chelating chromatographic matrix to form a bond between a metal ion (such as nickel, zinc or cobalt) and histidine residues present on histidine-tagged recombinant proteins. Upon washing the protein-

bound resin, histidine-tagged proteins will remain on the matrix while other proteins are removed. Subsequently, proteins were eluted with imidazole (Cat. No. I2399, Sigma). Imidazole competitively binds to the metal ions displacing the recombinant protein from the matrix. Recombinant proteins expressed in this study were soluble, thus they were purified under native conditions using BD TALON™ Metal Affinity Resins (Cat. No. 8901-1, Clontech Laboratories, Inc., Palo Alto, CA, USA). BD TALON™ resins are cobalt-based resins for the purification of histidine-tagged recombinant proteins. Two milliltres of resuspended resin (one-ml bed volume) was equilibrated with extraction/wash buffer pH 7.0 according to manufacturer's instructions. Twenty millilitres of supernatant prepared as described in Section 2.8.3.1 was added to a 50 ml polypropylene centrifuge tube containing the equilibrated resin. The mixture was incubated at room temperature for 30 min with gentle shaking to allow binding of histidine-tagged protein to the resin. The mixture was centrifuged at 1,200 rpm for 5 min and the supernatant removed. The resin was washed twice with 20 bed volumes of extraction/wash buffer pH 7.0 by shaking the suspension gently at room temperature for 10 min and centrifuging at 1,200 rpm for 5 min. The resin was resuspended in one bed volume of extraction/wash buffer pH 7.0 by vortexing, and the suspension was transferred to a 2-ml disposable gravity column (Cat. No. 8903-1, Clontech). After the resin had settled, buffer was allowed to drain and the column was washed once with 5 bed volumes of extraction/wash buffer pH 7.0. The recombinant protein was eluted and collected in fractions of 1 ml with 5 bed volumes of elution buffer pH 7.0 (50 mM sodium phosphate, 500 mM NaCl, 150 mM imidazole). Fractions were analysed by SDS-PAGE, and those containing the purified protein were pooled and stored at –80°C until required.

2.9 Protein analysis

2.9.1 Estimation of protein concentration

Protein concentration was estimated using the Bio-Rad Protein Assay (Bio-Rad), which is based on the method of Bradford (Bradford, 1976). This is a dye-binding assay that shows a variable colour change of the Coomassie® Brilliant Blue G-250 dye in response to different concentrations of the protein. The working dye was prepared by

diluting one part of the Bio-Rad Protein Assay Dye Reagent Concentrate (Cat. No. 500-0006) with 3 parts of distilled, deionized water (1:4 dilution). Bovine serum albumin (BSA; Cat. No. 23209, Pierce, Rockford, IL, USA) was used to prepare the protein standards. Five dilutions of BSA covering 1.2–10 μg/ml were prepared in distilled water to obtain a standard curve. Eight hundred microlitres of each standard and diluted sample solutions were added into separate disposable cuvettes. Two hundred microlitres of diluted dye reagent was then added into each cuvette and gently mixed. Each standard and sample was assayed in duplicate. The contents were incubated at room temperature for 10 min and the absorbance was read at 595 nm using the Helios α spectrophotometer.

2.9.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

A modified Laemmli (Laemmli, 1970) procedure was used to separate proteins on a discontinuous acrylamide gel consisting of a 4% stacking gel (0.125 M Tris-HCl pH 6.8, 0.1% [w/v] SDS, 4% [w/v] acrylamide/bis (Cat. No. 161-0121, Bio-Rad), 0.05% [w/v] ammonium persulfate (APS; Cat. No. 161-0700, Bio-Rad), 0.05% [v/v] N-N-N-N-tetramethylane diamine (TEMED; Cat. No. 161-0800, Bio-Rad)), and a 12% separating gel (0.375 M Tris-HCl pH 8.8, 0.1% [w/v] SDS, 12% [w/v] acrylamide/bis, 0.05% [w/v] APS, 0.05% [v/v] TEMED). Separation of proteins is based on the mass of the protein. Samples were mixed with an equal volume of 2 × SDS-PAGE gel loading buffer, and heated at 95°C for 10 min. Ten microlitres of Prestained SDS-PAGE Standard, broad range (Cat. No. 161-0318, Bio-Rad)/Precision Plus Protein™ All Blue Standard (Cat. No. 161-0373, Bio-Rad)/Precision Plus Protein™ Dual Colour Standard (Cat. No. 161-0374, Bio-Rad)/ Prestained BenchMark™ Protein Ladder (Cat. No. 10748-010, Invitrogen) was included in one lane as a size reference. Electrophoresis was carried out in a vertical mini-gel apparatus (Hoefer Scientific Instruments, San Francisco, CA, USA) containing running buffer pH 8.3 (0.192 M glycine, 0.02 M Tris base, 0.1% [w/v] SDS) for 2-3 h at 100V (PowerPac, Bio-Rad). Proteins were stained with gentle agitation for 30 min at room temperature with 0.125% (w/v) Coomassie Brilliant Blue R–250 (Cat. No. B0149, Sigma) in fixative 40% (v/v) methanol and 10% (v/v) acetic acid. Gels were then destained in 40% (v/v) methanol and 10% (v/v) acetic acid until the stained proteins were visible.

Subsequently, gels were dried in an Easy Breeze gel drier (Hoefer Scientific Instruments) for records.

2.9.3 Western immunoblots

Following gel electrophoresis, proteins in the gel were transferred onto BioTrace polyvinylidene difluoride membrane (PVDF; Cat. No. 66543, Pall Gelman Sciences) using a trans-blot electrophoretic transfer unit (Bio-Rad) containing transfer buffer (25 mM Tris base, 193 mM glycine, 20% [v/v] methanol, 0.015% [w/v] SDS) for 3-4 h at 100V. Alternatively, transfer was carried out overnight at 30V followed by 1 h at 100V. Transfer was confirmed by staining the membrane with 0.2% (w/v) Ponceau S (Cat. No. P3504, Sigma) in 30% (v/v) trichloroacetic acid for 10 min with shaking. The membrane was washed briefly in double-distilled water, and the non-specific sites on the membrane were blocked with 5% skim milk in Tris-buffered saline pH 7.4 (TBS; 20 mM Tris-HCl, 100 mM NaCl) supplemented with 0.1% Tween–20 (Cat. No. P1379, Sigma) overnight at 4°C, or for 2 h at room temperature. TBS containing 0.1% Tween-20 (TTBS) and 5% skim milk was used as the dilution buffer for reagents, and TBS with 0.1% Tween-20 was used as the washing buffer. The membrane was then cut into nine strips using a clean scalpel blade, and further incubations of each strip were carried out in individual reagent vessels (Cat. No. 783500, Biohit Corp, Helsinki, Finland). Each strip was incubated with 10 ml of diluted sheep/rabbit immune sera for 1 h at room temperature. For colorimetric detection, each strip was washed for four 5-10 min cycles in TTBS, and incubated with 10 ml of diluted alkaline phosphatase-labelled (AP-labelled) donkey anti-sheep (Cat. No. A5187, Sigma) or goat anti-rabbit (Cat. No. A3687, Sigma) whole IgG antibody for 1 h at room temperature. After another six 10-min-washes, the strips were incubated with 10 ml of colorimetric substrate prepared by dissolving 1 tablet of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT; Cat. No. B5655, Sigma) in water. When bands were observed, strips were washed twice in water, and dried between filter paper. Sera from a positive and a negative control sheep/rabbit were included in each blot for comparison. A separate Western blot was carried out to confirm the presence of histidine-tagged proteins post-purification. Briefly, a strip containing the fusion protein was incubated with 10 ml of 1:1,000 diluted anti-histidine mouse IgG monoclonal antibody (Cat. No. 34660, Qiagen), and 10 ml of 1:300 diluted peroxidase-labelled

sheep anti-mouse IgG (Cat. No. NXA931, Amersham Pharmacia Biotech). Each incubation step was carried out for 1 h at room temperature with a washing step as previously described. After another cycle of washing, the strip was incubated with 10 ml of peroxidase substrate 3,3′-Diaminobenzidine (DAB; Cat. No. D4418, Sigma) for colorimetric detection.

2.10 DNA and protein analysis

DNA sequences were edited and analysed using Chromas software (Technelysium Pty Ltd., Helensvale, Queensland, Australia). They were submitted to the GenBank®, a genetic sequence database that contains an annotated collection of all publicly available nucleotide and protein sequences. GenBank® is built by the National Centre for Biotechnology Information (NCBI) at the National Institutes of Health (NIH, Bethesda, MD, USA). Parameters including any identical/similar sequences and open reading frames (ORFs) of DNA inserts used in this study were obtained through the BLAST server at NCBI (http://www.ncbi.nlm.nih.gov/blast/) (Altschul *et al.*, 1997). *S.* Brandenburg exported proteins were analysed with the use of the ExPASy Molecular Biology Server (http://www.expasy.ch/). Other servers used in the analysis of *S.* Brandenburg insert DNA and amino acid sequences are shown in Table 2.6.

Table 2.6. Servers used for the analysis of *S.* Brandenburg insert DNA and amino acid sequences

Server	Prediction	References
PSORTb v. 2.0.4 http://www.psort.org/psortb/	Subcellular localization of proteins	Gardy & Brinkman, 2006; Gardy *et al.*, 2005
Neural Network (NN) and Hidden Markov Model (HMM) methods of SignalP 3.0 server http://www.cbs.dtu.dk/services/SignalP/	Presence and location of signal peptide cleavage sites	Bendtsen *et al.*, 2004; Nielsen & Krogh, 1998; Nielsen *et al.*, 1997
LipoP 1.0 server based on HMM http://www.cbs.dtu.dk/services/LipoP/	1. Lipoproteins 2. Discriminate between lipoprotein signal peptides, other signal peptides and N-terminal membrane helices	Juncker *et al.*, 2003
TatP 1.0 server http://www.cbs.dtu.dk/services/TatP/	Presence and location of Tat signals	Bendtsen *et al.*, 2005
TMHMM v. 2.0 based on (HMM) http://www.cbs.dtu.dk/services/TMHMM-2.0/	Transmembrane helices	Krogh *et al.*, 2001; Sonnhammer *et al*; 1998
HMMTOP v. 2.0 server http://www.enzim.hu/hmmtop/	Transmembrane helices and protein topology	Tusnády & Simon, 1998, 2001
Phobius http://www.phobius.cgb.ki.se/	Signal peptides and protein topology	Käll *et al.*, 2004
MEMSTAT v. 3.0 server http://www.bioinf.cs.ucl.ac.uk/psipred/	Protein topology	Jones, 1998; Jones *et al.*, 1994
TMpred server http://www.ch.embnet.org/software/TMPRED-form.html	Transmembrane helices and protein topology	Hofmann & Stoffel, 1993
TopPred server http://www.bioweb.pasteur.fr/seqanal/interfaces/toppred	Protein toplogy	Claros & von Heijne, 1994
PHDhtm at PredictProtein server http://www.predictprotein.org/	Protein topology	Rost, 1996; Rost *et al.*, 1996
Neural Network Promoter Predictor (NNPP) v. 2.2 http://www.fruitfly.org/seq_tools/promoter.html	Promoters	Reese, 2001

2.11 Sheep experiment with Salvexin^{TM}+B vaccine (Trial 2004)

Twelve four-month old Romney-ewe-hoggets from a farm without a previous history of *S.* Brandenburg were purchased through Agricultural Research Services of Massey University, Palmerston North. They were ear-tagged for identification, housed at the Massey University Animal Research Facilities, Palmerston North, and fed on pasture with water *ad libitum* throughout the study. Faecal samples were collected when they were 8 months of age, and thereafter every 2 weeks until they were 9 months. The faeces of all animals tested negative for *Salmonella* by culture. At 9 months, 6 randomly selected sheep were each vaccinated with 2 ml of Salvexin^{TM}+B commercial vaccine (Schering-Plough Animal Health Ltd.) subcutaneously in the anterior part of the neck, and were re-vaccinated after one month. Faeces, whole blood and sera were collected on day 0 before primary, and on day 30 before booster vaccine. Thereafter, samples were collected 3-weeks, and 2- and 3-months post-booster. Faeces, whole blood and sera collected prior to vaccination were tested to screen the experimental sheep for faecal shedding of *S.* Brandenburg, and reactivity to *S.* Brandenburg antigens. None of the sheep excreted *Salmonella* in faeces prior to, or during the experiment. Neither did they show the presence of antibodies against *Salmonella* antigens prior to the experiment. This work was approved by the Animal Ethics Committee of Massey University in Palmerston North. Table 2.7 shows the sheep used in the study.

Table 2.7. Sheep used in the Salvexin^{TM}+B experiment (Trial 2004)

Sheep number	
Control group	Vaccinated group
C005	V016
C006	V019
C015	V020
C017	V021
C022	V024
C026	V025

2.11.1 Faecal cultures

Approximately 1-2 grams of faeces were collected from the rectum of each animal into a uniquely identified sterile disposable container using a sterile gloved finger containing a non-bactericidal lubricant. The glove was changed before collecting each faecal sample. Faecal samples were kept on ice and processed within 4 h of collection. One gram of faeces was added to 10 ml of buffered peptone water, and incubated at 37°C for 24 h. Aliquots of 1-ml cultures were inoculated into 9 ml of tetrathionate broth supplemented with 200 µl of iodine-potassium iodide solution (30% w/v iodine, 25% w/v potassium iodide), and 9 ml of selenite F broth. The tetrathionate and selenite F broth cultures were incubated at 42°C and 37°C respectively for 24 h. A loopful of each broth culture was streaked onto XLD and MacConkey agar plates, and incubated at 37°C for 24 h. Any colonies suspected of being *Salmonella* species (while *Salmonella* colonies are red with a black centre on XLD agar, they are greyish on MacConkey agar) were inoculated into TSI agar, urca agar and lysine decarboxylase broth, and incubated at 37°C for 24 h. Suspected *Salmonella* colonies from TSI agar (most *Salmonella* isolates show a red slant, a yellow butt and H_2S production) were subjected to agglutination tests with *Salmonella* polyvalent O antiserum. Each assay contained a negative and a positive control. Faecal cultures of all 12 sheep used in the experiment were negative for *Salmonella* throughout the experiment.

2.11.2 IFN-γ assay

2.11.2.1 Antigen preparation

E. coli LPS present in recombinant antigen preparations were removed by passing over END-X® B15 Endotoxin Affinity Resins (Cat. No. R0026, Associates of Cape Cod, Inc., East Falmouth, MA, USA) according to the manufacturer's instructions. Protein preparations were incubated with 10 µg/ml polymyxin B sulfate (PMB; Cat. No. P4932, Sigma) at room temperature for 1 h to neutralize the biological activity of any remaining traces of LPS. PBS was used as a negative control. The non-specific T-cell activator, Concanavalin A (ConA; Cat. No. C5275, Sigma), was used at a final concentration of 20 µg/ml to check cell viability.

2.11.2.2 IFN-γ assay

IFN-γ assays were performed using a commercially available bovine IFN-γ capture ELISA kit (BOVIGAM™ EIA kit; Cat. No. 03000201, Commonwealth Serum Laboratories Ltd., Parkville, Victoria, Australia), according to the manufacturer's instructions. A monoclonal antibody (MAb) assay for bovine IFN-γ, using whole blood from animals incubated with *Mycobacterium bovis* purified-protein derivative (PPD) has been used in the bovine tuberculosis eradication programs (Whipple *et al.*, 1995). Due to cross-reactivity of the MAb to bovine IFN-γ with sheep IFN-γ (Rothel *et al.*, 1990), BOVIGAM™ EIA kit has been used to study IFN-γ responses of sheep infected with pathogens including *Mycobacterium paratuberculosis* (Dupont, 2002) and *Corynebacterium pseudotuberculosis* (Pépin *et al.*, 1997). Briefly, 20 ml of whole blood was collected from the jugular vein of each sheep into sodium heparin-containing 10 ml vacutainers (Cat. No. 366480, Becton Dickinson, Sparks, MD, USA) using 20 G × 1" vacutainer precision glide needles (Cat. No. 60214, Becton Dickinson), and mixed by gentle inversion several times. Blood and antigens were assayed in duplicates. One millilitre of blood was placed within 4 h of collection in each well of a 24-well plate (Nalgene Nunc International). One hundred microlitres of antigen or the control was added to each of 2 wells containing blood from each sheep, and the contents were mixed by shaking plates for 5 min on a rotating shaker. Plates were kept for 22 h at 37°C in a humidified incubator containing 5% CO_2. Two hundred microlitres of plasma from each well was removed into 96-well plates, and were stored at 4°C to be used on the following day, or at –20°C until used. Each plasma sample was assayed in duplicate for IFN-γ using BOVIGAM™ EIA plates according to the manufacturer's instructions. Three IFN-γ positive, and three IFN-γ negative controls included in the kit were used. Absorbance values were read at 450 nm using a MAXline VMax® kinetic ELISA reader (Molecular Devices Corp., Sunnyvale, CA, USA). Results were expressed as changes in the optical density, i.e. the mean OD of antigen-stimulated supernatants minus the mean OD of the PBS control, unstimulated supernatants.

2.11.3 Serological tests

For serological tests, blood samples were collected into non-heparinized 10 ml vacutainers (Cat. No. 366430, Becton Dickinson) and were left undisturbed at room temperature for approximately 3 h for clotting. Blood samples were centrifuged at 3,000 rpm for 20 min, sera were collected, aliquoted into 2 ml microcentrifuge tubes and stored at –70°C until assayed. Sera were tested for reactivity with *S.* Brandenburg antigens.

2.12 Other sources of sheep sera

2.12.1 Sheep naturally infected with *S.* Brandenburg

Sera were collected from 81 *S.* Brandenburg infected ewes approximately 3-4 weeks after abortions. Vaginal swabs collected from these ewes were subjected to routine bacteriological procedures to confirm *S.* Brandenburg infection. Furthermore, sera were collected from 80 sheep from farms without a history of *S.* Brandenburg infections. Faecal samples collected from these sheep were negative for *S.* Brandenburg. Samples were collected from sheep in South Island farms in NZ by Drs. John Smart (Clutha Vets Animal Health Centre, Balclutha, NZ) and Andrew Roe (Central Southland Veterinary Services, Winton, NZ).

2.12.2 Sera from sheep vaccination trial to compare Salvexin™ with Salvexin™+B (Trial 1999)

Ten sera collected from each group of sheep vaccinated with Salvexin™ and Salvexin™+B were used. Sera collected pre-vaccination, and 2-weeks post-booster were used in this study. This vaccine trial was conducted in 1999 at Massey University by Dr. Stanley Fenwick of Massey University, Palmerston North in collaboration with Schering-Plough Animal Health Ltd.

2.13 Rabbit sera

These sera were a generous donation from Jo Kerslake of Epicentre, Massey University, Palmerston North, NZ (Kerslake, 2003). The sera came from eight rabbits that were divided into 4 groups. On day 0 and 14, rabbits in each group were inoculated subcutaneously in the neck with 1 ml of an emulsion containing, either heat-inactivated *S.* Brandenburg, *S.* Typhimurium or *S.* Hindmarsh. One group that did not receive the emulsion served as a negative control. Blood samples were collected on day 0-, and 14-, 28- and 42-days post-inoculation. Sera stored at –70°C were used in this study.

2.14 Statistical analysis

Microsoft Excel (Microsoft Corp, Redmond, WA, USA) was used for basic calculations including means, standard deviations, and standard error of means. Results were analysed by the Student's *t*-test (Microsoft Excel) and considered significant when p values were <0.05.

Chapter 3 Identification of *S.* Brandenburg genes coding for exported proteins using a PhoA fusion screen

3.1 Abstract

Proteins that are exported out of the cytoplasm to the cell envelope, or that are secreted to the extracellular environment, are involved in cellular processes leading to virulence and immune responses. Alkaline phosphatase (PhoA) fusion technology has been widely used to create recombinant DNA libraries in order to identify such proteins from a number of pathogens. In this study, an *S.* Brandenburg genomic library was constructed in *E. coli* using the *phoA* reporter plasmid pJEM11 to identify the genes encoding exported proteins. Genomic DNA was partially digested, cloned into pJEM11, and transformed into *E. coli* DH10B cells. Subsequently, PhoA-fused exported proteins were selected as blue colonies on LB agar containing the colorimetric PhoA substrate, 5-bromo-4-chloro-3-indolylphosphate (BCIP). Expression of PhoA fusions was confirmed by Western blotting of selected recombinant *E. coli* cell lysates with PhoA-specific antibody. The DNA sequences of inserts from selected blue colonies were analysed using DNA and protein databases to identify those encoding exported proteins of interest.

3.1 Introduction

Pathogens including *Salmonella* encode exported proteins that are on the cell envelope (the inner membrane, the periplasm, and the outer membrane), or are secreted into the medium. These proteins have a number of functions including cell wall synthesis, pili and flagella biogenesis, nutrient uptake, and environmental sensing. Furthermore, exported proteins are involved in antibiotic resistance, virulence and immune responses due to their interaction with the host. Therefore, they have been the target for the development of vaccines, antimicrobials and diagnostic tests (Lewenza *et al.*, 2005).

Exported proteins contain a signal sequence at their amino terminus that targets them for export. Alkaline phosphatase gene fusion technology is a genetic tool to identify proteins that are translocated across the inner membrane. This technique has been used to study the exported proteins of a number of pathogens including *Actinobacillus actinomycetemcomitans* (Mintz & Fives-Taylor, 1999; Ward *et al.*, 2001), *Helicobacter pylori* (Bina *et al.*, 1997; Oliaro 2000), *Borrelia burgdorferi* (Kornacki & Oliver, 1998), *Mycobacterium tuberculosis* (Braunstein *et al.*, 2000; Lim *et al.*, 1995), *Mycobacterium avium* subspecies *paratuberculosis* (Dupont & Murray, 2001), *Treponema pallidum* (Blanco *et al.*, 1991), *Streptococcus pneumoniae* (Pearce *et al.*, 1993), Mycoplasma (Cleavinger *et al.*, 1995) and *Pseudomonas aeruginosa* (Lewenza *et al.*, 2005). *E. coli* alkaline phosphatase (PhoA) is a periplasmic enzyme that is encoded by the *phoA* gene. PhoA is synthesized as a monomer with a type I signal peptide at its amino terminus. After translocation into the periplasm, the signal peptide is removed, and disulfide bonds are formed with the help of disulfide bond formation proteins. Subsequently, PhoA folds and dimerises in the presence of four Zn2+ ions and two Mg2+ ions to form the enzymatically active conformation. PhoA is inactive in the reducing environment of cytoplasm due to the absence of intra-chain disulfide bonds (Kadokura *et al.*, 2001; Nakamoto & Bardwell, 2004). The pJEM11 vector (Figure 3.1) used in this Chapter contains a truncated *E. coli pho*A gene that does not possess a promoter, a ribosomal binding site, a start codon, and the signal sequence needed for the expression and export of PhoA. It is expressed when *phoA* is fused with a target gene in the correct orientation and reading frame. Subsequent subcellular localisation of the expressed protein depends on the presence or absence, and the type

of, the export signal. Export signals include cleavable signal sequences, and appropriately oriented non-cleavable signal sequences that remain as transmembrane segments of inner membrane proteins. If PhoA is fused to a protein that is in the inner membrane, periplasm, outer membrane or extracellular milieu, it will result in a PhoA positive colony. If it is fused to a cytoplasmic protein, it will result in a PhoA negative colony. Alkaline phosphatase activity can be detected on solid media containing BCIP, a colourless compound cleaved by PhoA to form a blue coloured compound. PhoA serves as a reporter for protein export signals.

This Chapter describes the construction of an *S.* Brandenburg secreted protein expression library in *E. coli* using the pJEM11 plasmid vector constructed by Lim and his co-workers (1995). *S.* Brandenburg inserts from selected PhoA positive blue colonies were sequenced to identify the proteins encoded by them, and their export signals.

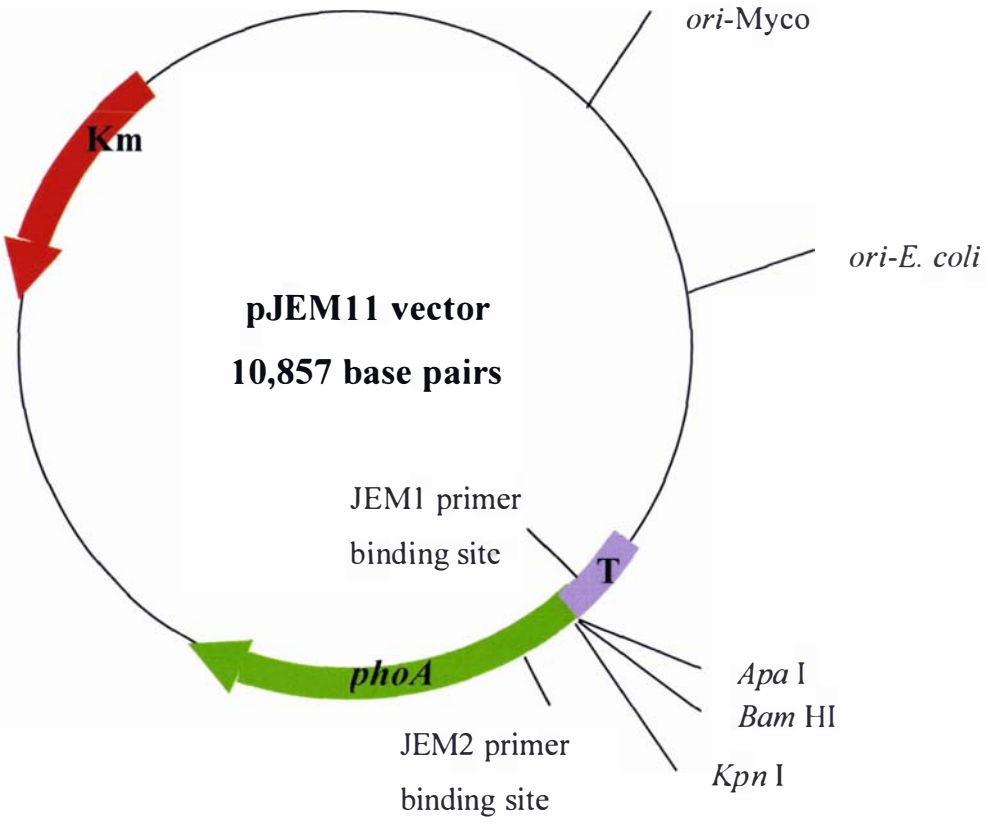

Figure 3.1. Map of the plasmid vector pJEM11. Features include origins of replication for *E. coli* (*ori–E.coli*) and mycobacteria (*ori*–Myco); kanamycin resistance marker (Km) for selection of transformants; truncated *E. coli phoA* gene that is devoid of the promoter, ribosomal binding site, signal sequence and the start codon needed for the expression and export of PhoA; multiple cloning site and the transcriptional terminator (T). Adapted from Lim *et al.*, 1995.

3.3 Materials and methods

3.3.1 Construction of an *S.* Brandenburg protein expression library

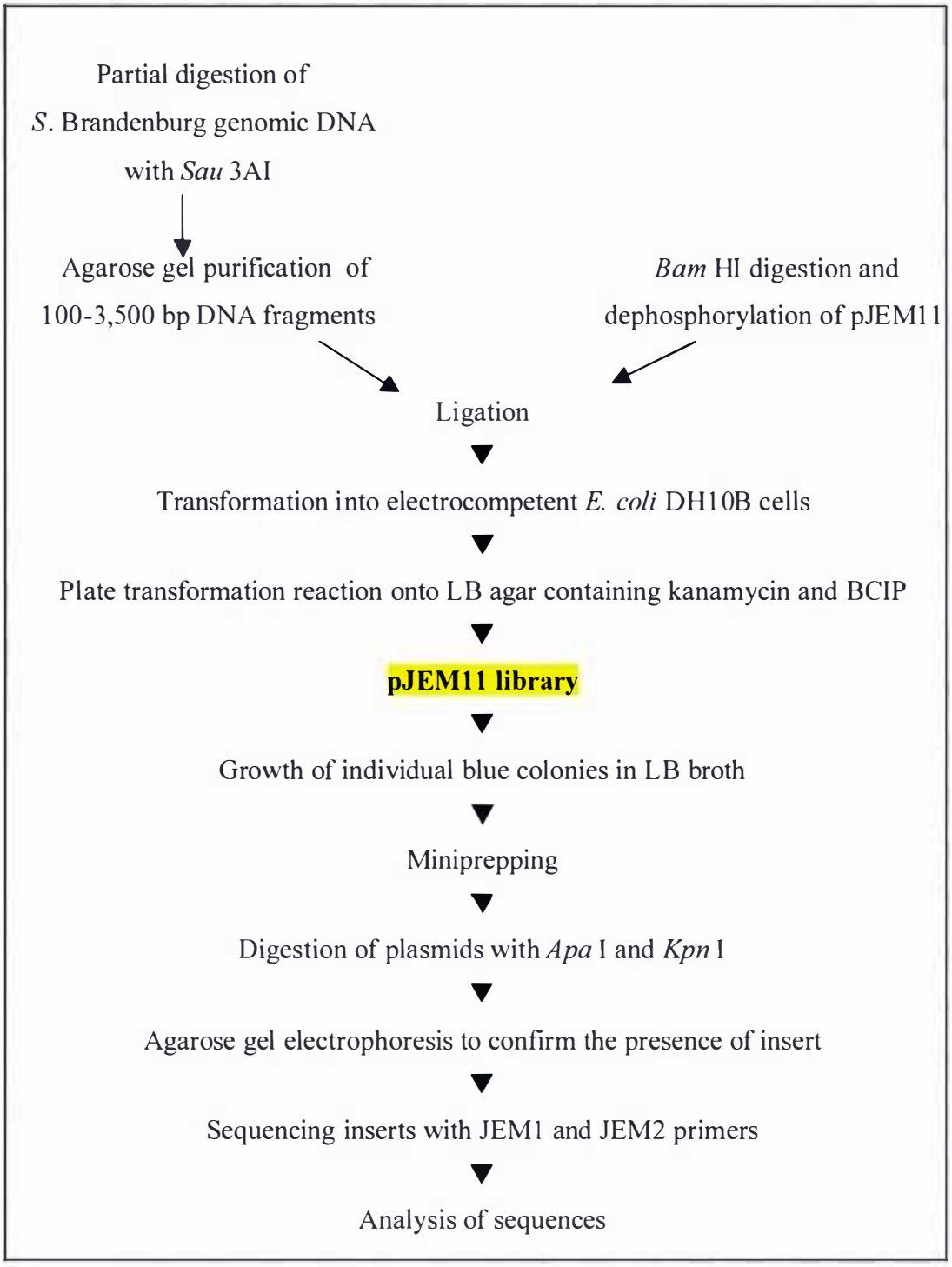

Figure 3.2. Flow chart showing steps in the construction of pJEM11 genomic library, and analysis of DNA inserts

3.3.1.1 Partial digestion of S. Brandenburg genomic DNA

For the preparation of an *S.* Brandenburg expression library, genomic DNA was isolated from strain S59 using phenol-chloroform method (Section 2.3.1.2), agarose gel-checked (Section 2.4.1) and quantitated (Section 2.4.3). To confirm that genomic DNA was from *Salmonella*, one μl of that was subjected to PCR amplification of *Salmonella*-specific *invA* gene. The two oligonucleotide primers *invA*-F and *invA*-R were from a paper published by Rahn and his co-workers (1992), and are shown in Table 3.1. PCR conditions were as described in Section 2.4.4.2, and consisted 30 cycles of denaturation (95°C for 1 min), annealing (60°C for 30 sec), elongation (72°C for 30 sec), and a final elongation (72°C for 7 min) step. The PCR product was gel purified (Section 2.4.2), and sequenced with *invA*-F and *invA*-R primers. Subsequently, genomic DNA was subjected to PFGE to confirm its source as *S.* Brandenburg. Following this, 4 μg of genomic DNA was partially digested with 1 unit of *Sau* 3AI (Cat. No. 709-743, Roche) in a reaction volume of 20 μl at 22°C for 5 min. Digestion was stopped by the addition of 5 μl of 0.5 M EDTA pH 8.0. The partial digest was electrophoresed on a 0.7% agarose gel, DNA fragments corresponding approximately to 100-3,500 bp region was excised, and agarose gel-purified (Section 2.4.2). DNA was eluted in 100 μl of EB buffer supplied with the kit, and 15 μl was gel checked. DNA concentration was quantitated by GeneQuant (Section 2.4.3), and the purified digest was stored at –20°C until used.

3.3.1.2 Preparation of pJEM11 plasmid DNA

A glycerol stock of *E. coli* DH10B cells containing pJEM11 plasmid was used for the propagation of the plasmid vector. A few microlitres of the glycerol stock was inoculated into two tubes each containing 5 ml of LB broth supplemented with kanamycin. After an overnight incubation at 37°C, plasmid was isolated using QIAprep Spin Miniprep Kit (Section 2.3.2), and quantitated (Section 2.4.3). Approximately 10 μg of plasmid was digested with 10 units of *Bam* HI (Cat. No. 567-604, Roche) at 37°C for 2 h. The plasmid was dephosphorylated with 2 units of CIAP at 37°C for 1 h. Subsequently, CIAP was inactivated and removed with QIAquick PCR Purification Kit (Section 2.5.1). Plasmid DNA was eluted in 50 μl of EB buffer (10

mM Tris.Cl, pH 8.5) supplied with the kit, and the efficiency of phosphatase treatment was determined. Briefly, 400 ng of vector was treated with 1 unit of T4 DNA ligase in a total volume of 10 µl at room temperature for 1 h. The ligation reaction was dialysed, and electroporated into 20 µl of *E. coli* DH10B cells as described in Section 2.6.1. Cells were recovered by the addition of 300 µl of LB medium, and 150 µl of the transformation reaction was plated on LB agar supplemented with kanamycin. As controls, the same procedure was carried out with undigested pJEM11 vector. Plates were incubated overnight at 37°C, and the number of colonies resulted from digested and undigested plasmids were counted.

3.3.1.3 Construction of the S. Brandenburg pJEM11 library

Seven hundred nanograms (10 µl) of gel purified *Sau* 3AI partial digest was ligated into 1 µg (10 µl) of *Bam* HI digested and CIAP treated pJEM11 vector using 1 unit of T4 DNA ligase in a 30 µl reaction at 14°C for 15 h. The vector: insert ratio was 1: 3 (Section 2.5.2). The ligation reaction was dialysed and electroporated into 100 µl of electrocompetent *E. coli* DH10B™ cells in a 0.1 cm cuvette (Section 2.6.1). One millilitre of SOC medium was added to the cuvette, and incubated at 37°C for 1 h. The transformation reaction was diluted with 3 ml of SOC medium, and 100 µl aliquots were plated onto 40 LB agar plates supplemented with kanamycin and BCIP. The plates were incubated at 37°C for 24 h. The number of colonies present in one quarter of a plate was counted to get the approximate number of total colonies of the library. The number of blue colonies in all 40 plates was counted. Each blue colony was transferred to a LB plate containing BCIP and kanamycin to reconfirm that it was a blue colony, and to get a pure culture. Subsequently, a representative blue colony from each pure culture was grown in 5 ml of LB broth supplemented with kanamycin at 37°C overnight. Eight hundred microliters of each overnight culture was mixed with 200 µl of sterile glycerol, and frozen at –80°C (Section 2.2.4). The remainder of each culture was used to isolate the plasmid using QIAprep Spin Miniprep Kit (Section 2.3.2). The number of colonies needed to represent a complete *S.* Brandenburg library was calculated using the following formula (Clarke & Carbon, 1976):

$$N = \frac{\ln (1-P)}{\ln [1-(I/G)]}$$

P= 99% probability (0.99)

N= number of clones necessary

G= size of target genome (4.8×10^6 bp)

I= average size of cloned inserts (1,400 bp)

3.3.2 Screening of the genomic library for the presence of *Salmonella*-specific sequences

An aliquot of total plasmid preparation isolated from the genomic library was subjected to PCR amplification of *invA* gene to confirm that the source of genomic DNA was *Salmonella*. Briefly, colonies in each of the 40 plates (Section 3.3.1.3) were resuspended in 1 ml of LB broth supplemented with kanamycin. Plasmids in each one-ml bacterial suspension were obtained using a QIAprep Spin Miniprep Kit. DNA in each column was eluted with 50 µl of EB buffer and pooled (Section 2.3.2). One microlitre of the total plasmid preparation was subjected to PCR amplification (Section 3.3.1.1), and the PCR product was sequenced (Section 2.7).

3.3.3 Analysis of *S.* Brandenburg expression library

3.3.3.1 Restriction endonuclease digestions of plasmids

Both the presence and the sizes of the inserts in the *S.* Brandenburg expression library were determined by restriction endonuclease digestion of plasmids obtained from 113 blue colonies. Two microlitres of each plasmid was digested with 1 unit of *Kpn* I (Cat. No. 15232-036, Life Technologies) in a reaction volume of 10 µl at 37°C for 1 h. Five microlitres of the digest was removed for gel checking, and the rest was subjected to digestion with 1 unit of *Apa* I (Cat. No. 15440-019, Life Technologies) in a reaction volume of 10 µl at 30°C for 1 h. Digests were electrophoresed on 1% agarose gels (Section 2.4.1).

3.3.3.2 Sequencing and analysis of DNA inserts

S. Brandenburg DNA inserts present in pJEM11 plasmids isolated from 95 blue colonies were sequenced either with 2 oligonucleotide primers JEM1 and JEM2 or JEM2 alone using ABI Prism® 377 DNA Sequencer (Section 2.7). Details of primers are shown in Table 3.1. JEM1 binds to the complementary sequence of the transcriptional terminator. JEM2 binds to the coding sequence of the *phoA* gene of the pJEM11 plasmid, and was used to sequence over the fusion joint and the 3′ end of the *S.* Brandenburg insert DNA. Nucleotide sequences were edited using the Chromas software package. Nucleotide and deduced amino acid sequence data were analysed using databases described in Section 2.10. Start codons of fusion proteins were predicted by analysing BlastP data. Ribosomal binding sites (RBS, Shine Dalgarno sequences, SD) were predicted by manual examination of the inserts upstream of the predicted start codon for consensus sequence GGAGG, core SD motifs GAGG, GGAG, AGGA, and for at least 3 bases that match part of the SD. Upstream sequences of inserts were examined for promoters manually, and promoter predictions by Neural Network (http://www.fruitfly.org/seq_tools/promoter.html).

Table 3.1. Oligonucleotide primers used to study *S.* Brandenburg expression library

Primer	Sequence (5′ to 3′)	Product size	Reference
InvA-F	GTGAAATTATCGCCACGTTCGGGCAA	~285	Rahn *et al.*, 1992
InvA-R	TCATCGCACCGTCAAAGGAACC		
JEM1	CGAGCTGCAGTGGGATGACC	Variable	Pasteur Institute
JEM2	TCGCCCTGAGCAGCCCGGTT		

3.3.3.3 Western blot analysis of exported proteins

Western blots were carried out with cell lysates to detect the alkaline phosphatase moiety of exported fusion proteins. SDS-PAGE and Western blotting were carried out as described in Sections 2.9.2 and 2.9.3 respectively. Briefly, approximately 10 μg of

lysate protein prepared from each recombinant *E. coli* colony (Section 2.8.1) was electrophoresed on a 12% SDS-PAGE gel, and transferred onto PVDF membrane. Commercial *E. coli* alkaline phosphatase (Cat. No. E2110Y, Amersham Pharmacia Biotech) was used as a positive control in one lane. Non-specific sites of the membrane were blocked, and the membrane was incubated with 1:20,000 diluted rabbit anti *E. coli* alkaline phosphatase IgG (Cat. No. 100-4134, Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) at room temperature for 1 h. The membrane was washed, and incubated with 1:5,000 diluted alkaline phosphatase-labelled goat anti-rabbit IgG (Cat. No. A3687, Sigma) at room temperature for 1 h. Subsequently, membrane was washed and incubated with alkaline phosphatase substrate BCIP/NBT to observe the bands of reaction between alkaline phosphatase moiety of fusion protein and anti *E. coli* alkaline phosphatase antibodies. The membranes were washed and dried for permanent records.

3.4 Results

3.4.1 Construction of an *S.* Brandenburg expression library in the pJEM11 vector

S. Brandenburg DNA sequences that encode exported proteins were identified by the preparation of a genomic library of *S.* Brandenburg *phoA* fusions in the pJEM11 vector, and subsequent expression in *E. coli.* The schematic diagram showing the basis of the expression library is shown in Figure 3.3. A diagram showing different *E. coli* phenotypes is shown in Figure 3.4.

PCR amplification of both genomic DNA used for the construction of the library, and total plasmid isolated from the expression library showed a PCR product approximately of 285 bp on an agarose gel (Figure 3.5). Sequencing confirmed that the amplified product was *invA*. PFGE of genomic DNA used for the construction of the library confirmed that the source of genomic DNA was *S.* Brandenburg (Figure 3.6). The total number of colonies of the library was estimated to be 19,840 by counting those in a quarter of one selected plate (124 × 4 × 40). According to the formula of Clarke and Carbon (Clarke & Carbon, 1976), the number of colonies needed to represent a complete *S.* Brandenburg library is 14,436. A total of 410 blue colonies were obtained. This represents 2% of the total genomic library. *Kpn* I and *Apa* I digestion of plasmids isolated from 113 blue colonies showed that inserts were present in 109 of them. The average insert size was 1,400 bp with a range of 100–3,500 bp. Inserts were absent in 4 blue colonies. Figure 3. 7 shows the digested products of some clones on an agarose gel.

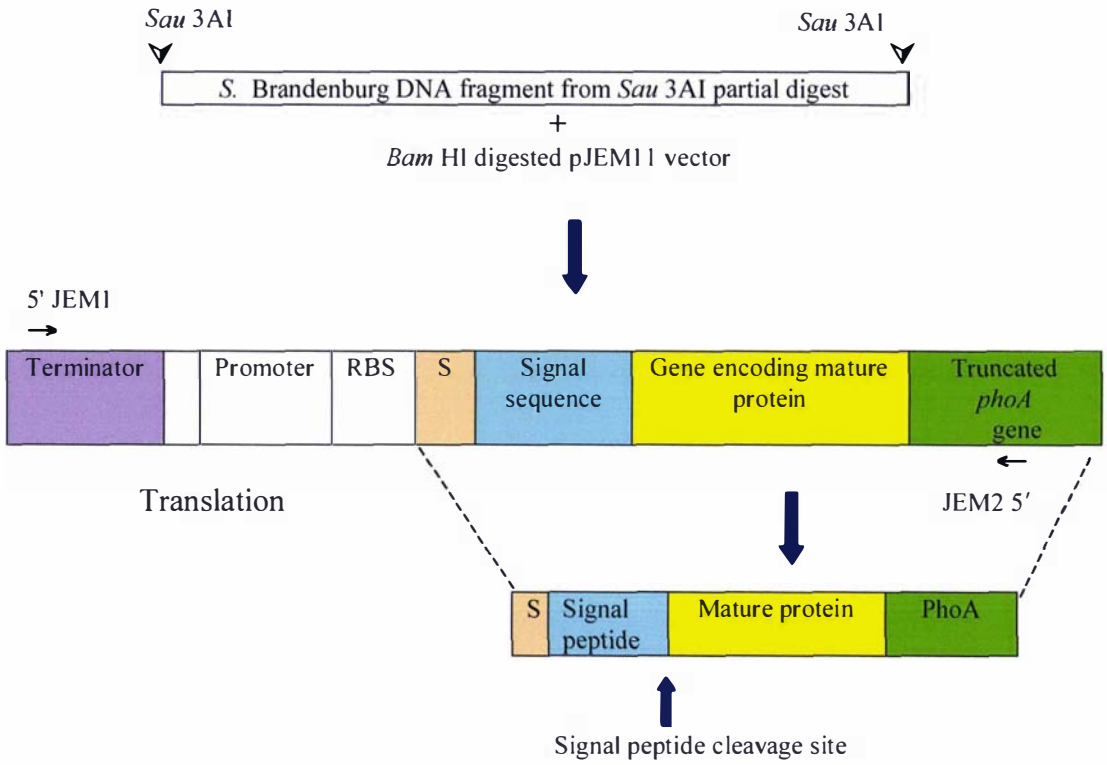

Figure 3.3. Schematic diagram showing the DNA elements needed for expression of PhoA fusion proteins, and the primers used for sequencing *S.* Brandenburg inserts in *phoA*+ constructs. DNA elements include a promoter, a ribosomal binding site (RBS), a start codon (S), a signal sequence, and a gene encoding the mature protein in frame with *phoA* gene of the pJEM11 vector. JEM1 binds to the complementary strand of the transcriptional terminator and JEM2 binds to the coding strand of the *phoA* gene.

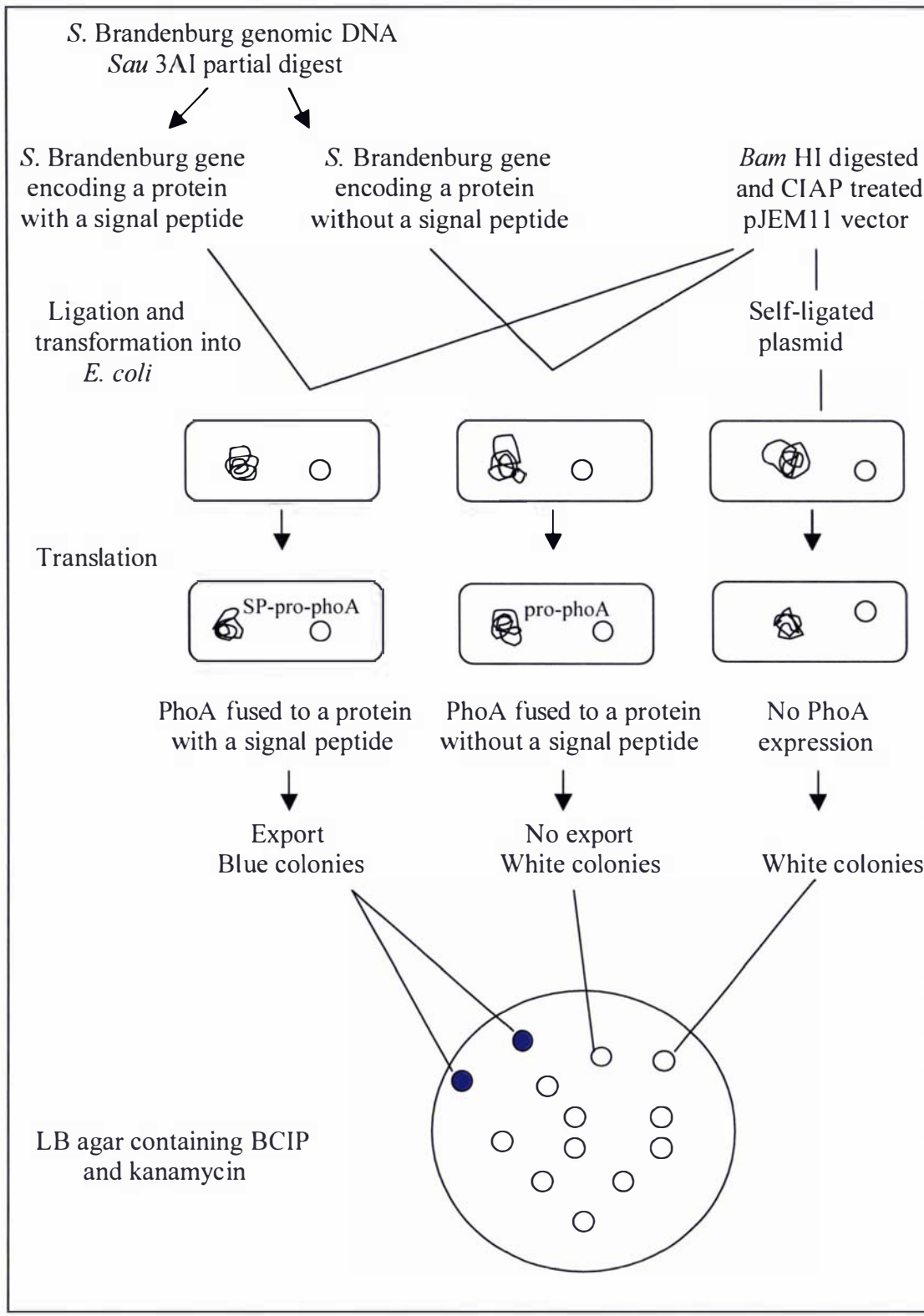

Figure 3.4. Schematic diagram showing different *E. coli* phenotypes of a *phoA* genomic library. SP, Signal peptide; pro, Mature protein.

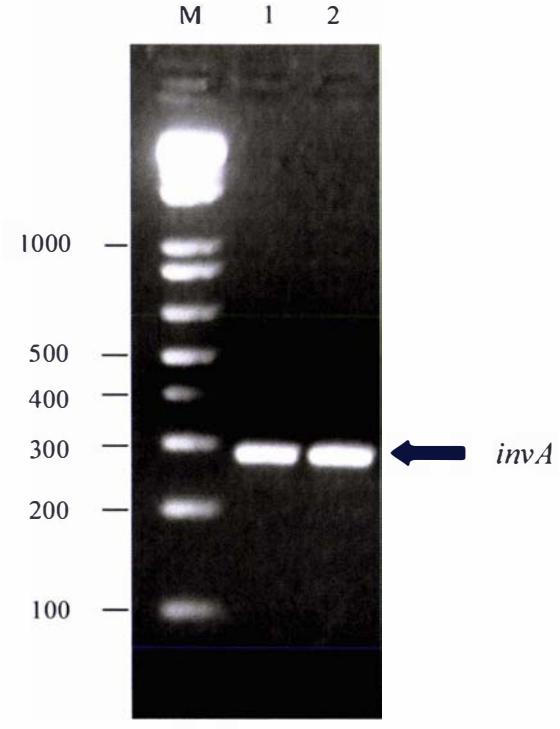

Figure 3.5. PCR amplification of *Salmonella*-specific *invA* gene from genomic DNA, and total plasmid isolated from the PhoA expression library. Lane M, 1-kb Plus DNA Ladder marker (bp); lane 1, PCR product from genomic DNA; lane 2, PCR product from total plasmid preparation. PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide and photographed under UV light. Blue arrow indicates the PCR product.

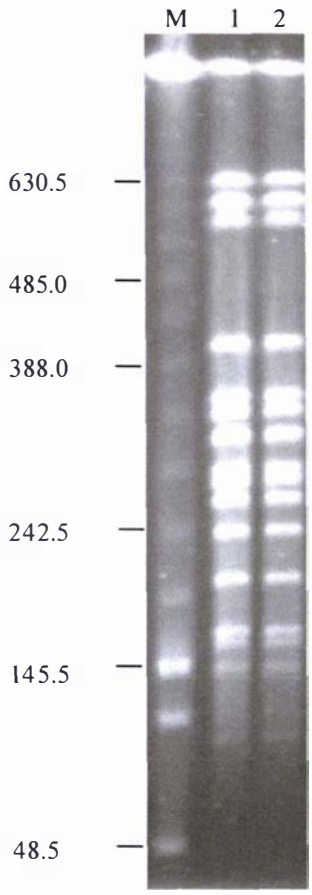

Figure 3.6. Pulsed-field gel electrophoresis (PFGE) profile of *S.* Brandenburg S59 genomic DNA used for the construction of pJEM11 library. Genomic DNA was digested with *Xba* I, and the digested products were electrophoresed on a 1% low melting agarose gel for 22 hours with an initial switch of 5 sec and a final switch of 50 sec. The gel was stained with ethidium bromide, and photographed under UV light. Lane M, Lambda Ladder (kbp) for molecular size comparison; lane 1, *S.* Brandenburg S59 genomic DNA; lane 2, *S.* Brandenburg reference strain genomic DNA.

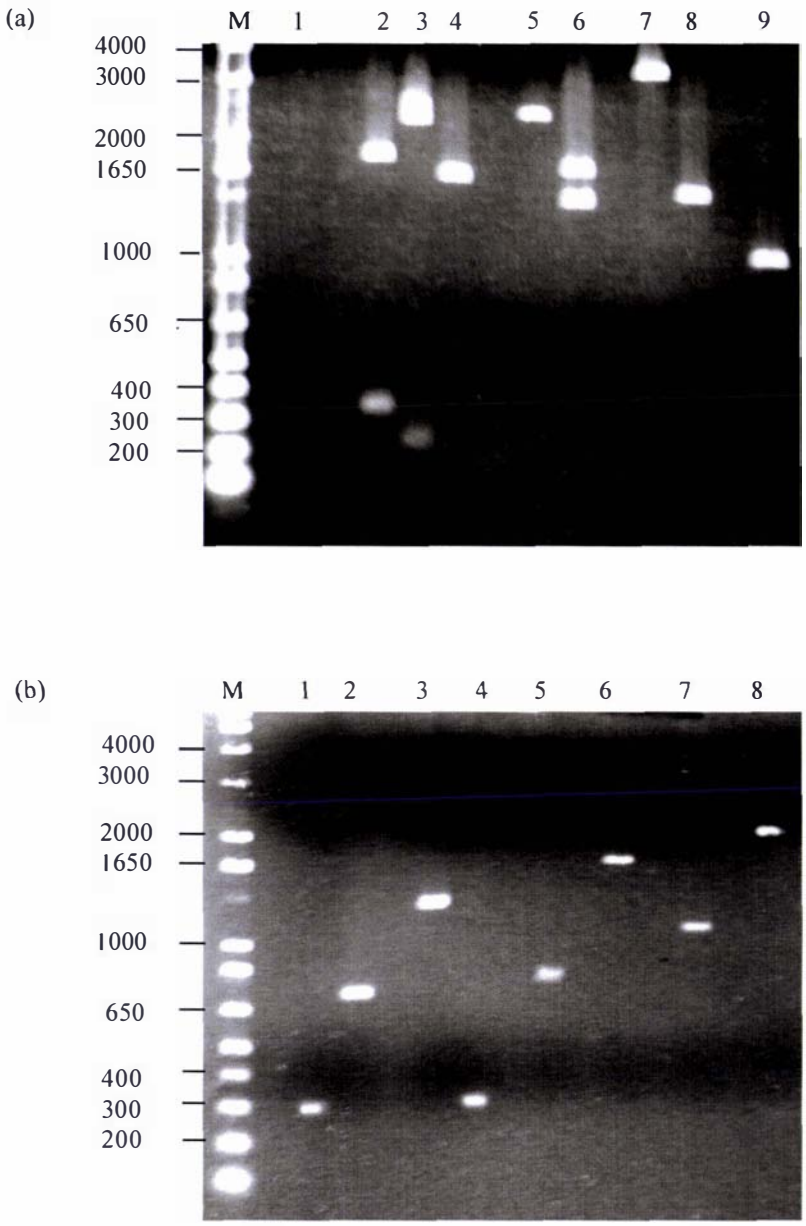

Figure 3.7. Restriction endonuclease digests of recombinant plasmids isolated from the expression library. Plasmids were digested with *Kpn* I and *Apa* I as described in Section 3.3.3.1. Digested products were electrophoresed on 1% agarose gels, stained with ethidium bromide, and photographed under UV light. Insert sizes were deduced from comparison with the DNA size marker. (a) Lane M, 1-kb Plus DNA Ladder marker (bp); lane 1, restriction endonuclease digest of plasmid from a white PhoA- colony; lanes 2 to 9, restriction endonuclease digests of plasmids from blue PhoA+ clones 16, 32, 44, 59, 70, 81, 89 and 103 respectively. (b) Lane M, 1-kb Plus DNA Ladder marker; lanes 1 to 8, restriction endonuclease digests of plasmids from blue PhoA+ clones 235, 318, 319, 320, 321, 333, 337 and 353 respectively.

3.4.2 Sequencing and analysis of *S.* Brandenburg DNA inserts encoding exported proteins

Ninety-five out of 109 plasmids that contained an insert were sequenced partially or completely to identify the genes encoding exported proteins. The cloned genes were followed by the sequence that encodes 22-amino-acid residue PhoA linker RIRTVPDSYTQVASWTEPFPFC, and then by the *phoA* gene that was lacking promoters, SD sequence, initiation codon, signal sequence, and the first 12 bases that encode the first 4 amino acids of the mature PhoA protein. The DNA and deduced amino acid sequences were analysed using DNA and protein databases to identify the open reading frames (ORF) responsible for the expression of PhoA fusions. Out of 95 PhoA fusions, 66 (69%) were unique while 29 (31%) were redundant. The ORF of all 66 unique fusion inserts were highly homologous (more than 94%) to those of various *Salmonella* strains available in the public databases. All were in frame with PhoA. Three groups of *PhoA* gene fusions were obtained. Forty-eight unique proteins listed in the first group (Table 3.2) contained the N-terminus and a predicted export signal. This group included 22 inner membrane, 16 periplasmic, 9 outer membrane and 1 extracellular protein. In this study, clone 46 contained the shortest fusion protein of 23 amino acids with homology to putative hemolysin-related protein YtfL. Clone 319 had the insert coding for the longest fusion protein of 306 residues with homology to periplasmic trehalase TreA. The second group of 7 unique proteins (Table 3.3) had the N-terminus without a known export signal. The protein sequences and upstream DNA sequences of these 7 proteins were manually examined for alternative start sites. The alternative translation products did not predict any export signal. BlastP of 5 of these proteins were unexpectedly homologous to known cytoplasmic proteins amidophosphoribosyltransferase PurF, response regulator in multi-component regulatory system TorR, invasion protein regulatory protein HilD, heat shock protein 33 HslO, and transcriptional activator CaiF. Two others had homology to phase-1 flagellin FliC and pathogenicity island-2 effector SseC. The third group of 11 proteins (Table 3.4) were partially sequenced so it was not possible to determine the N-termini, and the export signals. The complete ORF of these 11 proteins of other *Salmonella* strains obtained from the NCBI website showed the presence of export signals leading

7 of them to inner membrane, 2 to periplasm, and 2 to outer membrane. A summary of predicted subcellular localisation of 66 unique proteins is shown in Table 3.5.

Table 3.2. *S.* Brandenburg PhoA fusion proteins carrying predicted export signals

Clone No.	Insert size (bp)	Protein identity	Species	Gene [synonyms]	No. of fused amino acids	ORF full length	% ID	% SI	Signal[a]	Predicted location[b]
2 23	2600 300	AAL20563 Putative outer membrane lipoprotein	*S.* Typhimurium *S.* Typhi *S.* Paratyphi *S.* Choleraesuis	*ynbE*	40	63	100	100	LSP	Outer membrane
7	1825	AAL23053 Periplasmic maltose-binding protein/maltose transport	*S.* Typhimurium	*malE*	161	399	100	100	SSP	Periplasm
8 55	750 450	AAL21883 Putative POT family peptide transport protein	*S.* Typhimurium *S.* Typhi *S.* Paratyphi *S.* Choleraesuis	*ygdR*	34	72	100	100	LSP	Outer membrane
13 27 227 235	1400 300 450 300	AAL19598 A minor lipoprotein (rare lipoprotein B)	*S.* Typhimurium *S.* Choleraesuis *S.* Paratyphi *S.* Typhi	*rlpB*	37	196	97	100	LSP	Inner membrane
14 15 59 127	400 400 2500 400	YP_219414 Soluble lytic murein transglycosylase (exomuramidase)/ cell wall synthesis	*S.* Choleraesuis *S.* Typhimurium	*slt*	85	657	100	100	SSP	Periplasm

Table 3.2. Continued

16	2200	YP_215680 ABC superfamily glutamate/aspartate transporter	*S.* Choleraesuis *S.* Typhimurium *S.* Typhi *S.* Paratyphi	*gltI* [*ybej yzzk*]	48	308	100	100	SSP	Periplasm
18 74	1650 1650	CAD03184 Putative lipoprotein	*S.* Typhi	*yidQ* [*ecfL*]	72	111	95	98	LSP	Outer membrane
20 100	1100 1100	NP_459765 Putative pectinesterase (pectin methylesterase)/hydrolase involved in carbohydrate transport and metabolism	*S.* Typhimurium *S.* Choleraesuis *S.* Typhi *S.* Paratyphi	*ybhC*	30	427	96	100	LSP	Outer membrane
24	400	AAL20490 New outer membrane protein; predicted bacterial porin/outer membrane channels	*S.* Typhimurium *S.* Choleraesuis *S.* Paratyphi	*nmpC*	67	362	100	100	SSP	Outer membrane
28	400	YP_216956 Paral putative membrane component of transport system /predited transporter component	*S.* Choleraesuis *S.* Paratyphi *S.* Typhi *S.* Typhimurium	*yedE*	104	421	99	100	TMH (1)	Inner membrane
29	2200	YP_215655 Minor lipoprotein (rare lipoprotein A precursor)	*S.* Choleraesuis *S.* Paratyphi *S.* Typhimurium	*rlpA*	132	381	100	100	LSP	Outer membrane

Table 3.2. Continued

34	1400	YP_217920 Protease III/Insulinase like Zn-dependent peptidase	*S.* Choleraesuis	*ptr* [*ptrA*]	219	962	100	100	SSP	Periplasm
35 170	600 600	AAL22411 γ-glutamyltranspeptidase/ Catalysis of the reaction: (5-L-glutamyl)-peptide + an amino acid = peptide + 5-L-glutamyl-amino acid	*S.* Typhimurium *S.* Paratyphi *S.* Typhi	*ggt*	111	580	99	99	SSP	Periplasm
36	600	AAL22824 Putative outer membrane protein	*S.* Typhimurium	Not assigned	83	237	100	100	SSP	Outer membrane
38 238	2500 2500	AAL19154 Transpeptidase of penicillin-binding protein 1B (bifunctional murein transglycosylase and transpeptidase)/cell wall formation, drug resistance	*S.* Typhimurium *S.* Typhi	*mrcB* [*ponB*]	150	840	100	100	TMH (1)	Inner membrane
41 329	1300 1300	AAL22101 Putative periplasmic protein	*S.* Typhimurium *S.* Paratyphi *S.* Typhi	*yajC*	74	122	100	100	SSP	Periplasm

Table 3.2. Continued

43	650	YP_218473 Glycerol 3 phosphate binding periplasmic protein/glycerol 3 phosphate transporter	*S.* Typhi *S.* Paratyphi *S.* Choleraesuis	*ugpB* [*psiB*, *psiC*]	73	438	100	100	SSP	Periplasm
44 86	1650 1650	YP_215792 Putative permease/putative transport protein	*S.* Typhimurium *S.* Choleraesuis *S.* Paratyphi	*ybhL*	57	234	100	100	TMH (1)	Inner membrane
45 188	2300 2300	AAL21816 Aminopeptidase in alkaline phosphatase isozyme conversion	*S.* Typhimurium *S.* Choleraesuis *S.* Typhi	*iap*	112	348	100	100	SSP	Periplasm
46 52	3200 3200	AAL23227 Putative hemolysin-related protein	*S.* Typhimurium *S.* Choleraesuis *S.* Paratyphi *S.* Typhi	*ytfL*	23	447	100	100	TMH (1)	Inner membrane
47	1300	AAL20026 *Salmonella* pathogenicity island 5 encoded protein/secreted peptidase	*S.* Typhimurium	*pipD*	140	489	99	100	SSP	Extracellular
89	1400	AAL19208 Putative outer membrane lipoprotein. This is a NLPA lipoprotein. This family contains several antigenic proteins	*S.* Typhimurium *S.* Choleraesuis	*yaeC* [*metD*, *metQ*]	231	271	99	99	LSP	Outer membrane

Table 3.2. Continued

99 381	3500 3500	YP_218378 Glutathione-regulated potassium-efflux system protein	*S.* Choleraesuis *S.* Typhi *S.* Typhimurium *S.* Paratyphi	*kefB* [*trkB*]	56	601	100	100	TMH (2)	Inner membrane
103	1000	AAL19533 Putative inner membrane protein	*S.* Typhimurium *S.* Choleraesuis *S.* Paratyphi	*ybdJ*	41	82	100	100	TMH (1)	Inner membrane
115 306	1000 1000	AAL19997 Paraquat-inducible protein B/induced by paraquat	*S.* Typhimurium *S.* Choleraesuis	*pqiB* [*pqi5*]	62	546	100	100	TMH (1)	Inner membrane
119	2000	NP_463175| Putative inner membrane protein	*S.* Typhimurium *S.* Typhi *S.* Paratyphi	Not assigned	46	301	97	97	TMH (1)	Inner membrane
123	3100	AAL20148 Integral membrane protein ABC transporter, pedicted permease (contains LolE domain)/outer membrane lipoprotein transporter, LolA-dependent release of lipoproteins from inner membrane	*S.* Typhimurium	*ycfW* [*lolE*]	255	414	100	100	TMH (1)	Inner membrane
134	1500	AAL19390 Putative MFS family transporter/predicted transporter	*S.* Typhimurium *S.* Typhi	*yajR*	254	449	100	100	TMH (6)	Inner membrane

Table 3.2. Continued

142 353 393	500 2000 500	YP_219277 Murein peptide ligase/recycles cell wall peptidoglycan	*S.* Typhimurium *S.* Choleraesuis *S.* Typhi	*mpl* [*tpl*, *yjfG*]	50 182	457	100	100	SSP	Periplasm
151 399	1650 1650	AAL19802 Multidrug translocase/multidrug transport, antibiotic resistance	*S.* Typhimurium *S.* Choleraesuis	*mdfA* [*cmr*, *cmlA*]	36	410	100	100	TMH (1)	Inner membrane
167	750	AAV77817 Putative ABC transporter periplasmic binding protein/glutathione transporter	*S.* Typhimurium *S.* Paratyphi	*yliB* [*gsiB*]	166	512	98	98	SSP	Periplasm
173 183	750 750	YP_217104 Putative tyrosine-protein kinase in colanic acid export/autophosphorylating protein tyrosine kinase, a regulator of capsule production (mutants do not make capsules)	*S.* Choleraesuis *S.* Typhi	*wzc*	150	719	99	99	TMH (1)	Inner membrane
179 386	1650 1650	NP_461198 Periplasmic nitrate reductase/anaerobic respiration	*S.* Typhimurium *S.* Choleraesuis *S.* Paratyphi	*napC* [*yejX*]	101	200	100	100	TMH (1)	Periplasm (inner membrane associated)

Table 3.2. Continued

194	1500	CAD07494 Ecotin precursor/inhibits serine proteases including collagenase, trypsin, chymotrypsin, elastase and factor Xa	*S.* Typhimurium *S.* Typhi	*eco* [*eti*]	137	164	100	100	SSP	Periplasm
197	400	YP_215898 ABC superfamily Cytochrome-related transporter/cysteine transporter	*S.* Choleraesuis *S.* Typhimurium *S.* Typhi	*cydD* [*htrD*]	44	588	100	100	TMH (1)	Inner membrane
219	800	CAD09211 Maltose transport inner membrane protein/maltose transporter	*S.* Typhi *S.* Paratyphi	*malG*	163	296	100	100	TMH (3)	Inner membrane
226	1650	AAV76699 Putative fimbrial chaperone protein/pili assembly chaperone	*S.* Paratyphi	*stcB*	135	226	99	99	SSP	Periplasm
231	2000	CAD01823 Putative voltage gated chloride channel protein EriC/inorganic ion transport	*S.* Typhi *S.* Typhimurium	*eriC* [*clcA* *yadQ*]	136	429	99	99	TMH (3)	Inner membrane
251 315	750 750	AAL22139 Putative periplasmic protein/putative transport	*S.* Typhimurium *S.* Choleraesuis	*yraP* [*ecfH*]	128	191	100	100	SSP	Periplasm

Table 3.2. Continued

277	400	AAL21315 MFS superfamily xanthosine permease/xanthosine transporter	*S.* Typhimurium *S.* Typhi *S.* Choleraesuis	*xapB*	30	418	100	100	TMH (1)	Inner membrane
285 307	1800 1800	AAL23133 Putative periplasmic or exported protein	*S.* Typhimurium	STM 4309	226	466	100	100	SSP	Periplasm
318	750	NP_462546 Putative outer membrane lipoprotein (contains ompA motif)	*S.* Typhimurium *S.*Typhi *S.* Chleraesuis	*yiaD*	197	220	100	100	LSP (T)	Outer membrane
319	1300	AAV77047 Periplasmic trehalase/carbohydrate transport and metabolism	*S.* Paratyphi	*treA* [*osmA*]	306	570	99	100	Tat	Periplasm
320	350	YP_215504 Acridine efflux pump/acridine resistance	*S.* Choleraesuis	*acrB* [*acrE*]	27	1049	100	100	TMH (1)	Inner membrane
321	850	AAL22930 1,4-dihydroxy-2-naphthoate octaprenyltransferase (menaquinone biosynthetic protein)/coenzyme metabolism	*S.* Typhimurium *S.* Choleraesuis *S.* Typhi	*menA* [*yiiW*]	107	309	100	100	TMH (2)	Inner membrane

Table 3.2. Continued

368	2000	AAL19348 MFS family arabinose polymer transporter/arabinose polymer transporter	*S.* Typhimurium *S.* Paratyphi	*araJ*	44	390	100	100	TMH (1)	Inner membrane
387	200	YP_218756 ABC superfamily high affinity phosphate transporter/phosphate transporter	*S.* Choleraesuis *S.* Paratyphi *S.* Typhimurium	*pstA* [*phot phoR2b R2pho*]	46	296	100	100	TMH (1)	Inner membrane
391	800	YP_215471 Cytochrome o ubiquinol oxidase subunit I	*S.* Choleraesuis *S.* Paratyphi	*cyoB*	102	663	100	100	TMH (2)	Inner membrane

ORF, open reading frame; % ID, percent identity; % SI, percent similarity

[a]SSP, standard signal peptide; LSP, lipoprotein signal peptide; Tat, twin-arginine signal; TMH, transmembrane helix. Number of predicted TMHs is shown in parentheses.

[b]Location was predicted using PSORTb v. 2.0.4 server at http://www.psort.org/psortb/

Table 3.3. *S*. Brandenburg PhoA fusion proteins without identifiable export signals

Clone No.	Insert size (bp)	Protein identity	Species	Gene [Synonyms]	No. of fused amino acids	ORF full length	% ID	% SI	Predicted location[c]
145	1400	NP_461304 Amidophosphoribosyltransferase /transfers amide nitrogen	*S*. Typhimurium *S*. Choleraesuis *S*. Paratryphi	*purF* [*purC*, *ade(ub)*]	70	505	100	100	Cytoplasm
222	1000	YP_216408 Secretion system effector SseC, (Pathogenicity island 2 effector), translocation machinery component	*S*. Choleraesuis	*sseC*	138	484	98	99	Loosely associated with the outer membrane or secreted to the extracellular milieu.
273 383	400 300	AAL22683 Response regulator in multi-component regulatory system with TorS (sensory kinase) and TorT (periplasmic sensor)	*S*. Typhimurium *S*. Typhi	*torR*	50	230	100	100	Cytoplasm
298	850	AAR10677 Phase-1 flagellin/flagellar filament	*Salmonella enterica*	*fliC*	163	506	100	100	Extracellular

Table 3.3. Continued

333	1800	NP_461796 Invasion protein regulatory protein (transcription regulator HilD)/activator for invasion genes; depresses HilA expression	*S.* Paratyphi *S.* Typhimurium	*hilD*	133	309	100	100	Cytoplasm
337	1200	AAL22360 Heat shock protein 33/HSP33-like chaperonin	*S.* Typhimurium S. Choleraesuis	*hslO* [*yrfI*]	33	294	100	100	Cytoplasm
370	750	Transcriptional activator CaiF/transcriptional activation of cai operon	*S.* Typhimurium *S.* Choleraesuis *S.* Paratryphi *S.* Typhi	*caiF*	25	131	100	100	Cytoplasm

ORF, open reading frame; % ID, percent identity; % SI, percent similarity

[c]Location was predicted using PSORTb v. 2.0.4 server at http://www.psort.org/psortb/

Table 3.4. *S.* Brandenburg PhoA fusion proteins for which N-termini and signal sequences could not be predicted due to incomplete sequence data

Clone No.	Insert size (bp)	Protein identity	Species	Gene [Synonyms]	No. of fused amino acids	ORF full length	% ID	% SI	Predicted location[d]
11	1400	AAL19314 Cytochrome BD2 subunit I	*S.* Typhimurium *S.* Choleraesuis *S.* Typhi *S.* Paratyphi	Not assigned	217 72-288	467	100	100	Inner membrane
12	950	AAL21981 Periplasmic L-aspariginase II	*S.* Typhimurium *S.* Typhi *S.* Paratyphi	*ansB*	205 57-261	348	100	100	Periplasm
32 229	2700 2700	AAL23052 Maltose transport system permease protein/maltose transport	*S.* Typhimurium	*malF*	254 (20-273)	514	100	100	Inner membrane
62	1300	CAD09511 Putative membrane protein/required for invasion of K1 *E. coli* into brain microvascular endothelial cells	*S.* Typhi	*yijP*	228 100-327	577	100	100	Inner membrane

Table 3.4. Continued

69 266	2200 2200	YP_215939 Putative periplasmic protein/putative peptidoglycan binding domain	*S.* Choleraesuis	*ycbB*	164 238-401	615	98	98	Periplasm
70	3000	NP_462177 Putative transglycosylase	*S.* Typhimurium	*yraM*	197 (363-556)	680	100	100	Outer Membrane
81	3000	CAC86202 Putative endoglucanase/ production of cellulose that plays a role in biofilm formation	*S.* Typhimurium *S.* Typhi	*bcsG* [*yhjU*]	192 265-454	559	100	100	Inner membrane
112	2200	AAV78243 Ferrioxamine B receptor precursor/receptor for monomeric catechols	*S.* Paratyphi *S.* Typhi	*foxA*	252 117-368	696	99	100	Outer membrane
195	850	YP_217443 Putative transport protein, ethanol utilization	*S.* Choleraesuis *S.* Typhimurium *S.* Typhi	*eutH* [*yffU*]	124 72-195	408	100	100	Inner membrane

Table 3.4. Continued

225	2100	AAL21030 Putative HlyD family secretion protein, multidrug resistance protein mdtA precursor/mutidrug efflux system subunit of mdtABC, overexpreesion of mdtABC confers resistance to deoxycholate and novobiocin	*S.* Typhimurium	*yegM* [*mdtA*]	183 (182-363)	413	99	100	Inner membrane
228 343	1200 1200	AAL19763 Paral putative transport protein /predicted small conductance mechanosensitive channel	*S.* Typhimurium	*ybiO*	207 (94-300)	740	100	100	Inner membrane

ORF, open reading frame; % ID, percent identity; % SI, percent similarity

[d]Location was predicted using PSORTb v. 2.0.4 server at http://www.psort.org/psortb/

Table 3.5. Predicted subcellular localisation of *S.* Brandenburg fusion proteins

Table in which the proteins were listed	C	IM	P	OM	EC	Total
3.2	0	22	16	9	1	48
3.3	5	0	0	1	1	7
3.4	0	7	2	2	0	11
Total	5	29	18	12	2	66

C, cytoplasm; IM, inner membrane; P, periplasm; OM, outer membrane; EC, extracellular milieu.

Location was predicted using PSORTb v.2.0.4 server at http://www.psort.org/psortb/

The export signals, and the cleavage sites of the fusion proteins were predicted using SignalP 3.0, LipoP 1.0, TatP 1.0, TMHMM v.2.0, and HMMTOP v.2.0 servers (Table 2.6), and those of 48 fusions listed in Table 3.2 are shown in Appendix 1. It was not possible to predict the export signals of the 11 proteins listed in Table 3.4. However, one or more TMHs were predicted for 5 of them. These TMH predictions are shown in Appendix 2. Topology of inner membrane proteins were analysed by TMHMM v.2.0, HMMTOP v.2.0, Phobius, TopPred, MEMSTAT v.3.0, TMpred, and PHDhtm servers (Table 2.6), and are shown in appendices 1 and 2. All had their PhoA tail in the periplasmic side.

Start codons of fusion proteins were predicted by BlastP analysis. Forty-four PhoA fused proteins listed in Table 3.2, and all 7 listed in Table 3.3 had ATG as the start codon. Four of the PhoA-fused proteins listed in Table 3.2 had GTG as the start codon (Appendices 3 and 4). DNA sequences of the *phoA*-fused *S.* Brandenburg inserts shown in Tables 3.2 and 3.3 were examined for the SD sequences, and the –10 and –35 sites. Due to partial sequencing, it was not possible to predict the SD sequences of inserts in clones 134, 197 and 319, and the –10 and –35 sites of inserts in clones 7, 28, 34, 89, 123, 134, 173, 197, 318, 319 and 337. The neural network did not predict the –10 and –35 sites for the insert in clone 145. The remaining 43 inserts showed the presence of potential SD sequences, and the –10 and –35 sites (Appendices 3 and 4).

3.4.3 Detection of AP fusion proteins from recombinant *E. coli*

Presence of PhoA moiety of seven randomly selected fusion proteins was confirmed by immunoblotting recombinant *E. coli* cell lysates with PhoA-specific antibody. One or more bands were seen with all cell lysates. The bands showed molecular weights that are equal, or higher than that of *E. coli* PhoA (Figure 3.8).

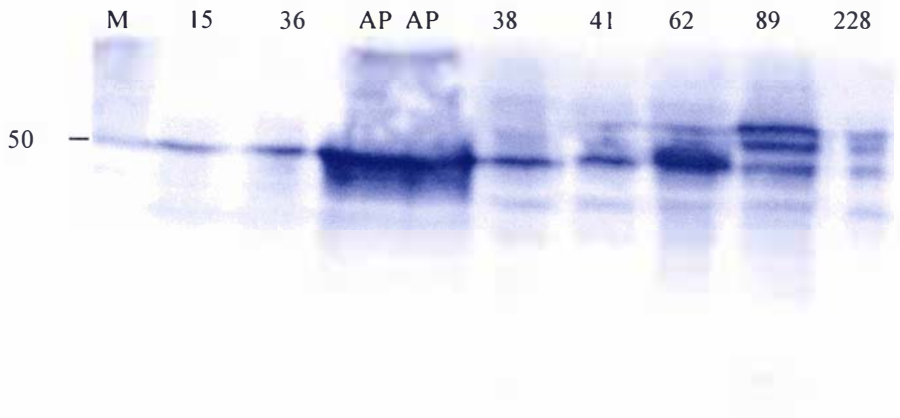

Figure 3.8. Western blot analysis of lysate protein from seven PhoA+ clones. Approximately 10 μg of lysate protein of each clone was separated on a 12% SDS-PAGE gel, and transferred to a PVDF membrane for Western blotting with anti-*E. coli* alkaline phosphatase IgG. Lane M, protein standard for molecular weight comparison (kDa); AP, commercial *E.coli* alkaline phosphatase control. Clone numbers are shown above each lane.

3.5 Discussion

In this study, an *S.* Brandenburg *phoA* fusion genomic library was prepared, and expressed in *E. coli* to identify and partially characterise the exported proteins of *S.* Brandenburg. The alkaline phosphatase fusion method is a genetic tool used to identify proteins that are transported across the inner membrane. Because PhoA is enzymatically active only after it is exported across the inner membrane, it acts as a sensor for proteins that carry export signals. This approach is valuable in the molecular analysis of pathogenesis since exported proteins that contribute to virulence and immunity can be identified and characterised.

According to Clarke and Carbon's formula (Clarke & Carbon, 1976), the number of colonies needed to represent a complete *S.* Brandenburg library is 14,436. Therefore, this library of 19,840 colonies is likely to be complete. A total of 410 blue colonies representing 2% of the expression library was obtained. This percentage of blue colonies is similar to those obtained in studies with other organisms (Kornacki & Oliver, 1998; Oliaro, 2000). The presence of PhoA moiety in the fusion proteins was confirmed by Western blotting a small sample. A number of bands (fusion proteins) with molecular weights of more than 50-kDa were seen in Western blots carried out with recombinant cell lysates. This may be due to proteolysis of fusion proteins. It was possible to predict export signals of 48 proteins (Table 3.2) that resulted in the translocation of PhoA-fused proteins through the inner membrane. Four types of signals, namely type I signal peptide, type II (lipoprotein) signal peptide, Tat signals, and transmembrane helices were identified. Thirty-five percent of predicted proteins had type I signal peptides, 2% had Tat signal peptides, and 17% had lipoprotein signal peptides. The remaining 46% had one or more transmembrane helices to anchor them to the inner membrane. In Gram-negative bacteria, lipoproteins are anchored to either the inner or outer membrane. It has been suggested that the amino acid in position +2 determines the final location of the lipoprotein (Juncker *et al.*, 2003). If a lipoprotein has a negatively charged amino acid at +2, it is predicted to be an inner membrane protein, otherwise an outer membrane protein (Yamaguchi *et al.*, 1988). Out of 8 lipoproteins, 6 could be further localised to the outer membrane based on this +2 amino acid rule. PsortB and BlastP data predicted the final destination of 48 proteins either to

inner membrane (22), periplasm (16), outer membrane (9), or to the extracellular milieu (1). The results obtained through PsortB, BlastP and lipoprotein +2 amino acid rule agree with the true subcellular localisation of these 48 proteins. In summary, 46% are predicted to be inner membrane, 33% are periplasmic, 19% are outer membrane, and 2% are extracellular proteins. Most of the fusion proteins identified in this study are of inner membrane type. This agrees with other studies that have shown most of the PhoA-fused proteins are of inner membrane (Braunstein *et al.*, 2000; Cleavinger *et al.*, 1995; Worley *et al.*, 1998). The presence of TMHs may alter the way the rest of the protein folds or inserts into the inner membrane. However, topological analysis showed that all 22 proteins with transmembrane helices had their PhoA tail in the extracytoplasmic domain to yield the PhoA+ phenotype. The N-termini and export signals of 11 proteins listed in Table 3.4 could not be determined due to only obtaining partial sequences from the inserts. PsortB and BlastP data showed that 7 (clones 11, 32, 62, 81,195, 225, 228) are inner membrane, 2 are periplasmic, and the other 2 are outer membrane proteins. Presence of one or more transmembrane helices in clones 11, 32, 62, 195 and 228 suggest that their final destination is the inner membrane. All these 5 had their PhoA tail in the periplasmic side to give the PhoA+ phenotype. Available stretches of 265-454 amino acid residues of BcsG of clone 81, and 182-363 of YegM of clone 225 did not show any transmembrane helices. However, analysis of whole wild type BcsG showed the presence of 4 TMHs (32-56, 69-86, 113-131, 138-155), and that of YegM showed the presence of one TMH (9-25) with their carboxy termini in the periplasmic side. This suggests that analysis would have shown the presence of these TMHs if the N-termini of these 2 proteins were available by full sequencing of the inserts. Therefore, these 7 inner membrane proteins appear to be genuine PhoA positives.

The PhoA system was designed to identify substrates of the Sec-pathway, which is the primary export pathway in bacteria. Export systems that are Sec-independent are also found in *Salmonella.* These include Tat pathway, type I (ABC transporters), type III and type V pathways. Tat signal peptides are similar to signal peptide 1, but the former contains the Tat motif RR/[FGAVML][LITMVF] upstream of their hydrophobic region (Chaddock *et al.*, 1995). Only one protein that is homologous to periplasmic trehalase was predicted to carry a Tat motif. This indicates that screening more blue colonies would likely identify more Tat substrates. A study carried out by Lewenza and his co-

workers (2005) identified the Tat motif in only one after screening 310 PhoA+ fusions. Type I and type III pathways translocate proteins directly from the cytoplasm to the outside or into the host cell cytosol. Because proteins exported through these pathways do not have N-terminal signal peptides and periplasmic intermediates, they are unlikely to be identified by the PhoA system. But, all the components of type I, and type III export apparatus travel through the Sec-pathway, and are identified through this system. The 7 proteins listed in the Table 3.3 did not show the presence of export signals at their N-termini. Wild type phase 1-flagellin, FliC does not contain a signal sequence. In *Salmonella*, It is exported from cytoplasm to the distal end of the growing flagella in a continuous channel through type III flagellar export system. It also leaks into the extracellular milieu. The export signal of FliC is unknown to date. However, a recent study showed that the 22-amino-acid stretch of N-terminal 26-47 segment of FliC possess the recognition signal for export through the flagellar export system (Vegh *et al.*, 2006). Wild type SseC is secreted through the type III non-flagellar secretion system. In this study, PhoA+ phenotype of the clones containing the N-terminal 163 amino acids of the FliC, and that of 138 amino acids of SseC proteins might have used unidentified export signals. PhoA+ colonies with unknown export signals have been reported (Lewenza *et al.*, 2005; Ward *et al.*, 2001). Fusion proteins expressed by five clones 145, 273, 333, 337 and 370 were 100% similar to known and predicted cytoplasmic proteins (PurF, TorR, HilD, HslO and CaiF respectively). Therefore, these 5 may be false positives associated with the PhoA fusion technique. False positives originating from cytoplasmic proteins have been reported previously (Lewenza *et al.*, 2005). Spontaneous lysis, folding of cytoplasmic PhoA to active conformation in cells that have stopped growing, leakage of cytoplasmic PhoA fusions from healthy cells are responsible for the generation of false positive colonies in the PhoA system (Derman & Beckwith, 1995; Lim *et al.*, 1995; Tullius *et al.*, 2001).

Sequences important for translation initiation include the initiation codon, and the SD sequence (Ma *et al.*, 2002; O'Donnell & Janssen, 2001). Since pJEM11 vector is devoid of these, it is necessary for the foreign inserts to possess these sequences to initiate translation in *E. coli*. Fifty-one (93%) proteins listed in Tables 3.2 and 3.3 had ATG as the start codon, while only 4 (7%) of those had GTG as the start codon. The most frequently used start codon in prokaryotes is ATG. About 8 percent start with

GTG, and 1% start with TTG (O'Donnell & Janssen, 2001). Therefore, the results obtained in this study agree with the above. The SD sequence acts as a translation signal for mRNA through its base-pairing to ribosomes. The SD sequence 5′ GGAGGT 3′ is located 5′ of the initiation codon of mRNA and is complementary to the anti-SD sequence 5′ ACCTCC 3′ near the 3' end of the 16S ribosomal RNA. The sequence 5′ GGAGG 3′, and variations of it with at least 3 nucleotides long that are complementary to parts of 5′ GGAGG 3′ are considered as SD sequences (Starmer *et al.*, 2006). Upstream sequences to predict SD sequences were available for 52 out of 55 *phoA*-fused inserts, and all these 52 harboured potential SD sequences.

Transcription initiates when RNA polymerase recognizes and binds to DNA sequences known as promoters. Prokaryotic promoters are two short hexanucleotide sequences centered at –10 and –35 relative to the initiation start point of a gene. The pJEM11 vector used in this study lacks a promoter upstream of *phoA*. Therefore, transcription of foreign inserts fused with *phoA* requires the presence of promoter sequences upstream of the transcription initiation site. It was not possible to predict –10 and –35 binding sites for 11 inserts in clones 7, 28, 34, 89, 123, 134, 173, 197, 318, 319 and 337 due to partial sequencing. The insert (*purF* gene) present in clone 145 did not show the presence of a potential promoter. Blast N analysis showed the presence of complete colicin V gene (*cvpA*) that ended 30 bases upstream of the start codon of *purF*. These 2 genes are arranged in an operon explaining the reason for the absence of –10 and –35 sites in *purF* gene. The other 43 inserts were predicted to harbour potential –10 and –35 sites to initiate transcription in *E. coli*. However, the actual location of the sites needs to be determined empirically.

BlastP analysis of the deduced amino acid sequences showed that all proteins had more than 95% similarity with proteins of different *Salmonella* serotypes, and showed an array of predicted functions. The outer membrane proteins included ones that function as transporters, porins, receptors and esterases. Periplasmic proteins included the binding components of ABC transporters, cell wall synthesis enzymes, proteases and chaperones. Inner membrane proteins included cell wall synthesis enzymes, efflux pumps, drug resistant proteins, and transporters. This study identified several virulence-associated and immunogenic proteins including putative hemolysin-related

protein, putative endoglucanase, penicillin-binding proteins, components of drug efflux systems, putative tyrosine-protein kinase in colanic acid export, serine protease inhibitor ecotin precursor, *Salmonella* pathogenicity island encoded proteins, outer membrane proteins, putative fimbrial proteins and flagellin proteins.

Almost all bacterial virulence factors are exported proteins that are either located on the bacterial surface, or are secreted into the surrounding extracellular milieu (Worley *et al.*, 1998). This study supports previous work showing that PhoA fusion technology is an economical and effective way of identifying genes encoding exported proteins. Phase-1 flagellin (FliC) of *S.* Brandenburg was identified as a blue colony (clone 298) through screening a number of PhoA positive colonies obtained from the *S.* Brandenburg expression library. It has been shown that FliC protein of other *Salmonella* serotypes elicits both humoral and cellular immune responses (McSorley *et al.*, 2000; Okamura *et al.*, 2003; Sbrogio-Almeida *et al.*, 2004; Strindelius *et al.*, 2004a). However, detailed studies on FliC of *S.* Brandenburg were not available at the time this work was undertaken. Therefore, *fliC* gene was selected for further studies that are described in Chapters 4, 5 and 6.

Chapter 4 describes the expression, and characterisation of humoral and cellular immune responses directed against phase-1 flagellin of *S.* Brandenburg.

Chapter 4 Cloning, expression, and immune responses directed against *S.* Brandenburg phase–1 flagellin (FliC)

4.1 Abstract

The phase-1 flagellin (*fli*C) gene that was identified from the *S.* Brandenburg PhoA expression library was sequenced. Phase-1 flagellin, part of its variable region (V456), and region IV (V4) were expressed as N-terminal histidine-tagged recombinant proteins, and purified by affinity chromatography. The recombinant proteins were evaluated in Western blots for humoral responses of sheep naturally infected with *S.* Brandenburg. All 81 naturally infected sheep had IgG antibodies against FliC, V456, and V4 recombinant proteins. While 9 out of 80 sera collected from sheep not exposed to *S.* Brandenburg reacted with FliC recombinant, none reacted with V456 and V4 recombinant proteins. Furthermore, sera collected from sheep vaccinated with Salvexin^{TM}+B (Trial 2004), and those vaccinated with Salvexin^{TM} and Salvexin^{TM}+B (Trial 1999) were evaluated in Western blots. While sera from Salvexin^{TM}+B-vaccinated groups reacted with FliC, V456, and V4 recombinant proteins, those from Salvexin^{TM}-vaccinated group reacted only with the FliC recombinant protein. All unvaccinated sheep were negative in the Western blots. The proteins were evaluated in an IFN-γ assay with whole blood for cell-mediated responses of sheep vaccinated with Salvexin^{TM}+B (Trial 2004). Recombinant proteins did not induce the secretion of IFN-γ in vaccinated sheep.

4.2 Introduction

The development and application of effective *S.* Brandenburg vaccines and diagnostic tools offer a potential means of reducing industry-associated losses and public health risks. However, development of these tools for *S.* Brandenburg infection in sheep has been hampered by the paucity of data on *S.* Brandenburg proteins that trigger immune responses. Hence, there is a need to identify and characterise the potential immunogenic proteins of *S.* Brandenburg. Porins, flagellar, fimbrial and outer membrane proteins are among the cell surface, and secreted proteins of *Salmonella* that activate immune cells (Cookson & Bevan, 1997; Finlay & Falkow, 1997; Mastroeni & Ménager, 2003; Secundino *et al.*, 2006).

The objective of this study was to express, purify and evaluate the immunogenic properties of *S.* Brandenburg phase-1 flagellin (FliC) protein that was identified from the alkaline phosphatase expression library described in Chapter 3. This protein was chosen because FliC of other *Salmonella* serotypes has been shown to induce both humoral and cell-mediated immune responses (Bergman *et al.*, 2005; McSorley *et al.*, 2002; Strindelius *et al.*, 2004a) that are needed for the effective clearance of *Salmonella* infections from the host. Strindelius and his co-workers (2002) showed that humoral responses of mice immunized with secreted proteins of *S.* Enteritidis were mainly directed against FliC and hook-associated protein 2. Flagellin has also been shown to result in the secretion of IFN-γ in mice immunized with live *S.* Typhimurium (Sbrogio-Almeida *et al.*, 2004).

In this study, *fliC* gene of *S.* Brandenburg was PCR-amplified to obtain the full DNA and deduced amino acid sequences. Subsequently, the complete ORF of *fliC*, and 2 different fragments of it were cloned into the pET14b vector, and expressed in *E. coli* BL21 (DE3) cells as N-terminal histidine-tagged fusion proteins to evaluate the IgG responses of sheep naturally infected with *S.* Brandenburg, and those vaccinated with Salvexin™ and Salvexin™+B vaccines. Cell-mediated immune responses of Salvexin™+B-vaccinated sheep against the fusion proteins were evaluated using an IFN-γ assay. Furthermore, reactivity of the fusion proteins with sera from rabbits immunized with *S.* Typhimurium, *S.* Hindmarsh and *S.* Brandenburg were evaluated.

4.3 Materials and methods

4.3.1 PCR amplification of the *fliC* gene from *S.* Brandenburg

Separate PCR reactions were performed to obtain the complete nucleotide sequence of *fliC*, and to clone the complete ORF, a part of the variable region (regions IV, V, VI), and region IV of *fliC* gene into the pET14b vector. In the remainder of the Chapter, the latter 2 regions will be identified as V456 and V4 respectively. The nucleotide sequence was obtained by PCR amplification of genomic DNA extracted from *S.* Brandenburg strain S59. Genomic DNA from another 9 *S.* Brandenburg strains S76, S79, S82, S84, S85, S196, S199, S207 and S229 was PCR-amplified to test the presence of *fliC* gene. Sources of strains are shown in Table 2.2. Genomic DNA was extracted by phenol-chloroform method (Section 2.3.1.2), checked by agarose gel electrophoresis for purity (Section 2.4.1), and used as the PCR template. Two oligonucleotide primers were designed to the conserved flanking regions of *Salmonella fliC* gene to amplify the complete *fliC* gene of *S.* Brandenburg. In addition, three pairs of oligonucleotide primers were designed for cloning complete ORF, *V456* and *V4* of *fliC* gene into the pET14b vector (Novagen). The forward primers were designed to be in frame with the N-terminal histidine-tag of the pET14b expression vector. The restriction site *Nde* I (5′CA$^{\downarrow}$TATG3′) was added to the 5′ ends of forward (*fliCNde*I-F, *V456Nde*I-F, *V4Nde*I-F) and reverse (*fliCNde*I-R, *V456Nde*I-R, *V4Nde*I-R) primers (after addition of the stop codon TTA to the 5′ end of the reverse primers of *V456Nde*I-R and *V4Nde*I-R). This was followed by the addition of eight extra bases to the 5′ ends to increase the efficiency of restriction endonuclease cleavage. The ATG codon from *Nde* I site was used as the start codon. Details of primers are shown in Table 4.1. PCR reactions were performed as described in Section 2.4.4.2 in a reaction volume of 50 µl consisting 1 µl of genomic DNA, 400 nM of each primer, 140 µM of each dCTP, dGTP, dATP and dTTP, 1× cloned *Pfu* DNA polymerase buffer, 2.5 units of *PfuTurbo*® DNA polymerase (Stratagene) and sterile distilled water. The reactions were subjected to a single cycle of denaturation at 95°C for 2 min, followed by 30 cycles of denaturation (95°C for 30 sec), annealing (60°C for 30 sec with *fli*C-F/*fli*C-R and *fliCNde*I-F/*fliCNde*I-F, 55°C for 30 sec with *V456Nde*I-F/*V456Nde*I-R, 56°C for 30 sec with *V4Nde*I-F/*V4Nde*I-R), elongation (72°C for 2 min), and a final elongation

(72°C for 10 min) in a GeneAmp Model 9600 thermocycler (Perkin-Elmer Cetus). A negative control without genomic DNA was included in each PCR assay. Ten microlitres of each PCR product was resolved by 1% agarose gel electrophoresis, stained with ethidium bromide, and imaged with a Gel Doc analyser (Section 2.4.1). The PCR products were purified using QIAquick Gel Purification Kit (Section 2.4.2), and the gel-purified products were submitted along with the primers (Table 4.1) to the Sequencing Facility of Massey University for sequencing on an ABI Prism® 377 DNA Sequencer (Section 2.7). To obtain readable sequence from the central region of *fliC*, an additional pair of internal primers (*fliC*Int-F/*fliC*Int-R) was used for sequencing the *fliC* gene. Purified DNA was stored at –20°C until used.

Table 4.1. Details of primers used to amplify, and sequence *S.* Brandenburg *fliC* gene, *V456,* and *V4*

Primer	Primer sequence[a] 5′ to 3′	Position at *fliC*
fliC-F	GATACAAGGGTTACGGTGAG	55-74 bp upstream of start codon
fliC-R	TTTCGCTGCCTTGATTGTG	48-30 bp downstream of stop codon
*fliC*Int-F	GCCAACGACGGTGAAACTATC	448-468
*fliC*Int-R	GAAGTTGTGACCAGCAGCTTTG	1188-1167
*fliCNde*I-F	GGGAATT●ATATGGCACAA GTCATTAATACAAACAGC	4-27
*fliCNde*I-R	GGGAATTCCATATGTTAACG CAGTAAAGAGAGGACG	1503-1482
*V456Nde*I-F	GGGAATTCCATATGAAGAGCG AAGCAGTTAAAAGCGG	544 -566
*V456Nde*I-R	GGGAATTCCATATGTTACAGT GCAGTTTTAGTGTTGCCG	1098-1077
*V4Nde*I-F	GGGAATTCCATATGAAGAGCG AAGCAGTTAAAAGCGG	544-566
*V4Nde*I-R	GGGAATTCCATATGTTA AGCACTAATACCACTGTCGG	912-893

[a]restriction site *Nde* I is indicated in the green box, and the stop codon in the yellow box. Extra bases shown in the pink box were added to the 5′ ends of *fliCNde*I-F, *fliCNde*I-R, *V456Nde*I-F, *V456Nde*I-R, *V4Nde*I-F and *V4Nde*I-R to increase the efficiency of restriction endonuclease cleavage.

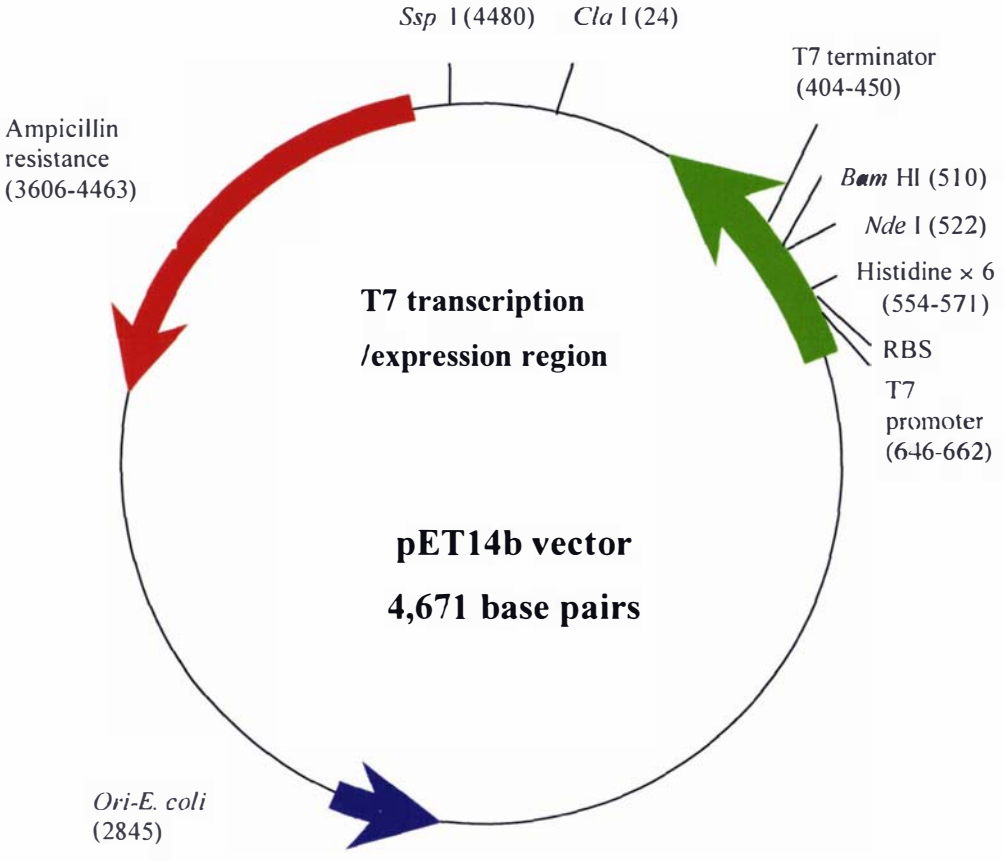

Figure 4.1. Map of the pET14b expression vector (Novagen). Important features include the presence of an *E. coli* origin (*Ori-E. coli*) of DNA replication for propagation of plasmid in *E. coli* (2845); bacteriophage T7 promoter (646-662); T7 transcription start (645); N-terminal histidine × 6 tag (554-571); multiple cloning site *Bam* HI-*Nde* I (510-526); T7 terminator (404-450), and ampicillin resistance gene (3606-4463). Adapted from the pET vector manual (Novagen).

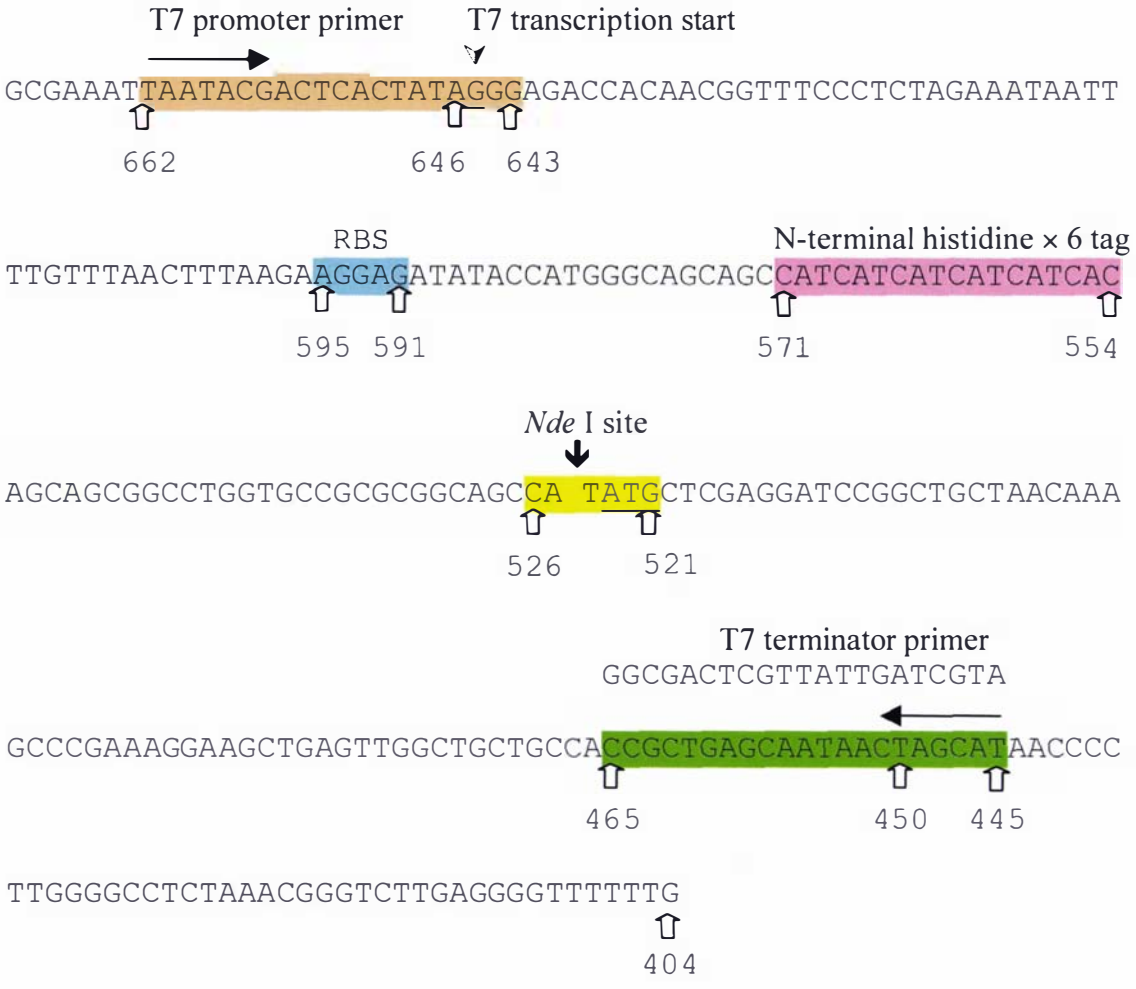

Figure 4.2. Detailed cloning/expression region of the coding strand of pET14b vector transcribed by T7 RNA polymerase. T7 promoter (646-662) and its primer (643-662), T7 transcription start (645), ribosomal binding site (RBS, 591-595), N-terminal histidine × 6 tag (554-571), *Nde* I site (CA↓TATG) of the multiple cloning site (521-526), and T7 terminator (404-450) and its primer (445-465) are shown. The codon ATG present in the *Nde* I site was used as the start codon for the expression of histidine-tagged recombinant proteins. Adapted from the pET vector manual (Novagen).

4.3.4 Cloning *fliC* gene, *V456*, and *V4* into the pET14b expression vector

The complete *fli*C ORF, *V456*, and *V4* of *fli*C were cloned into the pET14b expression vector to express them as histidine-tagged recombinant proteins in *E. coli*. Five hundred ng of gel-purified PCR products of *fli*C (1528 bp), *V456* (586 bp), and *V4* (400 bp) was each digested with 10 units of *Nde* I at 37°C for 2 h. Digests were subsequently cleaned with the QIAquick PCR Cleaning Kit (Qiagen), and each was eluted in 50 µl of EB buffer supplied with the kit. The PCR products and 20 ng of pET14b vector were ligated using 1:3 and 1:5 vector: insert ratios with 1 unit of T4 DNA ligase at 16°C for 16 h (Section 2.5.2). *E. coli* One Shot® BL21 (DE3) (Life Technologies) competent cells that carry the λDE3 lysogen containing T7 RNA polymerase under the control of *lac*UV5 promoter were transformed with 10 ng of ligation reaction by heat shock (Section 2.6.2). The transformation mixture was incubated with SOC medium at 37°C for 1 h with gentle shaking and plated on LB agar containing 50 µg/ml carbenicillin. The plates were incubated overnight at 37°C, and 6 colonies from each pET14b-*fliC*, pET14b-*V456*, and pET14b-*V4* transformation were subjected to colony PCR to confirm the presence of inserts. Briefly, genomic DNA was extracted from each colony by boiling method (Section 2.3.1.1), and the supernatant containing DNA was used for PCR amplification as described in Section 4.3.1. The complete *fli*C gene, *V456* and *V4* were amplified with primer pairs *fliCNde*I-F/*fliCNde*I-R, *V456Nde*I-F/*V456Nde*1-R and *V4Nde*I-F/*V4Nde*I-R respectively. Each of the colonies that had the insert was inoculated into 5 ml of LB broth containing 50 µg/ml carbenicillin, and incubated overnight at 37°C with shaking. Plasmid DNA was isolated as described in Section 2.3.2. One microlitre of pET14b-*fliC* was digested with 10 units of *Cla* I (Cat. No. 15416-050, Life Technologies), and two µl of each pET14b-*V456* and pET14b-*V4* plasmid was digested with 10 units of *Ssp* I (Cat. No. 15458-011, Life Technologies). Digests were in a reaction volume of 20 µl at 37°C for 1 h. Ten microlitres of each digest was analysed on a 1% agarose gel (Section 2.4.1) to select the plasmids carrying correctly orientated inserts. The reading frame and the sequence of the inserts were confirmed by sequencing (Section 2.7) a selected plasmid of each pET14b-*fliC*, pET14b-*V456* and pET14b-*V4* construct containing the correctly orientated insert. The plasmid-specific primers T7 promoter 5′ TAATACGACTCACT

ATAGGG 3′ and T7 terminator 5′ ATGCTAGTTATTGCTCAGCGG 3′ were used for sequencing. Colonies containing the correct insert were grown in LB broth (Section 2.2.2) and stored as glycerol stocks at –80°C (Section 2.2.4).

4.3.5 Expression and purification of recombinant proteins from *E. coli*

Glycerol stocks of *E. coli* One Shot® BL21 (DE3) cells carrying pET14b-*fliC*, pET14b-*V456,* and pET14b-*V4* plasmids were used for the expression of histidine-tagged fusion proteins as described in Section 2.8.2. Molecular weights of histidine-tagged proteins (combined size of FliC/V456/V4 plus upstream amino acid sequence MGSSHHHHHHSSGLVPRGSH for FliC/MGSSHHHHHHSSGLVPRGSHM for V456 and V4) were calculated using http://www.expasy.ch/tools/pi_tool.html. The expressed proteins were analysed by SDS-PAGE (Section 2.9.2), and the solubility of the recombinant proteins was tested as described (Section 2.8.2.1). The expressed proteins were soluble; hence they were purified under native conditions using BD TALON™ Metal Affinity Resins as described in Section 2.8.3. Fractions were analysed by SDS-PAGE and those containing the purified protein were pooled, and transferred to Spectra/Por® 6 dialysis membrane with a molecular-weight-cut-off (MWCO) of 25-kDa (Cat. No. 132552), 10-kDa (Cat. No. 132572) and 8-kDa (Cat. No. 182580) (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) for FliC, V456 and V4 respectively. Dialysis was conducted for 24 h at 4°C with constant stirring against three changes of 4 L of PBS pH 7.4 to remove salt and imidazole. The dialyzed samples were filter sterilized through a 0.22 μm syringe filter. Subsequently, the samples were gel checked (Section 2.9.2), and protein concentrations were estimated (Section 2.9.1). When the protein concentrations were low, purified recombinant proteins were concentrated using Centricon Centrifugal Filter Devices with MWCO of 30-kDa (YM-30; Cat. No. 4208), 10-kDa (YM-10; Cat. No. 4205) and 3-kDa (YM-3; Cat. No. 4202) (Millipore Corporation) for FliC, V456 and V4 recombinant proteins respectively. Protein preparations were stored at –80°C for future use in Western blots and IFN-γ assays.

4.3.6 Western blot analysis of recombinant proteins FliC, V456, and V4

Approximately 500 ng of purified protein was loaded on to each lane of a discontinuous polyacrylamide gel consisting of 4% stacking gel and 12% separating gel (Section 2.9.2), and the proteins were transferred onto PVDF membrane for Western blotting (Section 2.9.3). After confirming the presence of histidine-tagged proteins in Western blots with a MAb to histidine-tag (Section 2.9.3), animal sera were subjected to Western blots as described in Section 2.9.3. Briefly, each strip was incubated with 10 ml of diluted sheep/rabbit sera, and with 10 ml of diluted AP-labelled donkey anti-sheep/goat anti-rabbit whole IgG antibody. Each step was for 1 h at room temperature followed by a wash step. Sera from a positive and a negative control sheep were included in each blot for comparison. After another cycle of washing, the strips were incubated with 10 ml of BCIP/NBT substrate for colorimetric detection as described in Section 2.9.3. Western blots were done twice with each sample.

4.3.7 Serum Samples

Sera collected from 81 naturally infected and 80 non-infected sheep (Section 2.12.1), and 6 Salvexin^{TM}+B-vaccinated sheep (Trial 2004) (Section 2.11) were tested for the presence/absence of IgG antibodies against purified recombinant proteins in Western blots. Another source of sera was sheep vaccination trial to compare Salvexin^{TM} and Salvexin^{TM}+B vaccines (Trial 1999) (Section 2.12.2). Sera from rabbits immunized with *S.* Typhimurium, *S.* Hindmarsh and *S.* Brandenburg (Section 2.13) were also evaluated in Western blots.

4.3.8 IFN-γ assay

Cell-mediated immune responses of sheep vaccinated with Salvexin^{TM}+B (Trial 2004) (Section 2.11) against purified recombinant proteins FliC and V456 were tested by IFN-γ assays as described in Section 2.11.2. Each antigen was prepared as described in Section 2.11.2.1, and used at 1, 10 and 20 μg per well. Each blood sample and antigen was analysed in duplicate. Twelve sheep used for this assay were shown in Table 2.7.

4.4 Results

4.4.1 Sequence analysis of *S.* Brandenburg *fliC* gene

The 1625-bp PCR product was present in all the 10 *S.* Brandenburg isolates tested (Figure 4.3). PCR products from all the 10 isolates were sequenced, and the multiple alignments of the nucleic acid sequences showed a 100% identity to each other. BlastN analysis showed that the PCR product contained 1503-bp long *fliC* gene, and its flanking regions. The nucleic acid, and deduced amino acid sequences of *S.* Brandenburg *fliC* gene are shown in Figure 4.4. Wei and Joys (1985) divided *Salmonella* FliC into 8 regions I to VIII. A schematic diagram of 8 regions of *S.* Brandenburg FliC is shown in Figure 4.5. Multiple global alignment of FliC of *S.* Brandenburg with that of 12 other *Salmonella* serotypes showed identities varying from 53-77% (Appendix 5). Pair-wise comparison of different regions showed highly conserved N- (regions I and II) and C-domains (region VIII), and the central variable domain (regions III, IV, V, VI and VII). Regions IV, V and VI showed more variability than regions III and VII. Region IV of *S.* Brandenburg showed the highest variability with those of serotypes Typhimurium, Bovismorbificans, Montevideo, Oranienburg, Saintpaul, Enteritidis, Dublin and Anatum (Table 4.2).

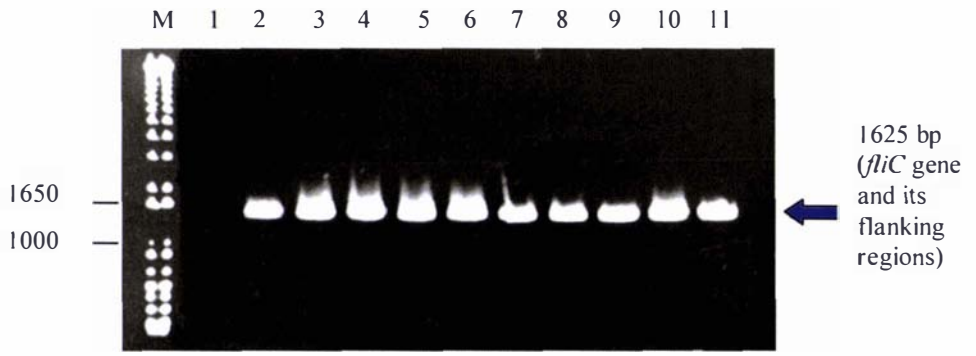

Figure 4.3. PCR amplification of the *fliC* gene from genomic DNA extracted from 10 *S.* Brandenburg isolates. Details are in Section 4.3.1. Ten microlitres of each sample was electrophoresed on a 1% agarose gel, stained with ethidium bromide and photographed under UV light. Lane M, 1-kb Plus DNA Ladder marker (bp); lane 1, negative control (water); lane 2, S59 (source unknown); lane 3, S76 (ovine faeces, 1999); lane 4, S79 (ovine faeces, 1999); lane 5, S 82 (bovine isolate, 1999); lane 6, S84 (avian isolate, 1999); lane 7, S85 (bovine isolate, 1999); lane 8, S196 (isolate from meat, 2001); lane 9, S199 (isolate from meat, 2001); lane 10, S207 (porcine faeces, 2001); lane 11, S229 (ovine uterus, 2001).

```
5'GATACAAGGGTTACGGTGAGAAACCGTGGGCAACAGCCCAATAACATCAAGTTGTAAT
TGATAAGGAAAAGATCATG GCA CAA GTC ATT AAT ACA AAC AGC CTG TCG
                M   A   Q   V   I   N   T   N   S   L   S
CTG TTG ACC CAG AAT AAC CTG AAC AAA TCC CAG TCT GCT CTG GGC
L   L   T   Q   N   N   L   N   K   S   Q   S   A   L   G
ACC GCT ATC GAG CGT CTG TCT TCC GGT CTG CGT ATC AAC AGC GCG
T   A   I   E   R   L   S   S   G   L   R   I   N   S   A
AAA GAC GAT GCG GCA GGT CAG GCG ATT GCT AAC CGT TTC ACC GCG
K   D   D   A   A   G   Q   A   I   A   N   R   F   T   A
AAC ATC AAA GGT CTG ACT CAG GCT TCC CGT AAC GCT AAC GAC GGT
N   I   K   G   L   T   Q   A   S   R   N   A   N   D   G
ATT TCT ATT GCG CAG ACC ACT GAA GGC GCG CTG AAC GAA ATC AAC
I   S   I   A   Q   T   T   E   G   A   L   N   E   I   N
AAC AAC CTG CAG CGT GTG CGT GAA CTG GCG GTT CAG TCT GCT AAC
N   N   L   Q   R   V   R   E   L   A   V   Q   S   A   N
AGC ACC AAC TCC CAG TCT GAC CTC GAC TCC ATC CAG GCT GAA ATC
S   T   N   S   Q   S   D   L   D   S   I   Q   A   E   I
ACC CAG CGC CTG AAC GAA ATC GAC CGT GTA TCC GGC CAG ACT CAG
T   Q   R   L   N   E   I   D   R   V   S   G   Q   T   Q
TTC AAC GGC GTG AAA GTC CTG GCG CAG GAC AAC ACC CTG ACC ATC
F   N   G   V   K   V   L   A   Q   D   N   T   L   T   I
CAG GTT GGT GCC AAC GAC GGT GAA ACT ATC GAT ATC GAT CTG AAG
Q   V   G   A   N   D   G   E   T   I   D   I   D   L   K
CAG ATC AAC TCT CAG ACC CTG GGT CTG GAT ACG CTG AAT GTG CAG
Q   I   N   S   Q   T   L   G   L   D   T   L   N   V   Q
AAA AAA TAT GAT GTG AAG AGC GAA GCA GTT AAA AGC GGC GGT GGG
K   K   Y   D   V   K   S   E   A   V   K   S   G   G   G
GCA ACA CTC AAT ACC ACT GGT CTT AAT GAT GCA GCT CTT AAA ACG
A   T   L   N   T   T   G   L   N   D   A   A   L   K   T
GGT GTT GGT GGT GCA ACA AAC GGT ACT GCT GCA ATT AAG GAT GGT
G   V   G   G   A   T   N   G   T   A   A   I   K   D   G
AAA GTC TTC TTC GAT GCA ACT GAT AAT AAA TAT TTT ATT GAA GTA
K   V   F   F   D   A   T   D   N   K   Y   F   I   E   V
GAA GGT TTA ACC GCT GGC GAC GCT ACT AAA AAT GGT GTT TAT GAA
E   G   L   T   A   G   D   A   T   K   N   G   V   Y   E
GTT AGT GTT GCA GAT GAT GGC ACT GTT ACA ATG CCG ACA ACC ACG
V   S   V   A   D   D   G   T   V   T   M   P   T   T   T
AAA GTG ACA GGA GGC ATG CCA GCC ACG GCG ACG GCA GTA ACC GAA
K   V   T   G   G   M   P   A   T   A   T   A   V   T   E
ACG CAG CCA AAA CCT GTA GCT CTC AGT ACA GCA GTT AAA GAT CAG
T   Q   P   K   P   V   A   L   S   T   A   V   K   D   Q
TTG ACC GAC AGT GGT ATT AGT GCT GCT GAT GCT GCA AAA GGC CAA
L   T   D   S   G   I   S   A   A   D   A   A   K   G   Q
TTA GTT ACG ATG TCT TAT ACG GAT AAA AAC GGT AAG ACT ATT GAT
L   V   T   M   S   Y   T   D   K   N   G   K   T   I   D
GGC GGT TTC GGT GTT AAA GTT GGG GCT AAT ATT TAT GCT GCA ACA
G   G   F   G   V   K   V   G   A   N   I   Y   A   A   T
```

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AAA AAT AAA GAT GGA TCG TTC AGC ATT AAC ACC ACT GAA TAT ACC
K   N   K   D   G   S   F   S   I   N   T   T   E   Y   T
GAT AAA GGC GGC AAC ACT AAA ACT GCA CTG AAC CAA CTG GGT GGC
D   K   G   G   N   T   K   T   A   L   N   Q   L   G   G
GCA GAC GGT AAA ACT GAA GTC GTT TCT ATC GAC GGT AAA ACC TAT
A   D   G   K   T   E   V   V   S   I   D   G   K   T   Y
AAT GCC AGC AAA GCT GCT GGT CAC AAC TTC AAA GCA CAG CCA GAG
N   A   S   K   A   A   G   H   N   F   K   A   Q   P   E
CTG GCT GAA GCG GCT GCT ACA ACC ACC GAA AAC CCG CTG GCT AAA
L   A   E   A   A   A   T   T   T   E   N   P   L   A   K
ATT GAT GCC GCG CTG GCG CAG GTT GAT GCG CTG CGT TCT GAC CTG
I   D   A   A   L   A   Q   V   D   A   L   R   S   D   L
GGT GCG GTA CAG AAC CGT TTC AAC TCC GCT ATC ACC AAC CTG GGC
G   A   V   Q   N   R   F   N   S   A   I   T   N   L   G
AAT ACC GTA AAT AAC CTG TCT TCT GCC CGT AGC CGT ATC GAA GAT
N   T   V   N   N   L   S   S   A   R   S   R   I   E   D
TCC GAC TAC GCG ACC GAA GTT TCC AAC ATG TCT CGC GCG CAG ATC
S   D   Y   A   T   E   V   S   N   M   S   R   A   Q   I
CTG CAG CAG GCC GGT ACC TCC GTT CTG GCG CAG GCG AAC CAG GTT
L   Q   Q   A   G   T   S   V   L   A   Q   A   N   Q   V
CCG CAA AAC GTC CTC TCT TTA CTG CGT TAA TCCGGCGATTGATTCACCG
P   Q   N   V   L   S   L   L   R
ACACGTGGTACACAATCAAGGCAGCGAAA 3'
```

Figure 4.4. DNA and deduced amino acid sequences of *S.* Brandenburg *fli*C gene, and the sequences used to design primers for PCR amplifications and sequencing. Amino acid sequence was obtained from the DNA sequence using ExPASy Molecular Biology Server at http://www.expasy.ch. Amino acids are shown in one-letter code, and the start (ATG) codon of the *fli*C gene is shaded blue. For cloning, *Nde* I site and extra bases were added to the 5′ ends of the primers (Table 4.1).

Orange boxed, sequences used to design primers in flanking areas to amplify *fli*C gene (*fliC*-F & *fliC*-R)

Pink boxed, sequences used to design primers for sequencing internal regions of *fli*C (*fliC*Int-F & *fliC*Int-R)

Yellow boxed, sequences used to amplify *fli*C gene for cloning (*fliCNde*I-F & *fliCNde*I-R)

Green boxed, sequences used to amplify *V456* region of *fliC* for cloning (*V456Nde*I-F & *V456Nde*I-R)

Underlined, sequences used to amplify *V4* of *fliC* for cloning (*V4Nde*I-F & *V4Nde*I-R)

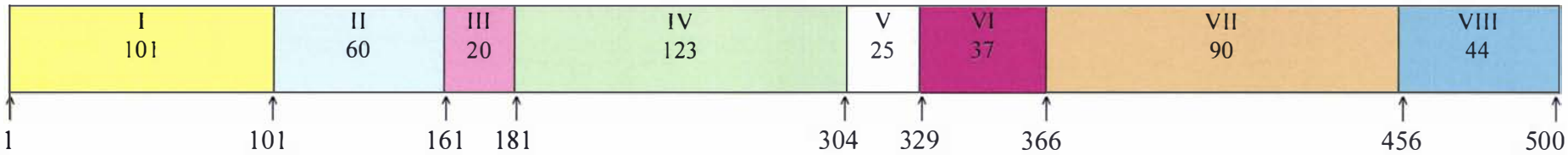


Figure 4.5. Schematic diagram showing eight regions of 500-residue *S*. Brandenburg FliC protein. Number of amino acid residues in each region is shown inside each box. Numbers below the multicoloured rectangle are the amino acid position numbers starting from the left-hand side.

Table 4.2. Pair-wise comparison of different regions of *S.* Brandenburg FliC with those of 13 other *Salmonella* serovars isolated from sheep in New Zealand

Serovar[a]	% Identity (No. of residues)							
	N-terminus		Variable region					C-terminus
	I	II	III	IV	V	VI	VII	VIII
Brandenburg (AAX24137)	(101)	(60)	(20)	(123)	(25)	(37)	(90)	(44)
Typhimurium (BAA02846)	100 (101)	100 (60)	90 (20)	34.1 (122)	56 (21)	64.9 (37)	91.1 (90)	100 (44)
Bovismorbificans (AAR10675)	100 (101)	100 (60)	90 (20)	36.5 (118)	56 (21)	64.9 (37)	91.1 (90)	100 (44)
Montevideo (CAA78778)	93.1 (101)	78.3 (60)	50 (20)	22.6 (129)	24 (23)	23.7 (38)	52.2 (90)	95.5 (44)
Oranienburg (CAA78779)	93.1 (101)	78.3 (60)	50 (20)	16.7 (132)	20 (23)	26.3 (38)	52.2 (90)	95.5 (44)
Saintpaul (AAT81611)	100 (101)	100 (60)	95 (20)	34.1 (124)	72 (25)	42.1 (38)	94.4 (90)	100 (44)
Senftenberg (CAA78781)	93.1 (101)	78.3 (60)	50 (20)	21.2 (129)	20 (23)	23.7 (38)	51.6 (91)	93.2 (43)
Enteritidis (CAA78777)	93.1 (101)	78.3 (60)	50 (20)	22.6 (129)	24 (23)	23.7 (38)	51.1 (90)	95.5 (44)
Dublin (CAA78776)	93.1 (101)	78.3 (60)	50 (20)	22.6 (129)	24 (23)	23.7 (38)	50.5 (91)	93.2 (43)
Anatum (AAT81609)	100 (101)	100 (60)	95 (20)	34.4 (124)	72 (25)	42.1 (38)	94.4 (90)	100 (44)
Agona (AAT81612)	93.1 (101)	78.3 (60)	50 (20)	22.6 (129)	20 (23)	23.7 (38)	52.2 (90)	95.5 (44)
Adelaide (AAA53490)	93.1 (101)	78.3 (60)	50 (20)	21.9 (129)	20 (23)	23.7 (38)	52.2 (90)	95.5 (44)
Derby (CAA78775)	93.1 (101)	78.3 (60)	50 (20)	21.9 (129)	20 (23)	23.7 (38)	52.2 (90)	95.5 (44)
Lille (AAR10700)	96 (101)	78.3 (60)	50 (20)	17.1 (68)	12 (21)	21.6 (24)	47.8 (83)	100 (44)
Range % Identity	93.1-100	78.3-100	50-95	16.7-36.5	12-72	21.6-64.9	47.8-90	95.5-100

Pairwise alignments were done at the LALIGN server at http://www.ch.embnet.org/software/LALIGN_form.html.

[a]GenBank accession numbers of FliC proteins of different *Salmonella* serovars are shown in parentheses.

4.4.2 Cloning *fliC* gene, *V456*, and *V4* into pET14b expression vector

To express recombinant proteins, *fliC* gene, *V456*, and *V4* were PCR amplified as described in Section 4.3.4. Primers were designed using the nucleic acid sequence of *fli*C gene obtained from this study. Subsequently, the PCR products were cloned separately into the pET14b vector, and the constructs were used to transform BL21 (DE3) *E. coli* cells. Figure 4.6 shows the linearized vector and inserts used for cloning. After transformation into *E. coli*, 6 colonies from each *fliC*, *V456* and *V4* transformations, were subjected to colony PCR. The inserts were present in all 12 *E. coli* colonies carrying *fliC* and *V456*, and 5 of those with *V4*. The plasmids were digested as described in Section 4.3.4, and gel electrophoresed to select the ones that contained inserts of the correct orientation (Figures 4.8, 4.10, 4.12). Figures 4.7, 4.9 and 4.11 show the details of restriction digests including fragment sizes of correctly and incorrectly orientated inserts. DNA sequencing of the inserts *fliC* (clone number 1), *V456* (clone number 8) and *V4* (clone number 8) confirmed that they were in the correct reading frame, and were the expected sequences.

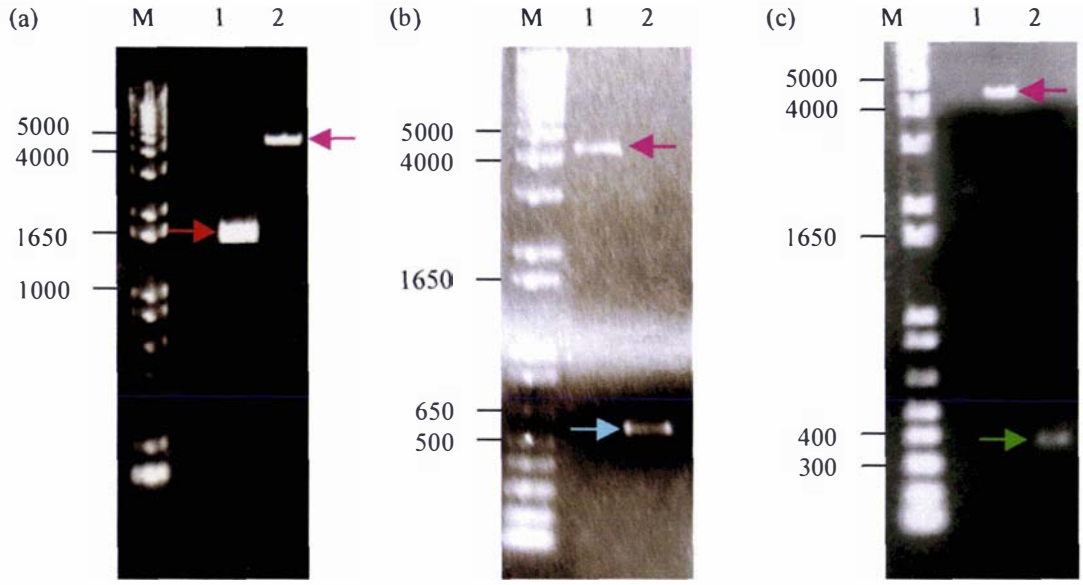


Figure 4.6. PCR products (*fliC*, *V456* and *V4*) and pET14b vector used for cloning. Samples were electrophoresed on 1% agarose gels, stained with ethidium bromide and photographed under UV light. (a) Lane M, 1-kb Plus DNA Ladder marker (bp); lane 1, 10 μl of *Nde* I digested and gel-purified PCR product (*fliC'*); lane 2, 2 μl of *Nde* I digested and dephosphorylated vector. (b) Lane M, 1-kb Plus DNA Ladder marker (bp); lane 1, 2 μl of *Nde* I digested and dephosphorylated vector; lane 2, 10 μl of *Nde* I digested and gel-purified PCR product (*V456*). (c) Lane M, 1-kb Plus DNA Ladder marker (bp); lane 1, 2 μl of *Nde* I digested and dephosphorylated vector; lane 2, 10 μl of *Nde* I digested and gel-purified PCR product (*V4*). Red, blue, green and pink arrows indicate PCR products *fliC'*, *V456*, *V4* and pET14b vector respectively.

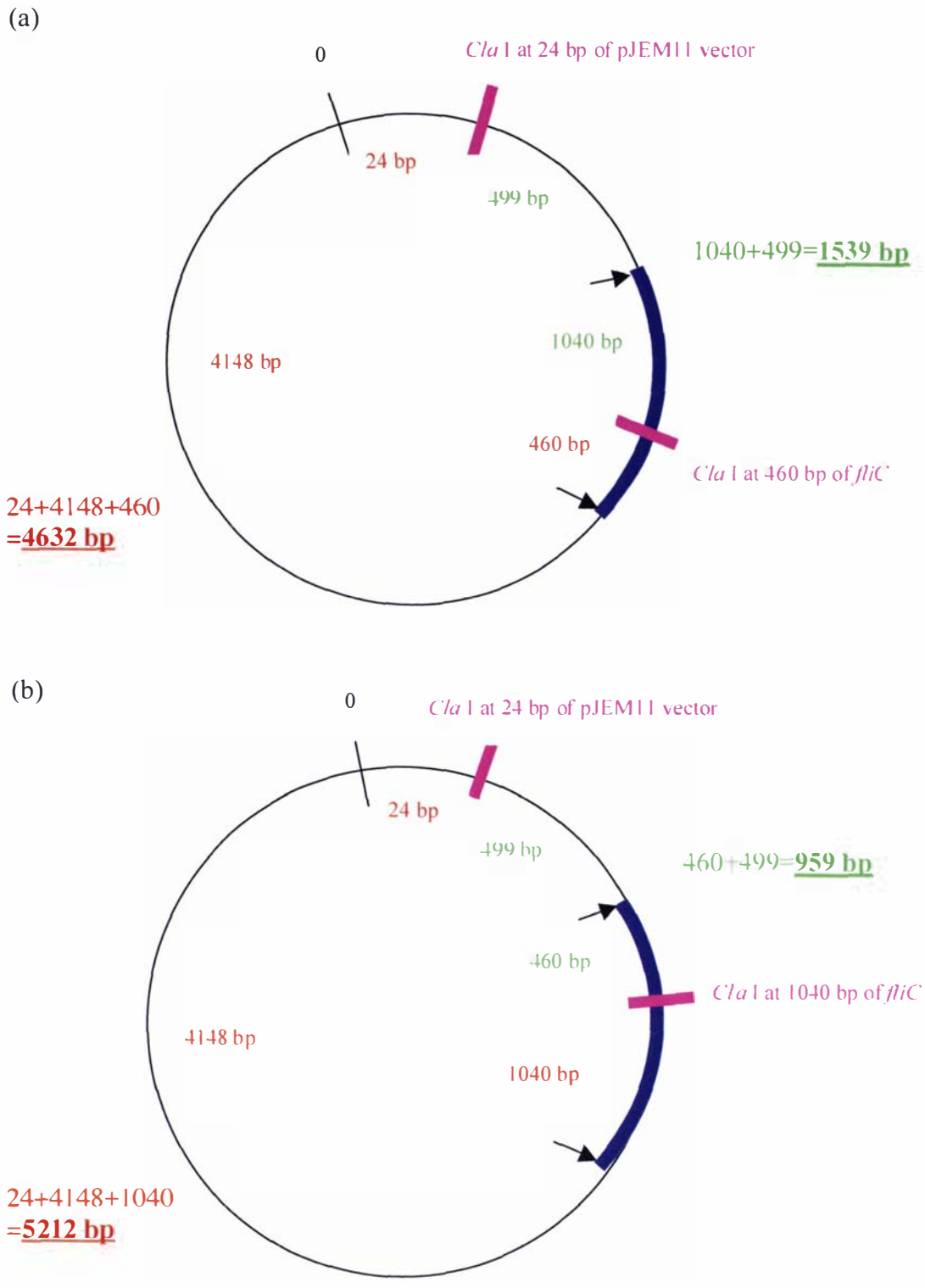


Figure 4.7. Schematic diagram showing *Cla* I digestion of 6171-bp pET14b-*fliC* construct. (a) Plasmids carrying correctly orientated inserts contain 2 fragments of 4632 (24+4148+460) and 1539 (1040+499) bp. (b) Plasmids carrying incorrectly orientated inserts contain 2 fragments of 5212 (24+4148+1040) and 959 (460+499) bp. Insert is shown in blue with black arrows. ATG of *Nde* I site was used as the start codon.

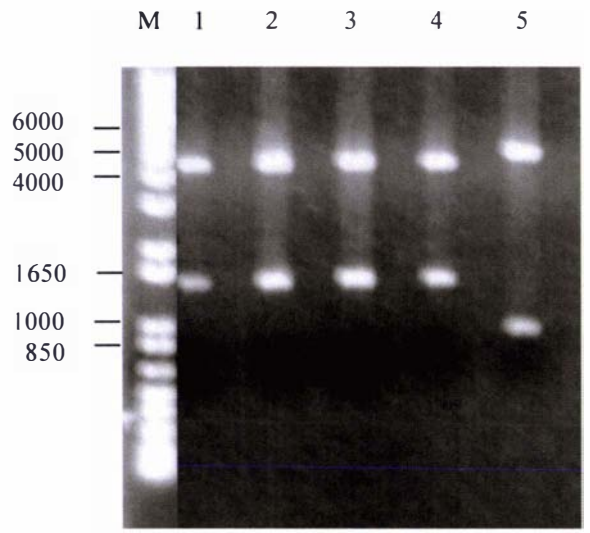


Figure 4.8. Restriction endonuclease digests of 6171-bp pET14b-*fliC* constructs. Plasmids from 5 recombinant *E. coli* colonies were digested with *Cla* I to select the ones carrying the correctly orientated inserts as described in Section 4.3.4. Samples of 10 μl were electrophoresed on a 1% agarose gel, stained with ethidium bromide and photographed under UV light. Digests of plasmids (clones 1, 2, 3 and 4) carrying correctly orientated inserts contain 2 fragments of 1539 and 4632 bp. Clone 5 carrying the incorrectly orientated insert contains 2 fragments of 959 and 5212 bp. Lane M, 1-kb Plus DNA Ladder marker (bp). Clone numbers are indicated at the top of the Figure.

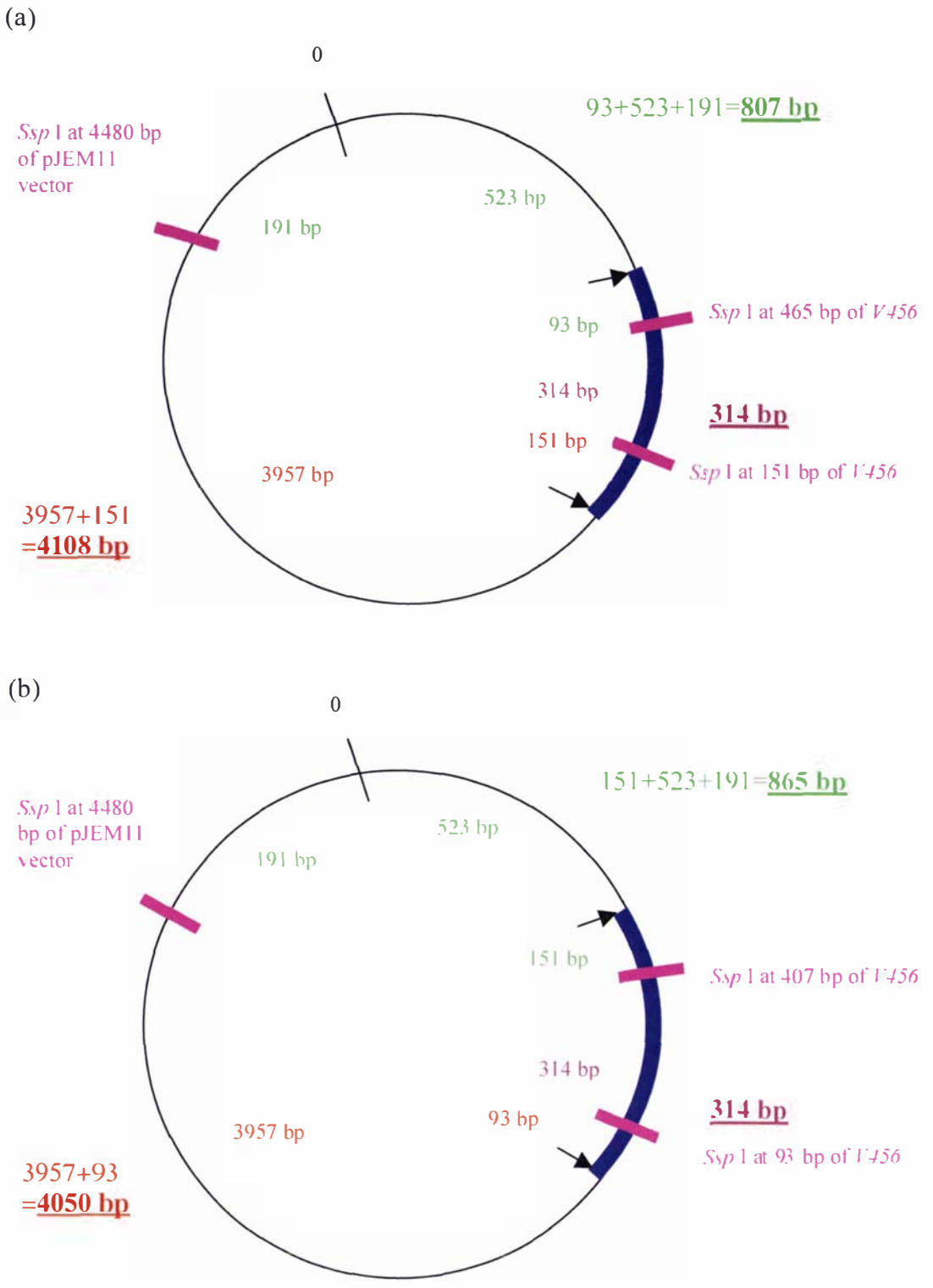


Figure 4.9. Schematic diagram showing *Ssp* I digestion of 5229-bp pET14b-*V456* construct. (a) Plasmids carrying correctly orientated inserts contain 3 fragments of 4108 (3957+151), 314 and 807 (93+523+191) bp. (b) Plasmids carrying incorrectly orientated inserts contain 3 fragments of 4050 (3957+93), 314 and 865 (151+523+191) bp. Insert is shown in blue with black arrows. *Ssp* I sites are shown in pink. ATG of *Nde* I site was used as the start codon.

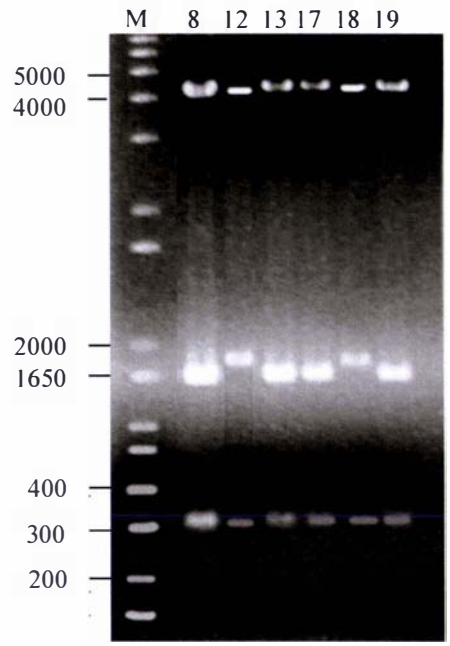


Figure 4.10. Restriction endonuclease digests of 5229-bp pET14b-*V456* constructs. Plasmids from 6 recombinant *E. coli* colonies were digested with *Ssp* I to select the ones carrying the correctly orientated inserts as described in Section 4.3.4. Samples of 10 μl were electrophoresed on a 1% agarose gel, stained with ethidium bromide and photographed under UV light. Digests of plasmids (clones 8, 13, 17 and 19) carrying correctly orientated inserts contain three fragments of 4108 (3957+151), 314 and 807 (93+523+191) bp. Clones 12 and 18 carrying the incorrectly orientated inserts contain three fragments of 4050 (3957+93), 314 and 865 (151+523+191) bp. Lane M, 1-kb Plus DNA Ladder marker (bp). Clone numbers are indicated at the top of the Figure.

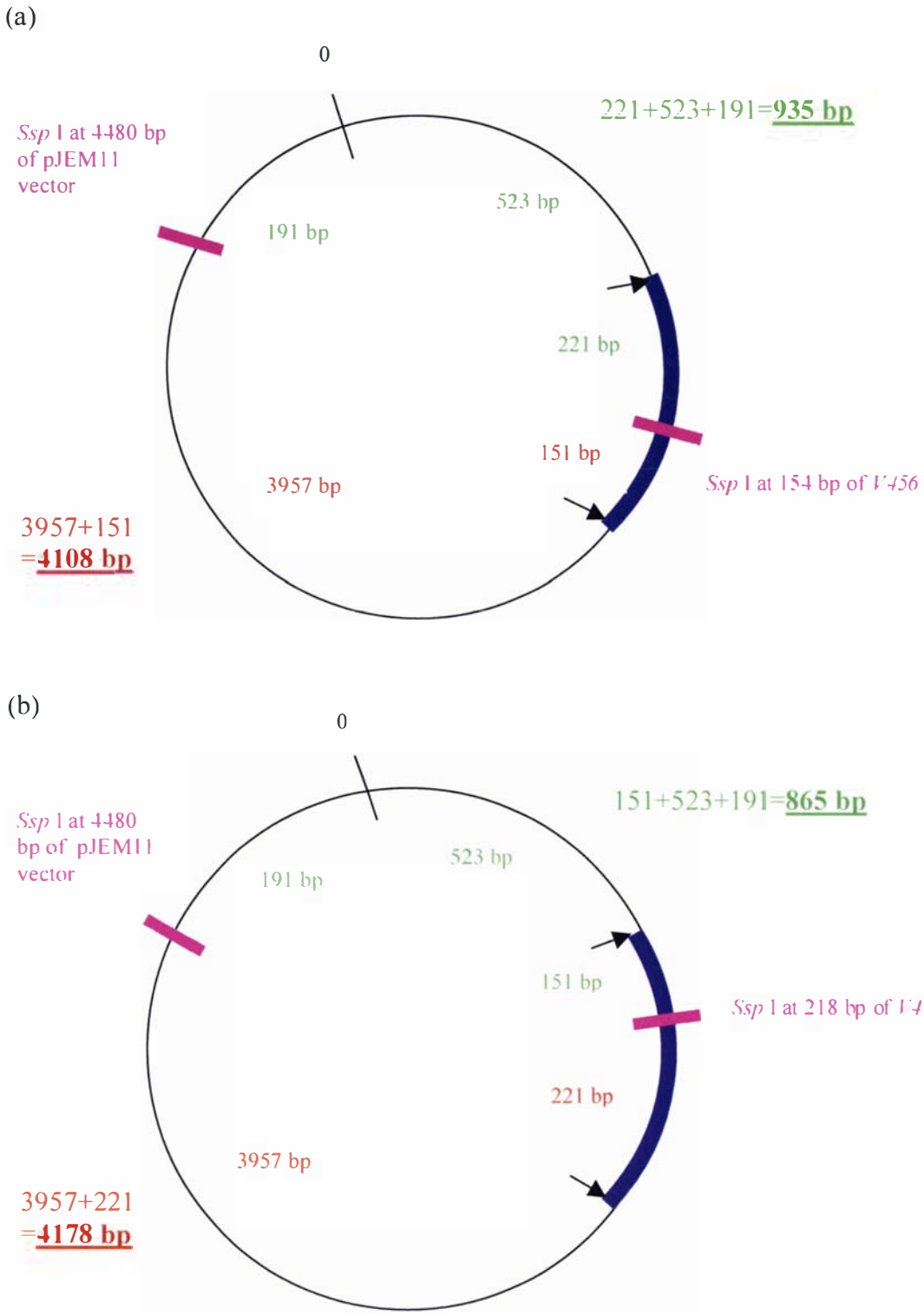


Figure 4.11. Schematic diagram showing *Ssp* I digestion of 5043-bp pET14b-*V4* construct. (a) Plasmids carrying correctly orientated inserts contain 2 fragments of 4108 (3957+151) and 935 (221+523+191) bp. (b) Plasmids carrying incorrectly orientated inserts contain 2 fragments of 4178 (3957+221) and 865 (151+523+191) bp. Insert is shown in blue with black arrows. *Ssp* I sites are shown in pink. ATG of *Nde* I site was used as the start codon.

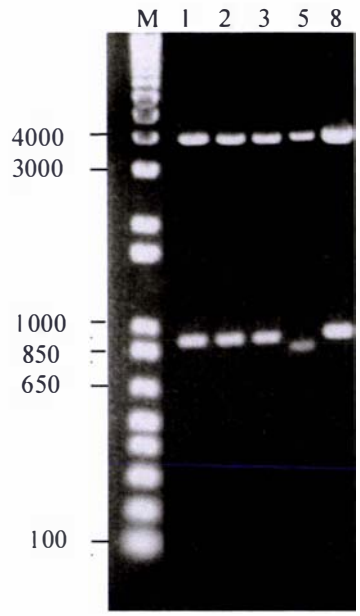


Figure 4.12. Restriction endonuclease digests of 5043-bp pET14b-*V4* constructs. Plasmids from 5 recombinant *E. coli* colonies were digested with *Ssp* I to select the ones carrying the correctly orientated inserts as described in Section 4.3.4. Samples of 10 µl were electrophoresed on a 1% agarose gel, stained with ethidium bromide and photographed under UV light. Digests of plasmids (clones 1, 2, 3 and 8) carrying correctly orientated inserts contain 2 fragments of 935 (221+523+191) and 4108 (3957+151) bp. Clone 5 carrying the incorrectly orientated insert contains 2 fragments of 865 (151+523+191) and 4178 (3957+221) bp. Lane M, 1-kb Plus DNA Ladder marker (bp). Clone numbers are indicated at the top of the Figure.

4.4.3 Expression and purification of recombinant proteins FliC, V456, and V4

Upon IPTG induction of *E. coli* BL21 (DE3) cells carrying plasmids pET14b–*fliC*, pET14b-*V456* and pET14b-*V4*, high levels of FliC, V456 and V4 were expressed as histidine-tagged fusion proteins of approximately 54-, 21- and 15-kDa respectively (Figure 4.13). The molecular mass of fusion proteins were calculated using ExPASY protein tool website (http://www.expasy.ch/tools/pi_tool.html). The calculated molecular masses of histidine-tagged FliC, V456 and V4 were 54.15-, 20.88- and 14.49-kDa respectively. The proteins were expressed with an N-terminal histidine tag for the detection in the Western blot, and purification through affinity chromatography. All three proteins were found to be soluble; hence they were purified from the soluble lysate by cobalt metal affinity chromatography, and eluted with elution buffer containing imidazole. SDS-PAGE analysis (Figure 4.14) and Western blotting with anti-histidine monoclonal antibody (Figure 4.15) revealed purified fusion proteins of the expected sizes.

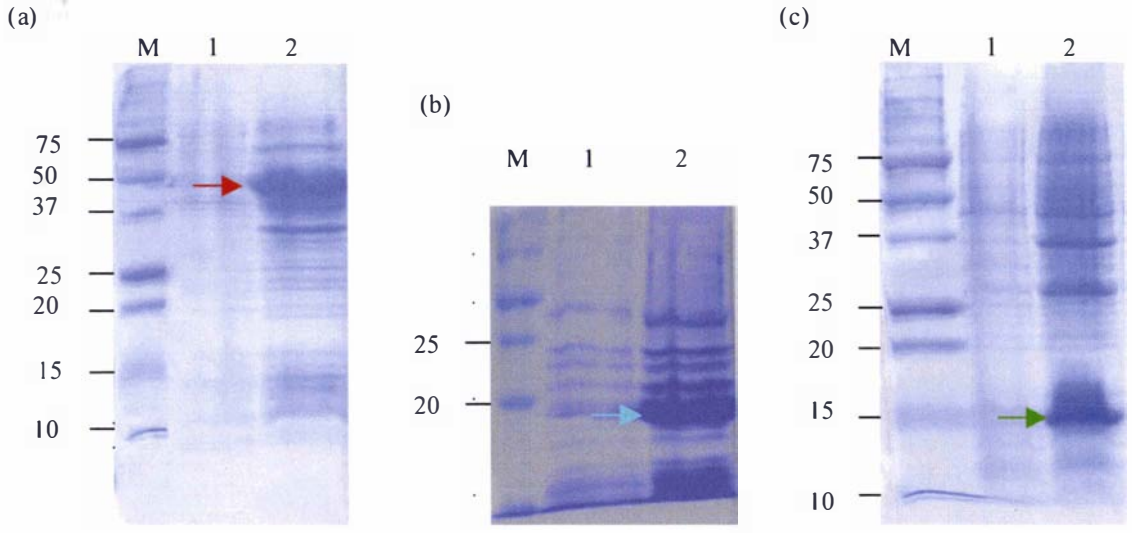


Figure 4.13. Expression of FliC, V456, and V4 as histidine-tagged recombinant proteins in pET14b from *E. coli*. Protein expression was induced with 1 mM IPTG, and the cells were harvested at pre- and 3-hour post-induction. Fifteen microlitres of resuspended cells from pre- and 3-hour post-induced samples were electrophoresed on 15% SDS-PAGE gels, and stained with Coomassie Blue. (a) FliC. Lane M, Precision Plus Protein™ All Blue Standard (kDa); lane 1, uninduced sample; lane 2, 3-hour induced sample. (b) V456. Lane M, Precision Plus Protein™ All Blue Standard (kDa); lane 1, uninduced sample; lane 2, 3-hour induced sample. (c) V4. Lane M, Precision Plus Protein™ All Blue Standard (kDa); lane 1, uninduced sample; lane 2, 3-hour induced sample. Red, blue and green arrows indicate the positions of recombinant proteins FliC, V456 and V4 respectively.

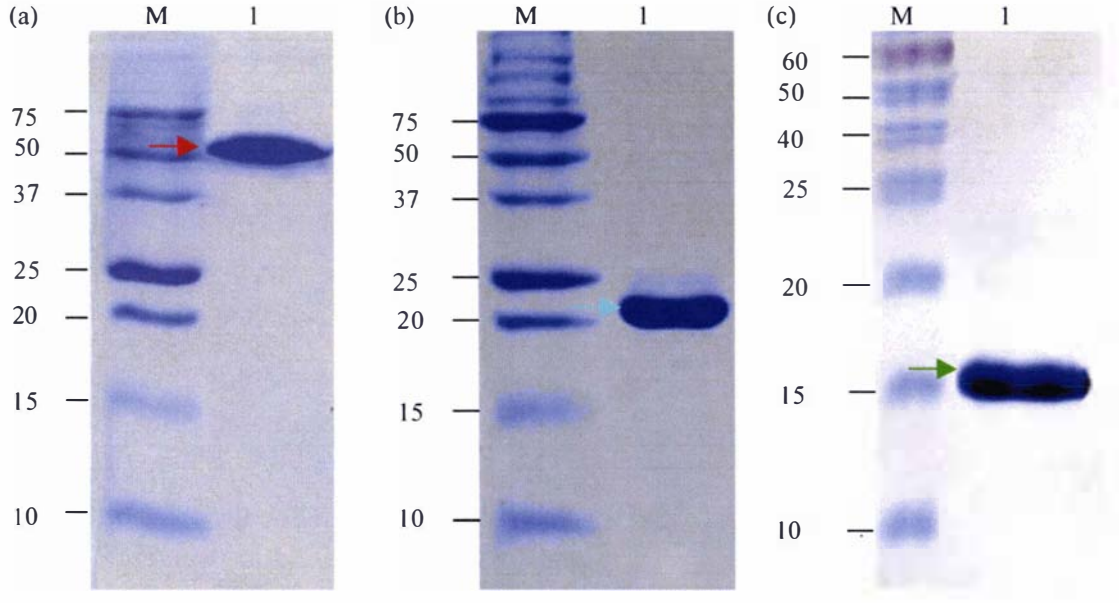


Figure 4.14. Purification of histidine-tagged FliC, V456, and V4 recombinant proteins by affinity chromatography. Sonicated cell lysates of *E. coli* carrying pET14b-FliC, pET14b-V456 and pET14b-V4 were applied to cobalt affinity columns, and the bound proteins were eluted with 150 mM imidazole. Five microlitres from each fraction was electrophoresed on 12% SDS-PAGE gels, and stained with Coomassie Blue. (a) Lane M, Precision Plus Protein™ All Blue Standard (kDa); lane 1, recombinant FliC protein. (b) Lane M, Precision Plus Protein™ All Blue Standard (kDa); lane 1, recombinant V456 protein. (c) Lane M, Prestained BenchMark™ Protein Ladder (kDa); lane 1, recombinant V4 protein. Red, blue, and green arrows indicate the positions of recombinant proteins FliC, V456, and V4 respectively.

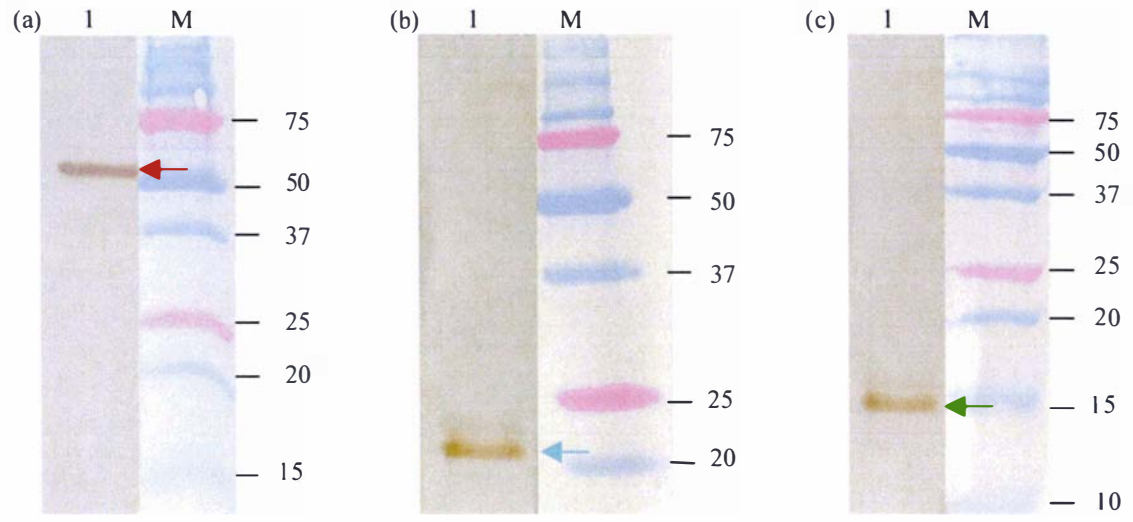


Figure 4.15. Western blot detection of purified FliC, V456, and V4 recombinant proteins with anti-histidine monoclonal antibody. A dilution of 1:1,000 anti-histidine mouse IgG monoclonal antibody, and that of 1:300 peroxidase-labelled sheep anti-mouse IgG were used. (a) Lane 1, FliC. (b) Lane 1, V456. (c) Lane 1, V4. Lane M of each contains Precision Plus Protein™ Dual Colour Standard (kDa). Red, blue, and green arrows indicate the positions of recombinant proteins FliC, V456, and V4 respectively.

4.4.4 Humoral immune responses to purified recombinant proteins

Reactivity of purified recombinant proteins FliC, V456, and V4 with sera was determined by Western blotting. Sheep sera obtained from a number of sources described in Sections 2.11, 2.12, and rabbit sera described in Section 2.13 were used in the blots. A summary of Western blot results is shown in Table 4.3.

4.4.4.1 Sera from naturally infected sheep

Eighty-one sera collected from naturally infected sheep (Section 2.12.1), reacted with all 3 recombinant proteins with different intensities. While 9 out of 80 sera collected from sheep not exposed to *S.* Brandenburg reacted with FliC recombinant, none reacted with V456 and V4 recombinant proteins. Individual blots representing naturally infected and non-infected groups are shown in Figures 4.16, 4.17 and 4.18.

4.4.4.2 Sera from Slavexin^{TM}+B-vaccinated sheep (Trial 2004)

In the sheep experiment described in Section 2.11, 6 sheep were used as controls, and 6 were vaccinated with commercially available Salvexin^{TM}+B. All the sera collected prior to vaccination, and those collected from control sheep throughout the experiment did not show any reactivity with all three recombinant proteins. All the sera collected 1-month post-priming, and 3-weeks, 2-, and 3-months post-booster from vaccinated group reacted with all three recombinant proteins (Figures 4.19, 4.20 and 4.21).

4.4.4.3 Sera from sheep vaccination trial to compare Salvexin^{TM} with Salvexin^{TM}+B (Trial 1999)

Sera collected from sheep vaccination trial to compare Salvexin^{TM} with Salvexin^{TM}+B (Section 2.12.2) were tested for their reactivity with the recombinant proteins. In this trial, 15 sheep were used as controls, 15 were vaccinated with Salvexin^{TM}, and the other 15 were vaccinated with Salvexin^{TM}+B. Ten samples from each group collected pre-vaccination and 2-weeks post-booster were used in the blots. All the pre-vaccination sera, and those collected from control sheep were negative. The samples from sheep

vaccinated with Salvexin™, and Salvexin™+B were positive with FliC, while only the latter group was positive with V456 and V4 (Figures 4.22 and 4.23).

4.4.4.4 Sera from vaccinated rabbits

Sera collected from rabbits (Section 2.13) immunized with heat-inactivated *S.* Brandenburg, *S.* Typhimurium, and *S.* Hindmarsh were used in the Western blots. Sera from control rabbits were negative with all 3 proteins. Samples from rabbits immunized with all 3 serotypes reacted with FliC, while those from rabbits immunized only with *S.* Brandenburg reacted with V456 and V4 (Figures 4.24, 4.25 and 4.26).

Table 4.3. Summary of serum IgG antibody detection in sheep and rabbits by Western blot

Source of serum samples	No. of sera tested	No. of sera positive in Western blot		
		FliC	V456	V4
(a) Sheep naturally infected with *S.* Brandenburg	81	81	81	81
(b) Sheep not exposed to *S.* Brandenburg (Section 4.4.4.1)	80	9	0	0
Trial 2004				
Sheep experimentally vaccinated with				
(a) Salvexin™+B	6	6	6	6
(b) Unvaccinated controls (Section 4.4.4.2)	6	0	0	0
Trial 1999				
Sheep experimentally vaccinated with				
(a) Salvexin™	10	10	0	0
(b) Salvexin™+B	10	10	10	10
(c) Unvaccinated controls (Section 4.4.4.3)	10	0	0	0
Rabbits experimentally immunized with				
(a) *S.* Brandenburg	2	2	2	2
(b) *S.* Typhimurium	2	2	0	0
(c) *S.* Hindmarsh	2	2	0	0
(d) Unvaccinated controls (Section 4.4.4.4)	2	0	0	0

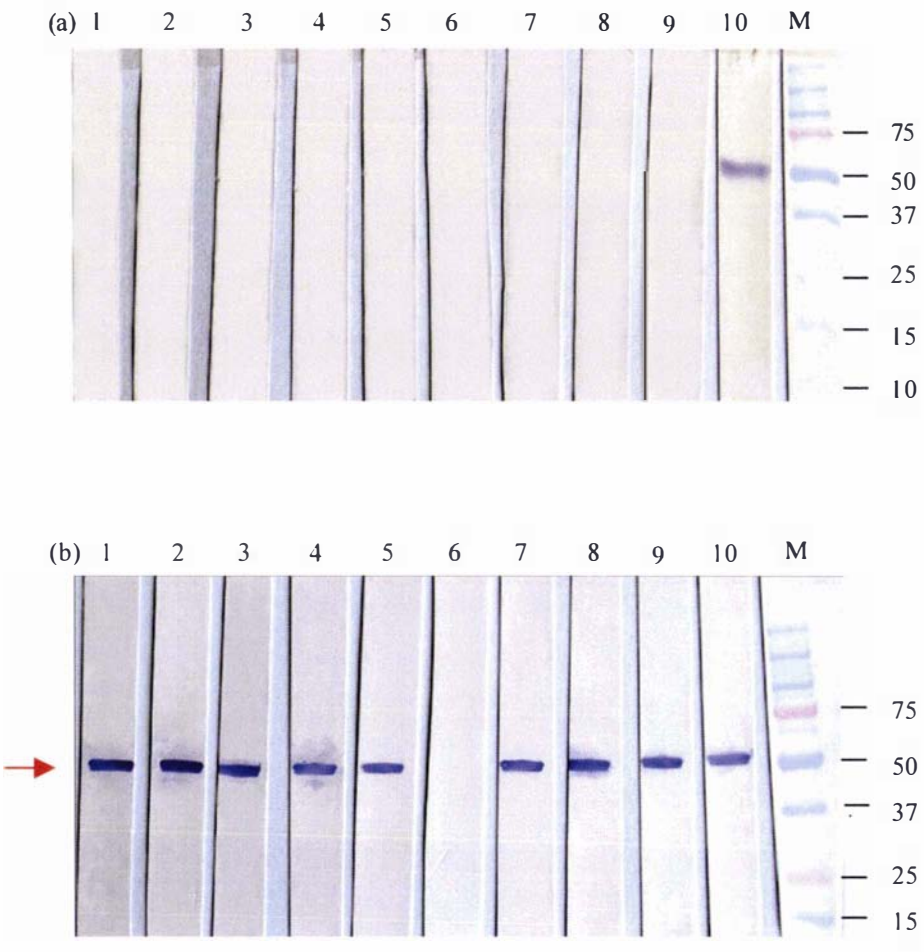


Figure 4.16. Western blot detection of serum IgG antibodies against recombinant FliC in sheep naturally infected with *S*. Brandenburg. A dilution of 1:500 of sheep sera, and that of 1:10,000 of AP-labelled anti-sheep IgG were used. (a) Lanes 1 to 8, sera from sheep not exposed to *S.* Brandenburg (sample numbers N1, N8, N17, N23, N35, N52, N54, N60 respectively); lane 9, negative control; lane 10, positive control; lane M, Precision Plus Protein™ Dual Colour Standard (kDa). (b) Lanes 1 to 5, sera from sheep naturally infected with *S*. Brandenburg (sample numbers S3, S4, S15, S29 and S55 respectively); lane 6, negative control; lane 7, positive control; lanes 8 to 10, sera from sheep naturally infected with *S*. Brandenburg (sample numbers S67, S78 and S77 respectively); lane M, Precision Plus Protein™ Dual Colour Standard (kDa). Red arrow indicates the position of recombinant FliC protein.

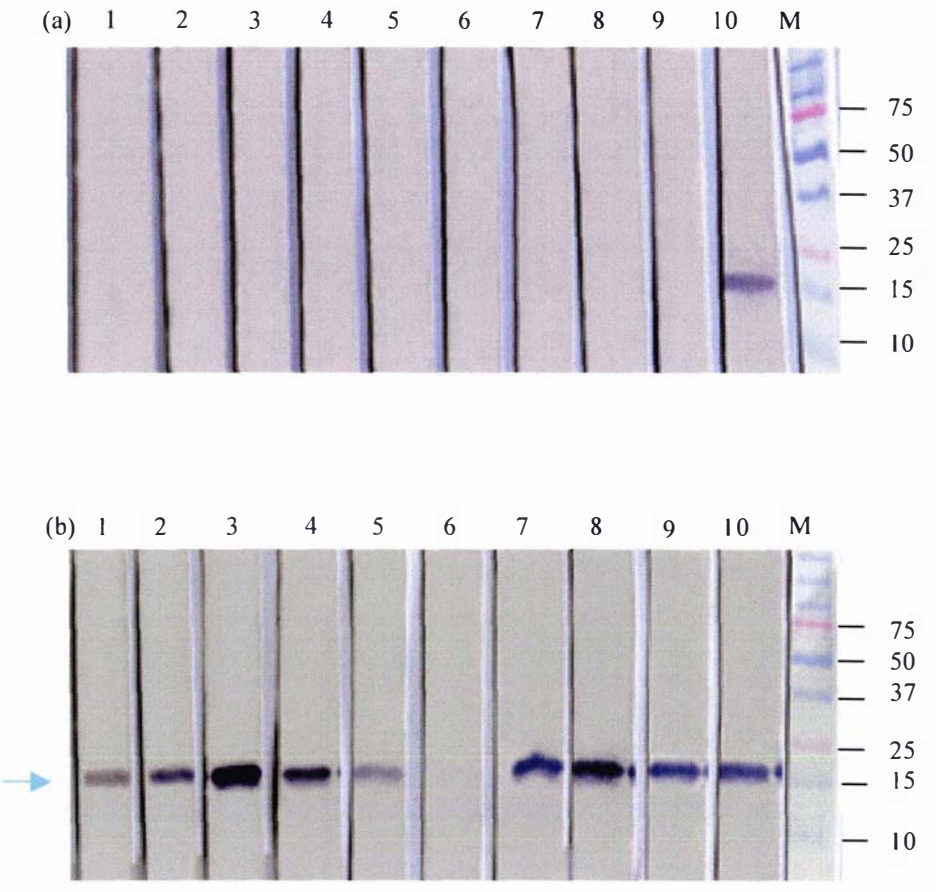


Figure 4.17. Western blot detection of serum IgG antibodies against recombinant V456 in sheep naturally infected with *S.* Brandenburg. A dilution of 1:500 of sheep sera, and that of 1:10,000 of AP-labelled anti-sheep IgG were used. (a) Lanes 1 to 8, sera from sheep not exposed to *S.* Brandenburg (sample numbers N1, N8, N17, N23, N35, N52, N54, N60 respectively); lane 9, negative control; lane 10, positive control; lane M, Precision Plus Protein™ Dual Colour Standard (kDa). (b) Lanes 1 to 5, sera from sheep naturally infected with *S.* Brandenburg (sample numbers S12, S4, S2, S54 and S69 respectively); lane 6, negative control; lane 7, positive control; lanes 8 to 10, sera from sheep naturally infected with *S.* Brandenburg (sample numbers S15, S9 and S81 respectively); lane M, Precision Plus Protein™ Dual Colour Standard (kDa). Blue arrow indicates the position of recombinant V456 protein.

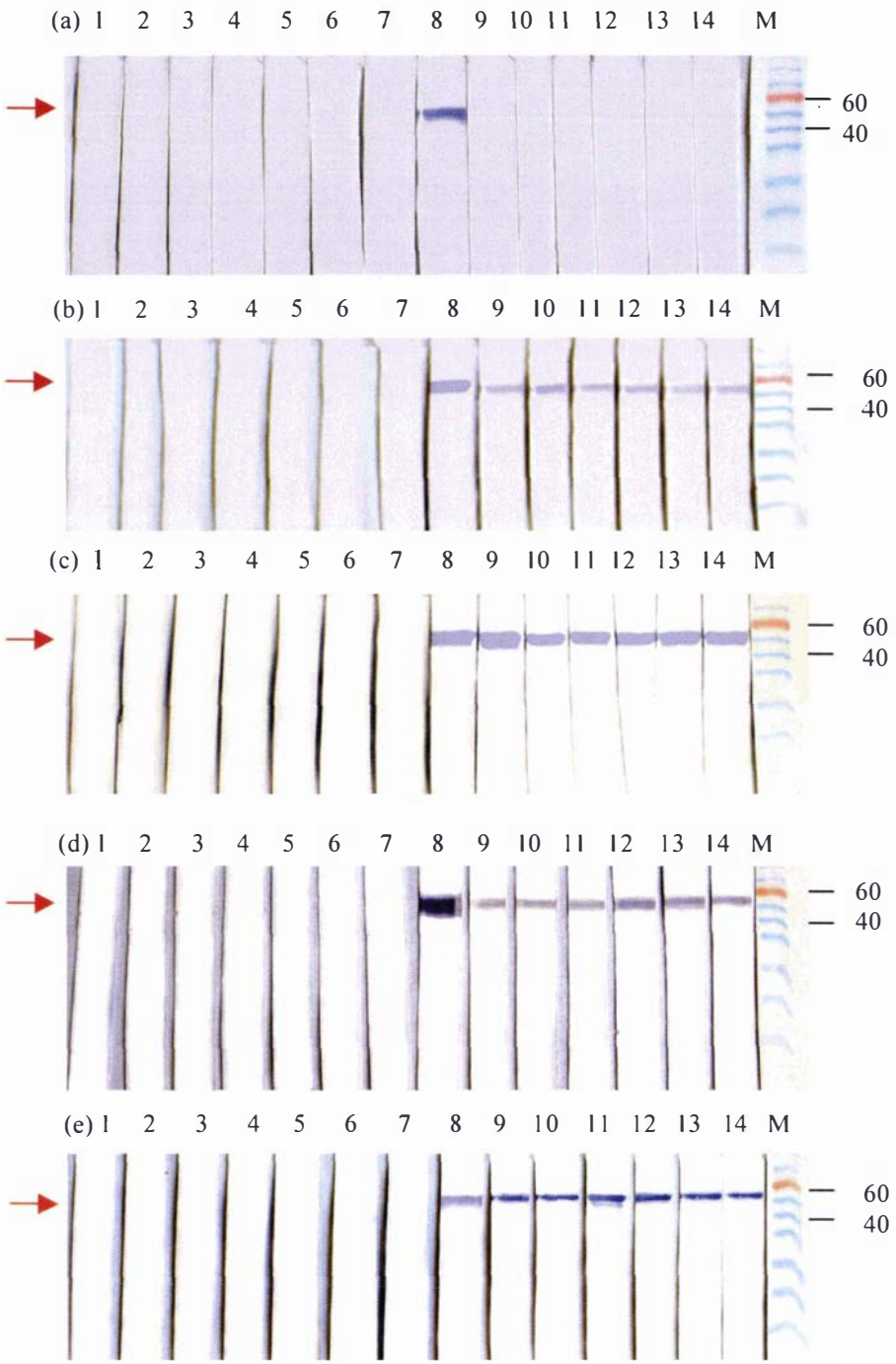


Figure 4.19. Western blot detection of serum IgG antibodies against recombinant FliC in sheep vaccinated with Salvexin™+B (Trial 2004). A dilution of 1:500 of sheep sera, and that of 1:10,000 of AP-labelled anti-sheep IgG were used. (a) Pre-vaccination sera. (b) One-month post-priming sera. (c) Three-weeks post-booster sera. (d) Two-months post-booster sera. (e) Three-months post-booster sera. Lanes 1 to 6, control sheep (animal numbers C005, C006, C015, C017, C022 and C026 respectively); lane 7, negative control; lane 8, positive control; lanes 9 to 14, vaccinated sheep (animal numbers V016, V019, V020, V021, V024 and V025 respectively); lane M, Prestained BenchMark™ Protein Ladder (kDa). Red arrow indicates the position of recombinant FliC protein.

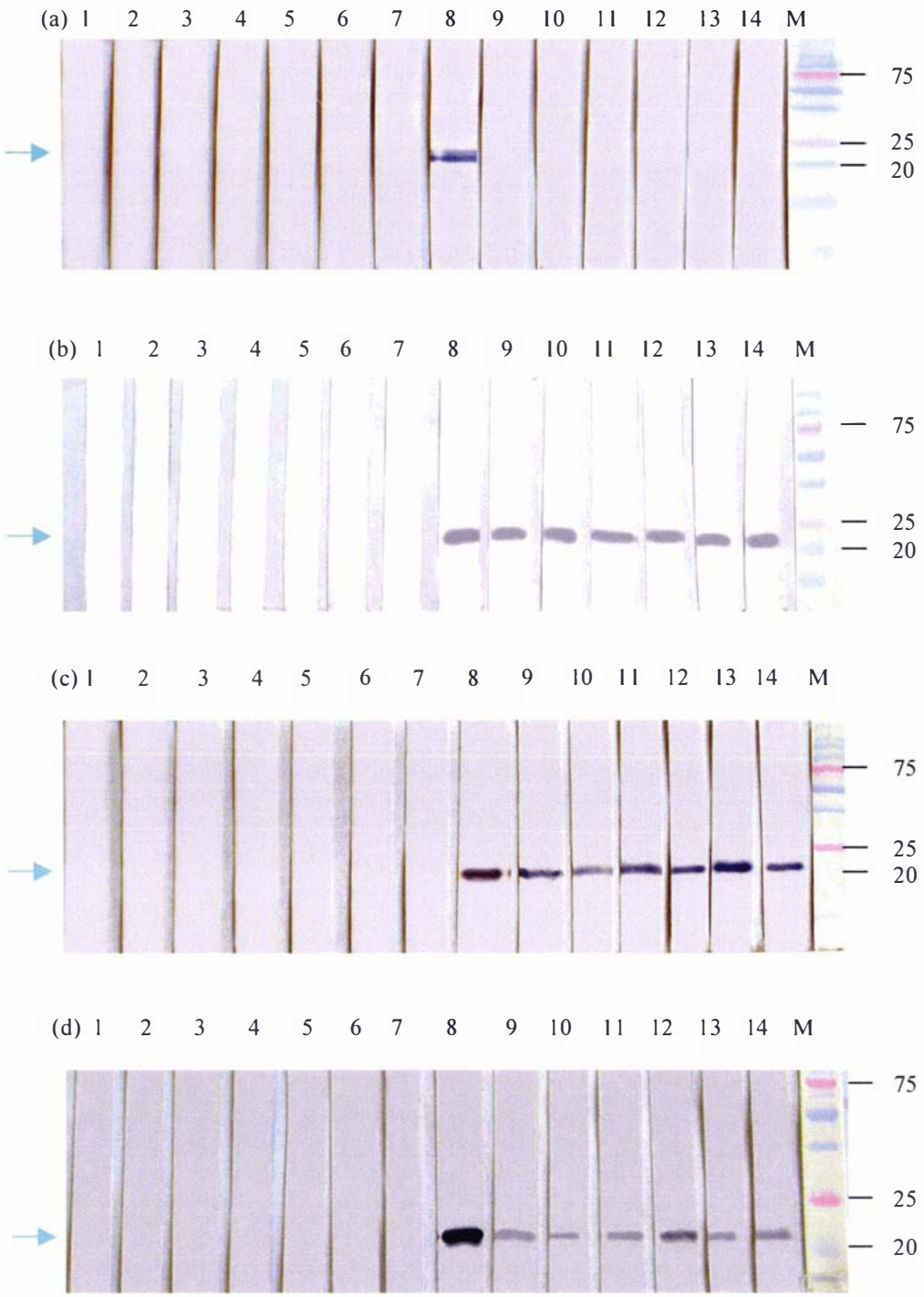


Figure 4.20. Western blot detection of serum IgG antibodies against recombinant V456 in sheep vaccinated with Salvexin™+B (Trial 2004). A dilution of 1:500 of sheep sera, and that of 1:10,000 of AP-labelled anti-sheep IgG were used. (a) Pre-vaccination sera. (b) Three-weeks post-booster sera. (c) Two-months post-booster sera. (d) Three-months post-booster sera. Lanes 1 to 6, control sheep (animal numbers C005, C006, C015, C017, C022 and C026 respectively); lane 7, negative control; lane 8, positive control; lanes 9 to 14, vaccinated sheep (animal numbers V016, V019, V020, V021, V024 and V025 respectively); lane M, Precision Plus Protein™ Dual Colour Standard (kDa). Blue arrow indicates the position of recombinant V456 protein.

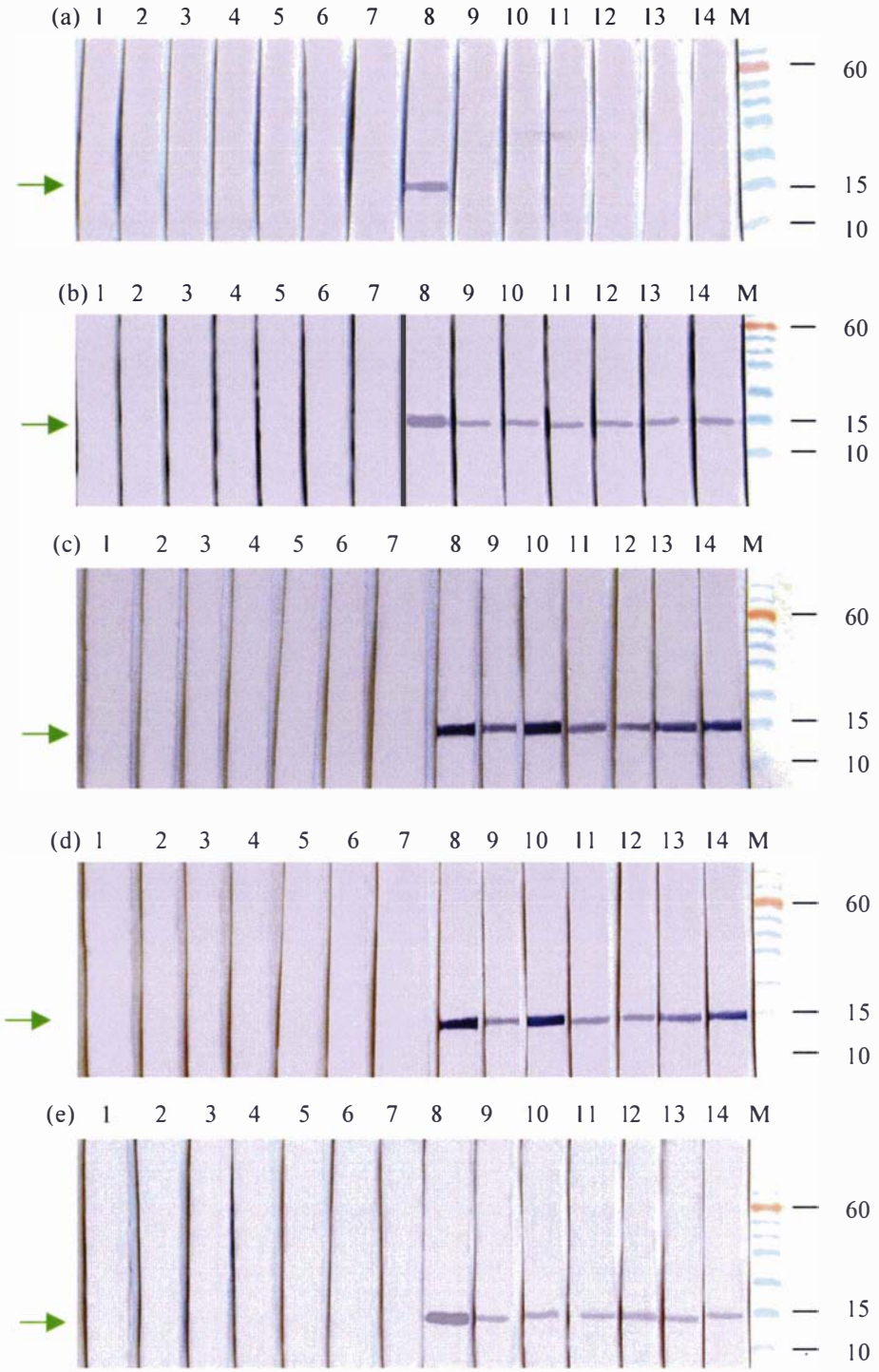


Figure 4.21. Western blot detection of serum IgG antibodies against recombinant V4 in sheep vaccinated with Salvexin^{TM}+B (Trial 2004). A dilution of 1:100 of sheep sera, and that of 1:10,000 of AP-labelled anti-sheep IgG were used. (a) Pre-vaccination sera. (b) One-month post-priming sera. (c) Three-weeks post-booster sera. (d) Two-months post-booster sera. (e) Three-months post-booster sera. Lanes 1 to 6, control sheep (animal numbers C005, C006, C015, C017, C022 and C026 respectively); lane 7, negative control; lane 7, positive control; lanes 9 to 14, vaccinated sheep (animal numbers V016, V019, V020, V021, V024 and V025 respectively); lane M, Prestained BenchMark^{TM} Protein Ladder (kDa). Green arrow indicates the position of recombinant V4 protein.

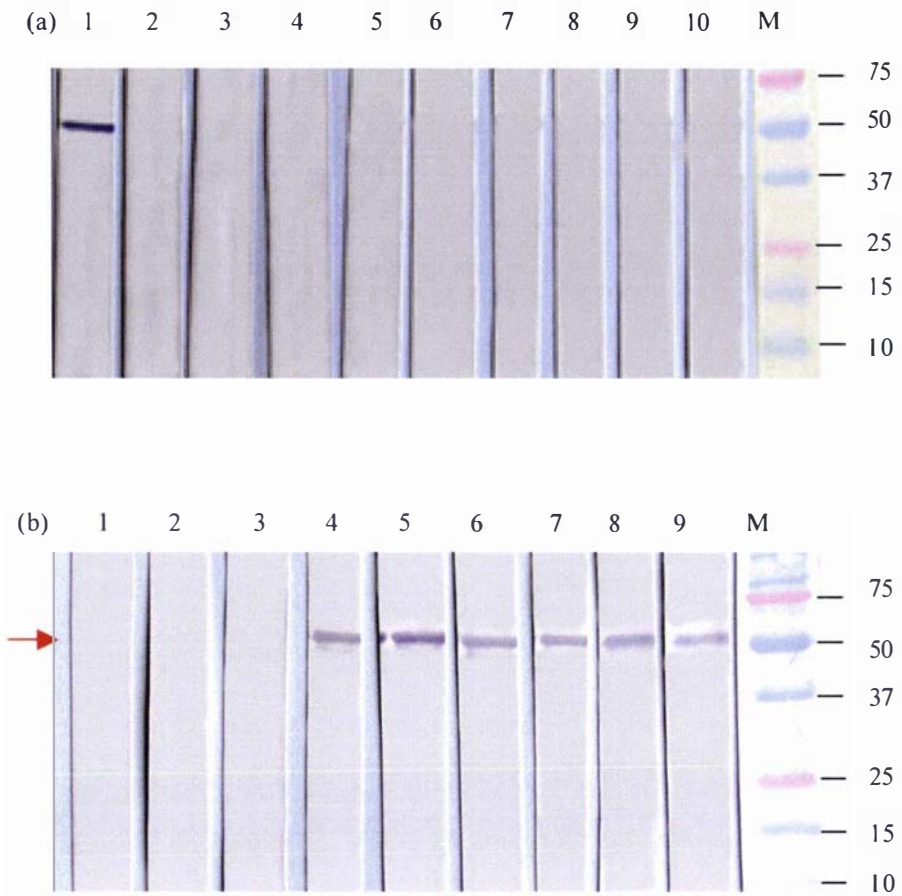


Figure 4.22. Western blot detection of serum IgG antibodies against recombinant FliC in sheep used in the vaccination trial to compare Salvexin™ with Salvexin™+B (Trial 1999). A dilution of 1:500 of sheep sera, and that of 1:10,000 AP-labelled anti-sheep IgG were used. (a) Pre-vaccination sera. Lane 1, positive control; lanes 2 to 10, pre-vaccination sera (sample numbers C1, C4, C7, Sa3, Sa4, Sa8, SB1, SB2 and SB5 respectively); lane M, Precision Plus Protein™ Dual Colour Standard (kDa). (b) Sera collected 2-weeks post-booster. Lanes 1 to 3, sera from unvaccinated sheep (sample numbers C1, C4 and C7 respectively); lanes 4 to 6, sera from sheep vaccinated with Salvexin™ (sample numbers Sa3, Sa4 and Sa8 respectively); lanes 7 to 9, sera from sheep vaccinated with Salvexin™+B (sample numbers SB1, SB2 and SB5 respectively); lane M, Precision Plus Protein™ Dual Colour Standard (kDa). Red arrow indicates the position of recombinant FliC protein.

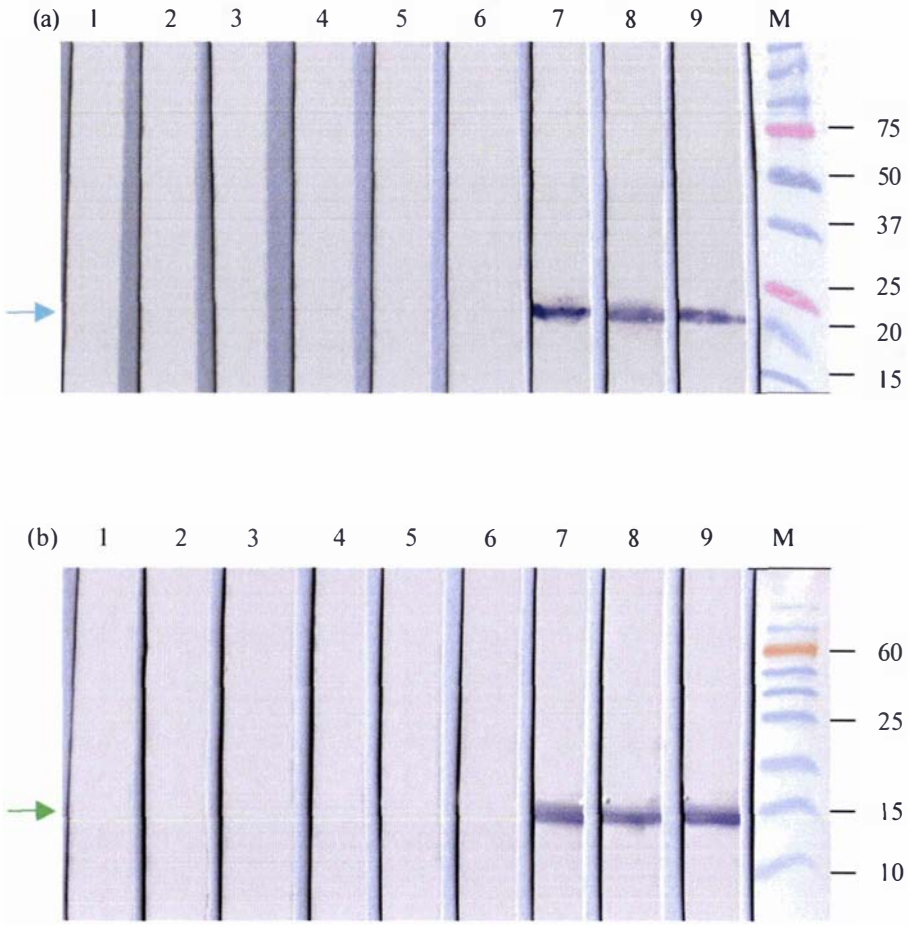


Figure 4.23. Western blot detection of serum IgG antibodies against recombinant V456 and V4 in sheep used in the vaccination trial to compare Salvexin™ with Salvexin™+B (Trail 1999). A dilution of 1:500 (a) and 1:100 (b) of sheep sera, and that of 1:10,000 AP-labelled anti-sheep IgG were used. Blood samples collected 2-weeks post-booster from ewes received Salvexin™ and Salvexin™+B vaccines were used. (a) V456. (b) V4. (a) and (b) Lanes 1 to 3, sera from unvaccinated sheep (sample numbers C1, C4 and C7 respectively); lanes 4 to 6, sera from sheep vaccinated with Salvexin™ (sample numbers Sa3, Sa4 and Sa8 respectively); lanes 7 to 9, sera from sheep vaccinated with Salvexin™+B (sample numbers SB1, SB2 and SB5 respectively). (a) Lane M, Precision Plus Protein™ Dual Colour Standard (kDa). (b) Lane M, Prestained BenchMark™ Protein Ladder (kDa). Blue and green arrows indicate the positions of recombinant V456 and V4 respectively.

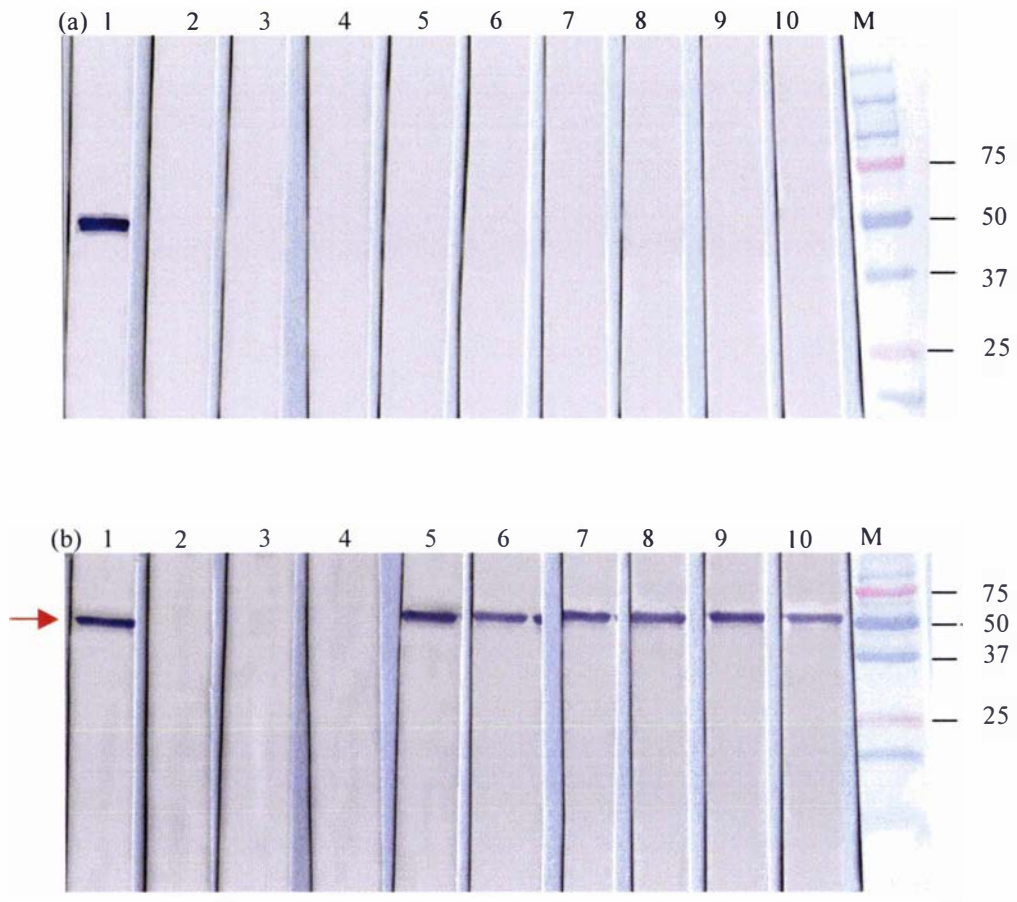


Figure 4.24. Western blot detection of serum IgG antibodies against recombinant FliC in rabbits. A dilution of 1:400 of rabbit sera collected (a) prior to vaccination (b) 2-weeks post-booster, and that of 1:10,000 AP-labelled anti-rabbit IgG were used. (a) Lane 1, positive control; lane 2, negative control; lanes 3 to 10, rabbit numbers 509, 510, 511, 512, 514, 525, 524 and 515 respectively; lane M, Precision Plus Protein™ Dual Colour Standard (kDa). (b) Lane 1, positive control; lane 2, negative control; lanes 3 and 4, unvaccinated rabbits (rabbit numbers 509 and 510 respectively); lanes 5 and 6, rabbits vaccinated with *S.* Hindmarsh (rabbit numbers 511 and 512 respectively); lanes 7 and 8, rabbits vaccinated with *S.* Typhimurium (rabbit numbers 514 and 525 respectively); lanes 9 and 10, rabbits vaccinated with *S.* Brandenburg (rabbit numbers 524 and 515 respectively); lane M, Precision Plus Protein™ Dual Colour Standard (kDa). Red arrow indicates the position of recombinant FliC protein.

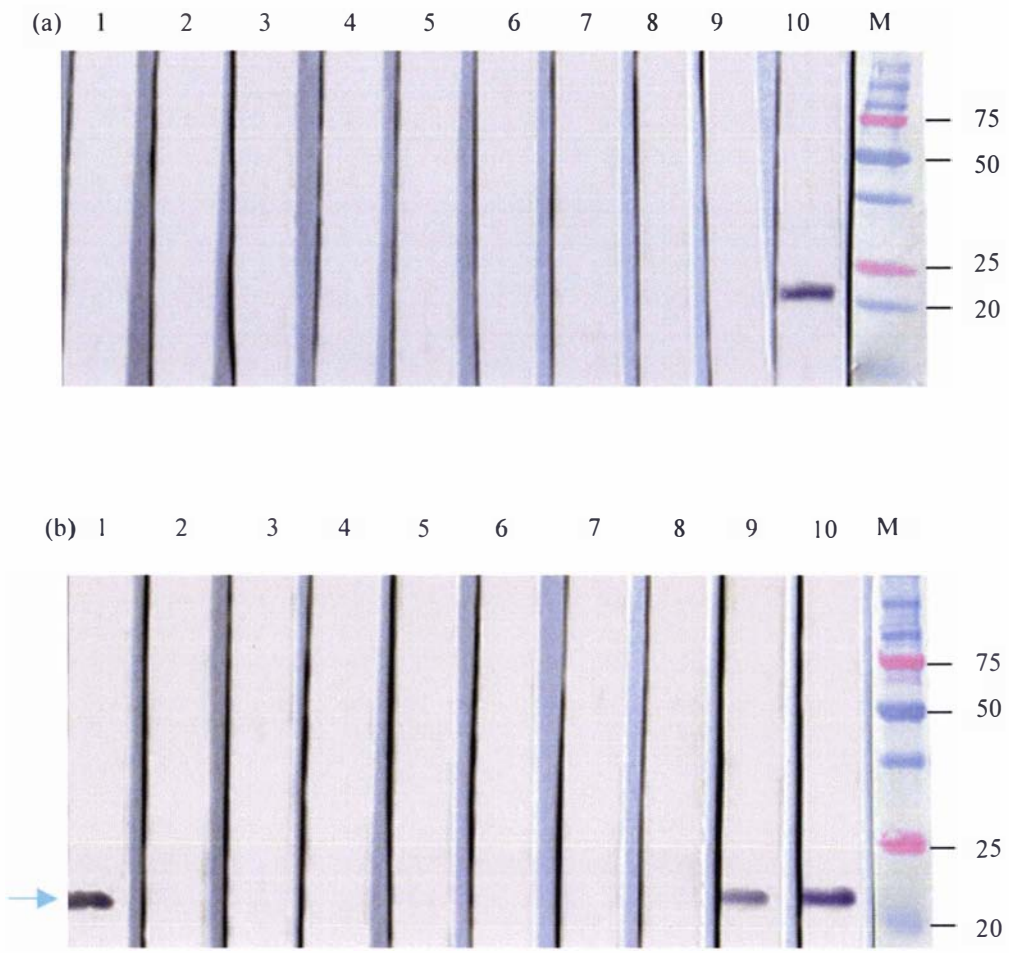


Figure 4.25. Western blot detection of serum IgG antibodies against recombinant V456 in rabbits. A dilution of 1:400 of rabbit sera collected (a) prior to vaccination (b) 2-weeks post-booster, and that of 1:10,000 AP-labelled anti-rabbit IgG were used. (a) Lanes 1 to 8, rabbit numbers 509, 510, 511, 512, 514, 525, 524 and 515 respectively; lane 9, negative control; lane 10, positive control; lane M, Precision Plus Protein™ Dual Colour Standard (kDa). (b) Lane 1, positive control; lane 2, negative control; lanes 3 and 4, unvaccinated rabbits (rabbit numbers 509 and 510 respectively); lanes 5 and 6, rabbits vaccinated with *S.* Hindmarsh (rabbit numbers 511 and 512 respectively); lanes 7 and 8, rabbits vaccinated with *S.* Typhimurium (rabbit numbers 514 and 525 respectively); lanes 9 and 10, rabbits vaccinated with *S.* Brandenburg (rabbit numbers 524 and 515 respectively); lane M, Precision Plus Protein™ Dual Colour Standard (kDa). Blue arrow indicates the position of recombinant V456 protein.

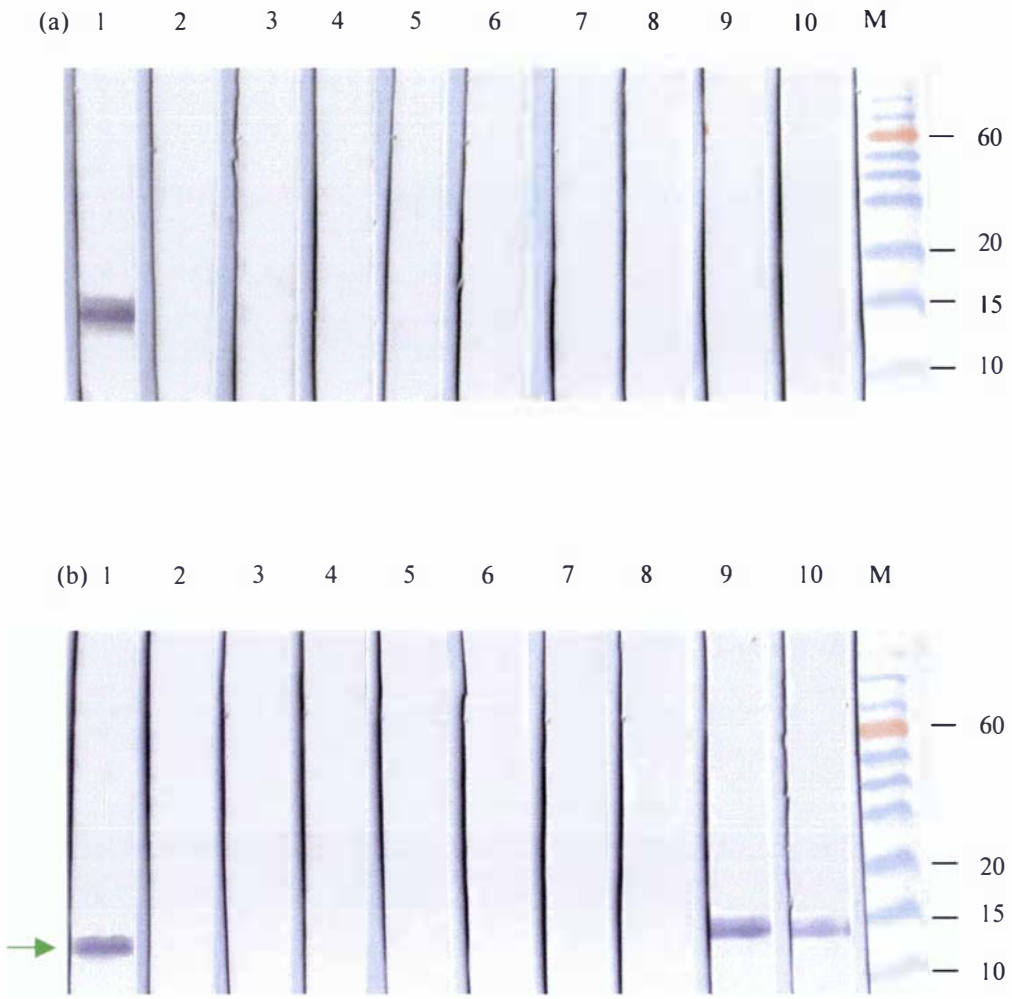
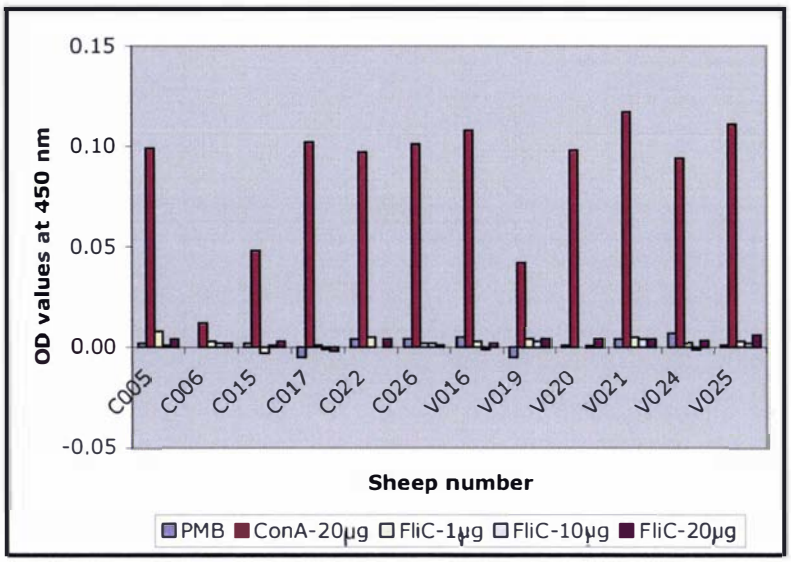


Figure 4.26. Western blot detection of serum IgG antibodies against recombinant V4 in rabbits. A dilution of 1:400 of rabbit sera collected (a) prior to vaccination (b) 2-weeks post-booster, and that of 1:10,000 AP-labelled anti-rabbit IgG were used. (a) Lane 1, positive control; lane 2, negative control; lanes 3 to 10, rabbit numbers 509, 510, 511, 512, 514, 525, 524 and 515 respectively; lane M, Prestained BenchMark™ Protein Ladder (kDa). (b) Lane 1, positive control; lane 2, negative control; lanes 3 and 4, unvaccinated rabbits (rabbit numbers 509 and 510 respectively); lanes 5 and 6, rabbits vaccinated with *S.* Hindmarsh (rabbit numbers 511 and 512 respectively); lanes 7 and 8, rabbits vaccinated with *S.* Typhimurium (rabbit numbers 514 and 525 respectively); lanes 9 and 10, rabbits vaccinated with *S.* Brandenburg (rabbit numbers 524 and 515 respectively); lane M, Prestained BenchMark™ Protein Ladder (kDa). Green arrow indicates the position of recombinant V4 protein.

4.4.5 Cell-mediated immune responses against FliC and V456 fusion proteins

Whole blood collected from control sheep, and those vaccinated with Salvexin™+B (Section 2.11) were subjected to IFN-γ assay with both FliC and V456 proteins (Section 2.11.2). Those from all sheep prior to vaccination, and sheep in the control group throughout the trial failed to produce any detectable IFN-γ in response to recombinant antigens. Furthermore, whole blood cells of immunized sheep cultured either in PBS or polymyxin B sulfate (PMB) alone failed to secrete any detectable IFN-γ. Vaccinated sheep did not produce IFN-γ following stimulation with recombinant proteins (Figures 4.27, 4.28, 4.29, 4.30 and 4.31).

(a)



(b)

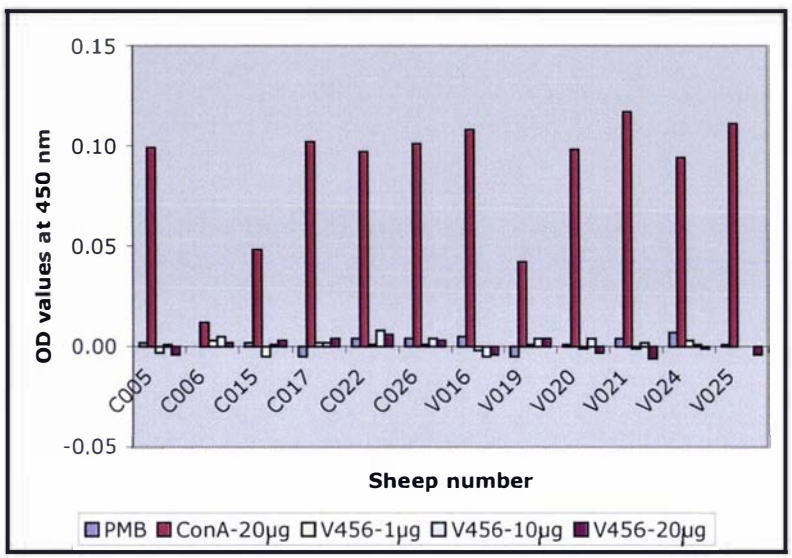
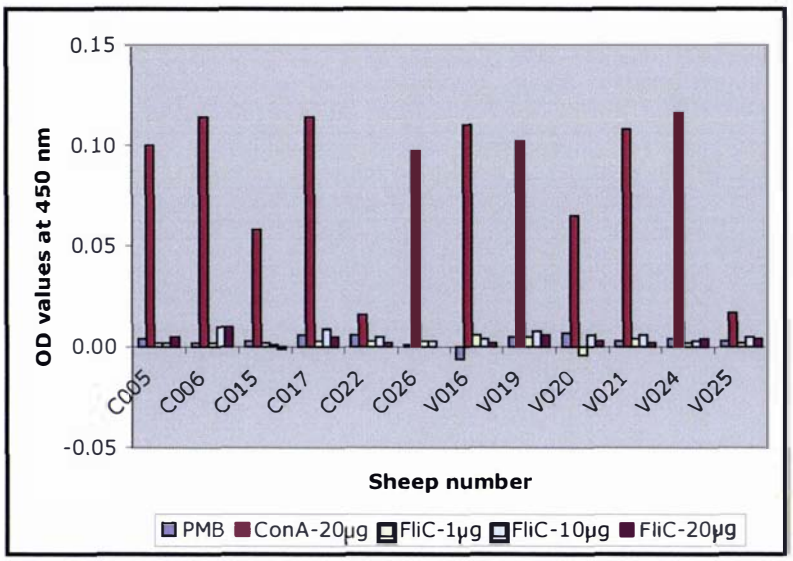


Figure 4.27. IFN-γ production in sheep vaccinated with Salvexin™ (Trial 2004): Pre-vaccination. Whole blood was incubated in duplicate wells with 1 µg/ml, 10 µg/ml and 20 µg/ml of (a) Recombinant FliC. (b) Recombinant V456. Recombinant proteins were incubated with 10 µg/ml PMB for 1 hr to remove LPS prior to their addition to whole blood. ConA was used as a non-specific T-cell activator to check the cell viability. Values of sheep used as controls (C005, C006, C015, C017, C022, C026) and, those used for vaccination (V016, V019, V020, V021, V024, V025) are shown. Each value represents the corrected absorbance at 450 nm. For each antigen concentration, PMB and ConA, corrected absorbance at 450 nm was the average absorbance of duplicate wells minus that of PBS for each sheep. See Appendix 6 for raw data.

(a)



(b)

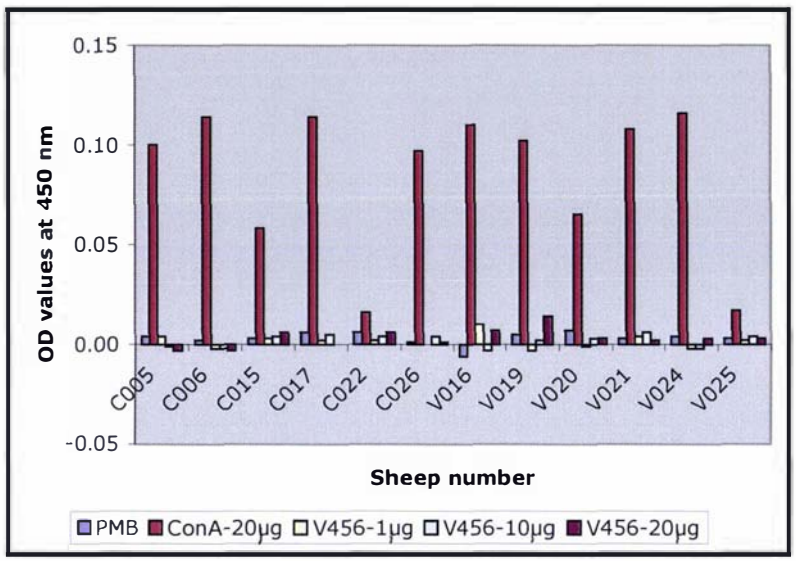
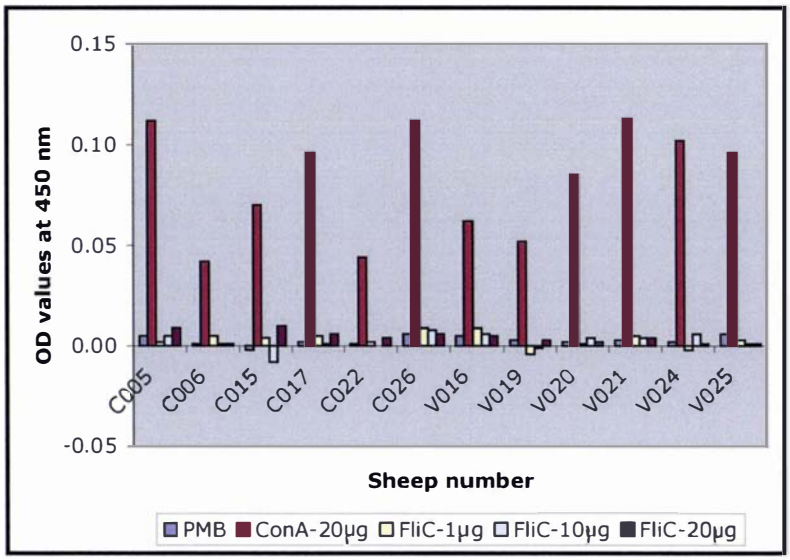


Figure 4.28. IFN-γ production in sheep vaccinated with Salvexin™ (Trial 2004): 1-month post-primary vaccination. Whole blood was incubated in duplicate wells with 1 µg/ml, 10 µg/ml and 20 µg/ml of (a) Recombinant FliC. (b) Recombinant V456. Recombinant proteins were incubated with 10 µg/ml PMB for 1 hr to remove LPS prior to their addition to whole blood. ConA was used as a non-specific T-cell activator to check the cell viability. Values of sheep used as controls (C005, C006, C015, C017, C022, C026) and, those used for vaccination (V016, V019, V020, V021, V024, V025) are shown. Each value represents the corrected absorbance at 450 nm. For each antigen concentration, PMB and ConA, corrected absorbance at 450 nm was the average absorbance of duplicate wells minus that of PBS for each sheep. See Appendix 7 for raw data.

(a)



(b)

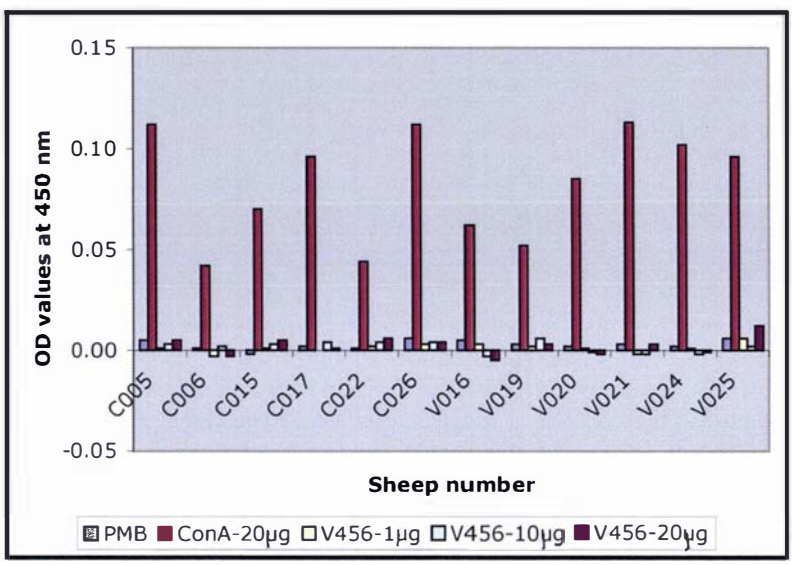
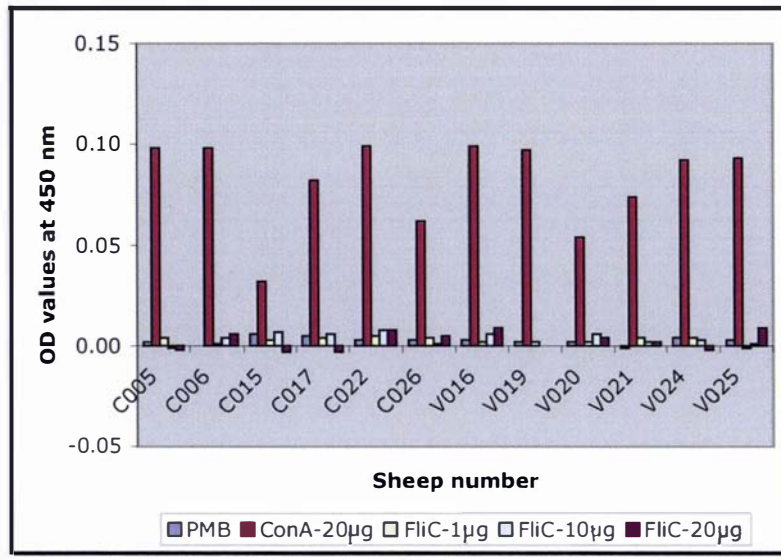


Figure 4.29. IFN-γ production in sheep vaccinated with Salvexin™ (Trial 2004): 3-weeks post-booster vaccination. Whole blood was incubated in duplicate wells with 1 µg/ml, 10 µg/ml and 20 µg/ml of (a) Recombinant FliC. (b) Recombinant V456. Recombinant proteins were incubated with 10 µg/ml PMB for 1 hr to remove LPS prior to their addition to whole blood. ConA was used as a non-specific T-cell activator to check the cell viability. Values of sheep used as controls (C005, C006, C015, C017, C022, C026) and, those used for vaccination (V016, V019, V020, V021, V024, V025) are shown. Each value represents the corrected absorbance at 450 nm. For each antigen concentration, PMB and ConA, corrected absorbance at 450 nm was the average absorbance of duplicate wells minus that of PBS for each sheep. See Appendix 8 for raw data.

(a)



(b)

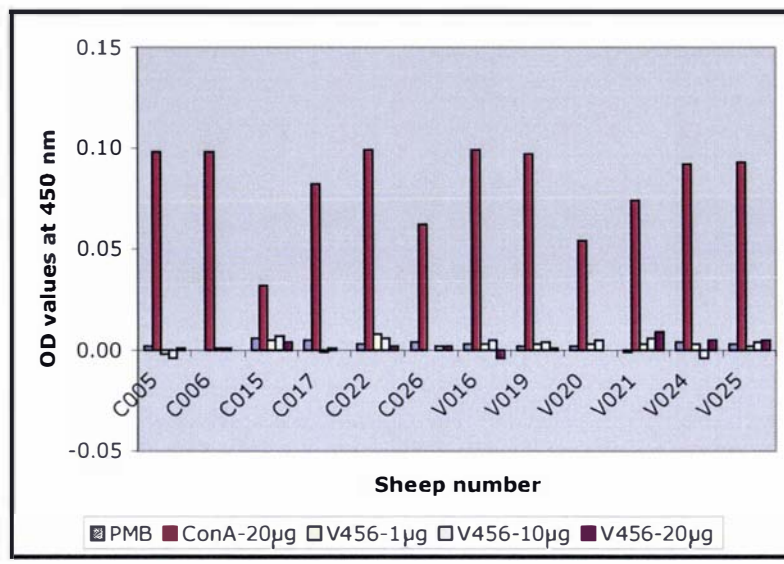
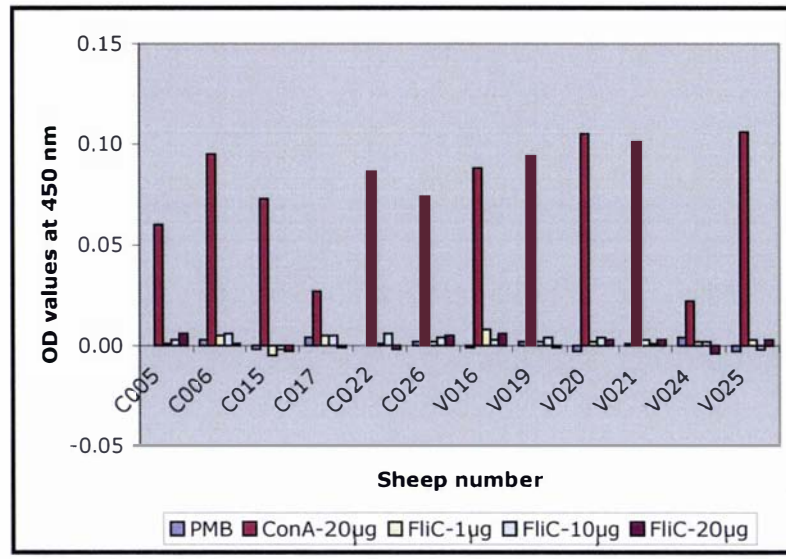


Figure 4.30. IFN-γ production in sheep vaccinated with Salvexin™ (Trial 2004): 2-months post-booster vaccination. Whole blood was incubated in duplicate wells with 1 µg/ml, 10 µg/ml and 20 µg/ml of (a) Recombinant FliC. (b) Recombinant V456. Recombinant proteins were incubated with 10 µg/ml PMB for 1 hr to remove LPS prior to their addition to whole blood. ConA was used as a non-specific T-cell activator to check the cell viability. Values of sheep used as controls (C005, C006, C015, C017, C022, C026) and, those used for vaccination (V016, V019, V020, V021, V024, V025) are shown. Each value represents the corrected absorbance at 450 nm. For each antigen concentration, PMB and ConA, corrected absorbance at 450 nm was the average absorbance of duplicate wells minus that of PBS for each sheep. See Appendix 9 for raw data.

(a)



(b)

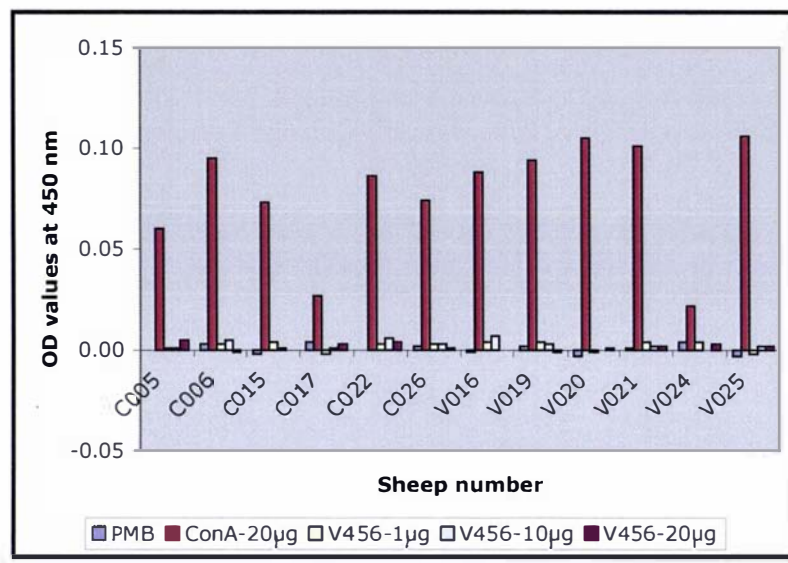


Figure 4.31. IFN-γ production in sheep vaccinated with Salvexin™ (Trial 2004): 3-months post-booster vaccination. Whole blood was incubated in duplicate wells with 1 µg/ml, 10 µg/ml and 20 µg/ml of (a) Recombinant FliC. (b) Recombinant V456. Recombinant proteins were incubated with 10 µg/ml PMB for 1 hr to remove LPS prior to their addition to whole blood. ConA was used as a non-specific T-cell activator to check the cell viability. Values of sheep used as controls (C005, C006, C015, C017, C022, C026) and, those used for vaccination (V016, V019, V020, V021, V024, V025) are shown. Each value represents the corrected absorbance at 450 nm. For each antigen concentration, PMB and ConA, corrected absorbance at 450 nm was the average absorbance of duplicate wells minus that of PBS for each sheep. See Appendix 10 for raw data.

4.5 Discussion

The complete *fliC* gene of *S.* Brandenburg was sequenced and compared with other *Salmonella fliC* genes deposited in the GenBank. This study showed that the *fliC* gene of *S.* Brandenburg is 1503-bp long encoding a 500-residue FliC protein. Alignment of amino acid sequences of FliC belonging to ten different *Salmonella* serovars including *S.* Brandenburg showed highly conserved regions I, II, VIII, intermediate variable regions III, VII, and highly variable regions IV, V, VI.

After analysing the amino acid sequence of *S.* Brandenburg FliC, the complete ORF, V456 (regions IV, V, VI), and V4 were expressed as separate histidine-tagged proteins to assess their immunogenic properties. Serum IgG responses of sheep against these 3 recombinant proteins were assessed by Western blots. Sera collected from sheep immunized with Salvexin™, and Salvexin™+B were positive against FliC protein while only the latter group was positive against V456 and V4. Therefore, responses against FliC of the Salvexin™-vaccinated sheep indicate that they are due to IgG antibodies directed towards the common epitopes located in N- and C-terminii of *S.* Typhimurium, *S.* Hindmarsh and *S.* Bovismorbificans FliC. Serotype-specific FliC epitopes are present in the variable region that is exposed on the outside of the flagella filament (Salazar-Gonzalez & McSorley, 2005). While sera from sheep vaccinated with Salvexin™+B reacted with V456 protein due to the presence of serotype-specific epitopes in V456, those of Salvexin™-vaccinated sheep did not react due to the absence of these epitopes in *S.* Typhimurium, *S.* Hindmarsh and *S.* Bovismorbificans. Serotype-specific FliC epitopes of these 3 serovars are i, r and r respectively. This shows that serotype-specific epitopes of *Salmonella* FliC are present in the amino acid sequence encompassing regions IV, V and VI. This is further evidenced by the reactivity between recombinant proteins, and of sera from rabbits immunized with *S.* Brandenburg, *S.* Typhimurium and *S.* Hindmarsh. While all rabbit sera reacted with FliC due to common epitopes, only those of *S.* Brandenburg-vaccinated rabbits reacted with V456 due to serotype-specific epitopes.

In response to immunization, all sera from Salvexin™+B-vaccinated sheep showed IgG antibodies to FliC, V456 and V4. Western blots had different intensities varying from

faint to strong bands. Sera collected 3-weeks post-booster showed the highest intensity due to peak IgG response. This agrees with the usual IgG response following vaccination where they reach high levels 2-3 weeks post-booster. While sera collected from 9 out of 80 sheep with no previous history of *S.* Brandenburg infection showed reactivity to FliC, no sera reacted with V456 and V4. Positive Western blots against FliC of these 9 sheep may be due to exposure of these farm-raised sheep to other salmonellae. All 81 sera from sheep naturally infected with *S.* Brandenburg reacted with FliC, V456 and V4 indicating the presence of both common- and *S.* Brandenburg-specific IgG antibodies. These samples showed different intensities in the blots indicating the presence of varying amounts of IgG antibodies in infected sheep. The flagellum IgG response in each sheep may be related to the dose of *S.* Brandenburg that they are exposed to. Alternatively, it may be due to natural variation in the magnitude of the responses to these antigens. These circulating IgG antibodies may play an important role in protection against *S.* Brandenburg infection.

This study showed that there was no IFN-γ production in Salvexin^{TM}+B-vaccinated sheep in response to stimulation with recombinant FliC and V456 proteins. IFN-γ is an important cytokine in the development of CMI responses against intracellular pathogens such as *Salmonella, Mycobacterium tuberculosis*, *Listeria monocytogenes* and *Leishmania* because of its ability to activate microbicidal activity of host macrophages. Antigen-specific activation of T-cells is considered as a mechanism in the production of IFN-γ during infection. The sources of IFN-γ include Th1 CD4+ T-cells, CD8+ T-cells and NK cells (Benbernou & Nauciel, 1994; Muotiala & Mäkelä, 1990; Ramarathinam *et al.*, 1991). In fact, flagellin has been described to activate DCs to release cytokines favouring a Th1-response (Salmon *et al.*, 2005). It has been shown that flagellin is a major antigen for activated CD4+ T-cells in mice orally infected with attenuated *S.* Typhimurium *aroA* strains. These mice exhibited CD4+ T-cell-dependent IFN-γ production, a step required for mounting protective immunity against virulent *Salmonella* strains (Sbrogio-Almeida *et al.*, 2004). Furthermore, it has been shown that splenic CD4+ T-cells from mice orally immunized with an attenuated *S.* Typhimurium *aroA* mutant secreted IFN-γ upon stimulation with recombinant proteins containing different FliC epitopes (residues 80-94, 339-350, 428-442 and 455-469) (Bergman *et al.*, 2005). Stimulation with flagella resulted in the secretion of IFN-γ from splenocytes

of chickens following vaccination with killed *S.* Enteriditis bacterin (Okamura *et al.*, 2004) and those of mice following vaccination with live attenuated *aroA S.* Dublin strains (Sbrogio-Almeida *et al.*, 2004). However, peripheral blood mononuclear cells (PBMC) of sheep vaccinated with live attenuated *aroA S.* Typhimurium strain S25/1 failed to produce IFN-γ upon re-stimulation with *Salmonella* antigens. In spite of this, these sheep showed only mild clinical signs upon challenge with the virulent *S.* Typhimurium wild-type strain S25/1. The same study showed that vaccinated mice showed IFN-γ production in response to *Salmonella* antigens, and protection upon challenge (Brennan *et al.*, 1994). These data indicate the presence of contrasting kinetics of IFN-γ production among different host species. Some mycobacterial proteins including heparin-binding haemagglutinin (HBHA) do not induce IFN-γ secretion when expressed in *E. coli* (Temmerman *et al.*, 2004). Therefore, another reason for the absence of IFN-γ responses shown in this study may be due to the inability to recognize the recombinant flagellin proteins.

While sera from sheep vaccinated with Salvexin^{TM}+B reacted with V4, those from Salvexin^{TM}-vaccinated sheep did not react with V4 fusion protein. Furthermore, sera from rabbits immunized with *S.* Brandenburg reacted with V4, those from *S.* Typhimurium and *S.* Hindmarsh immunized rabbits did not react with V4 fusion protein. Therefore, V4 appears to contain epitopes specific for *S.* Brandenburg.

Sera from 81 naturally infected sheep showed reactivity with recombinant V4 protein in the Western blots. Chapter 5 describes a study carried out to develop an ELISA using V4 as an antigen and assess the suitability of this assay for the serodiagnosis of *S.* Brandenburg infection in sheep.

Chapter 5 Development of an indirect enzyme-linked immunosorbent assay for the detection of serum IgG antibodies against region IV of phase-1 flagellin of *S.* Brandenburg in sheep

5.1 Abstract

Western blot results obtained from the study described in Chapter 4 showed that the recombinant region IV (V4) of phase-1 flagellin (FliC) is both immunogenic, and appears to contain epitopes specific for *S.* Brandenburg. Therefore, this recombinant protein was evaluated for its suitability as an antigen in an indirect ELISA for the detection of IgG antibodies in sheep sera. A cut-off value of 0.1 was calculated using 80 sera collected from sheep with no previous history of *S.* Brandenburg. In this Chapter, the results of ELISA with field sera collected from 81 sheep naturally infected with *S.* Brandenburg are discussed. The assay was able to detect antibodies belonging to IgG class with a sensitivity of 93.8% and a specificity of 100%. Thus, indirect ELISA using recombinant region IV (V4) of phase-1 flagellin may be a suitable screening tool for serological monitoring of sheep flocks infected with *S.* Brandenburg.

5.2 Introduction

Enzyme-linked immunosorbent assay (ELISA) is a rapid and a sensitive serological tool for the detection of infections caused by several pathogens in humans and animals. However, it is only recently that ELISAs have been used to detect *Salmonella* infections (Barrow, 2000). Some ELISAs are now in routine use, and a number are available commercially for serodiagnosis of salmonellosis. Antigens used in ELISAs include flagella (Dalby *et al.*, 2005; Gast *et al.*, 2002; Veling *et al.*, 2000), fimbriae (Hoorfar *et al.*, 1996; Rajashekara *et al.*, 1998), LPS (House *et al.*, 2005; Veling *et al.*, 2000), OMPs (Kim *et al.*, 1991), whole cell (Mizumoto *et al.*, 2004) and capsular (Ferry *et al.*, 2004) antigens.

Most of the serological methods use antigens made from crude extracts of *Salmonella* and require specific growth conditions for antigen preparation with difficulties in standardization. The antigenic components are difficult to reproduce from one bacterial culture preparation to the other. Thus, it is advantageous to develop a serological test with a more specific and a purified antigen preparation for the detection of antibodies against *S.* Brandenburg infection. Currently, recombinant proteins are used for the detection of serum antibodies against many bacterial diseases. These provide simple, rapid, highly sensitive, and inexpensive tools for antibody detection (Kwang & Littledike, 1995). Flagellae are immunodominant surface structures that elicit an antibody response upon infection making them suitable antigens for the serological detection of *Salmonella* infections in livestock (de Vries *et al.*, 1998).

ELISA tests using recombinant proteins containing phase-1 flagellin fragments for the detection of antibodies in poultry against *S.* Enteriditis have been reported (Mizumoto *et al.*, 2004; Yap *et al.*, 2001). To date, ELISAs based on recombinant flagellar antigens for the serodiagnosis of *S.* Brandenburg infection in sheep are not available. Chapter 4 described the expression, purification and reactivity of region IV fusion protein (V4) in Western blots. Sera of 81 sheep infected with *S.* Brandenburg reacted with the fusion protein confirming its immunogenicity. Furthermore, sera of sheep used in the vaccination trials described in Chapter 4 showed that region IV appears to be specific for *S.* Brandenburg that harbours l, v epitopes of FliC. The reactivity of

region IV with sera from sheep vaccinated with Salvexin^{TM}+B, and non-reactivity of it with those vaccinated with Salvexin^{TM} suggested that this may be a potential serodiagnostic antigen to differentiate Brandenburg infections from common sheep *Salmonella* serotypes Typhimurium, Bovismorbificans and Hindmarsh. Therefore, suitability of this recombinant protein as a diagnostic antigen in an indirect ELISA for the detection of serum IgG antibodies in sheep naturally infected with *S.* Brandenburg was investigated in this study.

5.3 Materials and methods

5.3.1 Preparation of recombinant region IV of *S.* Brandenburg FliC protein

The histidine-tagged recombinant region IV of *S.* Brandenburg FliC (V4) described in Chapter 4 (Sections 4.3.4 and 4.3.5) was used as the coating antigen in this study.

5.3.2 Serum samples

The following sera were used for ELISA.

Group 1: Sheep not exposed to *S.* Brandenburg (Section 2.12.1)

Sera were collected from 80 ewes in farms without a history of *S.* Brandenburg. These samples were used to determine the cut-off value in the ELISA.

Group 2: Sheep naturally infected with *S.* Brandenburg (Section 2.12.1)

Sera collected approximately 3-4 weeks post-abortions from 81 *S.* Brandenburg-confirmed ewes were used in the ELISA.

Group 3: Sheep vaccinated with Salvexin^{TM}+B (Trial 2004) (Section 2.11)

Sera collected 1-month post-priming, and 3-weeks, 2-months and 3-months post-booster from 6 Salvexin^{TM}+B-vaccinated sheep were used.

Group 4: Sheep vaccinated with Salvexin^{TM} and Salvexin^{TM}+B (Trial 1999) (Section 2.12.2). Sera collected 2-weeks post-booster from 10 ewes each group that received Salvexin^{TM} and Salvexin^{TM}+B vaccines were used. Sera from Salvexin^{TM}-vaccinated group were used to evaluate the specificity of ELISA.

Group 5: Rabbits immunized with *S.* Brandenburg, *S.* Hindmarsh and *S.* Typhimurium (Section 2.13). Sera collected 2- and 4-weeks post-booster from 2 rabbits each group were used. Sera from *S.* Hindmarsh- and *S.* Typhimurium-vaccinated groups were used to evaluate the specificity of ELISA.

5.3.3 Indirect ELISA

An indirect ELISA was carried out to detect IgG antibodies against region IV of FliC protein. Optimal concentrations of reagents were determined by checkerboard titrations (Crowther, 1995). Briefly, optimum concentration of the antigen was determined by titration of antigen with serial two-fold dilutions of a positive serum sample. The optimal dilution of sera was determined by titrating the previously optimised antigen with two-fold dilutions of positive and negative sera for *S.* Brandenburg antibodies. Purified recombinant protein was diluted in 0.1 M bicarbonate buffer pH 9.6 (0.06 M $NaHCO_3$, 0.04 M Na_2CO_3) to obtain a solution of 2.5 μg/ml. Ninety-six-well MaxiSorp™ microtitre plates (Cat. No. 439454, Nalgene Nunc International) were coated with 100 μl containing approximately 250 ng of antigen per well overnight at 4°C or 2 h at 37°C. The plates were washed three times with PBS pH 7.4 containing 0.05% (v/v) Tween 20, and once with PBS to remove unbound antigen. The non-specific sites were blocked by incubation with 200 μl of PBS containing 5% (w/v) skim milk per well for 1 h at 37°C or overnight at 4°C, and washed. One hundred microlitres of sera diluted (1:100) in PBS containing 5% skim milk was added to each well in duplicate, and incubated for 1 h at 37°C. After another cycle of washing, 100 μl of 1:10,000 diluted alkaline phosphatase-labelled (AP-labelled) donkey anti-sheep (Cat. No. A5187, Sigma) or goat anti-rabbit IgG (Cat. No. A3687, Sigma) antibody was added to each well, and incubated for 1 h at 37°C. After a final series of washes, 100 μl of substrate, prepared by dissolving two-5 mg *para*-nitrophenyl phosphate (pNPP; Cat. No. N9389, Sigma) tablets in 10ml of 1 M diethanolamine buffer pH 9.8 containing 0.5 mM $MgCl_2$ was added to each well and incubated for 30 min in the dark at room temperature. The resultant yellow colour reaction was stopped by the addition of 100 μl of 2 M Na_2CO_3 per well, and the optical density values at 405 nm (OD_{405}) were read using an ELISA plate reader. Negative control serum, positive control serum and dilution buffer were used on each plate as controls. The dilution buffer control was used to calculate the background OD. Each sample and control was assayed in duplicate. The OD value of each sample was calculated by subtracting the background OD from the mean OD of duplicates. The cut-off value of ELISA was determined as the mean OD of the 80 negative sera plus 3 standard deviations (SD). In addition to a single dilution, a separate ELISA was carried

out using 2-fold serum dilutions starting from 1:50 to determine the titre. The titre was expressed as the reciprocal of the highest serum dilution that produced an OD of ≥ cut-off value. Statistical differences were compared by use of the Student's *t*-test, and were declared significant at *p* values <0.05 throughout the study.

5.4 Results

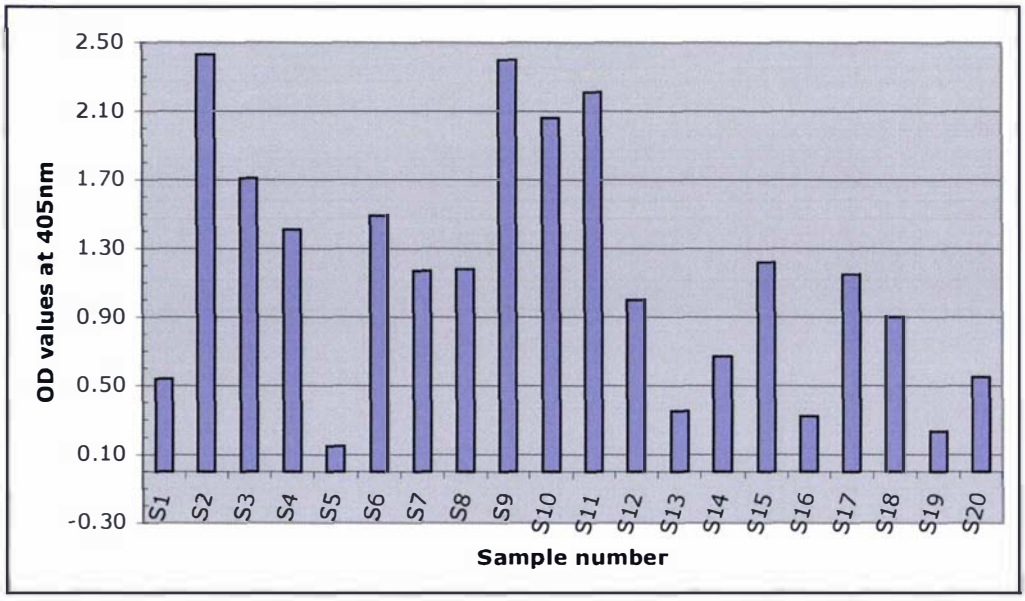
5.4.1 ELISA cut-off value

Immunogenicity of the purified region IV recombinant protein was determined by Western blotting prior to evaluating its suitability as a diagnostic antigen in an indirect ELISA format in this study. These results were shown in Section 4.4.4. During optimisation, checkerboard titrations indicated 250-ng of coating antigen, and a 1:100 dilution of sheep sera to be used in the ELISA. The mean background absorbance of the ELISA was 0.08. The OD value of each sample was calculated by subtracting the background OD from the mean OD of duplicates. All 80 sera collected from sheep not exposed to *S.* Brandenburg had OD values ranging from 0.002-0.071 (Appendix 11). This population had a mean of 0.033 and a SD of 0.021. The calculated cut-off value (mean plus 3 SDs of the absorbance values) from these 80 control sera was 0.096, and was set at 0.1.

5.4.2 Naturally infected sheep

Sera collected from 81 sheep naturally infected with *S.* Brandenburg (Section 2.12.1) were used to evaluate the sensitivity of ELISA. Sera with an OD value higher than 0.1 were scored as positive. Of the 81 sera, 76 had an OD value of more than 0.1 and were scored positive giving the test a sensitivity of 93.8%. For these 81 samples, OD values ranged from 0.06 to 2.43 (Figures 5.1 and 5.2, Appendix 12) with a mean of 0.71. Most had an OD in the range of 0.10-0.50 (Table 5.1). Five of the samples that had OD values less than 0.1 were scored negative. Figure 5.3 shows the distribution of OD values in these 81 sheep. The ELISA-negative 5 samples (sample numbers 31, 32, 43, 72 and 73) were Western blot-positive. The 76 ELISA-positive samples had a titre ranging from 100 to 6,400 (Figure 5.4). The 5 negative samples had a titre of 50.

(a)



(b)

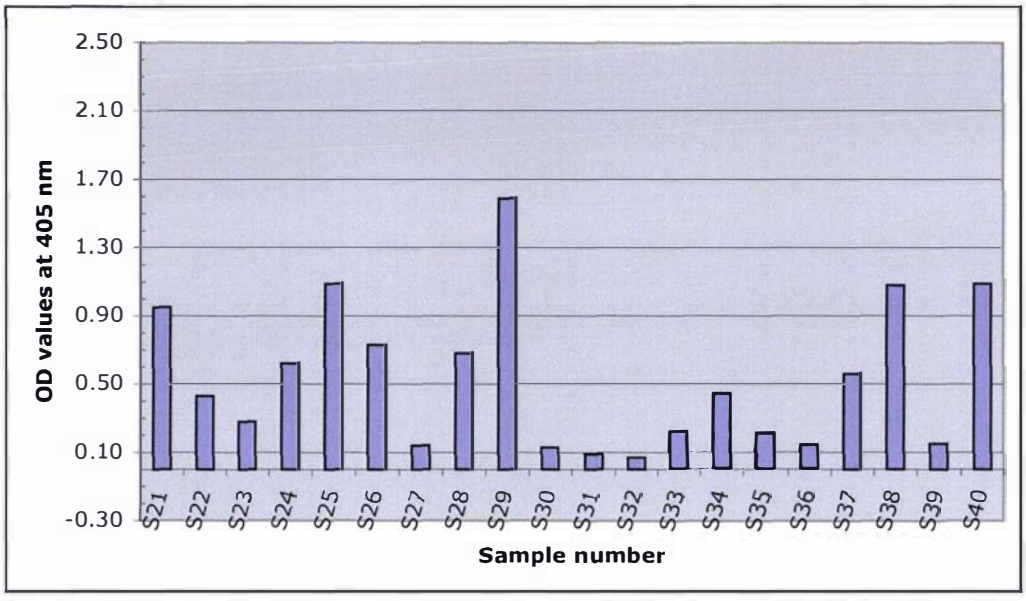
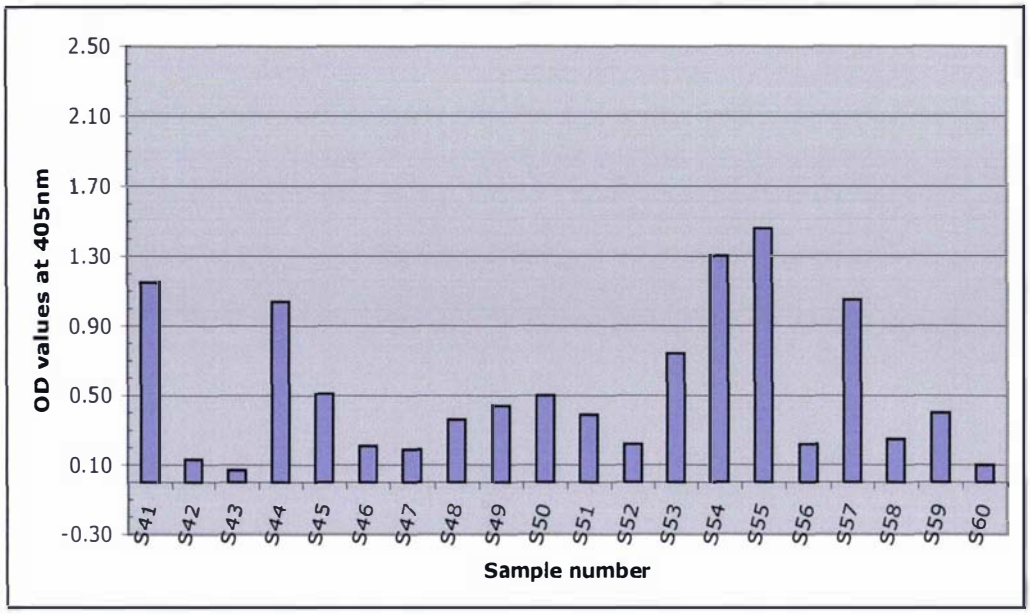


Figure 5.1. Detection of serum IgG antibodies against recombinant V4 in naturally infected sheep (sample numbers S1 to S40) by indirect ELISA. OD values of 40 sera collected 3-4 weeks post-abortions from *S.* Brandenburg-infected sheep. (a) Sample numbers S1 to S20. (b) Sample numbers S21 to S40. Each OD value represents the mean value of two determinations minus background OD at a serum dilution of 1:100. Data are shown in Appendix 12. Cut-off value was set at 0.1.

(a)



(b)

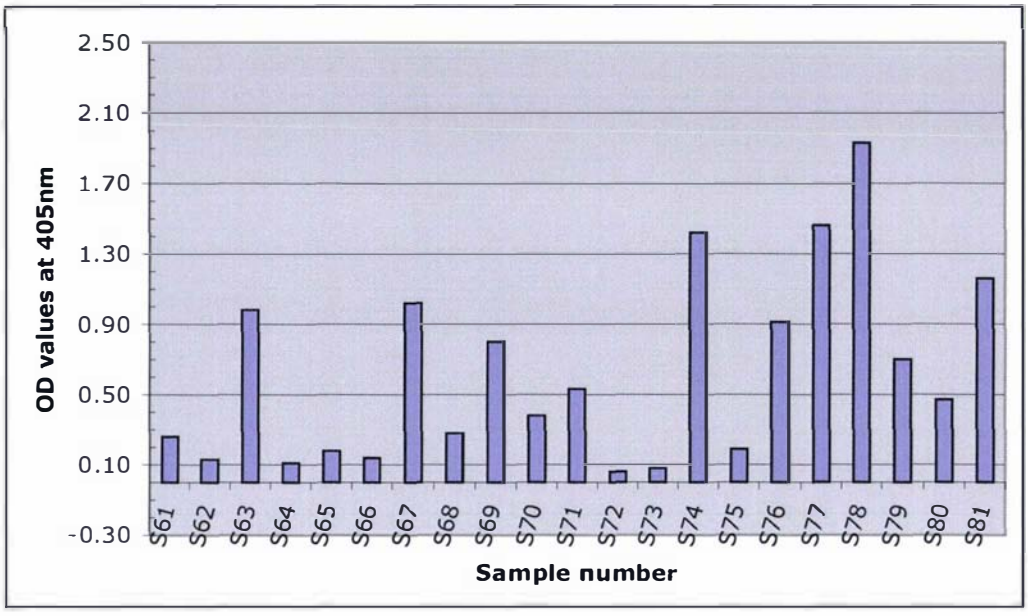


Figure 5.2. Detection of serum IgG antibodies against recombinant V4 in naturally infected sheep (sample numbers S41 to S81) by indirect ELISA. OD values of 41 sera collected 3-4 weeks post-abortions from *S.* Brandenburg-infected sheep. (a) Sample numbers S41 to S60. (b) Sample numbers S61 to S81. Each OD value represents the mean value of two determinations minus background OD at a serum dilution of 1:100. Data are shown in Appendix 12. Cut-off value was set at 0.1.

Table 5.1. Detection of serum IgG antibody response of naturally infected sheep against recombinant V4 protein by indirect ELISA. Sera collected 3-4 weeks post-abortions from 81 sheep naturally infected with *S.* Brandenburg were used at a dilution of 1:100. All 81 samples were Western blot-positive.

OD_{405} range	No. of samples
<0.10	5
0.10 – 0.50	34
0.51 – 0.90	13
0.91 – 1.30	17
1.31 – 1.70	6
1.71 – 2.10	3
2.11 – 2.50	3
Total	81

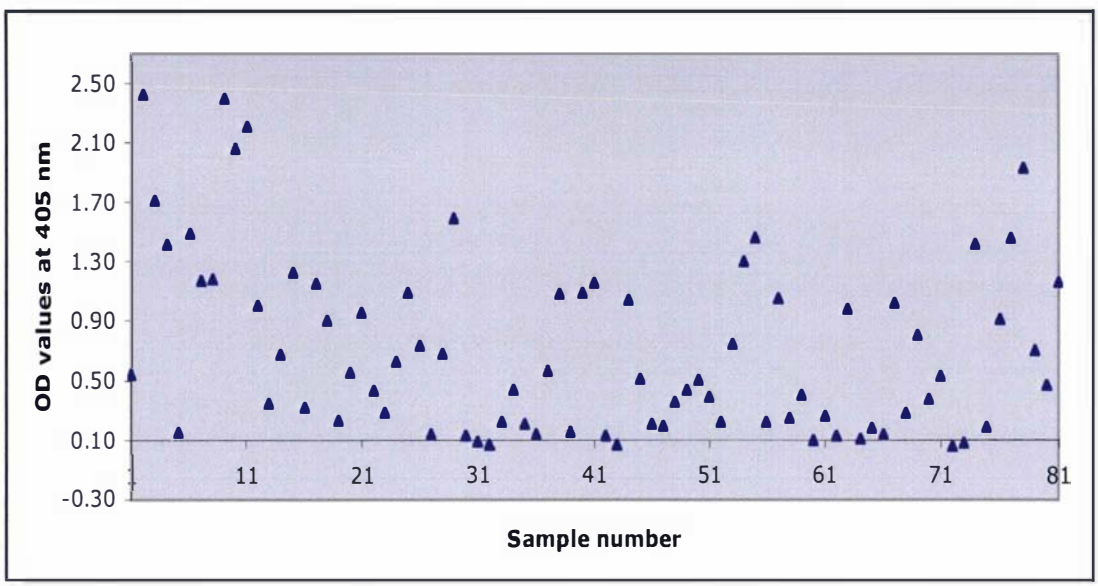


Figure 5.3. A scatter plot showing the distribution of OD values in 81 sheep naturally infected with *S.* Brandenburg. 1 to 11, samples S1 to S11; 11-21, samples S11 to S21; 21 to 31, samples S21 to S31; 31 to 41, samples S31 to S41; 41 to 51, samples S41 to S51; 51-61, samples S51 to S61; 61 to 71, samples S61 to S71; 71 to 81, samples S71 to S81. Data are shown in Appendix 12. Cut-off value was set at 0.1.

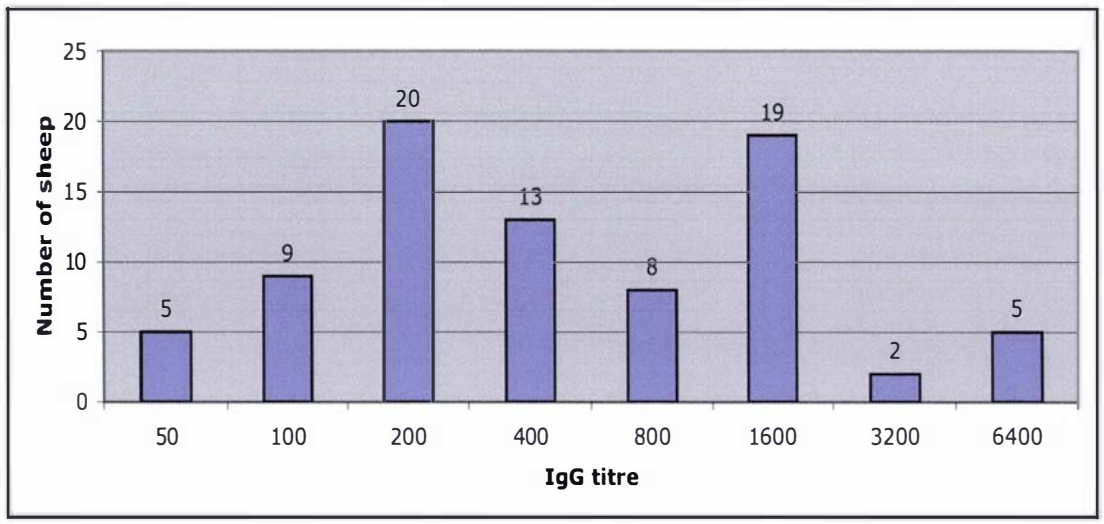
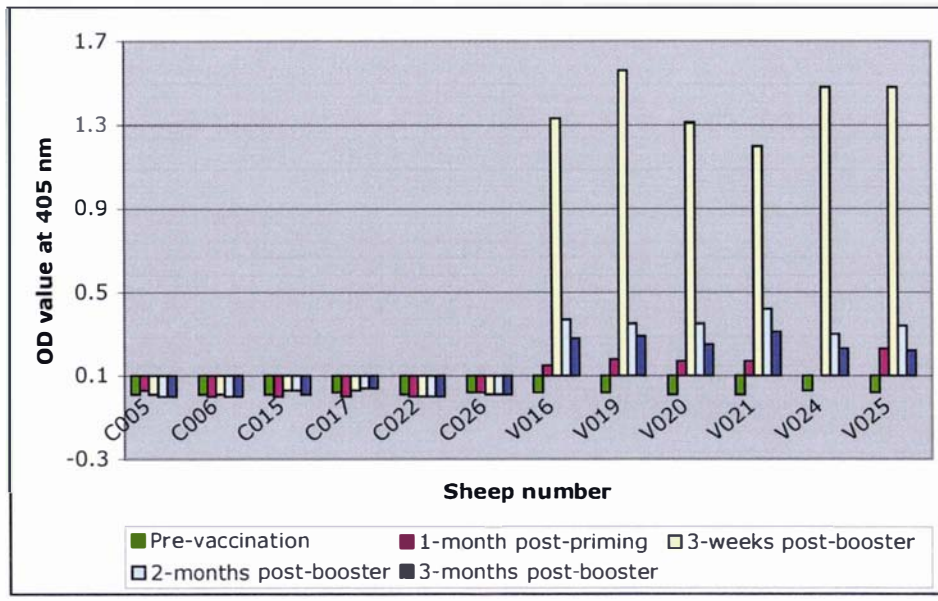


Figure 5.4. Titration of serum IgG antibodies against recombinant V4 in 81 naturally infected sheep by indirect ELISA. Data are shown in Appendix 12.

5.4.3 Sheep vaccinated with Salvexin™+B (Trial 2004)

Sera collected from 6 control and 6 Salvexin™+B-vaccinated sheep (Section 2.11) were used in the ELISA. All the sera collected pre-vaccination, and those of control sheep throughout the experiment had OD values less than 0.1, and were scored negative. All the sera collected from vaccinated-sheep 1-month post-priming, 3-weeks, 2-months and 3-months post-booster had OD values more than 0.1 (Figure 5.5, Appendix 13). The values peaked 3-weeks post-booster, and were in the OD range of 1.20-1.59. While the sera collected 2-months post-booster showed an OD range of 0.30-0.42, those collected 3-months post-booster had an OD range of 0.22-0.31. The vaccinated group showed a mean ELISA titre of 2100 with sera collected 3-weeks post-booster (Figure 5.6). Data were compared between the control and the vaccine groups using the Student's *t*-test. The results showed a significant difference ($p<0.001$) between sera from unvaccinated and Salvexin™+B-vaccinated sheep.

(a)



(b)

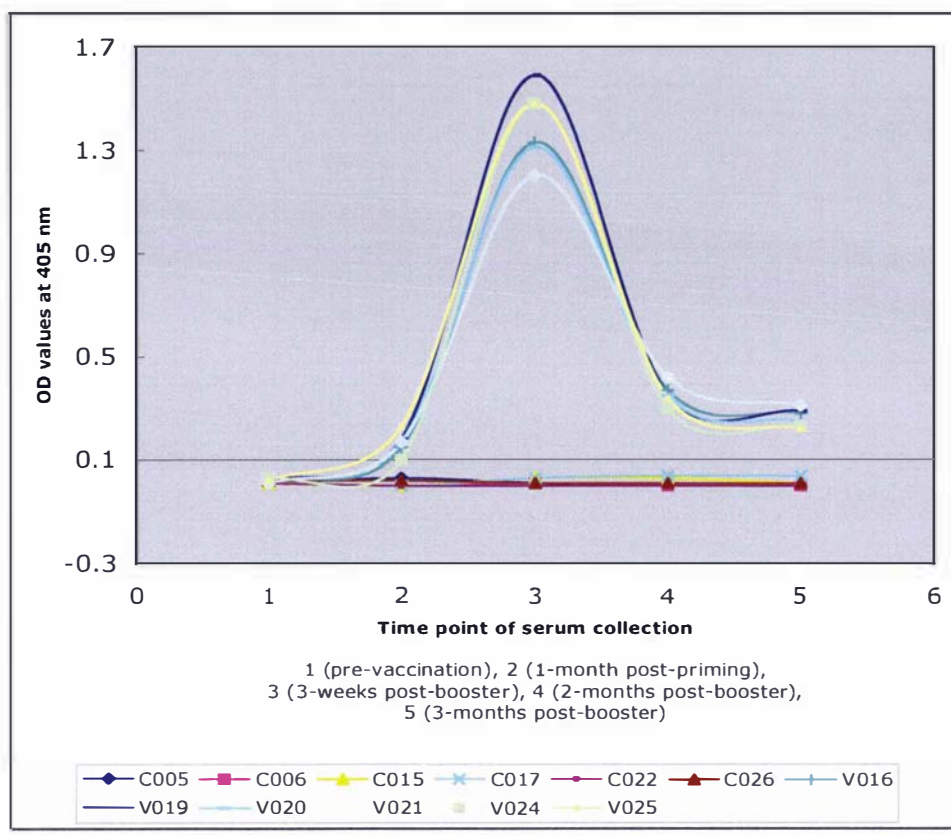


Figure 5.5. Detection of serum IgG antibody response against recombinant V4 in Salvexin^{TM}+B-vaccinated sheep (Trial 2004) by indirect ELISA. (a) and (b) A serum dilution of 1:100 and AP-labelled anti-sheep IgG dilution of 1:10,000 were used. Each OD value represents the mean value of two determinations minus background OD. Sera were from unvaccinated (animal numbers C005, C006, C015, C017, C022, C026), and vaccinated sheep (animal numbers V016, V019, V020, V021, V024, V025). Data are shown in Appendix 13. Cut-off value was set at 0.1.

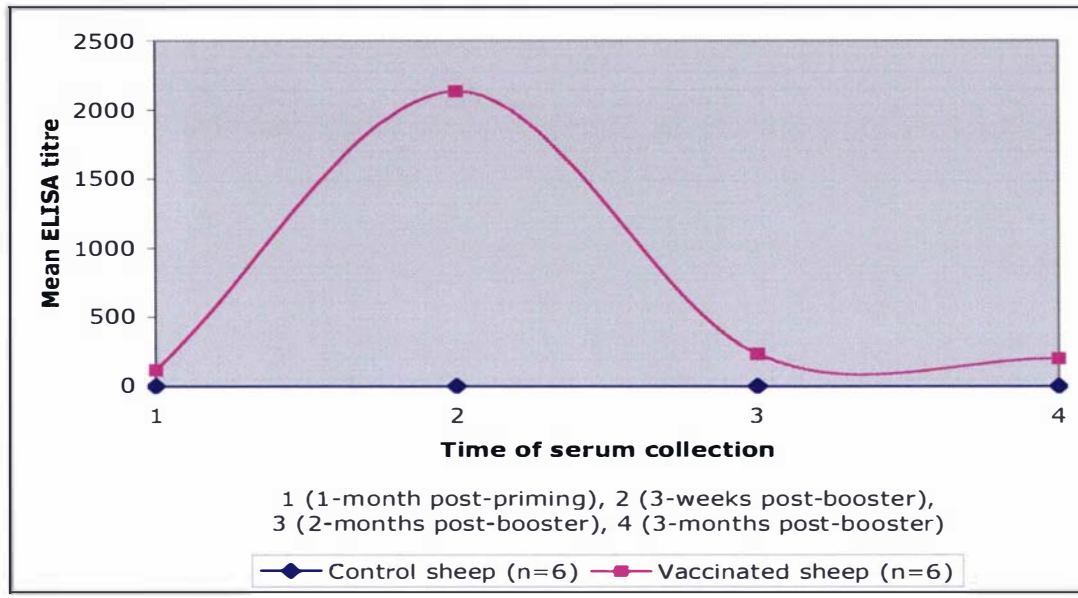


Figure 5.6. Titration of serum IgG antibody response against recombinant V4 in Salvexin^{TM}+B-vaccinated sheep (Trial 2004) by indirect ELISA. Two-fold serum dilutions and a 1:10,000 dilution of AP-labelled anti-sheep IgG were used. Each data point represents the mean IgG titre at each time point of serum collection. Data are shown in Appendix 13.

5.4.4 Sheep vaccinated with Salvexin™ and Salvexin™+B (Trial 1999)

Sera collected 2-weeks post-booster from ewes that received Salvexin™ and Salvexin™+B vaccines (Section 2.12.2) were used in the ELISA. Control sheep and sheep vaccinated with Salvexin™ had ELISA OD values less than 0.1, whereas sheep vaccinated with Salvexin™+B had values more than 0.1. For the latter group, values ranged from 0.28 to 1.82 with a mean of 0.72 (Figure 5.7, Appendix 14).

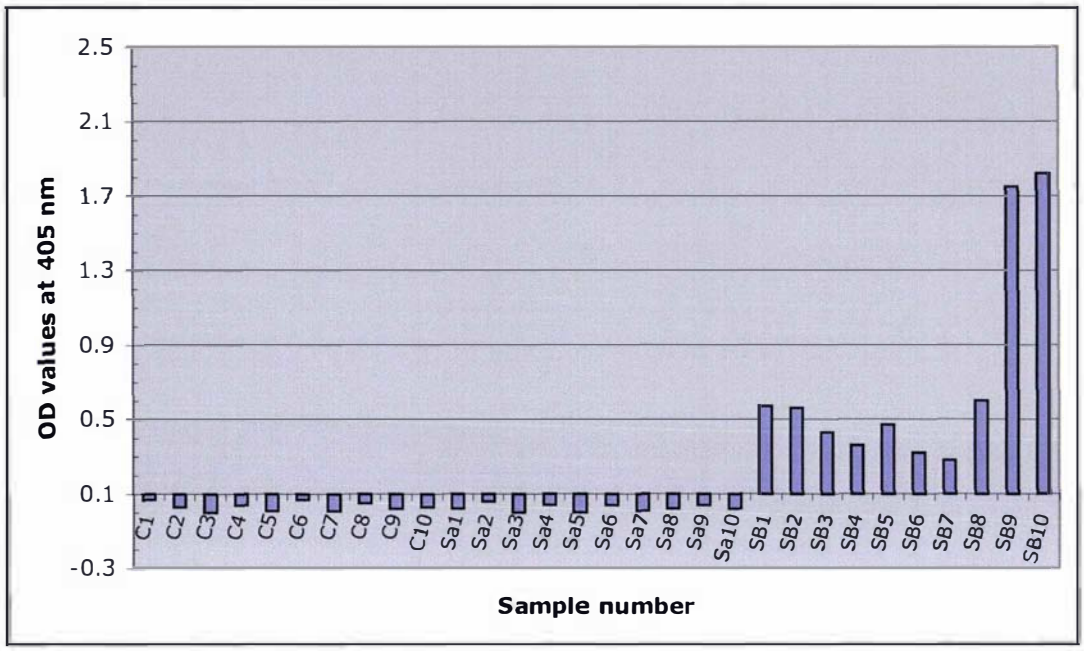


Figure 5.7. Detection of IgG antibody response against recombinant V4 protein in sera from sheep vaccination trial to compare Salvexin™ with Salvexin™+B (Trial 1999) by indirect ELISA. Sera collected 2-weeks post-booster from ewes received Salvexin™ (sample numbers Sa1, Sa2, Sa3, Sa4, Sa5, Sa6, Sa7, Sa8, Sa9 and Sa10), Salvexin™+B (sample numbers SB1, SB2, SB3, SB4, SB5, SB6, SB7, SB8, SB9 and SB10) and from ewes used as controls (C1, C2, C3, C4, C5, C6, C7, C8, C9 and C10) were used. A serum dilution of 1:100 and AP-labelled anti-sheep IgG dilution of 1:10,000 were used. Each OD value represents the mean value of two determinations minus background OD. Data are shown in Appendix 14. Cut-off value was set at 0.1. A significant ($p<0.001$) [Student's t-test] difference was found between the Salvexin™- and Salvexin™+B-vaccinated groups.

5.4.5 Rabbits vaccinated with *Salmonella* serotypes Typhimurium, Hindmarsh and Brandenburg

Sera collected 2- and 4-weeks post-booster from rabbits vaccinated with *S.* Brandenburg were ELISA-positive. Rabbit number 524 had OD values higher than rabbit number 515. Control group and the groups vaccinated with *S.* Hindmarsh and *S.* Typhimurium were ELISA-negative (Figure 5.8, Appendix 15).

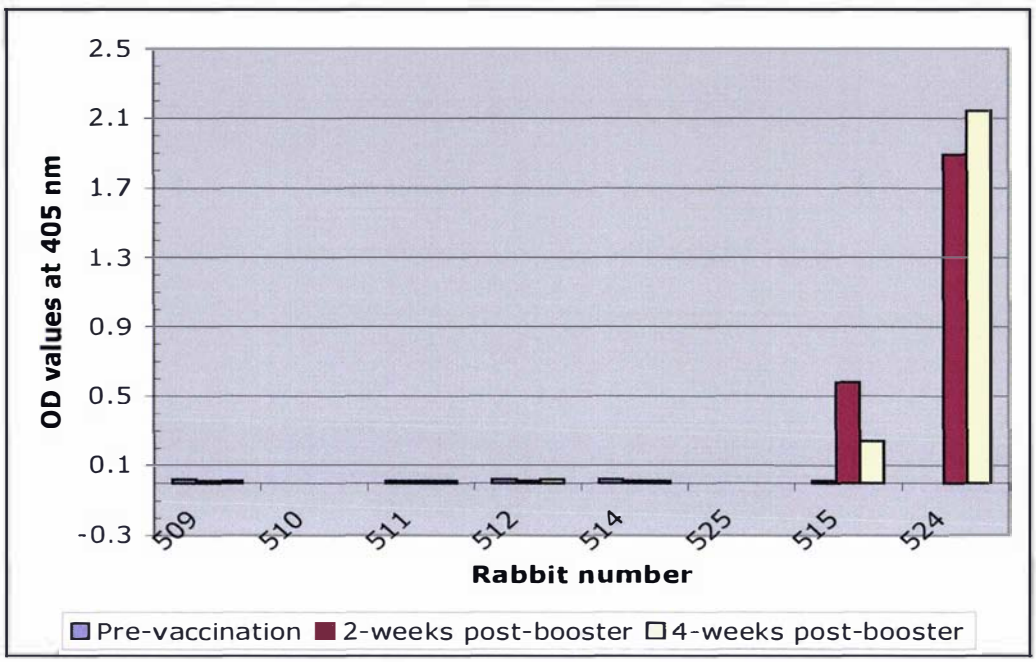


Figure 5.8. Detection of serum IgG antibodies against recombinant V4 protein in rabbits by indirect ELISA. Each group of rabbits separately received heat-inactivated *Salmonella* serotypes Hindmarsh (rabbit numbers 511 and 512), Typhimurium (rabbit numbers 514 and 525) and Brandenburg (rabbit numbers 515 and 524) suspensions on days 0 and 14. One group served as a negative control (rabbit numbers 509 and 510). A serum dilution of 1:100 and AP-labelled anti-rabbit IgG dilution of 1:10,000 were used. Pre-vaccination sera and sera collected 2- and 4-weeks post-booster were used. Each OD value represents the mean value of two determinations minus background OD. Data are shown in Appendix 15. A significant ($p<0.001$) [Student's *t*-test] difference was found between the *S.* Brandenburg- vaccinated group and other groups.

A summary of Western blot and ELISA results is shown in Table 5.2.

Table 5.2. Summary of serum IgG antibody detection against recombinant V4 protein in sheep and rabbits by indirect ELISA and Western blot

Source of samples (Time of sample collection)	No. of sera tested	No. of sera positive in Western blot	No. of sera positive in ELISA	OD mean (range)
Sheep naturally infected with *S.* Brandenburg (3-4 weeks post-abortions)	81	81	76	0.71 (0.06-2.43)
Trial 2004 Sheep experimentally vaccinated with Salvexin™+B (3-weeks post-booster)	6	6	6	1.4 (1.20-1.59)
Trial 1999 Sheep experimentally vaccinated with Salvexin™	10	0	0	0.03 (0-0.06)
Salvexin™+B (2-weeks post-booster)	10	10	10	0.72 (0.28-1.82)
Rabbits experimentally immunized with *S.* Brandenburg	2	2	2	1.24 (0.58-1.89)
S. Typhimurium	2	0	0	0.01 (0.00-0.01)
S. Hindmarsh (2-weeks post-booster)	2	0	0	0.01 (0.01)

5.5 Discussion

A serological test is a valuable additional tool in the detection of *S.* Brandenburg in sheep. This is the first reported ELISA to detect seroconversion of *S.* Brandenburg-infected sheep using a recombinant *Salmonella*-flagellin antigen. The negative reactions of the serum samples from *S.* Brandenburg-free sheep, together with the positive reactions of those from *S.* Brandenburg-infected sheep indicate the potential usefulness of recombinant region IV protein in an ELISA for screening *S.* Brandenburg-infected sheep. Flagellin is the major structural protein of flagella and carries serotype-specific H-antigenic determinants that are located in the region IV of the central variable domain of the FliC protein. These antigens induce the production of serotype-specific antibodies in the infected host. On the other hand, antibodies against the conserved N- and C-terminal domains of flagellin yield cross-reactions between *Salmonella* serotypes in serological tests that are based on whole purified flagellum antigen. Some of the flagellar antigens arc composed of a single antigenic factor (b, c, d, i, r) while others are composed of multiple antigenic factors (l, v; l, w; e, h; e, n, x; e, n, z15; 1, 2). Flagellar antigens are grouped into complexes depending on the antigenic factors they share. Examples are the E-complex that contains antigenic factor e (e, h; e, n, x; e, n, z15) and the L-complex that contains antigenic factor l (l, v; l, w; l, z13, l, z28) (Popoff *et al.,* 2003). Phase-1 flagellin of *S.* Brandenburg belongs to the L complex since it carries l, v epitopes.

Specificity of ELISA was determined by using sera collected from sheep vaccinated with Salvexin™/Salvexin™+B, and those from rabbits vaccinated with inactivated *S.* Typhimurium/*S.* Hindmarsh/*S.* Brandenburg. Only the sera from sheep vaccinated with Salvexin™+B, and those from rabbits vaccinated with *S.* Brandenburg were positive in ELISA. These results indicate that the specificity of this assay is 100% and there are no cross-reactions between different *Salmonella* serotypes that express flagellins belonging to complexes other than L complex. *Salmonella* serotypes that carry phase –1 or phase–2 flagellins belonging to the L complex would react with varying intensities in this ELISA due to shared epitopes among them. Examples of serotypes that possess phase-1 (H1) or phase-2 (H2) flagellin belonging to L complex include Ayton (H1: l, w), Bredeney (H1: l, v), Give (H1: l, v), Glidji (H1: l, w), Gloucester

(H2: l, w), Kimberly (H1: l, v), Livingstone (H2: l, w), London (H1: l, v), Ohio (H2: l, w), Potsdam (H1: l, v), Victoria (H1: l, w) and Wien (H2: l, w) (McQuiston *et al.*, 2004; Wang *et al.*, 2002). These serotypes are not common sheep pathogens and are not shown to cause ovine abortions. However, as a precautionary matter, it is important to complement positive ELISA results with history and bacteriological findings. Furthermore, more studies with sera from sheep/other animals infected with other *Salmonella* serotypes and bacterial strains need to be performed to increase the confidence of specificity.

Salvexin™+B-vaccinated sheep showed significantly higher serum IgG levels following both primary and secondary immunizations compared with age-matched non-vaccinated controls. Serum IgG levels peaked 3-weeks post-booster, which diminished gradually, but still remained higher than the controls at 3 months when the experiment ended. Similar kinetics have been observed in animals immunized with inactivated *Salmonella* vaccines (Muir *et al.*, 1998). Therefore, vaccinated sheep are expected to mount a good IgG response upon infection with *S.* Brandenburg.

In Denmark, until the end of 1994, pigs were tested for salmonellosis exclusively by bacteriological methods. Since 1995, *Salmonella* testing is based on a combination of both bacteriological and serological methods (Skov *et al.*, 2002). Currently, in Denmark, screening pigs for monitoring and controlling salmonellosis is by the identification of antibodies in serum and muscle juice using an indirect ELISA with a mixture of LPS as coating antigens (Mousing *et al.*, 1997). This program has reduced the human cases of pork-related salmonellosis (Korsak *et al.*, 2006). The purpose of this program is to identify common Salmonellae including *S.* Brandenburg without differentiating them. Since both *S.* Brandenburg and *S.* Typhimurium belong to serogroup B sharing LPS 'O' antigens 4 and 12, an LPS-based ELISA is not suitable to differentiate them. In New Zealand, *S.* Typhimurium and *S.* Hindmarsh are the most common causes of ovine salmonellosis leading to diarrhoea and mortality with occasional abortions (Clark *et al.*, 2002). *Salmonella* Oranienburg has also been isolated from aborted foetuses in sporadic outbreaks of abortions in New Zealand (Russel & Tannock, 1964). *Salmonella* serotypes Abortusovis, Dublin and Montevideo are causes of ovine abortions in other countries (Sharp *et al.*, 1983; Wray & Linklater, 2000). All these serotypes do not contain flagellar antigens of L complex. Therefore,

the ELISA described here is able to differentiate *S.* Brandenburg from the above serotypes, and this feature is important for disease monitoring purposes in New Zealand. A diagnostic test discriminating sheep infected with different *Salmonella* serotypes will be helpful for disease surveillance programs.

Infection with *Salmonella* leads to increased levels of serum IgG antibodies (Withanage *et al.*, 2005). Using a positive cut-off OD value of 0.1, the ELISA correctly classified 76 out of 81 sheep naturally infected with *S.* Brandenburg as positive giving the assay a sensitivity of 93.8%. ELISA-negativity of the other five samples may have been due to insufficient IgG at the time of sampling. The time interval between infection and blood sampling may be too short for these 5 sheep to result in sufficient IgG levels to give an OD of 0.1. The use of sensitive methods for the identification of *Salmonella* is important because information on the true *Salmonella* status in sheep flocks is essential for the control of *S.* Brandenburg infection. Bacteriological culture is the 'gold standard' for *Salmonella* diagnosis, and is the only one that enables identification of the specific serovars. Although bacteriological culture methods are highly specific, there are disadvantages (Funk *et al.*, 2005). The conventional bacteriological isolations from clinical samples are time-consuming and may not identify all infected animals due to intermittent shedding of *Salmonella.* The actual shedding of *Salmonella* indicates true infection and transmission, whereas the positive serology indicates also silent transmission within the herd (van Winsen *et al.*, 2001). Serology can be used to identify infected and carrier animals, and to aid surveillance and epidemiological studies. However, the presence of serum antibodies will not discriminate between previous and current exposure, and between vaccination and natural exposure. A paired serum sample testing may be beneficial in this regard.

Currently, there is no serological test to identify sheep infected with *S.* Brandenburg. The immunogenic characteristic of this recombinant flagellin elucidated by Western blots enabled the protein to be evaluated as a diagnostic antigen for incorporation into an ELISA for immunodetection of *S.* Brandenburg infection in sheep. This preliminary ELISA was carried out with sera from recently infected ewes (3-4 weeks post-abortion). In order to evaluate its suitability to detect carriers, it will be necessary to determine how long this ELISA remains able to detect antibodies in previously infected

sheep. It is believed that the antibody response against *Salmonella* flagellae is long-lasting (Frimpong *et al.*, 2000, Willke *et al.*, 2002).

Recombinant flagellin proteins are easily produced through heterologous expression systems, and therefore can be obtained in large quantities that are relatively pure. Therefore, inclusion of a recombinant protein with region IV in coating ELISA plates rather than the whole flagellin isolated from cultures has advantages such as purity, low cost and non-infectiousness. Moreover, it increases the specificity of the assay due to the absence of cross-reactions caused by conserved regions of whole flagellin. In conclusion, the ELISA described in this Chapter differentiates sheep infected with *S.* Brandenburg from those with other *Salmonella* serotypes that do not carry l, v specific epitopes on flagellin. The use of this ELISA in combination with bacteriological and/or molecular diagnostic methods would constitute an improved diagnostic assay for the detection of *S.* Brandenburg infection in sheep.

Salmonella Brandenburg carries the unique combination of phase-1 flagellin antigens (l, v), phase-2 flagellin antigens (e, n, z15), and group B specific O-4 antigen. Chapter 6 describes a PCR assay for the identification of *S.* Brandenburg by targeting the gene fragments encoding these 3 antigens, and *Salmonella*-specific InvA protein.

Chapter 6 Development of a PCR assay for the identification of *S.* Brandenburg

6.1 Abstract

Performance of a combined multiplex and a single polymerase chain reaction method for the identification of *S.* Brandenburg from pure cultures was investigated. Portions of *invA*, *rfbJ* (B), *fliC* (l, v), and *fljB* (e, n, z15) genes were targeted for amplification using four pairs of oligonucleotide primers. To validate the assay, genomic DNA from an array of 72 *Salmonella* strains representing 28 serotypes, and 5 non-*Salmonella* strains from 4 different genera was subjected to PCR. The targeted four genes were correctly amplified only from *S.* Brandenburg DNA with a specificity of 100%. All *S.* Brandenburg strains used in this study amplified all four genes with a sensitivity of 100%. These results indicate that this PCR assay is a simple, rapid, reliable and a reproducible method for the identification of *S.* Brandenburg that will aid in surveillance, prevention and control of this pathogen.

6.2 Introduction

In vitro amplification of DNA by PCR has become a powerful tool in diagnosis of pathogens (Delibato *et al.*, 2006; Malorny *et al.*, 2003). The advantages of PCR include rapidity, sensitivity and specificity (Stone *et al.*, 1994). A number of PCR assays utilizing different gene sequences have been developed for the detection of *Salmonella* at the genus level (Section 1.12.2). The most commonly used primer pair is one that was designed by Rahn and his co-workers (1992) to amplify *invA* gene (Arnold *et al.*, 2004; Malorny *et al.*, 2003). The commercially available PCR kits to identify *Salmonella* include Bax® *Salmonella* PCR Kit (Qualicon, Inc., Wilmington, DE, USA), Probelia™ PCR System (BioControl Systems, Inc., Bellevue, WA, USA), iQ-Check™ *Salmonella* Kit (Bio-Rad, Hercules, CA, USA), LightCycler® foodproof *Salmonella* Detection Kit (Roche, Mannheim, Germany), and GeneGen *Salmonella* Detection Kit (SY-LAB Geräte GmbH, Neupurkersdorf, Austria).

In routine bacteriology, final identification of *S.* Brandenburg is based on biochemical tests followed by serotyping. Serotyping is based on surface antigens, somatic O, phase–1 (H1) and phase-2 (H2) flagellar antigens. It requires a battery of high quality antisera, trained technicians, and is done in reference laboratories. Hence, evaluation of improved diagnostic techniques for the identification of *S.* Brandenburg is desirable in both animal and public health. As outlined in the previous paragraph, a number of PCR assays have been developed for the detection of *Salmonella* at the genus level. However, the number of published PCR assays to determine the *Salmonella* serotype is limited, and include those to detect serotypes Typhi, Paratyphi, Typhimurium, Enteritidis and Gallinarum (Table 6.1). To date, there is no serotype-specific PCR assay for the detection of *S.* Brandenburg. *S.* Brandenburg carries Group B specific O-4 antigen, H1 antigens l, v and H2 antigens e, n, z15. These are encoded by *rfbJ*(B), l, v alleles of *fliC* and e, n, z15 alleles of *fljB* genes respectively. Presence of *invA* is specific to *Salmonella,* and the combination of *rfbJ*(B), *fliC* (l, v) and *fljB* (e, n, z15) is specific to *S.* Brandenburg. Therefore, the amplification of *invA* gene will differentiate *Salmonella* from non-*Salmonella* isolates, and that of *rfbJ*(B), *fliC* (l, v) and *fljB* (e, n, z15) will differentiate *S.* Brandenburg from other *Salmonella* serotypes.

This Chapter describes the development of a rapid PCR method targeting *rfbJ*(B) in a single reaction, and *invA*, *fliC* (l, v), *fljB* (e, n, z15) genes in a multiplex reaction followed by agarose gel electrophoresis for the detection of *S.* Brandenburg at the serotype level from suspected *Salmonella* colonies.

Table 6.1. *Salmonella* serotype-specific PCR assays

Salmonella Serotype	Basis for PCR specificity	Type of PCR	References
Gallinarum	Single nucleotide difference at position 598 in the *rfbS* gene (allele-specific)	Conventional	Shah *et al.*, 2005
Typhi and Paratyphi	*tyv* (*rfbE*), *prt* (*rfbS*), *viaB*, and *fliC*	Multiplex	Hirose *et al.*, 2002
Typhi	*vexC* gene	Real-time	Farrell *et al.*, 2005
Typhi	*invA, viaB, fliC-d, prt* (*rfbS*)	Multiplex	Kumar *et al.*, 2006
Typhimurium	*mdh* gene that encodes malic acid dehydrogenase	Conventional	Leon-Velarde *et al.*, 2004
Enteriditis	Single nucleotide difference at position 272 in the *spvA* gene of virulence plasmid (allele-specific)	Conventional	Lampel *et al.*, 1996

tyv (*rfbE*), gene encoding CDP-tyvelose epimerase needed for the synthesis of O-antigen

prt (*rfbS*), gene encoding paratose synthase needed for the synthesis of O-antigen

viaB, gene encoding capsular polysaccharide (Vi) antigen

fliC, gene encoding phase-1 flagellin

vexC, gene encoding VexC needed for the translocation of Vi to the bacterial surface

invA, gene encoding invA protein of *Salmonella* type III secretion system

fliC-d, gene encoding d antigen of phase-1 flagellin

spvA, gene encoding SpvA

6.3 Materials and methods

6.3.1 Bacterial strains

Five non-*Salmonella* strains belonging to 4 different genera, and 72 *Salmonella* strains belonging to 28 different serotypes were used in this study (Tables 6.2 and 6.3). *Salmonella* serotypes carrying different O- and H-antigens were used. Reference numbers and sources of *Salmonella* and non-*Salmonella* strains are shown in Tables 2.2 and 2.3. All the *Salmonella* strains used in this study have been identified and serotyped by the Institute from which they were obtained. Bacterial cultures were grown as described in Chapter 2.2.2. Genomic DNA was extracted by the boiling method from cell suspensions of bacteria grown overnight on XLD agar at 37°C (Chapter 2.3.1.1).

Table 6.2. Non-*Salmonella* strains used in this study

Non-*Salmonella* strain	No. tested
Escherichia coli	1
Klebsiella aerogenes	1
Klebsiella edwardsii	1
Proteus mirabilis	1
Shigella flexneri	1

Table 6. 3. Properties of *Salmonella* strains used in this study

Salmonella serotype	Sero group	Antigenic formula[a] O: H1: H2 antigenic factors	No. tested
Brandenburg	B	[1], 4, [5], 12, [27]: l, v: e, n, z15	25
Typhimurium	B	[1], 4, [5], 12: i: 1, 2	4
Derby	B	[1], 4, [5], 12: f, g: [1, 2]	4
Agona	B	[1], 4, [5], 12: f, g, s: [1, 2]	4
Saintpaul	B	[1], 4, [5], 12: e, h: 1, 2	1
Liverpool	E4	1, 3, 19: d: e, n, z15	1
Potsdam	C1	6, 7, [14]: l, v: e, n, z15	1
Livingstone	C1	6, 7, [14]: d : l, w	1
Infantis	C1	6, 7, [14]: r: 1,5	1
Singapore	C1	6, 7: k: e, n, x	1
Newport	C2	6, 8, [20]: e, h: 1, 2	4
Hindmarsh	C3	8, [20]: r: 1,5	4
Gallinarum	D1	[1], 9, 12: -: -	1
Enteritidis	D1	[1], 9, 12: [f], g, m, [p]: [1, 7]	3
Azteca	B	4, [5], 12, [27]: l, v: 1, 5	1
Bredeney	B	[1], 4, 12, [27]: l, v: 1,7	1
Jos	B	[1], 4, 12, [27]: y: e, n, z15	1
Budapest	B	[1], 4, 12, [27]: g, t: -	1
Abortusovis	B	4, 12: c: 1, 6	1
Wien	B	[1], 4, 12, [27]: b: l, w	1
Ball	B	[1], 4, 12, [27]: y: e, n, x	1
Gloucester	B	[1], 4, 12, [27]: i: l, w	1
Mono	B	4, 12: l, w: 1,5	1
Togo	B	4, 12: l, w: 1, 6	1
Havana	G2	[1], 13, 23: f, g [S]: -	1
Cerro	K	[6], [14], 18: z4, z23: [1,5]	4
Anatum	E1	3, 10, [15] [15,34]: e, h : 1,6	1
Newington	E2	3, [15]: e, h: 1,6	1

[a]antigenic factors shown in parentheses may be present or absent

6.3.2 PCR primers

The target genes selected in this study were *inv*A (*Salmonella*-specific), *rfbJ*(B) (serogroup B), *fliC* (l, v) and *fljB* (e, n, z15). Oligonucleotide primers used for the amplification of *invA*, *rfbJ*(B) and *fljB* genes were from published articles (Rahn *et al.*, 1992; Luk *et al.*, 1993; Echeita *et al.*, 2002 respectively), while the pair used for that of *fliC* was designed using the DNA sequence of region IV of variable domain of *S.* Brandenburg *fliC* gene (GenBank accession number AY935580) (Figure 6.1). Primers were designed as described in Chapter 2.4.4.1. All primers were synthesized by Life Technologies in the desalted form and are shown in Table 6.4.

```
544 AAG AGC GAA GCA GTT AAA AGC GGC GGT GGG GCA ACA CTC
583 AAT ACC ACT GGT CTT AAT GAT GCA GCT CTT AAA ACG GGT
622 GTT GGT GGT GCA ACA AAC GGT ACT GCT GCA ATT AAG GAT
661 GGT AAA GTC TTC TTC GAT GCA ACT GAT AAT AAA TAT TTT
700 ATT GAA GTA GAA GGT TTA ACC GCT GGC GAC GCT ACT AAA
739 AAT GGT GTT TAT GAA GTT AGT GTT GCA GAT GAT GGC ACT
778 GTT ACA ATG CCG ACA ACC ACG AAA GTG ACA GGA GGC ATG
817 CCA GCC ACG GCG ACG GCA GTA ACC GAA ACG CAG CCA AAA
856 CCT GTA GCT CTC AGT ACA GCA GTT AAA GAT CAG TTG ACC
895 GAC AGT GGT ATT AGT GCT 912
```

Figure 6.1. DNA sequence of region IV of *S.* Brandenburg *fliC* gene. Underlined are the oligonucleotide sequences targeted to design primers to amplify a portion of *fliC* gene.

Table 6.4. PCR Primers (F for forward and R for reverse) used in this study

Target	Primer sequence (5′ to 3′)[a]	Position	Product size (bp)
invA			
invA-F	GTGAAATTATCGCCACGTTCGGGCAA	371-396	285
invA-R	TCATCGCACCGTCAAAGGAACC	655-634	
rfbJ(B)			
rfbJ(B)-F	AGAATATGTAATTGTCAG	15-32	
rfbJ(B)-R	TAACCGTTTCAGTAGTTC	897-880	883
FliC			
FliC (l, v)-F	CACTGGTCTTAATGATGCAGCTC	588-610	222
FliC (l, v)-R	CCTGTCACTTTCGTGGTTAT	809-790	
fljB			
fljB-F	GGCAACCCGACAGTAACTGGCGATAC	631-656	135
fljB-R	ATCAACGGTAACTTCATATTTG	765-744	

[a]Oligonucleotide primers used for the amplification of *invA*, *rfbJ*(B) and *fljB* genes were from published articles (Rahn *et al.*, 1992; Luk *et al.*, 1993; Echeita *et al.*, 2002 respectively).

6.3.3 DNA amplification and detection

Preliminary experiments to amplify *invA, fliC*, *fljB* and *rfbJ*(B) in a multiplex PCR did not yield consistent results with *rfbJ*(B) gene. The latter was either absent or was a faint band on agarose gels stained with ethidium bromide. Therefore, *invA, fliC* and *fljB* genes were amplified in a multiplex PCR, and the *rfbJ*(B) gene was amplified in a separate reaction. The multiplex PCR amplification was performed in a reaction volume of 50 µl consisting of 1 µl of genomic DNA, 200 nM of each primer *invA*-F, *invA*-R, *fliC* (l, v)-F, *fliC* (l, v)-R, *fljB*-F and *fljB*-R, 200 µM of each dCTP, dGTP, dATP and dTTP, 2 mM $MgCl_2$, 1× PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 1.25 units of Platinum® *Taq* DNA Polymerase (Life Technologies) and sterile distilled water. The reactions were subjected to a single cycle of denaturation at 95°C for 5 min, followed by 30 cycles of denaturation (95°C for 1 min), annealing (60°C for 30 sec),

elongation (72°C for 30 sec), and a final elongation (72°C for 7 min). The *rfbJ*(B) gene was amplified separately in a 50 µl reaction volume containing the same PCR reagents as the multiplex reaction replacing the primers with *rfbJ*(B)-F and *rfbJ*(B)-R. The thermocycler parameters were as above except that the annealing reaction was done at 55°C for 1 min, and elongation was at 72°C for 1 min. A negative control without genomic DNA replaced with sterile distilled water was included in each PCR assay. Reproducibility of the assay was confirmed by testing each strain in duplicate three times. Samples were stored at –20°C. Ten microlitres of multiplex PCR-amplified reaction was mixed with 10 µl of the single reaction, and the PCR products were visualised on 2.5% agarose gels stained with ethidium bromide (Chapter 2.4.1). One-kb Plus DNA Ladder marker was used as a size reference. Four PCR products obtained from *S.* Brandenburg amplification were agarose gel purified (Chapter 2.4.2), and sequenced on a capillary ABI3730 DNA Sequencer (Chapter 2.7). DNA sequences were edited using Chromas software, and analysed by BLASTN network server at http://www.ncbi.nlm.nih.gov.

6.3.4 Specificity and sensitivity

Genomic DNA extracted from a panel of *Salmonella* strains representing 28 different serotypes and non-*Salmonella* strains from four genera were PCR-amplified to evaluate the specificity and sensitivity of the PCR assay. The following formulas were used (Martin, 1984). Specificity % = true negative samples/(true negative samples + false positive samples) × 100, and sensitivity % = true positive samples/(true positive samples + false negative samples) × 100.

6.4 Results

In this study, portions of *invA*, *rfbJ*(B), *fliC* and *fljB* genes were amplified for the identification of *S.* Brandenburg. Figures 6.2 and 6.3 show the amplification products from *Salmonella* and non-*Salmonella* strains used in the study. While all *Salmonella* isolates amplified the *invA* gene, only Group B *Salmonella* isolates amplified the *rfbJ*(B) gene. While *fliC* primers amplified only the *Salmonella* isolates carrying l, v alleles, *fljB* primers amplified only those carrying e, n, z15 alleles. Non-*Salmonella* strains did not produce amplification products under these conditions. Only *S.* Brandenburg strains gave amplification products of all four genes with the expected sizes of 883 bp (*rfbJ*B), 285 bp (*invA*), 222 bp (*fliC*) and 135 bp (*fljB*). Sequencing and BlastN analysis of the amplicons of *S.* Brandenburg confirmed that only the correct gene fragments had been amplified. All twenty-five *S.* Brandenburg isolates used in this study amplified all four genes. Repeated PCR reactions gave identical results. A summary of results is shown in Tables 6.5 and 6.6.

Table 6.5. PCR results of non-*Salmonella* strains used in this study

Non-*Salmonella* strain	No. tested	No. of strains positive for target gene			
		InvA	*rfbJ*(B)	*fliC*	*fljB*
Escherichia coli	2	0	0	0	0
Klebsiella aerogenes	2	0	0	0	0
Klebsiella edwardsii	2	0	0	0	0
Proteus mirabilis	2	0	0	0	0
Shigella flexneri	1	0	0	0	0

Table 6. 6. PCR results of *Salmonella* strains used in this study

Salmonella Serotype	O Sero group	Antigenic formula[a] O: H1: H2 antigenic factors	No. tested	No. of strains positive for target gene			
				invA	*rfbJ(B)*	*fliC*	*fljB*
Brandenburg	B	[1],4,[5],12,[27]: l,v: e, n, z15	25	25	25	25	25
Typhimurium	B	[1], 4, [5],12: l: 1, 2	4	4	4	0	0
Derby	B	[1], 4, [5], 12: f, g: [1, 2]	4	4	4	0	0
Agona	B	[1],4,[5],12: f, g, s: [1, 2]	4	4	4	0	0
Saintpaul	B	[1], 4, [5], 12: e, h: 1, 2	1	1	1	0	0
Liverpool	E4	1, 3, 19: d: e, n, z15	1	1	0	0	1
Potsdam	C1	6, 7, [14]: l, v: e, n, z15	1	1	0	1	1
Livingstone	C1	6, 7, [14]: d: l, w	1	1	0	0	0
Infantis	C1	6, 7, [14]: r: 1,5	1	1	0	0	0
Singapore	C1	6, 7: k: e, n, x	1	1	0	0	0
Newport	C2	6, 8, [20]: e, h: 1, 2	4	4	0	0	0
Hindmarsh	C3	8, [20]: r: 1, 5	4	4	0	0	0
Gallinarum	D1	[1], 9, 12: -: -	1	1	0	0	0
Enteritidis	D1	[1], 9, 12: [f], g, m, [p]: [1, 7]	3	3	0	0	0
Azteca	B	4, [5], 12, [27]: l, v: 1,5	1	1	1	1	0
Bredeney	B	[1], 4, 12, [27]: l, v: 1,7	1	1	1	1	0
Jos	B	[1],4,12,[27]: y: e, n, z15	1	1	1	0	1
Budapest	B	[1], 4, 12, [27]: g, t: -	1	1	1	0	0
Abortusovis	B	4, 12: c: 1, 6	1	1	1	0	0
Wien	B	[1], 4, 12 [27]: b: l, w	1	1	1	0	0
Ball	B	[1], 4, 12, [27]: y: e, n, x	1	1	1	0	0
Gloucester	B	[1], 4, 12 [27]: i: l, w	1	1	1	0	0
Mono	B	4, 12: l, w: 1, 5	1	1	1	0	0
Togo	B	4, 12: l, w: 1, 6	1	1	1	0	0
Havana	G2	[1], 13, 23: f, g [s]: -	1	1	0	0	0
Cerro	K	[6], [14], 18: z4, Z23: [1, 5]	4	4	0	0	0
Anatum	E1	3, 10, [15] [15, 34]: e, h: 1, 6	1	1	0	0	0
Newington	E2	3, [15]: e, h: 1,6	1	1	0	0	0

[a]antigenic factors shown in parentheses may be present or absent

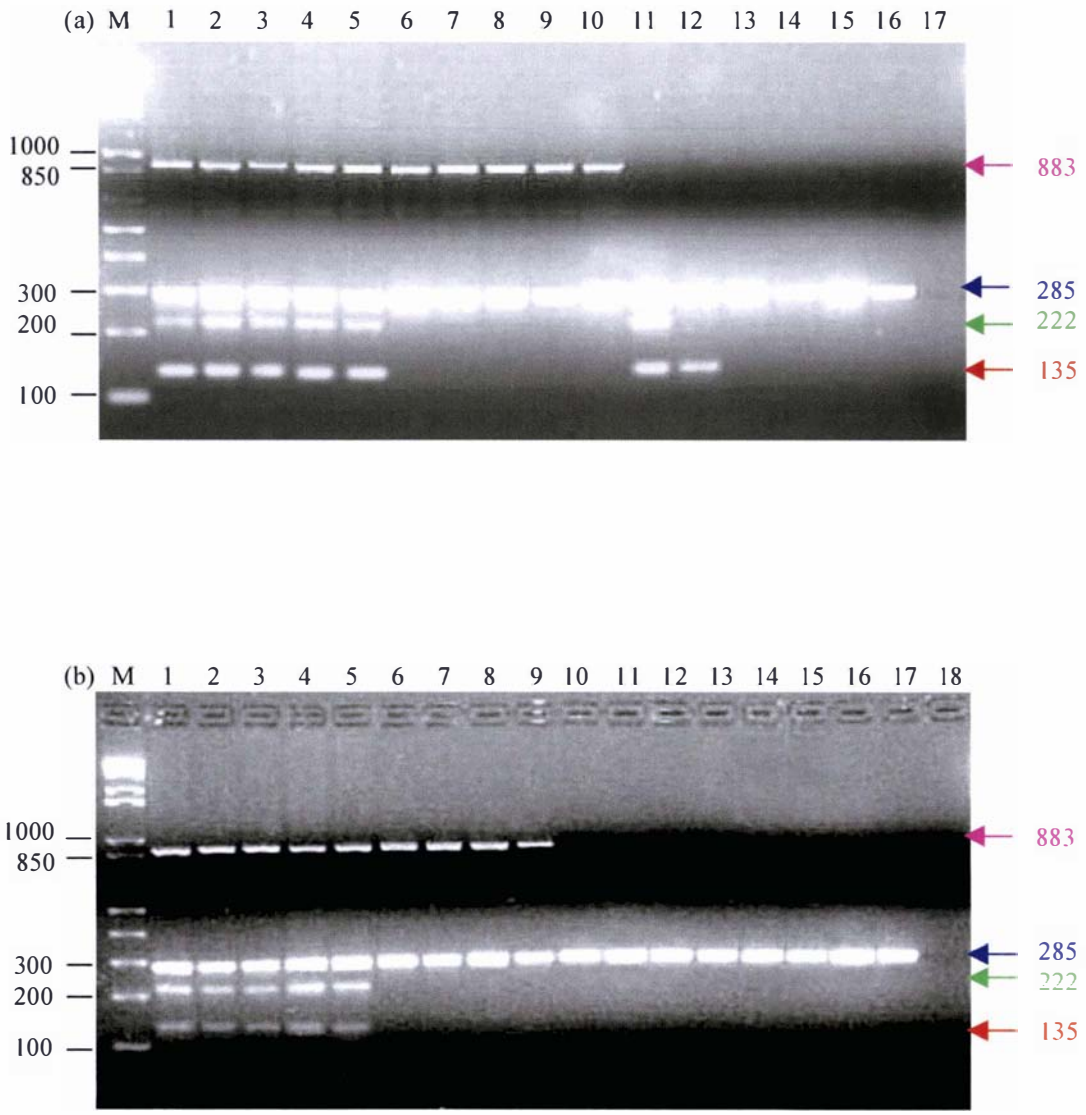


Figure 6.2. PCR amplification of *invA, rfbJ*(B), *fliC* (l, v), and *fljB* (e, n, z15) genes. PCR products were electrophoresed on a 2.5% agarose gel, stained with ethidium bromide and photographed under UV light. (a) M, 1-kb Plus DNA Ladder marker (bp); lanes 1 to 5, *S.* Brandenburg; lanes 6 to 9, *S.* Typhimurium; lane 10, *S.* St. Paul; lane 11, *S.* Potsdam; lane 12, *S.* Liverpool; lane 13, *S.* Livingstone; lane14, *S.* Infantis; lane 15, *S.* Singapore; lane 16, *S.* Gallinarum; lane 17, *E. coli.* (b) M, 1-kb Plus DNA Ladder marker (bp); lanes 1 to 5, *S.* Brandenburg; lanes 6 to 9, *S.* Derby; lane 10 to 13, *S.* Newport; lanes 14 to 16, *S.* Enteritidis; lane 17, *S.* Anatum; lane 18, *Klebsiella aerogenes*. Pink, blue, green and red arrows indicate *rfb*J(B), *inv*A, *fliC* and *fljB* amplicons respectively.

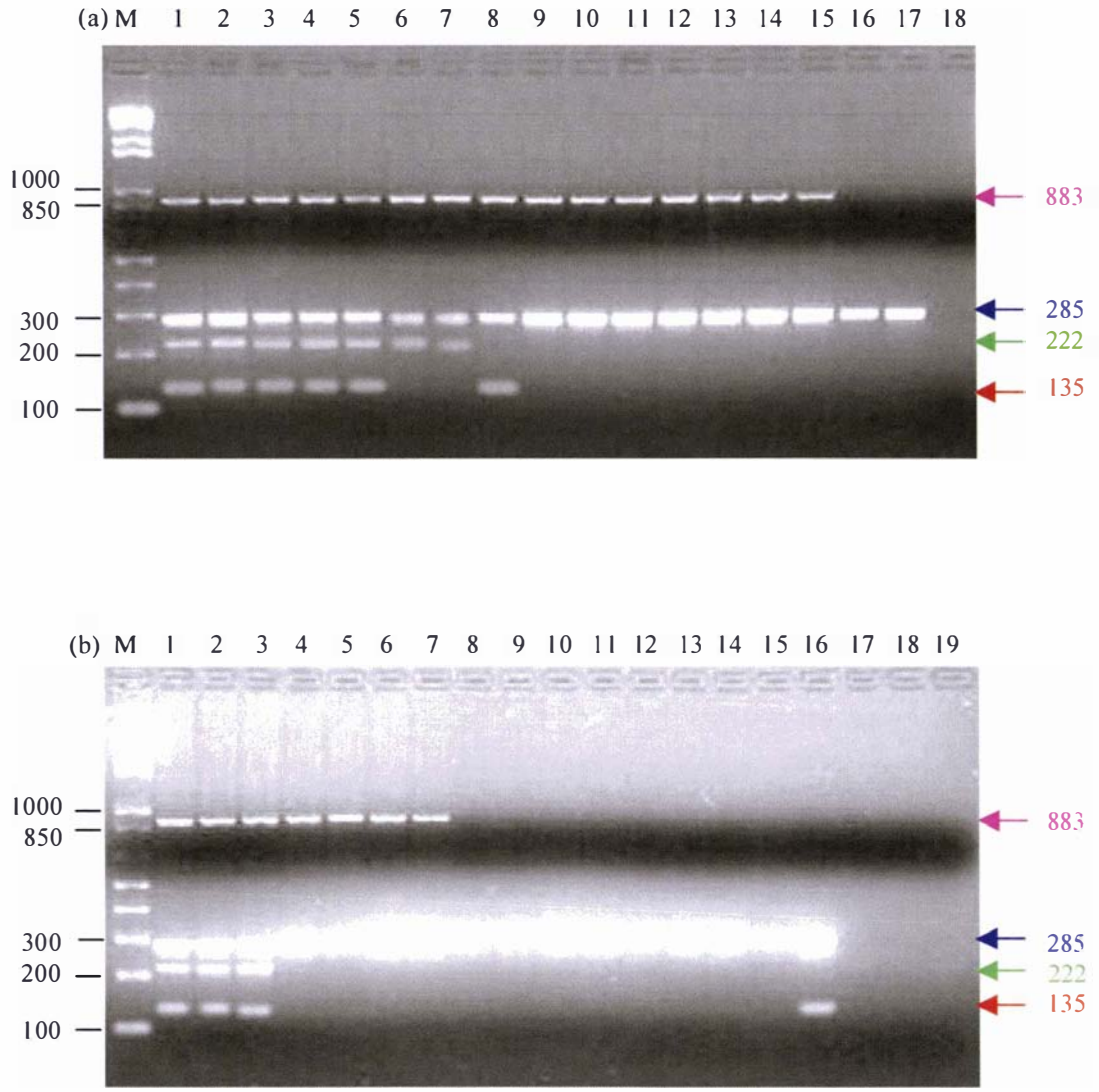


Figure 6.3. PCR amplification of *invA, rfbJ*(B), *fliC* (l, v), and *fljB* (e, n, z15) genes. PCR products were electrophoresed on a 2.5% agarose gel, stained with ethidium bromide and photographed under UV light. (a) M, 1-kb Plus DNA Ladder marker (bp); lanes 1 to 5, *S.* Brandenburg; lane 6, *S.* Azteca; lane 7, S. Bredney3; lane 8, *S.* Jos; lane 9, *S.* Budapest; lane 10, *S.* Abortusovis; lane 11, *S.* Wien; lane 12, *S.* Ball; lane 13, *S.* Gloucester; lane 14, *S.* Mono; lane 15, *S.* Togo; lane 16, *S.* Havana; lane 17, *S.* Newington; lane 18, *Klebsiella edwardsii.* (b) M, 1-kb Plus DNA Ladder marker (bp); lanes 1 to 3, *S.* Brandenburg; lanes 4 to 7, *S.* Agona; lanes 8 to 11, *S.* Hindmarsh; lanes 12 to 15, Cerro; lane 16, *S.* Liverpool; lane 17, *Proteus mirabilis*; lane 18 *Shigella flexneri;* lane 19, negative control (water). Pink, blue, green and red arrows indicate *rfb*J(B), *inv*A, *fliC* and *fljB* amplicons respectively.

6.5 Discussion

The PCR assay developed in this study was based on the amplification of *invA*, *rfbJ*(B), *fliC* and *fljB* genes directly from a single *Salmonella* colony. *InvA* gene was targeted for the diagnosis of *Salmonella* organisms at the genus level, and the combination of three polymorphic genes *rfbJ*(B), *fliC* and *fljB* was targeted for the identification of *S.* Brandenburg at the serotype level. *InvA* gene is present in all invasive strains of *Salmonella* (Galán, 1996) and absent from closely related genera such as *Escherichia* (Bäumler *et al.*, 1998). All 72 *Salmonella* strains used in the study amplified *invA* gene, while non-*Salmonella* strains belonging to other 4 genera were negative. These results agree with Rahn and his co-workers (1992) who designed and established the specificity of this widely used pair of primers. They showed that 630 *Salmonella* strains representing over 100 strains yielded the target band of 285 bp, whereas two strains of each serotype Litchfield and Senftenberg were negative. The strains that were shown to lack *invA* sequences were from environmental samples, and were not specifically associated with disease (Ginocchio *et al.*, 1997). Furthermore, this pair of primers was also used in a European research project in the validation and standardization of PCR for the detection of *Salmonella* species in food (Hoorfar, 1999). Malorny and his co-workers (2003) showed that one *S.* Saintpaul strain did not give the target band. It is speculated that *invA* gene is absent in these strains, which are not invasive or they might be using other invasive mechanisms. However, absence of *invA* gene in *Salmonella* seems to be rare (Malorny *et al.,* 2003).

The lipopolysaccharide O-antigen together with phase-1 and phase-2 flagellar antigens forms the basis for *Salmonella* serotyping (Popoff & LeMinor, 2001). A considerable amount of diversity is seen within *Salmonella* O-antigens, which are composed of multiple repeats of the O-subunit, and they contribute major antigenic diversity to the cell surface, which is used to serotype *Salmonella* isolates. The O-antigen specificity is based on the sugar composition and arrangement of sugars in the O-subunit (Fitzgerald *et al.,* 2003). The O-subunit of group B Salmonellae is an oligosaccharide of 4 sugar residues. Of these four sugars, 3 form a mannosyl-rhamnosyl-galactose backbone common to some other serogroups such as A and D. The fourth sugar is a dideoxyhexose substituted on the mannosyl residue. While the dideoxyhexose sugar

present in the O-subunit of serogroups A and D are paratose and tyvelose respectively, that of both serogroups B and C2 is abequose. Abequose confers on group B strains their specific O-4 antigen (Wyk & Reeves, 1989). The *rfb* gene cluster encodes the enzymes for biosynthesis of O-antigens. The *rfbJ* gene encodes abequose synthase that is responsible for the final specific step in the synthesis of abequose. There is a 44% difference between the nucleotide sequence of *rfbJ* gene of serogroup B [*rfbJ*(B)] and that of serogroup C2 [*rfbJ*(C2)] (Luk *et al.,* 1993; Wyk & Reeves, 1989). Luk and his co-workers (1993) designed a pair of primers targeting the serogroup B specific *rfbJ* gene that was used in this study and as expected, it amplified only the *rfbJ*(B) gene. In *S*. Brandenburg, phase–1 and phase-2 flagellins belong to L (1, v) and E complex (e, n, z 15) respectively. Region IV of *Salmonella* flagellin is the most variable, and is believed to carry the major serotype-specific epitopes of the flagellar antigens (Newton *et al.,* 1991; Wei & Joys, 1985). Primers used to amplify *fliC* and *fljB* were from region IV, and did not amplify other L- and E- complex genes of the *Salmonella* strains used in this study. This shows the specificity of these 2 pairs of primers for 1, v and e, n, z15 alleles of *fliC* and *fljB* genes respectively. Only *S.* Brandenburg strains gave all four amplification products of the expected sizes of 883 bp (*rfbJ*B), 285 bp (*invA*), 222 bp (*fliC*) and 135 bp (*fljB*). The absence of false positive reactions scored the assay a specificity of 100%.

All of the *S*. Brandenburg strains were obtained from clinical samples (uterine swabs, faeces, intestinal contents and meat) collected from sheep, cattle and pigs, and sheep yard dust. Pure cultures of *S*. Brandenburg were used for the initial evaluation of the assay because PCR assays are prone to inhibition by substances in the samples (Iijima *et al*., 2004). All the 25 *S*. Brandenburg cultures that were confirmed by serotyping were positive without any false negative reactions in the PCR giving the test a sensitivity of 100%. The PCR described here detected the combination of three *rfbJ*(B), *fliC* (1, v) and *fljB* (e, n, z15) genes that is unique to *S.* Brandenburg. With this PCR assay, identification of pure cultures of *Salmonella* to the serotype Brandenburg could be done in approximately 8 hours. This PCR assay combines the advantages of simple and rapid processing of PCR products with the use of primers that can be easily and cheaply synthesized on a large scale. Therefore, this PCR assay could easily be implemented into a routine microbiology diagnostic laboratory. Since, this assay

requires only genomic DNA, it does not depend on the expression of antigens thus making the need for time-consuming phase reversal of flagellin antigens unnecessary.

Chapter 7 General discussion

The main objective of the work presented in this thesis was to identify and characterise exported immunogenic proteins of *S.* Brandenburg that may be useful in diagnostic tests and subunit vaccines. This was achieved by the construction of an *S.* Brandenburg expression library in *E. coli* using alkaline phosphatase gene fusion technology. This technology allows genes to be identified on the basis of their protein products being exported from the cytoplasm.

This study describes the first *S.* Brandenburg expression library prepared in *E. coli* that led to the identification of a number of genes encoding exported proteins including outer membrane proteins, porins, fimbriae and phase-1 flagellin of *S.* Brandenburg. Thirty out of 66 proteins identified after sequencing 95 *phoA+* gene fusions had cleavable signal peptides needed for protein translocation through the inner membrane. This suggests that the PhoA technology identified proteins exported by the general secretory pathway. Limitations of the PhoA technology include the identification of a number of inner membrane proteins, the appearance of a few false positives, and the identification of partial gene sequences in most instances in lieu of full-length genes. In this study, 29 out of 66 proteins had one or more transmembrane helices to insert them to the inner membrane. Worley and his co-workers (1998) have described an invasin fusion technique to identify secreted proteins of *S.* Typhimurium excluding inner membrane proteins. They have constructed a genomic library by ligating *S.* Typhimurium chromosomal fragments to a deletion derivative of the outer membrane protein invasin gene of *Yersinia pseudotuberculosis.* This invasin fusion technology may be an alternative to PhoA technology if inner membrane proteins are to be excluded from the library. Construction of bacteriophage Lambda genomic libraries would facilitate the identification of full genes. However, this study suggests that in spite of these limitations, *phoA* gene fusion technology is a rapid technique to identify novel exported proteins.

Phase-1 flagellin (FliC) was identified through screening PhoA+ blue colonies and database searches, and FliC was selected for further characterisation due to its known immunogenic properties. The expression vector pET14b allowed the heterologous

expression of high levels of recombinant proteins in *E. coli*, and the cobalt affinity chromatography purification system resulted in highly pure recombinant proteins as evidenced by SDS-PAGE analysis. If needed, further purification of these recombinant proteins can be achieved by size exclusion column chromatography. FliC is one of the immunogenic antigens that is recognized by both B and T cells (Alaniz *et al.*, 2006). Western blot analysis described in Chapter 4 showed that naturally infected, and vaccinated sheep had serum IgG antibodies against FliC. This study indicated that Salvexin^{TM}+B-vaccinated sheep did not produce an IFN-γ response to recombinant FliC and V456. However, it is worthwhile to investigate the IFN-γ responses of naturally infected sheep against recombinant flagellin proteins. A recent study has shown that experimental mice immunized orally, intranasally and subcutaneously with *S.* Enteritidis flagellin alone, or with recombinant cholera toxin-B (rCTB) that is believed to increase antigen uptake across the mucosae, elicited high systemic (IgG) and mucosal (s-IgA) humoral responses. The group that received flagellin plus rCTB intranasally showed the highest IFN-γ in supernatants collected from splenocytes stimulated with flagellin. Upon challenge with virulent *S.* Enteritidis, the most protected groups were ones that were immunized orally with free flagellin, and intranasally with free flagellin plus rCTB (Strindelius *et al.*, 2004a). Furthermore, McSorley and his co-workers (2000) showed the protective nature of flagellin during a natural infection of mice with *S.* Typhimurium. Therefore, *S.* Brandenburg challenge studies in sheep immunized with recombinant FliC alone or with different adjuvants or agents like rCTB via different routes may provide information regarding flagellin as a potential candidate in a future subunit vaccine. Plasmid DNA vaccination, a strategy that allows for the development of a strong CMI response, has been recognized as an efficacious immunization route against intracellular bacteria (Chen *et al.*, 2006). Investigation of a DNA vaccine containing *fliC* as a potential subunit vaccine candidate may be worth pursuing.

To date, there are no published reports of diagnostic tools for the identification of *S.* Brandenburg infection other than routine bacteriological methods with a confirmatory serotyping step done according to the Kaufmann-White scheme. An easy and reproducible PCR assay for the confirmation of *S.* Brandenburg organism from pure cultures, and an indirect ELISA for the detection of *S.* Brandenburg antibodies in sheep

sera using flagellin proteins have been developed in this study. The immunogenic characteristic of recombinant region IV of FliC led to the evaluation of an indirect ELISA with a sensitivity of 93.8% and a specificity of 100% in the identification of sheep naturally infected with *S.* Brandenburg. The advantage of region IV antigen used in the ELISA is that it is not shared by other common pathogenic *Salmonella* serotypes in New Zealand sheep. However, further work is needed to define the sensitivity of the assay using sera from a number of sheep flocks naturally infected with *S.* Brandenburg, and to define specificity of the assay using sera from sheep infected with other *Salmonella* serotypes. Non-availability of sera from sheep infected with other *Salmonella* serotypes at the time of the study was a limitation. It is also important to investigate the background values of a large number of serum samples collected from sheep free of *S.* Brandenburg to evaluate cut-off values.

Use of separate phase-1 and phase-2 flagellin in a test allows discrimination between infections with *S.* Brandenburg, and other *Salmonella* serotypes that have only one of these antigens in common with *S.* Brandenburg. A combined preparation of region IV of FliC and FljB seems to be suitable to use as an antigen in an ELISA to increase the specificity to detect *S.* Brandenburg-specific antibodies. The indirect ELISA described in Chapter 5 could also be modified to detect IgM that appears early in infections, and also detection of antibodies from other sources such as meat juice and milk. Furthermore, it would be worthwhile using this ELISA to investigate the time course of the appearance and disappearance of antibodies against region IV of FliC in sheep naturally infected with *S.* Brandenburg. Other uses could include detection of serum antibodies in other animal species and humans using enzyme-labelled species-specific secondary antibody conjugates. Generation of monoclonal antibodies against antigenic epitopes located in the region IV may lead to the evaluation of different ELISA formats. For example, a competitive ELISA would detect antibodies against region IV of *S.* Brandenburg. ELISA plates coated with recombinant region IV are incubated with suspected sera, and subsequently with the MAb. Binding of the latter to the coated antigen depends on the presence and amount of antibodies against region IV. Subsequently, MAb binding can be detected by incubation with an enzyme-labelled (alkaline phosphatase- or peroxidase-labelled) anti-mouse immunoglobulins followed by the addition of the substrate. OD values are inversely related to the serum antibodies against region IV. Competitive ELISA removes the need for enzyme-

labelled species-specific secondary antibody conjugates enabling it to be used in all animal species and humans using enzyme-labelled anti-mouse immunoglobulin conjugates.

Chapter 6 described a PCR assay that was evaluated to confirm the identity of *S.* Brandenburg from a pure culture with a specificity of 100%, and a sensitivity of 100%. This assay is inexpensive, reproducible and has a high discriminatory power. In a routine diagnostic lab, a large number of samples can be investigated with this procedure. Pure cultures of *S.* Brandenburg were used for the initial evaluation of the PCR assay. Development of the assay directly with clinical or environmental samples was not done due to time limitation. One of the problems with a PCR assay for the direct detection of a pathogen in samples is the presence of PCR inhibitory substances such as bilirubin, bile salts, polysaccharides, DNases, and proteases (D'Souza & Jaykus, 2003; Iijima *et al.*, 2004). PCR facilitators, DNA extraction, and sample enrichment methods have been used to overcome these PCR-inhibitory substances (Arnold *et al.*, 2004; Malorny & Hoorfar, 2005). It has been shown that bovine serum albumin (BSA) acts as a PCR facilitator. Another strategy to overcome PCR inhibition is to use polymerases that are more resistant than *Taq* (Al-Soud & Rådström, 1998, 2000). DNA extraction methods, in addition to removing inhibitory substances, concentrate total genomic DNA (Stone *et al.*, 1994). Studies have shown that QIAmp Stool Kit (Qiagen) yield higher and less degraded DNA than the conventional phenol-chloroform method (Malorny & Hoorfar, 2005). Several authors have published PCR assays to detect *Salmonellae* from enriched broth samples with (Gentry-Weeks *et al.*, 2002; Malkawi & Gharaibeh, 2003; Oliveira *et al.*, 2003; Schrank *et al.*, 2001) or without DNA extraction (Chiu & Ou, 1996; Stone *et al.*, 1994). In most instances, an enrichment step has been necessary to remove PCR inhibitors and to increase sensitivity of the method. An optimal enrichment should inhibit the growth of background flora but simultaneously recover and multiply sub-lethally damaged *Salmonella* cells. Furthermore, it is important to include an internal amplification control (IAC) to detect false-negative results caused by PCR inhibitors. Comparison of the PCR system and the routine culture methods with different clinical and environmental samples with/without PCR facilitators/enrichment/DNA extraction methods would evaluate the sensitivity of the four pairs of primers used in this study for the detection of *S.* Brandenburg. A multifactorial design experiment would help to

investigate all parameters in a single experimental set up. A PCR-based assay would be most useful on culture-negative clinical samples from sheep with suspected *S.* Brandenburg infection. Clinical samples need to be investigated to evaluate the usefulness of this assay to identify carriers of *S.* Brandenburg. It is worthwhile to investigate the potential applicability of this PCR assay for the detection of *S.* Brandenburg from other sources such as environmental samples, food samples, meat products and slaughterhouse samples for more detailed epidemiological studies. Rapid detection methods may help in monitoring the contamination levels in processing plants and evaluating the performance of the Hazard Analysis and Critical Control Point (HACCP) system verification. How such technologies will be incorporated into the routine labs in the future, however, remains to be seen.

In this study, PCR products were qualitatively confirmed using agarose gel electrophoresis that is suitable to routine labs. Real-time PCR and PCR-ELISA are two other alternatives for gel electrophoresis. In recent years, a quantitative PCR method known as real-time PCR with an automatic confirmation phase has been developed (Delibato *et al.*, 2006). Real-time PCR is faster than gel electrophoresis, eliminates the use of hazardous ethidium bromide, but requires expensive equipment and reagents (Eyigor *et al.*, 2002; Schnidler *et al.*, 2002; Uyttendaele *et al.*, 2003). Therefore, evaluation of a real-time PCR with the primers used in this study may be worthwhile. PCR-ELISA techniques have been described for the detection of *Salmonella* in faecal and food samples (Gillespie *et al.*, 2003; Hong *et al.*, 2003; Luk *et al.*, 1997; Manzano *et al.*, 1998). PCR-ELISA involves incorporation of chemically tagged nucleotides into the PCR amplicon and subsequent detection of the PCR product with antibody-enzyme conjugate that recognizes the unique chemical label present in the incorporated nucleotides (Hong *et al.*, 2003). An ELISA format allows the screening of a large number of samples at one time. Automation of the PCR and ELISA procedures enables large-scale screening of samples in a short period of time. However, one of the disadvantages of the PCR-ELISA is the inability of this method to confirm the identity of the amplified PCR products. This shortcoming can be avoided by the use of oligonucleotide capture formats for the detection of PCR products in an ELISA. For example, digoxigenin-based PCR-ELISA (DIG-PCR-ELISA) requires a hybridisation step with a biotinylated oligonucleotide probe immobilized onto micro plate wells with Streptavidin (Luk *et al.*, 1997; Manzano *et al.*, 1998). The PCR system that was

described in Chapter 6 followed by an ELISA could be evaluated for the detection of *S.* Brandenburg infection in sheep.

Alternative proteins also may be evaluated as potential candidates for vaccines and serodiagnostic ELISAs. Clone 226 listed in Table 3.2 of Chapter 3 was predicted to be a putative fimbrial chaperone (protein/pili assembly chaperone) with an identity and similarity of 99% to that of *S.* Paratyphi. Fimbrial proteins of *Salmonella* have been shown to be immunogenic (Strindelius *et al.*, 2004b), and protective in poultry against challenge with *S.* Enteritidis (De Buck *et al.*, 2005; Li *et al.*, 2004). Furthermore, serodiagnostic ELISA formats have been evaluated with fimbrial proteins (Hoorfar *et al.*, 1996). Further screening of blue colonies obtained from the *S.* Brandenburg expression library may reveal immunogenic proteins that have the potential as vaccine candidates or diagnostic antigens.

In summary, the studies presented in this thesis have contributed to the general body of knowledge relating to immunogenic proteins of *S.* Brandenburg. The application of PhoA technology to this organism has resulted in the isolation of the *S.* Brandenburg *fliC* gene, which was sequenced and characterised. The information obtained was used to develop sensitive and specific PCR and ELISA assays to detect this important zoonotic pathogen.

Appendix 1. Predicted export signals for *S.* Brandenburg fusion proteins listed in Table 3.2

Clone No.	Amino acid sequence[a]	Export signal[b]
2	MKMSIAMLSALASFIVVG↓CTPRIEVAAPEQPITINMNVKIRIRTVPDSYTQVASWTEPEPFC	LSP 18
7	MKIKTGVGILALSALTTMMISAPALA↓KIEEGKLVIWINGDKGYNGLAEVGKKFEQDTGIKVTVEHPDKLEEKFPQVAAT GDGPDIIFWAHDRFGGYAQSGLLAEVTPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLVPNPPKTWEEI RIRTVPDSYTQVASWTEPEPFC	SSP 26
8	MKKWAVVISAVGLAFAVSG↓CSSDYVMATKDGRMIRIRTVPDSYTQVASWTEPEPFC	LSP 19
13	VRYLVTLLLSLAVLVTAG↓CGWHLRSTTQVPASMKTMIRIRTVPDSYTQVASWTEPEPFC	LSP 18
14	MMLHWITIEEVLVDRAKPFVWRLVAASVCLLTFCHLARA↓DSLEEQRNRYAQIKQAWDNRQMDVVEQMMPGLKDYPL YPYLEYRKIRIRTVPDSYTQVASWTEPEPFC	SSP 39
16	MIKELDMQLRKLTTAMLVMGLSAGLAHA↓EDGAPAAGSTLDKIAKNGVIRIRTVPDSYTQVASWTEPEPFC	SSP 28
18	VMIKNVLLTLIIWNGILLLGG↓CSSVMSHTGGKEGTYPGTRASATMIGNDETNWGTKSLAILDMPFTAVMDTI RIRTVPDSYTQVASWTEPEPFC	LSP 21
20	VNTLSVSRLALALAFGVTLSA↓CSSTPPDQIRIRTVPDSYTQVASWTEPEPFC	LSP 21
24	MKLKLVAVAVTSLLAAGVVNA↓AEVYNKDGNKLDLYGKVHAQHYFSDDNGSDGDKTYARLGFKGETQI RIRTVPDSYTQVASWTEPEPFC	SSP 21
28	VIGVVCALLVSILLSSEAKIIMSWQIIFKQTWLIKFWAPAPAVIAAGILSTYYFWAVTGITGTF GEFTRWGGQILQLFGVHAEQWGYYKLIILEGTPLTRIDGMMIRIRTVPDSYTQVASWTEPEPFC	TMH (1) 38-62

Appendix 1. Continued

29	MRKQLPVICVAAGIVLLAA↓CTNDGGQQQTTVAPQPAVCNGPTVEISGAEPRYEPLNPTANQDYQRDGKSYKIVQDP SRFSQAGLAAIYDAEPGSNLTASGEMFDPMQLTAAHPTLPIPSYARITNLANGRMI RIRTVPDSYTQVASWTEPEPFC	LSP 19
34	MPRSTWFKALLLLVALWGPAVQA↓DIGWHPLQETIRKSDKDTRQYQAIRLDNDMVVLLVSDPQAVKSLSALVVPVGSL EDPEAHQGLAHYLEHMCLMGSKKYPQADSLAEYLKRHGGSHNASTAPYRTAFYLEVENDALPGAVDRLADAIAAPLLD KKYAERERNAVNAELTMARTRDGMRMAQVSAETINPAHPGSHFSGGNLETLSDKPGNPVQQALI RIRTVPDSYTQVASWTEPEPFC	SSP 23
35	MKPTFMRWVAIAALLAGGTFSAVA↓NPPVTPPVSYGVEEDVFHPVRATQGMVASVDAMATQVGVDILKQGGNAV DAAVAVGYALAVTHPQAGNLGGGGFMLLRTKDGATTAIRIRTVPDSYTQVASWTEPEPFC	SSP 24
36	MKFKYALTSLALSVAILSSVPSTAFA↓IGGASGAKVDYQVQGKIGEVVMNPYDIAPLTAVIRNGGYQLRDVHVRIVPK ENGQEIRIRTVPDSYTQVASWTEPEPFC	SSP 26
38	MAGNDREPIGRKGKPSRPVKQKVSRRRQIIDDDYDDDYEDEEPMPRKGKGKGRKPRGKRGWLWLLLKLFIVFVVLFAI YGVYLDQKIRSRIDGKVWQLPAAVYGRMVNLEPDMPVSKNEMVKLLEATQYRLVTKMTRPGEFTVQANSIEMI RIRTVPDSYTQVASWTEPEPFC	TMH (1) 60-82
41	MKYRIALAITLFTLSAGSYA↓NSLCQEKEQDIQKEISYAEKHNNQRRIEGLNKALSEVRANCTDSKLRAEHQKKI RIRTVPDSYTQVASWTEPEPFC	SSP 20
43	MISLRHTALGLALSLAFTGQALA↓VTTIPFWHSMEGELGKEVDSLAQRFNQANPDYKIVPVYKGNYEQNLSAGI RIRTVPDSYTQVASWTEPEPFC	SSP 23
44	MDRFPRSDSIVQARSGLQTYMAQVYGWMTVGLLLTAFIAWYAANTPAVMMFVFSSKIRIRTVPDSYTQVASWTEPEPFC	TMH (1) 24 - 43
45	MFSATRRFAVILALGVGFILPAQA↓ASPGPGEIANTQARHIATFFPGRMTGSPAEMLSADYLRQQFTQMGYQSDIRTF NSRFIYTTKDNRKNWHNVTGSTVIAAHEGRVPQQIRIRTVPDSYTQVASWTEPEPFC	SSP 24

Appendix 1. Continued

46	MLNSIFIIFCLLAVSAFFSISEIRIRTVPDSYTQVASWTEPEPFC	TMH (1) 1-16
47	MKKYLAFAVTLLGMGKVIA↓CTTLLVGNQASADGSFIIARNEDGSANNAKHKVIHPVAFHQQGEYKAHRNNFSWPLPE TAMRYTAIHDFDTNDNAMGEAGFNSAGVGMSATETIYNGRAALAADPYVTKTGITEDAIESVIRIRTVPDSYTQVASWT EPEPFC	SSP 19
89	MAFKFKTFAAVGALIGSLALAG↓CGQDEKDPNHIKVGVIVGAEQQVAEVAQKVAKEKYGLDVELVTFNDYVLPNEALS KGDIDANAFQHKPYLDQQIKDRGYKLVSVGKTFVYPIAGYSKKIKSLDELKVGSQVAVPNDPTNLGRSLLLLQKVGLIKL KDGVGLLPTSLDIVENPKNLKIVELEAPQLPRSLDDAQIALAVINTTYASQIGLTPAKDGMFVEDKDSPYVNLIRIRTVPDS YTQVASWTEPEPFC	LSP 22
99	MEGADLLTAGVLFLFAAVAAVPLAARLGIGAVLGYLLAGIAIGPWGLGFISDVDEIRIRTVPDSYTQVA SWTEPEPFC	TMH(2) 6-25 32-50
103	MKHPLESLMTAAGILLMALLSSLLLPAPSLGLALAQKLVGIRIRTVPDSYTQVASWTEPEPFC	TMH (1) 1-33
115	MEPKKGEAKVQKVKNWSPVWIFPIVTALIGAWILFYHYSHQGPEVTLITTNAEGIEGGKTTIRIRTVPDSYTQVASWTEPEP FC	TMH (1) 19-36
119	MRYDISRGAICYGFFMRLLKRVIVVVLLGVILFMVRDDIRYVYQLIRIRTVPDSYTQVASWTEPEPFC	TMH (1) 13-35
123	MASPLSLLIGLRFSRGRRRGGMVSLISVISTIGIALGVAVLIVGLSAMNGFERELNNRILAVVPHGEIEAVNQPWTNWREA LAKVQKVPGIAAAAPYINFTGLVESGANLRAIQVKGVDPKQEQQLSALPSFVQNHAWDIIFKAGEQQIIIGKGVADALNV KQGDWVSIMIPNANADIIKLLQPKRVRLIIVIGILQLSGQLDIISFAMIPLEDAQQYLDMGSSVSGIALKVIIDVFNANKLVR DAGEVTNSYVYIKSWIRIRTVPDSYTQVASWTEPEPFC	TMH (1) (21-48)

Appendix 1. Continued

134	MTPGELRATWGLGTVFSLRMLGMFMVLPVLTTYGMALQGASEALIGIAIGIYGLAQAIFQIPFGLLSDRIGRKP LIVGGLAVFVAGSVIAALSHSIWGIILGRALQGSGAIAAAVMALLSDLTREQNRTKAMAFIGVSFGITFAIAMVLGPI VTHSLGLNALFWMIAALATLGILLTIWVVPNSTNHVLNRESGMVKGSFSKVLAEPRLLKLNFGIMCLHILLMSTFVA LPGQLADAGFPAAEHWKVYLATMVIRIRTVPDSYTQVASWTEPEPFC	TMH (6) 12-31 43-63 75-93 99-119 131-152 158-180 208-229
142	MRIHILGICGTFMGGLAMLARSLG↓HEVTGSDANVYPPMSTLLEKQGIDLIRIRTVPDSYTQVASWTEPEPFC	SSP 24
151	MQNRLQSGGRLGRQALLFPLCLVLYEFSTYIGNDMIRIRTVPDSYTQVASWTEPEPFC	TMH (1) 15-32
167	MTQFITHKWLPALGLASSIAAFPALA↓AKDVVVAVGSNFTTLDPYDANDTLSQAVAKSFYQGLFGLDKDMKVK NVLA EGYTVSDDGLTYTITLRQGVKFQDGADFDAAAVK ANLDRASNPDNHLKRYNLYKNIAKTEVVDPATVKITLKQPFSAF INILAHPATAMIRIRTVPDSYTQVASWTEPEPFC	SSP 26
173	MTEKVKQSAAVTGSDEIDIGRLVGTVIEARWWVLGTTAIFALCAVIYTFFATPIYTADALVQIEQNAGNSLV QDINSALAN KPPASDAEIQLIRSRLVLGKTVDDLDLDIAVTKNTFPLFGAGWERLMGRIINEMVKVTTFTRPETMSGQIRIRTVPDSYTQV ASWTEPEPFC	TMH (1) 31-53
179	MENSNRKPGWIKRVWRWWRSPSRLALGTLLLIGFIGGIIFWGGFNTGMEKANTEEFCISCHEMRNTVYEEYMET VHYNN RSGVRATCPDCHVPHEWGPKMIRIRTVPDSYTQVASWTEPEPFC	TMH(1) 21-43
194	MKMFVPAVVFAALASASAWA↓NNSDTAQPLEKIAPYRQAEKGMKRQVITLTPQQDESTLKVELLIGQTLNVDCNQHRLG GTLETKTLEGWGYDYYVFDNVTSPVSTMMACPEGKKEQKFVTAWLGEDGMLRYN SKLPIRIRTVPDSYTQVASWTE PE PFC	SSP 20
197	MNKTRQKELTRWLKQQSVISQRWLNISRLLGFMSGVLIVAQAWIRIRTVPDSYTQVASWTEPEPFC	TMH (1) 24-43

Appendix 1. Continued

219	MAMVQPKSQKLRLFITHLGLLIFIAAIMFPLLMVIAISLREGNFATGSLIPDKISWEHWRLALGFSVEHAIDGRVTPPFPVLL WLWNSVKIAGITAIGIVALSTTCAYAFARMRFPGKATLLKGMLIFQMFPAVLSLVALYALFDRLGQYIPFIGLNTHGGVI RIRTVPDSYTQVASWTEPEPFC	TMH (3) 12-39 90-109 121-143
226	MKRMIALCLACAAMPAWS↓GIYIYGTRIIYPAQKKDITVQLMNDGKRSSLIQAWIDNGDTSLPPEKLQVPFIMTPPVIRV AANSGQQLKIKKLANNLPGDRESLFYLNVLDIPPNSDENKDKNIIKFALQNRIKLIRIRTVPDSYTQVASWTEPEPFC	SSP 18
231	MHRLHAYPDLRTMFRRLLIATLIGILAALAVAAFRHAMQLLEWIFLSNDTGSLVNAAEGLSPWRRLITPALGGLAAGL LLWGWQKMNQQRPIIAPTDYMEALQTDGQFDVGASLVKSLASLLVVVSGSAIGREGAMIRIRTVPDSYTQVASWTEP EPFC	TMH (3) 17-34 65-82 113-130
251	MKAFSPLAVLISALLLQGCVAA↓AVVGTAAVGTKAATDPRSVGTQVDDGTLELRVSSALSKDEQIKKETRINVT AYQ GKVLLVGQSPNSELSARAKQIAMGVEGTTEVYNEIRQGQPIGLGTASNDTWIRIRTVPDSYTQVASWTEPEPFC	SSP 22
277	MGITSRLKVMSFLQYFIWGSWLVTLGSYMIRIRTVPDSYTQVASWTEPEPFC	TMH (1) 9-30
285	MKLNLITVSLATLVAAGAFPAHA↓GPQAHVVCGYHHTLGDDAIMMFGKANQAMWHDFFGNTHTDAVSTYQT LRAQ QPDTTCDNKADSSAYWAPSMKLPDGEIVNPAYQKTYYQSTNVAQYPLHPFPAGLELLAGDHHGTGPSSAITFLCANGK GYTNKVGEICGLRKAGDAVQFNIGIAFPNCWDGVNLKPTHSHNNAIYADHGKCSADYPVKIPTVNMNIAWVLPQIRIR TVPDSYTQVASWTEPEPFC	SSP 23
318	MKKRVFVIAAIVSGALAVSG↓CTTNPYTGEREAGKSGIGAGIGSLVGAGIGALSSSKKDRGKGALIGAAAGAALGGGV GYYMDVQEAKLRDKMRGTGVSVTRSGDNIILNMPNNVTFDSSSATLKPAGANTLTGVAMV LKEYPKTAVN VVGYT DSTGSHDLNMRLSQQRADSVASSLITQGVDASRIRTSGMGPANPIRIRTVPDSYTQASWTEPEPFC	LSP 20
319	MIPPEIRRSVLLQKAIKLALAGTLLTFASFSATA↓ADPSSDTETPQPPDILLGPLFNDVQNAKLFPDQKTFADAIPNSDPL MILADYRMQRNQSGFDLRHFVDVNFTLPKAGEKYVPPAGQSLREHIDGLWPVLTRSTKNVEKWDSLL PLPESYVVPG GRFREIYYWDSYFTMLGLAESGHWDKVADMVANFGYEIDAWGHIPNGNRTYYLSRSQPPFFAFMVELLAEHEGDDA LKEYLPQLQKEYAYWMEGVETLQPGQQNQRVVKLEDGSVLNRYWDDRDTPRPESWVEDIATAKSNPNRPATEIRIRT VPDSYTQASWWTEPEPFC	TAT 34

Appendix 1. Continued

320	MPNFFIDRPIFAWVIAIIIMLAGGLAIRIRTVPDSYTQVASWTEPEPFC	TMH (1) 13-30
321	MTEQQQISRTQAWLESLRPKTLPLAFAAIIVGTALAWWQGYFDPLVALLALITAGLLQILSNLANDYGDAVKGSDKPDRI GPLRGMQKGVITRQEMKRALIITVVLIRIRTVPDSYTQVASWTEPEPFC	TMH (2) 21-38 45-61 99-116
368	MKKVIFSLALGTFGLGMAEFGIMGVLTELARDVGITIPAAGHMIRIRTVPDSYTQASWTEPEPFC	TMH (1) 6-26
387	MATLDMQNTAQLAESRRKMQARRRMKNRIALTLSMATMAFGLFWLIRIRTVPDSYTQASWTEPEPFC	TMH (1) 29-46
391	MFGKLSLDAVPFHEPIVMVTIAAIIVGGLAILAAITYFGKWTYLWKEWLTSVDHKRLGIMYIIVAIVMLLRGFADAIMM RSQQALASAGEAGFLPPHHYDQIRIRTVPDSYTQVASWTEPEPFC	TMH (2) 17-38 57-79

[a]PhoA linker (underlined), for inner membrane proteins cytoplasmic loops (pink), transmembrane helices (orange), periplasmic loops (green).

[b]Export signals, and their cleavage sites of the fusion proteins were predicted using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/), LipoP 1.0 (http://www.cbs.dtu.dk/services/LipoP/), TatP 1.0 (http://www.cbs.dtu.dk/services/TatP/), TMHMM v.2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), and HMMTOP v.2.0 (http://www.enzim.hu/hmmtop/) servers. SSP, standard signal peptide 1; LSP, lipoprotein signal peptide; Tat, twin-arginine signal; TMH, transmembrane helix. The number and location of predicted TMHs are shown in parentheses. Cleavage sites of fusion proteins carrying SSP, LSP and Tat are indicated with a red arrow. Seventeen had type I signal peptides, 8 had type II lipoprotein signal peptides, 1 had Tat signal, and 22 contained one or more TMHs. Twenty-one of the latter group were located in the inner membrane, while the other one was a periplasmic protein attached to it through a TMH. Topology of inner membrane proteins were analysed by TMHMM v.2.0, HMMTOP v.2.0, Phobius (http://www.phobius.cgb.ki.se/), TopPred (http://www.bioweb.pasteur.fr/seqanal/interfaces/toppred), MEMSTAT v.3.0 (http://www.bioinf.cs.ucl.ac.uk/psipred/), Tmpred (http://www.ch.embnet.org/software/TMPRED-form.html), and PHDhtm (http://www.predictprotein.org/) servers.

Appendix 2. TMH predictions for *S.* Brandenburg fusion proteins listed in Table 3.4

Clone No.	Amino acid sequence[a]	Number of TMH predictions
11	LVMAYQFGTNWSGFSQFAGSITGPLLTYEVLTAFFLEAGFLGVMLFGWNKVGPGLHFLSTCMVALGTLMSTFWILAS NSWMHTPQGFEHHNGQVVPVDWFAVIFNPSFPYRLLHMSVAAFLSSAMFVGASAAWHLLKGNDTPAIRRMFSMAL WMAVVVAPVQALIGDMHGLNTLKHQPVKIAAIEGHWENTPGEPTPLTLVGWPD MEAERTRYALEIRIRTVPDSYTQ VASWTEPEPFC	4
32	IGLLGLLVGYLVVLMYVQGEYLFAIMTLILSSAGLYIFANRKTYAWRYVYPGLAGMGLFVLFPLVCTIAIAFTNYSST NQLTFERAQQVLMDRSYQAGKTYNFGLYPTGDEWQLALTDGETGKIIYLSDAFSFGGEQKLQLKETDALPGGERAN LRIITQNRLALNQITAVLPDESKVIMSSLRQFSGTRPLYTLADDGLLTNNQSGVKYRPNNDSGYYQSINADGSWGDEK LSPGYTVTIGAKNFTRVFTDEGIRIRTVPDSYTQVASWTEPEPFC	2
62	VLFVMFETNANEASEYLSQYFSLKIVLVALAYTVAAILLWTRLRPVYIPSPWRYLVSFALLYGLILHPIAMNTFIKHK SMEKTLDSLASRMEPAAPWQFITGYYQYRLQLASLNKLLNE NDALPPLANFQDHSGDAPRTLVLVIGESTQRGRMS LYGYPRETTPELDALHKTDPGLTVFNNVVTSRPYTIEILQQALTFADEKNPDWYLTKPSLMN MMKQAGYKTFWIRI RTVPD SYTQVASWTEPEPFC	2
81	LVINICSLSWSDVEAAGLMSHPLWSHFDILFKHFNSGTSYSGPAAIRLLRASCGQPSHTRLYQPADNECYLFDN LAKL GFTQHLMMDHNGEFGGFLKEVRENGGMQSELMNQSGLPTALLSFDGSPVYDDLAVLNRWLTGEEREANSRS ATFF NLLPLHDGNHFPGVSKTADYKIRAQKLFDELDAFFTELEKSGRKVMVVVVPEHGG ALKGDRM QIRIRTVPDSYTQV ASWTEPEPFC	0
195	LAHVLGPVIIPVYEMLGANPSMFAGTLLACDMGGFFLAKELAGGDVAAWLYSGLILGSMMGPTIVFSIPVALGIIEPS DRRYLALGVLAGIVTIPIGCIAGGLIAMYSGVQINGQPVEFTFALIRIRTVPDSYTQVASWTEPEPFC	3

Appendix 2. Continued

225	MKANVASAQLQLDWSRITAPVSGRVGLKQVDVGNQISSSDTAGIVVITQTHPIDLIFTLPESDIATVVQAQKAGKALV VEAWDRTNSHKLSEGVLLSLDNQIDPTTGTIKIKARFTNQDDTLFPNQFVNARMLVDTEQNAVVVPAAAVQMGNEG HFVWVLNDENNVSKKRVKIGIQDNRNVVIRIRTVPDSYTQVASWTEPEPFC	0
228	LENVTTVTRQYGDAFATRFAQLYRNITDAPIIKPFNSQTFTNALTHFLFLAVAVFGFYSVIRLCALPLYRKMGLWARK KNRERSNWLQLPAMIVGAFIIDLLLLALTLFIGQMLSDNFHAGSRTIAFQQSLFLNAFALIEFFKAILRLIFCPNVPELRP AIQDATARYWNRRMSSLSSLIGYGLIVAVPIISNQVNVQVGAMANVAIRIRTVPDSYTQVASWTEPEPFC	4

[a]PhoA linker (underlined), cytoplasmic loops (pink), transmembrane helices (orange), periplasmic loops (green).

Topology of inner membrane proteins were analysed by TMHMM v.2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), HMMTOP v.2.0 (http://www.enzim.hu/hmmtop/), Phobius (http://www.phobius.cgb.ki.se/), TopPred (http://www.bioweb.pasteur.fr/seqanal/interfaces/toppred), MEMSTAT v.3.0 (http://www.bioinf.cs.ucl.ac.uk/psipred/), Tmpred (http://www.ch.embnet.org/software/TMPRED-form.html), and PHDhtm servers (http://www.predictprotein.org/).

Appendix 3. Predictions of start codons, SD sequences, and the -10 and –35 sites for *S.* Brandenburg inserts listed in Table 3.2

Clone number (PS)[a]	DNA sequence of *S.* Brandenburg inserts[b]
2 (0.78)	GGATCGTTAACTTGAACTATACCCATGAAGAAAATGTGTTTACGCTGTGGCGCAGTTTGCGTTTTGGCGACAATTTACAGGCATGG CTTGAACAAAATACAGCGTTGCCGCAACCTCCTTGCCGGAAAGACAAGGACTGTGAGGATAAATA
7	AAGGATGGGAGATATG
8 (0.75)	GACTATTATCAGACATATGATTAACAGACTATTATCAGATGCAGGCTATGTGAGGACAAACGGATG
13 (0.80)	CATCTGGTGGCAAAATATCTTGATGGCGTTACCGTGCGTAAAGTGATTTACGTTCCGGGTAAACTCCTCAATCTGGTTGTTGGCTA AGCGCGGGAGGAAGC
14 (0.74)	GTAACGTGGCGCAAATGGCTATGACTGGTTAGCATAAATTCATTACGCGGCATG
16 (0.87)	GCACTTCGCAAAAATAAACAATATAGCAGCAAAATATTTACAACAAGCCACACTAAATGTTAACAAACACACATAACACTGCACGC GCAAGTTGCAGGCAATAACAACATCACAATAGCTATCAATGCGTCGACGGCGCAGATG
18 (0.98)	GTTATTTTTAGCAACGGATCGCTATAACTCATCATGCAAATAACGACAACCAACAGATGAATGAGTG
20 (0.72)	ACCTTTTCGCCTTCCCGTTTCGTTCAACTTAGTATAAAAAAGCAGGCTTCAATGGATGTCATTTAACTTTTTCAAGCCCGGAGCAA CCT GTG

Appendix 3. Continued

24 (0.89)	TATTATTGAACTTATGCCACTCCGTCATTTAAAAATAGTCTGCCATTGACAAACGCCTCGTTTAACAATGGTTGAGGAAACACGC TAAGAAAATTATAAGGATTATTAAA
28	AGGTGTAGGCAAATTCTGTG
29 (0.98)	GGGATTGTGATGTCGATCCACACCCACAGAAAAATGTTGTCGAAAAGCGTATAAGGGGTGCGCAATG
34	ATGAGGTCCGTGAATG
35 (0.99)	GCCTCTTTTTAAGTGTACCTCTATTTCGCCATGATGATGATTTTTTAAGCGTTGTGGACCAGGATTAAACGGGACATACACCCATT TATCTGGAGAAAAACAACG
36 (0.99)	CTTTGTATTGCGTGTAATAAATTAAGTAATATTATGTCCTGAGGCTATCAATAAAAATAGTTAATTATATATGCTTCATTTATTTT TATAAAAGAAACATAAAATGGAAAGTTAGTTATTTATTTATAAGATGGAGAGTGTTATG
38 (0.54)	TTCTTCTTCTGTCGTCTGGCGGAAGAATAGAGAATCGGGCCTTTGCGCCTGTATGTTGCGGAGAAAAAGCATG
41 (0.81)	TAAAGCGGTGTTTAACCCACCGCGTTATTATCGGGATCACCGCCCCGGACAACGGGTCCGAACATAAGGGAAAGTTCAGGAATG
43 (0.97)	TTTTTTTTGTAATAAAAAAGTTATTTTTCTGTCATTCGAGCATGTCATGTTACCCCCGCGAACATAAAACGCGTGATATCGCGCAT CCCGGCACAAAAAGAGAGATAACCG
44 (0.71)	GCGCGTTCGACGCCGGCCAGTTCATTATGGATTATCTGAAAACCCGTGCGCCGTTCTGGAAGCGTGAAGCCACGCCGGAAGGCGAT CGCTGGGTCGAAGCGCGCGACAGCGACCAGCAGTCGGCGAAACGTTGGTAGCAGAATGTGATACGCTTTAGGGTATCATTTCCACA CAGGAGTTCATC

Appendix 3. Continued

45 (0.80)	TACCGGATTGCCAGCATTTAGGAGCAAATGAAATTGTATTAACACGATCGCGGTTTCATACTAGCCACGTAAAATTTTGTTCTGTA TTTAAGGACTCATTATG
46 (0.96)	GGTTTTCATTAAACGTTACCACACGGATTTATAAACTTCCCTTCCGAGGATCTGGCGTTTCGCCGGATAAGATATG
47 (0.80)	ATATTGCTGGCTCGATCACTTTCTCGGTTTCCAATCCAGGGTCAATTCATCCCATAACGCGTTATTCAGTGGGAACCTTATG
89	TAAGGAAATAAGCATG
99 (0.50)	ATGTGCCGGATGCACTGGATGCCGCCCATTATTGTGTACTGGGCGCGGCGCCAGTCGCCGCAGACGCTGGCCAGTCATGCGAAAGC CTATGGCGAGTGGCTGGCGAATCCTGTGAGCGCGGGAGGCTATTGATG
103 (0.92)	ACATTGTGAAATCTGGGCGGGACAATAGACAGCATATTTGCCTTACCAGGCGATATCCCGGACAATGGCGTTTTAACTTACGTTTA ACAAAGCCATG
115 (0.65)	GCGTTGATGTTTGCTTTAGTCGTCATAATGACAATGTTTTCTGCGATGACCTTTGATCCGCGTCTGTCGTGGGATCGTGAATACGA ACCAGGCCATGAGGAGTCCTGATAGCATG
119 (0.98)	TCAATTTACAAAAAGGGTACGTTAGTGATTAAAATCGGGCTTTTTGTGATAACCCTCACTATTTCATTTTGCCTATGCTAATGGCG CTCGCCATAACCGAATAACGATTCTATCGGACATCGGCTTTATTTTTAAGAA
123	CCTGATGGGGGCTGAGTAATG
134	GATTATAAAATG

Appendix 3. Continued

142 (0.87)	ATGCGTTTCTCACTTGCCAGACATGTGCGTAAAATGGCTCGCAGATAAAAAAAGGATAGTGACGTATG
151 (0.90)	AAATAATGTCGATGATAACGGCGCAAACTTAAATTAACCTTAAAGTACGCAAGCGCTAAGTGGCTTTCTGTAAAAATGTGCG CTATTTCCCTTGCAGCCTCATAATTCGCTATCTCAGTTCCCCGACTTTCATTACACTCTGCGCGGTTTTTATTGGCGAGAGATGGT ATG
167 (0.87)	CCGTTTCATTGCAGTTGGTCGGGCCGGGACATTATGTTCGCGGCCGCTGCAGGATAAACGTCTCGCGCTTATAACATTCAGACAG GCAGGGTTTCAGGAGAACAACATG
173	GCAGGGAAAACCATG
179 (0.54)	CAGTCTGATACCGCGCCGATTATCGACAATACCTTTACCCCCTCGCAAGGTTACGGGAAATAAGAGGTCATTATG
194 (0.96)	TATTTTATTAATAATCTTGAAAATATGAGTAAATACAGATTAATAGATAATATGAATGTGTTAATCTGTTGTCCGTGCATACCGAA AACAGCAGAAAGGAAATACTGTGAACAATCAA
197	GCAATG
219 (0.77)	TCGTGAACCTGAAAGCCACGCGTATGAAGTTTGATTAAGGGAGATAATAACAATG
226 (0.89)	TTCTGTTGAATCTGTACTGACAATGACGGTATTAACTGATTAACAATCTTTTTTTGGCGGCTAAGCTTTTAGCCGCTTGTATCTCT TTTATAAGGTTATCGGCCATG
231 (0.79)	ATCATTATTGTACGCAATATTATATCGCACATAGTGCTTTTGCGTCTGACGCCATTTCCCCGCAGAATAGGCTATCCTGAAACTCC CTGTTTACCCGCTC
251 (0.85)	CATATGCTGACGGTAAACTGCCTGTGCGATTTGATCGATAACACGCTTTTTCCTCACCAGGATGATTAAGGAGTACACATG

Appendix 3. Continued

277 (0.56)	CACCAATCTGGCGGAAGGGCTGGGCGATGTAAAACTCTCCCATGCGCAAACGCTGGCTGCGGCTGAACTTTCTCGGCAGAACTTTA TAAATCTGATTTGCGGCTTTTTACGTAAGCTGGCCTAAACACAATGACTACGGCCCCGTATTGGGGCTTTATCTCACGGCAAGGAA TAAAAAATG
285 (0.85)	CAATTTGATCTGCTATTGCTGCTCCCACTTCTATTCAACCGGGCAGCAAAAAAATG
318	TAAGGATTTAAGATG
319	CAATG
320 (0.61)	TACGTCCTGGCGCACAGGTTAAAGTGCAGGAAATTACCGCGGATAACAAACAGCAAGCCGCAAGCGGTGATCAACCTGCTCAGCCC AGGTCTTAACTTAAACAGGAGCCGTTAAGACATG
321 (0.84)	CGCAGATGAAGATCTAAGCCGTTTTATCTTATAATCGCGTCCAATGCATTTTCATCACTGTTGATGGGGCTGAAAAGCCCCATTTT TATTGGCGCTAAATATG
368 (0.73)	TGGCTGTGACAATTCACAGCCACAGCGCGCAACATGCGCCTTTATTTTCGGCAGGTGGTATG
387 (0.17)	TTTATCGTCCTGGCGGCGTCTAAGTTCATGATTATGCGTCTGGCGAAGAACGAGGGAGCACGTTAATG
391 (0.75)	CAAGGTGGAATATTTCTCCAGCGTGAAACCAGATTTGTTCAAGGACGTTATTAACAAATTCATGGACCACGGTAAGAGCATGGACA TGACCCAACCTGAAGGTGGGCACAGCTCGCATGAAGGAATGGAAGGTATGGACATGAGCCACGCGGAATCCGCCAACTCCAAGGGT TGAGGAAGATAAGATG

[a]PS, Promoter score was predicted by neural network server at http://www.fruitfly.org/seq_tools/promoter.html.
[b]Start codon (green shaded), SD sequence (orange), transcription start site (pink underlined), -10 site (yellow shaded), -35 site (blue shaded). Due to partial sequencing, it was unable to predict the SD sequences of inserts in clones 134, 197 and 319, and the –10 and –35 sites of inserts in clones 7, 28, 34, 89, 123, 134, 173, 197, 318 and 319.

Appendix 4. Predictions of start codons, SD sequences, and the -10 and –35 sites for *S.* Brandenburg inserts listed in Table 3.3

Clone number (PS)[a]	DNA sequence of *S.* Brandenburg inserts[b]
145	AACAGGACGACAGGGCGTAAAATCGTGGGACATCTATG
222 (0.89)	GTGCGTATTTGCGGCGAGGTCAGTGAACATCAAATCCTCCGACTACGTGCAGAAAAGATGTTACAGCAGCTTTCTGACAGGAG CTAAAAATG
273 (0.64)	CACTGGTCAGGATGAACGGGATACGGCAATATATGAACAGATATGAACAGAAAGAGACCGCGCGCAGGATG
298 (0.90)	ACGGTGAGAAACCGTGGGCAACAGCCCAATAACATCAAGTTGTAATTGATAAGGAAAAGATCATG
333 (0.95)	TCAGATTTTTTCAGTAGGATACCAGTAAGGAACATTAAAATAACATCAACAAAGGGATAATATG
337	TGTCACCCGCAAGAGAGATG
370 (1.00)	TTAGTTTGGACAGGCCGTAGCCAGATTTTTAATTTGTGAAATGGTGTGCTTATG

[a]PS, promoter score was predicted by neural network server at http://www.fruitfly.org/seq_tools/promoter.html.
[b]Start codon (green boxed), SD sequence (orange), transcription start site (pink underlined), -10 site (yellow boxed), -35 site (blue boxed).
It was unable to predict –10 and –35 sites for the insert in clone 337 due to partial sequencing.
Neural network server did not predict –10 and –35 sites for the insert in clone 145.

Appendix 5. Multiple alignment of FliC belonging to 13 *Salmonella* serotypes isolated from sheep in New Zealand

```
Ade     MAQVINTNSLSLLTQNNLNKSQSSLSSAIERLSSGLRINSAKDDAAGQAIANRFTSNIKG 60
Der     MAQVINTNSLSLLTQNNLNKSQSSLSSAIERLSSGLRINSAKDDAAGQAIANRFTSNIKG 60
Ago     MAQVINTNSLSLLTQNNLNKSQSSLSSAIERLSSGLRINSAKDDAAGQAIANRFTSNIKG 60
Ent     MAQVINTNSLSLLTQNNLNKSQSSLSSAIERLSSGLRINSAKDDAAGQAIANRFTSNIKG 60
Dub     MAQVINTNSLSLLTQNNLNKSQSSLSSAIERLSSGLRINSAKDDAAGQAIANRFTSNIKG 60
Mon     MAQVINTNSLSLLTQNNLNKSQSSLSSAIERLSSGLRINSAKDDAAGQAIANRFTSNIKG 60
Sen     MAQVINTNSLSLLTQNNLNKSQSSLSSAIERLSSGLRINSAKDDAAGQAIANRFTSNIKG 60
Ora     MAQVINTNSLSLLTQNNLNKSQSSLSSAIERLSSGLRINSAKDDAAGQAIANRFTSNIKG 60
Lil     MAQVINTNSLSLLTQNNLNKSQSALGTAIERLSSGLRINSAKDDAAGQAIANRFTSNIKG 60
Typ     MAQVINTNSLSLLTQNNLNKSQSALGTAIERLSSGLRINSAKDDAAGQAIANRFTANIKG 60
Bov     MAQVINTNSLSLLTQNNLNKSQSALGTAIERLSSGLRINSAKDDAAGQAIANRFTANIKG 60
Brn     MAQVINTNSLSLLTQNNLNKSQSALGTAIERLSSGLRINSAKDDAAGQAIANRFTANIKG 60
Sai     MAQVINTNSLSLLTQNNLNKSQSALGTAIERLSSGLRINSAKDDAAGQAIANRFTANIKG 60
Ana     MAQVINTNSLSLLTQNNLNKSQSALGTAIERLSSGLRINSAKDDAAGQAIANRFTANIKG 60
        ************************:*.:*****************************:****

Ade     LTQASRNANDGISIAQTTEGALNEINNNLQRVRELSVQATNGTNSDSDLKSIQDEIQQRL 120
Der     LTQASRNANDGISIAQTTEGALNEINNNLQRVRELSVQATNGTNSDSDLKSIQDEIQQRL 120
Ago     LTQASRNANDGISIAQTTEGALNEINNNLQRVRELSVQATNGTNSDSDLKSIQDEIQQRL 120
Ent     LTQASRNANDGISIAQTTEGALNEINNNLQRVRELSVQATNGTNSDSDLKSIQDEIQQRL 120
Dub     LTQASRNANDGISIAQTTEGALNEINNNLQRVRELSVQATNGTNSDSDLKSIQDEIQQRL 120
Mon     LTQASRNANDGISIAQTTEGALNEINNNLQRVRELSVQATNGTNSDSDLKSIQDEIQQRL 120
Sen     LTQASRNANDGISIAQTTEGALNEINNNLQRVRELSVQATNGTNSDSDLKSIQDEIQQRL 120
Ora     LTQASRNANDGISIAQTTEGALNEINNNLQRVRELSVQATNGTNSDSDLKSIQDEIQQRL 120
Lil     LSQASRNANDGISIAQTTEGALNEINNNLQRVRELSVQAANGSNSDSDLKSIQDEIDQRL 120
Typ     LTQASRNANDGISIAQTTEGALNEINNNLQRVRELAVQSANSTNSQSDLDSIQAEITQRL 120
Bov     LTQASRNANDGISIAQTTEGALNEINNNLQRVRELAVQSANSTNSQSDLDSIQAEITQRL 120
Brn     LTQASRNANDGISIAQTTEGALNEINNNLQRVRELAVQSANSTNSQSDLDSIQAEITQRL 120
Sai     LTQASRNANDGISIAQTTEGALNEINNNLQRVRELAVQSANSTNSQSDLDSIQAEITQRL 120
Ana     LTQASRNANDGISIAQTTEGALNEINNNLQRVRELAVQSANSTNSQSDLDSIQAEITQRL 120
        *:***********************************:**::*.:**:***.*** ** ***
```

Appendix 5. Continued

```
Ade          EEIDRVSNQTQFNGVKVLSQDNQMKIQVGANDGETITIDLQKIDVKSLGLDGFNVNGPKE 180
Der          EEIDRVSNQTQFNGVKVLSQDNQMKIQVGANDGETITIDLQKIDVKSLGLDGFNVNGPKE 180
Ago          EEIDRVSNQTQFNGVKVLSQDNQMKIQVGANDGETITIDLQKIDVKSLGLDGFNVNGPKE 180
Ent          EEIDRVSNQTQFNGVKVLSQDNQMKIQVGANDGETITIDLQKIDVKSLGLDGFNVNGPKE 180
Dub          EEIDRVSNQTQFNGVKVLSQDNQMKIQVGANDGETITIDLQKIDVKSLGLDGFNVNGPKE 180
Mon          EEIDRVSNQTQFNGVKVLSQDNQMKIQVGANDGETITIDLQKIDVKSLGLDGFNVNGPKE 180
Sen          EEIDRVSNQTQFNGVKVLSQDNQMKIQVGANDGETITIDLQKIDVKSLGLDGFNVNGPKE 180
Ora          EEIDRVSNQTQFNGVKVLSQDNQMKIQVGANDGETITIDLQKIDVKSLGLDGFNVNGPKE 180
Lil          NEINRVAEQTDFNGKKVLSQDGQLTIQVGANDGETITIDLQKIDKTELGLDKLDVTKGIA 180
Typ          NEIDRVSGQTQFNGVKVLAQDNTLTIQVGANDGETIDIDLKQINSQTLGLDTLNVQQKYK 180
Bov          NEIDRVSGQTQFNGVKVLAQDNTLTIQVGANDGETIDIDLKQINSQTLGLDTLNVQQKYK 180
Brn          NEIDRVSGQTQFNGVKVLAQDNTLTIQVGANDGETIDIDLKQINSQTLGLDTLNVQKKYD 180
Sai          NEIDRVSGQTQFNGVKVLAQDNTLTIQVGANDGETIDIDLKQINSQTLGLDTLNVQKAYD 180
Ana          NEIDRVSGQTQFNGVKVLAQDNTLTIQVGANDGETIDIDLKQINSQTLGLDTLNVQKAYD 180
                :**:**: **:*** ***:**. :.********** ***::*:   **** ::*

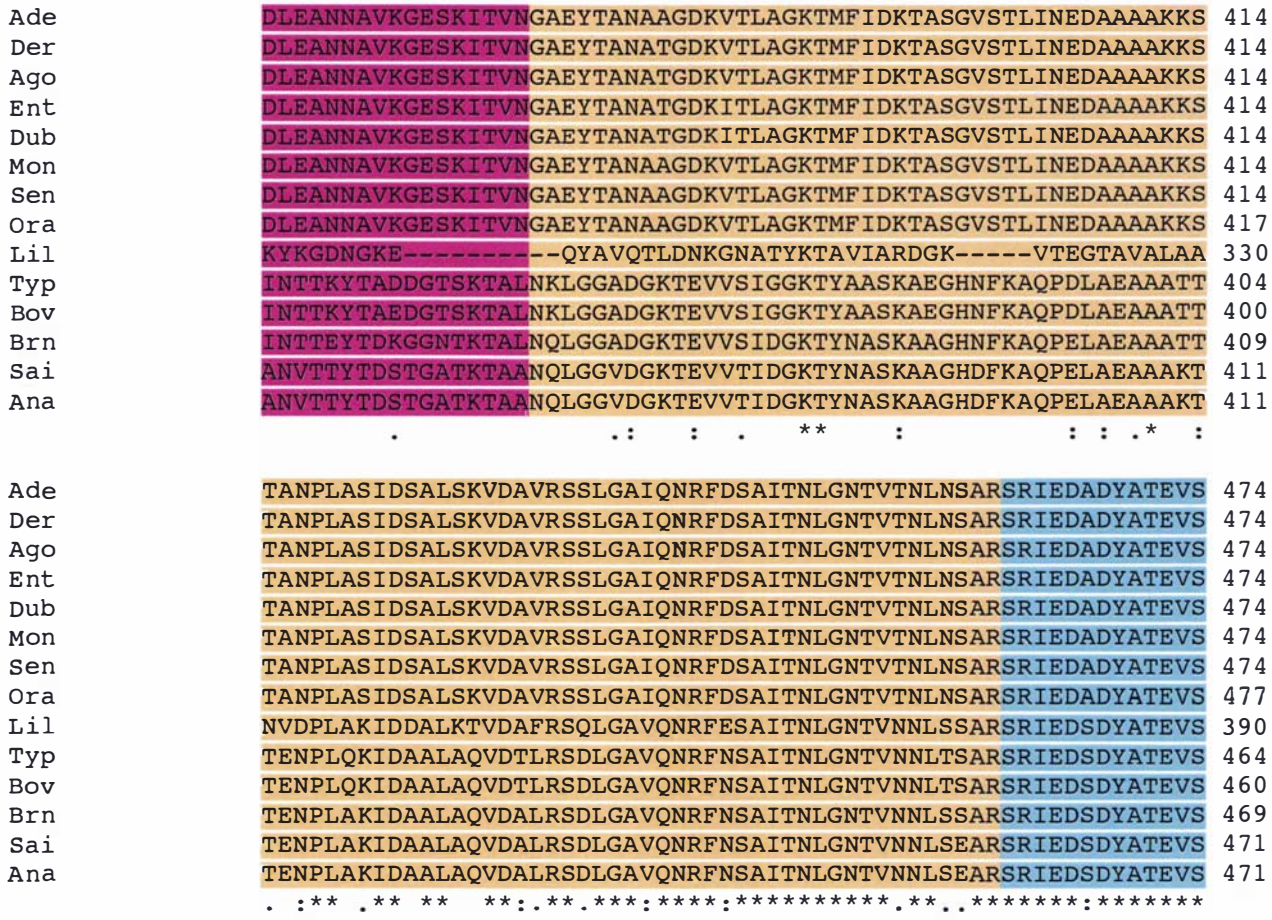
Ade          ATVGDLKSSFKNVTGYDT-YAAGADKYRVDINSGAVVTDAAAP---NKVYVNAANGQLTT 236
Der          ATVGDLKSSFKNVTGYDT-YAAGADKYRVDINSGAVVTDAVAP---NKVYVNAANGQLTT 236
Ago          ATVGDLKSSFKNVTGYDT-YAAGADKYRVDINSGAVVTDDAAP---DKVYVNAANGQLTT 236
Ent          ATVGDLKSSFKNVTGYDT-YAAGADKYRVDINSGAVVTDAAAP---DKVYVNAANGQLTT 236
Dub          ATVGDLKSSFKNVTGYDT-YAAGADKYRVDINSGAVVTDAVAP---DKVYVNAANGQLTT 236
Mon          ATVGDLKSSFKNVTGYDT-YAAGANKYRVDINSGAVVTDDAAP---DKVYVNAANGQLTT 236
Sen          ATVGDLKSSFKNVTGYDT-YAAGADKYRVDINSGAVVTDDAAP---DKVYVNAANGQLTT 236
Ora          ATVGDLKSSFKNVTGYDT-YAVGANKYRVDVNSGAVVTDTTAPTVPDKVYVNAANGQLTT 239
Lil          TTV---KEGTKLTADFAV-KDADFD----DKSTGTKVT---------------ANLELKQ 217
Typ          VSDTAATVTG-YADTT---IALDNSTFKASAT-GLGGTDQKIDG---DLKFDDTTGKYYA 232
Bov          VSDTAATVTG-YTDSA---TAIDKSTFAASAT-TLGGT-PAITG---DLKFDDTTGKYYA 231
Brn          VKSEAVKSGGGATLNT---TGLNDAALKTGVGGATNGTAAIKDG---KVFFDATDNKYFI 234
Sai          VSATAAMDPKSFTDGTKNLTAPDATAIKAALG-NPAATGDSLSA---TLSFKD--GKYYA 234
Ana          VSATAAMDPKSFTNGTKNLTAPDATAIKAALG-NPTATGDSLSA---TLSFKD--GKYYA 234
                ..         .        .              *               :
```

Appendix 5. Continued

```
Ade          DDAENNTAVDLFKTTKSTAGTAEAKAIAGAIKGGKEGDTFDYKGVTFTIDTKTGNDGNGK 296
Der          DDAENNTAVDLFKTTKSTAGTAEAKAIAGAIKGGKEGDTFDYKGVTFTIDTKTGNDGNGK 296
Ago          DDAENNTAVNLFKTTKSTAGTDEAKAIAGAIKGGKEGDTFDYKGVTFTIDTKTGNDGNGK 296
Ent          DDAENNTAVDLFKTTKSTAGTAEAKAIAGAIKGGKEGDTFDYKGVTFTIDTKTGDDGNGK 296
Dub          DDAENNTAVDLFKTTKSTAGTAEAKAIAGAIKGGKEGDTFDYKGVTFTIDTKTGDDGNGK 296
Mon          DDAENNTAVNLFKTTKSAAGTDEAKAIAGAIKGGKEGDTFDYKGVTFTIDTKTGDDGNGK 296
Sen          DDAENNTAVNLFKTTKSTAGTDEAKAIASAIKGGKEGDTFDYKGVSFTIDTKAGNDGNGT 296
Ora          ADAQNNTAVDLFKSTKSAAGTDDAKAIATSIKGGKVGDTFDYKGVSFTIDTKAGDDGNGT 299
Lil          DKSGN-----YFAYDKTAS----------------------KYYDATVDTATG------ 243
Typ          KVTVTG-G--TGKDGYYEVSVDKTNGEVTLAGGATSPLTGGLPATATEDVKNVQVANADL 289
Bov          DVSGT-----TAKDGVYEVTVAAD-GKVTLTGTPTGPITAGFPSTATKDVKQTQQENADL 285
Brn          EVEGLT-AGDATKNGVYEVSVADD-GTVTMP--TTTKVTGGMPATATAVTETQPKPVALS 290
Sai          TVAGYTNAADTSKNGKYEVNVDSATGAVTFN---AAPTKATVTGDTTVTKVQVNAPVAVS 291
Ana          TVAGYTNTADTSKNGKYEVNVDSATGAVTFN---AAPTKATVTGDTTVTKVQVNAPVAVS 291

Ade          VSTTINGEKVTLTVADITAGAANVDAATLQSSKNVYT--SVVNGQFTFDDKTKNESAKLS 354
Der          VSTTINGEKVTLTVADITGGAANVDAATLQSSKNVYT--SVVNGQFTFDDKTKNESAKLS 354
Ago          VSTTINGEKVTLTVADITGGAANVDAATLQSSKNVYT--SVVNGQFTFDDKTKNESAKLS 354
Ent          VSTTINGEKVTLTVADIATGATDVNAATLQSSKNVYT--SVVNGQFTFDDKTKNESAKLS 354
Dub          VSTTINGEKVTLTVADIAIGAADVNAATLQSSKNVYT--SVVNGQFTFDDKTKNESAKLS 354
Mon          VSTTINGEKVTLTVADIATGATDVNAATLQSSKNVYT--SVVNGQFTFDDKTKNESAKLS 354
Sen          VSTTINGEKVTLTVADITAGAANVNDATLQSSKNVYT--SVVNGQFTFDDKTKNESAKLS 354
Ora          VSTTINGEKVTLTISDIGASATDVNSAKIQSSKDVYT--SVVSGQFTFADKTKNESAKLS 357
Lil          --------KIEFTSG--TGKETTKDTSKLTDVTSLSK--EVTIDSGLTDDKS------LV 285
Typ          TEAKAALTAAGVTG----TASVVKMSYTDNNGKTIDGGLAVKVGDDYYSATQNK-DGSIS 344
Bov          TEAKAALTAAGVTG----TASVVKMSYTDNNGKTIDGGLAVKVGDDYYSATQNK-DGSIS 340
Brn          TAVKDQLTDSGISAADAAKGQLVTMSYTDKNGKTIDGGFGVKVGANIYAATKNK-DGSFS 349
Sai          TDVKKALEDGGVSNADATAAKLVKMSYTDKNGKSIDGGYALEAGGKYYAATYDEGTGKIT 351
Ana          TDVKKALEDGGVSNADATAAKLVKMSYTDKNGKSIDGGYALEAGGKYYAATYDEGTGKIT 351
```

Appendix 5. Continued



Appendix 5. Continued

```
                                                        % ID to S. Brandenburg
Ade          NMSKAQILQQAGTSVLAQANQVPQNVLSLLR 505    53
Der          NMSKAQILQQAGTSVLAQANQVPQNVLSLLR 505    53
Ago          NMSKAQILQQAGTSVLAQANQVPQNVLSLLR 505    53
Ent          NMSKAQILQQAGTSVLAQANQVPQNVLSLLR 505    53
Dub          NMSKAQILQQAGTSVLAQANQVPQNVLSLLR 505    53
Mon          NMSKAQILQQAGTSVLAQANQVPQNVLSLLR 505    53
Sen          NMSKAQILQQAGTSVLAQANQVPQNVLSLLR 505    53
Ora          NMSKAQILQQAGTSVLAQANQVPQNVLSLLR 508    50
Lil          NMSRAQILQQAGTSVLAQANQVPQNVLSLLR 421    59
Typ          NMSRAQILQQAGTSVLAQANQVPQNVLSLLR 495    77
Bov          NMSRAQILQQAGTSVLAQANQVPQNVLSLLR 491    77
Brn         NMSRAQILQQAGTSVLAQANQVPQNVLSLLR 500
Sai          NMSRAQILQQAGTSVLAQANQVPQNVLSLLR 502    75
Ana          NMSRAQILQQAGTSVLAQANQVPQNVLSLLR 502    75
              ***:****************************
```

Ade: *S.* Adelaide (AAA53490)

Der : *S.* Derby (CAA78775)

Ago: *S.* Agona (AAT81612)

Ent: *S.* Enteriditis (CAA78777)

Dub: *S.* Dublin (CAA78776)

Mon: *S.* Montevideo (CAA78778)

Sen: *S.* Senftenberg (CAA78781)

Ora: *S.* Oranienberg (CAA78779)

Lil: *S.* Lille (AAR10700)

Typ: *S.* Typhimurium (BAA02846)

Bov: *S.* Bovis-morbificans (AAR10675)

Brn: *S.* Brandenburg (AAX24137)

Sai: *S.* Saintpaul (AAT81611)

Ana: *S.* Anatum (AAT81609)

Alignment was executed with CLUSTALW server at http://www.ebi.ac.uk/clustalw/. Regions I to VIII of FliC are shown (I, II, III, IV, V, VI, VII, and VIII). % ID, percent identity. GenBank accession numbers of FliC proteins of different *Salmonella* serotypes are shown in parentheses.

Appendix 6. Raw data for IFN-γ assay-Figure 4.27 (Pre-vaccination)

Sheep No.	OD_{450}[a]								
	PBS	PMB	ConA 20 μg	V456			FliC		
				1 μg	10 μg	20 μg	1 μg	10 μg	20 μg
C005	0.063	0.065	0.162	0.060	0.064	0.059	0.071	0.064	0.067
C006	0.060	0.060	0.072	0.063	0.065	0.062	0.063	0.062	0.062
C015	0.080	0.082	0.128	0.075	0.081	0.083	0.077	0.081	0.083
C017	0.103	0.098	0.205	0.105	0.105	0.107	0.104	0.102	0.101
C022	0.073	0.077	0.170	0.074	0.081	0.079	0.078	0.073	0.077
C026	0.058	0.062	0.159	0.059	0.062	0.061	0.060	0.060	0.059
V016	0.063	0.068	0.171	0.061	0.058	0.059	0.066	0.062	0.065
V019	0.058	0.053	0.100	0.059	0.062	0.062	0.062	0.061	0.062
V020	0.071	0.072	0.169	0.070	0.075	0.068	0.071	0.072	0.075
V021	0.076	0.080	0.193	0.075	0.078	0.070	0.081	0.080	0.080
V024	0.154	0.161	0.248	0.157	0.155	0.153	0.156	0.153	0.157
V025	0.062	0.063	0.173	0.062	0.062	0.058	0.065	0.064	0.066

[a]Each OD_{450} value represents the average absorbance of duplicate wells

Appendix 7. Raw data for IFN-γ assay-Figure 4.28 (1-month post-priming)

Sheep No.	OD_{450}[a]								
	PBS	PMB	ConA 20 μg	V456			FliC		
				1 μg	10 μg	20 μg	1 μg	10 μg	20 μg
C005	0.071	0.075	0.171	0.075	0.070	0.068	0.073	0.073	0.076
C006	0.069	0.071	0.183	0.067	0.067	0.066	0.071	0.079	0.079
C015	0.069	0.072	0.127	0.072	0.073	0.075	0.071	0.070	0.068
C017	0.059	0.065	0.173	0.061	0.064	0.059	0.062	0.068	0.064
C022	0.067	0.073	0.083	0.069	0.071	0.073	0.070	0.072	0.069
C026	0.068	0.069	0.165	0.068	0.072	0.069	0.071	0.071	0.068
V016	0.105	0.099	0.215	0.115	0.102	0.112	0.111	0.109	0.107
V019	0.067	0.072	0.169	0.064	0.069	0.081	0.072	0.075	0.073
V020	0.073	0.080	0.138	0.072	0.076	0.076	0.069	0.079	0.076
V021	0.059	0.062	0.167	0.063	0.065	0.061	0.063	0.065	0.061
V024	0.063	0.067	0.179	0.061	0.061	0.066	0.065	0.066	0.067
V025	0.058	0.061	0.075	0.060	0.062	0.065	0.060	0.063	0.062

[a]Each OD_{450} value represents the average absorbance of duplicate wells

Appendix 8. Raw data for IFN-γ assay-Figure 4.29 (3-weeks post-booster)

Sheep No.	OD_{450}[a]								
	PBS	PMB	ConA 20 μg	V456			FliC		
				1 μg	10 μg	20 μg	1 μg	10 μg	20 μg
C005	0.058	0.063	0.170	0.059	0.061	0.063	0.060	0.063	0.067
C006	0.067	0.068	0.109	0.064	0.069	0.064	0.072	0.068	0.068
C015	0.149	0.147	0.219	0.138	0.140	0.142	0.153	0.141	0.159
C017	0.071	0.073	0.167	0.071	0.075	0.072	0.076	0.072	0.077
C022	0.059	0.060	0.103	0.061	0.063	0.065	0.061	0.059	0.063
C026	0.055	0.061	0.167	0.058	0.059	0.059	0.064	0.063	0.061
V016	0.121	0.126	0.183	0.077	0.071	0.069	0.130	0.127	0.126
V019	0.117	0.120	0.169	0.069	0.073	0.070	0.113	0.116	0.120
V020	0.081	0.083	0.166	0.082	0.080	0.079	0.082	0.085	0.083
V021	0.067	0.070	0.180	0.065	0.065	0.070	0.072	0.071	0.071
V024	0.073	0.075	0.175	0.074	0.071	0.072	0.071	0.079	0.074
V025	0.072	0.078	0.168	0.147	0.143	0.153	0.075	0.073	0.073

[a]Each OD_{450} value represents the average absorbance of duplicate wells

Appendix 9. Raw data for IFN-γ assay-Figure 4.30 (2-months post-booster)

Sheep No.	OD_{450}[a]								
	PBS	PMB	ConA 20 μg	V456			FliC		
				1 μg	10 μg	20 μg	1 μg	10 μg	20 μg
C005	0.063	0.065	0.161	0.061	0.059	0.064	0.067	0.062	0.061
C006	0.059	0.059	0.157	0.060	0.060	0.059	0.060	0.063	0.065
C015	0.054	0.060	0.086	0.059	0.061	0.058	0.057	0.061	0.051
C017	0.072	0.077	0.154	0.071	0.073	0.072	0.076	0.078	0.069
C022	0.057	0.060	0.156	0.065	0.063	0.059	0.062	0.065	0.065
C026	0.149	0.153	0.211	0.062	0.064	0.064	0.153	0.150	0.154
V016	0.068	0.071	0.167	0.071	0.073	0.064	0.070	0.074	0.077
V019	0.071	0.073	0.168	0.074	0.075	0.072	0.073	0.071	0.071
V020	0.058	0.060	0.112	0.061	0.063	0.058	0.060	0.064	0.062
V021	0.157	0.156	0.231	0.050	0.053	0.056	0.161	0.159	0.159
V024	0.065	0.069	0.157	0.068	0.061	0.070	0.069	0.068	0.063
V025	0.071	0.074	0.164	0.073	0.075	0.076	0.070	0.072	0.080

[a]Each OD_{450} value represents the average absorbance of duplicate wells

Appendix 10. Raw data for IFN-γ assay-Figure 4.31 (3-months post-booster)

Sheep No.	OD_{450}[a]								
	PBS	PMB	ConA 20 μg	V456			FliC		
				1 μg	10 μg	20 μg	1 μg	10 μg	20 μg
C005	0.058	0.058	0.118	0.059	0.059	0.063	0.059	0.061	0.064
C006	0.062	0.065	0.157	0.065	0.067	0.061	0.067	0.068	0.063
C015	0.153	0.151	0.226	0.157	0.154	0.153	0.148	0.151	0.150
C017	0.056	0.060	0.083	0.054	0.057	0.059	0.061	0.061	0.055
C022	0.073	0.073	0.159	0.076	0.079	0.077	0.074	0.079	0.071
C026	0.155	0.157	0.229	0.158	0.158	0.156	0.157	0.159	0.160
V016	0.072	0.071	0.160	0.076	0.079	0.072	0.080	0.075	0.078
V019	0.049	0.051	0.143	0.053	0.052	0.048	0.051	0.053	0.048
V020	0.067	0.064	0.172	0.066	0.067	0.068	0.069	0.071	0.070
V021	0.148	0.149	0.249	0.152	0.150	0.150	0.151	0.149	0.151
V024	0.059	0.063	0.081	0.063	0.059	0.062	0.061	0.061	0.055
V025	0.073	0.070	0.179	0.071	0.075	0.075	0.076	0.071	0.076

[a]Each OD_{450} value represents the average absorbance of duplicate wells

Appendix 11. Data used to calculate the ELISA cut-off value (Section 5.4.1)

Sample No.	OD_{405}	Sample No.	OD_{405}[a]	Sample No.	OD_{405}
N1	0.041	N28	0.067	N55	0.004
N2	0.037	N29	0.003	N56	0.053
N3	0.010	N30	0.022	N57	0.049
N4	0.025	N31	0.015	N58	0.003
N5	0.033	N32	0.021	N59	0.034
N6	0.070	N33	0.027	N60	0.031
N7	0.011	N34	0.068	N61	0.064
N8	0.069	N35	0.043	N62	0.070
N9	0.031	N36	0.031	N63	0.053
N10	0.026	N37	0.011	N64	0.032
N11	0.019	N38	0.037	N65	0.010
N12	0.037	N39	0.003	N66	0.021
N13	0.049	N40	0.013	N67	0.058
N14	0.025	N41	0.026	N68	0.041
N15	0.013	N42	0.041	N69	0.007
N16	0.022	N43	0.065	N70	0.019
N17	0.041	N44	0.016	N71	0.043
N18	0.067	N45	0.034	N72	0.061
N19	0.025	N46	0.067	N73	0.015
N20	0.002	N47	0.005	N74	0.027
N21	0.053	N48	0.011	N75	0.005
N22	0.030	N49	0.004	N76	0.037
N23	0.011	N50	0.018	N77	0.031
N24	0.029	N51	0.069	N78	0.051
N25	0.041	N52	0.024	N79	0.071
N26	0.070	N53	0.033	N80	0.069
N27	0.014	N54	0.011		

[a]Each OD_{405} value represents the mean value of two determinations minus background OD at 405nm.

Appendix 12. Data for indirect ELISA-Naturally infected sheep (Figures 5.1, 5.2, 5.3 and 5.4)

Sample	OD $_{405}$[a]	Titre	Sample	OD$_{405}$[a]	Titre
S1	0.544	400	S42	0.131	100
S2	2.434	6400	S43	0.065	50
S3	1.706	3200	S44	1.043	1600
S4	1.406	1600	S45	0.512	400
S5	0.148	200	S46	0.205	200
S6	1.493	1600	S47	0.191	200
S7	1.165	1600	S48	0.362	200
S8	1.179	1600	S49	0.439	400
S9	2.395	6400	S50	0.504	400
S10	2.062	6400	S51	0.392	200
S11	2.207	6400	S52	0.219	200
S12	1.001	1600	S53	0.736	800
S13	0.345	200	S54	1.296	1600
S14	0.674	400	S55	1.460	1600
S15	1.220	1600	S56	0.222	200
S16	0.320	200	S57	1.053	1600
S17	1.150	1600	S58	0.245	200
S18	0.901	800	S59	0.400	200
S19	0.227	200	S60	0.100	100
S20	0.551	400	S61	0.259	200
S21	0.947	800	S62	0.131	100
S22	0.430	400	S63	0.984	800
S23	0.275	200	S64	0.107	100
S24	0.621	400	S65	0.176	200
S25	1.085	1600	S66	0.137	100
S26	0.733	800	S67	1.022	1600
S27	0.140	100	S68	0.284	200
S28	0.683	400	S69	0.803	800
S29	1.590	3200	S70	0.381	200
S30	0.133	100	S71	0.526	400
S31	0.091	50	S72	0.059	50
S32	0.070	50	S73	0.082	50
S33	0.219	200	S74	1.420	1600
S34	0.440	400	S75	0.190	200
S35	0.206	200	S76	0.909	800
S36	0.143	100	S77	1.458	1600
S37	0.555	400	S78	1.931	6400
S38	1.082	1600	S79	0.703	800
S39	0.146	100	S80	0.474	400
S40	1.089	1600	S81	1.156	1600
S41	1.151	1600			

[a]Each OD_{405} value represents the mean value of two determinations minus background OD at 405nm.

Appendix 13. Data for indirect ELISA-Trial 2004 (Figures 5.5 and 5.6)

Sheep No.	OD_{405}[a]				
	Pre-vaccination	1-month post-priming	3-weeks post-booster	2-months post-booster	3-months post-booster
Control group					
C005	0.013	0.030	0.009	0.000	0.000
C006	0.008	0.000	0.013	0.000	0.000
C015	0.008	0.000	0.029	0.025	0.005
C017	0.021	0.000	0.028	0.037	0.037
C022	0.008	0.000	0.004	0.001	0.000
C026	0.021	0.020	0.011	0.013	0.011
Vaccinated group					
V016	0.021	0.150 (100)	1.329 (1600)	0.367 (200)	0.283 (200)
V019	0.022	0.180 (100)	1.585 (3200)	0.345 (200)	0.289 (200)
V020	0.006	0.169 (100)	1.309 (1600)	0.349 (200)	0.251 (200)
V021	0.007	0.166 (100)	1.202 (1600)	0.416 (400)	0.311 (200)
V024	0.025	0.103 (100)	1.480 (3200)	0.299 (200)	0.226 (200)
V025	0.017	0.231 (200)	1.484 (1600)	0.335 (200)	0.217 (200)
Mean titre		117	2133	233	200

[a]Each OD_{405} value represents the mean value of two determinations minus background OD at 405nm. Titres are shown in parantheses.

Appendix 14. Data for indirect ELISA-Trial 1999 (Figure 5.7)

Control Group		Salvexin™-vaccinated Group		Salvexin™+B-vaccinated group	
Sample No.	OD_{405}[p]	Sample No.	OD_{405}[a]	Sample No.	OD_{405}[a]
C1	0.071	Sa1	0.019	SB1	0.573
C2	0.032	Sa2	0.061	SB2	0.555
C3	0.009	Sa3	0.002	SB3	0.426
C4	0.044	Sa4	0.036	SB4	0.362
C5	0.012	Sa5	0.004	SB5	0.466
C6	0.073	Sa6	0.044	SB6	0.315
C7	0.010	Sa7	0.013	SB7	0.281
C8	0.052	Sa8	0.016	SB8	0.600
C9	0.021	Sa9	0.044	SB9	1.752
C10	0.031	Sa10	0.015	SB10	1.820

[a]Each OD_{405} value represents the mean value of two determinations minus background OD at 405nm.

Appendix 15. Data for indirect ELISA-Rabbit experiment (Figure 5.8)

Group of rabbits	Rabbit No.	OD_{405}[a]		
		Pre-vaccination bleed	2-weeks post-booster bleed	4-weeks post-booster bleed
Control	509	0.019	0.009	0.013
	510	0.000	0.00	0.004
S. Hindmarsh-vaccinated	511	0.012	0.014	0.009
	512	0.017	0.013	0.015
S. Typhimurium-vaccinated	514	0.023	0.009	0.006
	525	0.000	0.000	0.000
S. Brandenburg-vaccinated	515	0.006	0.577	0.243
	524	0.000	1.887	2.141

[a]Each OD_{405} value represents the mean value of two determinations minus background OD at 405nm.

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