The use of synthetic biology to engineer vaccines against Ebola and Zika Viruses using *Salmonella* based delivery systems

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Abstract

Ebola and Zika viruses have been brought to the world's attention in recent years due to widespread outbreaks and the devastating diseases they cause in humans. Hence there is an urgent need to develop vaccines which can protect from disease.

Salmonella vaccine strains have been genetically attenuated so as not to cause disease. These vaccine strains can be engineered to deliver heterologous antigens from viruses, bacteria, and worms to the host's immune system to elicit protective immunity. Such vaccines can be orally administered and would not need cold storage. A successful vaccine using this platform would have the additional advantages of being trivalent, protecting against *Salmonella,* Tetanus and Ebola or Zika virus.

The Ebola Glycoprotein (GP) and the Zika envelope protein (ZE) contain important protective epitopes, which are targets for neutralising antibodies, and therefore considered prime targets for vaccine development. Using the *Salmonella* based delivery systems we have successfully expressed these Ebola and Zika virus vaccine candidate proteins for delivery to the immune system.

A large panel of 19 variant constructs has been generated, which express these viral antigens, or sub-fragments containing important protective epitopes, as fusions to the C-fragment of Tetanus Toxin (TetC). TetC is a potently immunogenic molecule, which can be expressed to high levels by *Salmonella* and helps to solubilise the expressed fusion protein partner and act as an adjuvant. Expression of proteins in this vector is driven by the *in vivo* inducible promoter, *nirB*.

The panel of GP and ZE genes and cassettes were synthesised to be codon optimised, with toxic hydrophobic signal sequence and transmembrane regions removed, for expression in *Salmonella* Typhimurium. By testing with western blot, it was found that TetC rescued expression of the GP and ZE proteins, which were either expressed at much lower levels or not at all in constructs lacking the TetC fusion partner. The most promising candidates were then taken forward to *in vivo* testing. These strains have been shown to be stable both *in vitro* and *in vivo*, retaining the expression vector, and still expressing the antigens after passage in BALB/c mice.

Vaccination experiments were then carried out in BALB/c mice and have unfortunately shown that after a single immunisation, there was no significant IgG antibody response to the GP or ZE antigens.

An alternative non-living *Salmonella* vaccine delivery platform has also been investigated. By making *tolR* and *mlaA* gene knockouts in *Salmonella* Typhimurium strain SL1344, these strains have been shown to produce elevated amounts of outer membrane vesicles

(OMVs) than their wild type counterparts. These immunogenic vesicles could then be used to carry antigen as cargo for vaccine delivery and subsequently purified for immunisation.

Constructs were made to target the expression of the Zika envelope protein to the outer membrane or periplasm, using signal sequences from OmpA and DsbA respectively. The knockout strain SL1344 *ΔtolR*, harbouring these plasmid constructs, was able to express ZE in OMVs, hopefully either decorating the OMV surface, or contained within the lumen.

It is hoped that this could lead to an improved immune response compared to the live platform, as an adequate antigenic dose could be pre-determined and administered accordingly. The outer membrane components (such as LPS) which are naturally included in the OMVs, act as an adjuvant, and thus can increase the immunogenicity and efficacy of such a platform. A wider range of potential recipients could also be reached with such a vaccine, as vulnerable populations such as the very old, very young or immunocompromised patients cannot receive live vaccines. This study has shown the feasibility of such an approach of targeting antigen cargo to the OMVs, and will hopefully pave the way for future exciting research to determine its effectiveness

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Preface

This thesis is my own work and all experiments were carried out by myself unless otherwise stated. Any work carried out by or in collaboration with others has been acknowledged and stated explicitly.

Rebecca Kildani

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Abbreviations

µg	Microgram
µl	Microlitre
µM	Micro molar
aa	Amino acid
AAA	Aromatic amino acids
ABC	ATP binding cassette
ADE	Antibody dependent enhancement
Amp	Ampicillin
ampR	Ampicillin resistance gene
ATR	Acid tolerance response
bp	Base pairs
C	Capsid protein
CAMP	Cationic antimicrobial peptides
CFR	Case Fatality Rate
CFU	Colony forming units
ChAd3-ZEBOV	Chimpanzee adenovirus type 3 vaccine expressing Zaire Ebola GP
CNS	Central nervous system
CS	Circumsporozoite
DHB	2,3-dihydroxybenozate
DNA	Deoxyribonucleic acid
DOC	Sodium Deoxycholate
DRC	Democratic Republic of Congo

DsbA	Disulphide bond formation protein A
DsbAss	DsbA signal sequence
E	envelope protein
E. coli	*Escherichia coli*
EBOV	Ebola Virus
EBU	Evans-Blue Uranine
ELISA	Enzyme linked immunosorbent assay
EVD	Ebola Virus disease
FcR	Fc receptor
FRT	Flippase recognition target
g	grams
GABA	Gamma Aminobutyric acid
GALT	Gut-associated lymphoid tissue
GBS	Guillain-Barré Syndrome
Gly, Pr, Gly, Pro	Glycine, Proline, Glycine, Proline (pTECH2 hinge motif)
GMMA	Generalised Modules for Membrane Antigens
GP	Glycoprotein
Hbp	Haemoglobin protease
Hep B	Hepatitis B
HIV/AIDS	Human immunodeficiency virus / Acquired immune deficiency syndrome
hNPC	Human cortical neural progenitor cells
HRPO	Horse radish peroxidase
I.V	Intravenously
IFN	Interferon
IFNAR	Interferon-α/β receptor

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRF	Interferon regulatory factor
ISG	IFN-stimulated gene
ISGF	IFN-stimulated gene factor
Jak1	Janus kinase 1
Kan	Kanamycin
KanR	Kanamycin resistance gene
Kb	Kilobases
kDa	Kilodalton
L	L protein
LB	Lysogeny broth (Lennox broth)
LH	Low hydrophobicity
LPS	Lipopolysaccharide
M	Molar
M cells	Microfold cells
MCS	Multiple Cloning site
MHCII	Major histocompatibility complex II
ml	Millilitre
mRNA	Messenger RNA
MSR	Membrane spanning region
NADH	Nicotinamide adenine dinucleotide
NHP	Non-human Primate

NP	Nucleoprotein
NPC1	Niemann-Pick C1
ns	Not significant
NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5	Non-structural proteins 1, 2A, 2B, 3, 4A, 4B, 5
NTS	Non typhoidal *Salmonella*
OD_{450}	Optical density at 450nm absorbance
OD_{600}	Optical density at 600nm absorbance
Oligo	Oligonucleotide
OmpA	Outer membrane protein A
OmpAss	OmpA signal sequence
OMV	Outer membrane vesicle
PABA	Para-aminobenzoic acid
PAMP	Pattern associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHEIC	Public Health Emergency of International Concern
PQS	*Pseudomonas* quinolone signal
prM	Precursor-membrane protein
PRR	Pattern recognition receptor
R_0	Basic reproductive number
$r^- m^+$	Restriction negative, modification positive
RBS	Ribosome binding site
RNA	Ribonucleic acid
RPM	Revolutions per minutes
rRNA	Ribosomal RNA

S. enterica	*Salmonella enterica*
SCV	*Salmonella* containing vacuole
SD	Standard Deviation
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
sGP	Secreted GP
SPI	*Salmonella* pathogenicity island
SRP pathway	Signal recognition particle pathway
ss	Signal sequence
STAT	Signal Transducer and Activator of Transcription
T3SS	Type 3 secretion system
TCV	Typhoid conjugate vaccines
TEM	Transmission electron microscopy
TEMED	N,N,N'N'-Tetramethylethylenediamine
TetC	C-fragment of Tetanus toxin
T_H	T Helper cell
TLR	Toll-like receptor
TM	Transmembrane
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumour necrosis factor alpha
Tyk2	Tyrosine kinase 2
UDP	Uridine diphosphate
UTR	Untranslated region
UV	Ultraviolet
V	Volts
ViPS	Vi Polysaccharide

Vi-TT	Vi-Tetanus Toxoid
VLP	Virus like particle
VP35, VP40, VP30, VP24	Viral Proteins 35, 40, 30, 24
VSV	Vesicular Stomatitis Virus
VSV-EBOV	Vesicular Stomatitis Virus expressing vaccine Ebola GP
WHO	World health organisation
WT	Wild type
x g	Relative Centrifugal Force
ZE	Zika envelope protein
ZE(s) / (s193)	Zika envelope synthetic gene / truncated synthetic gene
ZIKV	Zika Virus

Chapter 1 – Introduction

1.1 Vaccines

Vaccines are often hailed as one of the most important inventions in modern medicine. From the now famous but humble beginnings in 1796 with Edward Jenner's Cowpox experiment (André, 2003), the use of vaccines is still the most effective way of combating infectious diseases, which is one of the leading causes of mortality worldwide. The eradication of smallpox in the late 1970's (Shchelkunova, & Shchelkunov, 2017) and the control of many other diseases including polio, mumps, measles and diphtheria, demonstrates the great potential of vaccinations and their importance in protecting much of the world's population from otherwise potentially fatal infections. The link between some infectious agents, for example Human Papilloma Virus, and cancer has also shown that it is possible to prevent further complications from infections using vaccines (Signorelli et al, 2017). It is also now evident that it may be possible to actually treat other diseases such as cancer or auto immune diseases using vaccines (Liu, 1999).

The protection offered by vaccines comes from the activation of the immune system by exposure to a (often attenuated) pathogen or antigen, priming the immune system and enabling a specific and more efficient secondary response when the pathogen is next encountered, thus preventing infection from taking hold. In second exposure, PAMPs, or Pathogen Associated Molecular Patterns, which are expressed on the surface of pathogens are recognized by pathogen recognition receptors (PRRs) on host innate immune cells such as macrophages and dendritic cells. This recognition interaction leads to a release of cytokines and chemokines by the innate immune cells and stimulates the adaptive immune response by way of T and B lymphocytes. These cells have receptors for specific epitopes found on the antigen and can then release cytokines and specific antibodies respectively, preventing the infection. Immune memory, arising from this priming of the immune system with a vaccine, is imperative as it allows for vaccines to be used prophylactically well before any exposure to the actual infectious agent occurs.

Vaccines come in many different forms; Toxoid vaccines such as the vaccine for tetanus; Killed or attenuated such as those for typhoid, polio and Hepatitis A; Subunit vaccines where a specific antigen from a pathogen is used, such as for Hepatitis B or Influenza (Baxter, 2007). DNA vaccines are also being investigated as a simple way of introducing an antigen to the host immune system (Liu, 1999).

Toxoid vaccines can protect against bacteria that are known to secrete toxins which are responsible for the disease symptoms, such as; tetanus, diphtheria, botulism and cholera among others (Baxter, 2007). These vaccines use inactivated (often by formaldehyde) bacterial toxins, which then elicit an immune response (Clem, 2011). For example, botulism is a rare, yet potentially life-threatening disease caused by neurotoxins produced by various *Clostridium* species, leading to paralysis symptoms. Vaccination against the toxins themselves can elicit neutralising antibodies which prevent their binding to pre-synaptic neuronal cell receptors and allow them to be cleared and degraded by phagocytes, thus preventing the botulism symptoms (Smith et al, 2009; Jones et al, 2008). Tetanus toxin also binds to receptors located on pre-synaptic nerve cells. This results in the improper function of GABA (gamma Aminobutyric acid) neurons which usually act to inhibit motor neurons. Without GABA neuron inhibition, increased activity of motor neurons results in severe muscle spasms. A vaccine which uses formaldehyde treated tetanus toxoid is able to elicit an adaptive immune response, where the toxoid is phagocytosed by dendritic cells and its antigens displayed on MHCII (major histocompatibility complex II) molecules on the cell surface. The cell then migrates to the draining lymph node and the corresponding T cell receptor, on the surface of T_H2 cells, binds to the MHCII-tetanus toxoid complex, activating the T cell to proliferate. The toxoid can also bind to a corresponding B cell receptor, also in the draining lymph node, again resulting in presentation of an MHC-toxoid complex. When this B cell encounters a T_H2 cell able to recognise this specific complex, the B cell is activated and then produces antibodies towards the toxoid. These antibodies create a large complex when bound to the toxin and therefore prevent receptor binding and the typical muscle spasms as seen with tetanus infection (Baxter, 2007).

Killed or inactivated vaccines are used against bacteria (e.g. typhoid) and viral pathogens (e.g. Hepatitis A). They are able to elicit an immune response to a wide range of antigens. The T_H2 cells recognise epitopes on these antigens and are activated to release cytokines which induce

B cell activation and thus proliferation and production of IgG antibodies and memory B cells, in preparation for future encounters with a live pathogen. This vaccination method is potentially less effective than others which use a more specific antigen directed approach, as booster immunisations are often needed due to the fact that the dead pathogen cannot replicate in the host, or may include antigens which can modulate the host immune system in a negative way and thus weaken the response (Baxter, 2007; Clem, 2011).

Subunit vaccines elicit an immune response to only antigenic components of a pathogen. By including only B or T cell epitopes, these vaccines have advantages over whole cell or virus vaccines as there is less chance of adverse reactions and no possibility of reversion to virulence (Clem, 2011). These carefully selected antigens can be proteins, which elicit a T cell dependent immune response, such as the vaccines for Hepatitis B or *Haemophilus influenzae*, or a polysaccharide, such as capsular polysaccharides from pneumococcal bacteria, which elicit a T cell independent response. Polysaccharide antigens are able to activate B cells with a specific receptor to produce antibodies without T cell contribution, as its large size and repeating structure is able to bind to multiple B cell receptors on one B cell. This response mainly produces IgM antibodies as without T cell help, there is restricted antibody isotype switching, resulting in only a limited amount of IgG antibodies and memory B cells produced. Protection here, comes from IgM activation of the complement system, lysing the *Streptococcus pneumoniae* bacteria (Baxter, 2007).

Live attenuated vaccines, in a similar way to killed or inactivated vaccines, use the whole pathogen to elicit an immune response. Viruses can be attenuated by passage in a foreign host, which then have a preferred virulence for the foreign host and not humans, or in conditions which are different to that of the human host (for example at a temperature lower than 37°C), resulting in mutants which are less able to thrive before the adaptive immune response can clear the infection (Baxter, 2007; Hanley, 2011). Bacterial pathogens can be genetically attenuated (see sections 1.3.1.1 and 1.3.2 for further details). There are limitations with these types of vaccines, for example, there is a potential to revert back to a virulent strain, particularly with the rapid rates of mutation of RNA viruses. It has been reported that some recipients of the attenuated oral polio vaccine, for example, can experience vaccine-associated paralytic poliomyelitis as the attenuated strain reverts back to virulence. This has

resulted in the alternative use of an inactivated rather than live attenuated vaccine in the United States in particular (Hanley, 2011).

DNA vaccines simply involve immunising with a plasmid encoding an antigen, allowing the host cells to produce the protein and introduce it to the immune system. There is no need for using live pathogens and therefore could be safer. Clinical trials for HIV, malaria and cancer vaccines have been carried out, but research to optimise potency are continuing (Liu, 1999).

1.1.1 Live recombinant vaccines

The use of attenuated bacteria or viruses as vectors to introduce heterologous antigen DNA into the host has been investigated to create recombinant vaccines. When designing vaccines for intracellular pathogens, including viruses, a recombinant vaccine may be a more attractive choice over live attenuated vaccines due to the risks of attenuation reversal and possible virulence in certain susceptible hosts. Recombinant vaccines, using safe attenuated vectors rely on an immune response directed at a particular antigen (which could be from any number of pathogens) which the vector has been engineered to express, and would elicit an immune response similar to that seen with subunit or toxoid vaccines (see above). An example of a successful recombinant vaccine against a virus is the Hepatitis B (Hep B) vaccine which uses yeast cells to express the Hep B surface antigen. The antigen can accumulate in virus like particles (VLPs) and elicit a protective immune response due to the high immunogenicity of these particles. Human papilloma Virus, which can be the cause of cervical cancer can also be protected against by use of VLPs made by the expression of the virus's main capsid protein in insect or yeast cells. These vaccines often need co-administration with an adjuvant to increase the immunogenicity of the antigens (Nascimento & Leite, 2012).

Bacterial vectors, such as has been investigated with attenuated *Salmonella* (as discussed in section 1.4), are able to express heterologous antigens by introduction of expression plasmids and can stimulate the immune system, also acting in an adjuvant capacity as the vector mimics a natural infection. To ensure a good immune response, the vector must be considered carefully and allow adequate expression of the antigen (Nascimento & Leite, 2012). The expression plasmid must also be considered to ensure it is stably retained by the vector, has an appropriate copy number and the promoter is strong enough for adequate expression. The

use of a vector also allows the generation of a multivalent vaccine towards both the vector and the pathogen to which the guest antigen belongs.

1.1.2 Salmonella as vaccines

Attenuated live *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) and *Salmonella* Typhi strains have been investigated for use as vaccines against a mouse model of Typhoid fever and human typhoid fever respectively (Garmory et al, 2002). They can also be engineered to carry heterologous antigens from viruses, bacteria, protozoa, and worms to elicit protective immunity against these pathogens (Khan et al, 1994a; Khan et al, 1994b). After oral inoculation with even a relatively small dose of attenuated *Salmonella*, it is able to invade and colonise lymphoid tissue, and can then induce systemic 'infection', resulting in T-cell memory and a long-lasting immune response (Curtiss et al, 2010). This will be discussed later in this thesis (see section 1.2.4).

The mucosal surfaces of the body are major routes of pathogen entry and depending on the immunisation route, localised protective immune responses can be induced. Examples of effective mucosal vaccines, administered orally, include live-attenuated poliovirus and Rotavirus vaccines which are able to induce both local mucosal IgA antibodies and $CD4^+$ and $CD8^+$ T cell mediated protective immune responses respectively (Azegami et al, 2014).
Importantly, it has been shown that an immunisation route which targets mucosal surfaces (such as oral administration), allows recombinant attenuated *Salmonella* vaccines to elicit both systemic and mucosal antibody responses (Clark-Curtiss & Curtiss III, 2018).
Salmonella enterica demonstrates a natural tropism for the mucosal surfaces of the intestinal tract including the payer's patches, vital components of the gut associated lymphoid tissue (GALT). This tropism lends itself well to an oral mucosal immunisation route (Gayet et al, 2017). Ty21a, a live attenuated strain of *Salmonella* Typhi Ty2, also taken orally, has been shown to elicit IL-17 producing *S*. Typhi specific T cell responses and also induce cross reactive immune responses against other serovars, namely *paratyphi* A and B, the causative agents of paratyphoid fever for which there are no current effective vaccines (Azegami et al, 2014). The Ty21a vaccine strain is discussed in further detail later in this thesis, in section 1.3.1.1.

Therefore, developing a recombinant multivalent vaccine for both typhoid fever and Ebola or Zika Viruses, as described in this thesis, may be possible in this way. Such a vaccine would be orally administered, thus the need for needles and cold storage facilities is no longer necessary. In the case of Ebola virus in particular, which is transmitted through mucosal contact with infected bodily fluids, a vaccine which is able to induce both systemic and localised mucosal protective immune responses could be very effective. This would allow for a much cheaper and easier to administer vaccine, which would be most welcome, especially in the developing countries such as West Africa and parts of the Americas where these viruses have caused such devastation.

1.2 *Salmonella* as human pathogens

Salmonella are Gram-negative facultative anaerobes belonging to the family *Enterobacteriaceae*. A major pathogen, *Salmonella* can cause gastroenteritis and typhoid fever in humans. Two species of *Salmonella* are known; *Salmonella bongori* and *Salmonella enterica*, the latter including in excess of 2600 serotypes (Coburn et al, 2007; dos Santos et al, 2018). *Salmonella enterica* are further divided into either typhoidal serovars (*S.* Typhi and *S.* Paratyphi) which are only able to infect humans, and non-typhoidal *Salmonella* (NTS), including *S.* Typhimurium, which are often zoonotic and includes some serovars with a wider range of hosts (Gordon, 2011). It has also been recently discovered, through genomic sequencing of ancient human tooth matter, that outbreaks of *Salmonella enterica* paratyphi C (which causes enteric fever) were likely a major contributing factor in the massive population decline of indigenous Mexican inhabitants in the 1500s and subsequently the collapse of the Aztec empire. It is thought that *Salmonella* paratyphi C was brought over during the European colonisation of the area by asymptomatic European carriers, which can be 1-6% of people infected (Vågene et al, 2017). The possibility of asymptomatic *Salmonella* Typhi carriers, was discovered in the case of Mary Mallon or “Typhoid Mary” in New York in the early 1900s, where she was determined to be exacerbating the spread of Typhoid fever in the area due to her unsanitary cooking practices (Marineli et al, 2013).

1.2.1 Salmonella Typhimurium as a human pathogen

One of the most frequently isolated serotypes of *Salmonella enterica* is the NTS serovar *Salmonella* Typhimurium, which can often be found in meat for human consumption such as chicken, pork and beef (dos Santos et al, 2018).

1.2.2 Salmonella Typhimurium infection

The most common route of infection by *Salmonella* Typhimurium is through contaminated food or water. Generally, infection in a healthy human entails a self-limiting bout of gastroenteritis, however in some more vulnerable patients, such as those with HIV/AIDS or malaria, particularly in sub-Saharan Africa, there can be the danger of life threatening systemic bacteraemia once infected (dos Santos et al, 2018; Lathrop et al, 2015).

1.2.3 Pathogenesis and invasion of Salmonella

The first obstacle that ingested *Salmonella* must face is the low pH of the stomach. To combat this, it activates the acid tolerance response (ATR) which acts homeostatically to keep the intracellular pH higher than the extracellular pH and allows survival in this environment (Fabrega & Vila, 2013). The *Salmonella* can then cross the intestinal wall's mucus layer, and with host-receptor interactions of adhesion factors on the bacterium surface, can adhere to the cells of the intestinal epithelium (dos Santos et al, 2018).

Salmonella have virulence genes encoded in pathogenicity islands (SPI) which allow them to invade host cells. The Type 3 secretion system 1 (T3SS1), encoded in SPI-1 allows the *Salmonella* to inject effector proteins into the host intestinal epithelial or M (microfold) cells (found in Peyer's patches, small areas of lymphoid tissue, in the wall of the ileum and often associated with macrophages) in the early stages of infection (Galan, 1999; dos Santos et al, 2018). These effector proteins, notably; SipA, SopB, SopA and Sop E/E_2, activate signal transduction pathways in the host cell and the actin cytoskeleton is rearranged. This leads to the 'ruffling' of the epithelial cell membrane and the *Salmonella* can be engulfed, similar to phagocytosis. The *Salmonella* is then contained in a *Salmonella* containing vacuole (SCV) in

the cell, where they are able to multiply (Zhang et al, 2018; Eng et al 2015; dos Santos et al, 2018).

The SCV is transported to the basolateral membrane of the intestinal epithelium and the *Salmonella* can enter the lymphatic system, be phagocytosed by cells such as macrophages, and from here develop a systemic infection (dos Santos et al, 2018).

1.2.3.1 Survival of Salmonella in Macrophages

Macrophages usually kill engulfed pathogens by acidifying the intracellular compartment, however *Salmonella enterica* have evolved to survive this and persist. It is thought that this acidification of the SCV acts as an induction signal for the expression of SPI-2 virulence proteins. This allows effector proteins to be delivered to the host cell and disrupt the host's immune response (Chakraborty et al, 2015).

Salmonella possess two virulence systems that allow survival after entry into macrophages and the SCV. These are the PhoP/Q two component regulatory system, and the SPI-2 encoded T3SS2. The sensor-kinase PhoQ senses when the *Salmonella* is in an acidified SCV such as in a macrophage, resulting in the phosphorylation of the response regulator PhoP. Phosphorylated PhoP signals to regulate proteins involved with the bacterial envelope. This results in the regulation of the outer membrane and increases the bacterium's resistance to cationic antimicrobial peptides (CAMP) and allowing survival in this environment (Dalebroux & Millar, 2014). In the phagosome environment, signals leading to the expression of T3SS2 proteins allows the secretion of effector proteins into the host cell which are needed for the *Salmonella's* survival.

Salmonella can cause the death of invaded macrophages by pyroptosis, by either T3SS1 dependent (early on after phagocytosis by the macrophage) or T3SS2 dependent means (occurring hours after phagocytosis). The early stage pyroptosis is mediated by the T3SS1 SipB effector protein and allows the activation of the inflammasome leading to the targeting of the macrophage for programmed cell death. The later stage pyrotopsis is mediated by the T3SS2 SpvB effector protein which depolymerises actin and causes cell death. This then allows the release of the intracellular *Salmonella* and it is able to disseminate in infected organs such as the liver and spleen (Fabrega & Vila, 2013).

It seems however that the phenotype of the invaded macrophage can affect the *Salmonella's* ability to replicate. (Lathrop et al, 2015).

1.2.4 Immune response to Salmonella infection

Mouse models of *Salmonella* infection are widely used to investigate the pathogenicity of salmonellosis and the resulting immune response (Mastroeni & Sheppard, 2004) however, it is unclear whether the murine model of *S.* Typhimurium, is a truly accurate model for human typhoid or non-typhoidal systemic salmonellosis (Pham & McSorley, 2015). *Salmonella* Typhimurium infection in mice appears to be similar to systemic *Salmonella* Typhi infection in humans, in that it penetrates the peyer's patches of the intestine and quickly disseminates to the liver, spleen and bone marrow. It is therefore the most commonly used animal model of typhoid disease (Moon & Mc Sorley, 2009).

The first line of defence against invading pathogens is the innate immune system. PAMPS on the bacterial surface, are recognised by pattern recognition receptors (PRRs) including Toll Like Receptors (TLRs) situated on the surface of resident macrophages and monocytes. For example, bacterial LPS is recognised by TLR 4 and flagellin recognised by TLR 5. This recognition initiates inflammation by the release of cytokines and the recruitment of neutrophils (Broz et al, 2012).

It appears that effective immunity to *Salmonella* relies on $CD4^+$ T helper cells and a Th type 1 response. A rapid, localised $CD4^+$ T cell response in Peyers' patches has been shown in mice orally inoculated with *Salmonella* Typhimurium (McSorley et al, 2002). Primary *Salmonella* infection elicits antibodies against LPS, and B cell deficient mice show impaired resistance to oral challenge with *Salmonella* Typhimurium, however this does not simply appear to be due a diminished antibody response, it seems that B cells are required to establish a long-lasting T cell memory response (Mastroeni et al, 2000 (a); Mastroeni et al, 2000 (b)).

1.3 Vaccines against typhoid fever

It is estimated that typhoid fever, caused by systemic *Salmonella* Typhi infection, causes over 21 million illnesses and 222,000 deaths annually, mainly in areas with inadequate water

sanitation and hygiene, such as South Asia and Sub-Saharan Africa. Vaccines for typhoid are important to control typhoid fever, especially in these areas where the infrastructure is not currently in place to improve water sanitation. It may be argued that here, prevention would be better than cure due to the rise in antibiotic resistance (Date et al, 2015).

Previously, heat killed or phenol inactivated whole cell vaccines were used, however these have been shown to be more toxic and induce more adverse events than newer typhoid vaccines such as Ty21a which is taken orally (see later) and the parenteral Vi Polysaccharide vaccines (Engels & Lau 1998). There are also newer vaccines against typhoid, which are at various stages of development. These include Typhoid conjugate vaccines (TCVs), where the Vi antigen, an immunogenic capsular polysaccharide found in *Salmonella* (Virlogeux-Payant & Popoff, 1996) is conjugated to a carrier protein, thus enhancing immunogenicity (Date et al, 2015). The Vi-TT (Vi-Tetanus Toxoid) conjugate vaccine for example has recently been recommended by the World Health Organisation (WHO) (Milligan et al, 2018).

The latest Cochrane review on typhoid vaccines (Milligan et al, 2018), includes these new vaccines and comments on their efficacy (See table 1.1). It appears that the currently licenced Ty21a and ViPS vaccines are certainly effective in preventing typhoid fever. The effectiveness of new vaccines, such as Vi-TT and other TCVs however, need to be more thoroughly investigated to determine effectiveness for children under 2 years of age, an especially vulnerable population in typhoid endemic countries (Milligan et al, 2018).

Vaccine type	Name	Route of administration	Comments
Live-attenuated	Ty21a	Oral, four doses (alternate days)	Approved for use in children over 6 years of age. Taken orally. E.g Vivotif (PaxVax).
Vi Polysaccharide	ViPS	Single parenteral dose	Approved for children 2 years of age and older, is not immunogenic in infants. e.g. Typherix (GSK) or Typhim (Sanofi Pasteur).
Typhoid Conjugate	Vi-rEPA	Two parenteral doses	Vi conjugated to non-toxic recombinant *Pseudomonas aeruginosa* exotoxin A (rEPA). Not currently commercialised, only been used in children over 2 years of age.
Typhoid Conjugate	Vi-TT	Two doses, intramuscular injection 6 weeks apart	Vi conjugated to Tetanus Toxoid. Licensed in India as - Peda Typh (BioMed) and Typbar TCV. Has been used in children over 6 months of age, however more data is needed to fully determine efficacy.

Table 1.1. Vaccines which have been developed against Typhoid (Milligan et al, 2018; Mitra et al 2016).

1.3.1 Live attenuated Salmonella vaccines

The use of killed or inactivated whole cell vaccines against typhoid has been discontinued due to the increasing reports of adverse effects and limited ability to induce cell mediated immunity upon vaccination. Thus, new vaccines have been developed including live attenuated *Salmonella* strains such as Ty21a which have been shown to be effective in immunising against typhoid fever. Live attenuated *Salmonella* can also be used to express heterologous antigens for delivery to the host immune system, and have therefore also been investigated as a vaccine delivery platform (Mastroeni et al, 2000 (a); Garmory et al, 2002).

1.3.1.1 Live attenuated Salmonella Typhi vaccine Ty21a

The Ty21a vaccine is an orally administered live attenuated strain of *Salmonella* Typhi Ty2. Attenuated by chemical mutagenesis using nitrosoguanidine in the 1970s, resulting in a mutation in the *galE* gene, which encodes the enzyme Uridine diphosphate (UDP)-galalctose-4-epimerase, it was shown to be safe and trigger protective responses against *Salmonella* Typhi infection when ingested. (UDP)-galactose-4-epimerase is responsible for the conversion of UDP-galactose to or from UDP-glucose. UDP-galactose allows the incorporation of galactose into the core of LPS and O-polysaccharide (O-Antigen), therefore the *galE* mutation results in a strain with rough LPS, lacking these parts. The O-Antigen is a major epitope on the *Salmonella* surface, and therefore to ensure an immune response, during production of Ty21a, there needs to be a limiting source of exogenous galactose to make UDP-galactose. This allows the bacteria to make full LPS, in a synthesis pathway that is non-*galE* dependent, giving rise to an immune response. Further mutations in Ty21a lead to a reduction in the levels of enzymes that can make UDP-galactose from exogenous sources, but *in vivo* when the exogenous galactose source is supplied, a build-up of galactose-1-phoshate and UDP-galactose inside the cell, leads to bacteriolysis and the infection is therefore not able to take hold. Further attenuation in Ty21a is also attributed to mutations resulting in the lack of synthesis of the Vi polysaccharide capsule. (Pham & McSorley, 2015; Kolecko et al, 2009; Germanier & Fürer, 1975).

1.3.2 Mutations resulting in attenuation of Salmonella

Salmonella have many virulence genes to enable colonisation of the host and establish infection. It is however possible to genetically attenuate the *Salmonella* by introducing mutations into these genes. This prevents their ability to cause disease, yet as they still retain capability of eliciting protective immunity, they are able to be used as a live vaccine (Hoiseth & Stocker, 1981).

1.3.2.1 Aromatic pathway mutations

One effective way of creating mutated strains which are attenuated, is to induce auxotrophy. Auxotrophic mutants require compounds essential for growth, which are not provided by the host, and that due to the mutation, the strain is unable to synthesise itself. A deletion in the *aroA* gene in *Salmonella* Typhimurium for example, results in a strain with an impairment in the aromatic biosynthesis pathway and is unable to synthesise aromatic amino acids (AAAs); tryptophan, tyrosine, phenylalanine and also the folate precursor para-aminobenzoic acid (PABA) and 2,3-dihydroxybenozate (DHB), the precursor for the bacterial iron acquisition compound; enterochelin.

These compounds are synthesised from chorismate, the final product of the aromatic (aro) biosynthesis pathway (see figure 1.1). PABA and DHB are not found in mammalian cells and so *Salmonella* must synthesise them using this pathway. A mutation in genes associated with this pathway, such as *aroA*, which encodes for the enzyme 5-Enolpyruvyl-shikimate-3-phosphate-synthase, disrupts the eventual synthesis of chorismate and therefore the *Salmonella* becomes auxotrophic for PABA, DHB and the AAAs. Thus, virulence is reduced due to the limit of PABA available and the *Salmonella* is unable to colonise the host due to its' self-limiting nature (Hoiseth & Stocker, 1981; Denich et al, 1993). The *aroA*$^{-}$ vaccine strain SL3261 (parent strain – SL1344) has been used as mentioned previously to sucessfully deliver recombinant antigens to the host and elicit immune responses (Khan et al, 1994a; Khan et al, 1994b; Ashby et al, 2005).

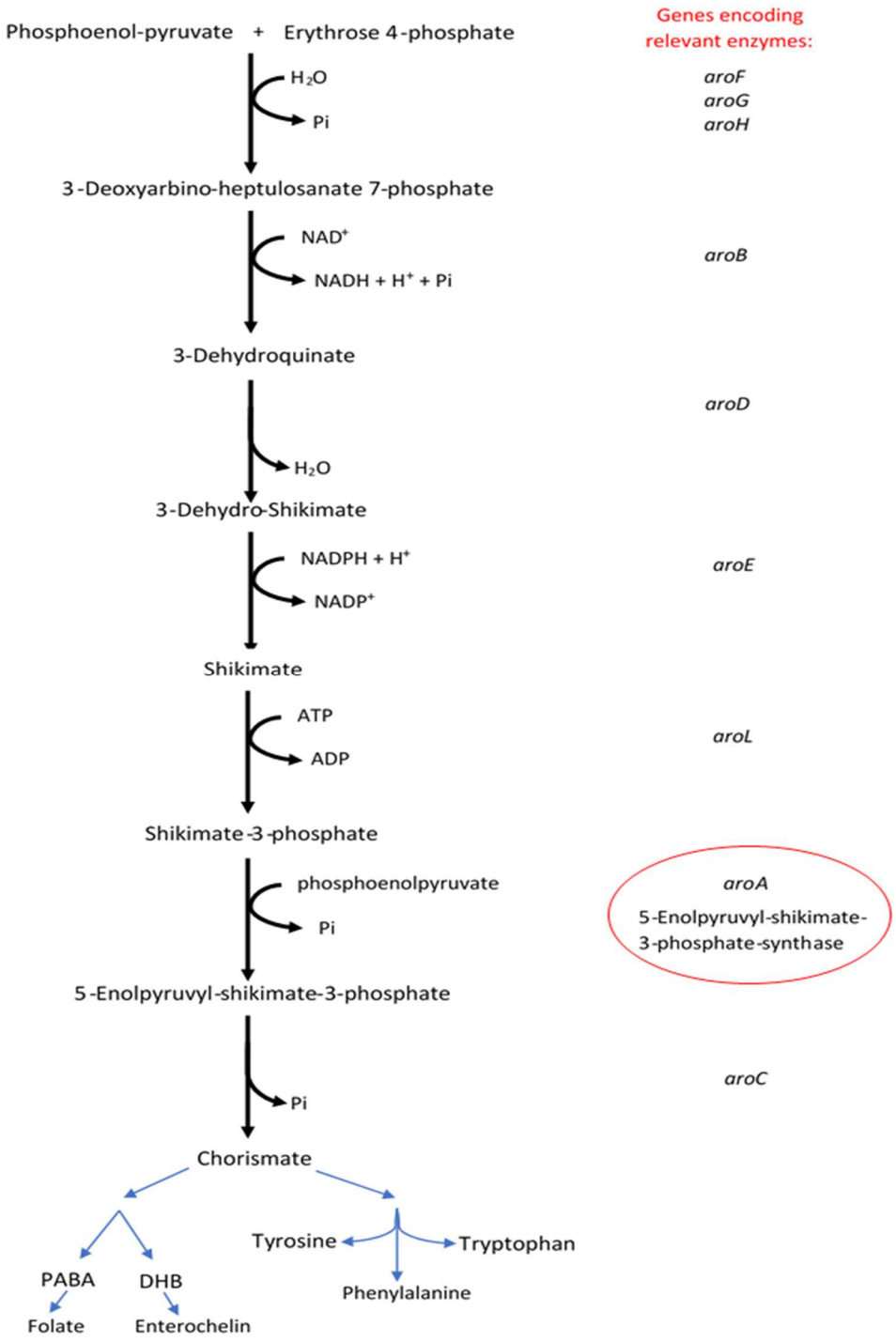


Figure 1.1. The aromatic (aro) synthesis pathway of *Salmonella.*
A mutation in the aroA gene, which encodes the enzyme 5-Enolpyruvyl-shikimate-3-phosphate-synthase (circled) results in an attenuated strain, unable to synthesise PABA, DHB or aromatic amino acids (adapted from: Hoiseth & Stocker, 1981; Tzin & Galili, 2010).

1.3.2.2 HtrA mutations

HtrA is a periplasmic stress induced protease found to be involved in the virulence of a number of bacterial pathogens. In *Salmonella* it allows survival in macrophages, strains with mutations in the *htrA* gene show increased sensitivity for oxidative stress (such as is found within the macrophage) and have been shown to be less virulent in mice (Lewis et al, 2009; Humphreys et al, 1999; Tacket et al, 1997). *Salmonella htrA* mutants have been shown to elicit an immune response in humans and mice and can also carry heterologous antigens for delivery to the immune system (Tacket et al, 1997; Chabalgoity et al, 1996).

1.4 Live recombinant *Salmonella* vaccines

As mentioned above, it has been shown that attenuated *Salmonella* can deliver heterologous antigens to the host immune system and elicit an immune response. This has been investigated with a number of antigens from other bacteria, viruses, protozoa and worms.

Antigens from parasitic flatworms *Schistosoma mansoni* (Khan et al 1994a; Khan et al, 1994b) and *Schistosoma haematobium* (Lee et al, 2000), glycoprotein D from herpes simplex virus (Chabalgoity et al, 1996), and a circumsporozoite protein (CS) from a malarial parasite (McKelvie et al, 2008), have all been successfully used in recombinant *Salmonella vaccines* and shown to elicit protective immune responses against their respective pathogens.

The p28 peptide of *Schistosoma mansoni* is the 28kDa glutathione S-transferase, and has been shown to induce protective immunity in a range of vaccinated hosts, containing important B and T cell epitopes, in particular, the immunogenic peptide aa115-131. This peptide, when expressed as a genetic fusion to the C-fragment of Tetanus Toxin (TetC), which will be discussed later in this thesis, has been shown to elicit antibody responses in vaccinated hosts when expressed in attenuated *Salmonella* (Khan et al, 1994a), in particular when the copy number of this short peptide is increased in tandem repeats (Khan et al, 1994b). Similarly, mice immunised with attenuated *Salmonella* Typhimurium strain C5 with a mutation in the stress protein gene *htrA*, were able to make an antibody response to tandem copies of aa 8-23 of the Herpes simplex virus glycoprotein D expressed by this strain (Chablgoity et al, 1996).

A double *Salmonella* Typhimurium vaccine strain mutant, with lesions in both the *aroA* and *waaN* genes, resulting in an auxotrophic mutant expressing modified Lipid A, seemed to show enhanced immune responses to a guest antigen, in this case TetC fused with CS from a malarial parasite, in vaccinated mice compared with the single *aroA* mutant. This could be due to the reduction in LPS toxicity in the double mutant, and therefore the ability to immunise with higher doses of *Salmonella*, thus increasing the antigenic (TetC-CS) dose (McKelvie et al 2008).

SL3261 expressing TetC, has also been shown to be safe when tested in rabbits and elicits a strong anti-TetC antibody response (Ashby et al, 2005).

More recently, studies have shown that recombinant *Salmonella* Typhimurium vaccine strains expressing heterologous O-antigen from other serovars (e.g. *Salmonella* Choleraesuis) may be able to elicit some heterologous protection in vaccinated mice against the other serovar and could lead to an alternative technique for expressing heterologous antigens (Zhao et al, 2017).

Vaccines such as these, once the expression of the antigen has been engineered to be optimal, can be cost effective and relatively easy to produce, potentially providing an easy way of immunising people in developing or poorer countries.

1.5 Expression of heterologous antigens in *Salmonella*

To allow the *Salmonella* to express heterologous antigens, expression plasmids engineered to carry these antigens are transformed into the bacterial cells. The most important issue to ensure has been addressed, is the ability of the *Salmonella* to retain the plasmid *in vivo*; its stability. Stable plasmids can be retained by the bacteria even in the absence of the selection antibiotics that are used when conducting the *in vitro* expression work. This is important as upon immunisation, there are no antibiotics available and loss of the plasmid could result in a lower antigenic dose, and therefore reduced or non-existent immune response. One way to improve the stability of such plasmids is by repeated passage (Chabalgoity, 1996). The copy number of a plasmid; how many copies are expected to be present in each cell, also can have an effect on its stability. A fine line must be met between the amount of antigen expressed – it must be sufficient for an immune response but also not too much so as to adversely affect

the cell, such as with toxic antigens. Using certain promoters can also help to optimise the vaccine. Promoters that are inducible rather than constitutively active could help to ensure that there is not an excessive amount of a toxic antigen being expressed, and could help direct expression only to the compartments where it is necessary. *In vivo* inducible promoters for example, could allow the antigen to only be expressed in response to certain signals, assisting with the efficiency of the vaccine.

1.5.1 Expression vector pTECH2

The plasmid pTECH2 (see figure 2.1 in Chapter 2, section 2.1.4) was adapted from the pTETnir15 vector and has been successfully used to express heterologous antigens in *Salmonella* (Chatfield et al, 1992; Khan et al, 1994a; Khan et al, 1994b; Chabalgoity et a 1996; Lee et al, 2000). A multiple cloning site consisting of; *XbaI*, *BamHI*, *EcoRV*, *HindIII* and *SpeI,* allows the insertion of a heterologous antigen gene which is expressed under the control of the anaerobically inducible promoter *nirB*, as a fusion to the atoxic C-fragment of tetanus toxin (see figure 1.2) To allow for selection of clones containing the correct plasmid, pTECH2 has an ampicillin resistance gene (AmpR).

Figure 1.2 Protein expression from pTECH2. Schematic diagram showing protein expression from the pTECH2 expression vector. Under the control of the anaerobically inducible promoter, nirB, the C-fragment of tetanus toxin is expressed (TetC). A multiple cloning site (MCS) allows the insertion of a heterologous antigen which results in the expression of a genetic fusion with the TetC protein, separated by a flexible hinge motif.

1.5.1.1 C-fragment of Tetanus toxin (TetC)

The highly immunogenic but non-toxic C-fragment of Tetanus Toxin (TetC) has been shown to be easily expressed by *Salmonella* and can help to modulate the immune response to a fused antigen, increasing antibody responses (Lee et al, 2000). Followed by a flexible 'hinge region'; a Gly, Pro, Gly, Pro motif, guest antigens can be cloned into the plasmid and will be expressed as a fusion protein to TetC. The TetC assists with solubilising and detoxifying these guest antigens and the hinge region allows for temporal and spatial separation between the two. The codons used for the Gly and Pro amino acids in the hinge are deliberately sub-optimal for expression in *Salmonella*, and therefore it is hoped that the ribosome is slowed here during translation, allowing time for correct folding of TetC.

1.5.1.2 In vivo inducible promoters

In order to maintain *in vivo* stability of plasmids, an *in vivo* inducible promoter can be used to direct expression of antigens. The *nirB* promoter, from the NADH-dependent nitrate reductase gene, has been shown to be activated during anaerobic growth and is regulated by nitrite and changes in oxygen tension. The *htrA* promoter, dependent on the σ^E transcription initiation factor, has been shown to increase expression of proteins in attenuated *Salmonella* where H_2O_2 is present, such as in the macrophage intracellular environment (Everest et al, 1995). Rather than using chemical induction such as with *trp* or *lac* promoters (induced by IPTG) which would not be possible in host tissues, *in vivo* inducible promoters allow protein expression to be turned on where it is needed in the host (Jayaraman et al, 1987; Chatfield et al, 1992).

1.5.2 Expression vector pBAD24

The pBAD24 vector (see figure 2.2 chapter 2, section 2.1.4) allows easy protein expression under the control of the L-arabinose inducible pBAD (araBAD) promoter (Guzman et al, 1995) The polylinker site allows cloning in of the antigen as well as signal sequences to direct expression to either the outer membrane or periplasm. An antibiotic resistance gene once again allowed for screening.

1.5.2.1 Arabinose inducible promoter

The pBAD24 vector allows expression of proteins under the control of the arabinose inducible promoter. The pBAD promoter of the arabinose operon (araBAD) is switched on in the presence of L-arabinose (Guzman et al, 1995). This can be used to regulate protein expression only as needed, especially when the protein is toxic to cells.

1.5.5 Salmonella Typhimurium SL5338

Prior to transformation of an expression plasmid into the attenuated *Salmonella* vaccine strain, plasmids can be initially transformed into the strain SL5338 (*galE* r^- m^+). This strain, which is restriction (r) negative and modification (m) positive, ensures that the transformed plasmid is protected as it will not be digested by certain restriction enzymes due to modification by methylation.

1.6 Codon usage and synthetic biology

The degeneracy of the genetic code means that there are multiple codons (sets of three nucleotides) that code for one amino acid.

1.6.1 Codon bias

It has been shown that different species tend to favour certain codons over others. Species can express proteins encoded by their preferred codons more easily than if less frequently used codons are used (Sharp et al, 2005). Codon usage tables are used to determine the frequency in which codons are used in different species.

Based on differences in codon usage in mammalian cells and *Salmonella*, it was decided that using synthetic genes coding for the Ebola Glycoprotein or Zika envelope would be advantageous, as they could be codon optimised for expression in *Salmonella* Typhimurium.

1.6.1.1 Codon optimisation parameters

In order to optimise the Ebola GP and Zika E gene sequences, commercial gene synthesis facility, Eurofins was used. Using their proprietary software; 'GENEius' along with the codon usage table for *Salmonella* Typhimurium, codons were chosen for optimal expression based on the following criteria:

- Very rare codons are avoided.
- 'Bad motifs' for example, unwanted restriction sites, are identified and replaced with suitable alternative codons.
- Direct and inverted repeats are changed to avoid unwanted RNA secondary structures, which can hinder DNA synthesis and reduce its stability.
- GC rich areas are avoided and these nucleotides are distributed as equally as possible.

These criteria mean that the most frequent codons for a certain species are not always the ones that are used in the optimised gene sequence. A balance is made to ensure good expression in the chosen species but avoiding certain codons which would have a negative effect on this (see footnote[1]).

[1] Note: for details on GENEius software see: https://www.eurofinsgenomics.eu/en/gene-synthesis-molecular-biology/geneius/

1.7 Ebola Virus

The Ebola Virus (EBOV) is an especially virulent pathogen and has one of the highest mortality rates of known human infectious diseases, causing severe haemorrhagic fever. It is also able to cause deadly infections in non-human primates. Part of the *Filovirus* family, which also contains Marburg Virus, EBOV includes individual species, named after the areas where they were discovered. In Africa, the known species are; Zaire, Sudan and Cote d'Ivoire (Feldmann & Geisbert, 2011). The Reston Ebola Virus was discovered in cynomolgus monkeys in a primate facility near Washington D.C, and traced back to the Philippines. While part of this group, Reston EBOV is not known to cause human disease. (Peters & Le Duc, 1999).

The Zaire Ebola virus was first discovered in 1976 and was named after the Ebola River in Zaire in what is now known as the Democratic Republic of Congo (DRC) (Emond et al, 1877; Johnson et al, 1977).

1.7.1 Ebola Virus outbreaks

The largest outbreak of Ebola virus ever seen started in West Africa in December 2013 through to the end of 2015. It is assumed that the index case for this outbreak was a 2 year old boy from the small village of Meliandou, Guinea, who contracted the infection via zoonotic transmission from a colony of insectivorous bats, living in a hollow tree (Saéz et al, 2015). This epidemic was particularly notable due to the dramatic symptoms of fatal haemorrhagic fever, and the record numbers of deaths from the virus, gaining widespread media coverage throughout.

The R_0, or basic reproductive number, of a pathogen gives an indication of how quickly an epidemic will spread. This is the calculated number of new cases, which an infected person will cause in a susceptible population. The higher the R_0 value, the faster this will be and an R_0 value less than 1 indicates that there is no epidemic. Implementation of interventions to control transmission of a pathogen can help to reduce the R_0 (Delgado & Simon, 2016; Woolhouse et al, 2016).

When comparing Ebola Virus with the R_0 of other viral pathogens, there are certainly those that have a higher average R_0 value during epidemics. The average R_0 for Ebola Virus at the height of the recent epidemic was 1.4 in September 2014, falling to 1.0 by that December. This is equivalent to the R_0 of seasonal influenza but far below those of untreated HIV ($R_0 = 6$) or measles ($R_0 = 17$, which is much more easily transmissible) (Kaner & Schaack, 2016). Again, the severe symptoms of Ebola haemorrhagic fever compared with these other diseases, combined with the threat of no available cure or vaccine, may have been a factor in the widespread panic that ensued regarding the disease. In comparison, the H1N1 Swine flu pandemic which lead to many more worldwide deaths (approximately 284,000) between April 2009 and August 2010, with a similar R_0 value of 1.4 – 1.6 (Dawood et al, 2012; Coburn et al, 2009; Leung & Nicoll, 2010) had similar media coverage and was also declared a public health emergency of international concern (PHEIC), but response to the outbreak was much quicker, compared to that of Ebola, where emergent cases were initially misdiagnosed as cholera or Lassa fever. An understanding of why delays in responding to outbreaks occur, could greatly help to allow much quicker responses in the future (Hoffman & Silverberg, 2018).

There is also a marked difference in case fatality rates (CFR) between the H1N1 and Ebola Viruses. Depending on the strain, Ebola virus can have a case fatality rate during an outbreak of between 25-50%, with some much higher into the range of 80%+. The recent West African outbreak, as detailed above was shown to be at about 40%, possibly due to the effectiveness of the Ebola Treatment Centres set up in response (De La Vega et al, 2015; Baseler et al, 2017). In the 2009 H1N1 pandemic, the CFR was much lower, of just 0.026% (Donaldson et al, 2009). Therefore, despite the much higher number of fatal H1N1 cases, the containment of the Ebola Virus outbreak was clearly much more effectively contained as it did not reach pandemic levels.

A new outbreak of Ebola Virus has been documented as recently as August 2018 in The DRC, with over 83 possible cases and 44 deaths already reported (Claude et al, 2018), highlighting the constant threat of a recurrence. One positive outcome of the response to the 2013 outbreak however, was a huge acceleration in vaccine development, and some headway has been made in that regard (see section 1.7.5.3). This hopefully means that an outbreak on the scale seen between 2013-2015 does not happen again.

1.7.2 The Ebola Virus genome

The Ebola virus has a 19kb, linear, negative sense RNA genome which codes for seven genes, but allows transcription of eight proteins by way of transcriptional editing (see figure 1.4). These proteins are:

Nucleoprotein (NP), Viral protein 35 (VP35), VP40, Glycoprotein (GP), secreted Glycoprotein (sGP), VP30, VP24 and Large protein (L).

Proteins NP, VP30, VP35 and L are associated with the viral RNA in a ribonucleoprotein complex, with the L protein acting as an RNA polymerase.

The virus's structural proteins are GP, which appears on the virus surface, and VP24 and VP40, which are presumed to be located on the inner side of the viral membrane (Feldmann et al, 1993) (see figure 1.3).

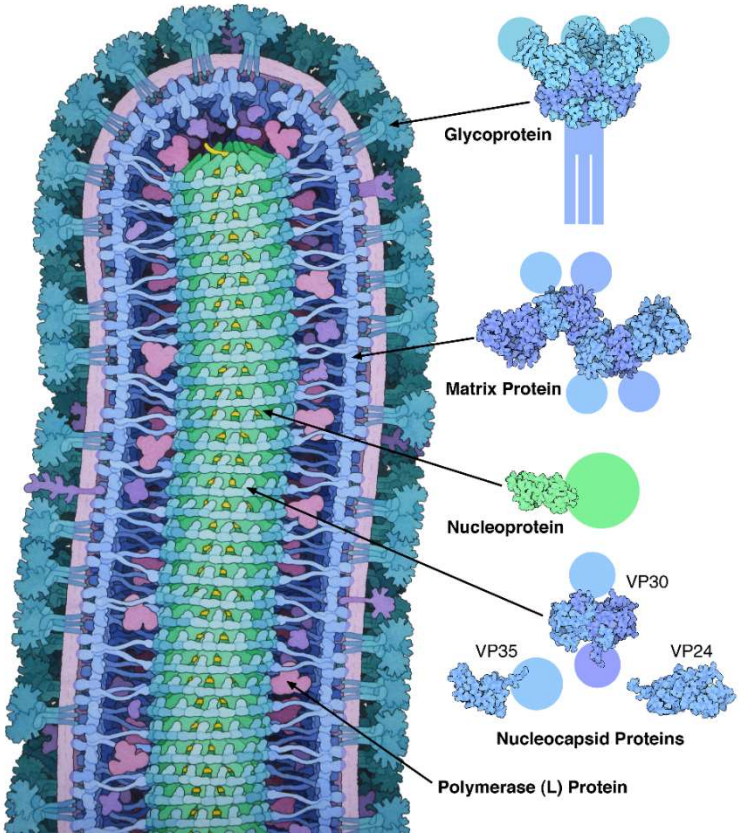


Figure 1.3. Known structures and location of Ebola Virus proteins. Diagram to display a cross section of the Ebola Virus showing the known structures and locations of viral proteins (green or blue) RNA (yellow), and viral membrane (purple). The Glycoprotein (GP) is shown as trimeric spikes decorating the membrane and is a key target for neutralising antibodies (Goodsell, 2014).

At both the 3' and 5' ends of the Ebola virus genome, there are also long non-coding 'leader' and 'trailer' regions of RNA (see figure 1.4). These regions have complementarity to each other and may form a 'pan handle' RNA structure. It is thought that this may be to act as a promoter as RNA secondary structure has been known in other virus species (Enterlein et al, 2009).

Figure 1.4. Map of Ebola virus genome. Schematic diagram of the Ebola virus negative sense genome showing each of its eight proteins; NP, VP35, VP40, GP (and sGP) VP30, VP24 and L. Transcriptional editing results in expression of two different Glycoproteins, sGP and GP1,2. The non-edited gene results in sGP and the addition of an extra adenosine at the editing site (dotted line) results in the fusion of the sGP and GP reading frames and the full-length GP is transcribed. Two long non-coding regions are situated at the 3' and 5' ends of the genome. (Adapted from Messaoudi et al, 2015).

1.7.3 Pathogenesis of Ebola Virus

Transmission of Ebola Virus between humans can occur either by the virus somehow entering the bloodstream (potentially inadvertently by injection or through wounds), or contact of the mucous membranes with infectious body fluids from an infected patient. It appears that the risk of infection is increased significantly with direct contact with infectious material (Baseler et al, 2017).

The recent outbreak in West Africa, included many local outbreaks in the area. In mid-2014 an outbreak commenced in Sierra Leone's Pujehun district which was finally declared Ebola free in early 2015. In this outbreak, it was noted that the majority of transmission events were between family members (Ajelli et al, 2015). Overall, it appears that directly caring for infected persons or contact with the body of a person who has died from Ebola Virus disease, due to traditional funeral practices, led to the widespread person to person transmission of the virus (Baseler et al, 2017).

In the infection of humans and non-human primates, the virus targets immune cells including dendritic cells, monocytes and macrophages. Before the onset of symptoms, these infected cells then travel to the lymph nodes where the virus replicates and is disseminated throughout the body, (Geisbert et al, 2003). The viral load increases exponentially in the early stages of infection and is able to overpower the innate immune system, leading to widespread dissemination of the virus in the bloodstream (Lanini et al, 2015).

1.7.4 Immune responses to Ebola Virus infection

It has been shown that patients who have recovered from Ebola Virus infection are able to produce antibodies to the Glycoprotein, some of which have been shown to be protective (Takada et al, 1997). During infection with Ebola Virus, there is an increase in the level of pro-inflammatory cytokines. Some of these, such as TNF-α have been shown to increase the permeability of human epithelial cells *in vitro* when released from EBOV infected monocytes or macrophages (Takada et al, 1997). These particular cells appear to be a target for the virus and the resultant over-production of these cytokines can lead to damage of the vascular system and result in leakage and haemorrhage (Feldmann et al, 1993). It seems apparent that patients who have an earlier, more controlled and balanced immune response to EBOV infections are generally given a better prognosis (Feldmann et al, 1993).

1.7.5 Treatment and current progress in Ebola vaccine development

After the scope of the most recent Ebola outbreak was realised, efforts were made to develop a safe and effective treatment for the many victims who contracted the infection, in an attempt to curb the spread of the virus. New efforts to develop an effective vaccine were also prompted so as to prevent such deadly epidemics in the future.

1.7.5.1 Small molecule drugs

Some existing small molecule drugs, such as brincidofovir and favipiravir, usually used to treat cytomegalovirus and influenza respectively, have been re-licenced for use in patients with Ebola Virus (Mendoza et al, 2016). They have been shown to have anti-Ebola Virus effects *in vitro* and favipiravir has been shown to treat Ebola Virus symptoms in immune deficient mice (Smither et al, 2014). It could potentially be used in patients in the early stages of Ebola Virus Disease (EVD) with lower viral loads (Liu et al 2017). This could be a useful drug discovery avenue to take, however more clinical trials in humans are necessary to fully determine the usefulness of small molecule drugs in treating EVD.

1.7.5.2 Antibody cocktails

During the recent outbreak in West Africa, it was deemed appropriate to issue antibody cocktails against Ebola Glycoprotein in emergency compassionate treatment to people infected with the virus. These were shown to be effective in the protection and treatment of Ebola Virus disease when tested in non-human primates (Muhlberger, 2007). The antibodies to Ebola glycoprotein that comprise this cocktail, dubbed ZMapp, were selected from previous monoclonal antibody cocktails, MB-003 and ZMAb (Qiu et al, 2014). ZMapp has been used to treat patients with Ebola Virus disease, notably two Americans who had contacted Ebola (Lyon et al, 2014). Again, more human clinical trials are needed to gain a better understanding of the efficacy and uses of such biological treatments.

1.7.5.3 Vaccines

There are currently no licenced vaccines against Ebola Virus, making development of a successful vaccine all the more pertinent. There have however, been numerous efforts to produce such a drug, including some projects that have reached clinical trial stages. Most particularly, ChAd3-ZEBOV (GSK) and VSV-EBOV (NIAID). VSV-EBOV is a live replicating viral vaccine, the likes of which have been shown to provoke both cellular and humoral immune responses against pathogenic viruses. Using the Vesicular Stomatitis Virus (VSV) to express

Ebola Virus glycoprotein, the vaccine has been shown to be effective in protecting against Ebola Virus challenge in non-human primates and has shown to be immunogenic in phase 1 clinical trials (Agnandji et al 2016).

ChAd3-ZEBOV, uses the chimpanzee adenovirus type 3 to express Ebola glycoprotein, but is replication deficient. In a human clinical trial, antibody titres to Ebola Glycoprotein were seen comparable to the VSV-EBOV vaccine (Ewer et al, 2016b).

Even more recently the VSV vaccine V920, expressing the Zaire Ebola Glycoprotein in lieu of the VSV glycoprotein, has been shown to be 100% effective in phase 3 trials and could be used imminently in the DRC, were a new outbreak has unfortunately been seen. It is hoped that the availability of this vaccine, which has been approved for use in a ring vaccination approach, in this new outbreak will be a great advantage and help to curb the spread before an epidemic on the scale of the one seen in 2013 occurs (Coller et al, 2017; Dyer et al, 2018; Claude et al, 2018).

It is clear that it is possible to produce an immune response to the Glycoprotein of Ebola Virus. This is encouraging and allows more exploration into different expression systems and administration methods, which could be used, allowing for even more effective vaccines.

1.7.6 Ebola Glycoprotein (GP) as a prime vaccine candidate

The Ebola Virus glycoprotein enables the Virus to facilitate fusion to host cell membranes (Takada et al, 1997), attach to, and enter host cells (Lee & Saphire, 2009). In order for this to occur, the Glycoprotein must bind to the Niemann-Pick C1 (NPC1) receptor, an endosomal membrane protein important in trafficking cholesterol (Wang et al, 2006). Posttranslational cleavage of the GP protein results in two subunits. GP subunit 1 (GP1) binds to this receptor, and GP subunit 2 (GP2) is considered the fusion subunit allowing host cell and viral membranes to connect (Takada et al, 1997). On the Virus surface, the glycoprotein is arranged in a trimer with a chalice shaped appearance. GP1 subunits form the bowl structure, and GP2 subunits the sides. Certain lysine residues near the centre of this bowl are believed to be important in attachment to host cell membranes (Lee & Saphire, 2009). Transcriptional editing of the Ebola Virus glycoprotein (GP) gene results in expression of different GP proteins. Secreted GP (sGP) is the most common product of non-edited translation of the GP gene, accounting for around 80% of GP translation (Feldmann et al, 2001; Tran et al, 2014). As its'

name would suggest, sGP is secreted from EBOV infected cells (Muhlberger, 2007). sGP shares the first 295 residues with the full-length GP, this is thought to act as a decoy as it competes with GP attached to the Virus for antibody binding (Cook & Lee, 2013). At the editing site, a sequence of seven uridines, the addition of an extra adenosine residue by L protein (the viral polymerase) (Volchkova et al, 2011) results in a frame shift which fuses the two sGP and GP overlapping open reading frames together, leading to the continuation of translation of the full-length GP1,2 (De la Vega et al, 2015; Feldmann et al, 1993).

While all viral components contribute to its pathogenesis, the type 1 membrane protein Glycoprotein (GP) is considered the most important. The singular surface protein of the Ebola Virus, it is currently the only known target for protective antibodies. GP is responsible for binding of the Virus and its entry into host cells (Lee & Saphire, 2009; Messaoudi et al, 2015) and acts as a catalyst to allow the Virus to attach to host cells (Lee & Saphire, 2009). It has been shown that GP from the Zaire Ebola strain will cause damage to endothelial cells in both human and non-human primate vessel models (Lee & Saphire, 2009). The ability to produce neutralising and protective antibodies towards this protein would allow protection against Ebola Virus and its haemorrhagic fever. Heavy N- and O- linked glycosylation on the surface of GP (see figure 1.5) acts to deter the interaction of antibodies to vital regions such as the receptor binding site, however some neutralising antibodies are able to bind to a non-glycosylated region near the base of the protein (Cook & Lee, 2013), meaning that glycosylation is not vital for the production of neutralising antibodies.

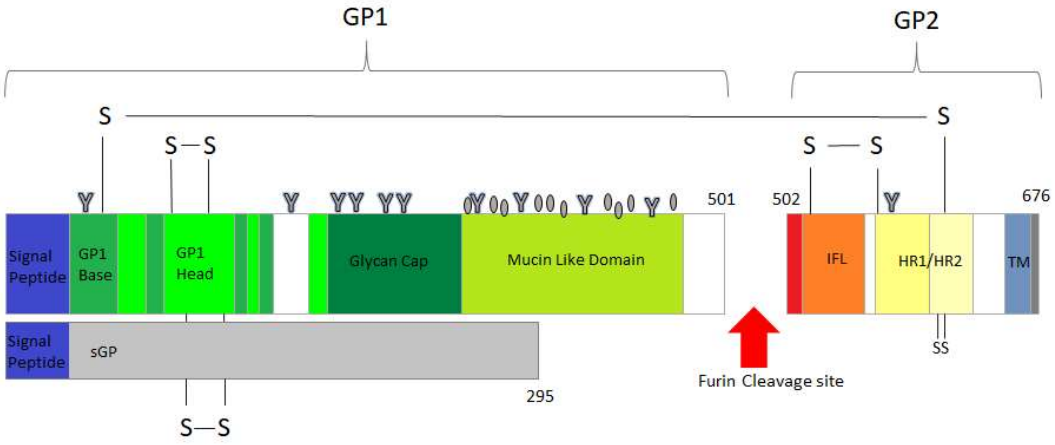


Figure 1.5. The domain organisation of the Ebola Glycoprotein. The furin cleavage site cleaves GP at residue 501 into the GP1 and GP2 subunits. The size (295 residues) and location of secreted GP (sGP) is also shown. Disulphide bonds are shown (S-S) to denote folding of the full protein. IFL – Internal Fusion Loop, HR 1/2 – Heptad Repeat 1 or 2, TM – Transmembrane domain which is followed by the cytoplasmic tail shown in grey. The Y symbol denotes N-linked glycosylation sites and the oval symbol O-linked glycosylation sites. sGP shares the first 295 aa of the full-length protein which is then transcribed after the addition of an extra adenosine residue. sGP is thought to act as a decoy for antibodies, while the heavy glycosylation at sites such as the mucin like domain is thought to shield epitopes from neutralising antibodies. Adapted from Lee et al, 2008; Cook & Lee, 2013.

1.8 Zika Virus

Zika virus is a *Flavivirus,* part of the *Flaviviridae* family, which also includes Dengue Virus, Yellow Fever and West Nile Virus. It was first isolated from a sentinel Rhesus macaque in the Zika forest of Uganda in 1947 (Dick et al, 1952). Thought to be a somewhat benign infection until an outbreak in the Federated States of Micronesia, on the Islands of Yap, and French Polynesia in 2007 and 2013 respectively, it then became much more well known. (Depoux et al, 2018; Musso & Gubler, 2016). The end of 2015 saw the rapid spread of Zika Virus throughout the Americas and along with it, connected incidences of foetal microcephaly (where a baby's head size is much smaller than normal) and Guillain-Barré syndrome. This brought Zika to the attention of the World Health Organisation (WHO), who concerned by its spread and these severe symptoms, declared it an international public health emergency in 2016 (Chitti et al, 2016; Tang et al, 2016). Phylogenetic analysis of different Zika Virus isolates has shown that there are two main lineages; African and Asian (Haddow et al, 2012).

1.8.1 Zika Virus outbreaks

Zika Virus is an arbovirus, meaning it is transmitted by arthropods, such as mosquitoes.
After it was first discovered, the first human case of Zika Virus was detected in the early 1950s in Nigeria. Since then, in Africa and South-East Asia, there have been a relatively small number of reported cases, before the outbreak in Yap, which affected almost 75% (5000 people) of its population. An epidemic in French Polynesia then followed which infected around 270,000 people in the space of approximately 6 months. In 2015, the virus was first suspected in Brazil, and was shown to be most closely related phylogenetically to the Asian strain from French Polynesia, possibly spread to Brazil from international sporting events held in Pacific countries around the time it was circulating there (late 2013-2014). By the end of 2017, 580,000 suspected and 221,000 confirmed cases of Zika Virus infection were reported in South and Central America and the Caribbean (Depoux et al, 2018).
The vectors of Zika Virus are dependent on the location. In The Americas, Pacific countries and South-East Asia, the mosquito *Aedes aegypti* is the most common vector, while in Africa it is *Aedes africanus*. The high competency of these vectors to disseminate the virus, means that it continues to spread in these areas. It has also now been shown that the virus can be

transmitted from person to person, such as through sexual intercourse or from mother to foetus (Vorou, 2016; Depoux, 2018).

1.8.2 The Zika Virus genome

The Zika Virus has an approximately 11Kb positive strand RNA genome which codes for a single polyprotein, translated in the cytoplasm of infected cells. This is then proteolytically cleaved by both viral and host proteases to result in 10 proteins. Three of these are structural, forming the virus particle; Capsid (C), Precursor-Membrane (prM) and envelope (E), and seven are non-structural; NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The non-structural proteins have functions related to polyprotein processing and viral genome replication and synthesis (Shi & Gao, 2017) (see figures 1.6 and 1.7).

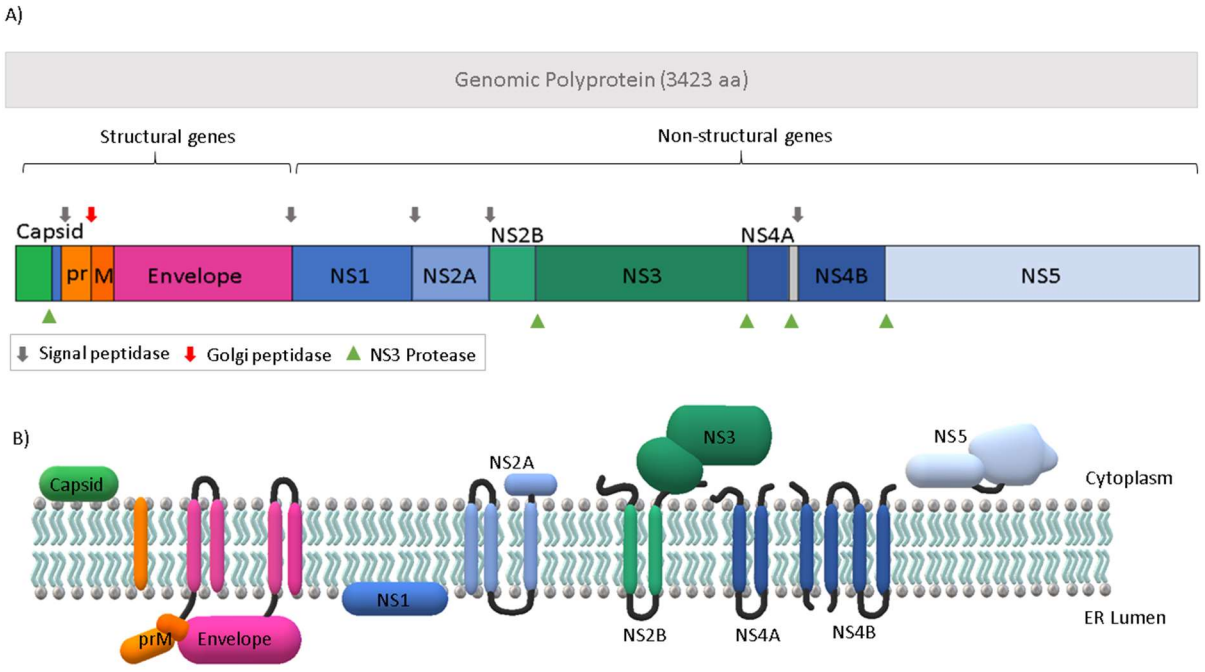


Figure 1.6. **Zika Virus genome organisation.** Schematic diagram showing the; Polyprotein (A) and processed proteins (B). A large polyprotein (A) is translated in the cytoplasm of infected cells and then cleaved by both host and viral (NS3) proteases to result in 10 proteins (B) (Adapted from Shi & Gao, 2017).

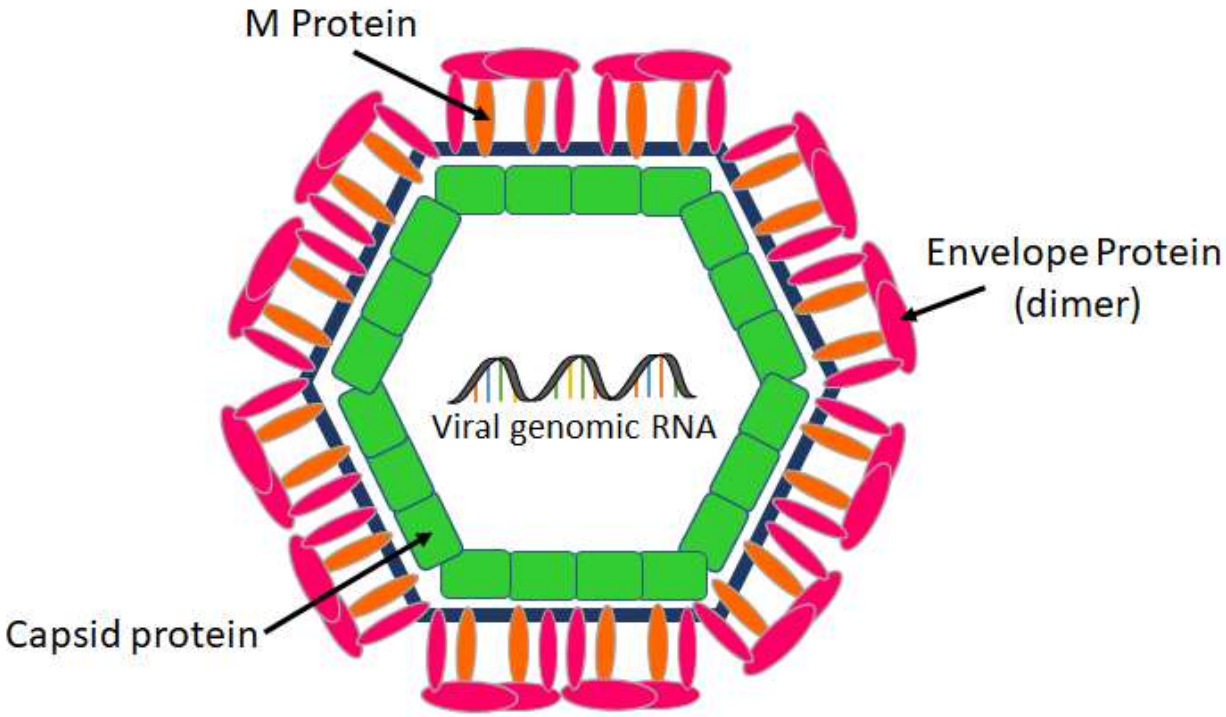


Figure 1.7. Locations of Zika Virus proteins. Schematic diagram of the locations of main structural Zika Virus proteins as a cross section. The Zika Virus has an icosahedral shape and the outside of the virus contains 180 copies of each of the envelope and M (Membrane) proteins (Shi & Gao, 2017).

1.8.3 Pathogenesis of Zika Virus

During a bite from an infected female mosquito (usually the *Aedes* genus), which has acquired Zika Virus through a viremic blood meal, the saliva of the mosquito, (containing the virus) is injected into the host (Musso & Gubler, 2016).

There is currently little that is known about the pathogenesis of Zika virus. It is thought that the virus replicates in nearby dendritic cells and can then spread to the lymph nodes and bloodstream. The majority of infected individuals are asymptomatic, however in those that do show symptoms, the virus is detectable in blood 3-4 days after their onset. In symptomatic patients, there is usually a mild fever, and reported rash, headache, conjunctivitis and joint and muscle pain (Krause et al, 2017).

The Zika Virus targets the interferon (IFN) anti-viral response. Usually, stimulation of interferon receptor subunits IFNAR1 and IFNAR2 by IFN activates the Janus kinases Jak1 and Tyk2 (Tyrosine kinase 2) by autophosphorylation. This then allows the phosphorylation of

STAT1 and STAT2 (Signal Transducer and Activator of Transcription) which form a heterodimer, which is able to bind IFN regulatory factor 9 (IRF9). This forms the heterotrimer; IFN-stimulated gene factor 3 (ISGF3) which translocates to the nucleus and acts as a transcription factor for hundreds of IFN stimulated genes (ISGs) which have antiviral properties (Fleming, 2016). All human flaviviruses that have so far been tested, have shown that the NS5 protein specifically antagonises IFN signalling. Depending on the flavivirus, the mechanism by which this occurs can be different. Zika Virus NS5 binds to human STAT2, resulting in its proteasomal degradation and thus inhibiting the Jak/STAT pathway described above. Interestingly, Zika NS5 does not bind with mouse STAT2, meaning that IFNAR knockout mice strains are needed to use as an animal model of Zika infection (Grant et al, 2016).

1.8.3.1 Microcephaly and Guillain-Barré syndrome

The recent outbreak of Zika virus, beginning in Brazil in 2015, has caused alarm due to the reported increase (of 20 times compared to previous years) of microcephaly cases, in babies born at this time (Wang et al, 2016). Foetal abnormalities including not only microcephaly, but also central nervous system issues, restricted growth and even death, were seen using ultrasound analysis, in 29% of a cohort of pregnant and Zika infected women, suggesting that infection during pregnancy may lead to adverse outcomes for the foetus (Brasil et al, 2016). Zika Virus has been shown to target human cortical neural progenitor cells (hNPCs), also found in the developing embryo brain, and this can cause apoptosis and dysregulation of the cell cycle, leading to attenuation of hNPC growth and proliferation. This tropism for neural progenitor cells, could in part explain a potential mechanism for microcephaly (Tang et al, 2016; Miner & Diamond, 2018). Zika virus has been shown to be present in the amniotic fluid of pregnant women with microcephalic foetuses, suggesting it can cross the placental barrier, strengthening the link between microcephaly and Zika Virus infection of pregnant women (Calvet et al, 2016).

In adults, Zika Virus infection has been linked with Guillain-Barré Syndrome (GBS) of which symptoms include paralysis and neuropathy. This may be due to potential autoimmune reactions as it has been shown that there is a large overlap between peptides of the Zika Polyprotein and human proteins linked to myelination, demyelination and axonal neuropathy

(Miner & Diamond, 2018; Lucehsse & Kanduc, 2016). Another possible mechanism could also be the fact that Zika Virus has been shown to be particularly detrimental to myelinating oligodendrocytes through infection, leading to a disruption in the myelin sheath and thus could contribute to the GBS symptoms (Cumberworth et al, 2017).

1.8.4 Immune responses to Zika Virus infection

Infection with Zika Virus triggers both the innate and cellular immune responses. Innate responses have been shown to involve production and signalling of type 1 interferons as described above (section 1.8.3). The cellular immune response to Zika virus has been shown in mice, NHPs and humans, to include $CD8^+$ and $CD4^+$ T cells. Strong $CD8^+$ T cell responses have been seen in Zika infected mice, along with the development of memory T cells. These recognise epitopes on the structural proteins prM, E and C.

It has also been reported that in the central nervous system (CNS) there is a Zika virus specific $CD8^+$ T cell response after infection of the neurons, even though this is an immune privileged site. While this may help to control the virus, the influx of $CD8^+$ T cells to the CNS may contribute to the incidence of GBS due to the cytotoxic killing of virus infected neurons (Richner & Diamond, 2018; Huang et al, 2017).

Protection against Zika Virus comes from the generation of potent neutralising antibodies, mainly against certain epitopes on the envelope protein (E) (Stettler et al, 2016; Sapparapu et al, 2016). It is however important to consider the phenomenon of antibody dependent enhancement of infection (ADE), which has been shown due to the antigenic cross reactivity of flaviviruses. In brief, phagocytes with Fc receptors (FcR) are able to clear pathogens coated with neutralising antibodies. Flaviviruses, in particular Dengue Virus, which this phenomenon has been mostly studied, can cause ADE during a subsequent infection with a similar but different strain or serotype. Antibodies produced in response to the first infection (particularly to pRM and E proteins) can bind to the second strain but are unable to act in a neutralising capacity. This means that FcR cells uptake the virus coated with the antibody but the virus can persist and replicate inside, rather than be cleared. The disease can then exacerbate and lead to the activation of cross reactive memory T cells and a subsequent cytokine storm, known as Dengue haemorrhagic fever or Dengue shock syndrome. It remains to be seen whether ADE is a problem in Zika Virus infections (particularly if a patient has been

previously infected with Dengue Virus) but it must be kept in mind in vaccine development, as there is a risk that disease could be exacerbated by a previous sub-optimal immune response (Richner & Diamond, 2018; Martin-Ascebes et al, 2018; Settler et al, 2016)

1.8.5 Prevention and current progress in Zika vaccine development

There is currently no commercially available prophylactic vaccines or treatment for Zika Virus, people in areas affected have been advised to use mosquito repellent and nets to avoid being bitten and pregnant women should aim to avoid Zika affected areas (Rather, 2017).
It has been noted however, that incredible progress has been seen in the development of a Zika vaccine since it was declared a major worldwide public health emergency in 2016, and a number of vaccines have reached clinical evaluation stages. For example, two DNA vaccines expressing the structural Zika prM and envelope proteins, were safe, well tolerated and elicited antibody responses allowing advancement to phase II clinical trials (Gaudinski et al, 2018). Other vaccine candidates have also been clinically tested, including those using inactivated Zika Virus, modified RNA candidates, and also a vaccine using a live measles vector (Barrett, 2018). This is encouraging as it will hopefully bring a safe and effective vaccine to market sooner rather than later.

1.8.6 Zika envelope protein (ZE) as a prime vaccine candidate

The Zika envelope protein (ZE) is the major virus surface protein and the main target for neutralising antibodies. It is a class-II fusion protein, responsible for attachment and entry into host cells and closely resembles the E protein of other flaviviruses (Settler et al, 2016; Zhang et al, 2017; Dai et al, 2016).
On the viral membrane, 90 anti-parallel homodimers of the E protein form a “raft-like structure”, enveloping the virus (see figure 1.7) . Each monomer has three domains; I, II, and III which are connected by flexible hinges. A helical transmembrane domain connects each monomer to the viral membrane. (Zhang et al, 2016; Shi et al, 2018).
It appears that some of the most potent monoclonal neutralising antibodies are directed to domain III (see figure 1.8) of the flavivirus envelope protein, particularly in the lateral ridge

region (Zhao et al, 2016). Other protective antibodies targeting the highly conserved hydrophobic fusion loop, situated at the tip of domain II, have also been discovered and while are less potent, do appear to be significantly present in the humoral immune response to flavivirus infection (Dai et al, 2016; Dejnirattisai et al, 2015). Furthermore, a unique quaternary epitope found on the dimer-dimer interface has been shown to elicit a potent broadly neutralising antibody (ZIKV-117) which was able to protect against vertical transmission of Zika Virus from mother to foetus (Sapparapu et al, 2016). An epitope for the broadly neutralising antibody 2A10G6 (also able to neutralise other flaviviruses), has also been found between residues 98 and 101 in the fusion loop area of the envelope protein (Dai et al, 2016) (see figure 1.8). There is clearly much evidence that many epitopes on the Zika envelope protein can elicit both cellular and humoral immunity, therefore suggesting that ZE is an attractive target for use in a vaccine.

Figure 1.8. The domain organisation of the Zika envelope protein. Schematic diagram of domains I, II and III, including approximate locations for some known protective epitopes. Adapted from Dai et al, 2016 (Sapparapu et al, 2016; Zhao et al, 2016).

1.9 Outer Membrane vesicles (OMVs) as a novel vaccine platform

Outer membrane vesicles (OMVs) are naturally occurring products secreted by Gram-negative bacteria. Formed by the blebbing of the outer membrane into small spherical packages, they contain periplasmic contents, including bacterial virulence factors, and nucleic acids, and outer membrane components, including antigens such as lipopolysaccharide (LPS) and proteins (Ellis & Kuehn, 2010). OMVs have many functions, from interbacterial communication, facilitating host-bacteria interactions, delivery of virulence factors and toxins to host cells, procurement of nutrients, biofilm formation and modulation of the immune response (Ellis & Kuehn, 2010; Jan, 2017).

1.9.1 Biogenesis of OMVs

All Gram-negative bacteria have been shown to produce outer membrane vesicles (Schwechheimer & Kuehn, 2015). The biogenesis of OMVs likely involves the weakening of the cross linkage and thus creating space between the outer membrane and peptidoglycan wall. The membrane bulges and the curvature eventually results in the fission of the membrane, creating the vesicle which contains periplasmic and outer membrane components (Kulp & Kuhen, 2010) (see figure 1.9). This curvature could be triggered by certain stressors such as antibiotics, which can result in an accumulation of misfolded protein waste in the periplasm, and thus OMV production may be primarily a stress response (Schwechheimer et al, 2013; Jan, 2017). OMV production may also be a part of cell wall synthesis, to release turgor pressure due to an increase in peptidoglycan production. An increase of certain molecules including LPS and phospholipids in the outer membrane, also results in changes in the curvature of the outer membrane and can increase OMV production. It has also been shown for example in *Pseudomonas aeruginosa,* that a quorum sensing molecule, PQS, when inserted into the outer membrane acts to induce increased membrane curvature (Jan, 2017).

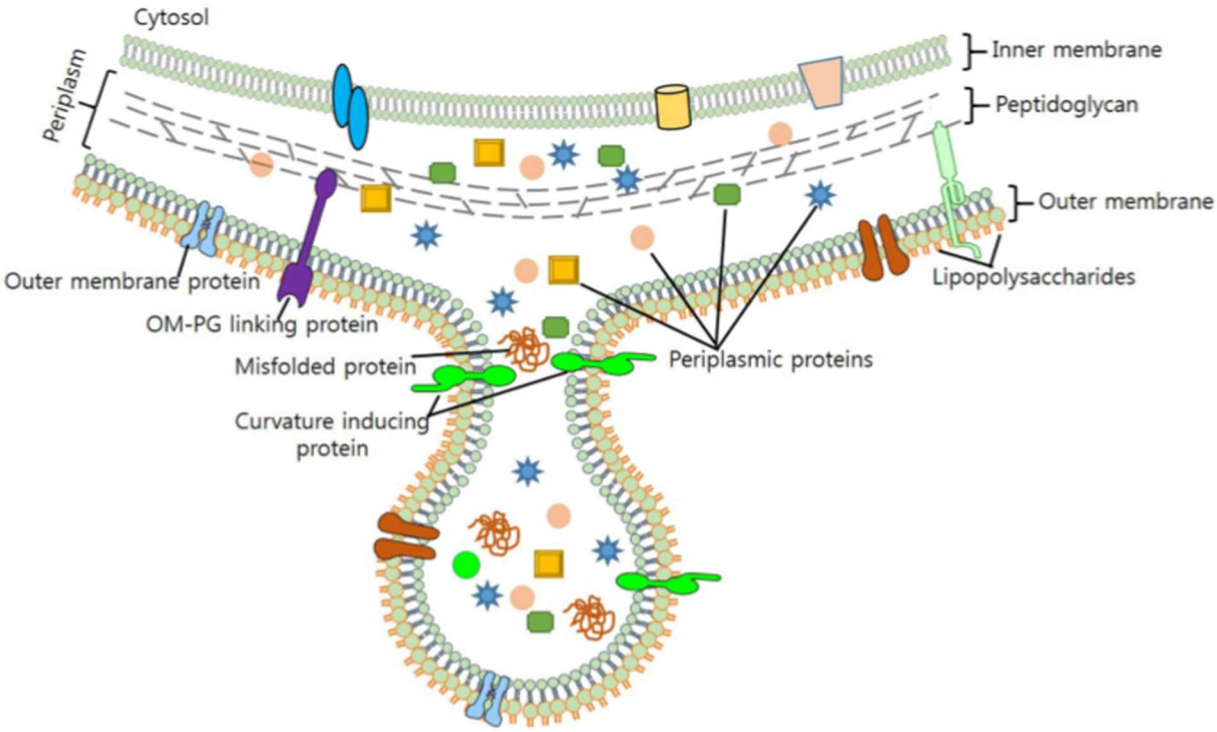


Figure 1.9. Biogenesis of Outer membrane vesicles (OMVs) in Gram-negative bacteria. The linkage between the outer membrane and peptidoglycan wall is reduced and the membrane bulges, resulting in the fission of a vesicle containing both outer membrane and periplasmic contents (Jan, 2017).

1.9.2 OMVs as immunogenic vehicles

OMVs are already a natural delivery system (Kulp & Kuehn, 2010) and have been shown to activate the host immune system due to the fact that they contain immunostimulatory molecules from the outer membrane, such as the aforementioned LPS and lipoproteins, thus stimulating host Toll-like receptors (TLRs). They have been shown to activate macrophages and stimulate dendritic cells to mature and produce cytokines such as TNF-α and IL-12. OMVs therefore, have been considered for use as vaccines (Alaniez et al, 2017; Rossi et al, 2016; Ellis & Kuehn, 2010). A vaccine against group B meningococcus, using purified OMVs from a *Neisseria meningitidis lpxL1* mutant, with detoxified lipooligosaccharide, has already been shown to be safe and immunogenic in human trials (Keiser et al, 2011).

OMVs are usually present in low quantities, so efforts have been made to genetically modify bacteria to increase the propensity of the cells to produce these vesicles for vaccine purposes. For example, in *Salmonella*, the deletion of the *tolR* gene does just this, as it disrupts the

linkage of the inner and outer bacterial membranes. The resulting 'Generalised Modules for Membrane Antigens', or GMMAs, could be a viable vaccine candidate with the added advantage of being cost effective to produce (Rossi et al, 2016; Meloni et al, 2015). A very recent study comparing the immunogenicity of *Salmonella* GMMAs with a glycoconjugate vaccine for *Salmonella* O-antigen (a component of LPS and known target of protective immunity) in mice, showed that the GMMAs were able to elicit a similar if not better immune response to O-antigen. This highlights the fact that the approach of using OMVs as vaccines is certainly very promising (Micoli et al, 2018). The bacterial strains could be engineered to allow expression of heterologous antigens with subsequent incorporation in the OMVs, allowing antigen cargo to be delivered to the host immune system (Gerritzen et al 2017). This would also be a non-living vaccine platform which would mean that unlike the living attenuated *Salmonella*, it may be possible to use in certain vulnerable patient groups where living vaccine strains could prove harmful, such as the very old, very young or immunocompromised.

1.9.3 Incorporating heterologous antigens into OMVs

One way to include heterologous antigens as cargo in purified OMVs could be to exploit protein expression mechanisms of the bacteria. Engineering expression plasmids which target heterologous protein antigen expression to specific cellular locations, such as the periplasm or outer membrane, would hopefully allow these antigens to be incorporated in the OMVs during biosynthesis.

1.9.3.1 Signal sequences for periplasmic expression of antigens

As OMVs contain bacterial proteins commonly found in the periplasm, it could be possible that guest antigens, such as the Zika envelope protein, can be engineered to be expressed and transported here and therefore contained within the OMVs when they bleb off. The OMVs would then be purified for potential use as an alternative delivery system than the living attenuated *Salmonella*.

1.9.3.1.1 The DsbA Signal sequence

It has been shown that cytoplasmically expressed bacterial proteins can be exported to the periplasm by the incorporation of the signal sequence of DsbA (Disulphide bond formation protein A), a bacterial thiol disulphide oxidoreductase. This directs the fused protein partner to the SRP pathway (Schierele et al, 2003).

1.9.3.2 Signal sequences for outer membrane expression of antigens

As the OMVs incorporate proteins situated on the bacterial outer membrane, it would be sensible to suggest that directing antigen expression there, would result in it being included when the OMVs bleb off.

1.9.3.2.1 The OmpA signal sequence

The OmpA protein (Outer membrane protein A) is a major Gram-negative outer membrane protein. It has been shown that by fusing an antigen to the OmpA leader sequence, it is possible to direct their expression to the bacterial outer membrane and also include them in OMVs (Ruppert et al, 1994; Fantappie et al, 2014).

1.10 Overall aims and scope of this thesis

This thesis aims to test *Salmonella* vaccine delivery platforms for developing effective vaccines against Ebola and Zika viruses.

A panel of variant constructs expressing the Ebola Glycoprotein, Zika envelope protein and sub-fragments thereof, will be designed and evaluated for antigen expression and plasmid stability.

The best *Salmonella* vaccine constructs, those which are able to express antigen at levels expected to be immunogenic, and which have been shown to be segregationally and genetically stable both *in vitro* and *in vivo*, will be used to immunise mice. The immune responses to these antigens will then be evaluated to assess the potential of this platform to develop effective vaccines to either Ebola aor Zika viruses.

An alternative non-living vaccine towards Zika virus will also be constructed. This will involve creating genetically modified *Salmonella* strains with a higher propensity to make immunogenic Outer Membrane Vesicles (OMVs). We aim to see if it is possible to direct the expression of the Zika envelope protein to these vesicles, which can then be purified, and hopefully used as a non-living vaccine.

It is hoped these prototype vaccines will elicit protective immune responses in mice and can therefore be a ‘proof of concept’ of a potentially cheap method by which to protect against such devastating viruses.

Chapter 2 – Materials and methods

2.1 Materials

All materials used in this study are documented below.

2.1.1 Chemicals

General laboratory chemicals and antibiotics were purchased from Sigma-Aldrich® (Dorset, UK) or Thermo Fisher Scientific (Loughborough, UK). Ingredients for bacterial growth media were purchased from BD (Oxford, UK).

2.1.2 Kits and Reagents

The Qiagen mini-prep kit, Qiagen PCR purification kit and Qiagen Gel extraction kit used to prepare DNA for cloning were purchased form Qiagen (Manchester, UK).

T4 DNA ligase, Quick-Load® 1 kb DNA Ladder, Restriction endonuclease enzymes and Phusion® High-Fidelity DNA polymerase PCR kit were purchased from New England Biolabs (NEB UK, Herts, UK). EmeraldAmp® GT PCR Master Mix was purchased from Clontech (Takara Bio Europe SAS, Saint-Germain-en-Laye, France).

Gel Red ™ (Biotium, Fremont, CA, USA) was used to detect DNA under UV light in agarose gels.

GeneRuler™1kb DNA Ladder and PageRuler™ Prestained Protein Ladder molecular weight markers were purchased from Thermo Fisher Scientific (Loughborough, UK).

Q5 Site Directed Mutagenesis kit was purchased from New England Biolabs (NEB UK, Herts, UK).

In-Fusion® HD cloning kit, used for ligation independent cloning was purchased from Clontech (Takara Bio Europe SAS, Saint-Germain-en-Laye, France).

2.1.3 Antibodies and recombinant proteins

Monoclonal antibodies

Antibody	Source
Mouse monoclonal anti-Ebola GP – 5E6	Professor Gary Kobinger, Public Health Agency of Canada
Mouse monoclonal anti-Ebola GP – 7G4	Professor Gary Kobinger, Public Health Agency of Canada
Mouse monoclonal anti-Ebola GP – 1H3	Professor Gary Kobinger, Public Health Agency of Canada
Mouse monoclonal anti-Ebola GP – 4G7	Professor Gary Kobinger, Public Health Agency of Canada
Mouse monoclonal anti Zika E	Aalto-Bioreagents (Dublin, Ireland)

Table 2.1. Details and sources of monoclonal antibodies used in this study.

Polyclonal antisera and secondary antibodies

Antibody	Source
Mouse anti-EBOV Serum from C57BL/6 mouse surviving Ebola challenge after treatment with mAb 5E6	Professor Gary Kobinger, Public Health Agency of Canada
Rabbit anti-Tet C Polyclonal sera	Professor Neil Fairweather, Imperial College London, UK
Rabbit anti-Zika envelope – R34 Serum from rabbit immunised with a peptide corresponding to residues 147-166 of Zika envelope (HGSQHSGMIVNDTGHETDENRAK)	Professor Alain Kohl, Glasgow University
Rabbit anti-Mouse HRPO	Abcam (Cambridge, UK)
Goat anti-Rabbit HRPO	Abcam (Cambridge, UK)

Table 2.2. Details and sources of polyclonal anti-sera and secondary antibodies used in this study.

Recombinant proteins

Protein	Strain	Source
Ebola Glycoprotein	Zaire Ebolavirus (strain Mayinga-76)	Professor Katie Ewer, Oxford University, UK (Ewer et al, 2016a) (Via Yper Hall, Public Health England)
Zika envelope	Derived from unspecified African strain, however all strains share high identity in this region.	Fitzgerald (Acton, MA, USA)

Table 2.3. Sources of recombinant proteins used in this study.

2.1.4 Plasmids

Plasmid	Genotype
pTECH2	Used to express Ebola and Zika virus antigens fused to Tet C, under control of *nirB* promoter, *AmpR*, TetC.
pBAD24	Arabinose-inducible expression vector. PBAD promoter, A*mp r*.
pKD46	Red helper plasmid. Derivative of pINT-ts, araC-ParaB and γ β exo, tL3 terminator downstream of exo. λ Red Recombinase gene, A*mpR*. Sensitive to temperature above 30°C.
pKD4	Derivative of pANTSγ with Kanamycin resistance (*KanR*) gene flanked by FLP recognition target (FRT) sites, A*mpR*
pCP20	*FLP+,* λ cI857+, λ *P*R Repts, A*mpR, CmR*. Sensitive to temperature above 30°C.

Table 2.4. Details of plasmids used in this study.

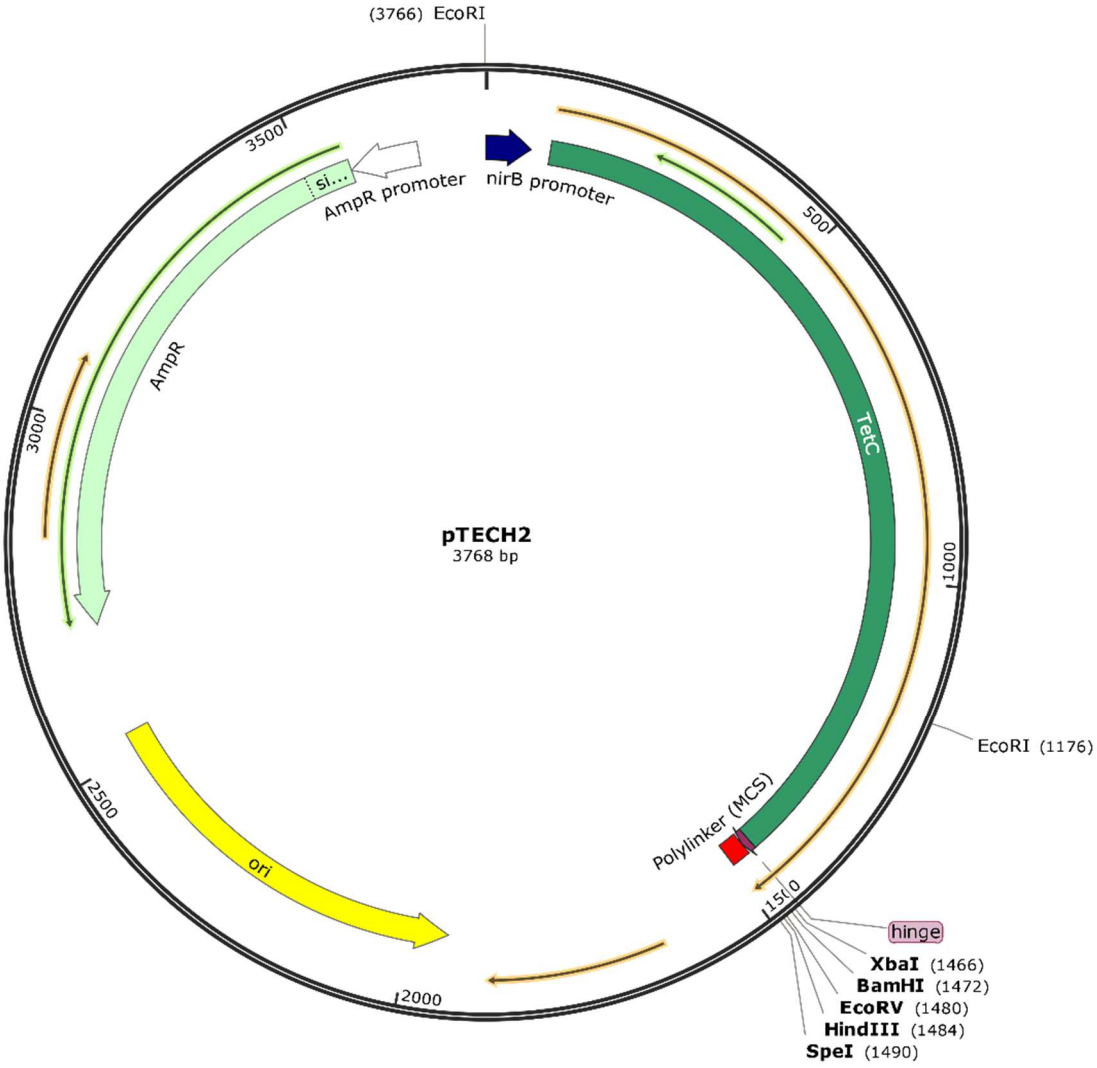


Figure 2.1. Plasmid map of the pTECH2 expression vector. Under the control of the *nirB* promoter, the atoxic C-fragment of Tetanus toxin TetC is expressed. A short Gly Pro Gly Pro hinge motif allows temporal and spatial separation between TetC and a guest antigen which can be cloned in at the polylinker site with a choice of five restriction enzyme sites. An ampicillin resistance gene allows for selection of colonies harbouring the plasmid. Plasmid map was made using the commercial software Snapgene.

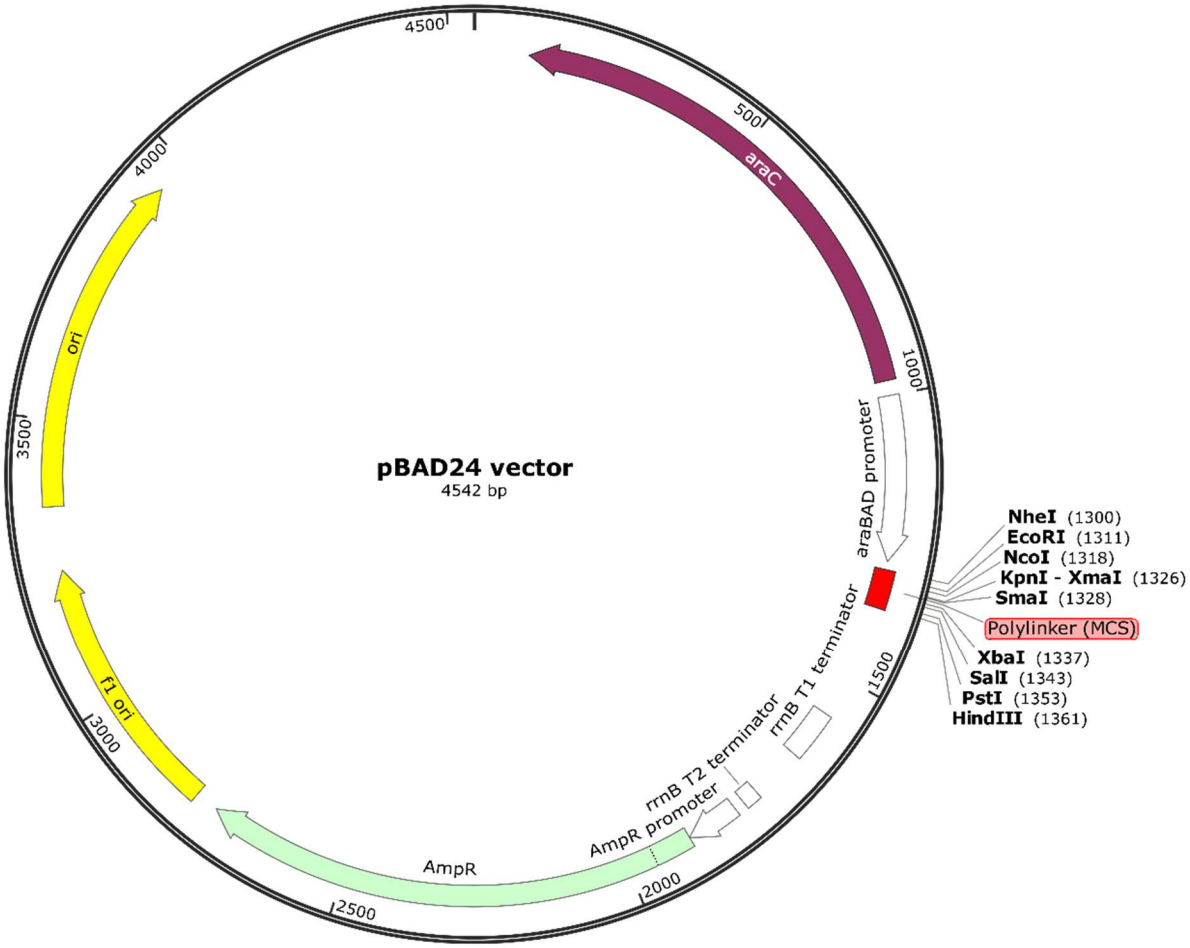


Figure 2.2. Plasmid map of the pBAD24 expression vector. Under the control of the pBAD (araBAD) promoter, in the presence of L-arabinose, the protein whose gene is cloned in the polylinker site can be expressed. An ampicillin resistance gene allows for selection of colonies harboring the plasmid and the vector also includes the pBR322 origin of replication. Plasmid map was made using the commercial software Snapgene.

2.1.5 Bacterial growth media

LB-Lennox broth

5g Tryptone, 2.5g Yeast extract, 5g NaCl, made up to 500ml with dH_20.

LB-Lennox Agar media

5g Tryptone, 2.5g Yeast extract, 5g NaCl, 7.5g Agar, made up to 500ml with dH_20.

EBU plates

LB-lennox agar (see above) plus 1.25g glucose, then when molten agar cooled to 55°C - 20ml 12.5% K_2HPO_4, 625µl 1% Evans Blue, 125µl 10% Sodium fluorescein (Uranine).

2.1.6 Buffers and Stock solutions

Antibiotic stock solutions

Ampicillin (1000x) - 50mg ampicillin sodium salt dissolved per 1 ml dH_20.

Working concentration: 50 µg/ml.

Kanamycin (1000x) - 50 mg kanamycin sulphate salt dissolved per 1 ml dH_20.

Working concentration: 50 µg/ml.

Chloro-naphthol (Western blot developer)

30mg 4-chloro-1-naphthol, 10ml methanol, 40ml PBS, 30µl hydrogen peroxide (30% w/w). Freshly prepared using cold ingredients immediately prior to use.

0.5M EDTA

93.06g EDTA made up to 500ml with dH_20. Adjusted to pH 8.0.

ELISA coating buffer

Phosphate buffered Saline (PBS) (see below).

LPS ELISA coating buffer (Reggiardo's buffer)

1.88g glycine (0.05M), 2.92g NaCl (0.1M), 0.19g EDTA (1mM), 1.05g NaF (0.05M), made up to 100ml in dH_20. Stored at 4°C, protected from light.

For use, dilute 1:5 in dH_20 plus DOC (Sodium Deoxycholate) to final concentration of 0.1%. Adjusted to pH 8 with NaOH.

ELISA block buffer

Blocker™ Casein in PBS (Thermo Scientific™).

Phosphate Buffered Saline (PBS)

PBS tablets (SIGMA), 1 tablet dissolved in 200ml dH_20.

SDS-PAGE running buffer

30.3 g Trizma® base (250 mM), 187.7 g glycine (2.5 M), 10 g sodium dodecyl sulphate (SDS) (1%), made up to 1 litre with dH_20.

TAE buffer (50X)

242 g Trizma® base, 57.1 ml acetic acid, 100 ml 0.5 M EDTA, made up to 1 litre with dH_20. Adjusted to pH 8.0.

TE buffer

10mM Tris-HCl pH 8.0, 10mM EDTA

Western blot transfer buffer

14.4 g glycine, 3.06 g Trizma® base, 200 ml methanol, made up to 1 litre with dH_20.

Freshly prepared and cooled to 4°C prior to use.

2.1.7 Bacterial strains

Strain	Genotype	Source
S. *enterica* serovar Typhimurium SL5338	*galE,* r – m + (Rough LPS mutant)	Khan lab strain
S. *enterica* serovar Typhimurium SL1344	Wild-type, derivative of LT2	Khan lab strain
S. *enterica* serovar Typhimurium SL3261	SL1344 *aroA*	Derek Pickard, Wellcome Trust Sanger Institute (London)
BRD 807	*aroA* and *htrA* double mutant of *Salmonella* Typhimurium SL1344	Derek Pickard, Wellcome Trust Sanger Institute (London)
BRD 509	*aroA* and *aroD* double mutant of *Salmonella* Typhimurium SL1344	Derek Pickard, Wellcome Trust Sanger Institute (London)
C5*htrA*	*htrA*::T*nphoA* insertion mutant of *Salmonella* Typhimurium C5	Derek Pickard, Wellcome Trust Sanger Institute (London)
***E. coli* NEB5α Chemically competent cells**	fhuA2 (argF-lacZ)U169 phoA glnV44 80 (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	New England Biolabs, Massachusetts, USA
***E. coli* Stellar ™ Competent cells**	F–, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ80d lacZΔ M15, Δ (lacZYA - argF) U169, Δ (mrr - hsdRMS - mcrBC), ΔmcrA, λ–	Clontech Laboratories, Inc. Califonia, USA

Table 2.5. Details and sources of bacterial strains used in this study.

2.1.8 Primers and Oligonucleotides

Synthetic oligonucleotide primers were designed and then purchased from Eurofins genomics and delivered as lyophilised samples. They were subsequently reconstituted in nuclease-free dH2O to a final concentration of 100µM and stored at -20°C until use.

Antigen genes

Gene	Strain	Source
Synthetic Ebola GP (V1)	Zaire Ebolavirus (strain Mayinga-76)	Eurofins Genomics (Ebersberg, Germany)
Synthetic Ebola GP (V2)	Zaire Ebolavirus (strain Mayinga-76)	Eurofins Genomics (Ebersberg, Germany)
WT Zika E	PE243 (Donald et al, 2016)	Dr Claire Donald (Glasgow University, UK)
Synthetic Zika E	PE243 (Donald et al, 2016)	DC Biosciences (Dundee, UK)

Table 2.6. Sources of antigen genes used in this study.

Cloning primers

Construct	Primer name	5' → 3' Sequence	Usage
Ebola Glycoprotein expression constructs			
pTECH2-GP (v1)	Synthetic GP FWD	CGC **TCT AGA** ATC CCG TTA GGC GTC ATT CA	*XbaI* cloning of synthetic GP(v1) into pTECH2 (minus signal peptide)
	Synthetic GP REV	ACT **AAG CTT** CTG TCG CCA GCC TGT CCA	*HindIII* cloning of synthetic GP(v1) into pTECH2 (minus transmembrane region)
pTECH2-GP (v1) section 1	Section 1 GP REV	TGC **AAG CTT** CGC AGG GGA TGG CTA CTA AA Used with Synthetic GP FWD	*HindIII* cloning of synthetic GP(v1) section 1 into pTECH2
pTECH2-GP (v1) section 2	Section 2 GP FWD	CGC **TCT AGA** CCT CAG GCT AAG AAA GAC TT	*XbaI* cloning of synthetic section 2 GP(v1) into pTECH2
	Section 2 GP REV	GCT **AAG CTT** TGG ACT TGT ACC ATC GCG GA	*HindIII* cloning of synthetic GP(v1) section 2 into pTECH2
pTECH2-GP (v1) section 3	Section 3 GP FWD	ACA **TCT AGA** GCC TCC GAA AAT TCC TCC GC	*XbaI* cloning of synthetic section 3 GP(v1) into pTECH2
	Section 3 GP REV	CAG **AAG CTT** TGA GCA TTG ACG ATC GCT TC	*HindIII* cloning of synthetic GP(v1) section 3 into pTECH2
pTECH2-GP (v1) section 4	Section 4 GP FWD	ATA **TCT AGA** CGG ACG CGT CGT GAA GCG AT Use with Synthetic GP REV	*XbaI* cloning of synthetic section 4 GP(v1) into pTECH2

Construct	Primer name	5' → 3' Sequence	Usage
pTECH2-GP (v2)	GP FWD	**GAT ATC** ATT CCG CTG GGC GTG A	*EcoRV* cloning of Synthetic GP (v2) full-length into pTECH2
	GP REV	**AAG CTT** TTG ACG CCA GCC GGT C	*HindIII* cloning of Synthetic GP (v2) full-length into pTECH2
pTECH2-GP MFL	GP MFL FWD	CAG AT**G GAT CC**A CGC CGG TCT ATA AGC TG Use with D-MFL REV to amplify sub-fragment 'MFL'	*BamHI* cloning of Synthetic GP (v2) sub-fragment MFL into pTECH2
pTECH2-GP D-MFL	GP D-MFL FWD	CAG AT**G GAT CC**C ACA AGG AAG GCG CAT TTTT C	*BamHI* cloning of Synthetic GP (v2) sub-fragment D-MFL into pTECH2
pTECH2-GP D-MFL **pTECH2-MFL**	GP D-MFL REV	CTG AT**A AGC TT**G CCA TCC TGA TTG TGC AT	*HindIII* cloning of Synthetic GP (v2) sub-fragments MFL or D-MFL into pTECH2
pTECH2-GP D	GP D REV	TAC TG**A AGC TT**T GCG AAA CAG CT Use with D-MFL FWD to amplify sub-fragment 'D'	*HindIII* cloning of Synthetic GP (v2) sub-fragment D into pTECH2
pTECH2-GP LH	GP LH FWD	CAG AT**G GAT CC**C CGC AGG CGA AGA AAG AT	*BamHI* cloning of Synthetic GP (v2) sub-fragment LH into pTECH2
	GP LH REV	TAC TG**A AGC TT**T TGC CCG ATG ACG CGC TTT C	*HindIII* cloning of Synthetic GP (v2) sub-fragment LH into pTECH2
pTECH10-GP	pTECH10 REV	CAG AAA GTC TCC TGT GGA	Used together with pTECH2-GP as a template to remove TetC gene with inverse PCR (Q5 site directed mutagenesis – NEB)
	pTECH10 FWD	ATG GAT ATC ATT CCG CTG GGC	
pTECH11-GP	pTECH11 REV	GTT GTC GAC CCA ACA ATC AAG	Used together with pTECH2-GP as a template to remove all but the first 30bases of the TetC gene with inverse PCR (Q5 site directed mutagenesis – NEB)
	pTECH11 GP FWD	GAT ATC ATT CCG CTG GGC GTG	

Construct	Primer name	5' → 3' Sequence	Usage
pTECH11-GP (155)	GP 155 FWD	CCG CAG GCG AAG AAA GAT TTC	Used with pTECH11 REV (pTECH2-GP template) - inverse PCR (Q5 site directed mutagenesis – NEB
Zika envelope Expression constructs			
pTECH2-synthetic ZE	ZEs FWD	TAG CC**G GAT CC**A TTC GTT GTA TCG GCG TA	*BamHI* cloning of Synthetic Zika E into pTECH2
	ZEs REV	**AAG CTT** TTT GCC GAT GGT GGA GCC	*HindIII* cloning of Synthetic Zika E into pTECH2
pTECH2-ZE(s193)	ZEs 193 REV	CTG AT**A AGC TT**A CGC GGT TCG CAA TCC AG	*HindIII* cloning of Synthetic Zika E (193) into pTECH2
pTECH2-ZE(WT)	ZE WT FWD	**GGA TCC** ATC AGG TGC ATA GGA GTC	*BamHI* cloning of WT Ze into pTECH2
	ZE WT REV	CGC **AAG CTT** TTA AGT GGC TTC AAA TGC	*HindIII* cloning of WT Ze into pTECH2
pTECH10-ZE(s)	pTECH10-ZE(s) FWD	ATG GGA TCC ATT CGT TGT ATC GGC Used with pTECH10 FWD	Used together with pTECH10 REV with pTECH2-ZE as a template to remove TetC gene with inverse PCR (Q5 site directed mutagenesis – NEB)
pTECH11-ZE(s)	pTECH11-ZE(s) FWD	GGA TCC ATT CGT TGT ATC GGC used with ZEs FWD	Used together with ptECH11 REV with pTECH2-ZE as a template to remove all but the first 30bases of the TetC gene with inverse PCR (Q5 site directed mutagenesis – NEB)
pTECH-ZE-DMFL	ZE-DMFL FWD	GCT TA**A AGC TT**G GTC CTG GTC CTC ACA AGG AAG GCG CAT TTT TC	*HindIII* cloning of Ebola GP DMFL into pTECH10-ZE(s)
	ZE-DMFL REV	CGC GT**A CTA GT**G CCA TCC TGA TTG TGC ATC A	*SpeI* cloning of Ebola GP DMFL into pTECH10-ZE(s)
pBAD24-ZE	pBAD24 ZE In-Fusion® FWD	CCG GGG ATC CTC TAG AAT GTC CAT TCG TTGTAT CGG CG	In-Fusion® Ligation independent cloning of ZE into pBAD24
	pBAD24 ZE In-Fusion® REV	CAA AAC AGC CAA GCT TTT ATT TGC CGA T GG TGG AGC C	

Table 2.7. Cloning primers. All primers used to PCR amplify Ebola GP or Zika E genes or gene fragments for cloning into expression vectors pTECH2 or pBAD24 as described. All primers were synthesised by Eurofins Genomics.

Signal sequence Oligonucleotides and In-Fusion® primers

Construct	Oligo/primer name	5' → 3' sequence	Usage
pBAD24 OmpA ss ZE	OmpA ss Sense	[PHO] **AAT TC**A TGA AAA AGA CAG CTA TCG CGA TTG CAG TGG CAC TGG CTG GTT TCG CTA CCG TAG CGC AGG CC**T**	Annealed oligo (sense and anti-sense strands) of OmpA signal sequence containing 'pre-digested' *EcoRI* and *XbaI* restriction sites, for cloning into pBAD24-ZE
	OmpA ss Anti-sense	[PHO] **CTA GA**G GCC TGC GCT ACG GTA GCG AAA CCA GCC AGT GCC ACT GCA ATC GCG ATA GCT GTC TTT TTC AT**G**	
pBAD24 OmpA ss MSR ZE	OmpA ss MSR In-Fusion® REV	ACA ACG AAT GGA CAT TTT AGC ACC AGC GTA CCA GGT GTT ATC TTT CGG AGC GGC CTG CGC TAC GGT AGC	In-Fusion® ligation independent cloning of OmpA Membrane spanning Region (MSR) into pBAD24-OmpA ss ZE, using pBAD24-OmpA-ZE as a template.
	OmpA ss MSR In-Fusion® FWD	ATG TCC ATT CGT TGT ATC GGC G	
pBAD24 DsbA ss ZE	DsbA ss Sense	[PHO] **AAT TC**A TGA AAA AGA TTT GGC TGG CGC TGG CTG GTA TGG TTT TAG CTT TTA GCG CCT CGG CAG CA**T**	Annealed oligo (sense and anti-sense strands) of DsbA signal sequence containing 'pre-digested' *EcoRI* and *XbaI* restriction sites, for cloning into pBAD24-ZE
	DsbA ss Anti-sense	[PHO] **CTA GA**T GCT GCC GAG GCG CTA AAA GCT AAA ACC ATA CCA GCC AGC GCC AGC CAA ATC TTT TTC AT**G**	

Table 2.8. Signal sequence Oligonucleotides and In-Fusion® primers.
Oligonucleotides used to construct OmpA and DsbA DNA signal sequences and In-Fusion® primers to allow the addition of the OmpA membrane spanning region (MSR). All primers were synthesised by Eurofins Genomics.

Construct screening primers

Construct	Primer name	5' → 3' sequence	Usage
Any pTECH2	pTETnir15 FWD	GAC CTG AAA ACC TAC TCT GT	pTECH2 screening
	pTETnir15 REV	CAG CGA GTC AGT GAG CGA G	
pTECH10/11	pTECH10/11 screen FWD	CGT CTT CAG AAT TCA GGT A	pTECH10/11 screening
Any pBAD24	pBAD screen FWD	TCT CGC TAA CCA AAC CGG TAA C	pBAD24 screening
	pBAD screen REV	CAG GAG AGC GTT CAC CGA CAA	
pTECH ZE-DMFL	pTECH ZE-DMFL screen FWD	CGTCTTCAGAATTCAGGTA	Used for DNA sequencing pTECH-ZE-DMFL construct
pBAD24-OmpA ss ZE **pBAD24-OmpA MSR ss ZE**	OmpA ss screen FWD	CAT GAA AAA GAC AGC TAT CGC G	Used for DNA sequencing to confirm insertion of OmpA signal sequence (with or without MSR) into pBAD24-ZE
pBAD24-DsbA ss ZE	DsbA ss screen FWD	CAT GAA AAA GAT TTG GCT GGC GC	Used for DNA sequencing to confirm insertion of DsbA signal sequence into pBAD24-ZE

Table 2.9. Construct screening primers. All primers used to screen putative clones as described, both with colony PCR and DNA sequencing (Eurofins). All primers were synthesised by Eurofins Genomics.

Knockout oligonucleotides

Strain	oligo name	5' → 3' sequence	Usage
SL1344 *ΔtolR*	*tolR* KO FWD	CCA GGC GTT TAC CGT AAG CGA AAG CAA CA AGGG GTA AGC CGT GTA GGC TGG AGC TGC TTC	Used in one-step gene disruption technique to knockout *tolR* in *Salmonella* SL1344
SL1344 *ΔtolR*	*tolR* KO REV	CCT GTT ACT CGC CGT CTT TCA AGC CAA CGG GAC GCA GAC TAT GGG AAT TAG CCA TGG TCC	
SL1344 *ΔmlaA*	*mlaA* KO FWD	ATG GCA ATC AGC GAT AGC CAT AAT TCA CAG GGA GAC ATC TGT GTA GGC TGG AGC TGC TTC	Used in one-step gene disruption technique to knockout *mlaA* in *Salmonella* SL1344
SL1344 *ΔmlaA*	*mlaA* KO REV	AAA AGG TGA GCA TTG CGC TCA CCT TTT TAT TTA CTG CCG TAT GGG AAT TAG CCA TGG TCC	

Table 2.10. Knockout oligonucleotides. All oligonucleotides (primers) used to PCR amplify the *KanR* gene from the pKD4 template to carry out the Lambda-red recombinase technique to knockout genes *tolR* or *mlaA* from *Salmonella* Typhimurium strain SL1344.

Knockout screening primers

Strain	Primer name	5' → 3' sequence	Usage
SL1344 *ΔtolR*	tolR check FWD	CGC CAG GCG TTT ACC GTA	Screening for successful *tolR* knockout
SL1344 *ΔtolR*	tolR check REV	GTT CGC CTG TTA CTC GCC G	
SL1344 *ΔmlaA*	mlaA check FWD	GGA TGG CAA TCA GCG AT	Screening for successful *mlaA* knockout
SL1344 *ΔmlaA*	mlaA REV	CTT TCT GAA ACG GAT CA	

Table 2.11. Knockout screening primers. List of all primers and oligonucleotides used in this study. All primers were synthesised by Eurofins Genomics.

2.1.9 SDS-PAGE gel

3.5% stacking gel

3.01ml dH_20, 1.2ml 1M Tris pH 6.8, 590µl 30% bis-acrylamide, 100µl 10% Sodium Dodecyl Sulphate (SDS), 150µl 10% Ammonium persulfate, 15µl N,N,N'N'-Tetramethylethylenediamine (TEMED).

10% separating gel

6.3ml dH_20, 3.75ml 1.5M Tris pH 8.8, 5.025ml 30% bis-acrylamide, 300 µl 10% Sodium Dodecyl Sulphate (SDS), 150µl 10% Ammonium persulfate, 15µl N,N,N'N'-Tetramethylethylenediamine (TEMED).

2.1.10 SDS PAGE Molecular weight marker

PageRuler^{TM} 10kDa-180kDa prestained protein ladder (ThermoScientific) was used as a molecular weight marker in SDS-PAGE gels and western blots to indicate the size of separated proteins (kDa) after fractionation by electrophoresis.

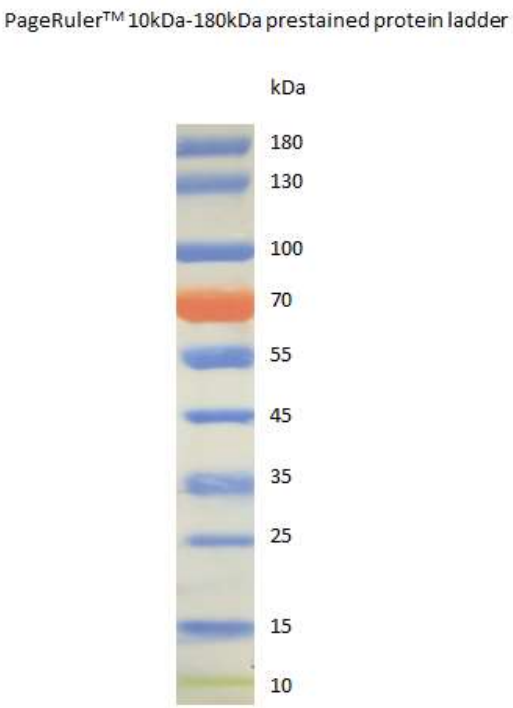


Figure 2.3. Protein molecular weight marker. Indicators of protein size (kDa) for PageRuler™ 10kDa-180kDa prestained protein ladder (ThermoScientific). Used in SDS-PAGE and western blots to determine size of fractionated proteins.

2.1.11 Agarose with Gel Red ™

0.7% agarose in TAE buffer, with 10000x Gel Red ™ (Biotium) to final 1x concentration, was used to fractionate DNA samples with electrophoresis and allowed the ability to view DNA bands under UV light with a UVP BioDoc-It™ imaging system.

2.1.12 Agarose Gel DNA Molecular weight marker

GeneRuler™ 1 kb DNA Ladder (ThermoScientific) or Quick-Load® 1 kb DNA Ladder (New England Biolabs) were used as DNA molecular size markers for fractionation of DNA in agarose gels.

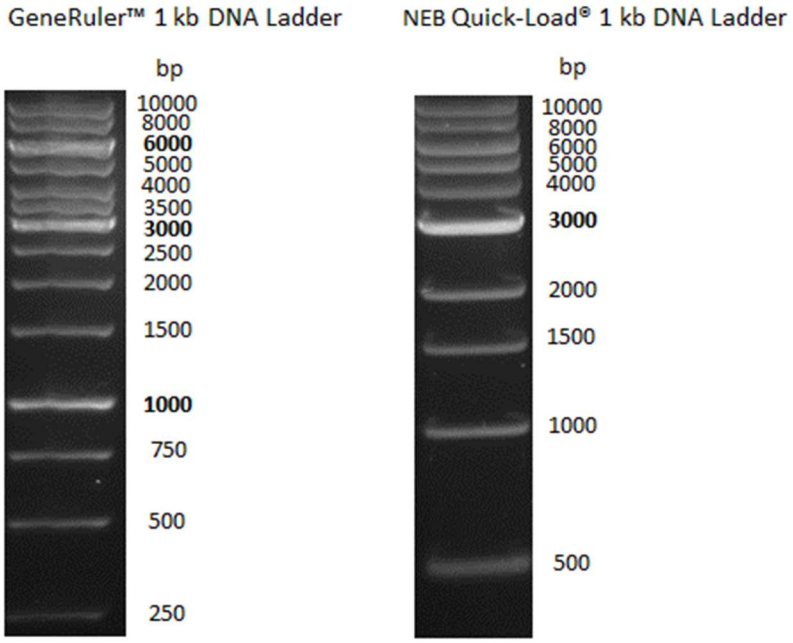


Figure 2.4. DNA molecular weight markers. Indicators of DNA fragment sizes for GeneRuler™ 1 kb DNA Ladder (ThermoScientific) or Quick-Load® 1 kb DNA Ladder (New England Biolabs). Used in agarose gel electrophoresis of DNA to determine size of DNA fragments.

2.2 Methods

All methods and protocols used in this study are documented below.

2.2.1 Growth conditions for Bacteria

Bacteria was grown in liquid LB broth or plated on LB agar with appropriate antibiotics if necessary. Ampicillin and Kanamycin were both added at 50µg/ml.

2.2.2 Plasmid DNA purification

Plasmid DNA was extracted from overnight bacterial cultures using the Qiagen mini-prep kit. 5ml overnight cultures were pelleted by centrifugation at 3220 x g for 10 minutes. Pellets were resuspended in 250µl Qiagen buffer P1 containing RNase, and 250µl Qiagen alkaline cell lysis buffer P2 was added. This was then mixed by inversion, prior to adding 350µl Qiagen neutralisation buffer N3. Samples were centrifuged at 16060 x g for 10 minutes and supernatant added to the provided QIAprep spin columns. The columns were centrifuged at 3220 x g for 1 minute and flow through discarded. The bound DNA remaining in the column was washed with 500µl Qiagen binding buffer, PB, followed by 750µl Qiagen wash buffer, PE with 1-minute spins in between at 3220 x g. Remaining wash buffer was removed with an extra 30 second spin at 3220 x g. The columns were then placed in clean 1.5ml microcentrifuge tube and the DNA was eluted with either 50µl of Qiagen Elution buffer, EB, or 50µl distilled DNase/RNase free water, depending on subsequent use. The purified plasmid was stored at -20°C.

2.2.3 Restriction enzyme digestion of DNA

Restriction endonuclease enzymes from New England Biolabs (NEB) were used according to the manufacturer's instructions using the provided NEB 10X CutSmart ® buffer.
Generally, 1µg of DNA was cut with 10 units of restriction enzyme and digested at 37°C for 1 hour. Following digestion, samples were purified using the QIAquick PCR purification kit

(Qiagen) according to manufacturer's instructions and visualised under UV light after agarose gel electrophoresis a UVP BioDoc-It™ imaging system.

2.2.4 PCR amplification and purification of DNA for cloning

DNA encoding the Ebola Glycoprotein and Zika envelope genes and sub-fragments were prepared for cloning into pTECH2 or pBAD24 using polymerase chain reaction (PCR) with NEB Phusion® High-Fidelity DNA polymerase PCR kit on a Biometra T1 thermocycler.
Regents were mixed on ice according to the manufacturer's instructions and the following cycling conditions were followed:

Cycle step	No. of cycles	Temp	Duration
Initial denaturation	1	98°C	30 seconds
Denaturation	30	98°C	10 seconds
Annealing		50°C	30 seconds
Extension		72°C	30 seconds per kb
Final extension	1	72°C	10 minutes
Hold	1	4°C	∞

Table 2.12. PCR amplification and purification of DNA for cloning. Thermal cycling conditions for polymerase chain reaction to amplify antigen gene fragments for cloning into expression vectors.

The PCR product, which included the restriction endonuclease sites from each primer, was then purified using the Qiagen PCR purification kit according to manufacturer's instructions.

2.2.5 Ligation

Ligation of DNA fragments and vector was carried out using T4 DNA Ligase (NEB). Following the creation of sticky ends by restriction enzyme digestion the following ligation mix was combined: 2µl 10X T4 DNA Ligase buffer (NEB), 1µl (400 units) T4 DNA ligase (NEB), calculated amounts of digested vector and insert DNA (approximately 150ng vector with a molar ratio of 3:1 vector to insert) Nuclease-free dH_2O up to 20µl.

Volumes of digested insert and vector DNA was calculated using the following equation:

$$\left(\frac{ng\ vector\ \times kb\ insert}{kb\ vector}\right) \times \frac{3}{1} = ng\ insert$$

Ligation reactions were mixed on ice and incubated at 16°C overnight using a Biometra T1 thermocycler. The following day, ligation reaction mixes were heat inactivated at 65°C for 10 minutes and transformed into chemically competent SL5338 *Salmonella* Typhimurium. Cells were spread onto LB agar at a range of dilutions with 50µg/ml ampicillin and incubated overnight at 37°C.

2.2.6 Colony PCR

To determine bacterial clones which had been successfully transformed with effectively ligated plasmids, single colonies from transformed cells were picked and transferred into 50µl TE buffer with 1% TritonX100. Samples were incubated at 95°C for 10 minutes and centrifuged at 16060 x g for 10 minutes. 1µl of this supernatant was then added to a master mix consisting of: 5µl Emerald master mix (Clontech), 1µl forward screening primer (10µM), 1µl reverse screening primer (10µM), 2µl nuclease-free dH_2O. Regents were mixed on ice according to the manufacturer's instructions and the following cycling conditions using a Biometra T1 thermocycler were followed:

Cycle step	No. of cycles	Temp	Duration
Initial denaturation	1	98°C	10 seconds
Denaturation	30	98°C	10 seconds
Annealing		52°C	30 seconds
Extension		72°C	60 seconds per kb
Final extension	1	72°C	5 minutes
Hold	1	4°C	∞

Table 2.13. Colony PCR. Thermal cycling conditions for polymerase chain reaction to amplify DNA from lysed bacteria to confirm the identity of putative clones. 1µl of the PCR mix was run on a 0.7% agarose gel and visualised under UV light with a UVP BioDoc-It™ imaging system.

2.2.7 Q5 Site-directed mutagenesis

To remove the *TetC* gene and create the pTECH-10 constructs, inverse PCR was carried out, using a Q5 Site-Directed Mutagenesis Kit (New England Biolabs). Primers were designed on either side of the *TetC* gene, so that the 5' ends annealed back to back on the new construct. The construct was then amplified using PCR. The reaction was mixed as follows: 12.5µl Hot Start High-Fidelity 2X Master Mix, 1.25µl of each primer (forward and reverse, to a final concentration of 0.5µM), 25ng template DNA (here, the pTECH2-GP or ZE plasmid), and 9µl nuclease-free dH_2O. The following cycling conditions using a Biometra T1 thermocycler were followed:

Cycle step	No. of cycles	Temp	Duration
Initial denaturation	1	98°C	30 seconds
Denaturation	30	98°C	10 seconds
Annealing		50-72°C	30 seconds
Extension		72°C	30 seconds per kb
Final extension	1	72°C	2 minutes
Hold	1	4°C	∞

Table 2.14. Q5 Site-directed mutagenesis. Thermal cycling conditions for inverse polymerase chain reaction using a Q5 Site-Directed Mutagenesis Kit (New England Biolabs).

The PCR product from the above reaction was then used in a KLD reaction, using the same kit. 1µl of PCR product was mixed with 5µl 2X KLD reaction buffer, 1µl KLD enzyme mix and 3µl nuclease free dH_2O. This reaction was incubated for 5 minutes at room temperature and 5µl used to transform NEB5α chemically competent *E. coli* cells (New England Biolabs) using the manufacturer's heat-shock protocol. Cells were spread onto LB agar at a range of dilutions with 50µg/ml ampicillin and incubated overnight at 37°C. Putative clones were screened using colony PCR (see section 2.2.6) and DNA sequencing (Eurofins).

2.2.8 In-Fusion® ligation independent cloning

The template vector was linearised by appropriate restriction enzyme digestion (see section 2.2.3) and the insert gene fragment was amplified by PCR using primers designed to have 15bp extensions complementary to the ends of the linearised vector, where the gene will be inserted.

100ng of template DNA was added to 1.2µl CloneAmp™ HiFi PCR premix, forward and reverse primers to a final concentration of 0.2µM and nuclease free dH_2O to a final volume of 25µl. The following cycling conditions using a Biometra T1 thermocycler were then followed:

Cycle step	No. of cycles	Temp	Duration
Denaturation	35	98°C	10 seconds
Annealing		55°C	15 seconds
Extension		72°C	5 seconds per kb
Hold	1	4°C	∞

Table 2.15. In-Fusion® ligation independent cloning. Thermal cycling conditions for polymerase chain reaction to amplify DNA for In-Fusion® cloning.

The resulting PCR product was then spin column purified using Nucleospin® PCR cleanup kit (Macherey-Nagal) according to manufacturer's instructions and the In-Fusion® cloning reaction was subsequently set up as follows: 100ng of purified DNA insert, 100ng purified linearised plasmid, 2µl 5X In-Fusion® HD Enzyme premix, and nuclease free dH_2O to a final volume of 10µl. The reaction was mixed and incubated for 15 minutes at 50°C using a Biometra T1 thermocycler, then placed on ice. 5ng of the reaction mixture was then immediately transformed into Stellar™ competent *E. coli* (Clontech) according to the manufacturer's instructions. Cells were spread onto LB agar at a range of dilutions with 50µg/ml ampicillin and incubated overnight at 37°C.

Putative clones were screened using colony PCR (see section 2.2.6) and DNA sequencing (Eurofins).

2.2.9 Annealing of oligonucleotide strands

The oligonucleotides encoding the *OmpA* and *DsbA* signal sequences were synthesised in sense and anti-sense strands and reconstituted in nuclease free and nuclease free dH_2O to a concentration of 100µM. To anneal the strands, 10µl of each reconstituted strand (making a final concentration of 10µM) was added to 80µl nuclease free dH_2O resulting in a final volume of 100µl. This mixture was heated to 95°C for 5 minutes using a Biometra T1 thermocycler and cooled at a rate of 1°C per minute.

2.2.10 Production of chemically competent Salmonella Typhimurium SL5338 and transformation

Salmonella Typhimurium strain SL5338 was cultured overnight at 37°C, with shaking at 200 RPM. The following morning the culture was diluted 1:100 in 100ml LB broth and placed at 37°C with shaking at 180 RPM until OD_{600} 0.4 – 0.6. Cells were chilled on ice for 20 minutes and then pelleted at 3220 x g at 4°C for 10 minutes.
Cells were resuspended in 50ml 0.1M $MgCl_2$ and pelleted at 3220 x g at 4°C for 10 minutes. The pellet was then resuspended in 50ml 0.1M $CaCl_2$ and pelleted as above. The pellet was finally resuspended in in 5ml 0.1M $CaCl_2$ and 250µl TES added.

Cells were chilled on ice for 30 minutes and the plasmid or ligation mix added to 200µl cells prior to chilling on ice for a further 30 minutes. Each 200µl reaction was heat shocked at 42°C for 2 minutes and left on ice to cool.

2ml warm LB broth was added and cells were placed at 37°C with shaking at 80 RPM for 30 minutes then 200 RPM for 1 hour. 100µl cells was plated on LB agar plates at a range of dilutions (1X to 1:100) with appropriate selective antibiotics.

2.2.11 Production of electro-competent Salmonella vaccine strains and electroporation

Salmonella Typhimurium vaccine strains were cultured overnight at 37°C, with shaking at 200 RPM. The following morning the culture was diluted 1:100 in 100ml LB broth and placed at

37°C with shaking at 180 RPM until OD_{600} 0.5. Cells were then washed in ice cold sterile dH_20 4 times in increasingly smaller volumes (25ml, 10ml, and 5ml until a final volume of 400µl). Cells were aliquoted, and plasmid was added to 100µl cells. Cells were left on ice for 30 minutes prior to electroporation at 2500V. 600µl warm SOC was added to each sample and incubated at 37°C for 1 hour. 100µl cells was plated on LB agar plates at a range of dilutions with appropriate selective antibiotics

2.2.12 Analysis of protein expression

Expression of the recombinant proteins in *Salmonella* Typhimurium strains SL5338, SL1344 or the various attenuated vaccine strains were examined by SDS-PAGE and western blot.

2.2.12.1 Cell Lysate preparation for analysis of recombinant proteins

5ml LB broth with 50µg/ml ampicillin was inoculated with the appropriate strain from a sterile loop and grown overnight at 37°C, with shaking at 200RPM. The following day, a reading was taken at OD_{600} to determine growth and the culture was pelleted by centrifugation at 3220 x g. Each pellet was resuspended in varying amounts of PBS-TX100 (resuspension buffer), depending on OD_{600} to allow for cell-number equilibration of samples.

The volume of resuspension buffer was calculated using the following method:
First, the OD_{600} of a 1:10 dilution of each of the samples was taken.
This value was converted to its antilog and the sample with the lowest value was identified. This would be the sample containing the smallest number of bacteria and would be resuspended in 150µl of the PBS-TX100 resuspension buffer. The resuspension volumes of the other samples were calculated as follows:

$$\left(\frac{antilog\ Sample\ OD_{600}}{antilog\ lowest\ OD_{600}}\right) \times 150\mu l\ =\ \mu l\ Resuspension\ buffer\ for\ sample$$

This ensured that if an equal volume of each resuspended sample was used for loading onto SDS-PAGE, there should be protein from an equivalent number of cells.

Once resuspended, 15µl of each sample was added to 5µl 4X Sample buffer (Expedeon) (for precast gels) or 15ul 2X Novex™ tris glycine buffer (Invitrogen) (for hand-made gels) with 5% beta-mercaptoethanol as a reducing agent.
Samples were boiled for 5 minutes and subsequently centrifuged at 16060 x g.

2.2.12.2 SDS-PAGE

The samples as prepared above were fractionated on either hand-made (see section 2.1.9) or NuPAGE™ 12% Bis-Tris protein gels (Expedeon), and run accordingly until the dye front reached the bottom of the gel. Following this, gels were stained with InstantBlue™ protein stain (Expedeon) to allow visualisation of protein bands.

2.2.12.3 Western blotting

Samples were prepared in the same way as for SDS-PAGE, however after running the gel, protein bands were transferred to nitrocellulose membrane (Amersham, GE healthcare) at 100V for 1 hour 30 minutes. The blot was then blocked with 5% milk powder (Sigma) in PBS 0.01% Tween (Sigma) for 1 hour at room temperature with constant rocking. The appropriate primary antibody was added, diluted in the milk blocking buffer and incubated overnight at 4°C. The blot was washed in PBS 0.01% Tween three times, for 5 minutes before the appropriate secondary antibody (with HRPO), again diluted in the milk buffer was added. The blot was incubated at room temperature for 2 hours before washing as above with PBS 0.01% Tween and developing with 4-chloro-1-naphthol to visualise bands.

2.2.13 In vitro stability of plasmids

The pTECH2 expression plasmid includes an ampicillin resistance gene to allow for easy selection of colonies. In order to carry out *in vivo* immunisation experiments, stability testing was carried out to ensure that the *Salmonella* vaccine strain would retain the plasmid, despite the fact that there was no advantage to the cell (no need to overcome antibiotic selection pressure) when antibiotics were not present.

SL3261 cells containing each plasmid were grown overnight in a 5ml LB liquid culture containing 50µg/ml ampicillin at 37°C shaking at 200RPM. These cultures were then diluted 1:100 in LB with either no ampicillin or 50µg/ml ampicillin and again grown overnight as above. The next day, a 10^{-6} dilution of each culture was made and 10µl was dropped (in triplicate) onto agar plates both with and without ampicillin. These were incubated overnight at 37°C and the CFU/ml calculated from each spot. From these, the plasmid stability was calculated from the CFU/ml of each sample from the overnight culture with no ampicillin (see red box in figure 2.5). As a control, the stability from the overnight culture with ampicillin was also calculated, but had no impact on the results.

To determine % stability, the following calculation was used:

$$\%\ Plasmid\ stability = \left(\frac{CFU/ml\ amp+}{CFU/ml\ amp-}\right) X\ 100$$

This work was carried out in collaboration with Bethany Gollan, MRes student under my supervision in the Khan lab, Newcastle University.

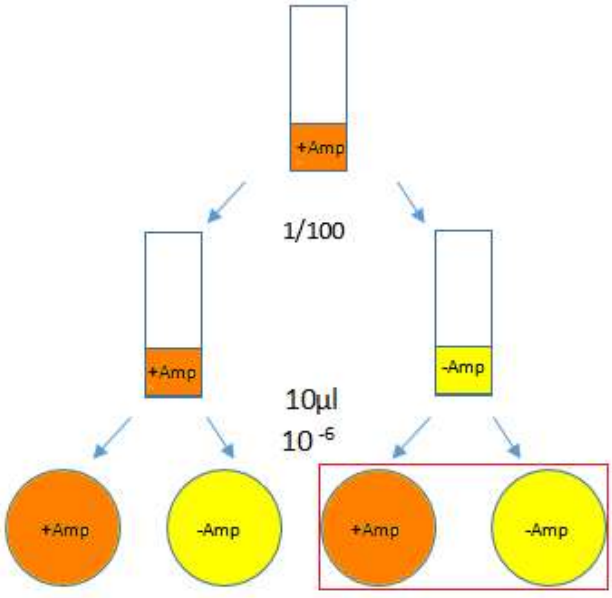


Figure 2.5. Method to determine recombinant plasmid stability *in vitro*.

2.2.14 In vivo stability of vaccine strains

After confirmation that the expression plasmids were stable without antibiotic selection *in vitro*, a pilot immunisation experiment was carried out to determine *in vivo* stability and to ensure that the *Salmonella* vaccine strain was still able to express the Ebola GP or Zika E proteins after passage *in vivo* when recovered from the livers and spleens of immunised mice. The work covered in 2.2.14.1 and 2.1.14.2 was very kindly carried out by Dr Omar Rossi, University of Cambridge.

2.2.14.1 Intravenous inoculation of mice with Salmonella vaccine strain

6-week-old BALB/c mice used in immunisation experiments were housed in standard facilities at the department of veterinary medicine, Cambridge University. They were allowed to acclimatise for 2 weeks and immunised at 8 weeks old. Mice were immunised intravenously (I.V) with Salmonella vaccine strains (see chapter 3, tables 3.3 and 3.4; and chapter 4 tables 4.2 and 4.3) and sacrificed 8 weeks post immunisation. Each plasmid was allocated 4 mice. Immunisations of the mice and bleeds were kindly carried out by Dr Omar Rossi, Cambridge University.

2.2.14.2 Determination of organ colonisation by Salmonella vaccine strains and plasmid stability

11 days post immunisation, the mice were sacrificed, and livers and spleens were dissected for analysis of colonisation. These organs were homogenised and resuspended in 5ml PBS. This was then diluted 1:100 and 50µl of this was spread onto LB agar plates both with and without ampicillin in triplicate. After incubation overnight at 37°C, the CFU/ml was calculated using the following calculation:

$$CFU/ml = (mean\ no.of\ colonies \times Dilution\ factor) \times (1000 \div 50\mu l)$$

From this, the total number of bacteria per organ could be determined by multiplying the CFU/ml by 5 (the total resuspension volume in ml for each organ).

From the total number per organ, the plasmid stability could be determined as before, using the colonies recovered from the organs grown on LB agar plates both with and without ampicillin, with the calculation:

$$\%\ Plasmid\ stability = \left(\frac{Organ\ total\ on\ amp + plate}{Organ\ total\ on\ amp - plate}\right) \times 100$$

2.2.14.3 Determination of recombinant protein expression after in vivo passage

10 colonies from each organ sample were collected on a loop and stored as stab agar culture in order to send to Newcastle for expression testing. Once arrived, streak plates were made from these stab agar cultures and following growth overnight on LB agar with 50µl/ml ampicillin, were used to make a 5ml LB broth liquid culture also with 50µl/ml ampicillin. Cells from this liquid culture were then processed as above for a western blot, probed with polyclonal Rabbit anti-Tet C sera followed by Goat anti Rabbit (HRPO) to determine expression of Tet C and Tet C fusion proteins from cells recovered from each mouse organ.

2.2.15 Investigation of immune response

IgG response to Ebola GP, Zika E or Tet C, constituents of the pTECH2 expression plasmids were assessed by ELISA.

2.2.15.1 ELISA to determine immune response to recombinant antigens or Tet C

Recombinant Ebola Glycoprotein, kindly provided by Oxford University and Public Health England (Ewer et al, 2016a), was coated 1µg/ml, Zika envelope protein (Fitzgerald) at 5µg/ml, and Tet C, kindly provided by Professor Neil Fairweather (Imperial College London) was coated at 1µg/ml onto 96 well Nunc MaxiSorp™ plates at in sterile PBS.
Plates were incubated at 4°C overnight before washing 6 times with 200µl/well wash buffer (PBS 0.05% Tween). Each well was blocked with 100µl Casein (Fisher) and the plate incubated at room temperature for 1 hour. Mouse sera dilutions were prepared to a volume of 50µl in

casein block buffer and placed in a Sterilin™ 96 well plate. Blocking buffer was washed from the experimental plate as above and sera samples transferred from the sample plate before incubating at room temperature for 2 hours. Plates were again washed as above and 50µl per well Rabbit anti-mouse HRPO secondary antibody (Abcam) diluted in casein was added. Plates were incubated for 1 hour at room temperature before washing as above. Assays were developed with 50µl per well TMB substrate (Sigma) and stopped with 50µl per well 0.5M H_2SO_4. Assays were read at 450nm with a BioTek plate reader.

2.2.15.2 ELISA to determine immune response to Salmonella Typhimurium LPS

Salmonella Typhimurium LPS (Sigma) was prepared by mixing in equal volumes with 0.5% Sodium Deoxycholate (DOC), making a solution of 0.5mg/ml LPS with 0.25% DOC. This was incubated for 15 minutes at 37°C and subsequently stored at -20°C until needed.

LPS in DOC was thawed and diluted to a final concentration of 5µg/ml in Reggiardo's buffer with 0.1% DOC, 50µl was added per well of a Nunc MaxiSorp™ 96 well plate and incubated at 37°C overnight. Wells were then emptied and washed with PBS 0.05% Tween (200µl/well, 2 quick rinses and then 3 times for 3 minutes each) and subsequently blocked with PBS 2% BSA, then incubated at 37°C for 1 hour.

Wells were emptied and washed as above, and serum diluted in PBS 0.05% Tween plus 1% BSA was added at 50µl per well. Plates were then incubated at 37°C for 2 hours before emptying and washing as above. 50µl per well Rabbit anti-mouse HRPO secondary antibody (Abcam) diluted in PBS 0.05% Tween plus 1% BSA was added and incubated for 1 hour at 37°C. Wells were emptied and washed as above before the assay was developed with 50µl per well TMB substrate (Sigma) and stopped with 50µl per well 0.5M H_2SO_4. Assays were read at 450nm with a BioTek plate reader.

2.2.15.3 Determination of neutralisation of Zika Virus by immunised mouse serum

The following assay was kindly carried out by Professor Arvind Patel and Ricardo Sachez-Velasquez, Glasgow University.

$7x10^3$ cells/well Vero cells were used to seed a 96 well plate in DMEM 10% FBS overnight.

The following day, three-fold dilutions of the experimental sera (1:20 – 1:43740) were mixed with 100 pfu of ZIKV Brazilian strain PE243 (Donald et al 2016) for 1 hour. The Vero cell medium was removed, and the sera/ZIKV mixture added onto the cells. The cells were topped up with 100µl of DMEM 2%FBS and incubated for 72 hours.

Cell supernatant was removed, and cells lysed with LB2 lysis buffer (20mM Tris-HCL (pH 7), 20 mM iodoacetamide, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, Complete ^{TM} protease inhibitors).

A 96 well plate (Immulon, 2HB – ThermoScientific) was coated with 3µg/well 4G2 anti Flavivirus antibody (Merk Millipore) and incubated overnight at room temperature. The following day, the antibody was removed and wells were blocked for 2 hours at room temperature with 2% skimmed milk powder in PBS-0.02% Tween-20.

100µl cell lysate added and incubated for 1 hour at room temperature. The plate was washed with PBS-0.02% Tween-20 and Rabbit anti-Zika envelope – R34 sera was added at 1:4500 and incubated for 1 hour at room temperature. The plate was washed as above and 1:10000 anti-rabbit IgG H&L (HRP) (Abcam) was added and again incubated for 1 hour at room temperature. The plate was washed as above and the assay was developed with TMB substrate (Sigma) for approximately 10 minutes before the reaction was stopped with 0.5 M H_2SO_4. Assays were read at 450nm with a Varioskan plate reader.

2.2.16 Salmonella mutagenesis

Knockouts of specific *Salmonella* Typhimurium genes were carried out to increase the propensity of the SL1344 strain to produce outer membrane vesicles.

2.2.16.1 One Step gene disruption technique, Lambda-red system

To create gene specific *Salmonella* knockout mutants, the Lambda-red recombinase technique was used (Datsenko & Wanner, 2000).

A DNA cassette containing a kanamycin-resistance gene (*KanR*) was amplified by PCR from the pKD4 plasmid. Primers were designed to include 40 bases from either the 5' or 3' end of the target gene (*tolR* or *mlaA*) on the SL1344 genome as well as 20 bases from the pKD4 plasmid, flanking the *KanR* gene and flippase recognition target (FRT) sites at the priming sites, P1 and P2.

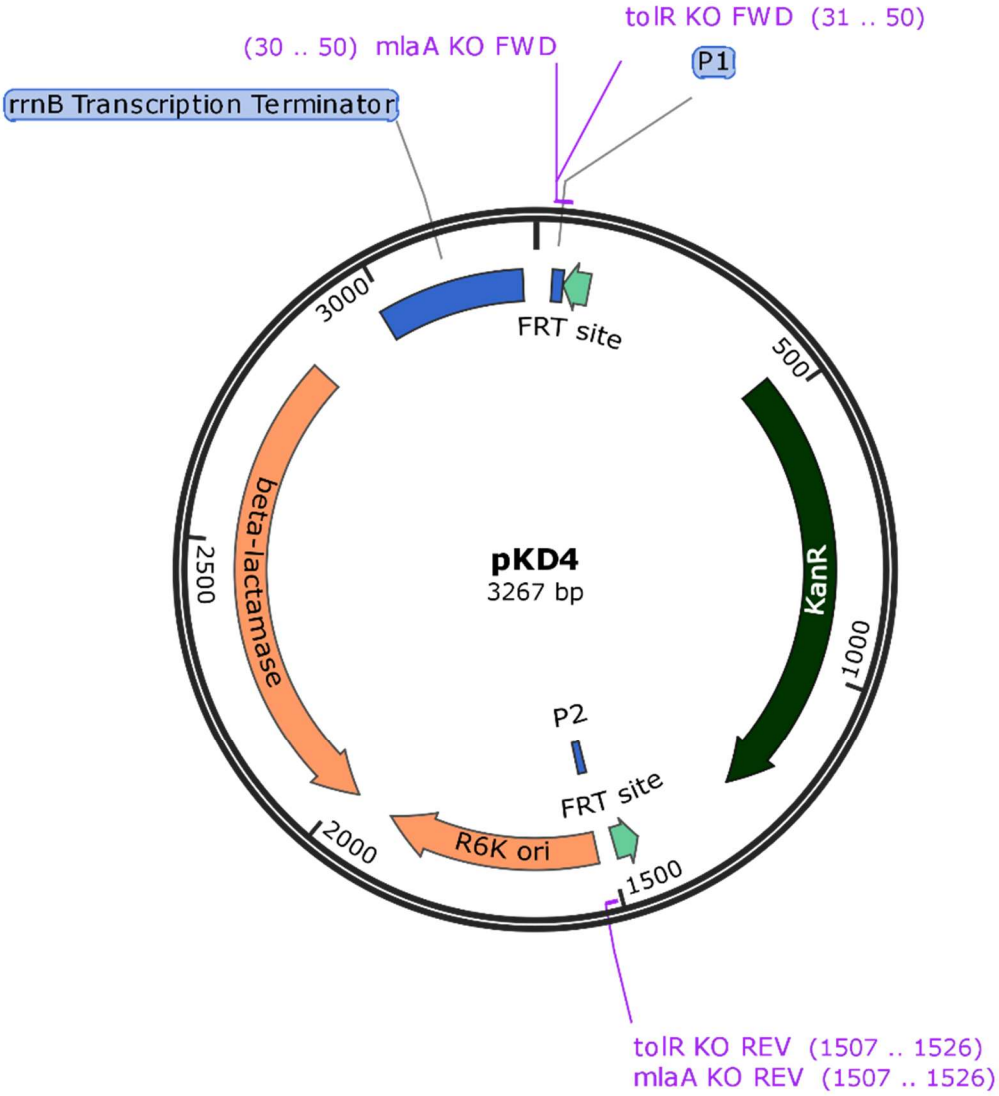


Figure 2.6. Plasmid map of pKD4. PKD4 contains the kanamycin resistance gene – KanR, found on bacterial transposon Tn5, encoding neomycin transphospherase, which is situated between two flanking FRT sites. Primers were made which included part of the *Salmonella* Typhimurium SL1344 genome, flanking the *tolR* or *mlaA* genes which were to be knocked out and also the P1 and P2 priming sites of pKD4. When amplified by PCR, the resulting cassette was transformed into parent SL1344 strains harbouring the pKD46, lambda-red recombinase enzyme expressing plasmid. Plasmid map was made using the commercial software Snapgene.

A)

B)

Figure 2.7. Gene sequences for *tolR* and *mlaA* genes in the *Salmonella* Typhimurium genome and corresponding primer oligonucleotide sequences. Oligonucleotides were used to amplify the *KanR* gene from the pKD4 plasmid (see figure 2.6). The genes are shown in blue with the flanking genome in black. The oligos contain 40 bases complementary to the flanking genome and 20 bases corresponding to priming sites 1 and 2 (P1, P2) on the pKD4 plasmid (shown in green). The resulting amplified PCR product is a cassette including the *KanR* gene, flanked by flippase recognition target (FRT) sites to allow for the subsequent lambda-red recombination reaction.

2.2.13.2 P22 Lysate production

Once the *KanR* gene and FRT site cassette had been amplified it was transformed into electro-competent *Salmonella* Typhimurium strain SL1344 which had previously been transformed with the pKD46 plasmid, expressing the Lambda-red recombinase enzyme. Homologous recombination allowed the *KanR* cassette to be inserted specifically at the *tolR* or *mlaA* gene sites. Knockout strains were then checked using colony PCR and successful knockouts transduced to a fresh WT SL1344 strain using P22 (see section 2.2.16.2).

2.2.16.2 P22 Transduction

Confirmed knockout mutants were transduced into a fresh wild type SL1344 background using P22 phage transduction.

2.2.16.2.1 P22 Lysate production

To prepare lysates of SL1344 *ΔtolR* or *ΔmlaA*, 10µl of P22 phage lysate was added to a 10ml LB with 50µg/ml kanamycin, of each of the knockout strains and grown overnight at 37°C with shaking at 200RPM. The following day, a few drops of chloroform were added and the culture forcefully shaken in order to release the phage from the now lysed cells. The solution was then divided into microcentrifuge tubes and centrifuged at 16060 x g for 3 minutes. The resulting supernatant was then removed and a few drops of chloroform added before filter sterilisation with 0.22µm syringe filters (Sartorius). The lysate was stored in sterile glass universal tubes at 4°C until required for transduction.

2.2.16.2.2 Transduction

The fresh wild type SL1344 strain was grown overnight in 5ml LB at 37°C with shaking at 200RPM. The following day, 1ml of this culture was removed and centrifuged at 16060 x g for 1 minute. The pellet was resuspended in 100µl of LB broth and 10µl of the P22 lysate (see section 2.2.16.2.1) was added and the mixture incubated at room temperature for 30

minutes. As a control a sample of wild type SL1344 was subject to the same conditions but without the addition of P22. 1ml of warm LB broth was then added to each sample before incubation at 37°C for 30 minutes. The cells were then once again centrifuged at 16060 x g for 1 minute and the pellet resuspended in 100µl of LB broth. This was then spread onto LB agar plates with 50µg/ml kanamycin and incubated overnight at 37°C.

2.2.16.2.3 Evans-Blue-Uranine (EBU) plates to remove P22 phage

A number of kanamycin resistant colonies of transduced SL1344 were picked from the LB agar plates and streaked onto EBU plates containing 50µg/ml kanamycin. The following day, cultures were checked for their colour; dark colonies still had phage present and light colonies were phage-free. The phage-free colonies were then screened with colony PCR to determine successful knockouts.

2.2.16.3 FLP recombination with pCP20

Phage-free SL1344 *ΔtolR* or *ΔmlaA* strains were then made electro-competent to allow transformation of the pCP20 plasmid, expressing FLP-recombinase. This resulted in recombination between the two FRT sites and the subsequent removal of the *KanR* gene.
The strains containing pCP20 were grown overnight with 50µg/ml ampicillin at 30°C with shaking at 200RPM. The following day, the culture was diluted 1:100 in 100ml LB and grown to OD_{600} 0.1, still at 30°C. The culture was then shifted to 42°C and grown until OD_{600} 0.8 – 1, to cure the strain of the heat-sensitive pCP20 plasmid. This culture was then streaked onto an LB agar plate and grown overnight at 37°C.
To confirm that the strain has been cured of the pCP20 plasmid, single colonies from the above plate were patched onto three separate LB agar plates; one with 50µg/ml ampicillin, one with 50µg/ml kanamycin and one with no antibiotics. The strains which are sensitive to both ampicillin and kanamycin should be successful knockout strains, cured of pCP20.
These colonies were then once again checked using colony PCR and DNA sequencing (Eurofins) to confirm successful knockout of *tolR* or *mlaA genes.*

2.2.17 Fractionation of Outer Membrane Vesicles

Cells were grown overnight with shaking at 37°C in 5ml LB broth with appropriate antibiotics. The following day, cells were diluted 1:100 in LB broth with appropriate antibiotics, and grown at 37°C with shaking to OD_{600} 0.2. Cells containing either of the pBAD plasmids were then induced with L-arabinose to a final concentration of 0.2%. The cells were then left to continue growing to OD_{600} 1. Cells were centrifuged twice at 3220x g at 4°C and the supernatant filtered with 0.22µm Stericup-GV Sterile Vacuum Filtration System (Merk Millipore). The cell pellet was retained and used for SDS-PAGE and western blot analysis.

The supernatants were then ultracentrifuged at 100,000 X g for 2 hours at 4°C and the resultant pellet was resuspended in 1ml sterile PBS before filter sterilising with 0.22µm syringe filters (Sartorius).

The OMV fraction in PBS was then prepared with appropriate loading buffer for SDS-PAGE and western blot.

2.2.18 Negative staining Transmission Electron Microscopy of Salmonella Outer Membrane Vesicles

5µl of purified OMV sample in PBS was adsorbed onto 300 mesh copper Formvar/carbon coated grids for 5 minutes. The grids were washed with drops of distilled water and dried with filter paper (Whatman). Grids were then treated 2% uranyl acetate in distilled water for 1 minute then blotted as above with filter paper and air dried. OMVs were observed with a Philips CM100 Transmission Electron Microscope operating at 100kV, recording electron micrographs at magnifications of 46000X and 92000X. EM images were acquired by Tracey Davey (Newcastle University Electron Microscopy Research services).

Chapter 3 - Expression of Ebola GP in *Salmonella* and evaluation of immune response in vaccinated hosts

The Ebola Virus is a deadly and highly virulent pathogen which has become well known in recent years due to large scale catastrophic outbreaks which highlighted the urgent need for an effective vaccine. Mostly problematic in poor African countries, a vaccine to protect against Ebola Virus would need to be cost effective as well as widely accessible in remote areas. The Glycoprotein (GP) is situated on the virus surface and is a major target for protective and neutralising antibodies. Using attenuated *Salmonella* vaccine strains to express and deliver the GP protein to the host immune system, could elicit these protective antibodies and be an affordable and easy to administer vaccine to prevent outbreaks on such a scale from occurring in the future.

3.1 Generating a panel of GP expression constructs

The Ebola virus glycoprotein (GP) is the major component of the viral surface and has been shown elicit protective neutralising antibodies (Lee & Saphire, 2009). This makes it an attractive target for use in a vaccine. It is hoped that using attenuated *Salmonella* Typhimurium to express GP or sub fragments of GP, this may elicit this protective response after these antigens are delivered to the immune system by the *Salmonella*. These gene fragments were first amplified by PCR and subsequently cloned into an expression vector using restriction enzymes and T4 ligation.

3.1.1 Synthetic codon optimised Ebola GP gene

A Synthetic Ebola GP gene was codon optimised for expression in *Salmonella* Typhimurium. By generating a synthetic Ebola gene sequence, using 'GENEius', the propriatory gene optimisation software of Eurofins Genomics, codons which are most frequently used by *Salmonella* Typhimurium were picked and unwanted sequences avoided. It was hoped that this would give the best chance of good levels of Ebola GP expression in the attenuated

vaccine strains. The wild type gene sequence was sent to Eurofins and was subsequently used to create the synthetic gene. The parameters for codon optimisation used by Eurofins are described in chapter 1, section 1.6.1.1.

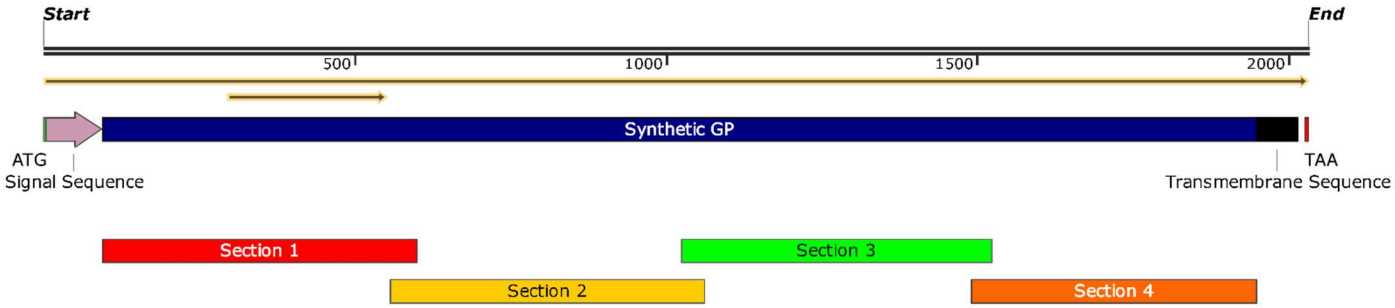


Figure 3.1. Map of the synthetic Ebola GP gene, including overlapping sub sections 1-4. Primers were designed to amplify the full-length Ebola GP, but devoid of the hydrophobic signal and transmembrane sequence and also 4 evenly sized sub fragments (sections 1-4). These PCR products were then cloned into the pTECH2 expression vector and once confirmed, checked for GP expression. Size of synthetic GP – 1854bp. Size of sub fragments - 1 (504 bp) 2 (504bp),3 (498bp), and 4 (459bp).

3.1.2 PCR amplification of Ebola GP and sub-fragments

Primers were designed to PCR amplify specifically demarcated regions of the Ebola GP gene (see figure 3.1), to determine whether smaller fragments (approximately equal sized) would be expressed better than the full-length GP. The PCR products from these reactions were then fractionated on a 0.7% agarose gel and visualised using UV light. Primers contained specific restriction sites allowing for cloning into the pTECH2 expression vector following digestion.

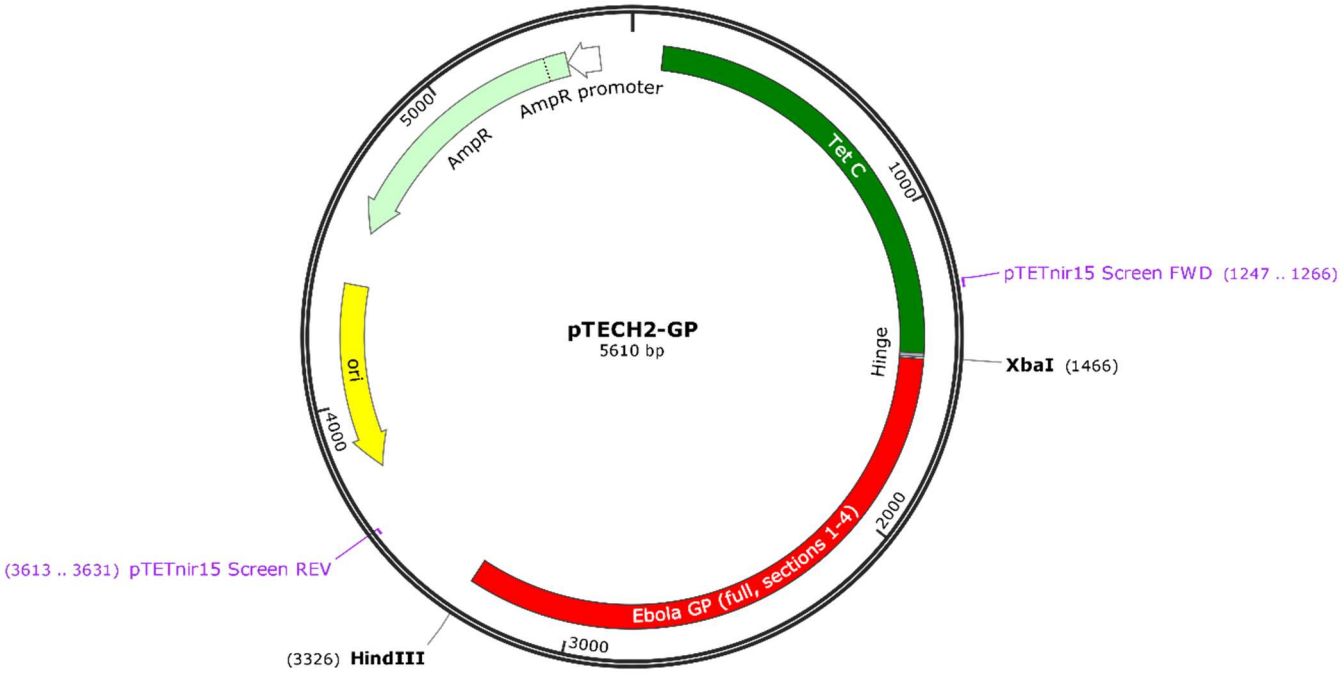


Figure 3.2. Plasmid map of the pTECH2-GP expression vector. Under the control of the *nirB* promoter, a TetC-GP fusion protein is expressed. The GP gene was cloned in using restriction enzymes *XbaI* and *HindIII*. Colony PCR was carried out on *Salmonella* Typhimurium SL5338 colonies after transformation using the pTETnir15 screening primers as shown to confirm putative clones containing the plasmid prior to expression analysis. Each of the smaller gene sections 1-4, as shown in figure 3.1 above (not pictured here), were cloned in place of the full GP gene to allow expression of shorter TetC-GP fusions. Plasmid map was made using the commercial software Snapgene.

3.1.2.1 PCR amplification of full synthetic Ebola GP

First, the full synthetic GP gene was amplified, giving a PCR product as expected of 1854bp (figure 3.3). This was the sequence of the full wild type Ebola GP, but devoid of the hydrophobic signal and transmembrane sequences at the N and C termini respectively.

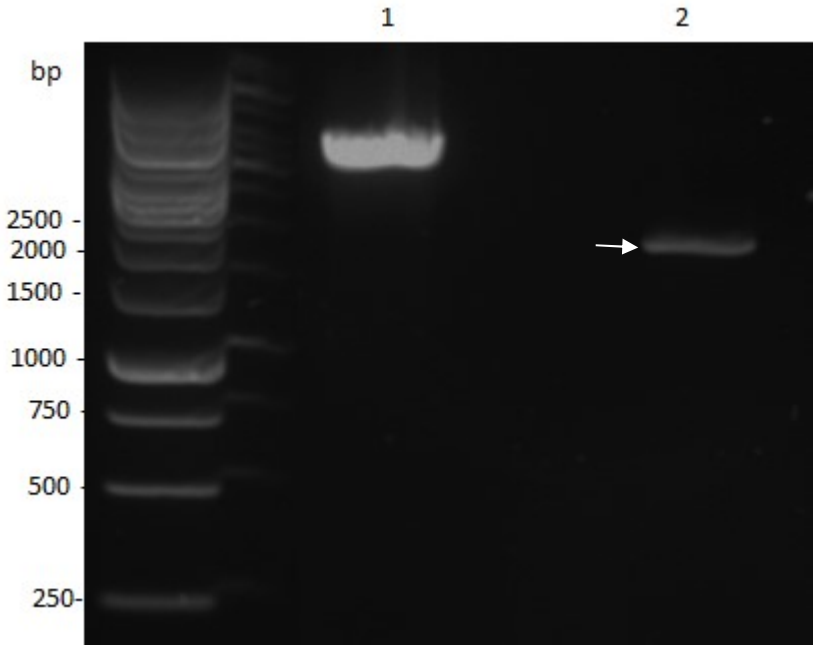


Figure 3.3. PCR amplification of synthetic GP. 0.7% agarose gel showing the PCR product of the synthetic GP gene, devoid of the hydrophobic signal and transmembrane sequences (lane 2) when amplified with corresponding primers containing restriction cloning sites for *XbaI* and *HindIII* (denoted by arrow). Lane 1 shows the pTECH2 vector, digested with *XbaI* and *HindIII* in preparation for ligation.

The 1854bp GP PCR product was then subsequently digested with *XbaI* and *HindIII*, spin column purified, and cloned into digested pTECH2 plasmid.

3.1.2.2 PCR amplification of Synthetic Ebola GP sub fragments 1-4

Since it has proved difficult for many groups to express the full-length Ebola GP in bacterial cells (Das et al, 2007; Zai et al, 2016) as an alternative strategy, it was then decided to generate constructs to attempt to express smaller sub-fragments of the Ebola GP gene, as this may facilitate expression of smaller defined proteins which may lack toxicity for the host cells. First, the gene was split into four roughly even approximately 500bp sections, 1 (504 bp) 2 (504bp), 3 (498bp), and 4 (459bp) (figure 3.4).

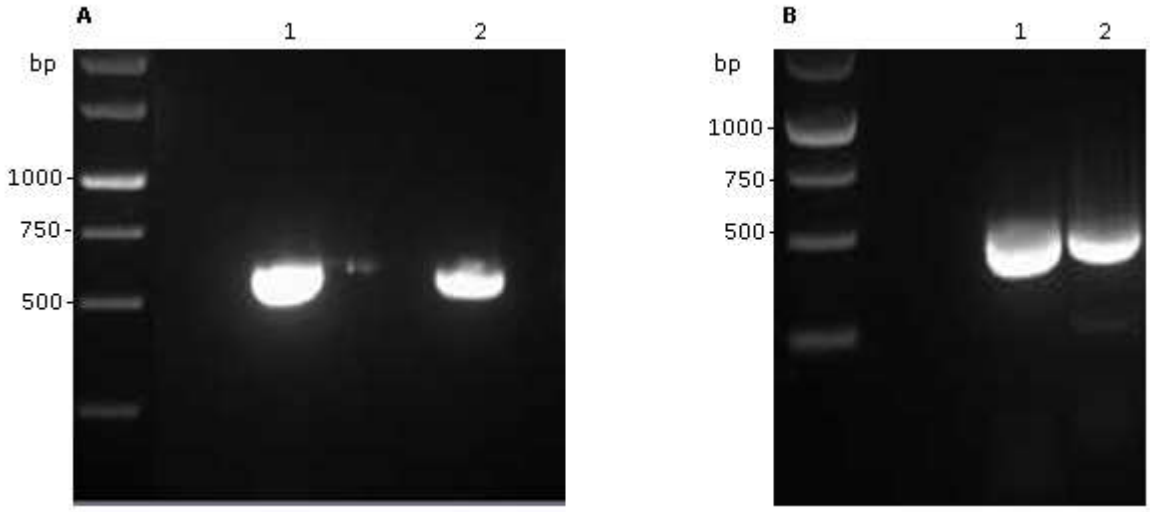


Figure 3.4. PCR amplification of synthetic GP sections 1-4. 0.7% agarose gel showing the PCR products of the synthetic GP gene split into 4 approximately equal sections. A1 - Synthetic GP section 1 (504bp), A2 - Synthetic GP section 2 (504bp), B1 – Synthetic GP section 3 (498bp), B2 – Synthetic GP section 4 (459bp). These products were subsequently digested with *XbaI* and *HindIII*, spin column purified, and cloned into digested pTECH2 plasmid.

3.1.3 Generation of pTECH2-GP constructs

The pTECH2 expression vector is derived from the pTETnir15 vector (Khan et al 1994 a; Khan et al 1994b). Under the control of the anaerobically inducible *nirB* promoter, the C-fragment of Tetanus toxin (TetC) is expressed. Guest antigen genes, such as Ebola GP can be cloned into the multiple cloning site following a flexible hinge region to allow expression of a TetC-antigen fusion protein. The Ebola GP gene and sub-fragments were successfully cloned into this vector with restriction cloning and T4 DNA ligation.

The mix from the ligation reaction was then transformed into the intermediate *Salmonella* Typhimurium strain SL5338 (r^- m^+). Colony PCR was performed on selected colonies to screen for putative recombinants. Screening primers flanking the cloning site of pTECH2 were designed to allow visualisation of successful insert cloning as this would result in a larger colony PCR product than if it were not present, a negative colony with no insert gives a PCR product of 543bp and those with the insert included are 543bp plus the insert size. The plasmid DNA from putative positive clones were then purified and digested with the cloning restriction enzymes to further confirm the presence of an insert of the correct size. Plasmids from putative clones determined by both of these methods were then sent for DNA sequencing (Eurofins), using the same oligonucleotide primers as in the colony PCR screen.

3.1.3.1 Construction of pTECH2-GP expression plasmid

The 1854 bp Ebola GP PCR product was cloned into the multiple cloning site of the pTECH2 vector using restriction enzymes *XbaI* and *HindIII* and ligation with T4 ligase. Colony PCR using pTECH2 vector screening primers (see table 2.9, chapter 2) and restriction enzyme digests and agarose gel fractionation of the resultant plasmid after transformation into SL5338 cells confirmed successful cloning (figure 3.5).

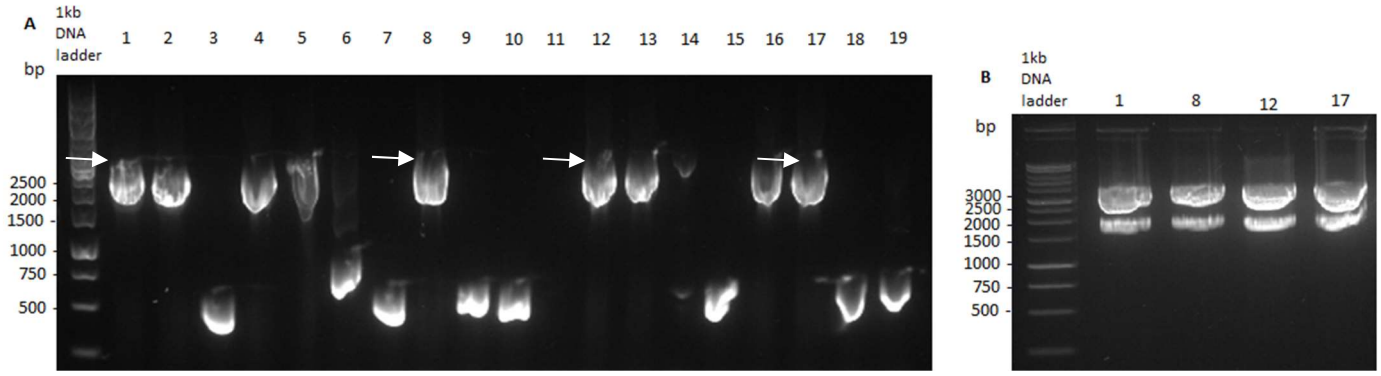


Figure 3.5. Colony PCR and restriction digest screens of putative pTECH2-GP clones. A) 0.7% agarose gel showing PCR products from colony PCR using pTECH2 screening primers to determine successful construction and transformation of the pTECH2 vector containing the 1854bp Synthetic GP into SL5338 *Salmonella* strain. Following transformation, colonies 1-19 (as shown on gel) were boiled before PCR amplification of the plasmid between each screening primer. Positive colonies which have successfully taken up the plasmid pTECH2 GP show a band at approximately 2.3kb (denoted by arrows). Colonies which have only taken up pTECH2 show a band at approximately 543bp. The agarose gel here is overloaded with sample, causing a rippling effect, however the differences in size between the samples are clearly apparent.

B) 0.7% agarose gel showing purified plasmid from positive colonies (see arrows on figure 3.5 A) digested with *XbaI* and *HindIII*, showing an insert band of 1854bp which corresponds to the size of the PCR product for the Ebola GP gene. Positive plasmids were then sent for DNA sequencing using pTETnir15 (pTECH2) screening primers.

3.1.3.2 Construction of pTECH2-GP sub sections 1-4 expression plasmids

Smaller fragments of the GP gene were cloned into the pTECH2 vector in the same way as the full-length GP. Successful cloning was determined again by colony PCR with the pTECH2 screening primers and restriction enzyme digestion. Here, also with the cloning enzymes *XbaI* and *HindIII* (figure 3.6).

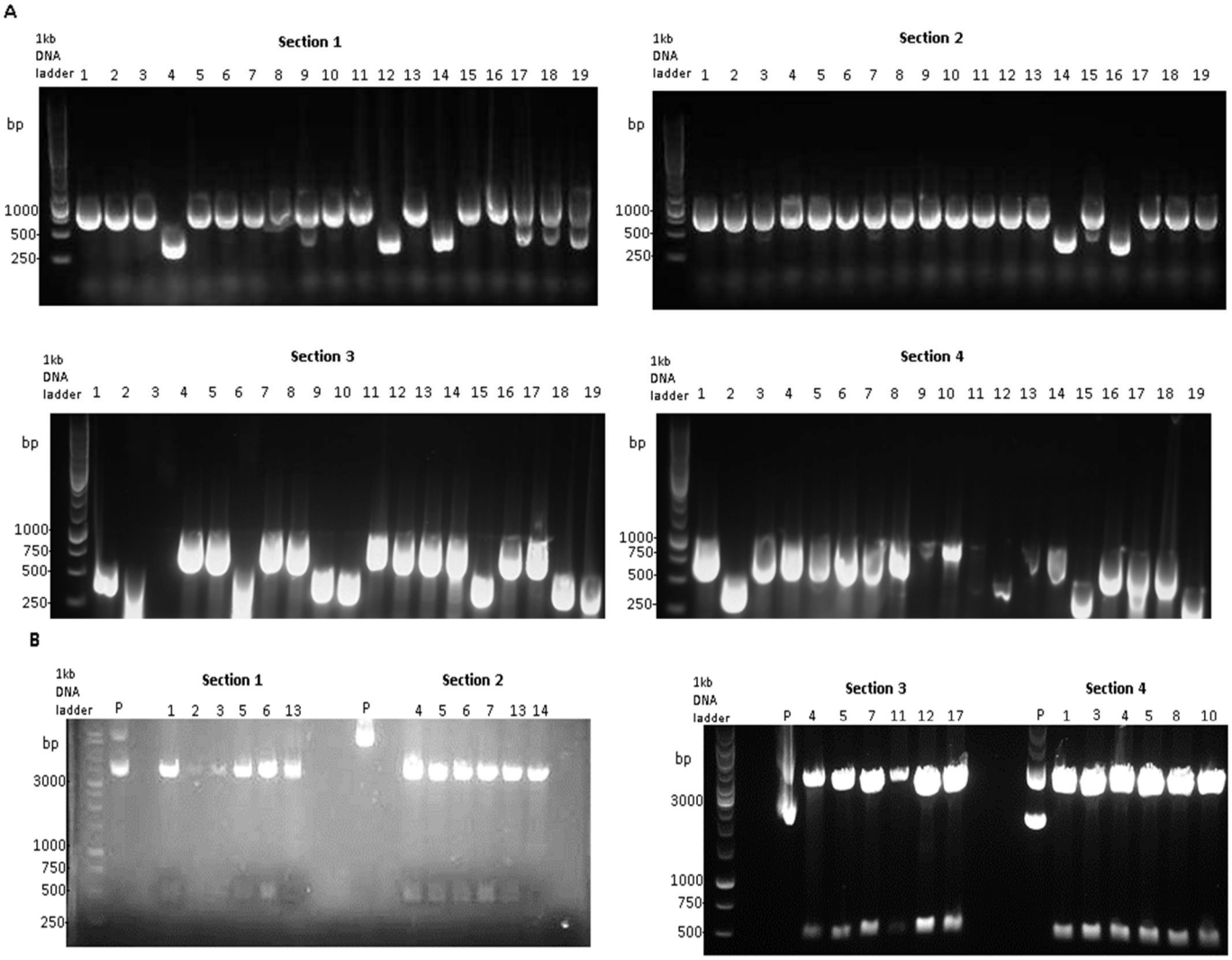


Figure 3.6. Colony PCR and restriction digest screens of putative pTECH2-GP 1-4 clones.
A) 0.7% agarose gel showing PCR products from colony PCR using pTECH2 screening primers to determine successful construction and transformation of the pTECH2 vector containing the 500bp Synthetic GP sections 1, 2, 3 and 4 into SL5338 *Salmonella* strain. Following transformation, colonies 1-19 (as shown on gel) were boiled before PCR amplification of the plasmid between each screening primer. Positive colonies which have successfully taken up the plasmid pTECH2 GP show a band at 1000bp. Colonies which have only taken up pTECH2 show a band at approximately 543bp. The agarose gels here are overloaded with sample, causing a rippling effect, however the differences in size between the samples are clearly apparent. **B)** 0.7% agarose gel showing purified plasmid from positive colonies (see figure 3.6 A) digested with *XbaI* and *HindIII*, showing an insert band of 500bp which corresponds to the size of the PCR product for each of these four Ebola GP sections. P is an uncut plasmid control. Positive plasmids were then sent for sequencing using the same screening primers as above.

3.1.4 Expression of TetC-GP fusion proteins in Salmonella intermediate strain SL5338 ($r^- m^+$)

Once the plasmid constructs of pTECH2-GP (or pTECH2-GP sub fragments 1-4) had been confirmed by colony PCR and informative digest (see figures 3.5 and 3.6), it then needed to be determined whether these could be used to express the TetC Ebola GP fusion protein in *Salmonella*. Chemical transformation of the plasmid into intermediate *Salmonella* strain SL5338 resulted in expression of these fusion proteins in bacterial culture.

To ensure that each sample contained an equivalent amount of cells, a dilution calculation was carried out using the OD600 of overnight cultures. Cell lysates were resuspended in varying amounts of PBS TX-100, depending on the OD600 value, and the same volume was taken from each of these diluted samples for preparation for SDS-PAGE. For the relevant calculations, please refer to Chapter 2, section 2.2.9.1.

3.1.4.1 Expression of TetC-GP fusion protein

The TetC-GP fusion protein expressed from the plasmid pTECH2-GP can be detected by polyclonal anti-TetC sera. The TetC protein is approximately 52 kDa in size and the GP protein (minus the hydrophobic N and C termini) is approximately 67 kDa. The resultant fusion protein is therefore approximately 121 kDa (figure 3.7). Other bands seen below the fusion protein, indicate either breakdown products or premature termination of translation, particularly where a band appears which is the same size as the TetC protein. This is not necessarily concerning as these may still contain important immunological epitopes of the GP protein (see figure 3.7). The full TetC-GP fusion band, appears to be relatively faint however, so this needs to be addressed to allow for adequate antigen availability during immunisation.

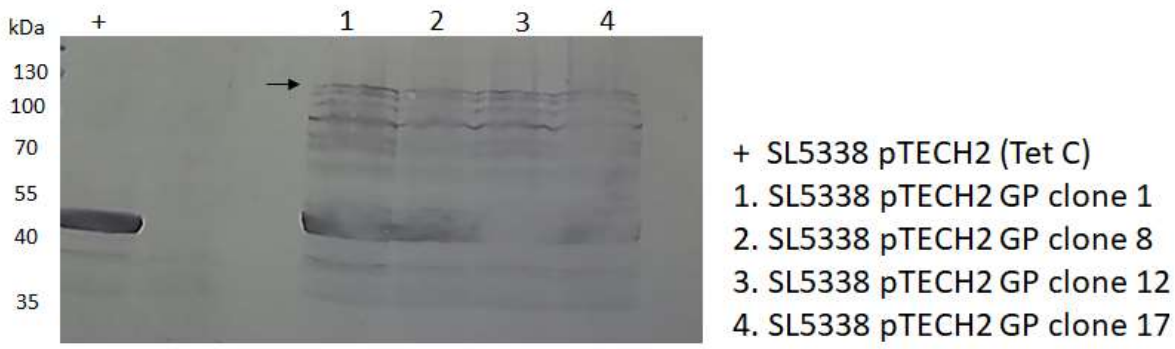


Figure 3.7. Expression of TetC-GP fusion protein. Western blot showing expression of TetC-GP fusion protein in cell lysates of 4 clones of *Salmonella* Typhimurium SL5338. The fusion is present as a band at approximately 121 kDa (shown by arrow). Bands below this indicate either fusion protein breakdown products or premature termination of translation, giving a 'ladder effect'. Blots were probed with polyclonal rabbit anti-TetC serum (1:1000) and Goat anti-rabbit HRPO (1:2000). As a positive control, SL5338 cells harbouring only the PTECH2 plasmid were used. This gives a single TetC band at approximately 52 kDa (denoted by lane marker +). The blot was developed with 4-chloro-1-napthol.

3.1.4.2 Expression of TetC-GP sub fragments 1-4 fusion proteins

After expression of the TetC-GP fusion protein was confirmed by western blot, the smaller sub fragments (each encoded by an approximately 500bp DNA cassette) were tested to determine if a smaller sized DNA cassette improved the expression levels of the fusion protein. Each sub-fragment was approximately 18 kDa, so the fusion with the 52 kDa TetC shows a band at approximately 70 kDa (see figure 3.8) It appears that while each of the four fusion proteins are expressed, there is variation between the sub fragments. The full fusion proteins are nowhere near as highly expressed as the TetC. There is an additional unexpected band the size of TetC which may be due to the premature termination of translation immediately after the TetC has been translated, possibly due to the toxicity of the GP protein.

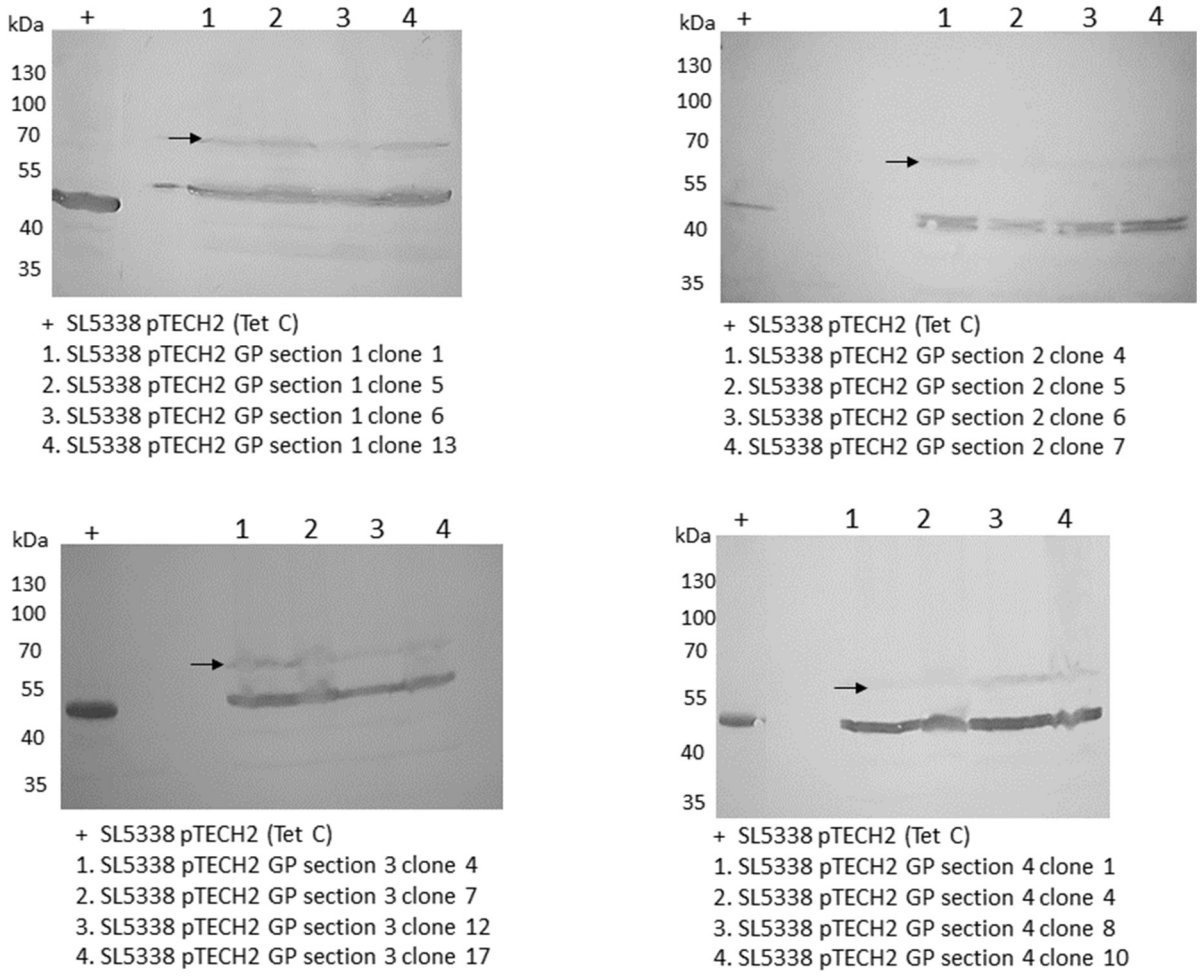


Figure 3.8. Expression of TetC-GP 1-4 fusion proteins. Western blot showing expression of TetC-GP sub fragments fusion proteins 1-4 in cell lysates of 4 clones of *Salmonella* Typhimurium SL5338. The fusions are present as a band in each blot at approximately 70kDa (shown by arrow).

Blots were probed with polyclonal rabbit anti-TetC serum (1:1000) and Goat anti-rabbit HRPO (1:2000). As a positive control, SL5338 cells harbouring only the PTECH2 plasmid were used. This gives a single TetC band at approximately 52 kDa (denoted by +). Blots were developed with 4-chloro-1-napthol.

3.2 Generating a panel of GP expression constructs with important immunological features – 'GP version 2'

Due to the fact that the expression levels of the TetC-GP fusion proteins appeared to be less than what is hoped for, especially in relation to the levels at which TetC is expressed, it was decided to get a new synthetic GP gene synthesised to see if a sequence using codons determined by an alternative algorithm, Genscript's OptimumGene™, would facilitate better expression. OptimumGene™ appears to use a wider variety of parameters than the Eurofins GENEius software, optimising transcription, translation and protein folding (see footnote [2]).

It was also decided that instead of evenly sized GP sub fragments, which did not seem to have improved expression compared to the full-length protein, the GP gene would be demarcated into sub fragments which were known to contain important immunological features (figure 3.9). These included known protective epitopes, sections which had been successfully expressed in bacterial cells (see section 'D') (Das et al, 2007), contained highly immunogenic regions (see section 3.2.1.3, MFL) (Wang et al, 2014), and also the area with the lowest concentration of hydrophobic residues (see section 3.2.1.4, LH).

These new sub fragments and the full GP were successfully cloned into the pTECH2 expression vector and subsequently expressed in *Salmonella*. Hereafter, only this 'version 2' GP sequence was used and therefore will be referred to simply as GP. For primers see Chapter 2 section 2.1.8 table 2.7 where the previous GP sequence is referred to as GP (v1), and this as GP.

[2] Note: for details on OptimumGene™, see https://www.genscript.com/codon-opt.html

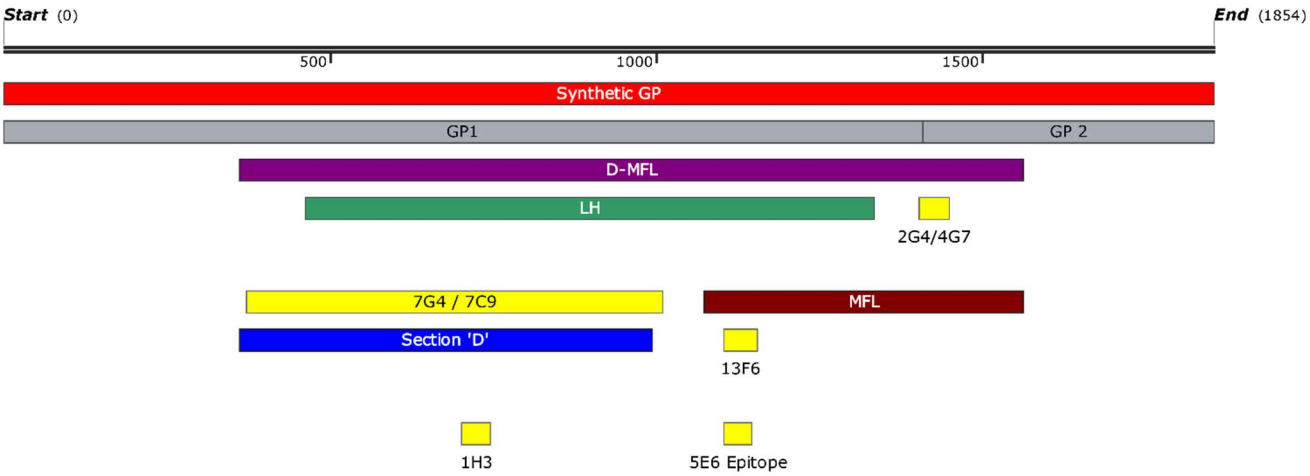


Figure 3.9. Map of the Ebola glycoprotein gene and sub fragments containing important immunological epitopes. The full-length synthetic GP is 1854bp long as is devoid of the wild type's hydrophobic signal and transmembrane sequences. The locations of the four sub fragments are shown, D, MFL, LH and a 'mini-gene' which incorporates both the D and MFL sub fragments. Locations of known protective epitopes are shown in yellow (Qiu et al, 2011) (see section 3.3.1.1 Conservation of known protective epitopes in Ebola GP). Made using the commercial software Snapgene.

3.2.1 PCR amplification of Ebola GP and sub-fragments

In the same way as above, the new synthetic GP gene was used and with primers specific to the full GP or various sub fragments, was PCR amplified and digested for subsequent restriction cloning into the pTECH2 expression vector (figure 3.10). A panel of constructs was created to enable evaluation of which expressed the GP protein best and would then be taken forward into *in vivo* testing (figure 3.11).

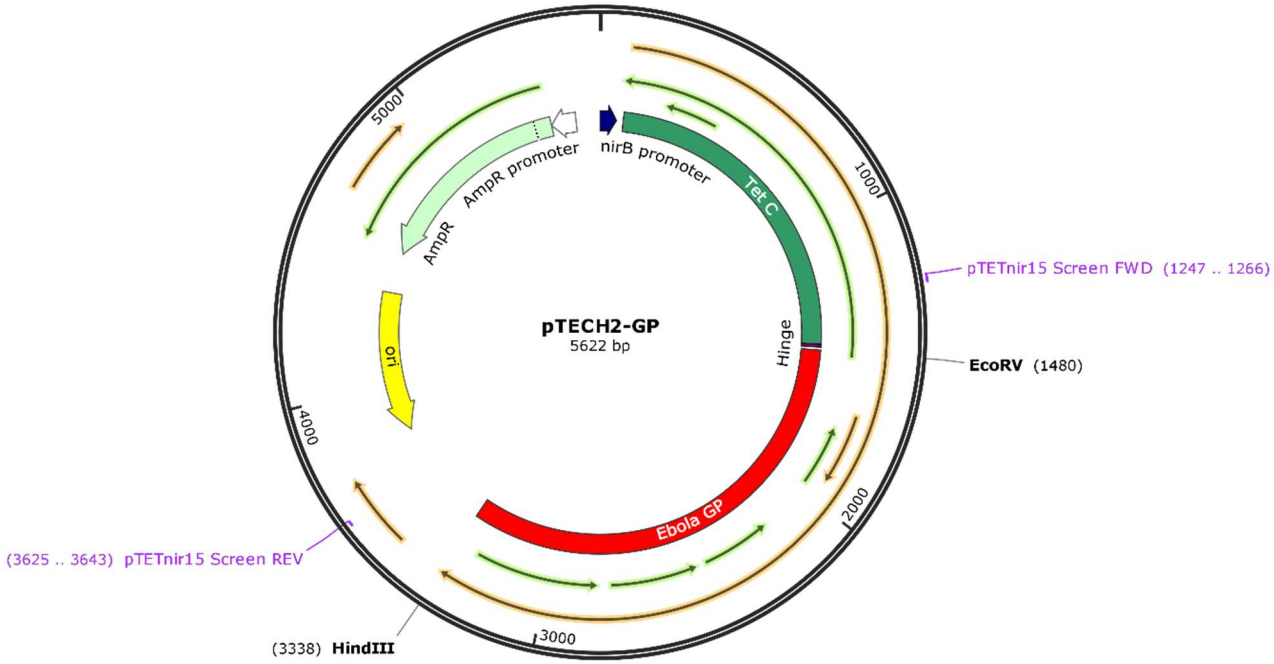


Figure 3.10. Plasmid map of the pTECH2-GP expression vector. Under the control of the *nirB* promoter, a TetC-GP fusion protein is expressed. The GP gene was cloned in using restriction enzymes *EcoRV* and *HindIII*. Colony PCR was carried out on *Salmonella* Typhimurium SL5338 colonies after transformation using the pTETnir15 screening primers as shown to confirm putative clones containing the plasmid prior to expression analysis. Each of the gene sub fragments (not pictured here) as shown in figure 3.11 below were cloned in place of the full GP gene to allow expression of shorter TetC-GP fusions. Plasmid map was made using the commercial software Snapgene.

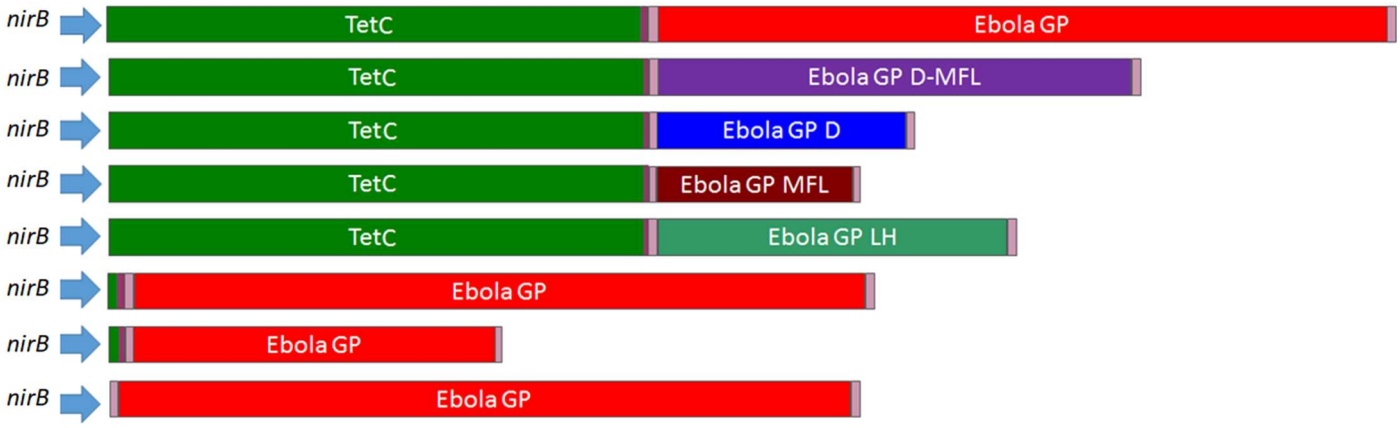


Figure 3.11. TetC-Ebola GP fusion protein constructs. Schematic presentation of the panel of Ebola GP fusion protein constructs generated in this study. Under the control of the *nirB* promoter, the full-length Ebola GP (devoid of hydrophobic signal and transmembrane sequences) and sub fragments thereof were expressed either as a fusion to the C-fragment of Tetanus Toxin (TetC), alone, or as a fusion to the full TetC ribosome binding site (RBS) in the pTECH2 expression vector in *Salmonella* Typhimurium. The TetC and Ebola GP fragments are separated by a flexible hinge, Gly Pro Gly Pro motif, which allows temporal and spatial separation between the two components to allow time for correct folding.

3.2.1.1 Synthetic Ebola GP (V2)

The new synthetic GP gene was amplified as above, using primers to remove the hydrophobic signal and transmembrane sequences at the N and C termini, giving a PCR product as expected of 1854bp. This PCR reaction also resulted in a smaller band showing at approximately 300bp. This could be due to the primers binding elsewhere on the gene sequence. This was rectified using gel extraction of DNA, where the correct top 1854bp band was excised and purified after digestion with the cloning restriction enzymes (see figure 3.12).

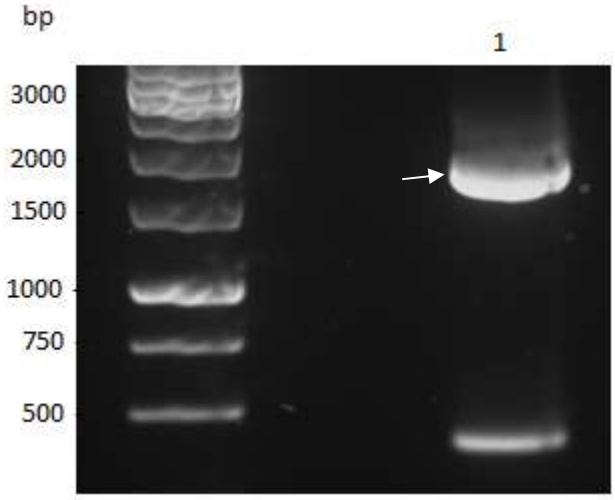


Figure 3.12. PCR amplification of synthetic GP (V2). 0.7% agarose gel showing the 1854bp PCR product of the 'version 2' full synthetic Ebola GP gene (1) when amplified with corresponding primers containing restriction cloning sites. This product was subsequently gel purified due to the presence of an extra band shown at approximately 300bp. It was then digested with the appropriate restriction enzymes, *EcoRV* and *HindIII*, spin column purified, and cloned into digested pTECH2 plasmid.

3.2.1.2 PCR amplification of GP Sub-fragment 'D'

The full-length Ebola GP has proved challenging for many groups to express in bacterial cells, particularly E. coli (Das et al, 2007; Zai et al, 2016) A sub fragment of the full-length wild type GP protein however, has been expressed at high levels in E. coli (Das et al, 2007). The 'D' gene sub fragment is 633bp (figure 3.13) and contains residues 157-368 of the GP protein, including some known protective epitopes.

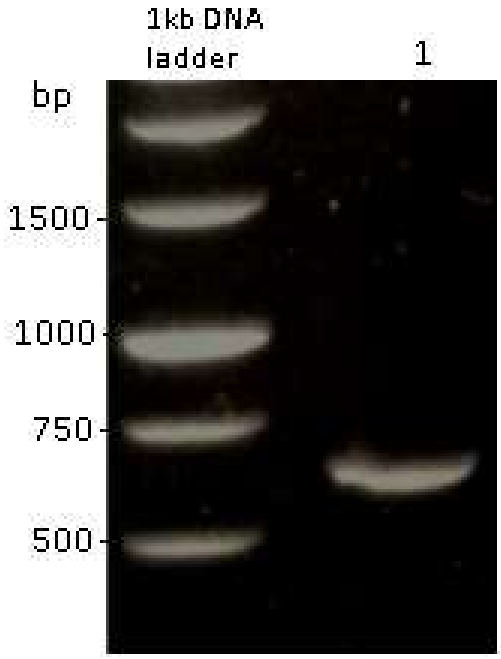


Figure 3.13. PCR amplification of synthetic GP sub fragment D. 0.7% agarose gel showing the 633bp PCR product 'D' Sub fragment of the synthetic Ebola GP gene (1) when amplified with corresponding primers containing restriction cloning sites. This product was subsequently digested with *BamHI* and *HindIII*, spin column purified, and cloned into into the digested pTECH2 plasmid (see figure 3.10)

3.2.1.3 PCR amplification of GP Sub-fragment 'MFL'

Sub fragment MFL is a highly immunogenic region, containing the mucin like domain (M) and fusion loop (FL) of the full GP. The wild type version has been shown to induce humoral and cellular immune responses and elicit neutralising antibodies towards this sub fragment (Wang et al, 2014). The 'MFL' gene sub fragment is 489bp (figure 3.14).

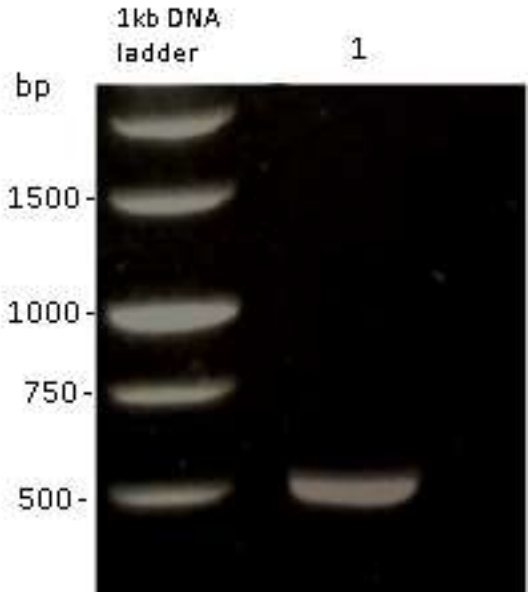


Figure 3.14. PCR amplification of synthetic GP sub fragment MFL. 0.7% agarose gel showing the 489bp PCR product 'MFL' Sub fragment of the synthetic Ebola GP gene (1) when amplified with corresponding primers containing restriction cloning sites. This product was subsequently digested with *BamHI* and *HindIII*, spin column purified, and cloned into the digested pTECH2 plasmid (see figure 3.10). This construct was made in collaboration with Bethany Gollan, a Masters student supervised by myself in the Khan laboratory.

3.2.1.4 PCR amplification of GP Sub-fragment 'LH'

There are potential difficulties encountered when expressing hydrophobic proteins, due to their tendency to aggregate, lower solubility and toxicity (Montigny et al, 2004). Therefore, a low hydrophobicity sub-fragment of Ebola GP was chosen using a Kyte-Doolittle Hydropathy plot (figure 3.15). It was thought that this sub-fragment which still contained some known epitopes may have improved expression, compared to its more hydrophobic counterparts. Gene sub fragment LH is 873bp (figure 3.16).

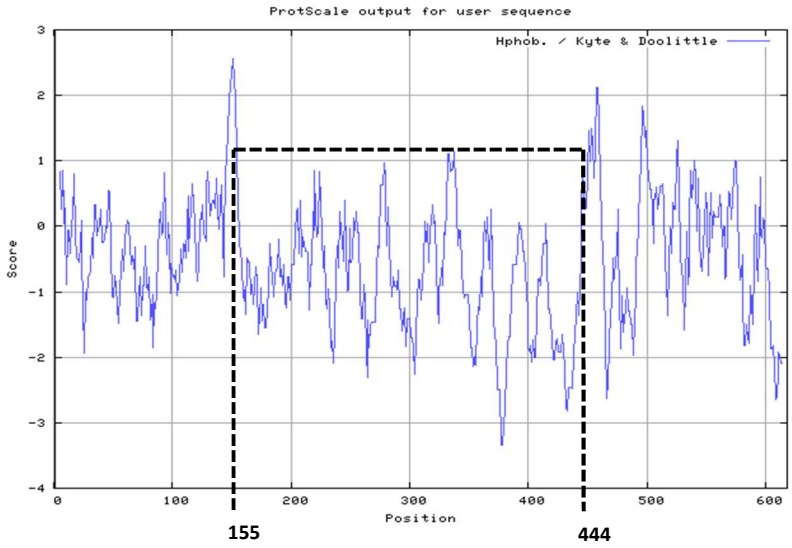


Figure 3.15 Kyte-Doolittle Hydrophobicity plot of the synthetic GP gene. The LH (low hydrophobicity) sub-fragment, containing the lowest concentration of hydrophobic residues is situated between residues 155 and 444.

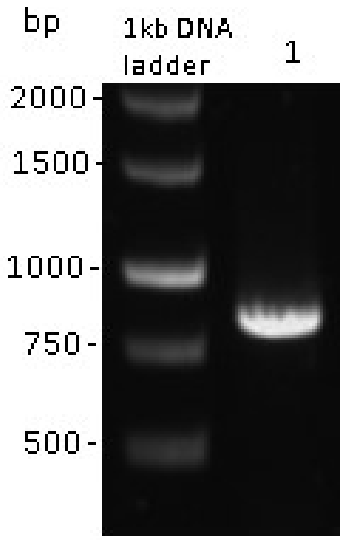


Figure 3.16. PCR amplification of synthetic GP sub fragment LH. 0.7% agarose gel showing the 873bp PCR product 'LH Sub fragment of the synthetic Ebola GP gene (1) when amplified with corresponding primers containing restriction cloning sites. This product was subsequently digested with *BamHI* and *HindIII*, spin column purified, and cloned into the digested pTECH2 plasmid.

3.2.1.5 PCR amplification of GP Sub-fragment 'D-MFL'

Since the wild type D and MFL sub fragments have been shown to be expressed in E. coli (Das et al, 2007; Wang et al, 2014), it was then decided to make a 'mini gene' combining 'D' sub fragment, which had been shown to express in bacterial cells (Das et al, 2007) and the adjacent highly immunogenic sub fragment 'MFL', which contains important immunological epitopes. It is hoped that that this mini gene allows for high expression of the protein and elicits a protective immune response in vaccinated hosts, due to the epitopes it contains. The D-MFL sub fragment is 1201bp (figure 3.17).

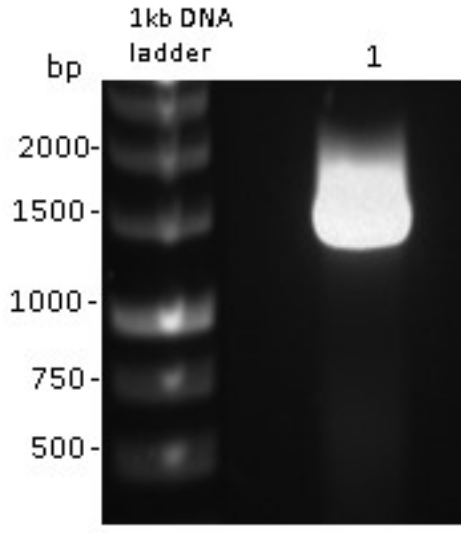


Figure 3.17. PCR amplification of synthetic GP sub fragment D-MFL. 0.7% agarose gel showing the 1201bp PCR product 'D-MFL' Sub fragment of the synthetic Ebola GP gene (1) when amplified with corresponding primers containing restriction cloning sites. This product was subsequently digested with *BamHI* and *HindIII*, spin column purified, and cloned into the digested pTECH2 plasmid. Although the agarose gel here is overloaded with sample the approximate 1201bp size of the band is apparent. This construct was made in collaboration with Bethany Gollan, a Masters student supervised by myself in the Khan laboratory.

3.2.2 Generation and expression of Ebola GP constructs, without TetC

In an attempt to evaluate the contribution of TetC to expression levels, a construct with the TetC gene deleted was made; pTECH10-GP (see figure 3.18).

3.2.2.1 Construction of pTECH10-GP expression plasmid

Primers were designed to allow inverse PCR, using Q5 site directed mutagenesis (NEB), to remove the TetC region of the pTECH2-Synthetic GP and insert a start codon at the beginning of the GP gene. This vector is designed to only express the synthetic GP as opposed to a TetC-GP fusion. Following chemical transformation into NEB5α E. coli cells (NEB), plasmid DNA from putative clones were purified and digested with the cloning restriction enzymes to visualise the GP insert on an agarose gel (see figure 3.19). Once the identity of the construct was confirmed, plasmid DNA was chemically transformed into *Salmonella* Typhimurium SL5338 cells. The construct was then further verified with DNA sequencing (Eurofins Genomics).

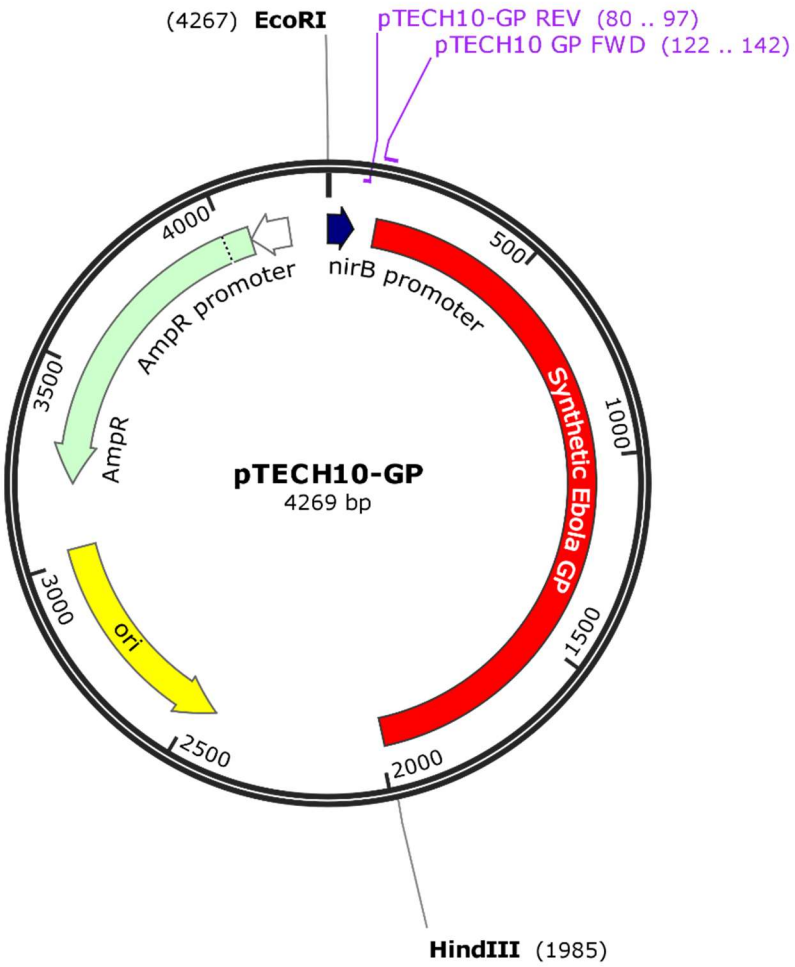


Figure 3.18. Plasmid map of the pTECH10-GP expression vector. This vector was constructed using Q5 Site Directed Mutagenesis (NEB) using the forward (FWD) and reverse (REV) primers shown. Inverse PCR allowed the removal of the TetC gene from the pTECH2-GP plasmid. Expression of Ebola GP is under the control of the *nirB* promoter and is expressed alone, without TetC as a fusion partner. Plasmid map was made using the commercial software Snapgene.

Plasmid DNA was purified from SL5338 cells after transformation of pTECH10-GP and digested with *EcoRI* and *HindIII* to ensure that the GP gene was present but the TetC was not. A band co-migrating with the synthetic GP PCR product shows that a band of the expected size for GP was present (see figure 3.19). A restriction fragment DNA band corresponding to the remainder of the plasmid itself is also present as expected, and is smaller in size than that of pTECH2-GP, suggesting that the TetC gene has been successfully deleted. Purified plasmid was then sent for DNA sequencing (Eurofins) for verification.

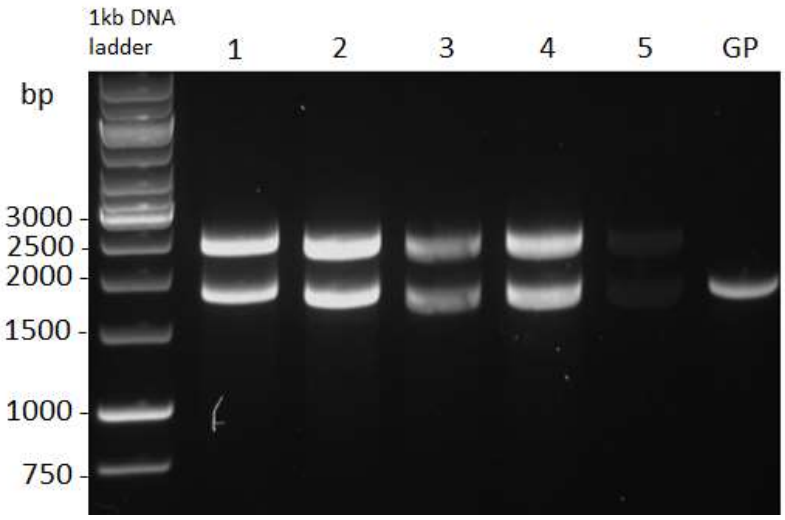


Figure 3.19. Restriction digest screens of putative pTECH10-GP clones. 0.7% agarose gel showing purified plasmid from 5 colonies of NEB5α E. coli suspected to contain the pTECH10-GP plasmid. The plasmid was digested with *EcoRI* and *HindIII* showing an insert band of 1854bp which co-migrated with the PCR product for the Ebola GP gene (labelled GP). The correct plasmid was then sent for sequencing (Eurofins) and once confirmed, transformed into *Salmonella* Typhimurium SL5338 cells for expression analysis.

3.2.2.2 Expression of Ebola GP alone in Salmonella strain SL5338

With confirmation that the plasmid pTECH10-GP had been constructed, it was then necessary to determine whether the Ebola GP could be expressed alone, without the TetC fusion partner. It appeared however that when probed with an anti-Ebola GP antibody there was no evidence of GP expression from SL5338 cell lysates containing the pTECH10-GP construct (see figure 3.20). Therefore, it seems that it is not possible to express Ebola GP alone in this system with no fusion partner. This could be due to the toxicity of the GP protein and thus the TetC may allow some detoxification when used as a fusion partner, and at least some of this is necessary for GP expression.

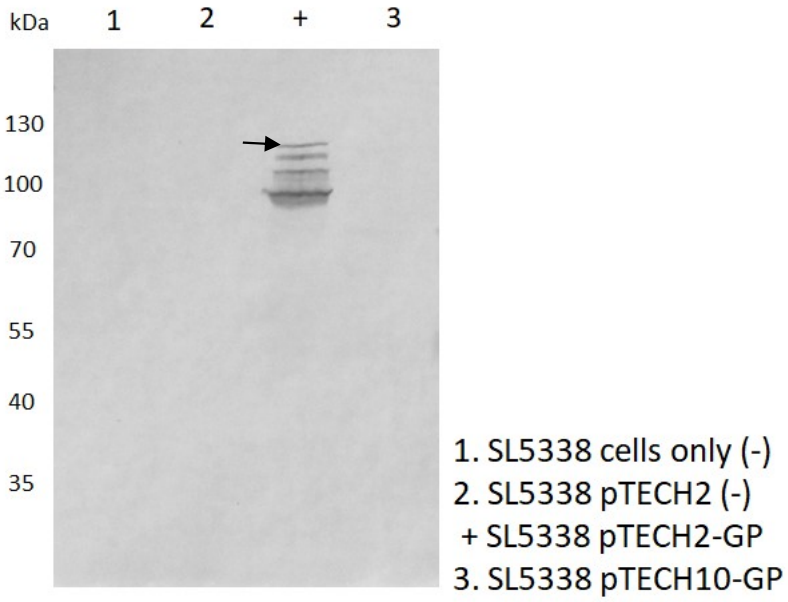


Figure 3.20. Expression of Ebola GP alone in *Salmonella* Typhimurium. Western blot to determine whether the plasmid pTECH10-GP is able to express Ebola GP in SL5338 cells with no fusion partner. Each lane contains cell lysates of *Salmonella* Typhimurium SL5338 with no plasmid (1), pTECH2 only (2), pTECH2-GP as a positive control (+) and pTECH10-GP. Blots were probed with polyclonal mouse anti-EBOV sera (From a C57BL/6 mouse which had survived Ebola virus challenge after treatment with monoclonal antibody 5E6) and Rabbit anti-mouse HRPO secondary antibody (Abcam). It is evident that there is no expression of GP from the pTECH10-GP plasmid which would show a band at approximately 69kDa. pTECH2-GP on the other hand, allows clear expression of a TetC-GP fusion protein, showing a band as expected at approximately 121kDa (+), denoted by arrow. The blot was developed with 4-chloro-1-napthol.

3.2.2.3 Construction of pTECH11-GP expression plasmid – Retention of the TetC ribosome binding site (RBS)

With the lack of Ebola GP expression from the pTECH10-GP construct, it was decided that retaining a small part of the TetC gene which could include the full ribosome binding site (RBS) may help with expression. The RBS is a region in the mRNA transcript, including the Shine-Dalgarno sequence situated upstream of the start codon (AUG), and encompasses some of the mRNA coding sequence downstream of the start codon between bases 1 and 18 (codons 1-6), this could be responsible for stabilisation of the *Salmonella* ribosomal RNA and translated mRNA complex during translation (Etchegaray & Inouye, 1999). This construct – pTECH11-GP was made in the same way as above, using Q5 site directed mutagenesis, however the first 10 amino acids (30 bases) of TetC were retained. This included the whole RBS of TetC, including the 5' UTR and Shine-Dalgarno sequence, necessary for regulation of protein synthesis. It has been postulated that when an mRNA sequence, named the 'Downstream box', situated downstream of the initiation or start codon, has some complementarity to bases 1534-1542 at the 3' end of the 16S ribosomal RNA (rRNA), it could act to enhance translation, with greater enhancement relating to greater complementarity with that section of the 16S rRNA. It is thought that the formation of the translation initiation complex could be brought about first by interaction between the 16S rRNA and the Shine-Dalgarno sequence, and then the ribosome-mRNA interaction is further stabilised by the pairing between the downstream box and its complementary 16S ribosomal RNA sequence (Etchegaray & Inouye, 1999). Therefore, this improved expression of the GP protein could be due to an increase in complementarity between the TetC mRNA just downstream of the start codon, and the Salmonella 16S rRNA.

Another smaller construct was also made which instead of the whole GP gene, a truncated version starting at residue 155 was used. From examining the hydrophobicity plot of the Ebola GP (see figure 3.15) it was evident that the middle portion of the protein contained the area of lowest hydrophobicity. Starting at P155, it is possible that this truncated version of Synthetic GP may be more easily expressed due to the lack of a large area of hydrophobic residues. Using Q5 site directed mutagenesis as above, all but the first 10 residues of TetC and

the remainder of GP after P155, resulted in the plasmid pTECH11-GP (155) confirmed once again by restriction digest using *EcoRI* and *HindIII* (figures 3.21 and 3.22).

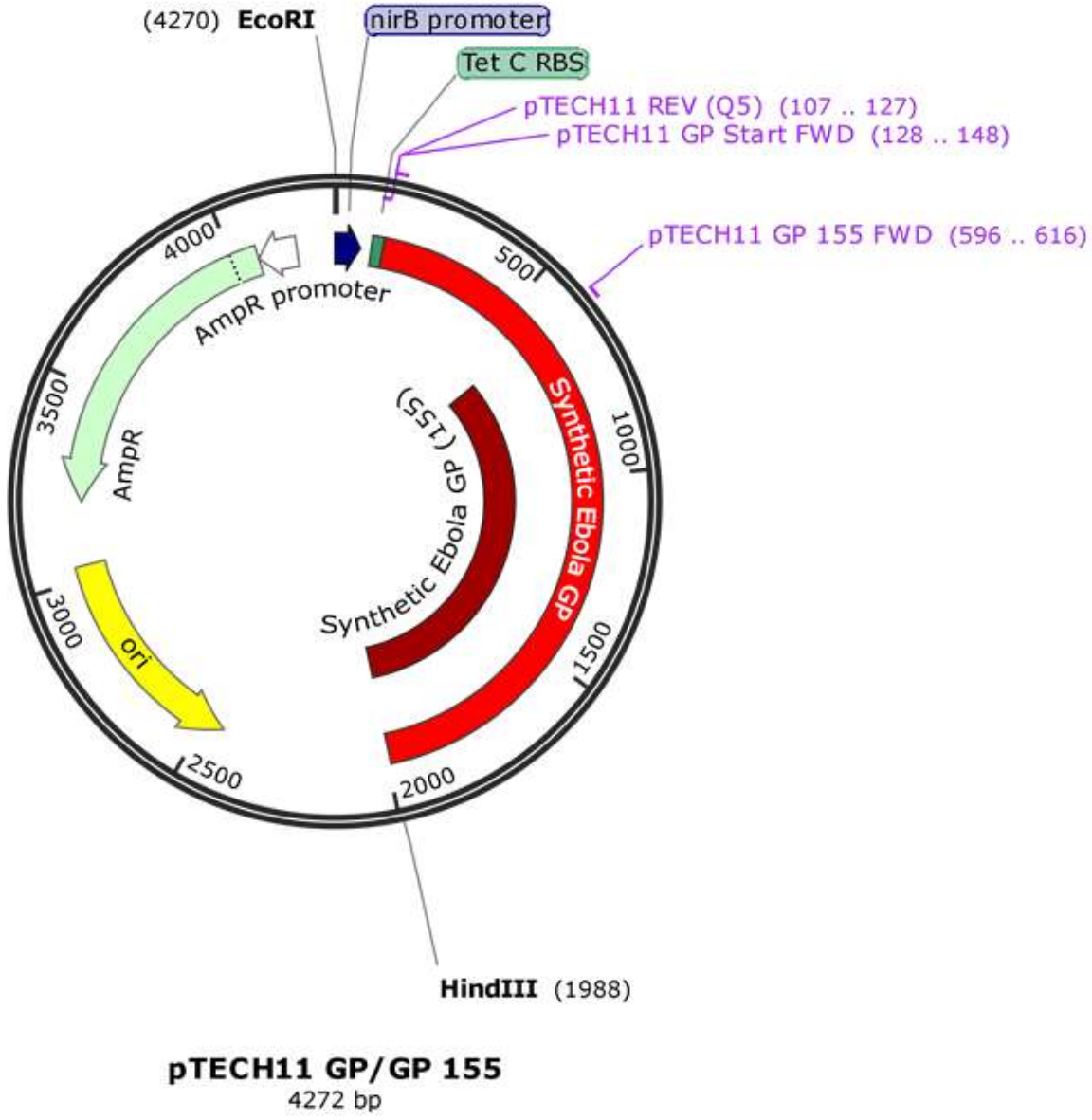


Figure 3.21. Plasmid map of the pTECH11-GP/GP (155) expression vector. This vector was constructed using Q5 Site Directed Mutagenesis (NEB) using the forward (FWD) and reverse (REV) primers shown. Inverse PCR allowed the almost complete removal of the TetC gene, apart from the first 10 amino acids, from the pTECH2-GP plasmid. Expression of Ebola GP is under the control of the *nirB* promoter and is expressed with the RBS of TetC as a small fusion partner. Plasmid map was made using the commercial software Snapgene.

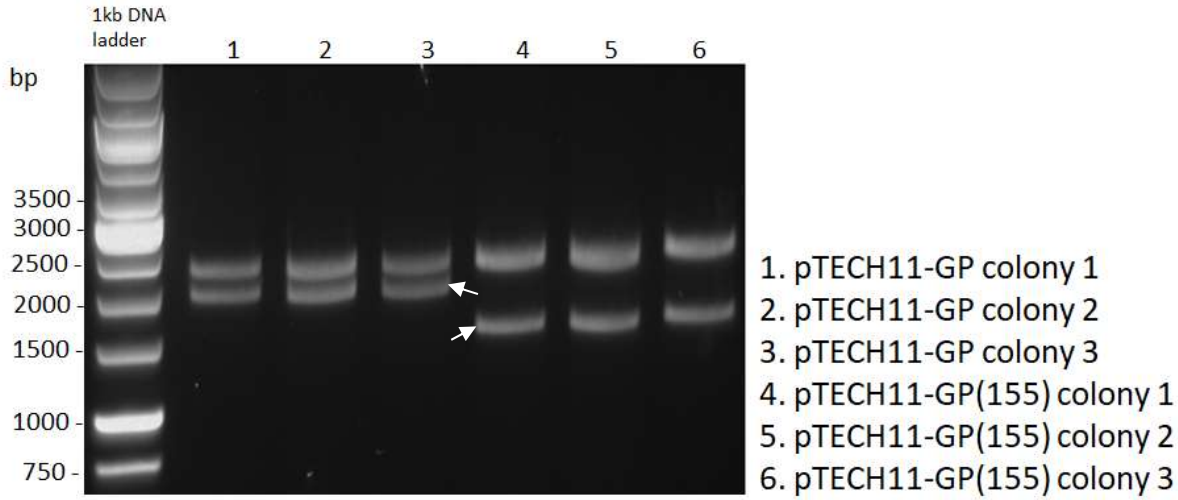


Figure 3.22. Restriction digest screens of putative pTECH11-GP/GP (155) clones. 0.7% agarose gel showing purified plasmid from 5 colonies of NEB5α E. coli suspected to contain the pTECH11-GP or pTECH11-GP (155) plasmid. The plasmid was digested with *EcoRI* and *HindIII* showing an insert band of 1854bp (full GP, lanes 1, 2 and 3) or 1389bp (GP 155, lanes 4, 5 and 6) as denoted by arrows. Bands corresponding to the size of the remainder of the plasmid are seen above as expected. The correct plasmids were then sent for sequencing (Eurofins) and once confirmed, transformed into *Salmonella* Typhimurium SL5338 cells for expression analysis.

3.2.2.4 Expression of Ebola GP with TetC RBS in Salmonella strain SL5338

With confirmation that the plasmids pTECH11-GP and pTECH11-GP (155) had been constructed (figure 3.22), it was then necessary to determine whether the Ebola GP could be expressed when only a small part of the TetC gene (the 10-amino acid or 30 base RBS) was present. Here, it appeared that SL5338 cell lysates containing these pTECH11-GP constructs probed with an anti-Ebola GP antibody, the addition of this RBS rescued expression of the GP protein, both full-length and truncated (see figure 3.23). Therefore, it seems that it is necessary to include the full TetC Ribosome Binding Site including the 30 bases downstream of the start codon to ensure that proper recruitment of all ribosome components and enzymes necessary for translation. It may be that the start of the synthetic GP gene sequence (which here is devoid of the hydrophobic and potentially toxic signal sequence) is suboptimal in maintaining stability of the ribosome and mRNA complex, as there could be less complementarity between the nucleotides downstream of the GP start codon, with the

Salmonella Typhimurium 16S ribosome. This could mean that the interaction between the mRNA and 16S rRNA is less stable and therefore the protein is unable to be expressed.

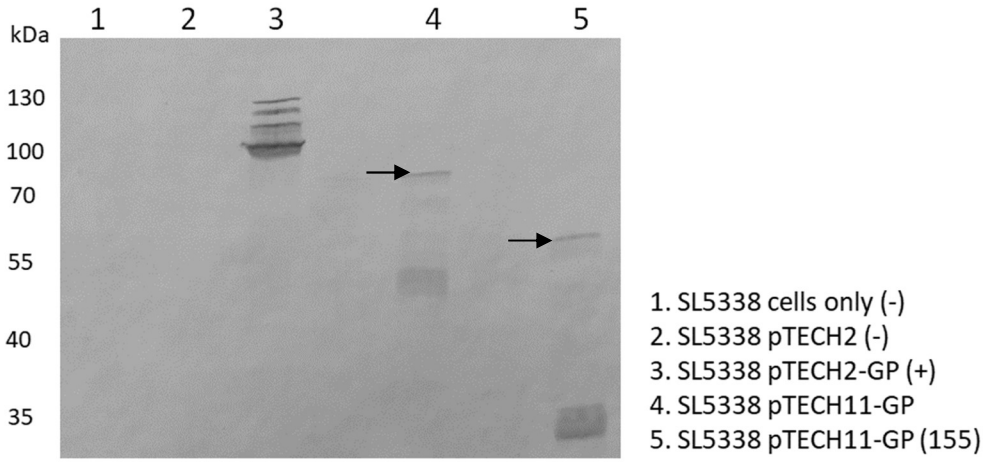


Figure 3.23. Expression of Ebola GP with TetC RBS in *Salmonella* Typhimurium. Western blot to determine whether the plasmid pTECH11-GP and pTECH11 GP (155) are able to express Ebola GP in SL5338 cells with only the RBS of TetC retained. Each lane contains cell lysates of *Salmonella* Typhimurium SL5338 with no plasmid (1), pTECH2 only (2), pTECH2-GP as a positive control (3) pTECH11-GP (4) and pTECH11-GP (155) (5). Blots were probed with polyclonal mouse anti-EBOV sera (From a C57BL/6 mouse which had survived Ebola virus challenge after treatment with monoclonal antibody 5E6) (1:2000) and Rabbit anti-mouse HRPO secondary antibody (Abcam) (1:4000). It is evident that there is expression of GP from both of the pTECH11 GP plasmids, but this is not as strong as the bands seen when GP is expressed with the full TetC protein (lane 3). The full GP plus the RBS of TetC is approximately 71Kda and the truncated GP protein which starts at p155, plus the TetC RBS, is approximately 54Kda (see arrows). The blot was developed with 4-chloro-1-napthol.

3.2.3 Screening putative clones after generation of pTECH2-GP expression plasmids (Colony PCR)

It appears that the Ebola GP protein cannot be expressed in this system without at least a small fusion partner of the TetC RBS. It is clear that expression is increased when the full TetC is present, and therefore to maximise protein expression and thus antigen delivery, it was decided that the full TetC would be kept as a fusion partner. It could also act as built in adjuvant to enhance the immune response to the vaccine. The constructs described in section 3.2 were therefore generated and expression was analysed by western blot to determine the best prospects to take forward for *in vivo* analysis of immune response.

3.2.3.1 Construction of pTECH2-GP (v2) expression plasmid

As with the previous pTECH2-GP construct, the expression plasmid containing the new, alternatively codon optimised GP (v2) was cloned into the pTECH2 plasmid by restriction cloning and ligation with T4 DNA ligase, followed by transformation into *Salmonella* SL5338. Single colonies were then picked, and colony PCR carried out using screening primers which were complimentary to inert flanking regions on pTECH2 (figure 3.24). This allowed visualisation of successful insertion of GP, as the PCR product shows a band at approximately 2354bp, rather than 543bp as would be amplified by the screening primers with the 'empty' pTECH2 vector as a template. Restriction digest using the cloning enzymes and DNA sequencing on putative clones acted to further confirm the successful generation of the desired pTECH2-GP construct.

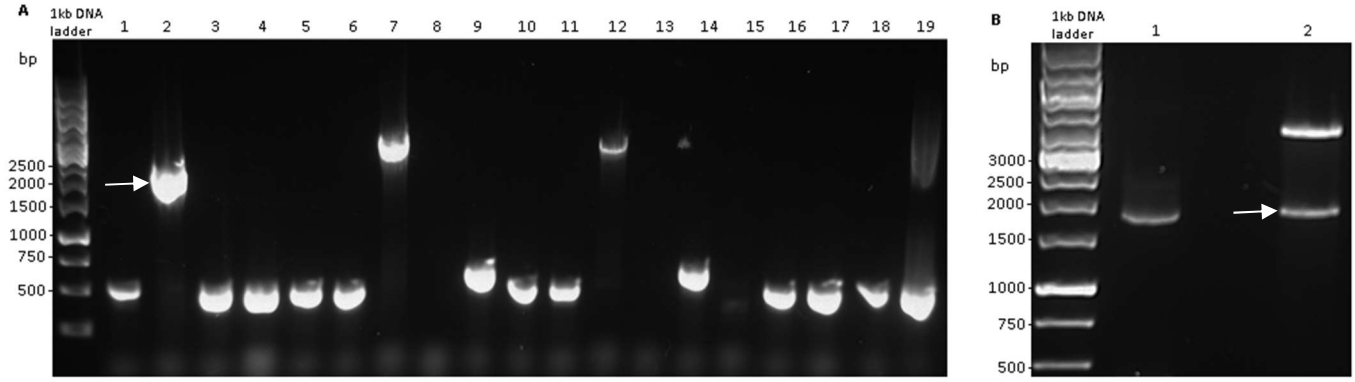


Figure 3.24. Colony PCR and restriction digest screens of putative pTECH2-GP (v2) clones. A) 0.7% agarose gel showing PCR products from colony PCR using pTECH2 screening primers to determine successful construction and transformation of the pTECH2 vector containing the 1854bp Synthetic GP into SL5338 *Salmonella* strain. Following transformation, colonies 1-19 (as shown on gel) were boiled before PCR amplification of the plasmid between each screening primer. Positive colonies which have successfully taken up the plasmid pTECH2 GP (notably colony 2) show a band at 2354bp (denoted by arrow). Colonies which have only taken up pTECH2 show a band at approximately 543bp.

B) 0.7% agarose gel showing purified plasmid from colony 2 (see figure 3.24 A) digested with *EcoRV* and *HindIII*, showing an insert band of 1854bp (lane 2, see arrow) which corresponds to the size of the PCR product for the Ebola GP gene (lane 1). This plasmid was then sent for sequencing using the same screening primers as described above

3.2.3.2 Construction of pTECH2-GP 'D' expression plasmid

Again, following transformation of *Salmonella* SL5338 with the ligation reactions inserting the 'D' fragment of the new optimised GP gene, colony PCR was used to screen for putative clones using screening primers as described above. Successful insertion of sub-fragment 'D', results in a band at approximately 1176bp (figure 3.25).

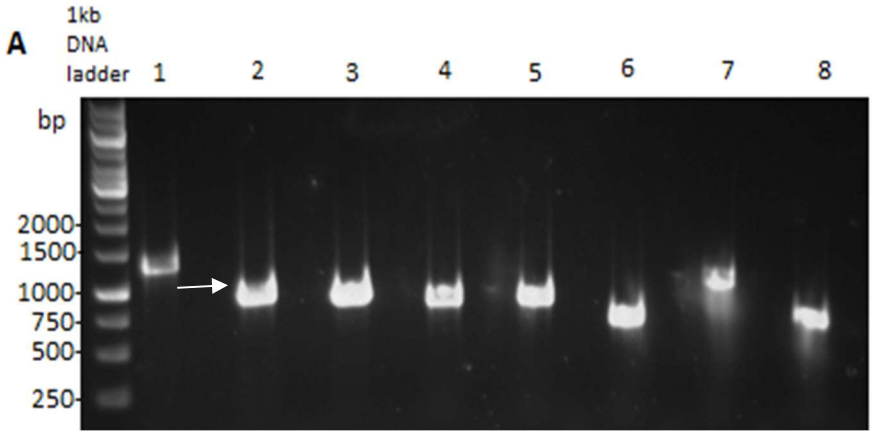


Figure 3.25. Colony PCR screen of putative pTECH2-GP D clones.
0.7% agarose gel showing PCR products from colony PCR using pTECH2 screening primers to determine successful construction and transformation of the pTECH2 vector containing the 633bp Synthetic GP section D into SL5338 *Salmonella* strain. Following transformation, colonies 1-8 (as shown on gel) were boiled before PCR amplification of the plasmid between each screening primer. Positive colonies which have successfully taken up the plasmid pTECH2 GP-D show a band at approximately 1176bp, as shown by arrow.

3.2.3.3 Construction of pTECH2-GP 'MFL' expression plasmid

Transformation of *Salmonella* SL5338 with the ligation reactions inserting the 'MFL' fragment of the new optimised GP gene was followed by colony PCR to screen for putative clones using the screening primers as described above. Successful insertion of sub-fragment 'MFL', results in a band at approximately 1032bp (figure 3.26)

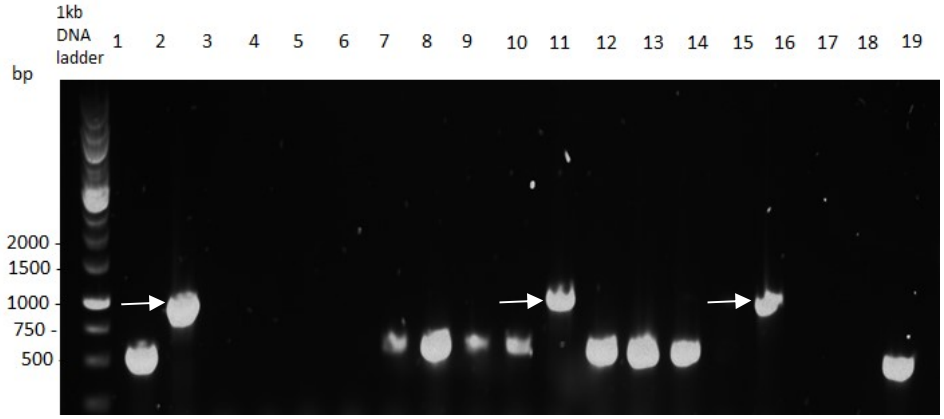


Figure 3.26. Colony PCR screen of putative pTECH2-GP MFL clones.
0.7% agarose gel showing PCR products from colony PCR using pTECH2 screening primers to determine successful construction and transformation of the pTECH2 vector containing the 489bp Synthetic GP section MFL into SL5338 *Salmonella* strain. Following transformation, colonies were boiled before PCR amplification of the plasmid between each screening primer. Positive colonies which have successfully taken up the plasmid pTECH2 GP-MFL show a band at approximately 1032bp, notably in lanes 2, 11 and 16 (denoted by arrows). The agarose gel here is overloaded with sample, causing a rippling effect, however the differences in size between the samples are clearly apparent

3.2.3.4 Construction of pTECH2-GP 'LH' expression plasmid

Colony PCR was used to screen for putative clones using screening primers as described above following the transformation of *Salmonella* SL5338 with the ligation reactions inserting the 'LH' fragment of the new optimised GP gene. Successful insertion of sub-fragment 'LH', results in a band at approximately 1416bp (figure 3.27)

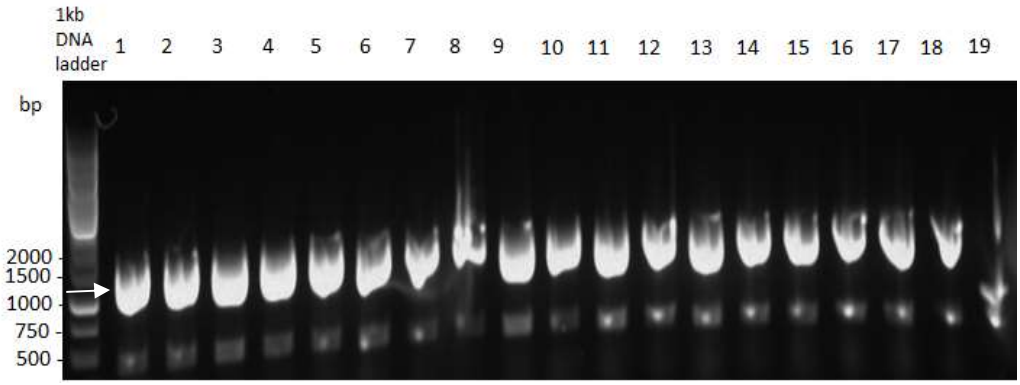


Figure 3.27. Figure 3.26. Colony PCR screen of putative pTECH2-GP LH clones.
0.7% agarose gel showing PCR products from colony PCR using pTECH2 screening primers to determine successful construction and transformation of the pTECH2 vector containing the 873bp Synthetic GP section LH into SL5338 *Salmonella* strain. Following transformation, colonies were boiled before PCR amplification of the plasmid between each screening primer. Positive colonies which have successfully taken up the plasmid pTECH2 GP-LH show a band at approximately 1416bp, as shown by arrow. The agarose gel here is overloaded with sample, causing a rippling effect, however the differences in size between the samples are clearly apparent.

3.2.3.5 Construction of pTECH2-GP 'D-MFL' expression plasmid

Salmonella SL5338 was transformed with the ligation reactions inserting the 'D-MFL' fragment of the new optimised GP gene, and colony PCR was used to screen for putative clones using screening primers as described above. Successful insertion of sub-fragment 'D-MFL', results in a band at approximately 1743bp (figure 3.28)

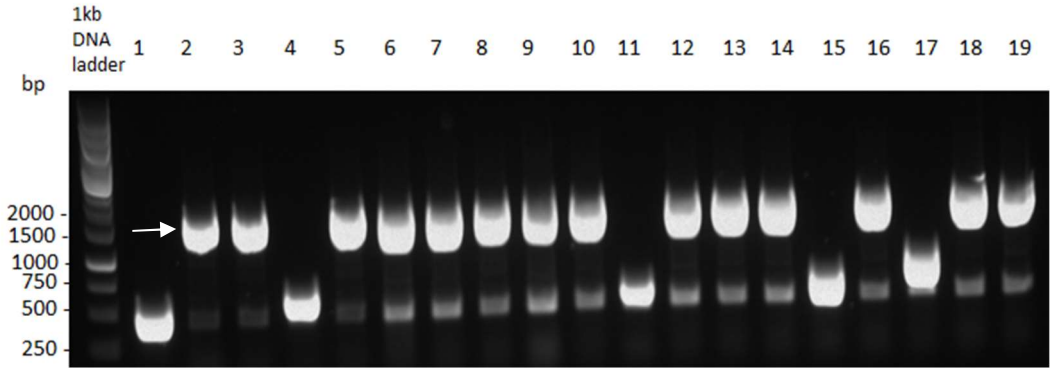


Figure 3.28. Figure 3.26. Colony PCR screen of putative pTECH2-GP DMFL clones.
0.7% agarose gel showing PCR products from colony PCR using pTECH2 screening primers to determine successful construction and transformation of the pTECH2 vector containing the 1200bp Synthetic GP section DMFL into SL5338 *Salmonella* strain. Following transformation, colonies were boiled before PCR amplification of the plasmid between each screening primer. Positive colonies which have successfully taken up the plasmid pTECH2 GP-DMFL show a band at approximately 1743bp, as shown by arrow. The agarose gel here is overloaded with sample, causing a rippling effect, however the differences in size between the samples are clearly apparent.

3.2.3.6 Informative digests of putative recombinant clones

Following the selection of colonies which through colony PCR had shown to have successfully taken up the recombinant plasmids, containing the sub fragments of the Ebola GP gene, the plasmid DNA from these colonies was purified and digested with the appropriate cloning restriction enzymes to allow visualisation of an insert at the expected size for each sub fragment (figure 3.29). From this, plasmid DNA from clones which were deemed to contain the correct insert was sent for sequencing using the pTET screening primers as used in the colony PCR tests to confirm that each of the sub fragments had been inserted in frame and downstream of the *nirB* promoter to allow their expression.

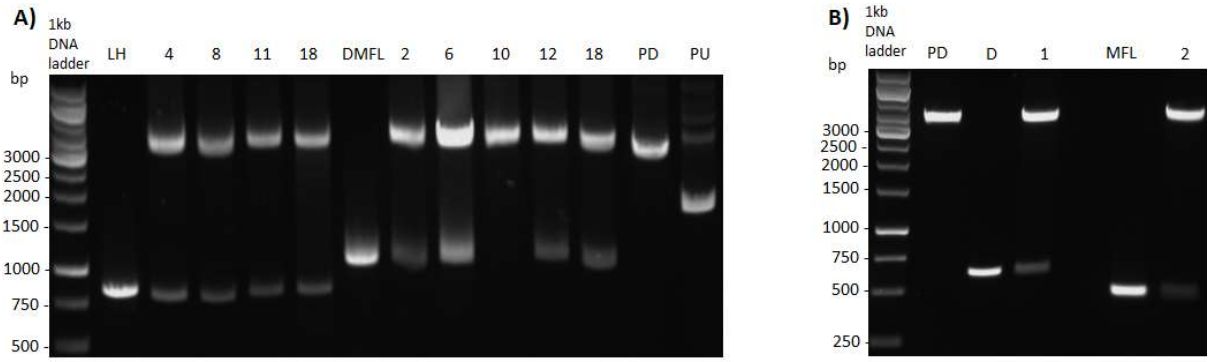


Figure 3.29. Restriction digest screens of putative recombinant pTECH2-GP sub fragment clones. 0.7% agarose gel showing purified plasmid from SL5338 colonies deemed (through colony PCR) to contain the Ebola GP sub fragment inserts. Plasmid DNA from these colonies were digested with the restriction enzymes used to clone these inserts (*BamHI* and *HindIII*). Successful cloning of the inserts into the pTECH2 plasmid resulted in a band of equivalent size to drop out when digested and visualised on an agarose gel. A) Plasmid DNA from clones containing the LH (873 bp) or DMFL (1200bp) inserts with the corresponding PCR products shown alongside the digested plasmid inserts to confirm size. B) As with A, however using plasmid DNA from clones containing the D (633bp, labelled 1) or MFL (489bp labelled 2) inserts. PD – pTECH2 digested with *BamHI* and *HindIII*. PU – un-digested pTECH2 plasmid.

3.3 Expression of TetC-GP fusion proteins

After a panel of constructs had been generated, expression analysis was carried out using western blot. Ensuring that each sample contained the same number of cells, calculated from the OD600 of overnight cultures, the expression levels of each construct could be determined and it could then be decided which to take forward, transform into vaccine strains and use in *in vivo* immunisations to determine immune response to Ebola GP.

3.3.1 Expression of TetC-GP fusion proteins in Salmonella intermediate strain SL5338

Firstly, to determine expression of the new Ebola GP a western blot was carried out on SL5338 cell lysates harbouring either pTECH2 alone, pTECH2-GP or no plasmid. When probed with polyclonal anti-TetC serum it was evident that the TetC-GP fusion protein was being expressed as with the previous GP sequence. Again, the fusion protein was expressed at a lower level than TetC and there was still evidence of breakdown products or premature termination of translation as seen in the lower bands (TetC-GP is shown at approximately 121kDa, see figure 3.30). It appears that with this alternatively optimised GP sequence, there are less of these lower bands present than as above with the first. This could suggest that there is less breakdown occurring.

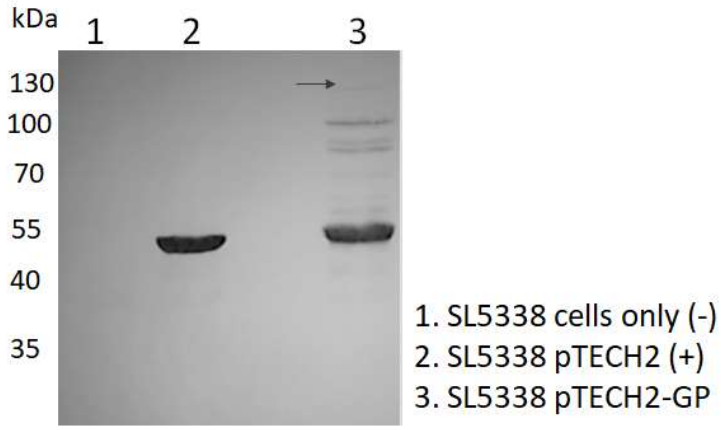


Figure 3.30. Expression of TetC-GP (v2) fusion protein Western blot showing expression of the TetC-GP fusion protein or TetC alone, in cell lysates of clones of *Salmonella* Typhimurium SL5338. The top band is the TetC-GP fusion protein at approximately 121kDa (see arrow.) The blot was probed with polyclonal rabbit anti-TetC serum (1:1000) and Goat anti-rabbit HRPO (1:2000). The blot was developed with 4-chloro-1-napthol.

After showing that the full new GP sequence was successfully expressed by *Salmonella*, it was then determined that the smaller constructs containing Ebola GP gene sub fragments should be tested for expression. A panel of various sub-fragment constructs was created to allow comparison of the expression levels, and therefore only take those which expressed the best through to the next stage of *in vivo* testing. Each sub-fragment was chosen for certain characteristics, either containing important immunological epitopes, has shown to be expressed previously in bacteria, or has a lower concentration of hydrophobic residues (figure 3.31).

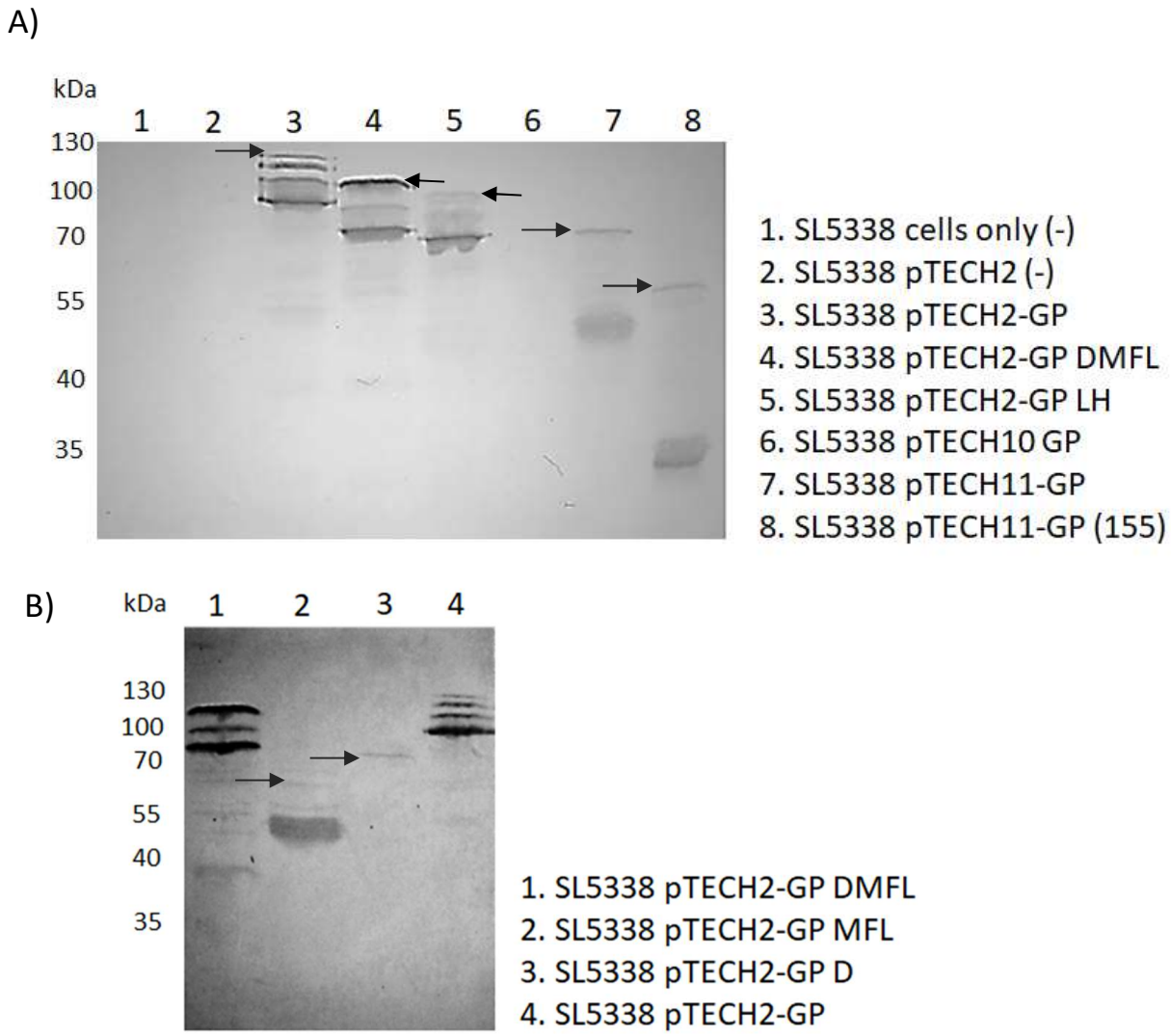


Figure 3.31. Expression of TetC-GP sub fragment fusion proteins.
Western blot showing all TetC-GP fusion proteins expressed in *Salmonella* Typhimurium SL5388. Cell lysates were fractionated on SDS-PAGE gel, cell numbers were corrected to the OD600 of the overnight cultures to ensure equivalent amounts are in each sample. Both blots were probed with mouse polyclonal anti EBOV sera (1:2000) and a secondary Rabbit anti mouse HRPO (1:4000). A) SL5338 cells only and SL5338 harbouring pTECH2 only were used as negative controls (lanes 1, 2). 3) TetC-full GP fusion at approximately 121kDa. 4) TetC-GP DMFL fusion at approximately 97kDa. 5) TetC-GP LH fusion at approximately 85kDa. 6) GP alone (no fusion) which did not express. 7) RBS of TetC fusion with GP at approximately 71kDa. 8) RBS of TetC fusion with truncated GP (155) at approximately 54kDa (as shown by arrows. B) Comparison of GP D and MFL fragments with full GP and GP DMFL. 1) TetC-DMFL fusion at approximately 97kDa. 2) TetC-GP MFL fusion at approximately 70kDa (see arrow). 3) TetC-GP D fusion at approximately 77kDa (see arrow). 4) TetC-full GP fusion at approximately 121kDa. Blots were developed with 4-chloro-1-napthol. Constructs pTECH2-GP DMFL and pTECH2-MFL were made in collaboration with Bethany Gollan, a Masters student supervised by myself in the Khan laboratory.

From these results it appears that when an equivalent number of cells are fractionated on an SDS-PAGE gel, and the expressed protein visualised by western blotting, the construct which allows greatest expression of the TetC-GP fusion protein, is that of pTECH2-GP DMFL. Sub fragment DMFL is a 'mini-gene' consisting of two sub fragments 'D' and 'MFL' which have been shown to express in E. coli and also contain important immunological epitopes (Das et a, 2007; Wang et al, 2014). It appears that this sub fragment expresses better than any of the others as a fusion to TetC. The two constituent parts of DMFL, sub fragments D and MFL, do express as a fusion to TetC, but at very low levels (see figure 3.31 B lanes 2 and 3).

Expressing the GP protein alone, with no fusion partner, does not appear to be possible in this system. No expression of GP was seen from the cells harbouring the pTECH10-GP construct. Adding a small 10 amino acid fragment of TetC (the RBS), rescued this expression, although not to the levels seen with the full TetC fusion proteins.

Antigen expression from *Salmonella* vaccine strains, must be optimised to ensure that a suitable antigenic dose is administered which can elicit a protective immune response. Therefore, the constructs which have shown to express the TetC-GP fusion proteins more efficiently, will be taken further for expression analysis in *Salmonella* vaccine strains and then *in vivo*, to allow the best chance of success.

3.3.1.1 Conservation of known protective epitopes in TetC-Ebola GP fusion proteins

Prior to testing these constructs for expression in *Salmonella* vaccine strains, further analysis was carried out to determine whether known protective epitopes were present in these fusion proteins. The 'ladder' effect of multiple bands seen in the western blot, suggests that there could be some breakdown of the fusion proteins. Monoclonal antibodies directed to these epitopes (kindly provided by Professor Gary Kobinger, see table 3.1) were used in a western blot to show that these are still present even in these breakdown or shortened protein products.

Antibody	Epitope	Category	Notes
5E6 (IgG2a)	$_{401}$ ATQVEQHHRRTDND $_{415}$ Towards the start of the MFL sub fragment	Linear	Protective
7G4 (IgG1)	Between residues 157-369 Includes the D sub-fragment	Conformational	Protective
4G7 (IgG2b)	$_{501}$ REAIVNAQPKCNPNL $_{515}$ Towards the end of the MFL sub fragment	Linear	Neutralising
1H3 (IgG2a)	$_{267}$ SNTTGKLIWKVNPEI $_{280}$ Towards the middle of the D sub fragment.	Conformational	Neutralising

Table 3.1. Monoclonal antibodies to Ebola GP. Provided by Professor Gary Kobinger (Université Laval, Quebec, Canada), these were used to determine whether the TetC-GP fusion proteins expressed in *Salmonella* contained these protective epitopes. The conformational epitopes could allow us to determine if the GP protein is folding correctly (Qiu et al, 2011; Gonzalez-Gonzalez, 2015). Also refer to figures 3.9 and 3.3.2 for epitope locations on Ebola GP.

The use of a selection of antibodies which recognised a variety of linear or conformational epitopes would also help to determine if the GP protein has folded correctly. Conformational epitopes are non-continuous along the protein and require a certain (folded) tertiary conformation in order to be recognised by their specific antibody.

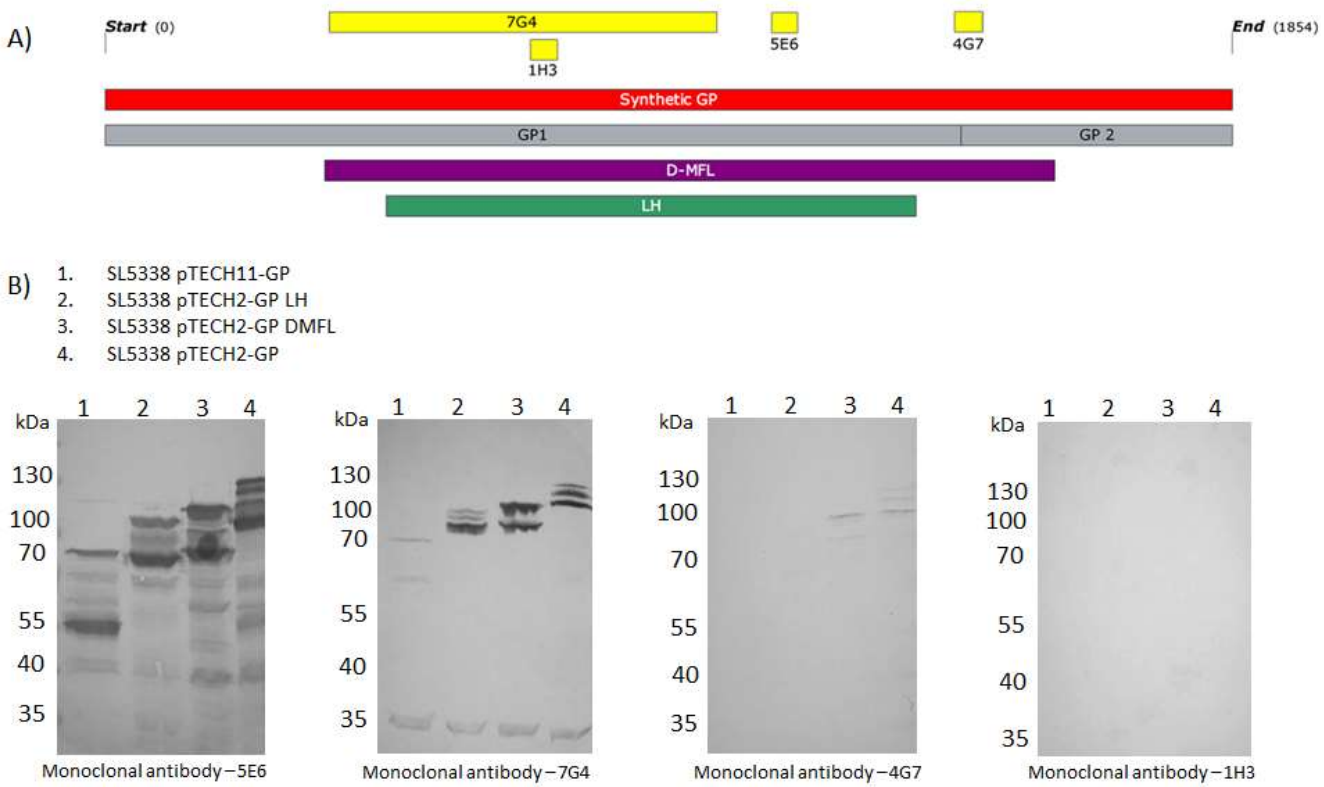


Figure 3.32. Conservation of known epitopes in TetC-Ebola GP fusion proteins.
A) Schematic diagram showing epitope locations (in yellow) for relevant mouse monoclonal antibodies in relation to sub-fragments of Ebola GP and the full-length protein (Qiu et al, 2011; Gonzalez-Gonzalez, 2015). Also refer to table 3.1. **B)** Western blot showing expression of 4 different TetC-GP fusion proteins in cell lysates of *Salmonella* Typhimurium SL5338 probed with a selection of 4 different monoclonal antibodies to Ebola GP, the epitopes of which are described above in table 3.1. In each sample, the top band is the size expected for the full-length fusion protein. 1) Tet RBS plus GP – approximately 70kDa. 2) TetC-GP LH – approximately 85kDa. 3) TetC-GP DMFL – approximately 97kDa. 4) TetC-full GP – approximately 121kDa. With breakdown products forming a 'ladder' underneath. Blots were probed with 1:5000 monoclonal Mouse anti Ebola GP antibodies (kindly provided by Professor Gary Kobinger) as labelled, and 1:4000 Rabbit anti-mouse HRPO. Blots were developed with 4-chloro-1-napthol.

Bands were seen at the expected size for each of the TetC-GP and sub-fragment fusions (see figure 3.32) when blotted with 3 of the 4 monoclonal antibodies and importantly, the conformational epitope recognising antibody, 7G4, detected bands in each of the samples. This suggests that the GP protein here is still folded in a way that this antibody can bind. Importantly, the smaller bands seen in each blot are still detectable by antibodies to specific epitopes on Ebola GP, suggesting that despite potential protein degradation, these smaller fragments may still be able to elicit antibodies *in vivo*. The antibody 4G7 recognises a linear

epitope at the end of the MFL sub fragment, so the fusion proteins without this will not be recognised. The conformational epitope recognising antibody 1H3 did not appear to bind to any of the fusion proteins, this could be due to either protein mis-folding around this epitope, or that the antibody itself was defective. None the less, protective epitopes on Ebola GP are clearly present in the TetC-GP fusion proteins as shown using the small panel of antibodies which were available.

3.3.2 Expression of TetC-GP fusion proteins in Salmonella vaccine strains

TetC-GP fusion proteins successfully expressed in *Salmonella* strain SL5338 contained protective linear and conformational epitopes of Ebola GP. SL5338 is non-attenuated, so to develop these constructs into a vaccine, they must be transformed into attenuated *Salmonella* vaccine strains.

In order to determine which vaccine strain to use in *in vivo* experiments, the constructs which appeared to express the TetC-GP fusion proteins best were then electroporated into a small panel of attenuated *Salmonella* vaccine strains and evaluated by western blot to determine which expressed the fusion proteins best (figure 3.33). The plasmids chosen to take forward to this stage were: pTECH2 (as a TetC control), pTECH2-full GP, pTECH2-GP DMFL and pTECH2-LH.

The attenuated strains used for this testing were as follows:

Strain	Mutation
SL3261	*aroA* mutant of *Salmonella* Typhimurium SL1344
BRD 807	*aroA* and *htrA* double mutant of *Salmonella* Typhimurium SL1344
BRD 509	*aroA* and *aroD* double mutant of *Salmonella* Typhimurium SL1344
C5*htrA*	*htrA*::T*nphoA* insertion mutant of *Salmonella* Typhimurium C5

Table 3.2 Attenuated *Salmonella* vaccine strains used in this study (Roberts et al, 2000; Chabalgoity et al, 1996).

It appears that the strain SL3261 expresses the fusion proteins best and therefore this will be used in the *in vivo* immunisation studies to determine immune response to Ebola GP.

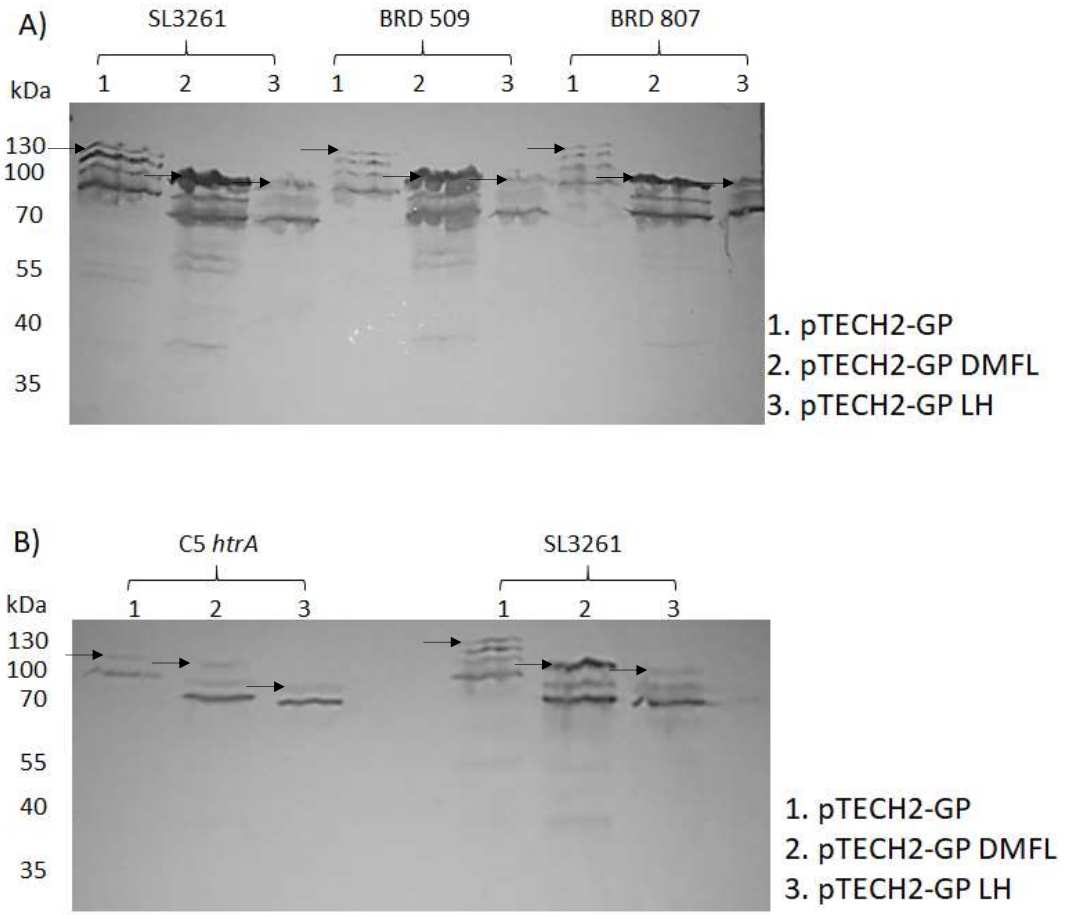


Figure 3.33. Expression of TetC-GP fusion proteins in *Salmonella* vaccine strains.
Western blot showing expression of TetC-GP fusion proteins in four different *Salmonella* Typhimurium attenuated vaccine strains; SL3261, BRD 509, BRD 807 and C5*htrA*. Cell lysates were fractionated on SDS-PAGE and transferred to nitrocellulose. Blots were probed with polyclonal mouse anti Ebola serum (1:2500) and Rabbit anti mouse HRPO (1:4000). The lanes are as follows: 1) TetC-GP showing a band at approximately 121kDa, 2) TetC-GP DMFL showing a band at approximately 97kDa, 3) TetC-GP LH showing a band at approximately 85kDa all denoted by arrows. It appears that the strain which expresses the GP fusions best is SL3261 due to the stronger bands seen in these samples. It was ensured that a equivalent numbers of cells were used in each sample, so the variation in band strength is therefore due to variation in expression levels between each strain. TetC-GP 121kDa, TetC-DMFL 97kDa, TetC-LH 85kDa.

3.4 *In vitro* stability of pTECH2-GP plasmids in *Salmonella* Typhimurium vaccine strain SL3261

In order to go forward with *in vivo* immunisation experiments to determine to the immune response to Ebola GP, it was vital to first determine the stability of the pTECH2 expression plasmids. When used *in vivo*, there are no antibiotics present to allow for selection and the ampicillin resistance gene present on the pTECH2 vector is not necessary. Therefore, the *Salmonella* vaccine strain must be able to stably retain the expression plasmid with no antibiotic selection pressure. This was first carried out *in vitro* using culture with and without ampicillin and the resultant CFU/ml which was determined allowed the calculation of plasmid stability. The results show that pTECH2-GP is 100% stable and pTECH2-GP DMFL 91.5% stable when grown in the absence of ampicillin and can therefore be taken forward for *in vivo* analysis. The pTECH2 control vector is 100% stable as expected.

Constructs	Overnight Growth media	Plate growth media	CFU/ml	% Stability
pTECH2	LB	LB	8×10^8	
		LB + AMP	8×10^8	100%
pTECH2-GP	LB	LB	1.37×10^9	
		LB + AMP	1.37×10^9	100%
pTECH2-GP DMFL	LB	LB	1.4×10^9	
		LB + AMP	1.53×10^9	91.5%

Table 3.3. *In vitro* Stability of pTECH2 constructs expressing Ebola GP. Calculated to show stable retention of the pTECH2 plasmids expressing Ebola GP or the sub fragment DMFL in the absence of selective pressure from ampicillin. This work was carried out in collaboration with Bethany Gollan, a Masters student supervised by myself in the Khan laboratory.

3.5 *In vivo* stability of pTECH2-GP plasmids in *Salmonella* Typhimurium vaccine strain SL3261

After confirmation that the pTECH2-GP expression plasmids were stable without antibiotic selection *in vitro*, a pilot immunisation experiment was carried out to determine *in vivo* plasmid stability and to ensure that the *Salmonella* vaccine strain was still able to express the Ebola GP or Zika E proteins after passage *in vivo* when recovered from the livers and spleens of immunised mice. The work covered in 3.5.1 and 3.5.2 was very kindly carried out by Dr Omar Rossi, University of Cambridge.

3.5.1 Initial inoculation of mice with Salmonella vaccine strain SL3261 expressing TetC-GP fusion proteins

BALB/c mice were split into groups of 4 and immunised with *Salmonella* Typhimurium vaccine strains harbouring the pTECH2 expression plasmids. These are; pTECH2, the TetC only expressing control and pTECH2-GP and pTECH2-GP DMFL which express the Ebola GP and GP sub fragment DMFL. Each mouse was immunised intravenously with 5 X 10^5 CFU SL3261 in 0.2ml PBS.

Group	Immunisation	n BALB/c =
TETC	SL3261 pTECH2	4
TETC-GP	SL3261 pTECH2-synthetic EBOV GP	4
TETC-DMFL	SL3261 pTECH2-synthetic EBOV GP DMFL	4

Table 3.4. Mice used in GP plasmid stability inoculation experiment. Mice immunised in initial inoculation experiment to determine plasmid stability and TetC-GP fusion protein expression after *in vivo* passage.

3.5.2 Plasmid stability after in vivo passage in Salmonella Typhimurium vaccine strain SL3261

11 days post immunisation, the inoculated mice were sacrificed, and the livers and spleens analysed for colonisation by *Salmonella* Typhimurium SL3261 vaccine strain harbouring the pTECH2, pTECH2-GP and pTECH2-GP DMFL plasmids. These vaccine strain cells were recovered from the organs and plated on LB agar both with and without ampicillin and a count for SL3261 numbers in each organ was calculated. The percentage difference in these totals between those grown in the presence of ampicillin and those without, allowed a % value for plasmid stability (cells which retained the plasmid without ampicillin selection pressure) was calculated. Mice were immunised with pTECH2 only as a control and these results show that after *in vivo* passage, the cells harbouring this plasmid were able to stably retain it despite the absence of ampicillin as expected. It seems that the pTECH2-GP plasmid is somewhat less stable as a smaller percentage of cells recovered from the livers and spleens were able to retain the plasmid with no antibiotic selection. The pTECH2-GP DMFL plasmid on the other hand, appears almost as stable as the pTECH2 control plasmid (figure 3.34)

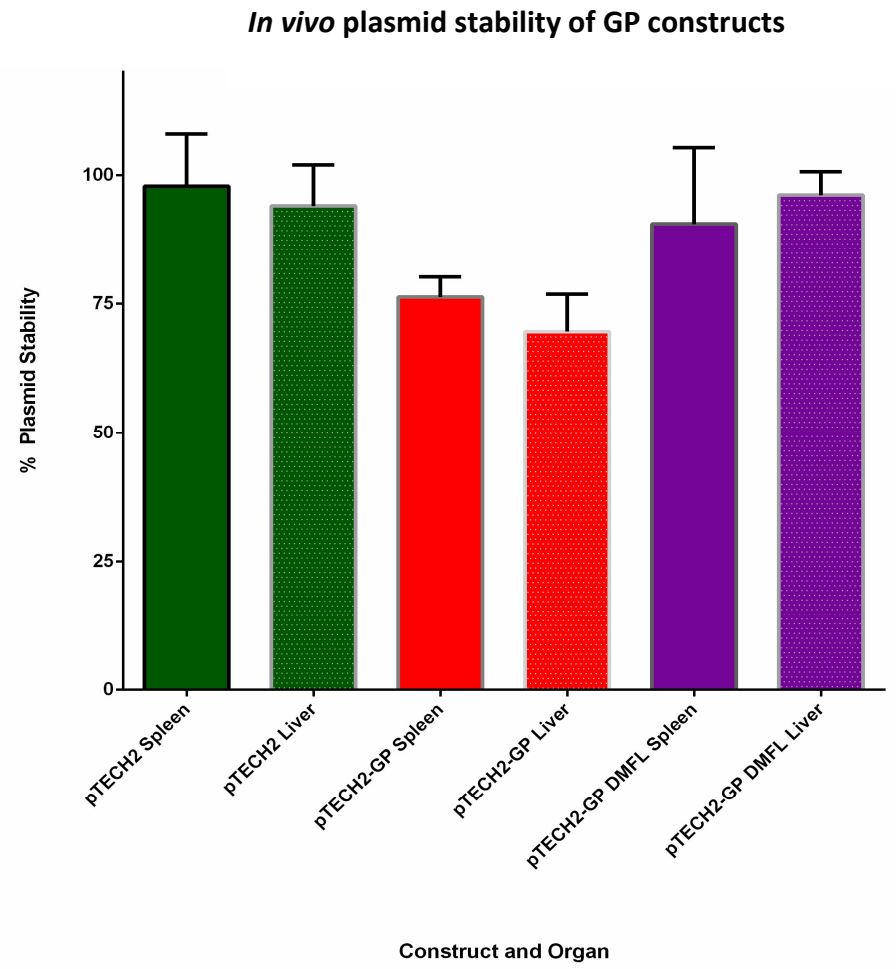


Figure 3.34. Plasmid stability of recombinant Ebola GP constructs *in vivo*.
Graph to show plasmid stability % of pTECH2 constructs expressing GP or the GP DMFL sub fragment after *in vivo* passage and recovery from the livers and spleens of inoculated mice. Results are presented as the mean percentage of all mice in each group plus SD.

3.5.3 Expression of TetC- GP fusion proteins after in vivo passage

Following confirmation that the plasmids are retained stably in SL3261 cells when recovered from inoculated mice livers and spleens after *in vitro* passage, it was then necessary to determine whether these cells were still able to express the TetC or the TetC-GP fusion proteins. Cells recovered from these organs were plated onto LB agar with ampicillin and a loop of colonies of each were picked for transport to Newcastle as agar stabs by Dr Omar Rossi, Cambridge University. Upon arrival these samples were streaked out onto agar plates containing ampicillin and after growth overnight, a sample of these cells were taken for processing for SDS-PAGE and western blot (figure 3.35).

Cells recovered from the livers and spleens were all able to express either TetC (SL3261 harbouring pTECH2) or the TetC-GP or TetC-GP DMFL (pTECH2-GP or pTECH2-GP DMFL) fusion proteins and showed an equivalent pattern of bands as SL3261 cells from stock agar plates harbouring the same plasmids, which had not been subject to *in vivo* passage.

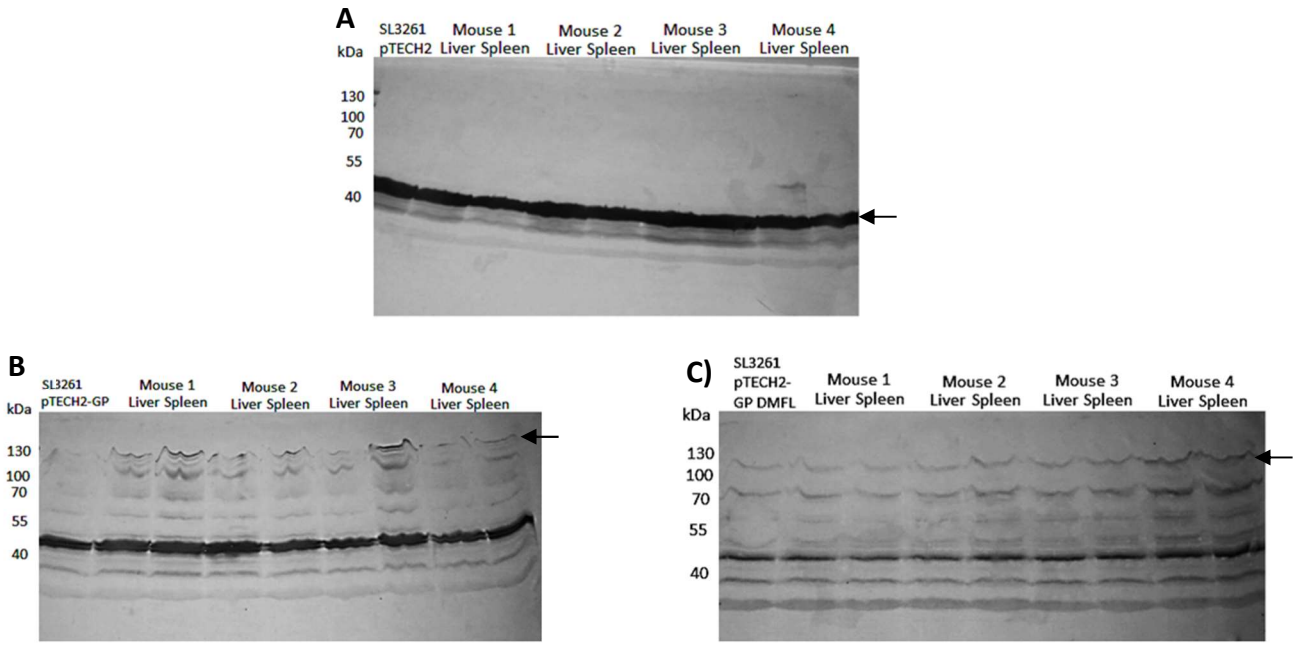


Figure 3.35. Expression of TetC-GP fusion proteins after *in vivo* passage.
Western blot showing expression of TetC and TetC-Ebola GP fusion proteins from *Salmonella* Typhimurium SL3261 cell lysates after recovery from immunised mouse livers and spleens. Cell lysates were fractionated on SDS-PAGE before transfer to nitrocellulose. Blots were probed with Blots were probed with polyclonal rabbit anti-TetC serum (1:1000) and Goat anti-rabbit HRPO (1:3000). The TetC protein is clearly seen at approximately 50 kDa (A) and the TetC-GP (B) and TetC-GP DMFL (C) fusion proteins at 121kDa and 97kDa respectively, as shown in line with arrows. Cells from a stock plate which had not been subject to *in vivo* passage were used as a positive control (shown in the first lane of each blot).

3.6 Immune responses to *Salmonella* based Ebola Virus vaccine

Stability of the pTECH2 expression plasmids both *in vitro* and *in vivo* was confirmed and expression of the TetC-GP fusion proteins has been shown in SL3261 vaccine strains when recovered from livers and spleens of immunised mice 11 days post inoculation. It could then be determined whether these vaccine strains expressing the TetC-GP fusion proteins would elicit a protective immune response in mice.

3.6.1 Inoculation of mice with Salmonella vaccine strain SL3261 expressing TetC-GP fusion proteins

As with the initial pilot immunisation to determine *in vivo* plasmid stability, mice were once again inoculated by intravenous injection with the SL3261 *Salmonella* Typhimurium vaccine strain expressing TetC or the TetC-GP fusion proteins.

Group	Immunisation	n BALB/c =
TETC	SL3261 pTECH2 6.41 log10 CFU in 0.2ml PBS	6
TetC-GP	SL3261 pTECH2-GP 6.32 log10 CFU in 0.2ml PBS	6*
TetC-GP DMFL	SL3261 pTECH2-GP DMFL 6.24 log10 CFU in 0.2ml PBS	6
NEG	Not immunised	4

Table 3.5. Mice used in GP immunisation experiment. Inoculation experiment to determine immune response to TetC-GP fusion proteins. 6 mice were immunised with the SL3261 vaccine strain expressing either TetC or the TetC-GP fusion proteins. 4 mice were not immunised so as to allow a baseline reading for normal mouse serum. * One mouse died before the conclusion of the experiment, due to a UTI, not related to immunisation.

8 weeks post immunisation, the mice were sacrificed, and blood was collected from each mouse. This was allowed to coagulate before centrifugation to allow collection of serum only. Sera was kindly collected and processed by staff at Cambridge University and sent to Newcastle to be tested for immune response.

Once received, sera was aliquoted and stored at -80 °C prior to use in ELISA assays.

3.6.2 IgG antibody response to Ebola GP

Firstly, the IgG response of immunised mouse serum to Ebola GP was determined by ELISA. ELISA plates were coated with 2µg/ml recombinant Ebola GP kindly provided by PHE, Oxford. Sera was diluted 1:25 and the IgG response was measured at 450nm with Rabbit anti-mouse HRPO (Abcam) as the secondary antibody. The ELISA was developed with TMB and stopped with H2SO4 prior to reading. Disappointingly, it does not seem from these results (see figure 3.36) that there is an apparent IgG antibody response to Ebola GP above the background controls. There is perhaps one outlier in the TetC-DMFL group with a higher reading than all others, but in general, the response is still quite low.

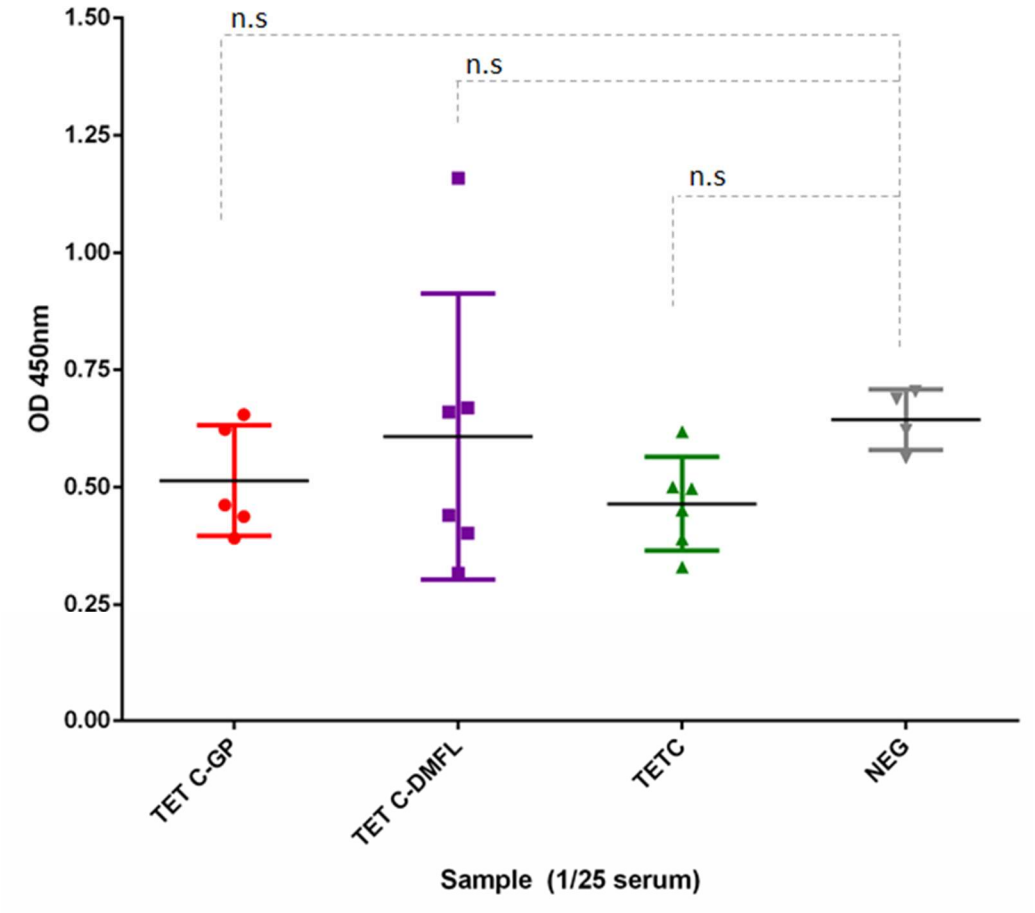


Figure 3.36. IgG antibody response to Ebola GP in GP immunised mice.
Graph showing IgG response in immunised mouse sera as measured by ELISA with plates coated with 2µg/ml recombinant Ebola GP. Readings are presented as an average of triplicate wells + SD. Statistical significances compared to normal mouse sera (NEG) were analysed by unpaired t test. P values <0.05 were considered significant. Here, none of the means of any of the immunised groups (TETC-GP, TETC-DMFL and TETC) were significantly different from normal mouse sera (ns = not significant).

3.6.3 IgG antibody response to TetC

It was then determined that the IgG response to TetC should be determined as a control for IgG response in general of all immunised mice. As each of the fusion proteins contain TetC, and this is a potent immunogen, it would be assumed that all mice immunised would elicit antibodies towards TetC. ELISA plates were coated with 1µg/ml TetC (kindly provided by Professor Neil Fairweather, Imperial College London), sera once again diluted to 1:25 and Rabbit anti-mouse HRPO used as a secondary antibody. Here (see figure 3.37) it is obvious that the mice immunised with SL3261 expressing TetC only have a clear IgG response to the TetC protein and no response is seen from the non-immunised mice (NEG) as expected. Interestingly, there is a much lower response seen in mice immunised with SL3261 expressing the TetC-GP or TetC-DMFL fusion proteins. It would be expected that a strong IgG response to TetC would also be seen in these mice as it is present in the fusion proteins. This reduced response to TetC in these mice could be indicative of an overall dampened immune response, and this may also indicate why there was no IgG response to Ebola GP.

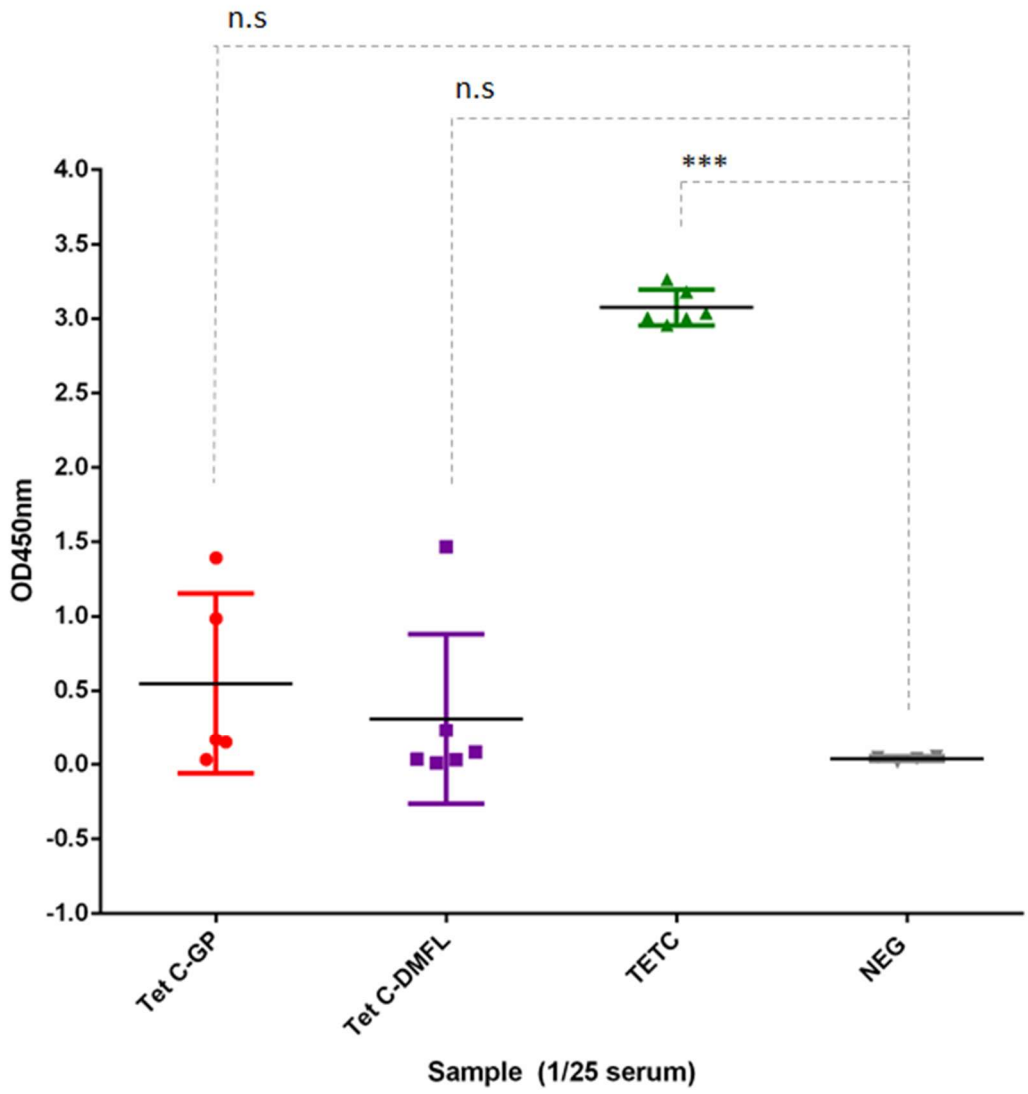


Figure 3.37. IgG antibody response to TetC in GP immunised mice.
Graph showing IgG response in immunised mouse sera as measured by ELISA with plates coated with 1/ml TetC protein. Readings are presented as an average of triplicate wells + SD. Statistical significances compared to normal mouse sera (NEG) were analysed by unpaired t test. P values <0.05 were considered significant. Here, neither of the means of the groups immunised with SL3261 cells expressing the TetC-GP fusion proteins (TETC-GP, TETC-DMFL) were significantly different from normal mouse sera (ns = not significant). Mice immunised with SL3261 cells expressing TetC only however showed a significant IgG response to TetC compared with non-immunised mice (P = <0.0001 - ***).

3.6.4 IgG antibody response to Salmonella Typhimurium LPS

It appeared that there was a dampened IgG response to TetC from the mice immunised with SL3261 cells expressing the TetC-GP fusion proteins. This was unexpected as the mice immunised with the TetC only expressing cells showed a strong IgG response to TetC. It was postulated that this may be due to a potential immunosuppressive effect of the vaccines (Lee et al, 1985). To determine if this was the case, the IgG response to *Salmonella* Typhimurium LPS was measured again using ELISA (figure 3.38). *Salmonella* Typhimurium LPS (Sigma) was coated onto ELISA plates at 5μg/ml and pooled sera from each of the groups was titrated from a 1:50 to a 1:6400 dilution. The secondary antibody was once again Rabbit anti-mouse HRPO and the assay was developed as before with TMB and H2SO4. It appears that from these results that there is not a clear difference between the anti LPS IgG titre in mice immunised with SL3261 cells expressing only TetC compared to those immunised with SL3261 expressing the fusion proteins. Compared to the non-immunised mice, which as expected did not show an IgG response to LPS as expected, this suggests that the overall immune response is not suppressed and therefore there may be alternative reasons as to why the IgG response to Ebola GP was so low.

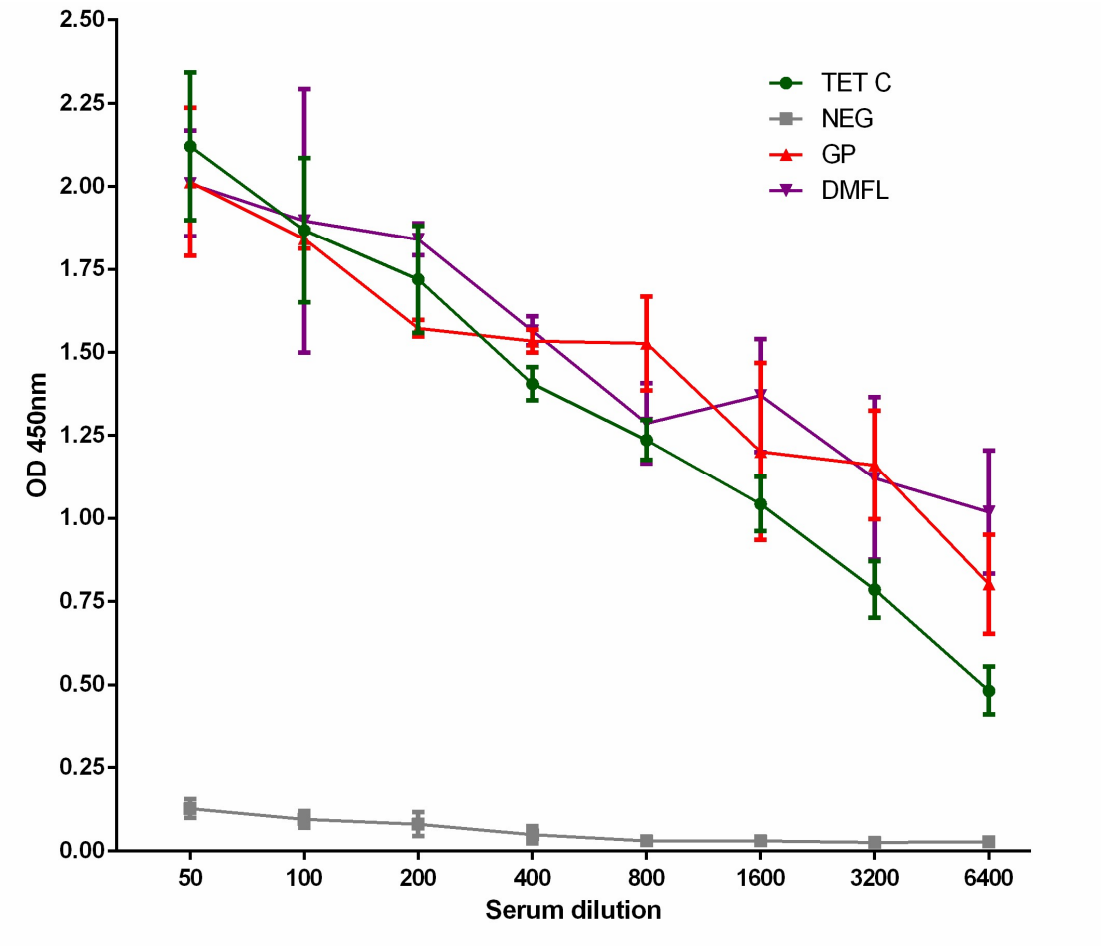


Figure 3.38. IgG antibody response to *Salmonella* Typhimurium LPS in GP immunised mice. Graph showing titration of IgG response against *Salmonella* Typhimurium LPS, from 1:50 to 1:6400 dilution, of pooled sera from mice immunised with *Salmonella* Typhimurium SL3261 vaccine strain expressing TetC or TetC-GP fusion proteins. Non- immunised mice (NEG) were used as a control. Readings are presented as an average of triplicate wells + SD. Statistical significances compared to TetC immunised sera (TETC) were analysed by unpaired t test. P values <0.05 were considered significant and at all dilutions, none were significantly different.

3.7 Discussion

A panel of constructs were generated to allow expression of the Ebola Glycoprotein by attenuated *Salmonella* vaccine strains. From this panel, constructs which had shown to be expressed best were selected for use in *in vivo* immunisation experiments to determine immune response in mice to Ebola GP. The constructs expressing the fusion proteins of the C-fragment of tetanus toxin (TetC) and Ebola GP were based on the pTECH2 expression plasmid.

TetC-GP (the full-length GP protein) and TetC-GP DMFL (containing known epitopes for protective antibodies) were deemed to be qualitatively expressed best, using western blotting, in SL3261, an *aroA* mutant of *Salmonella* Typhimurium. Attempts were also made to express the Ebola GP protein without the addition of the TetC fusion partner (figure 3.20). This was more difficult than expected and no GP expression was seen in *Salmonella* Typhimurium harbouring the pTECH10-GP expression vector, which did not contain any of the TetC sequence. Expression of GP was rescued by the addition of the first 30 bases (10 residues) of the Tet C sequence upstream of the GP gene (figure 3.23), possibly due to an increase in the stability of the mRNA and 16S ribosomal RNA interaction, which has potentially been improved with increasing complementarity to a sequence in the 16S ribosomal RNA downstream of the Shine-Dalgarno recognising sequence. By creating a large panel of variant constructs, the differences in expression between each construct could be assessed and the ones allowing the best levels of GP expression then brought forward for further testing.

The pTECH2-GP and pTECH2-GP DMFL plasmids were shown to be stably retained in the absence of selective ampicillin both *in vitro and* following *in vivo* passage in mice (figures 3.34 and 3.35). Recovery of strains from livers and spleens, showed that the strains were still expressing the TetC-GP fusion proteins (figure 3.35). This allowed an immunisation experiment to determine mouse immune responses to Ebola GP to be carried out. Mice were immunised with intravenous SL3261 harbouring the pTECH2, pTECH2-GP and pTECH2-GP DMFL plasmids and after 8 weeks, sera analysed by ELISA to determine IgG responses (figures 3.36, 3.37, 3.38). Unfortunately, there was no significant IgG response to Ebola GP from either of the groups immunised with the *Salmonella* expressing the TetC-GP fusion proteins. In addition, the mice immunised with *Salmonella* harbouring the pTECH2 only plasmid, as

expected, had a strong IgG response to TetC, however the mice immunised with the vaccine strains expressing the TetC-GP fusion proteins elicited a lower response to TetC. It was thought that potentially, this may have been due to an immunomodulatory effect which has been reported with attenuated *Salmonella* vaccine strains, potentially resulting in an overall lower IgG response (Lee et al, 1985). To test this theory, ELISA assays were carried out to determine the IgG responses from the immunised mice against *Salmonella* Typhimurium LPS. There was not a significant difference in the responses seen in mice immunised with SL3261-TetC control and the other SL3261-TetC-GP fusion experimental groups. This suggests that the overall immune response from the mice was not lowered in the TetC-GP fusion experimental groups.

This lack of response could be due to a variety of reasons, including *in vivo* expression levels of the fusion proteins. This is discussed in more detail in chapter 6, section 6.4.

Chapter 4 - Expression of Zika Envelope in *Salmonella* and evaluation of immune response in vaccinated hosts

The Zika virus, carried by *Aedes* species mosquitoes, has recently seen rapid spread throughout the Americas. It has also been determined to be the cause of a large increase in incidences of foetal microcephaly and central nervous system issues when the pregnant mother is infected. In adults, infection with Zika Virus has been connected with Guillain-Barré Syndrome (GBS), which can cause neuropathy and paralysis. These alarming complications resulting from an infection that otherwise causes relatively mild symptoms, has highlighted the need for a safe and effective vaccine against Zika Virus, to reduce the impact it can have on vulnerable populations. The envelope glycoprotein (ZE) is situated on the virus surface and has epitopes that are able to elicit cellular and humoral immune responses and is therefore an attractive target for use in a vaccine. Using attenuated *Salmonella* vaccine strains to express and deliver the ZE protein to the host immune system, could elicit protective antibodies and be a cost effective easy to administer vaccine hopefully preventing such devastating complications from this rapidly spreading virus in the future.

4.1 Generating a panel of Zika envelope expression constructs

The Zika Virus envelope protein (ZE) is the major protein situated on the viral surface and has been shown elicit protective neutralising antibodies (Dai et al, 2016). This makes it an attractive target for use in a vaccine. It is hoped that using attenuated *Salmonella* Typhimurium to express ZE may elicit a protective immune response to Zika infection. Zika E genes were first amplified by PCR in the same way as with Ebola GP in chapter 3, and subsequently cloned using restriction enzymes and T4 ligation.

4.11 Synthetic codon optimised and wild type Zika envelope genes

A Zika envelope gene was codon optimised for expression in *Salmonella* Typhimurium. The gene sequence was generated using Eurofins Genomics' proprietary software 'GENEius', utilising codons most frequently used by *Salmonella* Typhimurium and avoiding unwanted motifs. It was hoped that this would allow optimum expression of the Zika envelope protein. The synthetic gene sequence was then sent to DC Biosciences (Dundee, UK) for synthesis.

With this synthetic Zika E gene sequence which has been codon optimised for expression in *Salmonella* Typhimurium we can compare its expression and immunogenicity, with a wild type (WT) (non-optimised) ZE, which was kindly provided by Dr Claire Donald and Professor Alain Kohl, Glasgow University.

The gene sequence used (both WT and synthetic) was from the Zika Virus Isolate KX197192, strain PE243 (Donald et al, 2016).

4.1.2 PCR amplification of Zika envelope and sub-fragments

Both the synthetic, codon optimised and wild type Zika Envelope genes were PCR amplified with primers specific to the full ZE or a truncated version consisting of the first 193 amino acids. These amplified gene products were then digested for subsequent restriction cloning into the pTECH2 expression vector (figure 4.1). A panel of constructs was created to enable evaluation of which expressed the ZE protein best and would then be taken forward into *in vivo* testing (figure 4.2).

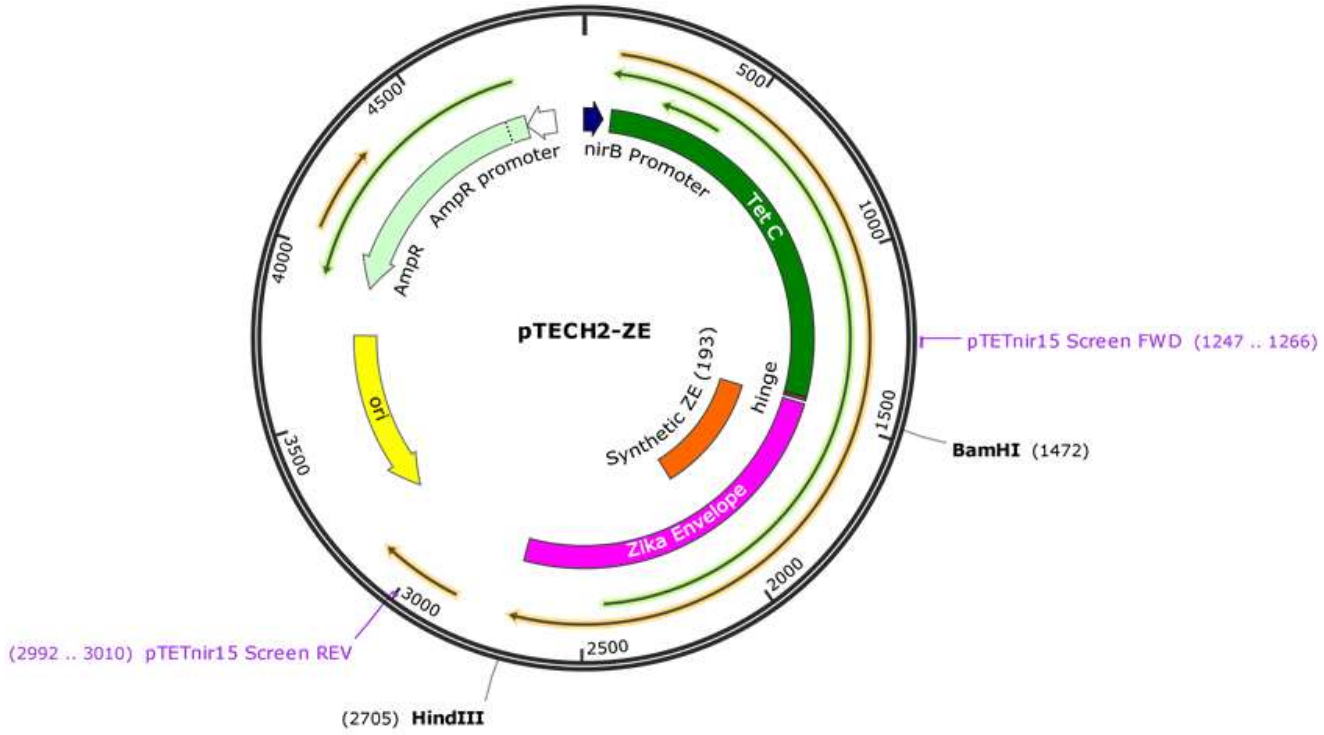


Figure 4.1. Plasmid map of the pTECH2-ZE expression vector. Under the control of the *nirB* promoter, a TetC-GP fusion protein is expressed. The GP gene was cloned in using restriction enzymes *BamHI* and *HindIII*. Colony PCR was carried out on *Salmonella* Typhimurium SL5338 colonies after transformation using the pTETnir15 screening primers as shown to confirm putative clones containing the plasmid prior to expression analysis. The full-length ZE shown in pink represents either the codon optimised synthetic gene (ZEs) or the WT gene sequence (ZE WT). The location of the smaller synthetic gene fragment (ZE 193) is shown and was cloned in place of the full ZE gene to allow expression a shorter TetC-ZE fusion. Plasmid map was made using the commercial software Snapgene.

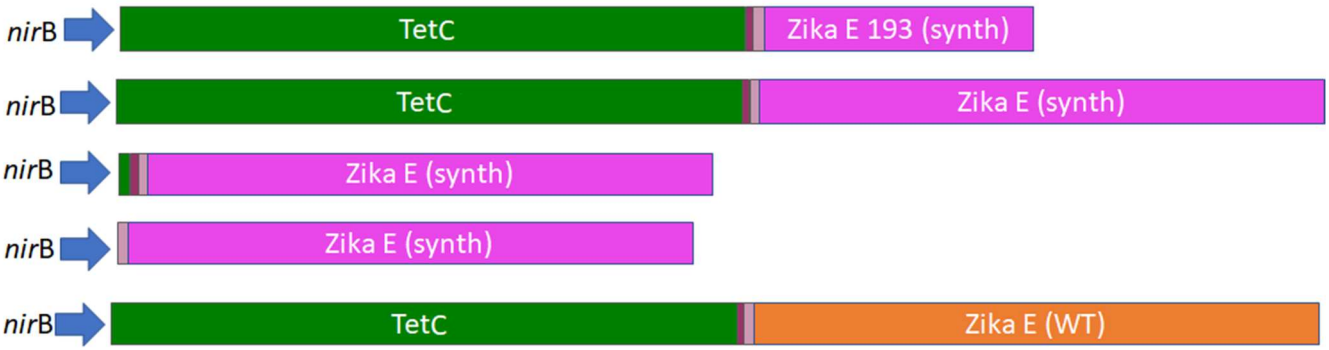


Figure 4.2. TetC-Zika envelope fusion protein constructs.
Zika envelope Fusion protein constructs. Schematic presentation of the panel of Zika envelope fusion protein constructs generated in this study. Under the control of the *nirB* promoter, the full-length Zika envelope was expressed either as a fusion to the C-fragment of Tetanus Toxin (TetC), alone, or as a fusion to the full TetC ribosome binding site (RBS) in the pTECH2 expression vector in *Salmonella* Typhimurium. The TetC and Zika E fragments are separated by a flexible hinge, Gly Pro Gly Pro motif, which allows temporal and spatial separation between the two components to allow time for correct folding. A short fragment of the synthetic Zika E gene (the first 193 residues) was also expressed as a fusion to TetC to determine if length may have an effect on expression levels. The wild type ZE sequence (Zika E WT) was also incorporated into a pTECH2 construct to determine the difference in expression between this sequence and the synthetic sequence codon optimised for expression in *Salmonella* Typhimurium (Zika E synth).

First, each of the Zika envelope genes (Synthetic optimised – ZE(s) or Wild type – ZE(WT)) and the truncated version of the synthetic ZE (the first 193 aa – ZE(s193)) were amplified by PCR, with the cloning restriction enzyme sites included on each end (*BamHI* and *HindIII*). Each PCR product was the expected size; ZE(s) 1242bp, ZE(s193) 579bp and ZE(WT) 1218bp (figure 4.3). In the case of ZE(WT), a smaller band (under 250bp) was seen when the PCR product was visualised on an agarose gel, potentially due to the presence of primer dimers. This was easily rectified by the use of the QIAquick ® Gel Extraction kit (Qiagen), where the correct band (1218bp) was excised and purified prior to further processing to prevent contamination with the smaller unwanted piece of amplified DNA.

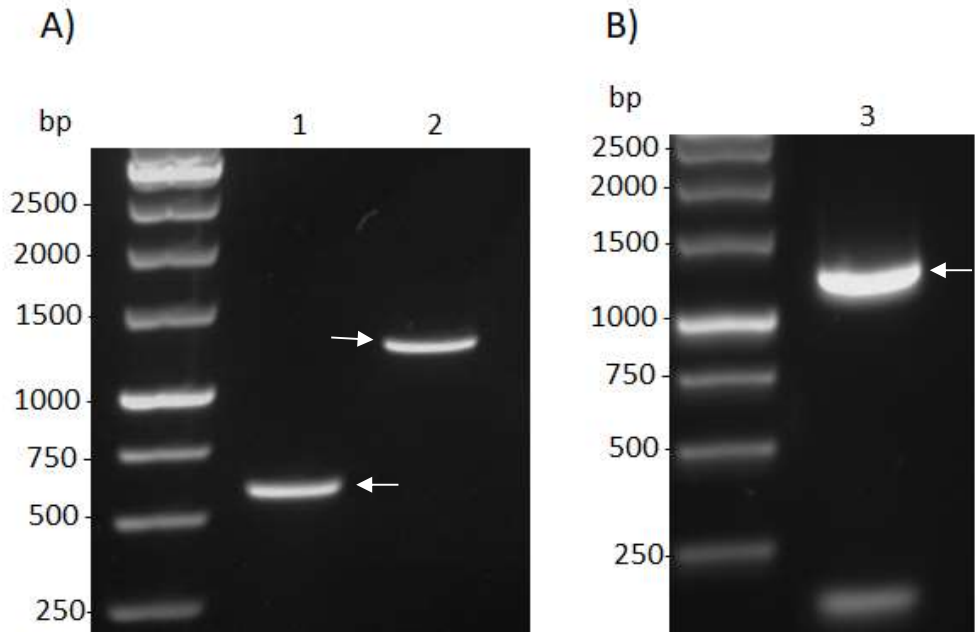


Figure 4.3. PCR amplification of Zika envelope.
0.7% agarose gel showing PCR products of the Zika envelope gene when amplified with corresponding primers containing restriction cloning sites for *BamHI* and *HindIII*. Lane 1 is the truncated synthetic Zika E (193), consisting of the first 193aa of the envelope protein at 579bp. Lane 2 is the full-length synthetic, codon optimised Zika E gene at 1242bp and lane 3 is the WT Zika E gene (non-codon optimised) at 1218bp, all denoted by arrows. In lane 3, an extra smaller band is also seen possibly due to the presence of primer dimers, in this case, the top correct band (1218bp, denoted by arrow) was excised and purified using the Qiagen Gel purification kit.

These PCR products were subsequently digested with *BamHI* and *HindIII*, spin column purified, and cloned into digested pTECH2 plasmid.

4.1.3 Generation of pTECH2-ZE constructs

As mentioned previously, guest antigens can be cloned into the multiple cloning site of pTECH2. This allows the expression of a protein consisting of the C-fragment of Tetanus Toxin (TetC) and the guest antigen as a genetic fusion, under the control of the *nirB* promoter. Here, the PCR products for the Zika envelope constructs were digested with restriction enzymes *BamHI* and *HindIII* and cloned into the digested pTECH2 vector using T4 ligase. The ligation mix from this reaction was then transformed into chemically competent *Salmonella* Typhimurium SL5338 cells. Colonies were screened using pTETnir15 screening primers to determine putative clones containing one of the pTECH2-ZE plasmids. The screening primers amplify a 543bp region on the pTECH2 plasmid which includes the multiple cloning site (See figure 4.1). When the correct gene is inserted into the cloning site, the resultant PCR product from a colony PCR is the insert plus 543bp in total. Negative colonies that do not include the ZE insert give a product of only 543bp. Plasmid DNA from positive clones from this screen was then purified and digested with the cloning enzymes to visualise the insert band and further confirm successful cloning (figure 4.4)

4.1.3.1 Construction of pTECH2-Full synthetic ZE expression plasmid

The 1242bp synthetic Zika envelope PCR product was cloned into the multiple cloning site of the pTECH2 vector using restriction enzymes *BamHI* and *HindIII* and ligation with T4 ligase. Colony PCR using vector screening primers showing a band at 1785bp and informative digests of the resultant plasmid after transformation into SL5338 cells confirmed successful cloning (figure 4.4).

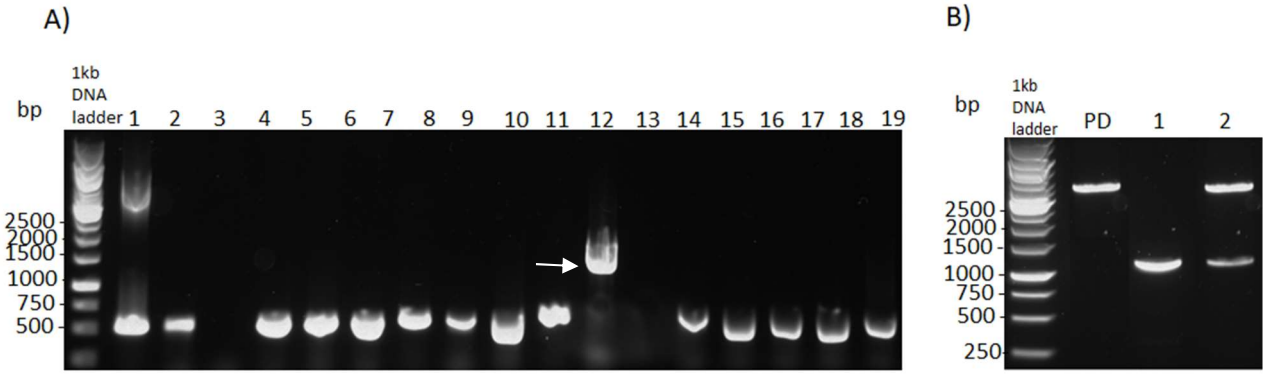


Figure 4.4. Colony PCR Colony PCR and restriction digest screens of putative pTECH2-ZE (s) clones. A) 0.7% agarose gel showing PCR products from colony PCR using pTETnir15 screening primers to determine successful construction and transformation of the pTECH2 vector containing the 1242bp Synthetic ZE gene into SL5338 *Salmonella* strain. Following transformation, colonies 1-19 (as shown on gel) were boiled before PCR amplification of the plasmid between each screening primer. Positive colonies which have successfully taken up the plasmid pTECH2-ZE(s) (notably colony 12 – denoted by arrow) show a band at 1785bp. Colonies which have only taken up pTECH2 show a band at approximately 543bp.

B) 0.7% agarose gel showing purified plasmid from colony 12 (see figure 4.4 A) digested with *BamHI* and *HindIII*, showing an insert band of 1242bp (lane 2) which corresponds to the size of the PCR product for the Zika envelope gene (lane 1). PD is plasmid pTECH2 digested with the same restriction enzymes as a negative control. This putative plasmid from colony 12 was then sent for sequencing using the same screening primers as above This construct was made in collaboration with Bethany Hunter, a Masters student supervised by myself in the Khan laboratory.

4.1.3.2 Construction of pTECH2-Truncated (193aa) Synthetic ZE (pTECH2-ZE193) expression plasmid

The 579bp truncated synthetic Zika envelope (193) PCR product was cloned into the multiple cloning site of the pTECH2 vector using restriction enzymes *BamHI* and *HindIII* and ligation with T4 ligase. Colony PCR using vector screening primers, showing a band at 1122bp, and informative digests of the resultant plasmid after transformation into SL5338 cells confirmed successful cloning (figure 4.5)

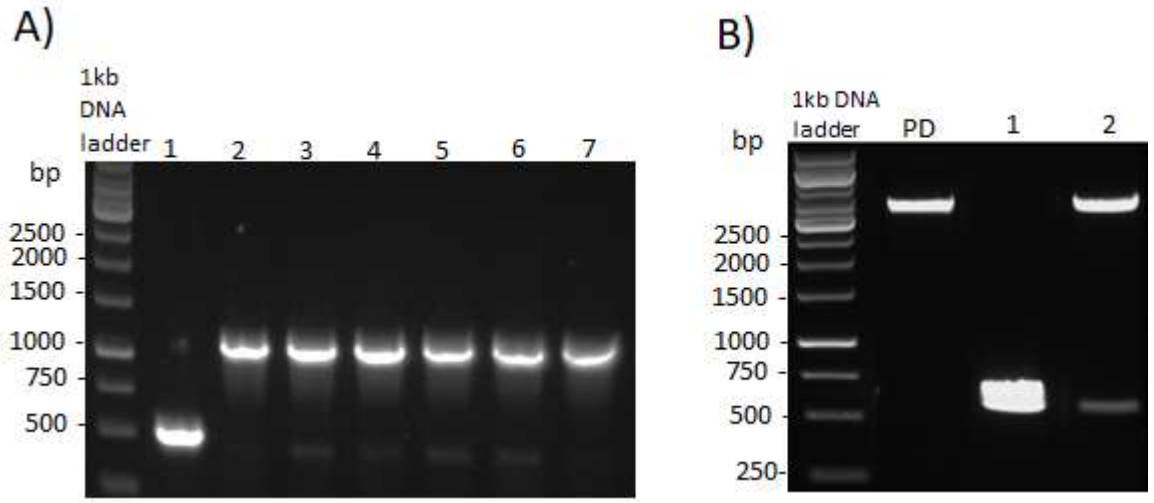


Figure 4.5. Colony PCR and restriction digest screens of putative pTECH2-ZE (s193) clones. A) 0.7% agarose gel showing PCR products from colony PCR using pTETnir15 screening primers to determine successful transformation of the pTECH2 vector containing the 579bp Synthetic ZE (193) gene fragment into SL5338 *Salmonella* strain. Following transformation, colonies 1-7 (as shown on gel) were boiled before PCR amplification of the plasmid between each screening primer. Positive colonies which have successfully taken up the plasmid pTECH2-ZE(193)show a band at 1122bp (denoted by arrow). Colonies which have only taken up pTECH2 show a band at approximately 543bp, as seen in lane 1.

B) 0.7% agarose gel showing purified plasmid from colony 2 (see figure 4.5 A) digested with *BamHI* and *HindIII*, showing an insert band of 579bp (lane 2) which corresponds to the size of the PCR product for the truncated Zika envelope gene (lane 1). PD is plasmid pTECH2 digested with the same restriction enzymes as a negative control. This putative plasmid from colony 2 was then sent for sequencing using the same screening primers as in figure 4.4 A. This construct was made in collaboration with Bethany Hunter, a Masters student supervised by myself in the Khan laboratory.

4.1.3.3 Construction of pTECH2-Full Wild type ZE (pTECH2-ZE WT) expression plasmid

The 1218bp Wild Type Zika envelope PCR product was cloned into the multiple cloning site of the pTECH2 vector using restriction enzymes *BamHI* and *HindIII* and ligation with T4 ligase. Colony PCR using vector screening primers, showing a band at 1761bp, and informative digests of the resultant plasmid after transformation into SL5338 cells confirmed successful cloning (figure 4.6)

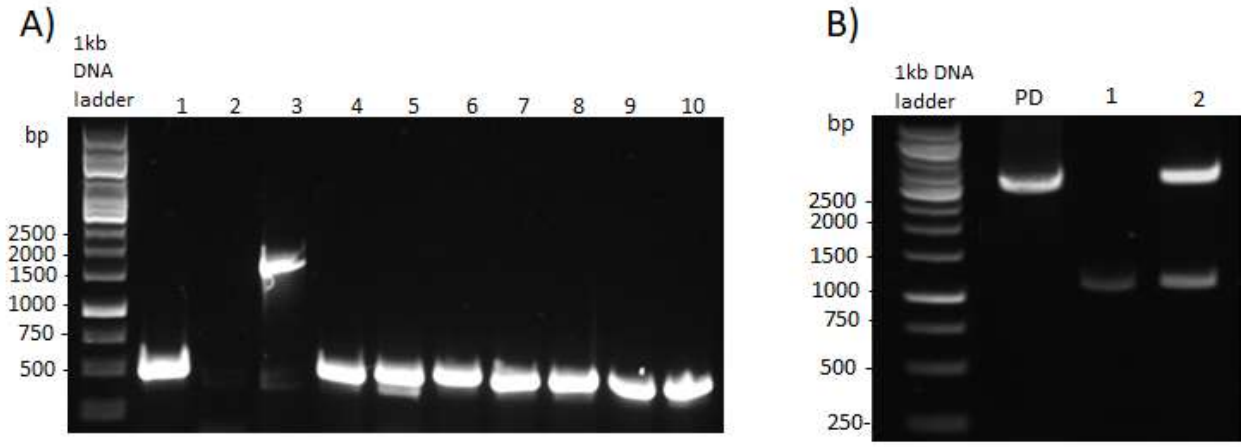


Figure 4.6. Colony PCR and restriction digest screens of putative pTECH2-ZE (WT) clones. A) 0.7% agarose gel showing PCR products from colony PCR using pTETnir15 screening primers to determine successful construction and transformation of the pTECH2 vector containing the 1218bp Wild Type Zika E (ZE WT) gene into SL5338 *Salmonella* strain. Following transformation, colonies 1-10 (as shown on gel) were boiled before PCR amplification of the plasmid between each screening primer. Positive colonies (see lane 3) which have successfully taken up the plasmid pTECH2-ZE(WT) show a band at 1761bp. Colonies which have only taken up pTECH2 show a band at approximately 543bp.

B) 0.7% agarose gel showing purified plasmid from colony 3 (see figure 4.6 A) digested with *BamHI* and *HindIII*, showing an insert band of 579bp (lane 2) which corresponds to the size of the PCR product for the truncated Zika envelope gene (lane 1). PD is plasmid pTECH2 digested with the same restriction enzymes as a negative control. This putative plasmid from colony 3 was then sent for sequencing using the same screening primers as above. This construct was made in collaboration with Bethany Hunter, a Masters student supervised by myself in the Khan laboratory.

4.2 Expression of TetC-ZE fusion proteins in *Salmonella* intermediate strain SL5338

Using colony PCR and informative digest of putative clones, the pTECH2-ZE constructs had been confirmed (pTECH2-ZE(s), pTECH2-ZE(s193), pTECH2-ZE(WT)). These were then transformed into *Salmonella* Typhimurium SL5338 cells and checked for expression of the Zika envelope protein (or truncated fragment 193) in fusion with TetC (figure 4.7). To ensure that each sample on the SDS-PAGE gel prepared for western blot contained an equivalent number of cells, the dilutions of cell lysates were corrected for cell density (OD_{600}) to ensure equal cell numbers. For the relevant calculations, please refer to Chapter 2, section 2.2.9.1. Following confirmation of expression in strain SL5338, the plasmids were then electroporated into *Salmonella* Typhimurium vaccine strains to ensure continued expression and to use in *in vivo* experiments to determine immune response to ZE.

4.2.1 Expression of TetC-ZE fusion proteins in Salmonella intermediate strain SL5338

The TetC – Zika E fusion proteins can be detected on western blot by both anti TetC polyclonal sera and monoclonal anti-Zika E antibodies. The TetC protein is approximately 52kDa and both the synthetic and wild type Zika envelope proteins are approximately 45kDa, resulting in a 97kDa fusion protein expressed (including the hinge region and rest of the poly-linker site). The truncated ZE(s193) is approximately 21kDa, resulting in a 74kDa fusion protein (also including the hinge and poly-linker). Again, a western blot probed with polyclonal anti-TetC mouse sera, show that these constructs clearly express the protein at expected size in SL5338, and although some breakdown products are visible, the fusion proteins (97kDa, 97kDa and 74kDa respectively) are clearly seen at the expected size (see figure 4.7 A). When the same samples are probed with a monoclonal anti Zika envelope antibody, the same bands at 97kDa are extremely clear, and some breakdown products are also visible, suggesting that the epitope (currently unknown) is still present in these shorter products and may still elicit an immune response (see figure 4.7 B). It appears that the truncated synthetic ZE protein does not contain the epitope for this monoclonal antibody as no bands are visible in this sample (figure 4.7 B lane 3). The epitope is therefore situated after the 193rd amino acid of the ZE protein.

In both of these blots, when cells harbouring the pTECH2-ZE plasmids (both synthetic and wild type) it appears that a stronger band is seen in the samples which contain the plasmid with the synthetic codon optimised Zika envelope gene. This is probably to be expected as the codons used for this construct are more favourably used by *Salmonella* Typhimurium.

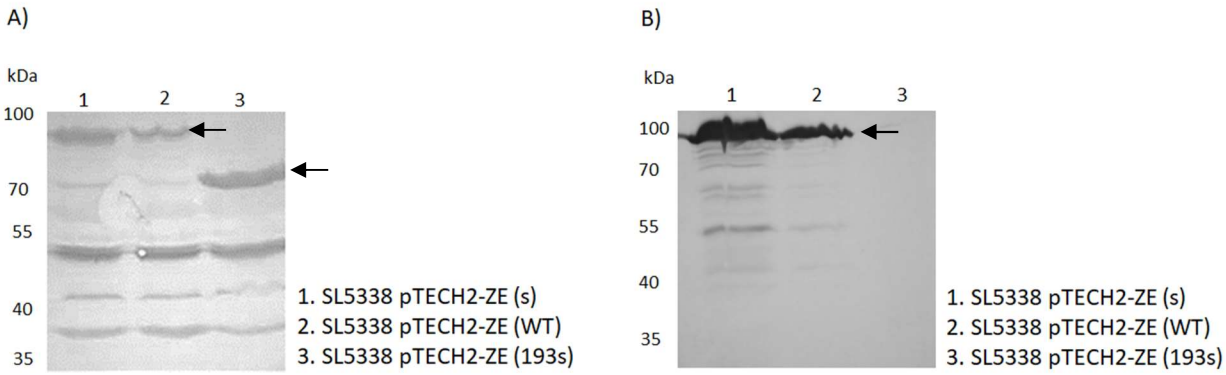


Figure 4.7. Expression of TetC-GP fusion proteins.
Western blot showing expression of TetC-ZE fusion proteins (as denoted by arrows) in cell lysates of *Salmonella* Typhimurium SL5338 probed with polyclonal rabbit anti TetC serum (1:1000) followed by Goat anti rabbit HRPO (1:3000) (A) and mouse monoclonal anti ZE (1:10000) followed by rabbit anti-mouse HPRO (1:4000) (B). Lane 1 shows the TetC-ZE (s) fusion at approximately 97kDa, lane 2 the TetC-ZE (WT) fusion also at approximately 97kDa and lane 3 the TetC-ZE(s193) fusion at approximately 74kDa. It appears that while the TetC-ZE(s193) fusion is visible when cells containing this plasmid are probed with anti-TetC, the epitope for this particular monoclonal anti Zika E antibody used in (B) is not present, suggesting the epitope for this antibody is more towards the C terminal of the envelope protein (or at least after the 193rd amino acid). This construct was made in collaboration with Bethany Hunter, a Masters student supervised by myself in the Khan laboratory.

4.2.2 Expression of TetC-ZE fusion proteins in Salmonella vaccine strains

After expression of TetC- Zika envelope fusion proteins was confirmed in *Salmonella* Typhimurium strain SL5338, the relevant expression plasmids then had to be transformed into an attenuated strain so as to allow for *in vivo* experiments. The constructs were electroporated into two vaccine strains: SL326 and C5*htrA* to determine which would be used *in vivo* (see chapter 2, section 2.1.7, table 2.5 for strain details). Cell lysates from each of these strains harbouring either pTECH2-ZE(s) or pTECH2-ZE(WT) were evaluated by western blot to determine which background expressed the fusions best)figure 4.8). Again, ensuring that equivalent cell numbers were included in each sample, it appears that the SL3261 strain expressed the fusion slightly better than C5*htrA*.

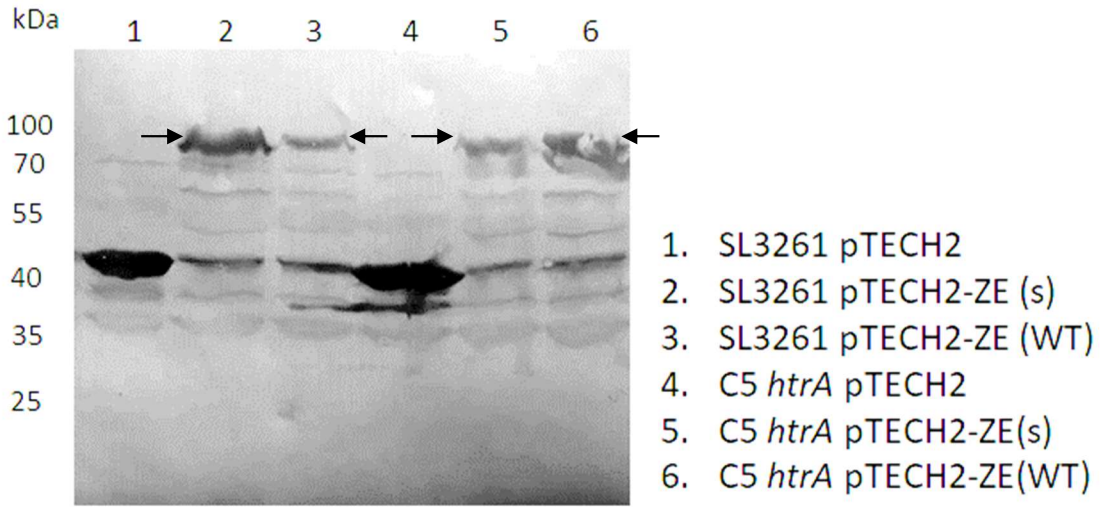


Figure 4.8. Expression of TetC-GP fusion proteins in *Salmonella* vaccine strains.
Western blot showing TetC and TetC-ZE fusion proteins (as denoted by arrows at approximately 97kDa) expressed in either SL3261 (lanes 1-3) or C5*htrA* (lanes 4-6) *Salmonella* Typhimurium vaccine strains. TetC is also clearly shown expressed by both strains, with a band at approximately 50kDa (lanes 1 and 4). Cell lysates were fractionated on SDS-PAGE which was then transferred to nitrocellulose. The blot was probed with polyclonal rabbit anti-TetC serum (1:1000) followed by Goat anti-Rabbit HRPO (1:3000). It appears that strain SL3261 expresses the fusion proteins slightly better (in particular when the synthetic codon optimised gene sequence is used – lane 2) due to the stronger bands seen in these samples. This work was carried out in collaboration with Bethany Hunter, a Masters student supervised by myself in the Khan laboratory.

4.3 Generation and expression of Zika envelope constructs, without TetC

After the three pTECH2 Zika envelope plasmids were confirmed, two alternative constructs were made in order to evaluate the contribution of the TetC fusion partner to expression levels. These plasmids; pTECH10-ZE(s) expressing ZE alone with no fusion partner and pTECH11-ZE(s) which contained only the ribosome binding site of TetC (the first 10 amino acids) fused to the synthetic ZE gene, were made using site directed mutagenesis (NEB) to delete all or most of the TetC gene (as in chapter 3 with the Ebola GP constructs). It has been shown previously (see chapter 3, section 3.2.2) that the Ebola GP protein could not be expressed without at least the TetC RBS fusion partner in this system, therefore the pTECH11-ZE construct was also included here in case the same was true for the Zika envelope protein.

4.3.1 Construction of pTECH10-ZE expression plasmid

Primers were designed to remove the TetC gene from the pTECH2-ZE(s) plasmid using Q5 Site Directed Mutagenesis (NEB). The resulting plasmid – pTECH10-ZE(s) (figure 4.9) was chemically transformed into NEB5α E. coli cells (NEB). Plasmid DNA from putative clones were then purified and digested with *EcoRI* and the cloning enzyme *HindIII*. Plasmid DNA from a positive clone would result in two bands visible when the digest mix was fractionated on an agarose gel. DNA from a negative clone, i.e. if the plasmid had retained TetC, would result in three bands visible on an agarose gel due to an *EcoRI* restriction site present in the TetC gene sequence (figure 4.10).

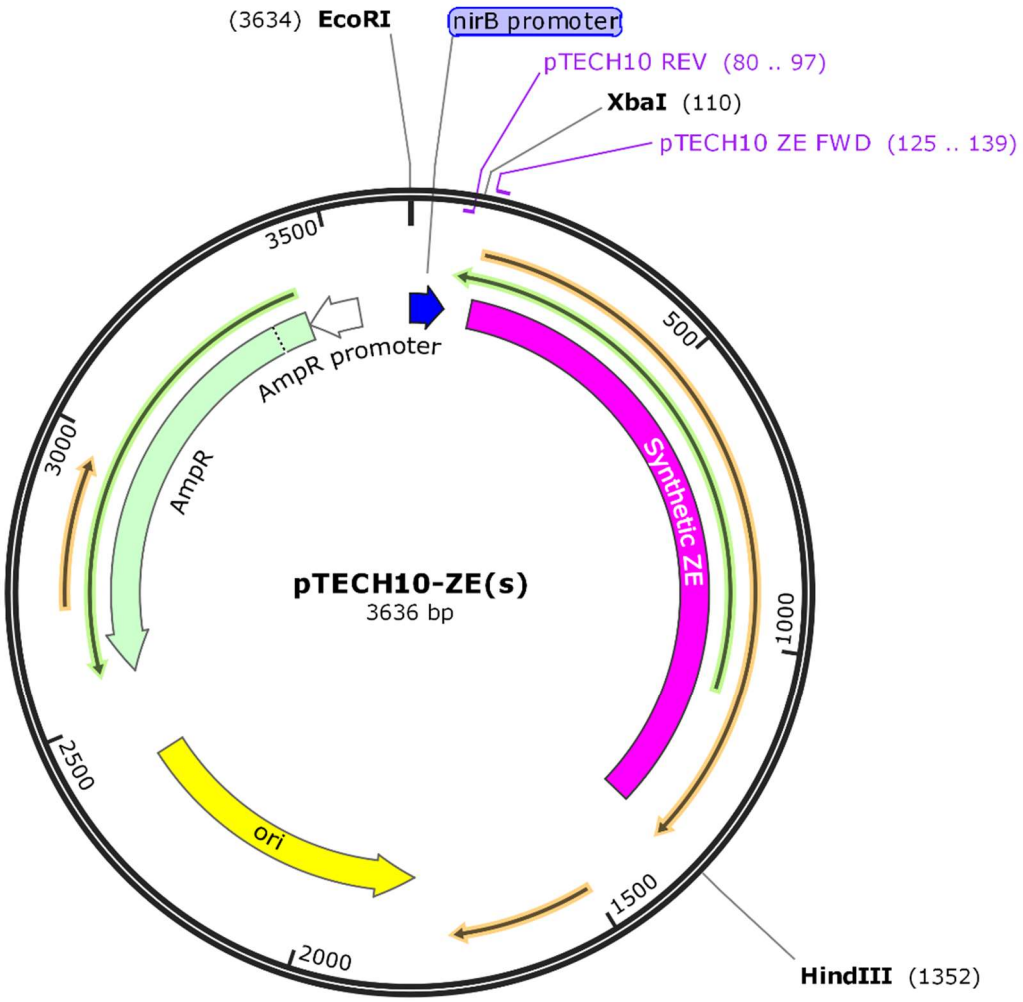


Figure 4.9. Plasmid map of the pTECH10-ZE(s) expression vector.
This vector was constructed using Q5 Site Directed Mutagenesis (NEB) using the forward (FWD) and reverse (REV) primers shown. Inverse PCR allowed the removal of the TetC gene from the pTECH2-ZE(s) plasmid. Expression of Zika envelope is under the control of the *nirB* promoter and is expressed alone, without TetC as a fusion partner. Plasmid map was made using the commercial software Snapgene.

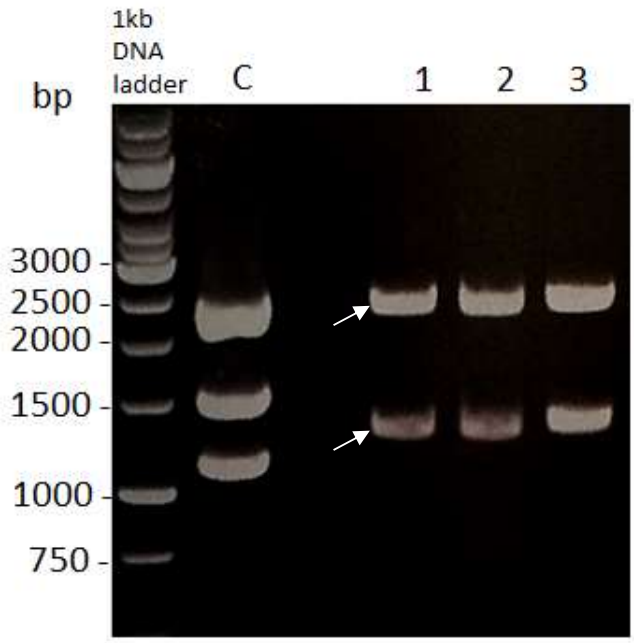


Figure 4.10. Restriction digest screens of putative pTECH10-ZE clones.
0.7% agarose gel showing purified plasmid from 3 colonies of NEB5α E. coli suspected to contain the pTECH10-ZE(s) plasmid (lanes 1-3). The plasmid was digested with *EcoRI* and *HindIII* showing two distinct bands at 1353bp and 2282bp (as denoted by arrows). An extra *EcoRI* site situated towards the C terminal of the TetC gene means that a digested plasmid which has retained the TetC gene would show 3 bands (such as lane C – pTECH2-ZE(s) negative control, also digested with the same enzymes).

Purified plasmid from colony 1 (lane 1, figure 4.10) was then sent for sequencing (Eurofins) and once confirmed, transformed into *Salmonella* Typhimurium SL5338 cells for expression analysis.

4.3.2 Construction of pTECH11-ZE expression plasmid - Retention of the TetC ribosome binding site (RBS)

As reported in chapter 3, the Ebola GP protein did not express without the TetC fusion partner. This was rescued somewhat by retaining the TetC RBS, including 30 bases downstream of the start codon (this has been described in more detail in Chapter 3, section 3.2.2.3). It was therefore decided to create an equivalent construct in the event that the same was true for the expression of Zika E. This construct was also made using Q5 site directed mutagenesis (NEB) to remove all but the first 10 amino acids of TetC and thus retain the full RBS and additional downstream sequence after the initiation codon (figure 4.11).

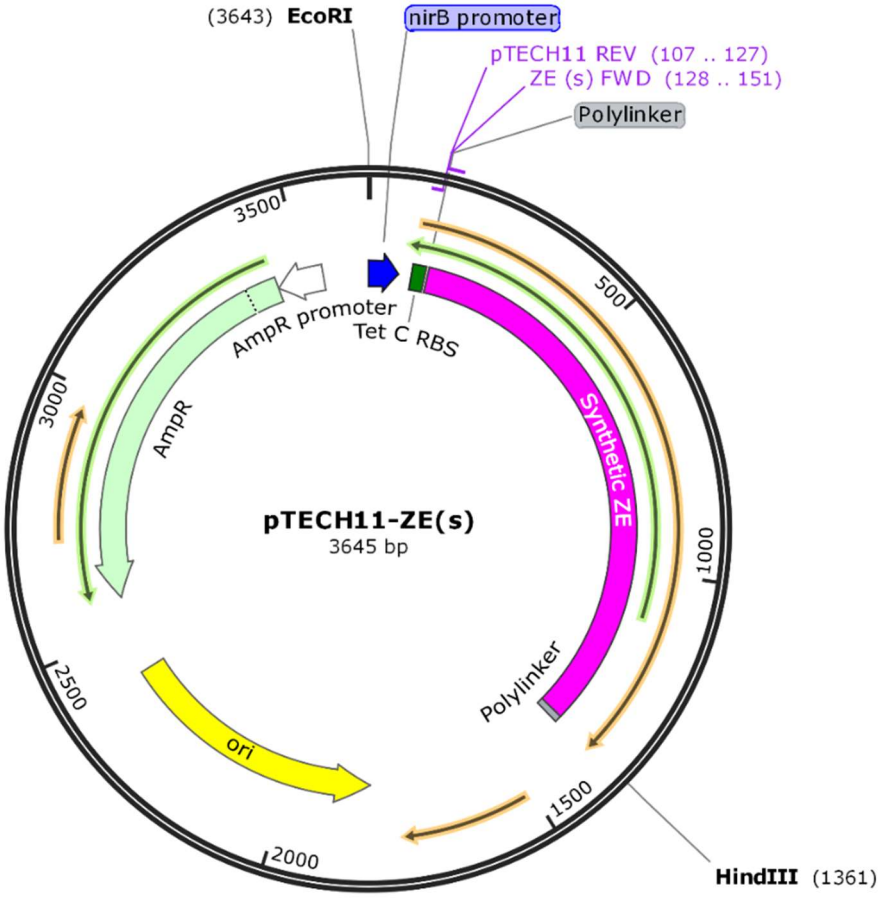


Figure 4.11. Plasmid map of the pTECH11-ZE(s) expression vector. This vector was constructed using Q5 Site Directed Mutagenesis (NEB) using the forward (FWD) and reverse (REV) primers shown. Inverse PCR allowed the removal of the majority of the TetC gene from the pTECH2-ZE(s) plasmid, except the first 10 amino acids (RBS). Expression of Zika envelope is under the control of the *nirB* promoter and is expressed alone, without TetC as a fusion partner. Plasmid map was made using the commercial software Snapgene.

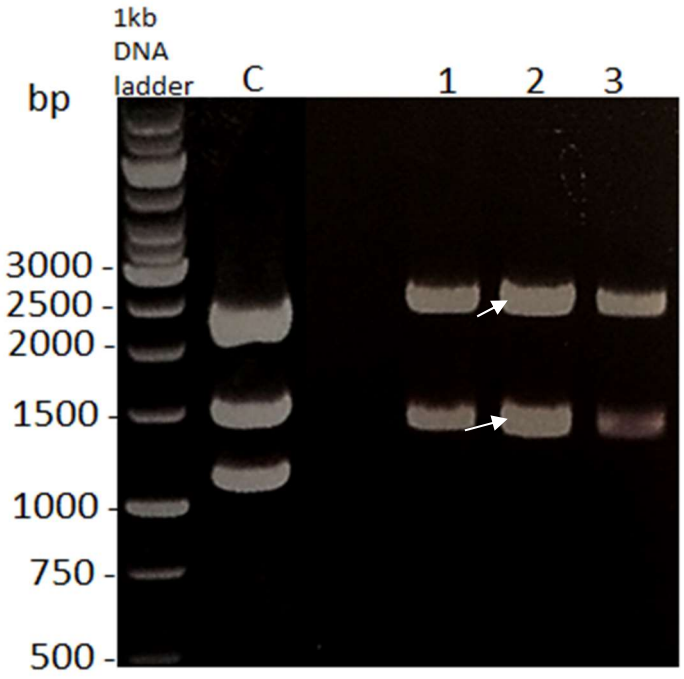


Figure 4.12. Restriction digest screens of putative pTECH11-ZE clones.
0.7% agarose gel showing purified plasmid from 3 colonies of NEB5α E. coli suspected to contain the pTECH11-ZE(s) plasmid (lanes 1-3). The plasmid was digested with *EcoRI* and *HindIII* showing two distinct bands at 1363bp and 2282bp (as denoted by arrows). An extra *EcoRI* site situated towards the C terminal of the TetC gene means that a digested plasmid which has retained the TetC gene would show 3 bands (such as lane C – pTECH2-ZE(s) negative control, also digested with the same enzymes).

Purified plasmid from colony 2 (lane 2, figure 4.12) was then sent for sequencing (Eurofins) and once confirmed, transformed into *Salmonella* Typhimurium SL5338 cells for expression analysis.

4.3.4 Expression of ZE alone in Salmonella strain SL5338

After confirming through sequencing that the pTECH10-ZE(s) and pTECH11-ZE(s) plasmids had been successfully constructed, additional constructs were made in an attempt to allow *Salmonella* Typhimurium to express the Zika envelope protein when no TetC fusion partner was present.

SL5338 cell lysates were fractionated on SDS-PAGE gel and transferred for western blot to nitrocellulose. When probed with a monoclonal mouse anti-Zika E antibody (Aaltobioreagents) both of these constructs did express the Zika envelope protein but not to the level of which was seen when as a fusion with the full TetC (see figure 4.13). This could mean that the TetC RBS and additional downstream sequence is not necessarily contributing to increasing the expression of Zika E, as similar levels of expression are seen in the pTECH10-ZE(s) samples, where this is not present. The stability of the interaction between the *Salmonella* Typhimurium 16S rRNA and RBS of the ZE mRNA could therefore be similar to that of TetC, and it could be that the huge increase in expression seen when the full TetC is present (pTECH2-ZE(s)) is due to other areas on the TetC mRNA. This indicates that the full TetC protein may help to rescue the Zika E expression.

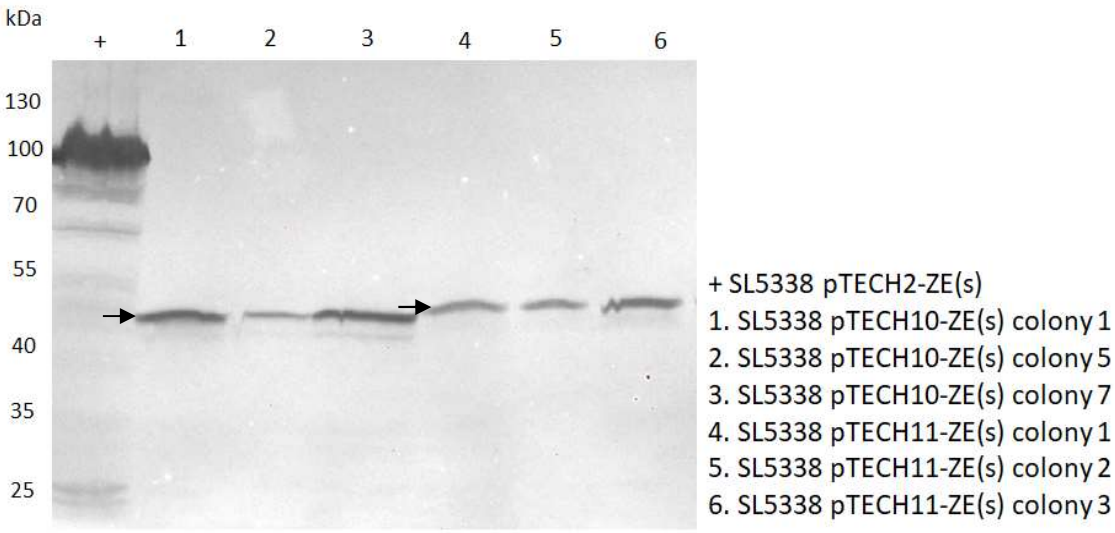


Figure 4.13. Expression of Zika E alone in *Salmonella* Typhimurium.
Western blot showing Zika envelope expressed in *Salmonella* Typhimurium SL5338 with no TetC fusion partner (pTECH10 – lanes 1-3) at approximately 45kDa (see arrow) or with only the small TetC RBS fusion partner (pTECH11 – lanes 4-6) at approximately 46kDa (see arrow). Cell lysates of SL5338 colonies harbouring either of these plasmids (three of each) were fractionated on SDS-PAGE and transferred to nitrocellulose. The blot was probed with mouse monoclonal anti-ZE (aaltobioreagents) (1:10000) followed by Rabbit anti-mouse HRPO (abcam) (1:4000). The positive control lane (+) shows the TetC-ZE(s) fusion protein at approximately 97kDa expressed by SL5338 cells as a comparison. Each sample was calculated to ensure an equivalent number of cells in each, therefore these results suggest that fusion to the whole TetC rescues expression of ZE. This construct was made in collaboration with Bethany Hunter, a Masters student supervised by myself in the Khan laboratory.

4.4 Generation of a Zika E-Ebola GP fusion protein construct

As shown in chapter 3, it was not possible to express the Ebola GP protein without the presence of at least the ribosome binding site (RBS) of TetC as a fusion partner. With the full TetC present, the expression of the Ebola GP protein was much higher. In this chapter, the same has been explored for the Zika envelope protein and was shown to express both with the TetC RBS fusion and alone, but again not to the levels as seen in fusion with the full TetC. It was therefore decided to see if rather than TetC, we could use the Zika E as a fusion partner to Ebola GP sub fragment DMFL and express a ZE-EBOVGP fusion protein (figure 4.14)

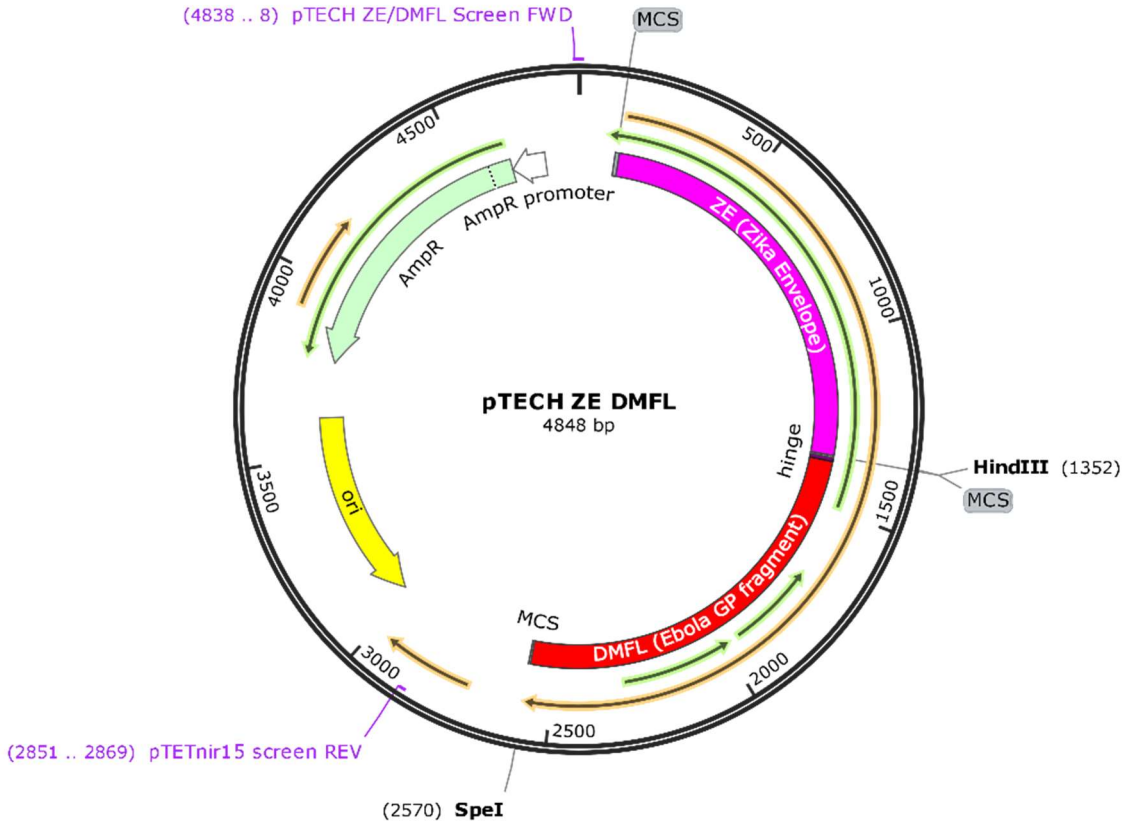


Figure 4.14. Plasmid map of the pTECH ZE-GP DMFL expression vector. Under the control of the *nirB* promoter, a Zika E-GP DMFL fusion protein is expressed. The GP DMFL gene was cloned in using restriction enzymes *SpeI* and *HindIII*. Colony PCR was carried out on *Salmonella* Typhimurium SL5338 colonies after transformation using the pTECH ZE/DMFL forward and pTETnir15 reverse screening primers as shown to confirm putative clones containing the plasmid prior to expression analysis. The full-length ZE shown in pink represents the codon optimised synthetic gene (ZEs) and the Ebola GP DMFL gene sub fragment is shown in red. Plasmid map was made using the commercial software Snapgene.

4.4.1 Generation of pTECH ZE-EBOVGP DMFL expression plasmid

The pTECH10-ZE plasmid was digested with restriction enzymes *SpeI* and *HindIII*. The 1200bp Ebola GP DMFL was then amplified by PCR (see chapter 3) however with new primers to allow the addition of *SpeI* and *HindIII* restriction sites, digested with these enzymes and purified. This DMFL insert was then ligated into the digested plasmid using T4 Ligase (NEB) to create the pTECH2-ZE-GP DMFL plasmid, expressing a Zika envelope-Ebola GP DMFL fusion protein. The ligation mix was transformed into chemically competent SL5338 cells and the resultant colonies were picked and screened using colony PCR. A new screening primer was made for this construct to amplify a section of the plasmid which would include the ZE and DMFL gene sequences when used with the pTETnir15 REV primer as mentioned previously. If the Ebola GP DMFL gene sub fragment has been successfully incorporated into the plasmid, the resultant PCR product would be 2880bp, if the DMFL gene is not present (pTECH2-ZE only), the product would be 1680bp (figure 4.15).

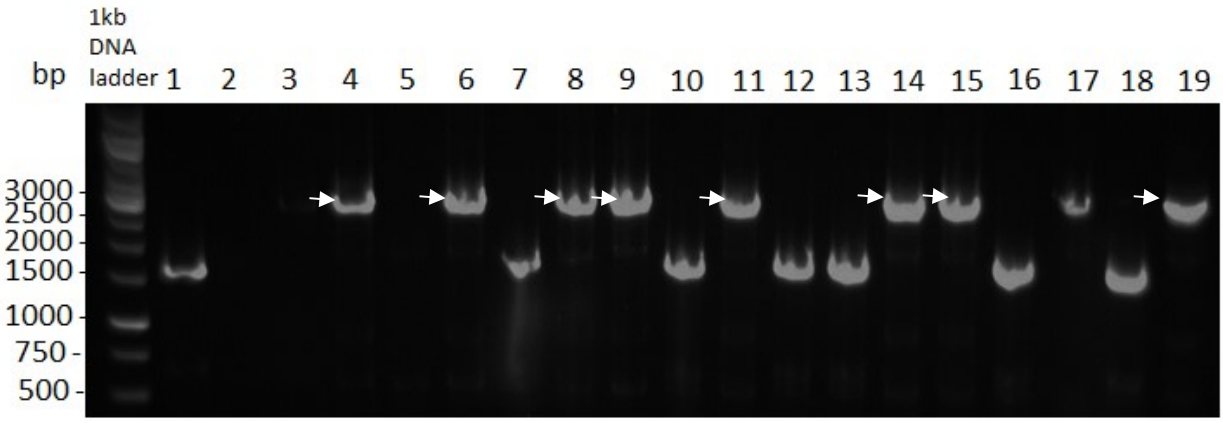


Figure 4.15. Colony PCR screen of putative pTECH-ZE-DMFL clones.
0.7% agarose gel showing PCR products from colony PCR using pTECH screening primers to determine successful construction and transformation of the pTECH vector containing both the Zika envelope (synthetic, codon optimised) gene and the Ebola GP DMFL gene sub fragment. Screening primers amplify a product of 2880bp when the DMFL sub fragment is present and 1680 when it is not. This shows 10 colonies out of 19 tested are positive (as shown by arrows), some of which will be tested for expression of the fusion.

4.4.2 Expression of ZE-GP fusion protein in Salmonella Typhimurium

Eight putative clones (4, 6, 8, 9, 11, 13, 14 and 19) were then prepared for western blot analysis and 7 out of these were shown to express the ZE-GP DMFL fusion protein when probed with both anti-Ebola GP and anti-Zika E monoclonal antibodies by western blot (see figure 4.16). While there is clearly expression of this fusion, it seems that the Zika envelope protein does not have the same effect when rescuing expression of Ebola GP as the TetC fusion partner. It is therefore decided that this construct will not be taken further into *in vivo* testing as the expression levels may be too low to administer an appropriate antigenic dose.

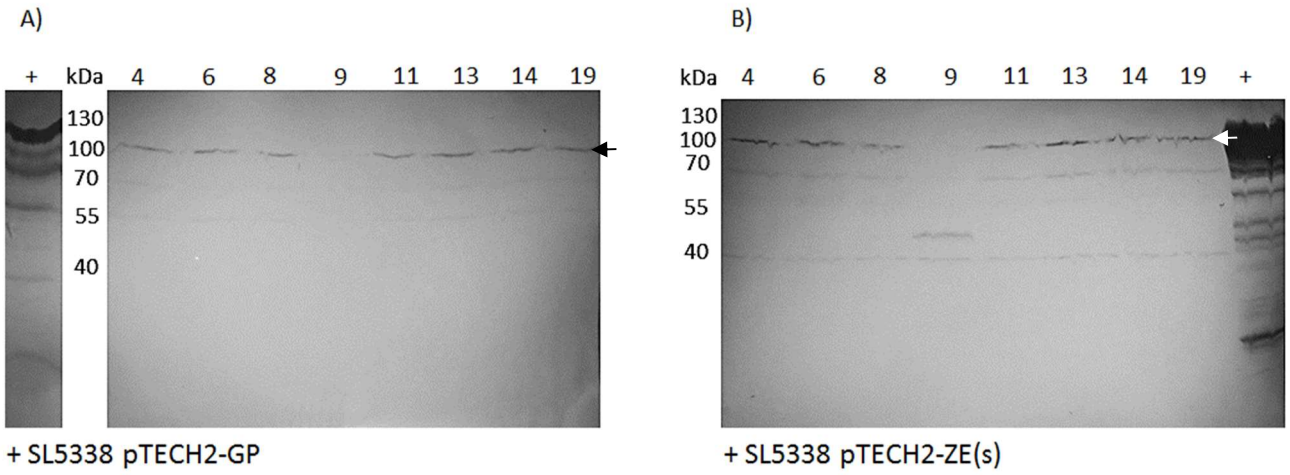


Figure 4.16. Expression of ZE-GP fusion protein in *Salmonella* Typhimurium.
Western blot showing ZE-GP DMFL fusion proteins *Salmonella* Typhimurium strain SL5338. Cell lysates of SL5338 cells which had been shown by colony PCR to potentially contain the pTECH ZE-GP DMFL expression plasmid were fractionated on SDS-PAGE and transferred to nitrocellulose. Blots were probed with either polyclonal mouse anti-EBOV sera (A) or monoclonal mouse anti Zika envelope (aaltobioreagents) (B) followed by Rabbit anti mouse HRPO (abcam). In 7 out of the 8 colonies tested, there is clear expression of the ZE-GP DMFL fusion as bands were seen at approximately 89kDa (as shown by arrows) when probed with both antibodies. This expression however was nowhere near as strong as that of TetC-GP or TetC-ZE(s) as shown in lanes labelled with +. This suggests that while ZE may rescue the expression of Ebola GP, TetC is a better fusion partner when considering total protein expression.

4.5 *In vitro* stability of pTECH2-ZE plasmids in *Salmonella* Typhimurium vaccine strain SL3261

As is the case with the pTECH2 plasmids expressing the Ebola GP proteins, the same must be done for the Zika E expression plasmids to ensure that the *Salmonella* vaccine strain can stably retain the plasmid in the absence of ampicillin selection pressure, due to the fact that when used *in vivo*, there will be no antibiotics present. Using the pTECH2 plasmid as a control, which shows 100% stability, the ZE(s) and ZE (s193) expressing constructs showed 81.8% and 100% stability respectively, allowing these to move forward for *in vivo* testing.

Constructs	Overnight Growth media	Plate growth media	CFU/ml	% Stability
pTECH2	LB	LB	8×10^8	
		LB + AMP	8×10^8	100%
pTECH2-ZE(s)	LB	LB	1.17×10^9	
		LB + AMP	1.43×10^9	81.8%
pTECH2-ZE(s193)	LB	LB	1.3×10^9	
		LB + AMP	1.2×10^9	108.3% (100%)

Table 4.1. *In vitro* Stability of pTECH2 constructs expressing Zika envelope. Stability of pTECH2 constructs expressing Zika envelope. Calculated to show stable retention of the pTECH2 plasmids expressing ZE (s) or the truncated ZE (s193) in the absence of selective pressure from ampicillin.

4.6 *In vivo* stability of pTECH2-ZE plasmids in *Salmonella* Typhimurium vaccine strain SL3261

After confirmation that the pTECH2-ZE expression plasmids were stable in the absence of antibiotic selective pressure, an immunisation experiment was carried out in order to determine plasmid stability *in vivo* and ensure expression of the TetC-ZE fusion proteins was still possible after *Salmonella* was recovered from the livers and spleens of immunised mice. The work covered in sections 4.6.1 and 4.6.2 was very kindly carried out by Dr Omar Rossi, University of Cambridge.

4.6.1 Initial inoculation of mice with Salmonella vaccine strain SL3261 expressing TetC-ZE fusion proteins

As with the Ebola GP vaccines, BALB/c mice were split into groups of 4 and immunised with *Salmonella* Typhimurium vaccine strains harbouring the pTECH2 expression plasmids. These are; pTECH2, the TetC only expressing control and pTECH2-ZE(s) which express the TetC-Zika envelope fusion protein. Each mouse was immunised intravenously with 5 X 10^5 CFU SL3261 in 0.2ml PBS.

Group	Immunisation	n BALB/c =
TETC	SL3261 pTECH2	4
TETC-ZE(s)	SL3261 pTECH2-synthetic Zika envelope	4
TETC-ZE(s193)	SL3261 pTECH2-synthetic Zika envelope 193	4

Table 4.2. Mice used in ZE plasmid stability inoculation experiment. Mice immunised in initial inoculation experiment to determine plasmid stability and TetC-ZE fusion protein expression after *in vivo* passage.

4.6.2 Plasmid stability after in vivo passage

11 days post immunisation, mice were sacrificed and livers and spleens analysed for colonisation by *Salmonella* Typhimurium SL3261 vaccine strain harbouring the pTECH2, and pTECH2-ZE(s) plasmids. Vaccine strain cells were recovered from the organs and plated on LB agar with and without ampicillin. A count for SL3261 numbers in each organ was calculated and the percentage difference between totals grown with and without ampicillin allowed a % value for plasmid stability to be calculated (figure 4.17). As in chapter 3, section 3.5.2, mice were immunised with pTECH2 as a control (this group is the same TetC group as featured in 3.5.2). It seems that the pTECH2-ZE(s193) plasmid is stable after *in vivo* passage, with results comparable to the pTECH2 control at about 95%. Plasmid pTECH2-ZE(s) however appears less stable at only around 50%. This could again be due to the full ZE fusion partner, which may make expression more difficult due to its larger size than the truncated s193 fragment.

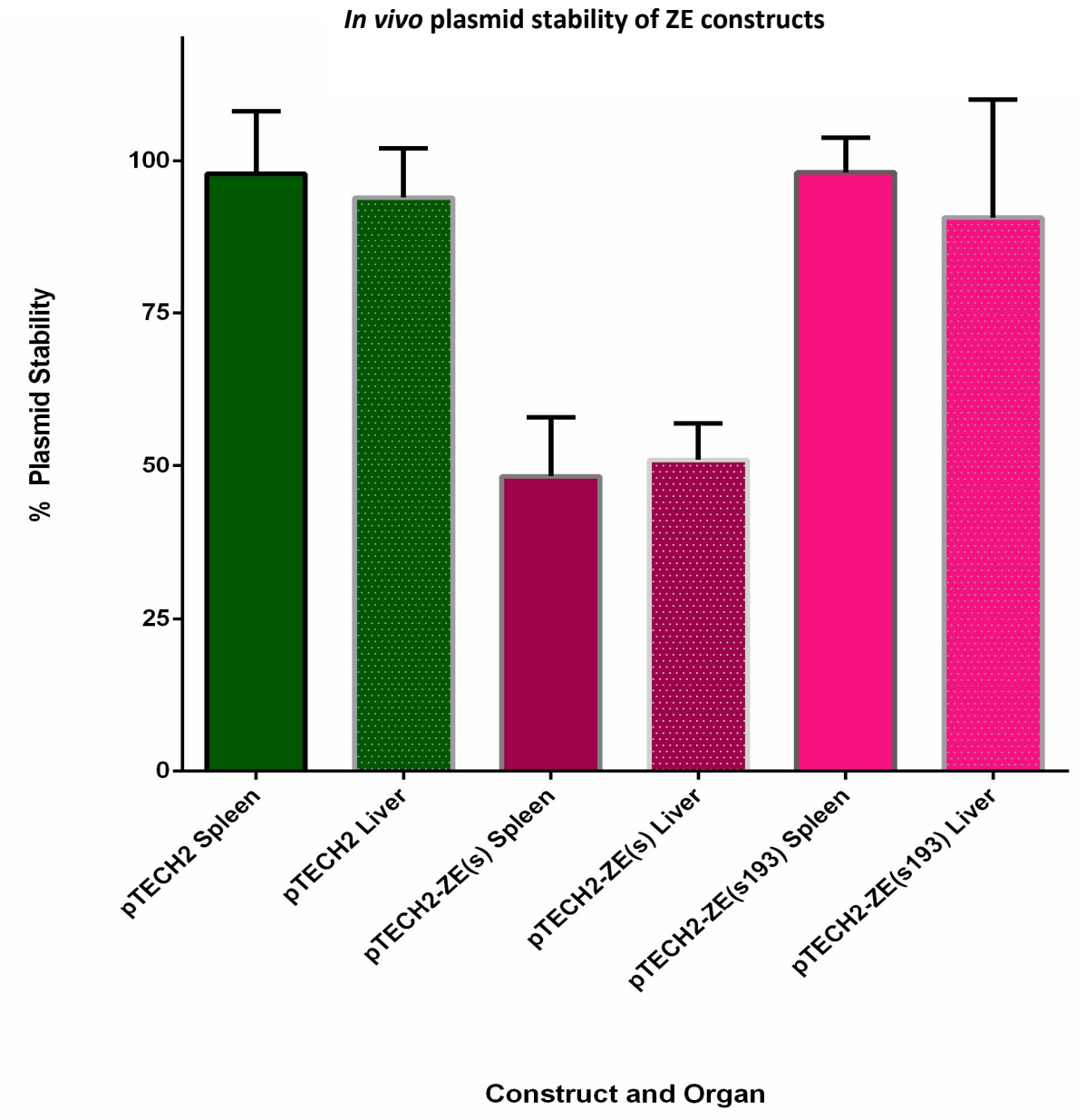


Figure 4.17. Plasmid stability of recombinant Zika envelope constructs *in vivo*.
Graph to show plasmid stability % of pTECH2 constructs expressing Zika E after *in vivo* passage and recovery from the livers and spleens of inoculated mice. pTECH2 controls shown are the same groups as in Chapter 3, 3.5.2. Results are presented the mean percentage of all mice in each group plus SD.

4.6.3 Expression of TetC-ZE fusion proteins after in vivo passage

The pTECH2 and pTECH2-ZE(s193) plasmids were shown to be stable *in vivo* at over 90% when recovered from inoculated mouse livers and spleens. The pTECH2-ZE(s) plasmid however was only shown to have around 50% stability. It was decided to check for expression of the TetC-ZE fusion proteins from the cells recovered from these organs by western blot of cell lysates after overnight growth on LB agar containing ampicillin following transport to Newcastle as agar stabs.

Cells recovered from the livers and spleens were all able to express either TetC (SL3261 harbouring pTECH2 – also shown in chapter 3, section 3.5.3) or the TetC-ZE(s) or TetC-ZE(s193) fusion proteins and on western blots, showed an equivalent pattern of bands as SL3261 cells from stock agar plates harbouring the same plasmids, which had not been subject to *in vivo* passage (figure 4.18).

In the case of the pTECH2-ZE(s) plasmid, which showed reduced stability compared to the pTECH2 control and also pTECH2-ZE(s193), the TetC-ZE(s) fusion protein is still expressed as detected by the anti-TetC antibody, however there seems to be variation between the expression levels of each mouse. It was therefore decided that when carrying out the main immunisation experiment (see 4.7.1) to determine immune response to the vaccine, SL3261 cells recovered from the spleen of mouse 1 in the TETC-ZE(s) group (figure 4.18 B) would be used to immunise new mice, due to the fact that repeated passage can increase plasmid stability when there are no selective antibiotics present (Chabalgoity et al, 1996) and cells from this sample appear to express the fusion at higher levels than samples from the other 3 mice in this group.

Vaccine strains for the main immunisation (4.7.1) for the other groups however were from the same stocks as this pilot experiment as the stability and expression from these was shown to be sufficient.

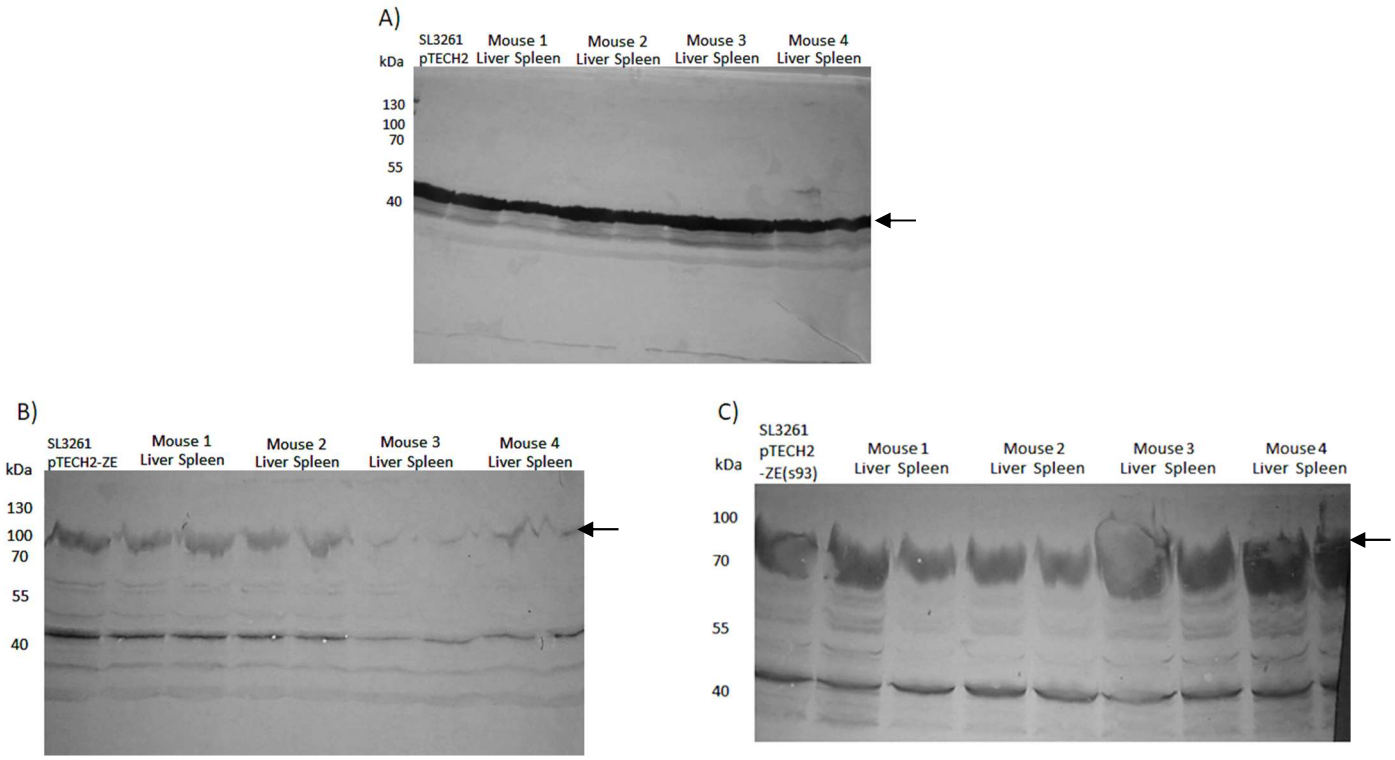


Figure 4.18. Expression of TetC-ZE fusion proteins after *in vivo* passage.
Western blot showing expression of TetC and TetC-Zika E fusion proteins from *Salmonella* Typhimurium SL3261 cell lysates after recovery from immunised mouse livers and spleens. Cell lysates were fractionated on SDS-PAGE before transfer to nitrocellulose. Blots were probed with Blots were probed with polyclonal rabbit anti-TetC serum (1:1000) and Goat anti-rabbit HRPO (1:3000). The TetC protein is clearly seen at approximately 50 kDa (A) and the TetC-ZE(s) (B) and TetC-ZE(s193) (C) fusion proteins at 97kDa and 74kDa respectively as shown in line with arrows. Cells from a stock plate which had not been subject to *in vivo* passage were used as a positive control (shown in the first lane of each blot).

4.7 Immune responses to *Salmonella* based Zika Virus vaccine

It was confirmed through western blot that *Salmonella* Typhimurium vaccine strain SL3261 could still express the TetC-ZE fusion proteins following *in vivo* passage, a new immunisation experiment was therefore carried out to determine whether the vaccine strains expressing the ZE fusion proteins would elicit an immune response in mice.

4.7.1 Inoculation of mice with Salmonella vaccine strain SL3261 expressing TetC-ZE fusion proteins

Mice were once again inoculated by intravenous injection with *Salmonella* Typhimurium vaccine strain SL3261 expressing TetC (control) or TetC-ZE fusion proteins.

Group	Immunisation	n BALB/c =
TETC	SL3261 pTECH2 6.41 log10 CFU in 0.2ml PBS	6
TetC-ZE(s)	SL3261 pTECH2-ZE(s) 6.22 log10 CFU in 0.2ml PBS Taken from Mouse 1, Spleen (after *in vivo* passage)	6
TetC-ZE (s193)	SL3261 pTECH2-ZE(s193) 6.22 log10 CFU in 0.2ml PBS	6
TetC-ZE(WT)	SL3261 pTECH2-ZE(WT) 6.26 log10 CFU in 0.2ml PBS	6
NEG	Not immunised	4

Table 4.3. Mice used in ZE immunisation experiment. Mice immunised in inoculation experiment to determine immune response to TetC-GP fusion proteins. 6 mice were immunised with the SL3261 vaccine strain expressing either TetC or the TetC-ZE fusion proteins. 4 mice were not immunised so as to allow a baseline reading for normal mouse serum. Note: groups TETC and NEG are the same control groups as shown in chapter 3 section 3.6.1.

8 weeks post immunisation, the mice were sacrificed, and blood was collected from each mouse. This coagulated before centrifugation and serum only was collected. Sera was kindly collected and processed by staff at Cambridge University and sent to Newcastle to be tested for immune response. Once received, sera was aliquoted and stored at -80 °C prior to use in ELISA assays.

4.7.2 IgG antibody response to Zika envelope protein

The IgG response of immunised mouse serum to Zika envelope was determined by ELISA. Plates were coated with 5µg/ml Zika envelope protein (Fitzgerald) and sera diluted 1:25. The IgG response was measured at 450nm with Rabbit anti-mouse HRPO (Abcam) as the secondary antibody. The assay was developed with TMB and stopped with H2SO4 prior to reading. Again, as was seen with the ELISA assay results from the immunisation with Ebola GP expressing vaccine strains, it seems that there is no significant immune response to Zika E (figure 4.19). The commercial recombinant Zika Envelope used to coat the ELISA plate was, according to the manufacturer (Fitzgerald), derived from an unspecified African strain, however all strains share very high identity of this sequence. Monoclonal antibodies directed to the Zika Envelope were able to bind to both the commercial and TetC-ZE fusion proteins when tested prior to carrying out the ELISA (data not shown).

There are some potential outliers in the TetC-ZE(s) group, but the TetC negative control group, which it would be thought would give a low response to ZE, seems much higher than expected.

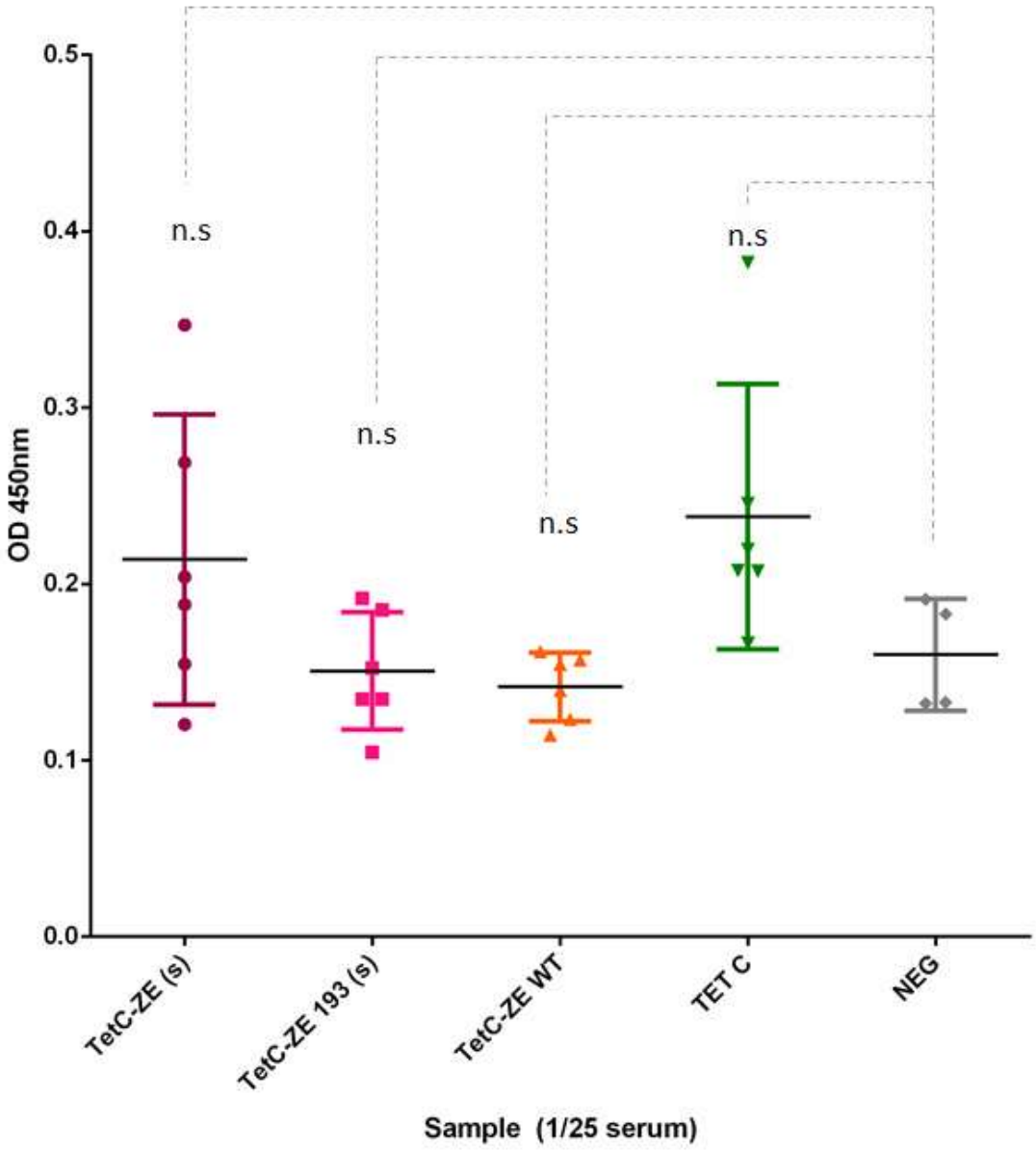


Figure 4.19. IgG antibody response to Zika envelope in ZE immunised mice.
Graph showing IgG response in immunised mouse sera as measured by ELISA with plates coated with 5µg/ml recombinant Zika envelope. Readings are presented as an average of triplicate wells + SD. Statistical significances compared to normal mouse sera (NEG) were analysed by unpaired t test. P values <0.05 were considered significant. Here, none of the means of any of the immunised groups (TETC-ZE(s), TETC-ZE(s193), TETC-ZE(WT) and TETC) were significantly different from normal mouse sera (ns = not significant).

4.7.3 IgG antibody response to TetC

As in chapter 3, it was then decided that the IgG response to TetC should be analysed so that a general IgG response could be determined in all immunised mice. It is clear that the TetC only immunised control mice, respond as expected well and the non-immunised (NEG) mice do not show any IgG response to TetC. The TetC-ZE(s) immunised mice however, also do not appear to show any notable response to TetC and the same can be said for the TetC-ZE(s193) and TetC-ZE(WT) immunised mice, except for some outliers (figure 4.20) Again however, the mean responses of all of the immunised groups (apart from TETC control) are not significantly different to non-immunised mice (NEG). The lower IgG response seen in the fusion immunised groups was also seen in chapter 3 with the TetC-Ebola GP immunised mice. Assays to determine if the overall immune response was lower, potentially due to immunosuppression, were therefore carried out by determining IgG response to *Salmonella* Typhimurium LPS.

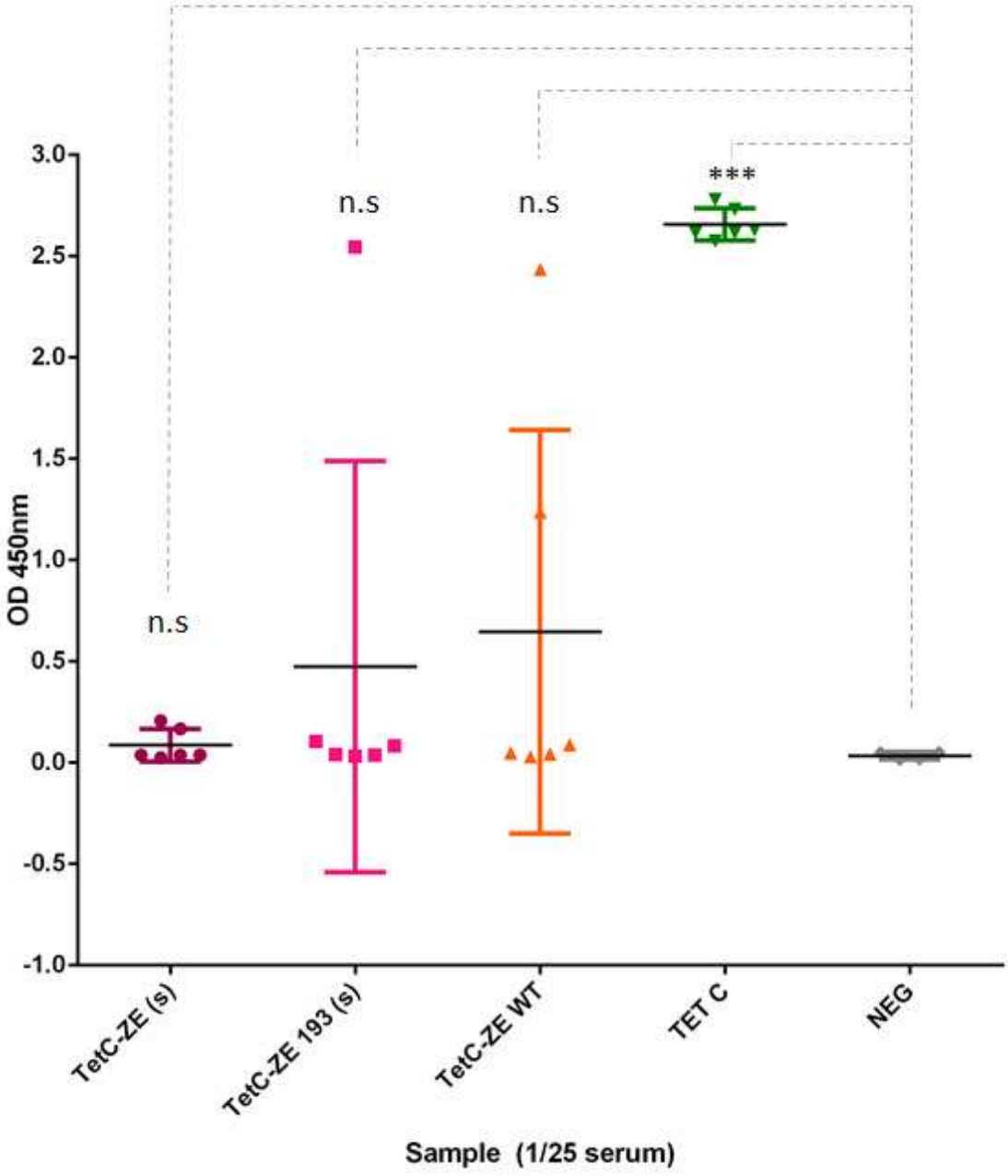


Figure 4.20. IgG antibody response to Tet C in ZE immunised mice.
Graph showing IgG response in immunised mouse sera as measured by ELISA with plates coated with 1µg/ml TetC protein. Readings are presented as an average of triplicate wells + SD. Statistical significances compared to normal mouse sera (NEG) were analysed by unpaired t test. P values <0.05 were considered significant. Here, neither of the means of the groups immunised with SL3261 cells expressing the TetC-GZE fusion proteins (TETC-ZE(s), TETC-ZE(s193) and TETC-ZE(WT) were significantly different from normal mouse sera (ns = not significant). Mice immunised with SL3261 cells expressing TetC only however showed a significant IgG response to TetC compared with non-immunised mice as expected (P = <0.0001 - ***)

4.7.4 IgG response to Salmonella Typhimurium LPS

As there appeared to be a lower IgG response to TetC in the mice immunised with SL3261(pTECH2-ZE) constructs, expressing the TetC-ZE fusion proteins, it was decided to try and elucidate an overall immune response by analysing IgG response to *Salmonella* Typhimurium LPS (figure 4.21). This response, which is unrelated to the expression of TetC or TetC-ZE fusion proteins could show whether there is an immunosuppressive effect of the vaccine strains.

Salmonella Typhimurium LPS (Sigma) was coated onto ELISA plates at 5µg/ml and pooled sera from each of the groups was titrated from a 1:50 to a 1:6400 dilution. The secondary antibody was again Rabbit anti-mouse HRPO and the assay was developed as before with TMB and H2SO4. There does not seem to be a clear difference between the anti LPS IgG response in mice immunised with SL3261 cells expressing only TetC compared to those immunised with SL3261 expressing the TetC-ZE fusion proteins. Compared to the non-immunised mice, which as expected did not show an IgG response to LPS as expected, this suggests that the overall immune response is not suppressed and therefore there may be alternative reasons as to why the IgG response to the Zika envelope protein in any of the three fusion groups, is not as high as was expected, when taking the relatively high expression levels of the TetC-ZE fusions into consideration.

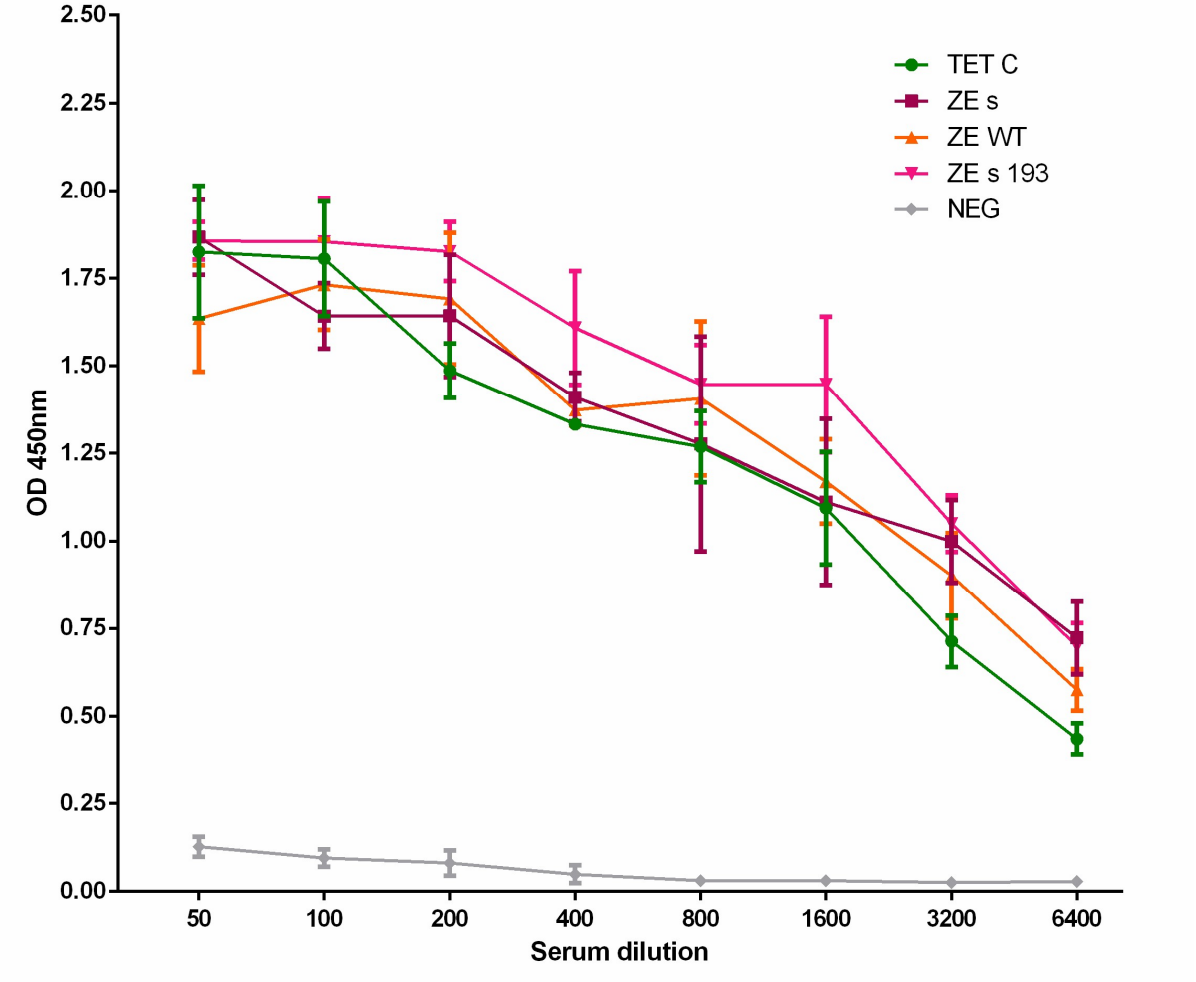


Figure 4.21. IgG antibody response to *Salmonella* Typhimurium LPS in ZE immunised mice. Graph showing titration of IgG response against *Salmonella* Typhimurium LPS, from 1:50 to 1:6400 dilution, of pooled sera from mice immunised with *Salmonella* Typhimurium SL3261 vaccine strain expressing TetC or TetC-ZE fusion proteins. Non- immunised mice (NEG) were used as a control. Readings are presented as an average of triplicate wells + SD. Statistical significances compared to TetC immunised sera (TETC) were analysed by unpaired t test. P values <0.05 were considered significant and at all dilutions, none were significantly different.

4.8 Determination of Zika Virus Neutralisation activity in immunised mice

The ELISA data had shown that while there did not appear to be a significant IgG response to Zika envelope in immunised mouse sera, there was some outlying samples which may have hinted at a response. In order to test this further, sera samples were sent to Professor Arvind Patel and Ricardo Sanchez Velazquez (Glasgow University) who kindly carried out micro-neutralisation assays to determine if the sera from these immunisations had any virus neutralising capacity (figure 4.22).

Vero cells were infected with Zika virus Brazilian strain PE243 (Donald et al, 2016) mixed with the experimental sera and incubated for 72 hours. A sandwich ELISA was then carried out to determine expression of Zika E from infected cells. From this, the % infection was calculated. A lower % infection was related to a lower amount of ZE expressed and thus a higher neutralisation capacity of the serum.

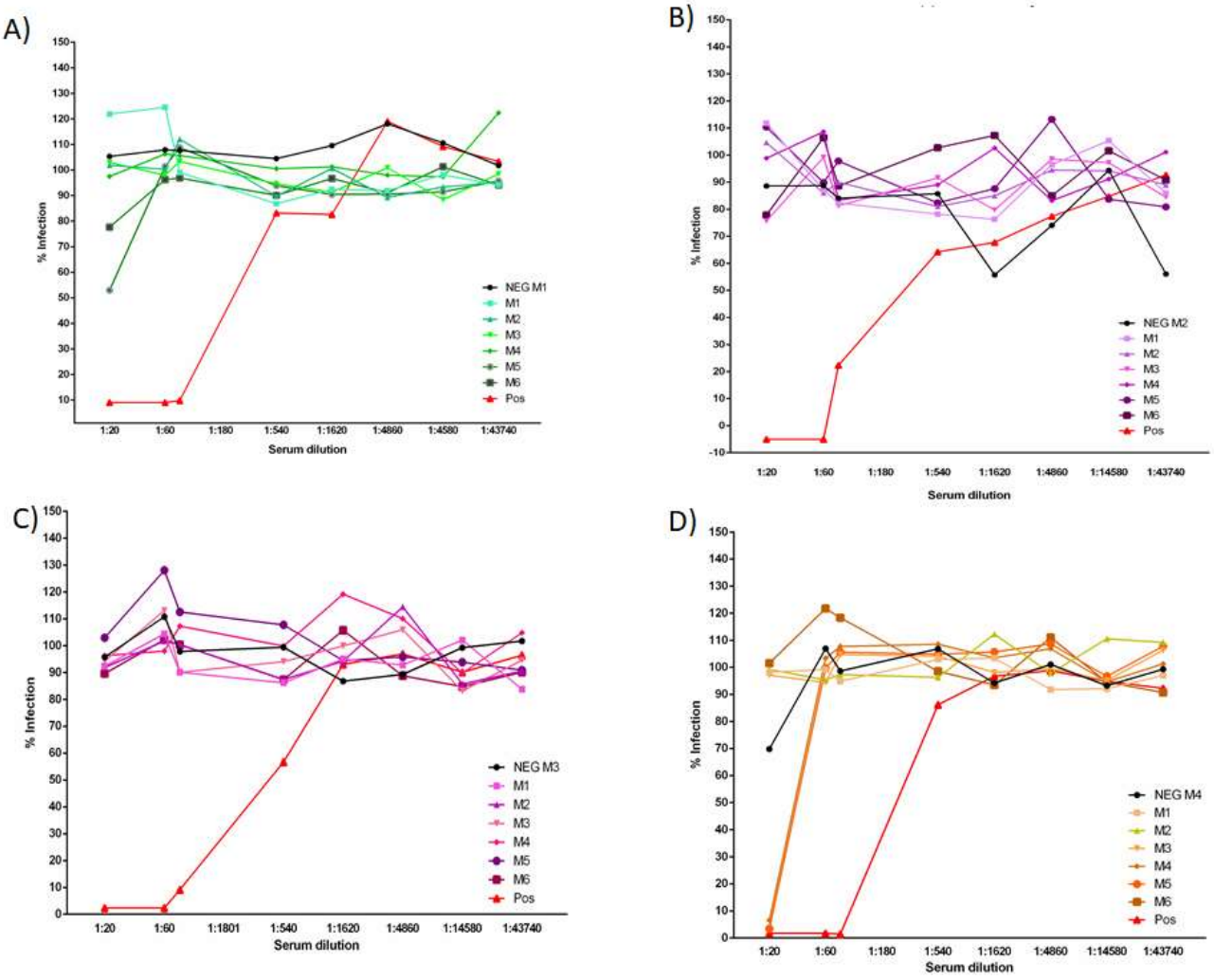


Figure 4.22. Zika Virus neutralisation activity in ZE immunised mice.
Graphs showing results of micro-neutralisation assays against Zika Virus by immunised mouse sera. Each sample was used in conjunction with ZIKV (PE243) in a series of 3-fold dilutions (1:20 – 1:43740) to infect Vero cells. A sandwich ELISA to detect the presence of Zika E by these cells was then carried out and the OD450 was converted into a value for % of infection. As a Positive control, mouse sera from a mouse immunised with Domain III of ZE was used (shown in red on each graph - Pos). Mice from the NEG group (non-immunised) are used as a negative 'normal mouse serum' control. Mouse groups are A) TETC, B) TETC-ZE(s), C) TETC – ZE(s193), D) TETC – ZE(WT). Results from each mouse are presented separately (M1-6). This experiment was very kindly carried out by Professor Arvind Patel and Ricardo Sanchez Velazquez (Glasgow University).

It appears that there is little in the way of neutralising activity by the immunised mouse sera against Zika envelope. There are two potential outliers (see fig 4.22 D), mice M4 and M5 immunised with TetC-ZE(WT), which show some neutralising activity at high serum concentration of 1:20 dilution. These interestingly do not correspond with any of the mice shown to have a slight IgG response to Zika E. Assays using these samples were then repeated however, and unfortunately shown to have no neutralising activity.

4.8 Discussion

A panel of constructs were generated to allow the expression of the Zika envelope protein by attenuated *Salmonella* vaccine strains. From this panel, the constructs which had shown to best express the Zika envelope protein were selected for investigation in *in vivo* immunisation experiments to determine the immune response in vaccinated mice towards the Zika envelope. These constructs, which facilitated expression of fusion proteins of the C-fragment of Tetanus toxin (TetC) and Zika E, from the pTECH2 expression vector, were transformed into attenuated *Salmonella* vaccine strains and used to immunise mice.

TetC-ZE(s); TetC fused to the full-length ZE protein utilizing the codon optimised synthetic gene for expression in *Salmonella* Typhimurium, and TetC-ZE(193); TetC fused to a truncated 193aa ZE protein were both expressed well by the *Salmonella* vaccine strain SL3261. TetC-ZE(WT); TetC fused to the ZE protein which used the WT codons was also tested to determine if there was an advantage to using synthetic codon optimization to make these constructs. It appears that the codon optimised ZE fusion protein was better expressed than the WT. Expression was assessed by the western blotting of *Salmonella* Typhimurium vaccine strain SL3261 cell lysates harbouring the relevant plasmids.

Attempts were also made to express the Zika envelope protein without the addition of the TetC fusion partner. Unlike Ebola GP (as reported in chapter 3, section 3.2.2.2), the ZE protein was expressed by *Salmonella* Typhimurium alone in the pTECH10-ZE expression vector. The level of expression however was nowhere near that of the pTECH2-ZE construct which has the full-length TetC fusion partner. The addition of the sequence of 30 bases downstream of the TetC start codon, which had been shown to rescue the expression of Ebola GP (see chapter

3, section 3.2.2.3), did not appear to improve the expression of the Zika envelope compared to its expression alone. This may be due to a more stable interaction between the *Salmonella* 16S rRNA and the ZE mRNA, in contrast to the Ebola GP mRNA, due to potentially higher complementarity, and therefore facilitation of expression.

An alternative construct was also generated which used the Zika envelope as a fusion partner to the Ebola GP DMFL sub-fragment. Due to the fact that ZE was successfully expressed alone, and GP was not, it was thought that the ZE could perhaps replace the TetC and rescue expression of the Ebola GP DMFL sub fragment. The ZE-GP DMFL fusion protein was successfully expressed, however not to levels seen with the TetC fusion partner.

It was concluded that the TetC fusion partner allowed much higher levels of expression of the fusion proteins and therefore the pTECH2 constructs would be brought forward for further testing.

The pTECH2- ZE plasmids were shown to be stably retained in the absence of antibiotic selection both *in vitro* and *in vivo*, and still expressed the TetC-ZE fusion proteins following *in vivo* passage (figures 4.17 and 4.18). This allowed an immunisation experiment to determine mouse immune responses to Zika envelope to be carried out. Mice were immunised with intravenous SL3261 harbouring the pTECH2, pTECH2-ZE(s), pTECH2-ZE(193) and pTECH2-ZE(WT) plasmids and after 8 weeks, sera was analysed by ELISA to determine IgG responses (figures 4.19, and 420). Unfortunately, there was no significant IgG response to the Zika envelope protein from either of the groups immunised with the *Salmonella* expressing the TetC-ZE fusion proteins. In addition, the mice immunised with *Salmonella* harbouring the pTECH2 only plasmid, as expected, had a strong IgG response to TetC, however the mice immunised with the TetC-ZE fusion expressing plasmids showed a lower response to TetC.

Overall IgG antibody response to *Salmonella* Typhimurium LPS was also tested (figure 4.21), to determine if the lack of response seen towards Zika envelope in the TetC-ZE fusion immunised mice was due to an overall dampened immune response or immunomodulatory effect of the attenuated vaccine strains. However, there were no significant differences between the responses seen in mice immunised with the SL3261-TetC control or the SL3261-TetC-ZE fusion experimental groups.

Some potential outliers were found of TetC-ZE immunised mice that showed a hint of an IgG response to ZE. The difficulty in procuring a recombinant Zika Envelope protein for use in these assays, meant that a commercial protein, expressed using a Baculovirus-insect cell expression system was used. This was derived from the African strain which shares high identity to all other strains for the E protein. This could have been an explanation for the poor IgG response seen in these assays, however the same samples were then subject to virus neutralisation assays (figure 4.22) and unfortunately were shown to be unable to neutralise the Zika Virus PE243 strain.

This lack of response could be due to a variety of reasons, including *in vivo* expression levels of the fusion proteins. This is discussed further in chapter 6, section 6.5.

Chapter 5 - Exploiting *Salmonella* Typhimurium Outer Membrane Vesicles (OMVs) as a novel vaccine delivery platform

5.1 Introduction

Gram-negative bacteria produce naturally occurring vesicles formed from the blebbing of the outer membrane – Outer Membrane Vesicles (OMVs). These spherical packages contain periplasmic and outer membrane located molecules (such as virulence factors) which can be delivered to host cells (Kulp & Kuehn, 2010).

It has been considered that these vesicles, due to their immunostimulatory properties and cargo delivery abilities, could be used as a non-living vaccine. OMVs contain potent pathogen associated molecular patterns (PAMPS) such as lipopolysaccharide (LPS) which are recognised by pattern recognition receptors (PRRs) such as TLR-4 (Rossi et al, 2016) and activate the innate immune system. Genetic modification of *Salmonella*, producing knockout strains of certain genes has shown to increase the propensity of these cells to produce OMVs (Rossi et al, 2016). This combined with the engineering of plasmid constructs to express antigens, such as the Zika envelope, could provide a novel vaccine platform to allow delivery of ZE to host cells without the need to use living *Salmonella* vaccine strains.

OMVs carrying Zika envelope could be purified and used to immunise. This method would have the advantage of being able to quantify antigenic dose compared to the living platform, and thus could be a more efficient way of antigen delivery at concentrations needed to elicit a protective immune response. This vaccine delivery platform could also have the advantage of increasing access to these vaccines, due to the contraindications of administering live vaccines to vulnerable people, for example, those with compromised immune systems or the very young and very old.

5.2 Gene Knockouts to increase OMV production

The yield of OMVs naturally produced by Gram-negative bacteria is usually in low quantities. To exploit this mechanism in order to develop vaccines, it is important to increase this yield to ensure that an adequate antigenic dose is administered.

Targeted genetic modification to knockout certain genes of some bacteria, for example those responsible for enabling the linking of inner and outer bacterial membranes, has shown that this yield increase is possible due to an induction of 'hyperblebbing' by the cells as a result of the knockout (Rossi et al, 2016)

5.2.1 Constructing gene knockout of tolR in Salmonella Typhimurium strain SL1344

The protein TolR is part of the *tol-pal* system, which is highly conserved between Gram-negative bacterial species and involved in upholding outer membrane stability. It has been shown that *E. coli tol* mutants produce an increased quantity of outer membrane vesicles (Lazzaroni et al, 1999; Bernadac et al, 1998).

TolR is part of a complex of proteins in this system which also includes TolQ and TolA and is found in the cytoplasmic membrane. TolR is anchored to the inner membrane with the majority of the protein situated in the periplasm and helps to facilitate linkage between the inner and outer membranes of the bacteria (Lazzaroni et al, 1999; Rossi et al, 2016).

In *Salmonella* Typhimurium, deletion of the *tolR* gene, and therefore disrupting this system, results in a strain in which the integrity of the outer membrane is altered and more likely to bleb off creating vesicles (Berlanda Scorza et al, 2012; Rossi et al, 2016).

It was therefore decided to create a *Salmonella* Typhimurium Δ*tolR* strain to determine if it was possible to firstly, purify an adequate quantity of OMVs, and secondly to express the Zika envelope protein in such a way that it was packaged in these vesicles, showing a viable method for which to create this non-living Zika vaccine.

Using the lambda-red recombinase technique (Datsenko & Wanner, 2000!) the *tolR* gene was knocked out of *Salmonella* Typhimurium strain SL1344 and replaced with the kanamycin resistance gene – *KanR*, found on bacterial transposon Tn5, encoding neomycin

transphospherase and taken here from the plasmid PKD4. A colony PCR was carried out with 'tolR check' primers to confirm a successful knockout (figure 5.1). If the *tolR* gene was still present in the SL1433 genome, the PCR would simply amplify a 515bp product which included the 429bp *tolR* gene. If the knockout, by way of replacing *tolR* with the kanamycin resistance cassette was successful, the primers would amplify a product of 1.5kb.

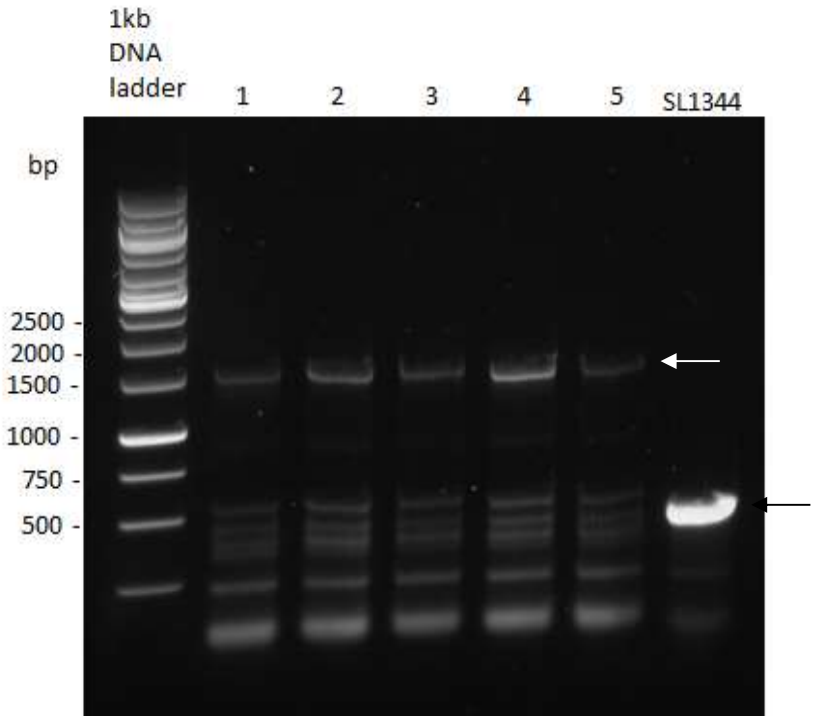


Figure 5.1. Colony PCR screen to determine *tolR* knockout of SL1344.
0.7% agarose gel showing PCR products from colony PCR using *tolR* screening primers of the SL1344 genomic DNA to determine successful knockout and replacement of the *tolR* gene with *KanR*. Colonies 1-5 (as shown on gel) were boiled before PCR amplification of the genome between each screening primer. Positive colonies in which the knockout has been successful show a band at approximately 1.5kb (see white arrow). A negative control, SL1344 in which the *tolR* gene is present, shows a band at 515bp (see black arrow).

5.2.2 Constructing gene knockout of mlaA in Salmonella Typhimurium strain SL1344

An ATP binding cassette (ABC) transport system, the Mla pathway, is found to be highly conserved between gram-negative bacterial species. It is thought that this pathway helps to prevent phospholipid accumulation at the outer leaflet of the outer membrane (OM) and is involved in maintaining the asymmetry of lipids between the inner membrane (IM) and OM (Malinverni & Silhavy, 2009).

Mutations resulting in the repression or deletion of components in this pathway can increase the production of OMVs by gram-negative bacteria, as phospholipids accumulate in the OM (Reidl, 2016).

MlaA (also known as VacJ), is a lipoprotein found on the OM, thought to act as a translocation channel for phospholipids (Abellón-Ruiz et al, 2017). MlaA works in this system with Yrb proteins, trafficking phospholipids from the OM to the IM to prevent accumulation. Deletion of the *MlaA* gene for example, leads to over accumulation of OM outer leaflet phospholipids, resulting in asymmetric expansion of the outer leaflet which eventually blebs off to become an OMV (Roier et al, 2016).

It was therefore decided to make SL1344 *ΔMlaA* as an alternative to *ΔtolR*, to determine which would be the best strain to use for the OMV vaccine platform.

Again, a colony PCR was carried out with 'mlaA check' primers to confirm a successful knockout (figure 5.2). If the *mlaA* gene was still present in the SL1433 genome, the PCR would simply amplify an 855bp product which included the 756bp *mlaA* gene. If the knockout, by way of replacing *mlaA* with the Kanamycin resistance cassette was successful, the primers would amplify a product of 1.5kb.

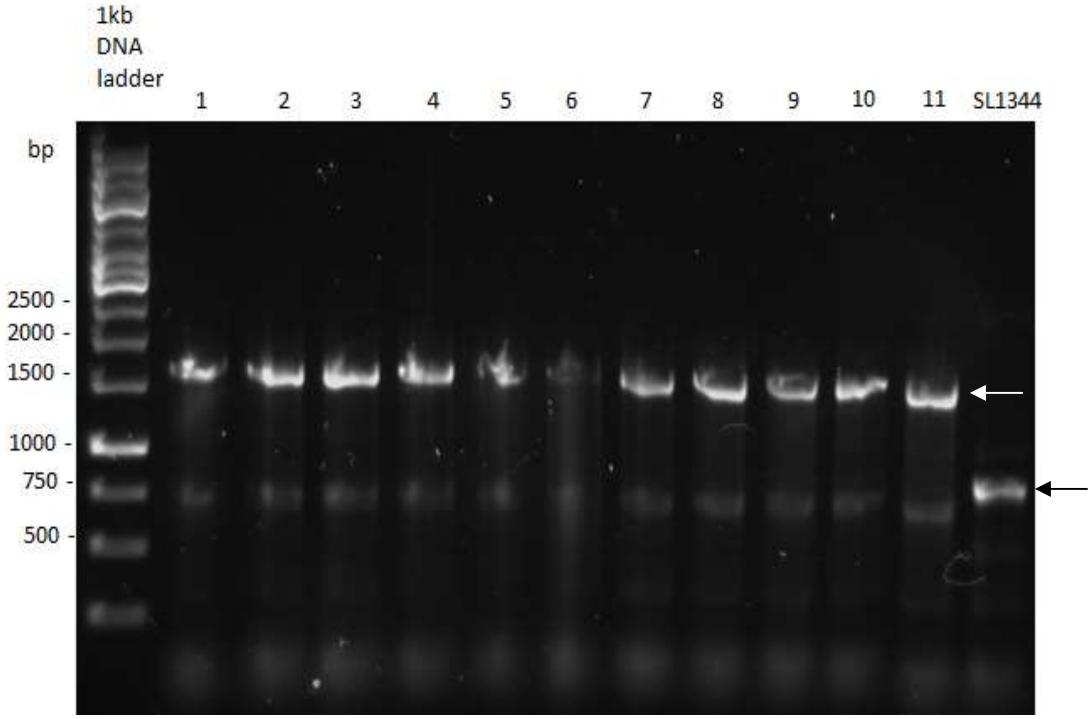


Figure 5.2. Colony PCR screen to determine *mlaA* knockout of SL1344.
0.7% agarose gel showing PCR products from colony PCR using *mlaA* screening primers of the SL1344 genome to determine successful knockout and replacement of the *mlaA* gene with *KanR*. Colonies 1-11 (as shown on gel) were boiled before PCR amplification of the genomic DNA between each screening primer. Positive colonies in which the knockout has been successful show a band at approximately 1.5kb (see white arrow). A negative control, SL1344 which has the *mlaA* gene, shows a band at 855bp (see black arrow).

Following successful construction of gene knockouts, the *KanR* gene was removed using the FLP-out technique with transformation of the temperature sensitive pCP20 plasmid, expressing FLP recombinase. The removal of the *KanR* gene was checked using sequencing on kanamycin sensitive colonies. Each of the confirmed knockout mutant strains were transduced by P22 phage containing the genetic knockout lesion, into a fresh SL1344 strain to lower the risk of secondary mutations occurring. This resulted in the strains SL1344 Δ*tolR* and SL1344 Δ*mlaA*, which had sensitivity to kanamycin and ampicillin, allowing for further selection of strains when transformed with ZE expression plasmids.

5.3 Directing expression of antigens to the cell membrane or periplasm

Once confirmation of the *tolR* and *mlaA* knockouts had been carried out, plasmid constructs were made to direct expression and targeting of the Zika envelope protein to either the outer membrane or periplasm of the SL1344 cells. This would hopefully lead to the incorporation of the ZE protein into the OMVs, either on the vesicle surface, or packaged within the lumen.

5.3.1 Expression plasmid pBAD24-ZE

Firstly, a new expression plasmid was created using the In-Fusion® ligation independent cloning kit (Clontech). Using primers which had a 15-base overlap with the ends of the Zika envelope insert and the linearised pBAD24 cloning vector, the pBAD24-ZE plasmid was successfully constructed and transformed into Stellar® competent *E. coli* (Clontech) (figure 5.3).

This was then checked by informative digest (figure 5.4) and sequencing (Eurofins) with pBAD screening primers to confirm the plasmid to allow for further cloning of the protein directing signal sequences. The Zika envelope protein would then be expressed under the control of the araBAD, arabinose inducible promoter.

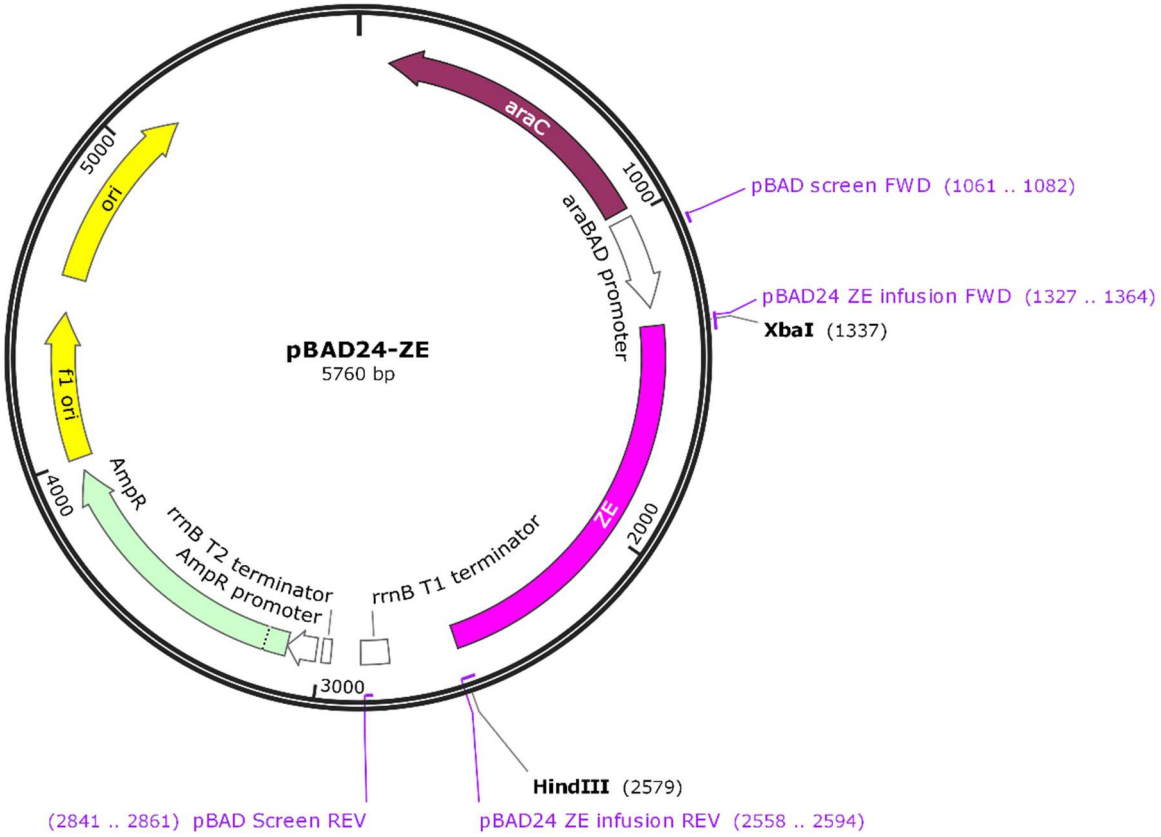


Figure 5.3. Plasmid map of the pBAD24-ZE expression plasmid.
The ZE gene was inserted into the pBAD24 vector using the In-Fusion® cloning kit (Clontech). The Zika envelope protein is under the control of the arabinose inducible promoter (araBAD promoter).

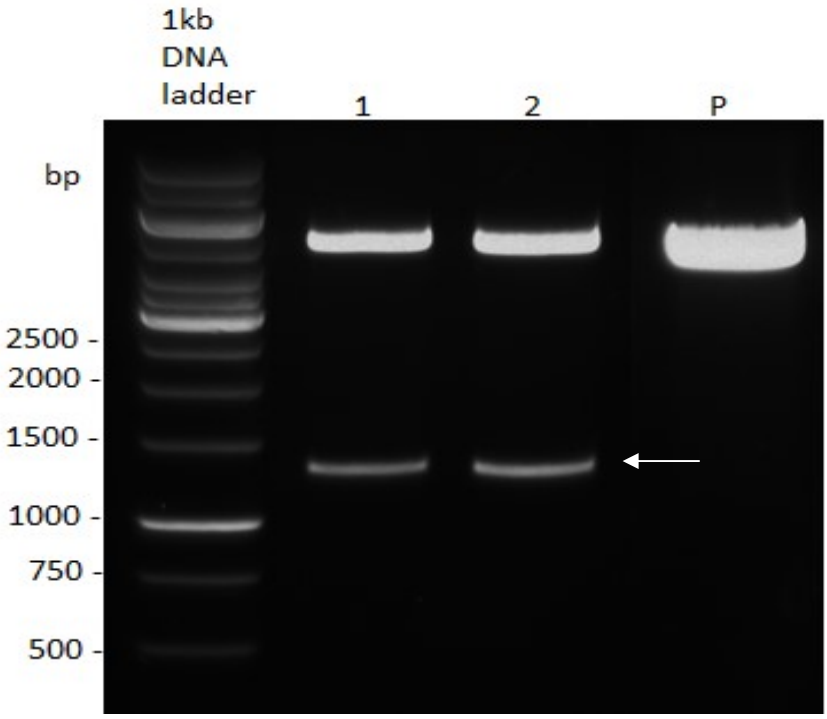


Figure 5.4. Restriction digest screen of putative pBAD42-ZE clones.
0.7% agarose gel, showing pBAD24-ZE digested with *Xba* and *HindIII* from two colonies of Stellar® competent *E. coli*, transformed with the In-Fusion® reaction mix. This gives an insert band approximately 1.2kb, corresponding to the Zika envelope gene (lanes 1 and 2, see arrow). P is uncut plasmid. The pBAD24-ZE plasmid was then further confirmed by sequencing (Eurofins) using the pBAD screening primers (see figure 5.3).

5.3.2 Utilising the OmpA Signal sequence to target protein expression to the bacterial outer membrane

OmpA, Outer Membrane Protein A, is a multifunctional protein, highly conserved amongst Gram-negative bacteria, including *Salmonella* (Krishnan & Prasadarao, 2012). As the name would suggest, it is situated on the outer membrane of these bacteria. By using the signal sequence of OmpA, it could act to direct the Zika envelope protein to the *Salmonella* outer membrane and therefore would be included in the OMVs.

The *Salmonella* Typhimurium OmpA signal sequence is: MKKTAIAIAVALAGFATVAQA.

5.3.3 Constructing pBAD24 vector to express Zika envelope with OmpA signal sequence (OmpAss)

First, a construct was made inserting the OmpA signal sequence into the pBAD24-ZE plasmid, at the N terminal end of the ZE gene, before the start codon (see figure 5.5 for the resultant expressed protein). Oligos of the sense and anti-sense strands of the OmpA sequence were synthesised by Eurofins, with inclusion of the nucleotides from 'digested' restriction sites *XbaI* and *EcoRI*, which sit 20 bases apart on the pBAD24 vector. Each OmpA oligo was annealed and ligated into the pBAD24-ZE vector using T4 DNA ligase.

Figure 5.5. Schematic presentation of the OmpAss Zika E construct.
In the pBAD24 vector, under the control of the arabinose inducible araBAD promoter, the Zika envelope protein is expressed with the addition of the signal sequence from *Salmonella* Typhimurium OmpA (OmpA SS). This would hopefully act to direct ZE expression to the outer membrane and subsequent incorporation in OMVs.

NEB5α *E. coli* cells (NEB) were transformed with the reaction mix and ampicillin resistant colonies were checked with colony PCR with OmpA Forward and pBAD Screen reverse primers, a positive colony giving a band at approximately 1.5kb (figure 5.6).

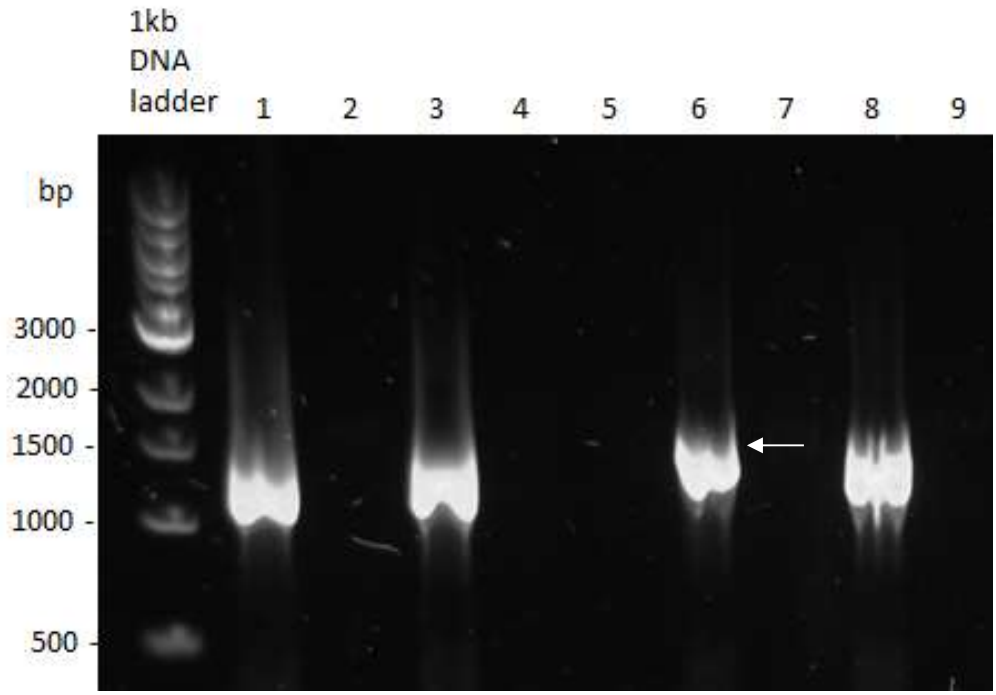


Figure 5.6. Colony PCR screen of pBAd24-OmpAss ZE putative clones.
0.7% agarose gel showing PCR products from colony PCR of transformed NEB5α competent *E. coli* (NEB), using OmpA forward and pBAD reverse screening primers. Positive colonies which include the ompA signal sequence give a band at approximately 1.5kb (see arrow). Colony 6 was chosen for further screening by sequencing (Eurofins) using pBAD screening primers and was shown to read the Zika E gene through in frame with the OmpA signal sequence and araBAD promoter.

5.3.4 Constructing pBAD24 vector to express Zika envelope with OmpA signal sequence (OmpAss) membrane spanning region (MSR) of OmpA

It was also decided after successfully constructing the pBAD24-OmpA ss-ZE plasmid, that expression and targeting of ZE to the outer membrane could potentially be improved by the addition of an extra few bases of the OmpA protein. This would include the first 12 amino acids after the signal sequence which in WT *Salmonella* would be the first membrane spanning region (MSR) of OmpA. This would hopefully act to securely anchor the Zika E protein to the outer membrane.

The MSR was added to the pBAD-OmpA ss-ZE plasmid using the In-Fusion® kit with the MSR included on the reverse primer. The reaction mix was transformed into Stellar® competent *E. coli* cells and colonies picked the following day after incubation on LB agar with ampicillin. Plasmid DNA from a selection of these colonies was purified and sent for sequencing (Eurofins) with pBAD screening primers to confirm the inclusion of the MSR. With only a small addition of nucleotides, it would have been difficult to differentiate the band size difference on a colony PCR gel, so this was not carried out at this time. The sequencing results confirmed the successful addition of the MSR to this plasmid.

The sequence of the *Salmonella* Typhimurium OmpA MSR is: APKDNTWYAGAK.

The resulting construct allowed the expression of the Zika Envelope fused to the OmpAss and MSR (figure 5.7).

Figure 5.7. Schematic presentation of the OmpAss MSR Zika E construct.
In the pBAD24 vector, under the control of the arabinose inducible araBAD promoter, the Zika envelope protein is expressed with the addition of the signal sequence and membrane spanning region from *Salmonella* Typhimurium OmpA (OmpA SS). This would hopefully act to direct ZE expression and anchor to the outer membrane and subsequent incorporation in OMVs.

5.3.5 Utilising the DsbA Signal sequence to target protein expression to the bacterial periplasm

Another potential way of incorporating the Zika envelope in the OMVs would be to direct expression to the periplasm. A gel-like matrix situated between the inner and outer membrane, it contains proteins involved in multiple functions. When OMVs are produced, some of the periplasmic material is incorporated in the lumen of the vesicle. By directing expression to the periplasm, it is hoped that the Zika envelope protein would also be included. It has been shown that proteins can be directed to the periplasmic space by the DsbA, a bacterial thiol disulphide oxidoreductase, signal sequence (Schierle et al, 2003).

The *Salmonella* Typhimurium DsbA signal sequence is: MKKIWLALAGMVLAFSASAA.

5.3.6 Constructing pBAD24 vector to express Zika envelope with DsbA signal sequence (DsbAss)

As with the OmpAss construct, the DsbA signal sequence was inserted into the pBAD24-ZE plasmid, at the N terminal end of the ZE gene, before the start codon (see figure 5.8 for the resultant expressed protein). Oligos of the sense and anti-sense strands of the DsbA sequence were once again synthesised by Eurofins, with inclusion of the nucleotides from 'digested' restriction sites *XbaI* and *EcoRI*, situated 20 bases apart on the pBAD24 vector. Each DsbA oligo was annealed and ligated into the pBAD24-ZE vector using T4 DNA ligase, before transformation into NEB5α competent *E. coli* (NEB).

Figure 5.8. Schematic presentation of the DsbAss Zika E construct.
In the pBAD24 vector, under the control of the arabinose inducible araBAD promoter, the Zika envelope protein is expressed with the addition of the signal sequence from *Salmonella* Typhimurium DsbA (DsbA SS) This would hopefully act to direct ZE to the periplasmic space and subsequent incorporation in the lumen of the OMVs.

Successful incorporation of the DsbA signal sequence was confirmed by colony PCR using DsbA forward and pBAD screening reverse primers, with positive colonies giving a PCR product at approximately 1.5kb (figure 5.9).

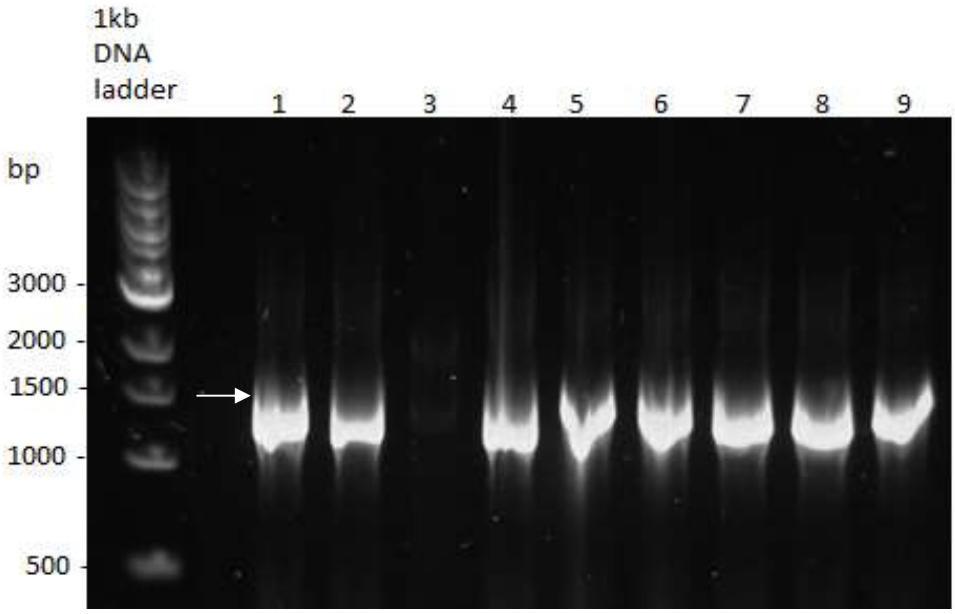


Figure 5.9. Colony PCR screen of pBAd24-DsbAss ZE putative clones.
0.7% agarose gel showing PCR products from colony PCR of transformed NEB5α competent *E. coli* (NEB), using DsbA forward and pBAD reverse screening primers. Positive colonies which include the DsbA signal sequence give a band at approximately 15.kb. Colony 1 (see arrow) was chosen for further screening by sequencing (Eurofins) using pBAD screening primers and was shown to read the Zika E gene through in frame with the DsbA signal sequence and araBAD promoter.

5.3.7 Expression of Zika E in SL1344 ΔtolR and ΔmlaA knockout strains

The confirmed plasmids expressing the Zika envelope and the OmpA or DsbA signal sequences, were then transformed into the *Salmonella* Typhimurium knockout strains, SL1344 *ΔtolR* and SL1344 *ΔmlaA*. Successful transformation was again confirmed by sequencing (Eurofins) purified plasmid using pBAD screening primers.

To determine whether the newly constructed signal sequence plasmids would express the Zika envelope protein, cells which had successfully taken up the new plasmids, were grown overnight and the following day induced with L-arabinose. Cells were pelleted and lysed in preparation for SDS-PAGE and western blot with mouse monoclonal anti Zika envelope (figure 5.10).

The plasmids that were successfully transformed and strains tested here are:

SL1344 *ΔmlaA* pBAD24 OmpAss-ZE

SL1344 *ΔmlaA* pBAD24 OmpAss MSR-ZE

SL1344 *ΔtolR* pBAD24 OmpAss MSR-ZE

SL1344 *ΔtolR* pBAD24 DsbAss-ZE

Each showing expression of the approximately 45kDa Zika envelope protein.

Unfortunately, the DsbA plasmid was not successfully transformed into SL1344 *ΔmlaA* and the OmpA (no MSR) was not successfully transformed into SL1344 *ΔtolR*.

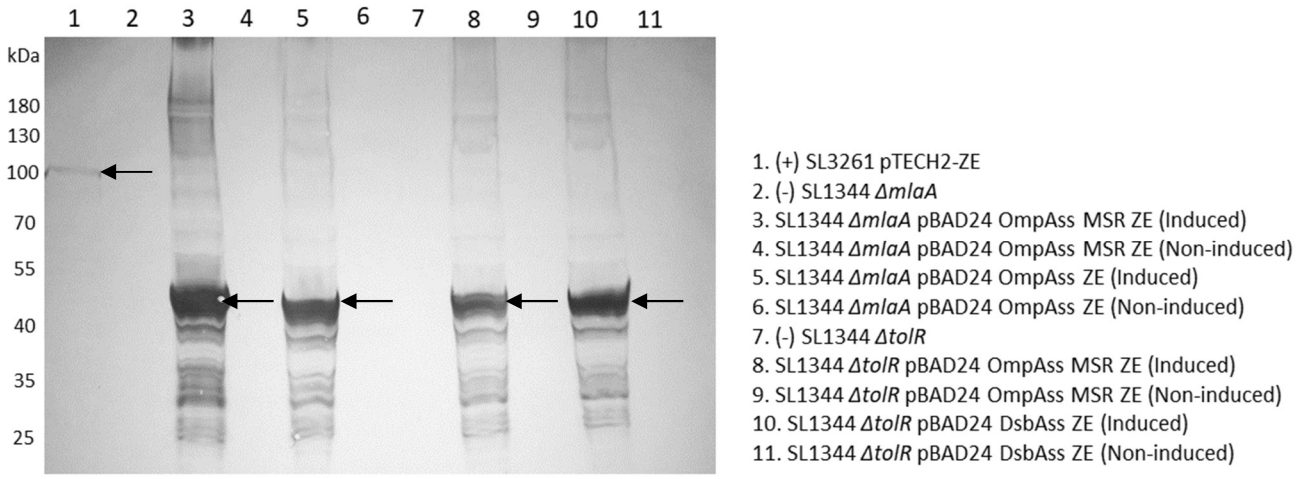


Figure 5.10. Expression of Zika E in SL1344 ΔtolR and ΔmlaA knockout strains.
Western blot showing expression of Zika envelope protein in SL1344 Δ*mlaA* and SL1344 Δ*tolR* cell lysates harbouring pBAD24 plasmids expressing ZE with the OmpA and DsbA signal sequences or OmpA signal sequence plus the membrane spanning region (MSR) of OmpA. The blot was probed with monoclonal mouse anti-Zika envelope. Lane 1 is a positive control of SL3261 lysate harbouring the pTECH2-ZE plasmid as described in chapter 4, expressing a Tet C-ZE fusion protein (approximately 97kDa, see arrow). Lanes 2 and 7 are SL1344 Δ*mlaA* and SL1344 Δ*tolR* cell only negative controls respectively. Each strain harbouring each plasmid was run both with (lanes 2, 5, 8 and 10) and without (lanes 4, 6, 9 and 11) L-arabinose induction. The Zika E protein is clearly visible as a band at approximately 45kDa in the arabinose induced lysates.

5.4 Outer Membrane Vesicle production

Following confirmation that the SL1344 Δ*mlaA* and SL1344 Δ*tolR* strains were able to express the Zika envelope protein harbouring the pBAD24 expression plasmids with the OmpA or DsbA signal sequences included, the Outer Membrane Vesicles were purified from spent supernatant from induced SL1344 strains to determine if the Zika envelope would be incorporated.

5.4.1 Fractionation of OMVs

Firstly, the purified OMV fraction from each of the knockout strains was run on SDS-PAGE gel to allow comparison between each strain to determine which would have the potential to produce more OMVs. After ultracentrifugation of filter sterilised supernatant, the OMV pellet was resuspended in 1ml sterile PBS. The same volume of sample from each strain was then prepared for SDS-PAGE (figure 5.11)

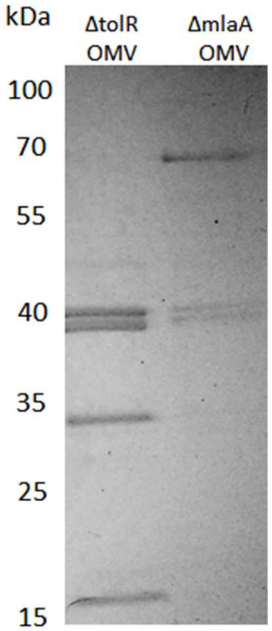


Figure 5.11. SDS-PAGE of the purified Outer Membrane Vesicle fraction from SL1344 knockout strains.
The pellet resulting from ultracentrifugation of spent supernatant from overnight growth of SL1344 Δ*tolR* and SL1344 Δ*mlaA* strains was resuspended in PBS. Equal volumes of sample were boiled with reducing sample buffer before running SDS-PAGE. The gel was subsequently stained with InstantBlue™ (Expedeon).

It was then decided to proceed with the SL1344 Δ*tolR* strain as it appeared, due to the band intensity on the InstantBlue™ stained gel, that there was a higher amount of OMV production from this strain than SL1344 Δ*mlaA*.

As shown above, SL1344 Δ*tolR* cells were transformed with the pBAD24 plasmids expressing Zika E with the OmpA signal sequence, plus MSR or the DsbA signal sequence. The OMVs were purified from spent supernatant of arabinose induced cultures and run on SDS-PAGE, stained with InstantBlue™ to visualise the OMV fraction versus the cell lysates (figure 5.12).

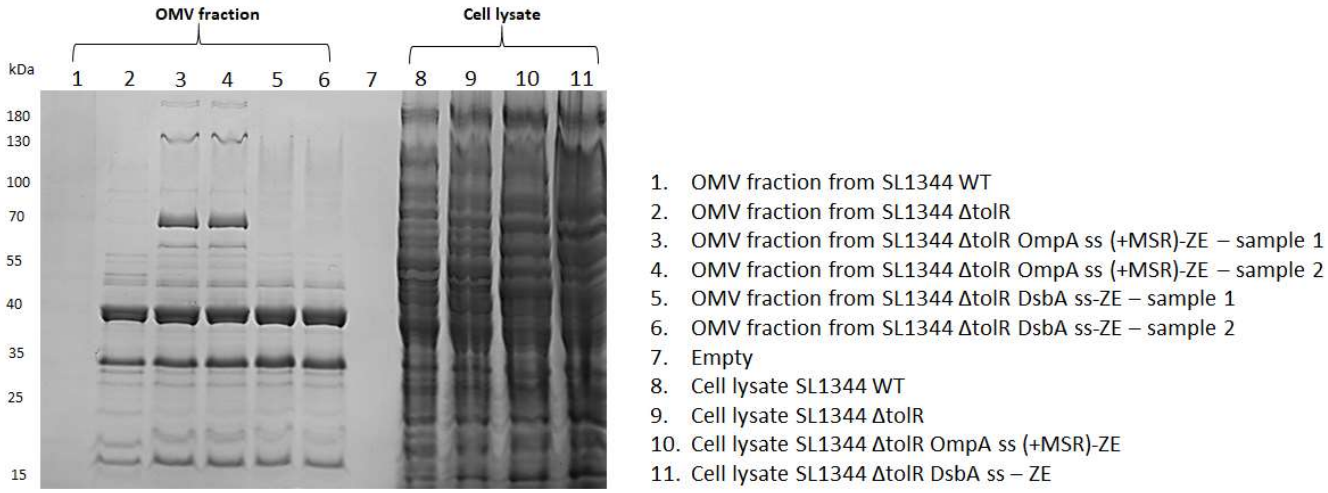


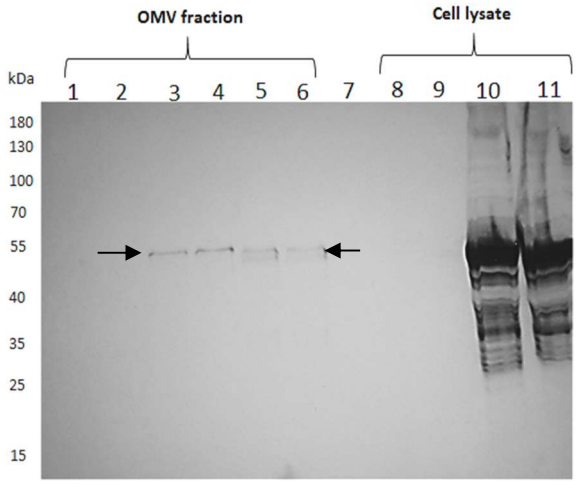
Figure 5.12. SDS-PAGE of purified outer membrane vesicles from SL1344 Δ*tolR* cells.
OMVs were fractionated by ultracentrifugation of spent supernatant and run on SDS-PAGE. Cell lysates were prepared from the pellet of the same cultures. The gel was stained with InstantBlue™. Lane 2 shows the OMV fraction from WT SL1344 cells and lane 2 from SL1344 Δ*tolR* cells. Lanes 3-6 show OMVs purified SL1344 Δ*tolR* cells harbouring the pBAD24-OmpAss(MSR)-ZE or pBAD24-DsbA-ZE plasmids. Lanes 8-10 show cell lysates from each of these samples.

It is clear to see, when compared to the protein profile of purified OMVs, that the SL1344 Δ*tolR* cells produce far more than the WT strain, where only very faint bands are visible (figure 5.12 lane 1). Each sample of the *tolR* knockout strain, both with and without plasmid, show intense bands, showing OMV proteins. These are similar to those shown by Bai et al, when OMVs were purified after *Salmonella* Typhimurium was grown in LB media (Bai et al, 2014). The cell lysates from the same samples were also included as a comparison, the decrease in the number of bands in the OMV fraction compared to the total cell lysates is very clear and shows successful purification of the vesicles.

Interestingly, a strong band of approximately 70kDa, appears in the OMV samples of SL1344 Δ*tolR* strain, when harbouring the construct using the OmpA signal sequence plus MSR (figure 5.12 lanes 3 & 4). This is not seen when the strain harbours the construct using the DsbA signal sequence (figure 5.12 lanes 5 & 6). This protein is not detected with anti-Zika envelope antibodies (see figure 5.14), so at present, it is not clear what this is. This could be elucidated using MALDI-TOF mass spectrometry to carry out peptide mass fingerprinting and determine the sequence of the protein.

5.4.2 Expression of Zika envelope in OMVs

Following confirmation of successful purification of outer membrane vesicles, the same samples were again run on SDS-PAGE and transferred for western blot, to determine whether the OMVs contained the directed Zika envelope protein. When probed with monoclonal mouse anti ZE, bands were seen at the expected size of the combined OmpAss (plus MSR) or DsbAss and ZE proteins (figure 5.13).



1. OMV fraction from SL1344 WT
2. OMV fraction from SL1344 ΔtolR
3. OMV fraction from SL1344 ΔtolR OmpA ss (+MSR)-ZE – sample 1
4. OMV fraction from SL1344 ΔtolR OmpA ss (+MSR)-ZE – sample 2
5. OMV fraction from SL1344 ΔtolR DsbA ss-ZE – sample 1
6. OMV fraction from SL1344 ΔtolR DsbA ss-ZE – sample 2
7. Empty
8. Cell lysate SL1344 WT
9. Cell lysate SL1344 ΔtolR
10. Cell lysate OMV fraction from SL1344 ΔtolR OmpA ss (+MSR)-ZE
11. Cell lysate OMV fraction from SL1344 ΔtolR DsbA ss – ZE

Figure 5.13. Expression of Zika envelope in SL1344 outer membrane vesicles.
Western blot showing expression of Zika envelope protein in Outer Membrane vesicles purified from spent supernatant of SL1344 Δ*tolR* cells harbouring pBAD24 expressing the Zika envelope protein with the OmpA (plus MSR) or DsbA signal sequences. Lanes 1 and 2 are negative controls from cells containing no plasmid, lanes 3 and 4 and lanes 5 and 6 are two samples of each set of purified OMVs from SL1344 Δ*tolR* cells harbouring pBAD24-OmpAss(MSR)-ZE (3&4) or pBAD24-DsbAss0ZE (5&6). Lanes 8-10 are cell lysates from the same cells, with negative controls in lanes 8 and 9. Blot was probed with 1:2000 monoclonal mouse anti Zika envelope (Aaltobioreagents) followed by 1:4000 Rabbit anti-mouse HRPO. Bands are seen at approximately 50kDa corresponding to the size of the Zika envelope (and in the case of lanes 3 and 4, the extra membrane spanning amino acids of *OmpA*), denoted by arrows.

5.4.3 TEM of OMVs produced from SL1344 ΔtolR Salmonella strain

After producing protein profiles which seemed to correspond with successful OMV purification (figures 5.11 and 5.12), it was decided that the vesicles should be visualised using transmission electron microscopy (TEM). The samples (in PBS) of purified OMVs from SL1344 Δ*tolR* cells were adsorbed to Formvar grids for negative staining with uranyl acetate and observed with a Philips CM100 Transmission Electron Microscope. These images clearly show an abundance of OMVs in the samples from SL1344 Δ*tolR* cells, both with and without plasmid, and much less from SL1344 WT (figure 5.14).

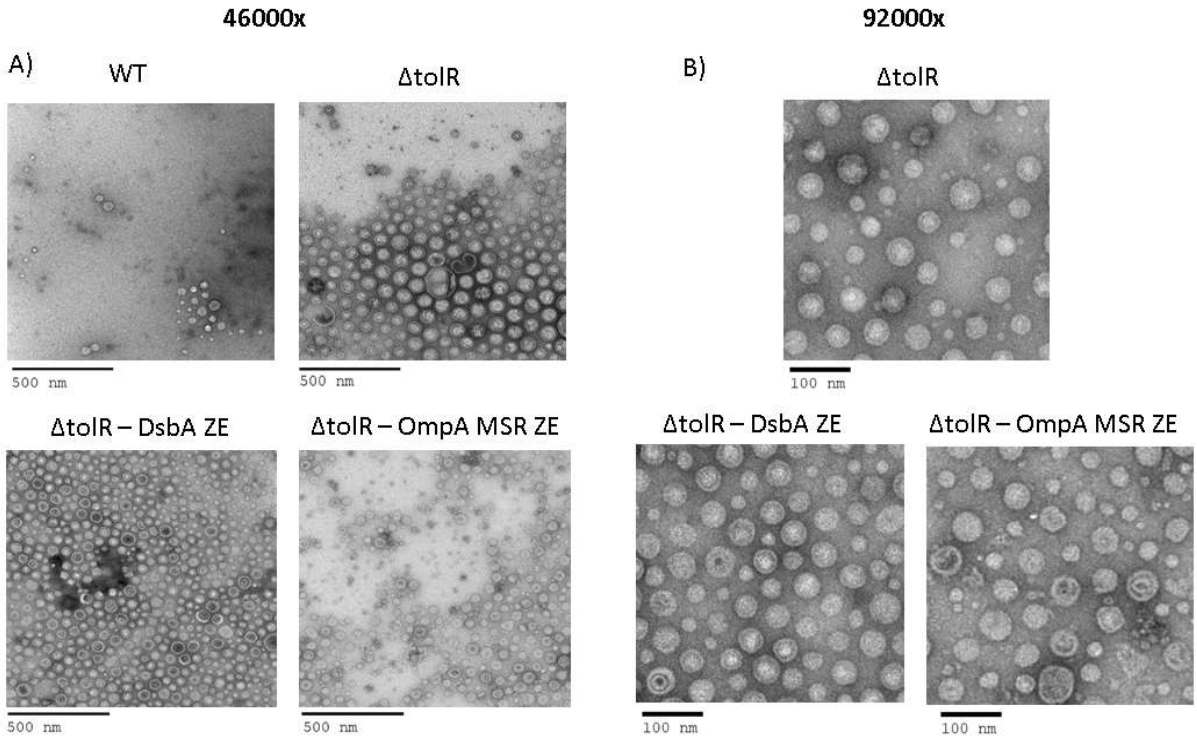


Figure 5.14. Transmission Electron microscopy of OMVs.
OMVs purified from SL1344 (WT) or SL1344 Δ*tolR* (ΔtolR) cells with and without the Zika envelope expressing pBAD plasmids. Magnification was either 46000x (A) or 92000x (B). Bars are 500nm and 100nm respectively. Images were acquired by Tracey Davey (Newcastle University Electron Microscopy Research services).

5.5 Discussion

By generating knockouts of the *tolR* and *mlaA* genes of *Salmonella* Typhimurium SL1344, strains were successfully able to produce a much higher yield of outer membrane vesicles than the wild type strain. Using the pBAD24 expression vector, the Zika envelope protein was expressed by these knockout cells genetically fused with the signal sequences from either the *Salmonella* Typhimurium OmpA or DsbA proteins. This allowed the targeting of expression of ZE to the outer membrane or periplasm respectively, and subsequent incorporation in the OMVs. These OMVs were successfully purified from spent supernatant by ultracentrifugation and visualised with transmission electron microscopy.

From Western blot analysis of purified OMVs, it is clear to see that Zika envelope is detected only in the OMV fractions purified from cells harbouring the pBAD plasmids expressing ZE (figure 5.13). This suggests that the expression of the Zika envelope protein has been successfully directed to either the outer membrane, with the OmpA signal sequence, or the periplasm, with the DsbA signal sequence, and that is has been incorporated into the OMVs themselves. This shows the potential of this method in producing vesicles which could deliver the antigen to the host without the need for a live bacterial delivery system as described in chapters 3 and 4. Improvements to expression would need to be investigated so as to allow for maximum antigenic dose, however as a proof of concept, it has been shown to be a promising possible avenue for further investigation. This will be discussed further in chapter 6.

Chapter 6 – Discussion and future work

The development of vaccinations to some of the most deadly or problematic pathogens the world has ever seen is an endeavor that should be continuously explored. The lack of effective vaccines licensed for protection against Ebola or Zika Viruses, highlighted with the recent widespread outbreaks, have increased efforts into research and clinical trials.

This project aimed to investigate the uses of *Salmonella* as vaccine delivery platforms, in an attempt to create a prototype vaccine that would have the eventual advantages of oral administration, no need for cold storage and also be cost effective to develop and produce in comparison with other vaccine strategies.

6.1 *Salmonella* based vaccine strategies

Live attenuated *Salmonella* vaccines have been shown to be more effective in providing protection against typhoid than heat killed or phenol inactivated strains, due to their ability to grow and persist in intracellular compartments. These strains can also express heterologous antigens from a variety of pathogens for delivery to the host immune system (Khan et al, 1994a; Khan et al, 1994b; Chabalgoity et al, 1996; Lee et al, 2000; Mckelvie et al, 2008). It was hoped that similar results would be seen in this study, expressing Ebola GP and Zika envelope proteins from *Salmonella* vaccine strains.

Mucosal vaccines allow for the induction of both a local mucosal response, followed by a more widespread systemic response. A live *Salmonella* based vaccine would be an ideal vehicle for this as, when ingested, the bacterium first infects the intestinal mucosa before propagating and persisting systemically. A successful vaccine using this delivery route, would be extremely attractive as it would stimulate a protective immune response at the mucosa, and would hopefully block pathogen entry at this site (Gayet et al, 2017).

In the case of Ebola Virus infection, which is primarily transmitted through mucosal surfaces (Baseler et al, 2017), this is certainly a logical avenue to take and could be very effective in preventing mucosal transmission in the first place. While Zika Virus is a mosquito-borne virus, and thus transmitted primarily by arthropod vectors, it has been shown to be present in the

body fluids (for example; saliva, tears, breast milk, semen and urine) of those infected and reports have been made showing the possibility of sexually transmitted infection. There is therefore a suggestion that there could be a risk of Zika Virus transmission via the mucosal route, particularly from patients with a high viral load (Newman et al, 2017). An effective vaccine using this system, allowing both mucosal and systemic protection would also have the advantages of being very easy to administer (orally) and cost effective to produce.

The live-attenuated poliovirus vaccine, which as discussed in section 1.1.2, is able to provide mucosal and systemic protection against the virus is an example of a very successful vaccine which is administered orally. The advantage here of the *Salmonella* based system for vaccine delivery, is that this could be taken in pill form (such as Ty21a) and not as a liquid (as with the polio vaccine) which can be harder to administer and is known to have a strong and unpleasant taste. Thus, a pill could be seen as more acceptable and does not require any specialist equipment or training to administer as opposed to parenteral methods of vaccination.

While live vaccines can be effective, and are shown to be less likely to induce adverse events in patients than killed *Salmonella* (Engels & Lau, 1998), they are often unsuitable for certain patient groups such as the very young (under 2 years of age), the elderly or the immunocompromised (Milligan et al, 2018). A non-living vaccine delivery platform, may help to widen the access for these vaccines.

Another potential advantage of using non-living vaccines could be the delivery of the antigenic dose. With live vaccines, expressing the antigen as in this study, the dose is limited by the *in vivo* expression within the *Salmonella* vaccine strain, and the impact that the expression of the heterologous antigen has upon the physiological health of the host cell. With a non-living vaccine delivery platform, the antigen is already present and can be administered in as much quantities as necessary to elicit a protective immune response without the need to rely on *in vivo* expression.

One potential alternative vaccine delivery system could be the use of synthetic liposomes, which can be engineered to incorporate a wide range of antigens and subsequently can elicit strong protective immune responses. This system would likely be administered via

intramuscular injection, yet could be used for intranasal mucosal immunisation depending on the liposome composition (Schwendener, 2014). Such a vaccine would be safer than using live attenuated pathogens but could be a less cost-effective method than the use of naturally occurring bacterial outer membrane vesicles (OMVs) for example.

The use of outer membrane vesicles from Gram-negative bacteria as a vaccine delivery system is currently receiving growing attention. As well as the potential to carry heterologous antigens, they also act as a self-adjuvant due to their incorporation of immunostimulatory molecules naturally occurring on the bacterial surface (Van der pol et al, 2015; Tan et al, 2018). OMVs have been successful in conferring protection in mice against pathogens such as *Bordetella pertussis* (Roberts et al, 2008), *Haemophilus influenzae* (Roier et al, 2012), *Streptococcus pneumoniae* from heterologous Pneumococcal Protein PspA (Muralinath et al, 2011) and elicit neutralising antibodies towards heterologous *Chlamydia muridarum HtrA* (Bartolini et al, 2013). An OMV vaccine against *Neisseria meningitidis* has also been proven safe and effective in humans (Holst et al, 2009). The OMV platform clearly has some advantages over the live attenuated *Salmonella* vaccine, yet to allow for a mucosal administration route, which could be more effective depending on the pathogen, it is likely that a much higher dose would be required compared to an injectable vaccine (Holst et al, 2009). If this was optimised however, then it is possible that OMVs can be used as a potentially safer mucosal vaccine alternative to a live delivery system, mitigating the risk of attenuated strains regaining virulence and widening access to these vaccines in vulnerable patient groups.

It is hoped that a protective immune response from *Salmonella* OMVs containing Zika envelope will be elicited in immunised mice to further test this proof of concept idea.

6.2 Expression of Ebola GP in attenuated *Salmonella* vaccine strains

The development of a panel of constructs, allowed exploration of a variety of ways in which to try to get the best possible expression of Ebola GP, a feat which has proved difficult for many groups. The panel included not only the full-length GP (devoid of the hydrophobic signal and transmembrane regions), but also sub fragments of the protein containing known epitopes recognised by known protective or neutralising antibodies, including those used in therapeutic cocktails (Qiu et al, 2014; Gonzalez-Gonzalez, 2015). This approach, targeting known B-cell epitopes, which have been shown to be vital in protection against Ebola Virus infection would hopefully ensure that the peptides expressed in the vaccine are able to elicit these protective and neutralising antibodies (see figure 6.1).

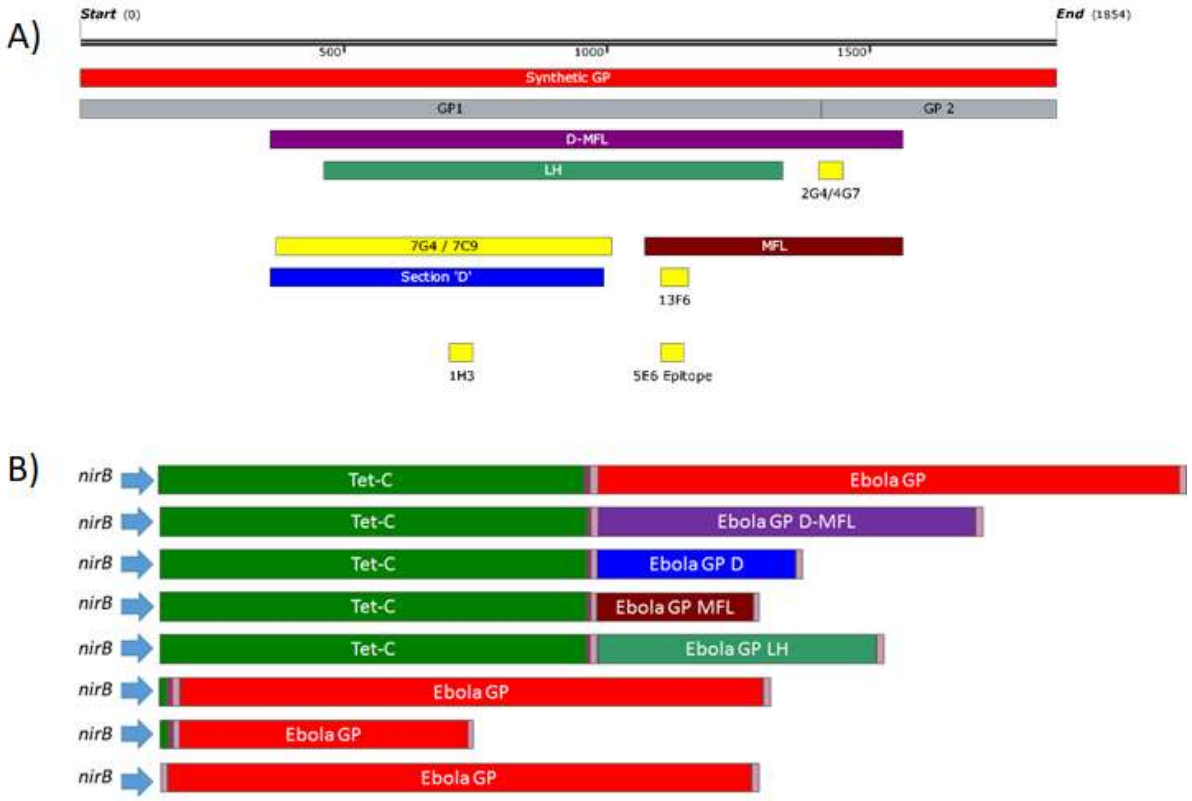


Figure 6.1. Ebola GP sub fragments, conserved epitopes and panel of generated constructs. A) Schematic diagram showing epitope locations (in yellow) for relevant mouse monoclonal antibodies in relation to sub-fragments of Ebola GP and the full-length protein. Epitopes to monoclonal antibodies used in therapeutic cocktails such as 2G4 and 4G7 (Zmapp) and 13F6 (MB-003) are also shown. (Qiu et al, 2011; Gonzalez-Gonzalez, 2015). **B)** Schematic presentation of the panel of Ebola GP fusion constructs generated in this study.

Referring to figure 3.31 in section 3.3.1, it was shown that there was a variation in expression levels between each of the GP expression constructs made. *Salmonella* harbouring the pTECH10-GP plasmid, which was an attempt to express the GP protein alone, did not express any GP protein detectable by western blot. This was somewhat rescued by the addition of the TetC full RBS, including 30 bases downstream of the initiation codon, resulting in the pTECH11-GP plasmids, which *Salmonella* were able to use to express GP, potentially due to an increase in stability of the interaction between the 16S rRNA and GP mRNA (Etchegaray & Inouye, 1999) (see section 3.2.2.3 for more detail). The addition of the full TetC protein as a fusion partner however, dramatically increased protein expression of the full-length GP.

The Ebola GP gene was subsequently demarcated into smaller sub fragments (see figure 6.1) with differing results in regards to expression. The D and MFL sub fragments, so chosen due to their incorporation of known protective epitopes and ability of expression in bacteria (Das et al, 2007; Wang et al, 2014) did express in *Salmonella* as fusions to TetC, but not to the levels that were anticipated. The amalgamation of these sub fragments, into the larger DMFL sub fragments showed a much higher level of expression when fused to TetC, even it would seem higher than the full-length GP. This fragment also incorporates the mucin like domain, which has been shown to include an IgG specific immunodominant region, with B-cell epitopes eliciting protective antibodies in survivors of Ebola virus infection (Becquart et al, 2014). The sub fragment LH also expressed relatively well as a TetC fusion partner.

The varying levels of expression seen in *Salmonella* between these constructs made the choice of which to take forward to the next stage of development relatively easy. When transformed into attenuated *Salmonella* vaccine strains, the cells harbouring pTECH2-GP and pTECH2-GP DMFL consistently gave the strongest bands on western blot when probed with an anti-Ebola GP antibody. It was then therefore decided to use vaccine strain SL3261 (which showed better expression compared with other vaccine strains – figure 3.33) with these two constructs in further stability and then *in vivo* testing.

In all of the samples tested, the western blots, probed with both anti-TetC and anti-Ebola GP, the proteins expressed showed a ladder-like pattern of multiple bands in each lane. These bands of smaller proteins, which appeared lower than the full-length fusion proteins could be the result of either protein breakdown or premature termination of translation. These have

shown however, to still contain protective epitopes, both linear and conformational, when probed with specific monoclonal antibodies to Ebola GP (see section 3.3.1.1, table 3.1, figure 3.3.2 and section 6.2, figure 6.1). This then is not necessarily a concern, as these smaller fragments could still elicit protective antibodies upon immunisation.

6.2.1 Stability of Ebola GP expression plasmids in vitro

In vitro stability testing was carried out to determine whether *Salmonella* vaccine strains stably retained the pTECH2-GP plasmids in the absence of antibiotic selection (here, ampicillin). It was shown that when compared with the pTECH2 control plasmid, which was shown to be 100% stable, the pTECH2-GP and pTECH2-GP DMFL plasmids were stable at 100% and 91.5% respectively (Table 3.3). This was encouraging as when testing these strains in *in vivo* immunisation experiments, it would not be practical to select with ampicillin.

6.2.2 Stability of Ebola GP expression plasmids in vivo

Once the plasmids had been shown to be stable *in vitro,* a pilot immunisation experiment was carried out to ensure stability *in vivo*. BALB/c mice were immunised intravenously with the *Salmonella* SL3261 vaccine strain harbouring the GP and GP DMFL expressing plasmids. *Salmonella* recovered from the livers and spleens of the animals were grown in the absence of ampicillin selection and compared to cells harbouring the pTECH2 plasmid control, which had a stability of 95-98%, the cells harbouring the GP expressing plasmids were lower. The cells harbouring the pTECH2-GP DMFL plasmid showed good stable retention of approximately 90-96%, however the retention of the pTECH2-GP plasmid with no antibiotics was 70-76% (figure 3.34).

The higher retention of the pTECH2-GP DMFL plasmid compared to its full-length GP expressing counterpart, pTECH2-GP, could be due to the potential inherent toxicity of the GP protein. It has been shown that the full-length GP is difficult to express in bacterial cells (Zai et al, 2016; Das et al, 2007). This could mean that the DMFL sub-fragment is less toxic than

the full-length GP and therefore the plasmid expressing DMFL, is more likely to be retained by the *Salmonella* when no antibiotic is present. Cells may therefore be less likely to retain the pTECH2-GP plasmid in the absence of ampicillin selection.

These results show that overall, the plasmids are quite stable after *in vivo* passage, albeit with varying results, and importantly, *Salmonella* strains show continued expression of the TetC and TetC-GP fusion proteins after recovery from the livers and spleens (figure 3.35). The strains harbouring these plasmids would therefore be testable in a larger scale immunisation experiment to determine any immune response to the GP antigen.

6.3 Expression of Zika envelope in attenuated *Salmonella* vaccine strains

As with the Ebola GP, a panel of constructs were made in an attempt to facilitate as much expression as possible of the Zika envelope protein (figure 6.2). With the increase in expression of Ebola GP shown by the constructs expressing TetC-GP fusion proteins it was thought that this may also be the case for ZE.

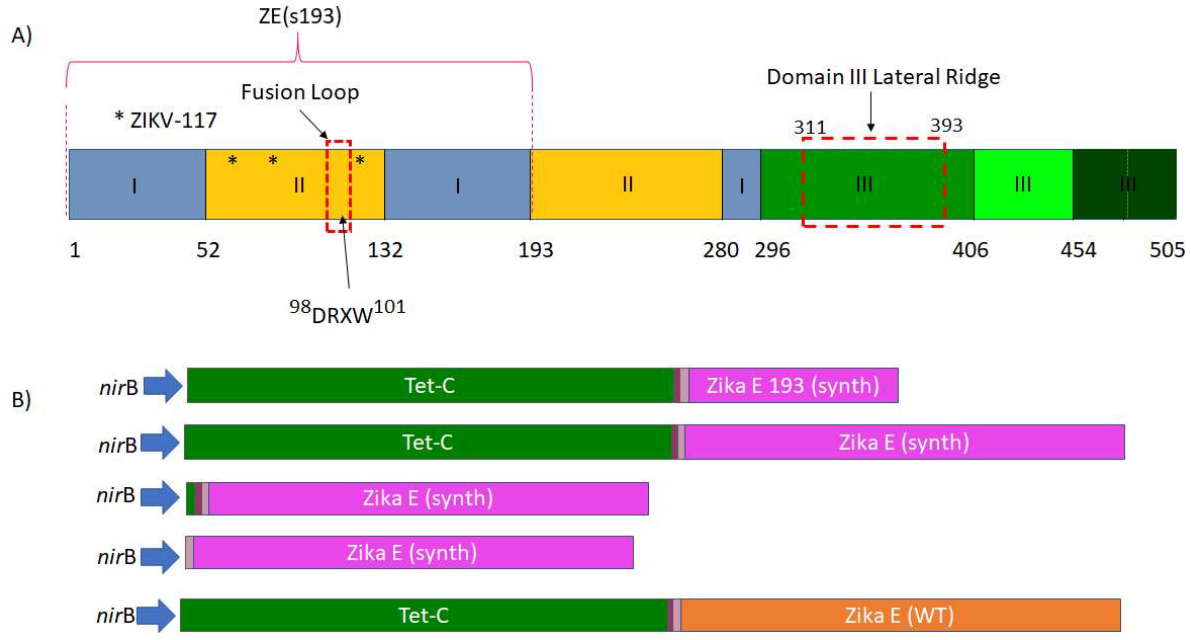


Figure 6.2. Zika Envelope sub fragments, conserved epitopes and panel of generated constructs. A) Schematic diagram of domains I, II and III of Zika E, including approximate locations for some known protective epitopes (shown by red boxes). Adapted from Dai et al, 2016 (Sapparapu et al, 2016; Zhao et al, 2016). **B)** Schematic presentation of the panel of Zika E fusion constructs generated in this study.

Three TetC-Zika envelope expressing constructs were made and showed some variation in expression. The most notable, being the clear difference between the constructs containing the wild type ZE gene sequence and the synthetic, codon optimised sequence. It was clear to see from western blot analysis when probed with both anti-TetC and monoclonal anti-ZE (see chapter 4, section 4.2.1, figure 4.7), the codon optimised ZE sequence offers better expression than the wild type. The enhanced expression of the synthetic optimised sequence, designed to use as many *Salmonella* Typhimurium favoured codons as possible, shows that this is one simple method that can act to improve heterologous protein expression in bacterial systems such as this.

The construct expressing the TetC - truncated Zika envelope fusion protein, the first 193 amino acids –pTECH2- ZE 193s, appears to express about as well as the full-length synthetic ZE. This construct was not recognised however by the monoclonal anti-ZE that was used (aaltobioreagents). It was not possible to determine what the epitope for this antibody was as it has not been mapped, however we can ascertain from these results that it lies towards the C terminal of the ZE protein, or at least after the first 193 amino acids.

Again, as with the Ebola GP constructs, the ZE constructs were expressed in a panel of *Salmonella* Typhimurium vaccine strains to determine which to use for the *in vivo* analysis. As the best expression for the Ebola GP constructs came from the C5*htrA* and SL3261 strains, these were tested with the ZE constructs. Once again, it was determined that slightly better expression was seen in the SL3261 strain and this was therefore chosen to take forward.

Expression of the TetC-ZE fusion proteins had been confirmed, and additionally, seemed to express better than the Ebola GP fusion proteins. This could be due to the ZE protein being less toxic to cells and therefore easier to express. It was decided that it would also be tested whether the ZE protein could be expressed alone, with no TetC fusion partner. Constructs were made which allowed this, pTECH10-ZE(s) and pTECH11-ZE(s) which contained none or only the RBS plus a 30bp downstream sequence of TetC respectively. In contrast to the pTECH10-GP construct, where no GP expression was seen at all, the Zika envelope protein was expressed successfully with no fusion partner. This again could be due to the lower toxicity of the ZE compared with Ebola GP. The pTECH11 construct, which had rescued the Ebola GP expression however, did not appear to improve expression for the Zika envelope.

With both of these constructs, while ZE was still expressed by the *Salmonella,* it was again not to the level of TetC-ZE fusion protein expressed by the pTECH2 constructs. Therefore, it was decided not to take these constructs forward for *in vivo* testing.

As it had been shown that the Zika envelope could be expressed alone in this system, it was thought that it may be possible to rescue the expression of the Ebola GP by way of constructing a ZE-GP fusion. In this system, GP is unable to be expressed with no fusion partner, as shown with the pTECH10-GP construct, however expression is rescued by fusion with TetC. By creating the construct pTECHZE-DMFL, it was shown that a Zika E-Ebola GP DMFL fusion protein could be expressed by the *Salmonella* cells (see section 4.4.2 figure 4.16). Once again however, this was nowhere near the level of expression seen with the TetC-GP or TetC-ZE fusions and therefore it was decided not to pursue this construct further as TetC is considered a superior fusion partner when total protein expression is considered.

6.3.1 Stability of Zika envelope expression plasmids in vitro

In vitro stability testing was carried out to determine whether *Salmonella* vaccine strains stably retained the pTECH2-ZE plasmids in the absence of antibiotic selection (here, ampicillin). It was shown that when compared with the pTECH2 control plasmid (100% stable), the pTECH2-ZE(s) and pTECH2-ZE(s193) plasmids were stable at 81.8% and 100% respectively (table 4.1). This again showed that the plasmids were stably retained by the *Salmonella* vaccine strain even without exogenous ampicillin selection and would allow the pilot *in vivo* immunisation to be carried out without the fear that the plasmids would be lost.

6.3.2 Stability of Zika envelope expression plasmids in vivo

Once the plasmids had been shown to be stable *in vitro,* a pilot immunisation experiment was carried out to ensure stability *in vivo*. BALB/c mice were immunised intravenously with the *Salmonella* SL3261 vaccine strain harbouring the ZE(s) and ZE(s193) expressing plasmids. *Salmonella* recovered from the livers and spleens of the animals were grown in the absence of ampicillin selection and compared to cells harbouring the pTECH2 plasmid control, which

had a stability of 95-98%, the cells harbouring the GP expressing plasmids were lower. The cells harbouring the pTECH2-ZE(s193) plasmid showed good stable retention of approximately 91-98%, however the retention of the pTECH2-ZE(s) plasmid with no antibiotics was approximately 50% (figure 4.17). This could mean that the plasmid expressing the truncated ZE protein is more stably retained as expression of the smaller protein may not negatively impact the physiology of the host cell, compared to the full-length ZE.

The *Salmonella* recovered from the organs was tested for expression of the TetC-ZE fusion protein and all samples were shown to express when analysed on western blot, probed with anti-TetC (figure 4.18). There did appear to be variation in expression between samples recovered from different mice, so it was therefore decided that the sample which showed the highest expression of TetC-ZE (Mouse 1, spleen) would be used to immunise mice in the main experiment, as it has previously been shown that further passage *in vivo* can improve plasmid stability, (Chabalgoity et al, 1996) an issue seen with this construct. The expression of the stable TetC-ZE(s193) fusion protein was shown to be high, which hopefully would mean that there would be a good antigenic dose at immunisation.

6.4 Immunogenicity of Ebola GP expressed in attenuated *Salmonella* vaccine strains

From the results of the ELISA assays, it appears that there was no significant IgG response to Ebola GP in mice immunised with *Salmonella* Typhimurium vaccine strain SL3261 expressing either of the TetC-GP fusion proteins (figure 3.36)

It was initially postulated, as there was also a decreased IgG response to TetC (figure 3.37), this may be due to an overall immunosuppressive effect from the vaccine strain. This has been shown before by Eisenstein and colleagues, who reported that mice immunised with the *aroA*$^{-}$ *Salmonella* Typhimurium strain SL3235, showed notable mitogenic suppression, while also being highly immune to *Salmonella* challenge. This effect assumed to be immunomodulatory, rather than a poor overall response, appeared to be mediated by macrophages (Lee et al, 1985). It could be, that this immunomodulatory effect, may result in a lower IgG response due to the lower mitogenesis of B and T cells.

However, when the same sera were tested for IgG response against *Salmonella* Typhimurium LPS (figure 3.38), there was no obvious lack of response seen. This therefore could mean that other factors are the cause of this unexpected lack of response. As has been shown, the expression plasmids continue to express the TetC and both TetC-GP fusion proteins after *Salmonella* recovery from immunised mouse livers and spleens, 11 days post immunisation. As these cells were grown on LB agar post organ recovery however, it could be that expression of the fusion proteins is for some reason decreased when the *Salmonella* is actually persisting *in vivo*. It could also be that the fusion proteins themselves are somehow degraded, resulting in a lower antigenic dose and thus lack of IgG response. It could also be that the known cytotoxicity of the Ebola GP protein is making the *Salmonella* cells sick and thus unable to persist for long enough *in vivo* in the absence of the selective antibiotic advantage.

IgG response was only examined in these assays, given the time limitations of this project. Further immunological insights could be provided by determining the cytokine profiles in the sera of the immunised mice. This would indicate if there was a T cell response, and depending on which cytokines present, what kind of T cell response this may be.

To determine if the apparent lack of response is due to minimal *in vivo* expression, the organs from the immunised mice could be analysed to determine antigen expression, for example using immunohistochemistry on liver and spleen tissue. It may be possible that this would show a difference in expression from *Salmonella* cells growing *in vivo* to those growing *in vitro* (even after recovery from the organs). If this was the case, then this may explain that the lack of response is due to a sub-optimal antigenic dose.

6.5 Immunogenicity of Zika envelope expressed in attenuated *Salmonella* vaccine strains

From the results of the ELISA assays, it appears that there was no significant IgG response to Zika envelope in mice immunised with *Salmonella* Typhimurium vaccine strain SL3261 expressing either of the TetC-ZE fusion proteins. In the group immunised with pTECH2-ZE(s) there are potentially one or two mice that show some response, however when taking into account the surprisingly high values from the sera from TetC only immunised mice, it would be difficult to say that this was a true response (figure 4.19).

Again, as with the GP immunised mice, there is a marked decrease in immune response to TetC (figure 4.20), although interestingly one mouse from each of the TetC-ZE (193s) and TetC-ZE(WT) groups show an IgG response to TetC, comparable to what is seen in the TetC only negative control mice. These results could again point to the fact that the expression of the fusion protein *in vivo* is in some way decreased or the protein is degraded and thus lowering the antigenic dose to sub-sufficient levels. Sera was also tested for response to *Salmonella* Typhimurium LPS (figure 4.21), to determine if there was an overall immunosuppression, however as with the Ebola GP groups, this did not appear to be the case and IgG response to LPS was not significantly different in fusion protein immunised groups, compared with the TetC only immunised control group.

Again, further analysis on the immunised mouse sera or organs could be carried out to provide insight into the cytokine profiles and true *in vivo* expression levels, as discussed in section 6.4.

6.5.1 Measurement of neutralisation activity by Zika E immunised mouse sera

Despite the overall lack of response in immunised mouse sera to Zika E, there were some samples which appeared to be outliers and show a slightly stronger response. It was decided, with the kind help of Professor Alain Kohl and Professor Arvind Patel (Glasgow University) micro-neutralisation testing could be carried out to determine if any of the mice had produced neutralising antibodies to Zika envelope (figure 4.22). It appeared however that this was not the case and no virus neutralisation was seen in any samples from the immunised mice. This again could be due to a sub-sufficient antigenic dose, possibly due to a lack of sufficient *in vivo* expression of the fusion proteins. It could be that taking a more epitope focused approach when designing the Zika envelope constructs may have yielded more favourable results in terms of virus neutralisation.

6.6 Potential improvements to enhance immune response – Future work

It appears in this study, that there were unfortunately no protective immune responses generated towards either the Ebola Glycoprotein or Zika envelope protein, in mice immunised with *Salmonella* vaccine strains expressing these antigens. Given more time, other avenues could be explored which may act to improve the immune response seen here.

One potential method of increasing immunogenicity is to use tandem repeats of known protective epitopes, rather than the full-length protein. Previous studies have shown that by using this approach, antibody responses to a peptide of the P28 Glutathione S-Transferase, from the parasitic worm *Schistosoma mansoni*, increased when more copies of the epitope were fused to the TetC in the pTECH2 plasmid, with an 'octameric repitope' eliciting the strongest response (Khan et al, 1994 a). Also, genetic fusions which expressed 2 or 4 repeated epitopes from glycoprotein D of Herpes Simplex virus (HSV), again in the pTECH2 plasmid as used in here, resulted in an antibody response to HSV, however fusions expressing only one epitope copy only elicited a response to TetC (Chabalgoity et al, 1996). In this study, known protective epitopes of either Ebola GP or Zika E could be cloned repeatedly into the pTECH2 plasmid which may make for a more directed and specific immune response to these antigens. This would be a relatively simple avenue to explore given more time.

It has also been shown that using an alternative vaccine strain could have an effect on the levels of expression seen of heterologous guest antigens. It has been shown that the C5*htrA* strain may express longer antigens better than the *aroA*$^{-}$ SL3261 strain. SL3261 has been shown to be unable to elicit an antibody response to the guest peptide of P28 Glutathione S-Transferase, compared to C5*htrA* (see above) in immunised mice (Chabalgoity et al, 1996). In this study, there was no evidence *in vitro* of the C5*htrA* strain showing better expression of the TetC fusion proteins (with either Ebola GP or Zika E), however this was not tested *in vivo* and a further immunisation study to compare between the two strains could elicit more favourable immune responses.

Another possible element to consider is the promoter by which expression of the fusion protein is controlled. There does need to be a balance between antigen expression (yet ensuring a sufficient antigenic dose) and potential toxicity to the *Salmonella* which could

reduce its effectiveness, so unregulated antigen expression, which may be high, may in fact be detrimental. This could be solved by the use of *in vivo* inducible promoters. In this study, the pTECH2 expression vector utilised the *nirB* promoter to express the TetC-GP or TetC-ZE fusion proteins, aed anaerobically and when the *Salmonella* enters host cells, including macrophages (Everest et al, 1995). Possible alternative promoters could be used to try and increase *in vivo* expression of antigens. For example, the *htrA* promoter, which is dependent on the σ^E transcription initiation factor, was shown to increase expression of LacZ in attenuated *Salmonella* in the presence of H_2O_2, which could be seen in the macrophage environment (Everest et al, 1995). Another study has shown that the use of the *dmsA3* promoter (which like the *nirB* promoter is part of the FNR dependent family and induced by anaerobic conditions), to express TetC in *Salmonella* Typhi vaccine strain CVD 908-*htrA*, does not show an apparent difference in TetC expression, compared to the use of the *nirB* promoter, however elicits a significantly higher IgG response to TetC in immunised mice (Orr et al, 2001).

An alternative method of heterologous antigen expression, may also improve the immune response seen in immunised animals. It could be that retaining the heterologous antigen in the cytoplasm of the *Salmonella*, as seen here with the live vaccine approach, may not be optimal in the elicitation of high antibody titres. It could be that targeting expression of the heterologous antigen to allow it to be displayed on the *Salmonella* surface, may be more successful in inducing higher antibody titres (Clark-Curtiss & Curtiss III, 2018). This approach has been considered when designing constructs for the Outer Membrane Vesicle, non-living vaccine platform (see section 5.3), however incorporating this strategy in the live vaccine could yield more favourable results.

A booster vaccination after a certain time point, for example when the *Salmonella* infection is expected to have been cleared, could also potentially increase the immune response seen to the guest antigens in this study. For example, Doggett and colleagues showed that mice immunised with a *Salmonella* Typhimurium vaccine strain expressing either a single copy or three tandem repeats of the *Streptococcus sobrinus* antigen SpaA, showed an increase in serum anti-SpaA IgG titres when boosted intranasally 26 weeks after first immunisation. This was despite the fact that mice immunised initially with the single antigen copy strain did not show a significant IgG titre (Doggett et al, 1993). Again, time limitations prevented this from

being investigated in this case, but this also highlights the potential of using recombinant tandem repeats of antigen to significantly increase antibody response.

Given more time in this project, the methods mentioned above could have been investigated, in an attempt to improve the expression of the guest antigens and subsequent immune response in vaccinated mice. It was thought at the time that the level of expression seen of both the Ebola GP and Zika envelope fusion proteins, would have been sufficient to elicit a protective response, but this was unfortunately not the case. The lessons learned here would be to create an even wider ranged panel of constructs, focusing on adding tandem epitope repeats, instead of single peptides (sub-fragments) or full-length proteins, and investigating the use of alternative promoters at the construct design stage and a variety of vaccine strains in the immunisation study. This would potentially allow a more varied approach by 'casting a wider net' and may have a higher chance of a construct which is successful in eliciting a protective immune response.

6.7 *Salmonella* Typhimurium Outer Membrane vesicles (OMVs) as a non-living vaccine platform

Alongside the live vaccine work, another avenue was investigated to determine the feasibility of using *Salmonella* Typhimurium outer membrane vesicles as a non-living vaccine delivery platform. This platform could have numerous advantages over the live *Salmonella* delivery system, not least of which, the immunisation would not rely on the survivability and antigen expression capacity of the *Salmonella*, but antigen could be purified (in vesicles) to specific quantities, allowing control over antigenic dose. As a non-living vaccine delivery platform, it could also potentially be safer and possibly allow previously contra-indicated groups, such as infants, the very old or immunocompromised to be vaccinated with this platform.

6.7.1 Knockouts to increase OMV production (tolR vs mlaA)

As with most Gram-negative bacteria, *Salmonella* naturally produce OMVs, however at relatively low levels. It has been shown however that certain gene specific knockouts can increase the propensity of strains to produce these vesicles. Knockouts of the genes *tolR* and *mlaA* have been shown to have this effect in a variety of Gram-negative organisms (Berlanda

Scorza et al, 2012; Rossi et al, 2016; Lazzaroni et al, 1999; Bernadac et al, 1998; Reidl, 2016; Roier et al, 2016).

Using the one step gene disruption, lambda-red recombinase technique (Datesnko & Wanner, 2000), *ΔtolR* and *ΔmlaA* strains of *Salmonella* Typhimurium SL1344 were made and OMVs successfully purified from spend supernatant. When these OMV fractions were viewed using transmission electron microscopy and negative staining it was immediately apparent that the two knockout strains produced more OMVs than the wild type SL1344 (see figure 5.1.3, chapter 5 section 5.4.2). When the OMV fractions from both knockout strains were analysed using SDS-PAGE, it appeared that there was more protein and therefore likely more OMVs produced by the *ΔtolR* strain compared with the *ΔmlaA* strain. It was therefore decided that SL1344 *ΔtolR* would be taken forward for further consideration as the more vesicles produced, the easier it may be to purify a sufficient antigenic dose.

6.7.2 Targeting of the Zika envelope protein for inclusion in OMVs

Following successful purification of OMVs, the question of antigen presentation then needed to be answered. It was decided that a cost-effective and relatively easy method would be to engineer a plasmid which would hopefully direct the expression of the Zika envelope antigen so that it would be contained in the OMVs.

By engineering expression plasmid, pBAD24-ZE, made using In-Fusion® ligation independent cloning (Clontech), to include signal sequences from known *Salmonella* proteins situated either on the outer membrane or periplasm. It was decided to use the signal sequences for the outer membrane situated OmpA and periplasmically situated DsbA. It was hoped that the Zika E protein, directed by these signal sequences would be either anchored to the outer membrane, and thus displayed on the outside of the OMV, or situated in the periplasm and therefore contained within the lumen of the OMV which contains periplasmic material.

6.7.2.1 Expression of Zika envelope in OMVs

Signal sequence constructs were successfully made, their identity confirmed by DNA sequencing, and transformed into the SL1344 knockout strains. These were then tested for expression of Zika E by western blot of cell lysates and this was shown to be very effective.

Following confirmation of Zika E expression by the *Salmonella* cells themselves, the OMVs were purified from the spent supernatant of these transformed strains and analysed by western blot, probed with monoclonal anti-Zika E to determine, showing that the Zika envelope protein was present in the OMV fraction. This suggests that outer membrane targeting by the OmpA by the signal sequences was successful. The addition of 12 extra residues, corresponding with the membrane spanning region (MSR) of the OmpA protein, hopefully would assist with anchoring the fused ZE to the membrane.

6.7.3 Future work for the OMV platform

The initial results from the OMV platform are very encouraging. It has been shown here that a simple gene knockout can vastly increase the production of OMVs in *Salmonella* as expected and described by other groups. Here however, it has also been shown that these OMVs could be potentially used to make multi-valent vaccines by way of utilising them as a non-living antigen delivery system. The antigen 'cargo', here Zika E, is loaded into the OMVs using signal sequences to direct its expression from a plasmid to the outer membrane or periplasm of the *Salmonella*, subsequently appearing in the OMVs when they are formed.

To further confirm evidence of the expression of Zika E, either displayed on the outside of the OMVs or contained within the lumen, the next step would be to use immuno-gold staining on OMV fractions in TEM to visualise the Zika E in these locations. Further work here, given more time, could also be to use the pBAD24-ZE plasmid as a non-SS control, transforming this into the knockout strains should result in no ZE being detected in the OMV fraction, thus providing evidence that the ZE seen here in the OMV fraction is not due to cytoplasmic contamination of the sample.

It would then need to be determined whether these ZE carrying OMVs, would elicit an immune response when used as a vaccine. OMVs themselves are highly immunogenic due to the outer membrane components such as LPS that they contain, which are known to be potent activators of the innate immune system, in particular, from recognition by Toll-like receptors (TLR). TLR4 recognises LPS, an abundant and very potent immunostimulatory component of the OMV membrane. This potency could lead to unwanted and dangerous reactions such as fever or even septic shock in humans when immunised with certain doses

of OMVs (Rossi et al, 2014). In order to progress to *in vivo* studies, steps would therefore need to be taken in order to reduce the reactogenicity and potential toxicity of the LPS. This would allow a higher OMV dose and thus a higher and hopefully sufficient antigenic dose.

Lipid A anchors the LPS to the membrane and is the endotoxic region of the molecule (Rossi et al, 2014). Modifications, again using specific gene knockouts, have been shown to address its reactogenicity and activation of TLR4. The classic hexa-acylated structure of Lipid A, is also its most endotoxic. Inactivation of the *msbB* and *pagP* genes in *Salmonella*, result in a solely penta-acylated Lipid A with a reduction in the stimulatory potentials of their OMVs, thus possibly resulting in a safer vaccine, where the immunogenic dose is not yet known (Rossi et al, 2016).

Further strategies to increase the expression of the Zika envelope (such as mentioned in section 6.6) could be investigated to increase the chances of a protective immune response. The expression of ZE repitopes (tandem repeats) or even just smaller peptides containing known protective epitopes and displaying these, rather than the full-length ZE, on the OMV surface or contained in the periplasm may help to promote a stronger immune response.

Following these modifications and investigations into the ZE expression, the ZE carrying OMVs could then be used to immunise mice and determine immune response. One recent study by Martins and colleagues has shown that a fusion of OMVs from *Neisseria meningitidis* and Zika Virus expressed from infected C6/36 cells is immunogenic in mice, eliciting antibodies and both TH1 and TH2 responses towards the Zika Virus (Martins et al, 2018). While this method did not utilise molecular biology to target the expression of the antigen, it still highlights the possibility of using OMVs from bacterial pathogens in a multivalent vaccine, affording protection against other pathogens, Zika Virus in particular.

Another interesting method which could be employed to decorate the OMV surface with a heterologous antigen is to exploit natural bacterial autotransporter proteins, usually used to allow gram negative bacteria to translocate proteins across the cell envelope and secrete virulence factors. Daleke-Schermerhorn and colleagues have shown that the *E. coli* autotransporter, haemoglobin protease (Hbp) has side domains which are not necessary for translocation. These side domains can be replaced with heterologous polypeptides, in this case a selection of antigens from *Mycobacterium tuberculosis*, which can then be displayed

on the surface of OMVs if the Hbp-antigen fusions are expressed in hypervesiculating bacterial mutants (such as the tolR knockout mutants as mentioned above) (Daleke-Schermerhorn et al, 2014)

If a response to ZE is seen when these OMVs containing the Zika Envelope antigen are used to immunise, this would be an incredibly attractive and cost-effective vaccine platform not only for Zika Virus but could be engineered to carry antigens from a variety of other pathogens.

6.8 Overall discussion

As outlined in chapter 1, section 1.10, the overall aims and scope of this thesis was to test both living and non-living *Salmonella* vaccine delivery platforms for Ebola and Zika viruses. These were met by the generation of panels of constructs, allowing expression of the both the full-length and sub-fragments of, the Ebola Glycoprotein and Zika envelope protein, either with or without a fusion partner of the C-fragment of Tetanus Toxin (TetC). These proteins were selected as target antigens as they are known targets for protective and neutralising antibodies (Stettler et al, 2016; Sapparapu et al, 2016; Takada et al, 1997; Cook & Lee, 2013), and successful expression of these antigens was observed in *Salmonella* Typhimurium.

It has been shown that bacterial expression of Ebola GP is particularly difficult, with no reports on successful expression of the full-length protein alone or at 37°C possibly due to its toxicity (Zai et al, 2016). Here, the full Ebola GP protein was expressed with only a very small fusion partner (the first 10 residues of the TetC protein), albeit at levels which are probably insufficient for use in a vaccine. This success however, could lead to further ideas for strategies to improve expression of challenging proteins.

The constructs which were determined to be the best expressed by the *Salmonella* vaccine strain SL3261 were those which included the full TetC fusion partner. These strains were therefore taken forward into *in vivo* experiments where plasmid stability and continued fusion protein expression were confirmed. Following this, a larger scale immunisation

experiment was carried out to determine immune response to the Ebola or Zika antigens, however unfortunately there did not appear to be any significant IgG response to these.

The fact that mice are so widely used in biological research, yet do have significant differences in immune development and response compared to humans, could also be a factor when considering the immune response to these vaccine platforms. There are differences in expression of Ig receptors and different Ig isotypes in mice and humans, and also in B and T cell development (Mestas & Hughes, 2004). It may therefore be unwise to assume that the response (or lack of) to experimental vaccines in mice would be the same as seen in humans. Clearly there are ethical barriers towards the testing of drugs in humans before efficacy is seen in an animal model, but perhaps a more humanized mouse model, or alternative animal model altogether could give different and more favourable results.

An alternative vaccine delivery platform, exploiting *Salmonella* Typhimurium Outer Membrane Vesicles (OMVs) was also investigated. It was found that generating knockout strains of SL1344, *ΔtolR* and *ΔmlaA,* made it possible for the cells to produce a much higher yield of OMVs than a wild type strain. This, combined with generating new plasmid constructs to target expression of Zika envelope to the bacterial outer membrane or periplasm, by use of OmpA or DsbA signal sequences, allowed the incorporation of ZE into purified OMVs. This could be a more effective strategy as antigen dose can be more accurately determined and as a non-living platform, could widen access to such a vaccine in vulnerable patient populations who are not able to receive living vaccines.

All in all, while there was a lack of immune response seen here with the living platform, this thesis has allowed insight into how it may be best to go about creating live attenuated *Salmonella* vaccine delivery platforms for emerging viral pathogens and potentially, which strategies to avoid. There are encouraging developments, particularly in the now growing field of Outer Membrane Vesicle based vaccines, which could lead to exciting future research into *Salmonella* based vaccine delivery systems. This non-living vaccine platform could have the potential to widen access to bacterial vaccines and act as a safe delivery system for antigens of other pathogens.

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